Published in final edited form as:

Nat Neurosci. ; 14(10): 1267–1275. doi:10.1038/nn.2922.

***Drosophila* Rae1 controls the abundance of the ubiquitin ligase Highwire in post-mitotic neurons**

Xiaolin Tian¹, Jing Li¹, Vera Valakh², Aaron DiAntonio², and Chunlai Wu¹

¹Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, Louisiana, USA

²Department of Developmental Biology, Washington University School of Medicine, St. Louis, Missouri, USA

Abstract

The evolutionarily conserved Highwire (Hiw)/*Drosophila* Fsn E3 ubiquitin ligase complex is required for normal synaptic morphology during development and axonal regeneration after injury. However, little is known about the molecular mechanisms that regulate the Hiw E3 ligase complex. Using tandem affinity purification techniques, we identified *Drosophila* Rae1 as a previously unknown component of the Hiw/Fsn complex. Loss of *Rae1* function in neurons results in morphological defects at the neuromuscular junction that are similar to those seen in *hiw* mutants. We found that Rae1 physically and genetically interacts with Hiw and restrains synaptic terminal growth by regulating the MAP kinase kinase kinase Wallenda. Moreover, we found that the Rae1 is both necessary and sufficient to promote Hiw protein abundance, and it does so by binding to Hiw and protecting Hiw from autophagy-mediated degradation. These results describe a previously unknown mechanism that selectively controls Hiw protein abundance during synaptic development.

A series of *in vivo* studies in a variety of organisms have found that the evolutionarily conserved PHR proteins, including Hiw (fly), Phr1 (mouse), RPM-1 (worm) and Esrom (zebrafish), regulate the development and repair of the nervous system¹. In *Drosophila*, *hiw* mutants exhibit a marked synaptic terminal overgrowth at the larval neuromuscular junctions (NMJs) with an increased number of boutons and a decreased bouton size^{2,3}, and a similar anatomical overgrowth of the giant fiber–tergotrochanteral motoneuron synapse in the CNS⁴. Aberrant synaptic morphology has also been identified in *rpm-1* mutant worms^{5,6} and *Phr1* mutant mice^{7,8}. In addition, disrupting the function of *hiw* orthologs in worm, zebrafish and mouse results in prominent axon guidance defects^{5,7,9–11}. More recently, *hiw*, *rpm-1* and their downstream target *wallenda* (fly) or *dlk-1* (worm) have been shown to regulate axonal regeneration after nerve injury^{12–14}. Thus, PHR proteins are important in a broad range of neuronal processes, including axon guidance, synaptic development and

© 2011 Nature America, Inc. All rights reserved.

Correspondence should be addressed to C.W., cwu@lsuhsc.edu.

Note: Supplementary information is available on the Nature Neuroscience website.

AUTHOR CONTRIBUTIONS

C.W. and A.D. initiated the project and conducted the Hiw structure and function analysis. C.W. directed the rest of the studies. X.T. and C.W. conceived and designed the experiments. X.T., J.L. and C.W. performed the experiments. V.V. contributed to the characterization of the Rae1 mutant phenotype. C.W. and X.T. wrote the manuscript with input from A.D.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at <http://www.nature.com/reprints/index.html>.

axonal regeneration. However, it is unclear how these diverse functions of PHR proteins are achieved. An important step toward answering this question is to understand the molecular mechanisms that regulate PHR proteins.

Hiw and its orthologs are enormous proteins that share a number of highly conserved functional domains, including an RCC1 domain^{15–17}, two PHR repeats¹⁸, a Myc-binding domain¹⁶ and a C-terminal RING-H2 finger E3 ubiquitin ligase domain^{9,19}. Studies in worms, flies and mice have found that the PHR E3 ubiquitin ligases associate with a highly conserved F-box protein—FSN-1 in worm, Fsn in fly or Fbox45 in mouse—and together they function as a SCF-like E3 ubiquitin ligase complex to regulate neural development^{20–22}. An important downstream target of the ubiquitin ligase complex is the MAP kinase kinase kinase (MAPKKK) Wallenda (Wnd), which activates a MAP kinase cascade to control synaptogenesis^{23,24}. Although considerable progress has been made in understanding the PHR-associated ligase complex and its downstream signaling cascade, very little is known about how PHR proteins are regulated. Autophagy can negatively regulate the abundance of Hiw protein²⁵ in fly, but it is unclear how autophagy, a general protein-degradation pathway, can be controlled to modulate Hiw protein levels during synaptic development. Identifying Hiw cofactors that regulate Hiw activity and abundance is necessary to determine the mechanisms by which *hiw* functions are controlled.

We identified one such Hiw cofactor, *Drosophila melanogaster* Rae1. Rae1 encodes a 346 amino-acid protein that belongs to an evolutionary conserved WD40 repeat protein family. Three major functions of Rae1 have been reported in different organisms. First, Rae1 associates with microtubules in the cytoplasm and regulates the organization of the cytoskeletal network during mitosis²⁶. Second, Rae1 binds to Nup98 to facilitate the transportation of poly-A RNA from the nucleus to the cytosol²⁷. Third, Rae1 is an anaphase-promoting complex (APC)-associated protein that inhibits the targeting of Securin by the APC ubiquitin ligase complex, thereby regulating entry into anaphase during mitosis²⁸. In cultured *Drosophila* SL2 cells, knockdown of Rae1 by RNA interference induces arrest in G1 phase, but results in no mRNA transport defects, suggesting that Rae1 is involved in cell cycle regulation, but not in mRNA export, in these cells²⁹. Although Rae1 binds microtubules in cultured mammalian neurons³⁰, the function of Rae1 in the nervous system has not yet been studied. We found that Rae1 is a binding partner and positive regulator of Hiw. Rae1 and Hiw physically and genetically interacted and worked together to restrain synaptic terminal growth. These results indicate that the function of Rae1 in the Hiw E3 ubiquitin ligase complex is to prevent the autophagy-mediated degradation of Hiw protein in post-mitotic neurons. Our findings, and those of others (B. Grill, L. Chen, E.D. Tülgren, S.T. Baker, W. Bienvenut *et al.*, personal communication), suggest an evolutionarily conserved role for Rae1 in the regulation of neural development.

RESULTS

Drosophila Rae1 associates with Hiw

To identify Hiw cofactors, we used a two-step purification method called tandem affinity purification (TAP) to isolate Hiw-interacting proteins from the *Drosophila* adult head³¹, and then used liquid chromatography–tandem mass spectrometry (LC-MS/MS) to identify the proteins contained in the Hiw-associated complex (see Online Methods). We previously identified *Drosophila* Fsn as a Hiw-interacting protein and found that Hiw participates in a SCF-like E3 ubiquitin ligase complex^{20,21}. Here we identified another Hiw-binding partner, *Drosophila* Rae1. Rae1 was first identified from the ~40-kDa protein band migrating on the SDS-PAGE gel (Fig. 1a) on the basis of the recovery of four unique peptides (MFGATQSTNR, EPNPMMTINLPER, GLIYSLQNSPTEYK and VAIQYVNPNGNPK). In addition, using a shotgun proteomic approach that is capable of identifying all of the trypsin-

digested peptides from the Hiw-associated protein complex (see Online Methods), we found numerous peptides from Rae1 (Supplementary Table 1), which further validated the presence of Rae1 in the protein complexes that were pulled down by N-terminal TAP-tagged Hiw. As an independent test of the association between Rae1 and Hiw, we performed a reverse TAP using neuronally expressed transgenic TAP-tagged Rae1 as the bait to isolate Rae1-interacting proteins in the brain. We then resolved Rae1 and its associating proteins by electrophoresis on a SDS-PAGE gel (Fig. 1b) and used shotgun proteomics analysis to identify the associated proteins. As expected, Hiw was identified with very high confidence (Supplementary Table 2), along with Fsn, which further confirmed the association of Rae1 with the Hiw/Fsn E3 ubiquitin ligase complex.

We then tested the interaction between endogenous Hiw and Rae1 proteins in 3rd instar larval brains. We immunoprecipitated endogenous Hiw using an antibody to Hiw (Hiw2b)¹⁹ and found that Rae1 co-immunoprecipitated with Hiw, but was absent in control immunoprecipitation from *hiw*-null larval brains (Fig. 1c). Furthermore, the Hiw-Rae1 interaction remained unaffected in *Fsn* mutant brains, indicating that Fsn is not required for the binding of Rae1 to Hiw. Although Fsn was not required for the Hiw-Rae1 interaction, Rae1 was detected in Fsn-immunoprecipitated complexes (Supplementary Fig. 1), further supporting the existence of a Rae1/Hiw/Fsn protein complex *in vivo*. Taken together, our data suggest that Rae1 associates with the Hiw complex in post-mitotic neurons.

Rae1 is required to restrain synaptic terminal growth

The strong association of Rae1 and Hiw in neurons led us to postulate that Rae1 may work with Hiw during synaptic development as either a positive or negative regulator of the Hiw/Fsn ligase complex. To assess the function of Rae1 in synaptic development, we carried out a *Rae1* loss-of-function study. We first generated *Rae1* imprecise excision alleles by mobilizing a transposable P-element (P{GawB}NP3499) located ~400 bp upstream of the first exon of the *Rae1* locus. After screening over 800 excision events, we recovered two independent *Rae1* imprecise excision mutants that survived to the second instar larval stage, *Rae1*^{EX28} and *Rae1*^{EXB12}. The mutant larvae started wandering out of food with a body size close to second instar. They then continued to wander for several days without a further increase in their body size and eventually died without pupating. PCR of the genomic DNA extracted from *Rae1*^{EX28} and *Rae1*^{EXB12} mutants revealed that deletions of the *Rae1* locus in both mutants encompassed almost the entire *Rae1* promoter region and a small region of the 3' untranslated region (UTR) of the neighboring gene *pirk* (Fig. 2a). Western blot analysis showed that Rae1 protein levels were reduced to less than 10% of the wild-type level in *Rae1*^{EX28}, *Rae1*^{EX28}/*Df*(2R)5764 and *Rae1*^{EXB12}/*Df*(2R)5764 mutants (Fig. 2b,c). Given that *Rae1*^{EX28} mutants had a very similar level of residual Rae1 protein to *Rae1*^{EX28}/*Df*(2R)5764 mutants and carried a smaller lesion on the neighboring gene *pirk*, we focused our analysis on the *Rae1*^{EX28} mutants.

Morphological analysis of the NMJs from *Rae1*^{EX28} and *Rae1*^{EX28}/*Df*(2R)5764 larvae revealed a morphological defect that was similar to that seen in the *hiw* mutant NMJ: synaptic terminal overgrowth and decreased bouton size (Fig. 3a). We quantified this phenotype at the NMJ of muscle 6/7 on segment A3, although all phenotypes were qualitatively similar at all of the other type I NMJs. The average muscle sizes of all of the quantified NMJs were similar (muscle 6 on segment A3 (×1,000 μm²): wild type, 27.1 ± 1.9; *Rae1*^{EX28}/*Df*(2R)5764, 23.4 ± 1.4; *Rae1*^{EX28}, 26.9 ± 0.9; *Rae1*^{EX28} neuronal rescue, 25.6 ± 1.2, *Rae1*^{EX28} and *wnd* suppression, 23.2 ± 0.8; *hiw*^{ΔN}, 27.3 ± 2.0). However, the number of boutons was increased by almost twofold (120 ± 5 versus 67 ± 5 in wild type) and bouton size was decreased by ~50% (4.4 ± 0.4 μm² versus 7.9 ± 0.5 μm² in wild type) in homozygous *Rae1*^{EX28} mutants compared with wild-type larvae with similar body size (Fig. 3). The *Rae1*^{EX28}/*Df*(2R)5764 mutant's phenotype was very similar to that of the

homozygous *Rae1*^{EX28} mutant, suggesting that *Rae1*^{EX28} is a strong loss-of-function allele. These findings suggest that *Rae1* acts to restrain synaptic growth at the NMJ.

On the basis of the *Rae1* mutant phenotype and the association between *Rae1* and *Hiw*, we predicted that *Rae1* functions in the presynaptic motoneuron to restrain the synaptic growth at NMJs, as both *Hiw* and *Fsn* regulate synaptic development on the presynaptic side^{19,21}. To test this idea, we expressed the *UAS-TAP-Rae1* transgene under the control of *elav-Gal4*, a pan-neuronal Gal4 driver, in the *Rae1* mutant flies. We found that both the increased bouton number and the reduced bouton size were restored to almost wild-type levels by neural expression of *Rae1* in these larvae (Fig. 3). These data indicate that the synaptic phenotype caused by *Rae1*^{EX28} mutation is a result of the loss of *Rae1*, but not *pirk*, and that *Rae1* is required in the presynaptic motoneurons to control the growth of synapse.

Rae1* genetically interacts with *hiw

Our biochemical data and *Rae1* loss-of-function analysis strongly suggest that *Rae1* and *Hiw* function in the same complex to restrain synaptic overgrowth. To further test this hypothesis, we analyzed genetic interactions between *Rae1* and *hiw*. We reasoned that if *Rae1* and *Hiw* function together in a complex, then partial loss-of-function of *Rae1* should enhance the intermediate synaptic terminal overgrowth caused by the *hiw* hypomorphic mutant, *hiw*^{ND512,3}. A partial loss-of-function of *Rae1*, *Rae1* heterozygosity (*Rae1*^{EX28/+}), resulted in a ~50% reduction of *Rae1* protein (Fig. 2) and a significant increase in the number of boutons on synapses on muscle 6/7 ($P < 0.001$; Supplementary Fig. 2), but did not change the number of boutons on muscle 4 (Fig. 4). However, when we combined *hiw*^{ND51} with *Rae1* heterozygosity, we observed a marked enhancement of NMJ overgrowth on muscle 4 (Fig. 4). The dominant enhancement of a weak *hiw* allele by the heterozygous *Rae1* mutant is consistent with the hypothesis that the two genes directly interact in the same genetic pathway. Consistent with this model, we found that a null allele of *hiw*, *hiw*^{ΔN}, was not further enhanced by *Rae1* heterozygosity (Fig. 4). Together, our data provide strong genetic evidence that *Rae1* and *hiw* collaborate to restrain synaptic terminal growth.

The *Hiw*/*Fsn* E3 ligase complex regulates synaptic terminal growth by downregulating *Wnd*²³. Loss of *wnd* function suppressed the morphological defects in either *hiw* or *Fsn* mutant NMJs. Given that we found that *Rae1* interacts with *Hiw* to restrain synaptic growth, one would expect that *Rae1* should also work through *Wnd* in this process. To test this hypothesis, we assessed the ability of *wnd* to suppress the morphological defects caused by *Rae1*^{EX28}. We found that *wnd* suppressed both the increased bouton number (84.1 ± 3.9 in *wnd* suppression versus 119.8 ± 5.2 in *Rae1*^{EX28}) and decreased bouton size ($7.2 \pm 0.3 \mu\text{m}^2$ in *wnd* suppression versus $4.3 \pm 0.3 \mu\text{m}^2$ in *Rae1*^{EX28}) seen in the *Rae1* mutant at the NMJ (Fig. 3). Collectively, our data indicate that *Rae1* is required in motoneurons and works together with *hiw*, upstream of *wnd*, to restrain synaptic growth at the *Drosophila* NMJ.

A structure and function analysis of *Hiw* functional domains

Hiw and its homologs are huge proteins with multiple conserved domains. The identification of *Hiw* binding partners, such as *Medea*³², *Fsn*²¹ and *Rae1*, and their functional relevance to *Hiw* activity suggest that different *Hiw* functional domains may interact with different cofactors and together modulate *Hiw*'s action. To study the functional requirement of these conserved domains and to develop tools for dissecting domain-specific interactions, we performed a systematic structure and function analysis on *Hiw*. We previously found that a full-length *hiw* transgene rescues the *hiw* mutant phenotype when expressed in neurons and that a similar transgene with point mutations in its RING-finger domain fails to do so, indicating that the E3 ligase domain is essential for *Hiw* function¹⁹. We then generated additional truncated *hiw* transgenes lacking the RCC1 domain, the PHR repeats, the C-

terminal half of the Hiw protein or the N-terminal half of the Hiw protein. In addition, we generated a transgene encoding the 1,000 amino-acid fragment of the Hiw C terminus containing the E3 ubiquitin ligase domain (Fig. 5a). We next confirmed the effective expression levels of all of the *hiw* mutant transgenes in larval brains by western blot, and found that all of the Hiw deletion mutants were expressed with expected molecular weights at levels close to or higher than the wild type *hiw* transgene (Fig. 5b). When expressed in neurons in *hiw* mutants, only the full-length *hiw* transgene completely rescued the *hiw* phenotype¹⁹; none of the truncated *hiw* transgenes were able to rescue the synaptic terminal overgrowth phenotype (Fig. 5a and Supplementary Fig. 3). We next examined Wnd protein levels in these larval brains, we found a consistent correlation between elevated Wnd protein levels and the inability of the mutated *hiw* transgenes to rescue *hiw* morphological phenotype, in contrast with the fully restored Wnd protein levels and synaptic terminal size that were observed during neuronal expression of the *hiw* full-length transgene (Supplementary Figs. 3 and 4). The inability of the *hiw* mutant transgenes to restore Wnd protein level and to rescue the *hiw* morphological phenotype is unlikely to be a result of insufficient or excessive expression levels of these transgenes because most of the presented mutant *hiw* trans-genes were expressed at higher levels than the wild-type *hiw* transgene and because we analyzed multiple insertion lines of each transgene that had similar expression levels as the wild-type *hiw* transgene and found that none of them rescued the *hiw* overgrowth phenotype (data not shown). Thus, the results of our structure and function analysis on Hiw suggest that an intact Hiw protein with all of the functional domains is required for the proper control of Wnd protein expression and presynaptic arborization at the fly NMJ.

The inability of all the truncated Hiw proteins to rescue the *hiw* morphology may be a result of disruptions of the normal Hiw function or a gross alteration in protein folding, stability and/or localization. Although none of the mutant transgenes rescued the *hiw* morphology, some did act in a dominant-negative manner. When we used a strong neuronal Gal4 driver, *GS-elav-Gal4*, to express each of the *hiw* trans-genes in a *hiw* heterozygous background (*hiw*^{ND8/+}, no overgrowth phenotype), four *hiw* transgenes resulted in synaptic terminal overgrowth. Among them, *hiw-phr* overexpression resulted in a mild overgrowth, whereas Hiw-RCC1 and Hiw-HindIII overexpression resulted in an intermediate phenotype, and Hiw-CT overexpression resulted in notable *hiw*-like synaptic terminal overgrowth (Fig. 5c,d). The dominant-negative effects likely arise in relation to *hiw*, as, when expressed in a wild-type background using the same driver, each of the four *hiw* transgenes resulted in a milder overgrowth phenotype than that observed in the *hiw* heterozygous background (data not shown). These data indicate that the four truncated Hiw proteins can act as dominant-negative mutants, likely by sequestering important Hiw cofactors. Collectively, our data suggest that all of the Hiw functional domains are necessary for the ability of Hiw to restrain synaptic terminal growth. The fact that each individual domain is necessary for Hiw action suggests that these domains coordinate with each other, potentially by binding to different factors, and work together to modulate the activity of a Hiw complex.

Rae1 interacts with a Hiw C-terminal domain

Except for Hiw-HindIII, the Hiw transgenes were tagged with TAP at their amino-terminus (Fig. 5a), allowing us to map the domain(s) of Hiw that mediate its interaction with Rae1. To do this, we first expressed Hiw deletion transgenes in neurons and performed the first step of TAP pull-down experiments with 3rd instar larval brain lysates. We used immunoblotting to determine whether Rae1 co-precipitates with different Hiw truncated proteins. All of the transgenic proteins, except for Hiw N-terminal half (Hiw-NT) and Hiw very C terminus (Hiw-CT1000), strongly associated with Rae1, whereas Hiw-NT completely and Hiw-CT1000 nearly completely lost their ability to pull-down Rae1 (Fig.

6a,b and Supplementary Figs. 1 and 5). These results suggest that the domain required for the Hiw-Rae1 interaction resides primarily in the C-terminal portion of Hiw outside of the Hiw-CT1000 region (amino acids 2,885 to 4,082). Our data are consistent with a detailed structure and function analysis (B. Grill, L. Chen, E.D. Tulgren, S.T. Baker, W. Bienvenut *et al.*, personal communication), which mapped the Rae1-binding motif on Hiw to the corresponding protein fragment, amino acids 2,932–2,959.

Notably, neuronal expression of Hiw-CT, which contains the Rae1-binding domain, had prominent dominant-negative effects on synaptic terminal growth at the NMJs (Fig. 5c,d). On the basis of the results of our domain-mapping study, we postulated that the dominant-negative effect of Hiw-CT is partly caused by its ability to sequester Rae1 and consequently prevent the formation of functional Hiw-Rae1 complexes. We further predicted that increasing Rae1 expression would suppress the dominant-negative effects of Hiw-CT. To test this hypothesis, we coexpressed Hiw-CT and Rae1 or a control GAP-GFP protein, a fusion of the myristoylation sequence from GAP43 to GFP that targets GFP to the cell membrane, in neurons. We found that the synaptic overgrowth phenotypes caused by Hiw-CT expression were completely suppressed by coexpression of Rae1, but not by GAP-GFP (Fig. 6c,d). These data confirm that Rae1 is a cofactor that is required for the function of the Hiw complex, which is diluted out by this dominant-negative Hiw fragment. This notion is also supported by the fact that all of the mutant *hiw* transgenes that contain the Rae1-binding region exhibited various levels of dominant-negative effect when overexpressed in the wild-type background (Fig. 5a).

Rae1 promotes Hiw protein abundance

To understand how Rae1 positively regulates Hiw function at the molecular level, we first tested whether neuronal expression of Hiw can rescue the *Rae1* morphological phenotype and vice versa. We found that neither GFP-Hiw nor N-terminal TAP-tagged Rae1 can rescue the mutant phenotype of the other (Fig. 7a), suggesting that they are required for each other and the Hiw-Rae1 interaction is essential for a functional E3 ubiquitin ligase complex. To exclude the possibility that this inability to rescue is caused by insufficient expression levels of either genes, we examined the expression levels of both transgenes in wild-type and mutant larval CNS. Although we detected no substantial difference in the expression levels of *elav-Gal4*-driven Rae1 in *hiw* mutant and wild-type larval CNS (data not shown), *elav-Gal4*-driven GFP-Hiw expression in *Rae1* homozygous mutant brains was markedly diminished compared with the broad neuronal cell body expression of GFP-Hiw in *Rae1* heterozygous mutant brains (Supplementary Fig. 6). To confirm that transgenic GFP-Hiw protein is downregulated in *Rae1* mutant brains, we analyzed the expression level of *BG380-Gal4*-driven GFP-Hiw protein in *Rae1^{EX28}* and wild-type larval brains by immunocytochemistry. Again, we observed a marked reduction of GFP-Hiw expression in *Rae1^{EX28}* brains compared with wild-type brains (Fig. 7b). These data suggest that Rae1 may function to regulate the abundance and stability of Hiw.

To test this hypothesis, we examined whether the endogenous Hiw protein is also reduced in *Rae1* mutants. We assayed Hiw protein levels in the brains of wild-type, *hiw^{ΔN}*, *Rae1^{EX28}* and *Rae1^{EX28}* larvae with neuronal rescue by a *Rae1* transgene. We extracted total proteins from dissected larval brains using a lysis buffer that contains urea, SDS, and a comprehensive panel of protease and proteasome inhibitors to ensure the maximal preservation of total neuronal proteins in the lysates. Hiw immunoblot of these brain lysates revealed that endogenous Hiw protein in *Rae1* mutant brain lysate was markedly reduced compared with that in wild-type brains (Fig. 7c). The reduction of Hiw protein level seemed to depend on the dose of *Rae1*, as the *Rae1* heterozygous mutants also showed a mild decrease in Hiw expression (Supplementary Fig. 7). The neuronal expression of the *NT-Rae1* transgene completely restored Hiw protein level (Fig. 7c), consistent with its ability to

rescue the synaptic terminal overgrowth phenotype at the *Rae1* mutant NMJs (Fig. 3). In contrast with the markedly reduced protein level of Hiw that we observed in *Rae1* mutant brain lysate, Rae1 protein level was not altered in *hiw* mutants (Figs. 1c and 7c,d and Supplementary Fig. 7), indicating that Rae1 is not a Hiw ubiquitination target. We next tested whether neuronal overexpression of transgenic Rae1 protein is sufficient to elevate endogenous Hiw protein level in wild-type background. We consistently detected a ~70% increase in the level of endogenous Hiw protein when we expressed transgenic NTAP-Rae1 protein in wild-type brains at about the same level as endogenous Rae1 (driven by a pan-neuronal driver C155-Gal4; Fig. 7d,e). These data indicate that Rae1 is both necessary and sufficient to promote Hiw protein abundance.

Given the established role of Rae1 in regulating mRNA export in yeast²⁷, the observed effect of Rae1 on Hiw protein levels could be a result of a general defect in mRNA export when *Rae1* is mutated. If this were true, we would predict a gross change in protein levels for a broad range of molecules. However, none of the neuronal proteins that we tested, Futsch, CamKII, β -tubulin, Syntaxin and the neural form of β -catenin, showed decreased protein levels in *Rae1* mutant or increased protein levels in Rae1-expression brain lysate (Fig. 7c,d and Supplementary Fig. 7). Hence, both the reduction of protein level in the *Rae1* mutant brains and the increase of protein level in Rae1-expression brains are selective to Hiw. Rae1's regulation of Hiw protein abundance is therefore unlikely mediated by general defects in mRNA transportation. This observation is also consistent with the previous finding that knockdown of Rae1 activity by RNA interference in cultured *Drosophila* cells does not lead to mRNA transport defects²⁹.

The direct association of Rae1 to Hiw complex strongly suggests that Rae1 regulates the abundance of Hiw at the post-translational level. However, given the broad functions of Rae1 homologs in other systems, Rae1 could also regulate *hiw* transcription or *hiw* mRNA stability. To test these possibilities, we examined *hiw* mRNA levels in wild-type and *Rae1* mutant brains using quantitative reverse-transcription PCR (RT-PCR). We found that there was no significant change in *hiw* transcript levels in Rae1 mutant brains compared to wild-type brains (Δ Ct ratio of specific transcript to 18S rRNA internal control transcript: *Rae1*^{EX28} 17.36 ± 0.58 , versus wild type 17.48 ± 0.19 ; $P > 0.5$; see Online Methods). These data therefore exclude the possibility that Rae1 regulates *hiw* transcription or mRNA stability. In addition, *Rae1* mutants were unable to sustain a comparable level of the transgenic GFP-Hiw protein expressed via the UAS or Gal4 systems in larval CNS (Fig. 7b). The UAS-GFP-*hiw* transgene contains the full-length Hiw coding sequence without introns and UTRs. Given that RNA-binding proteins often target 3' UTRs to regulate mRNA stability, degradation, nuclear export, subcellular localization and translation efficiency^{33,34}, it is unlikely that Rae1 regulates the transgenic *hiw* mRNA via these mechanisms. Thus, Rae1 controls endogenous Hiw protein expression via post-translational regulation. Altogether, these findings strongly suggest that Rae1 regulates Hiw protein expression through direct protein-protein interactions with the Hiw E3 ligase complex.

Rae1 suppresses autophagy-mediated degradation of Hiw

How does Rae1-Hiw interaction promote the abundance of Hiw protein? How does it interact with known mechanisms that regulate Hiw protein level? Autophagy regulates synaptic development by promoting the degradation of Hiw protein²⁵. It is thus possible that Rae1-Hiw interaction may protect Hiw from being degraded by the autophagy pathway. If this were true, we would expect that reducing autophagic activity in *Rae1* mutant should suppress both the reduction of Hiw protein level and the associated synaptic terminal overgrowth. We tested this possibility by crippling autophagic activity via removal of one copy of three different essential autophagy genes³⁵, *atg1*, *atg2* or *atg18*, respectively, in the *Rae1* mutant background. We found mutations of these essential autophagy genes can each

dominantly suppress the *Rae1* mutant morphological phenotype (Fig. 8a,b), while heterozygous mutants of *atg1*, *atg2* or *atg18* alone have no synaptic overgrowth phenotype (Fig. 8b). Furthermore, this suppressions correlates with Hiw protein levels; there was a significant increase in the expression of Hiw protein in *Rae1* mutant brains when one copy of *atg1*, *atg2* or *atg18* was removed ($P < 0.05$; Fig. 8c,d). These data indicate that Hiw is rapidly degraded via autophagy in the absence of *Rae1*.

Neuronal expression of *Atg1* induces high levels of autophagy and causes synaptic terminal overgrowth by downregulating Hiw²⁵. If the interaction between *Rae1* and Hiw protects autophagy-mediated degradation of Hiw, coexpression of *Rae1* with *Atg1* should suppress *Atg1*-induced overgrowth. We coexpressed NTAP-*Rae1* with *Atg1* and found that the *Atg1*-induced NMJ overgrowth was fully suppressed (Fig. 8e,f). This suppression is not a result of a simple dilution of Gal4, as coexpression of GAP-GFP had no effect on *Atg1*-induced NMJ overgrowth. Together, these findings indicate that an important function of *Rae1* in the Hiw E3 ligase complex is to prevent autophagy-mediated degradation of Hiw.

DISCUSSION

Molecular pathways mediated by Hiw and its orthologs are important for neural development and regeneration. By identifying and characterizing the Hiw-interacting protein *Rae1*, we identified a previously unknown function of *Rae1* in the post-mitotic neuron: regulating synaptic development by binding to Hiw and protecting Hiw from autophagic degradation.

Rae1 associates and functions with Hiw in neurons

We provide three pieces of evidence that *Rae1* is a bona fide Hiw-binding protein. First, *Rae1* and Hiw were co-purified from adult head homogenate in reciprocal TAPs using either Hiw or *Rae1* as the tagged proteins, respectively. Second, endogenous *Rae1* co-immunoprecipitated with Hiw in fly larval brains. Finally, coexpressing *Rae1* suppressed the dominant-negative effects caused by overexpression of the truncated Hiw protein (Hiw-CT) that contains the *Rae1*-binding region.

In addition to *Rae1*, a number of other molecules have been identified that bind Hiw and its orthologs. These factors include the fly medea/SMAD (a component of the TGF- β signaling pathway³²), adenylate cyclase (an enzyme controlling cAMP levels^{15,36}), Tuberin (a component of the TSC complex that regulates TOR signaling³⁷), GLO-4 (a positive regulator of a Rab GTPase pathway³⁸) and FSN-1/Fsn/Fbox45 (an F-box protein^{20–22}). Of these interactions, only the Fsn-PHR and *Rae1*-PHR (our results and those of B. Grill, L. Chen, E.D. Tulgren, S.T. Baker, W. Bienvenut *et al.*, personal communication) interactions have been shown to be conserved across species. Studies of the PHR-FSN interaction have defined a common mechanism in the action of PHR proteins, RING E3 ligases working in an SCF-like ubiquitin ligase complex^{20–22}. Similarly, the PHR-*Rae1* interaction may reveal a previously unknown and conserved mechanism that positively regulates the action of PHR proteins in post-mitotic neurons.

Drosophila nucleoporin (Nup98) is a binding partner of *Rae1* involved in many biological processes, such as facilitating mRNA export and the *Rae1*-APC interaction^{27,28}. Our *Rae1* TAP experiment also pulled down Nup98 with high confidence (Fig. 1b and Supplementary Table 2), suggesting that Nup98 strongly associates with *Rae1* in *Drosophila* neurons. However, Nup98 was not detected in the TAP-Hiw pulldown complex. This observation suggests that the interaction between Hiw and *Rae1* may not require Nup98 and that *Rae1* and Nup98 may carry out functions in neurons that are independent of Hiw.

We investigated the biological relevance of the Hiw-Rae1 interaction in post-mitotic neurons. Rae1 has multiple important functions in mitotic cells, but its role in post-mitotic neurons is unknown. Our loss of function analysis of *Rae1* at the *Drosophila* larval NMJ revealed that Rae1 functions in post-mitotic motor neurons to restrain synaptic growth. Rae1 is unlikely to be a ubiquitin target of the Hiw E3 ubiquitin ligase complex, as *Rae1* functions in the same direction as *hiw*, and Rae1 protein expression was unchanged in *hiw* null mutants compared with wild-type animals. Instead, our data suggest that Rae1 works as a positive regulator to facilitate the function of the Hiw E3 ligase complex. This notion is supported by the similar loss of function phenotype in *Rae1* and *hiw* null mutants, the strong genetic interactions between *Rae1* and *hiw*, and the ability of *wnd*, a known Hiw downstream target, to suppress *Rae1* phenotypes. Thus, our biochemical data, together with our genetic analysis, suggest that Rae1 acts in the post-mitotic neurons to restrain the growth of synaptic terminals through its interaction with the Hiw E3 ubiquitin ligase complex.

The role of Rae1 in stabilizing Hiw protein

The Rae1-Hiw protein interaction could promote the function of Hiw ubiquitin ligase by regulating Hiw's localization, enzymatic activity or substrate specificity, or protein stability. Our results favor the last possibility, that Rae1 regulates Hiw protein abundance and stability. We found that Hiw protein levels were markedly reduced in the *Rae1^{EX28}* mutant brains and substantially increased in the brains of flies expressing Rae1, and that both the reduction and increase were selective for Hiw, as the levels of many other neuronal proteins were unaltered. Thus, an important function of Rae1 in the post-mitotic neuron is to dynamically regulate Hiw protein expression. To further understand the molecular basis of how Rae1 promotes Hiw protein abundance, we have excluded the possibilities that reduction of Hiw protein in the *Rae1* mutants could be due to defects in mRNA export in general, or reduced *hiw* transcription or mRNA stability. It is also unlikely that Rae1 controls the translation of *hiw*, as the promoter and 3' UTR region of *hiw* were unnecessary for Rae1's regulation of Hiw abundance. These data, together with the finding that Rae1 physically associates with Hiw, strongly suggest that Rae1 promotes Hiw protein stability via its association with Hiw and prevents Hiw's degradation.

Protein clearance and quality control in eukaryotic cells are executed by two major pathways, ubiquitin-proteasome-mediated degradation and autophagy. The former is generally anticipated to be a more specific targeting mechanism than the latter. However, recent studies have suggested that selective autophagy can be achieved through a number of autophagic adaptor proteins, although the molecular mechanisms remain to be elucidated³⁹. Autophagy was recently implicated in the regulation of Hiw protein levels in *Drosophila*²⁵, but it is unclear whether this regulation is selective and how the selectivity is achieved. We found, both genetically and biochemically, that the association of Rae1 and Hiw prevents Hiw from being degraded by autophagy. Our results suggest a mechanism for regulating selective autophagy: specific factor(s) can bind to the target protein and prevent it from being selected for autophagy-mediated degradation.

Although our data do not exclude a functional requirement of Rae1 for Hiw activity and localization, they clearly indicate that Rae1 is essential for controlling Hiw protein levels during development. Rae1, a microtubule-binding protein with multiple cellular functions, is of great interest as a regulator of synaptic development. Our data suggest that decreasing and increasing Rae1 from its basal protein levels drives corresponding changes in Hiw protein levels. It is intriguing to speculate that Rae1 functions as a molecular switch in response to either environmental cues or microtubule networks to rapidly and precisely regulate Hiw protein levels during neural development and regeneration.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/natureneuroscience/>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are particularly grateful to D. Sitterlin for providing the antibody to Rae1. We would like to thank the A.J. Siteman Cancer Center at the Washington University School of Medicine and Barnes-Jewish Hospital in St. Louis, Missouri, and the Department of Physiology at the Louisiana State University Health Sciences Center in New Orleans for the use of their proteomic core facilities. We are grateful to T. Neufeld and the Bloomington Stock Center for fly stocks, and the Developmental Studies Hybridoma Bank for antibodies. We also thank B. Grill and Y. Jin for helpful comments. This work is supported by grants from the US National Institutes of Health (NS070962 to C.W. and DA020812 to A.D.).

References

- Po MD, Hwang C, Zhen M. PHRs: bridging axon guidance, outgrowth and synapse development. *Curr Opin Neurobiol.* 2010; 20:100–107. [PubMed: 20079626]
- DiAntonio A, et al. Ubiquitination-dependent mechanisms regulate synaptic growth and function. *Nature.* 2001; 412:449–452. [PubMed: 11473321]
- Wan HI, et al. Highwire regulates synaptic growth in *Drosophila*. *Neuron.* 2000; 26:313–329. [PubMed: 10839352]
- Uthaman SB, Godenschwege TA, Murphey RK. A mechanism distinct from highwire for the *Drosophila* ubiquitin conjugase bendless in synaptic growth and maturation. *J Neurosci.* 2008; 28:8615–8623. [PubMed: 18716220]
- Schaefer AM, Hadwiger GD, Nonet ML. rpm-1, a conserved neuronal gene that regulates targeting and synaptogenesis in *C. elegans*. *Neuron.* 2000; 26:345–356. [PubMed: 10839354]
- Zhen M, Huang X, Bamber B, Jin Y. Regulation of presynaptic terminal organization by *C. elegans* RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron.* 2000; 26:331–343. [PubMed: 10839353]
- Bloom AJ, Miller BR, Sanes JR, DiAntonio A. The requirement for Phr1 in CNS axon tract formation reveals the corticostriatal boundary as a choice point for cortical axons. *Genes Dev.* 2007; 21:2593–2606. [PubMed: 17901218]
- Burgess RW, et al. Evidence for a conserved function in synapse formation reveals *Phr1* as a candidate gene for respiratory failure in newborn mice. *Mol Cell Biol.* 2004; 24:1096–1105. [PubMed: 14729956]
- D'Souza J, et al. Formation of the retinotectal projection requires Esrom, an ortholog of PAM (protein associated with Myc). *Development.* 2005; 132:247–256. [PubMed: 15590740]
- Culican SM, Bloom AJ, Weiner JA, DiAntonio A. Phr1 regulates retinogeniculate targeting independent of activity and ephrin-A signaling. *Mol Cell Neurosci.* 2009; 41:304–312. [PubMed: 19371781]
- Lewcock JW, Genoud N, Lettieri K, Pfaff SL. The ubiquitin ligase Phr1 regulates axon outgrowth through modulation of microtubule dynamics. *Neuron.* 2007; 56:604–620. [PubMed: 18031680]
- Yan D, Wu Z, Chisholm AD, Jin Y. The DLK-1 kinase promotes mRNA stability and local translation in *C. elegans* synapses and axon regeneration. *Cell.* 2009; 138:1005–1018. [PubMed: 19737525]
- Xiong X, et al. Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. *J Cell Biol.* 2010; 191:211–223. [PubMed: 20921142]
- Hammarlund M, Nix P, Hauth L, Jorgensen EM, Bastiani M. Axon regeneration requires a conserved MAP kinase pathway. *Science.* 2009; 323:802–806. [PubMed: 19164707]

15. Scholich K, Pierre S, Patel TB. Protein associated with Myc (PAM) is a potent inhibitor of adenylyl cyclases. *J Biol Chem.* 2001; 276:47583–47589. [PubMed: 11590159]
16. Guo Q, Xie J, Dang CV, Liu ET, Bishop JM. Identification of a large Myc-binding protein that contains RCC1-like repeats. *Proc Natl Acad Sci USA.* 1998; 95:9172–9177. [PubMed: 9689053]
17. Garbarini N, Delpire E. The RCC1 domain of protein associated with Myc (PAM) interacts with and regulates KCC2. *Cell Physiol Biochem.* 2008; 22:31–44. [PubMed: 18769030]
18. Abrams B, Grill B, Huang X, Jin Y. Cellular and molecular determinants targeting the *Caenorhabditis elegans* PHR protein RPM-1 to perisynaptic regions. *Dev Dyn.* 2008; 237:630–639. [PubMed: 18224716]
19. Wu C, Wairkar YP, Collins CA, DiAntonio A. Highwire function at the *Drosophila* neuromuscular junction: spatial, structural, and temporal requirements. *J Neurosci.* 2005; 25:9557–9566. [PubMed: 16237161]
20. Liao EH, Hung W, Abrams B, Zhen M. An SCF-like ubiquitin ligase complex that controls presynaptic differentiation. *Nature.* 2004; 430:345–350. [PubMed: 15208641]
21. Wu C, Daniels RW, DiAntonio A. Dfsc collaborates with Highwire to down-regulate the Wallenda/DLK kinase and restrain synaptic terminal growth. *Neural Dev.* 2007; 2:16. [PubMed: 17697379]
22. Saiga T, et al. Fbxo45 forms a novel ubiquitin ligase complex and is required for neuronal development. *Mol Cell Biol.* 2009; 29:3529–3543. [PubMed: 19398581]
23. Collins CA, Wairkar YP, Johnson SL, DiAntonio A. Highwire restrains synaptic growth by attenuating a MAP kinase signal. *Neuron.* 2006; 51:57–69. [PubMed: 16815332]
24. Nakata K, et al. Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell.* 2005; 120:407–420. [PubMed: 15707898]
25. Shen W, Ganetzky B. Autophagy promotes synapse development in *Drosophila*. *J Cell Biol.* 2009; 187:71–79. [PubMed: 19786572]
26. Brown JA, et al. A mutation in the *Schizosaccharomyces pombe* rae1 gene causes defects in poly(A)⁺ RNA export and in the cytoskeleton. *J Biol Chem.* 1995; 270:7411–7419. [PubMed: 7706287]
27. Pritchard CE, Fornerod M, Kasper LH, van Deursen JM. RAE1 is a shuttling mRNA export factor that binds to a GLEBS-like NUP98 motif at the nuclear pore complex through multiple domains. *J Cell Biol.* 1999; 145:237–254. [PubMed: 10209021]
28. Jeganathan KB, Malureanu L, van Deursen JM. The Rae1-Nup98 complex prevents aneuploidy by inhibiting securin degradation. *Nature.* 2005; 438:1036–1039. [PubMed: 16355229]
29. Sitterlin D. Characterization of the *Drosophila* Rae1 protein as a G1 phase regulator of the cell cycle. *Gene.* 2004; 326:107–116. [PubMed: 14729268]
30. Kraemer D, Dresbach T, Drenckhahn D. Mrnp41 (Rae1p) associates with microtubules in HeLa cells and in neurons. *Eur J Cell Biol.* 2001; 80:733–740. [PubMed: 11831386]
31. Rigaut G, et al. A generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol.* 1999; 17:1030–1032. [PubMed: 10504710]
32. McCabe BD, et al. Highwire regulates presynaptic BMP signaling essential for synaptic growth. *Neuron.* 2004; 41:891–905. [PubMed: 15046722]
33. Conne B, Stutz A, Vassalli JD. The 3' untranslated region of messenger RNA: a molecular 'hotspot' for pathology? *Nat Med.* 2000; 6:637–641. [PubMed: 10835679]
34. Bolognani F, Perrone-Bizzozero NI. RNA-protein interactions and control of mRNA stability in neurons. *J Neurosci Res.* 2008; 86:481–489. [PubMed: 17853436]
35. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell.* 2008; 132:27–42. [PubMed: 18191218]
36. Pierre SC, Hausler J, Birod K, Geisslinger G, Scholich K. PAM mediates sustained inhibition of cAMP signaling by sphingosine-1-phosphate. *EMBO J.* 2004; 23:3031–3040. [PubMed: 15257286]
37. Murthy V, et al. Pam and its ortholog highwire interact with and may negatively regulate the TSC1. TSC2 complex *J Biol Chem.* 2004; 279:1351–1358.

38. Grill B, et al. *C. elegans* RPM-1 regulates axon termination and synaptogenesis through the Rab GEF GLO-4 and the Rab GTPase GLO-1. *Neuron*. 2007; 55:587–601. [PubMed: 17698012]
39. Johansen T, Lamark T. Selective autophagy mediated by autophagic adapter proteins. *Autophagy*. 2011; 7:279–296. [PubMed: 21189453]

Figure 1.

Rae1 and Hiw interact with each other in neurons. **(a)** The Hiw-associated complex was purified by TAP (see Online Methods) and analyzed by one-dimensional SDS-PAGE gel followed by Sypro Ruby staining. Mass spectrometry identified the full-length Hiw, β -tubulin, Rae1 and Fsn, indicated by arrows. **(b)** The Rae1-associated complex purified from fly brains by TAP was analyzed by one-dimensional SDS-PAGE gel followed by Coomassie blue G-250 staining. Mass spectrometry identified Hiw, Nup98 and Rae1, indicated by arrows. **(c)** Larval brain lysate of wild-type (WT), *hiw* null mutant (*hiw* ^{ΔN}) and *Fsn* mutant (*Fsn*^{f06595/Df(2R)7872}) flies was subject to co-immunoprecipitation with antibody to Hiw (Hiw2b). Both the input and the immunoprecipitated complexes were analyzed by western blots with antibodies to Hiw or Rae1. The membrane was also blotted with antibody to β -tubulin to ensure equal amount of total proteins in the input samples. Full-length blots are presented in Supplementary Figure 8.

Figure 2.

Generation of *Rae1* mutant alleles. **(a)** The gene structure of *Rae1*. The black-arrowed boxes indicate exons. A transposable element, P{GawB}NP3499, located ~400 bp upstream of the first exon of *Rae1* was used to generate imprecise excision mutants of *Rae1*. PCR analysis indicates that ~900 bp and a ~1.5-k bp DNA fragment in the *Rae1* promoter region are missing in *Rae1^{EX28}* and *Rae1^{EXB12}*, respectively. **(b)** Western blot analysis on Rae1 protein in the brain lysate of wild-type, *Df(2R)5764/+*, *Rae1^{EX28}/+*, or late second instar *Rae1^{EX28}*, *Rae1^{EX28}/Df(2R)5764(/Df)* and *Rae1^{EXB12}/Df(2R)5764* larvae. β-tubulin was used as the loading control. Full-length blots are presented in Supplementary Figure 9. **(c)** Quantification of Rae1 protein levels in the *Rae1* heterozygous and homozygous mutant brains. Rae1 protein levels were first normalized to β-tubulin protein levels and are presented as percentage of wild-type level. **P* < 0.05, ***P* < 0.001. Error bars denote s.e.m.

Figure 3.

Rae1 is required to restrain synaptic terminal growth at the NMJ. (a) Representative confocal images of segment A3 muscle 6/7 synapses stained for both DVGLUT (green) and FasII (red), in late 2nd/early 3rd instar wild-type, *Rae1^{EX28}*, *Rae1^{EX28}* presynaptic rescue (*Rae1^{EX28}; elav-Gal4/UAS-NTAP-Rae1*) and *Rae1^{EX28}* *wallenda* suppression (*Rae1^{EX28}; wnd³*) larvae. Scale bar represents 10 μ m. (b,c) Quantification of bouton number (b) and size (c) in wild-type, *Rae1^{EX28}/Df*, *Rae1^{EX28}*, *Rae1^{EX28} rescue*, *Rae1^{EX28}; wnd³* suppression and *hiw^{ΔN}* larval NMJs (segment A3 muscle 6/7; $n = 20, 21, 18, 20, 20$ and 11, respectively, for bouton number; $n = 271, 465, 548, 726, 486$ and 826, respectively, for bouton size). There was no significant difference in either bouton number or bouton size between *Rae1^{EX28}* and *Rae1^{EX28}/Df* ($P > 0.1$ for both comparisons). $*P < 0.001$. Error bars denote s.e.m.

Figure 4.

Rae1 genetically interacts with *hiw* to restrain synaptic terminal growth. **(a)** Representative confocal images of muscle 4 synapses stained for both DVGLUT (green) and FasII (red) in wild-type, *Rae1^{EX28/+}*, *hiw^{ND51}*, *hiw^{ND51}; Rae1^{EX28/+}*, *hiw^{ΔN}* and *hiw^{ΔN}; Rae1^{EX28/+}* larvae. Scale bar represents 10 μm. **(b)** Quantification of bouton number at muscle 4 NMJs in wild-type, *Rae1^{EX28/+}*, *hiw^{ND51}*, *hiw^{ND51}; Rae1^{EX28/+}*, *hiw^{ΔN}* and *hiw^{ΔN}; Rae1^{EX28/+}* larvae ($n = 27, 23, 42, 46, 23$ and 25 cells, respectively). There was a significant increase in the number of boutons formed in *hiw^{ND51}; Rae1^{EX28/+}* compared with *hiw^{ND51}* 3rd instar larval NMJs ($*P < 0.001$). The number of boutons in the *hiw^{ΔN}; Rae1^{EX28/+}* double mutant was not significantly different from that in *hiw^{ΔN}* ($**P > 0.5$), demonstrating a lack of enhancement of the *hiw^{ΔN}* phenotype by *Rae1^{EX28/+}*. Error bars denote s.e.m.

Figure 5.

A structure and function analysis of Hiw functional domains. **(a)** Schematic presentation of NTAP-tagged (NT-) and HM-tagged (HM-) *hiw* transgenes encoding full-length, mutated or truncated Hiw proteins. Hiw functional domains are marked with colored boxes. The positions of amino-acid substitution in NT-*Hiw*ΔRING and the added amino acid residue necessary to maintain the reading frame in NT-*Hiw*-RCC1 and NT-*Hiw*-PHR are indicated. The right column summarizes the ability of the given *hiw* transgene to rescue the synaptic overgrowth phenotype in *hiw* mutants, to cause dominant negative overgrowth phenotype when expressed in wild-type background and to bind to Rae1. **(b)** Western blot analysis of fly brain lysate using antibody to TAP (PAP, Sigma) or Myc revealed the expression of wild-type and mutant *hiw* transgenes in predicted size. **(c)** Representative confocal images of muscle 4 synapses stained for DVGLUT (green) and FasII (red) in wild-type (+/*hiw*^{ND8}; +/+; *GS-elav-Gal4*/+), NT-*Hiw* overexpression (+/*hiw*^{ND8}; *UAS-NT-hiw*/+; *GS-elav-Gal4*/+), NT-*Hiw*-NT overexpression (+/*hiw*^{ND8}; *UAS-NT-hiw-NT*/+; *GS-elav-Gal4*/+), NT-*Hiw*-CT1000 overexpression (+/*hiw*^{ND8}; +/+; *GS-elav-Gal4*/*UAS-NT-hiw-CT1000*), NT-*Hiw*-PHR overexpression (+/*hiw*^{ND8}; +/+; *GS-elav-Gal4*/*UAS-NT-hiw-PHR*), HM-*Hiw*-HindIII overexpression (+/*hiw*^{ND8}; *UAS-HW-hiw-HindIII*/+; *GS-elav-Gal4*/+), NT-*Hiw*-RCC1 overexpression (*NT-hiw-RCC1*/*hiw*^{ND8}; +/+; *GS-elav-Gal4*/+), or NT-*Hiw*-CT overexpression (+/*hiw*^{ND8}; +/+; *GS-elav-Gal4*/*UAS-NT-hiw-CT*) 3rd larval NMJs. Overexpression is indicated by an up arrow. Scale bar represents 10 μm. **(d)** Quantification of the number of boutons at muscle 4 NMJs in wild-type larvae or larvae overexpressing wild-type or mutant *hiw* transgenes ($n = 23, 24, 26, 27, 26, 21, 25$ and 27 , respectively; * $P < 0.001$). Error bars denote s.e.m.

Figure 6.

Rae1 interacts with a fragment in the Hiw C-terminal region, and coexpression of Rae1 with NT-Hiw-CT suppresses the NT-Hiw-CT–induced dominant-negative overgrowth phenotype. **(a)** Indicated NTAP-tagged *hiw* transgenes (described in Fig. 5) and a TAP only transgene were expressed in neurons under the control of the *BG380-Gal4* driver. Larval brain lysates from each sample were subject to IgG pulldown. Both the inputs and the IgG pulldown complexes were analyzed by western blot using antibody to Rae1. Rae1 was present in all of the pulldown complexes except for those from TAP only, NT-Hiw-CT1000 and NT-Hiw-NT. Full-length blots are presented in Supplementary Figure 10. **(b)** Quantification of the interaction between various Hiw transgenic proteins and Rae1. Rae1 intensities in TAP pulldown blots were normalized to both the intensities and molecular weights of the corresponding TAP pulldown proteins. **(c)** Representative confocal images of muscle 4 synapses stained for both DVGLUT (green) and FasII (red) in wild-type (*BG380/Y; +/+; +/+*), NT-Hiw-CT and GAP-GFP (*BG380/Y; UAS-GAP-GFP/+; UAS-NT-hiw-CT/+*), and NT-Hiw-CT and Rae1 (*BG380/Y; +/+; UAS-NT-hiw-CT/UAS-GFP-Rae1*) larvae. Scale bar represents 10 μm. **(d)** Quantification of number of boutons at muscle 4 NMJs in wild-type, NT-Hiw-CT overexpression (*BG380/Y; +/+; UAS-NT-hiw-CT/+*), NT-Hiw-CT and GAP-GFP, and NT-Hiw-CT and Rae1 ($n = 24, 27, 32$ and 26 , respectively) larvae. Error bars denote s.e.m.; * $P < 0.001$.

Figure 7.

Rae1 promotes Hiw abundance. **(a)** Neuronal expression of neither Hiw nor Rae1 rescues the synaptic terminal overgrowth phenotype caused by the loss of function of the other gene. Quantification of the number of boutons in wild-type, *Rae1^{EX28}*, *Rae1^{EX28}; Hiw* rescue (Res) (*Rae1^{EX28}; elav-Gal4/UAS-GFP-hiw*), *hiw^{ΔN}* and *hiw^{ΔN}; Rae1* rescue (*hiw^{ΔN}; elav-Gal4/UAS-NTAP-Rae1*) larval NMJs (segment A3 muscle 6/7; $n = 12, 12, 12, 7$ and 11 , respectively). **(b)** Representative confocal images of wild-type (*BG380-Gal4/+; +/+; UAS-GFP-hiw/+*) or *Rae1* mutant (*BG380-Gal4/+; Rae1^{EX28}; UAS-GFP-hiw/+*) ventral ganglia with *UAS-GFP-hiw* transgene expressed in neurons. The larvae were stained with antibodies to horseradish peroxidase (HRP, red) and GFP (green). Scale bar represents 10 μ m. **(c)** Western blots of total proteins extracted from wild-type, *hiw^{ΔN}*, *Rae1^{EX28}* and *Rae1^{EX28} rescue* (*Rae1^{EX28}; elav-Gal4/UAS-NTAP-Rae1*) larval brains probed with indicated antibodies. Full-length blots are presented in Supplementary Figure 11. **(d)** Western blot analysis on total proteins extracted from *hiw^{ΔN}*, wild-type (*C155-Gal4/+*) and *Rae1* expression (*C155-Gal4/+; UAS-NTAP-Rae1/+*) larval brains. Full-length blots are presented in Supplementary Figure 12. **(e)** Quantification of Hiw protein levels in the wild-type and the *Rae1* expression larval brains ($*P < 0.001$, based on seven independent experiments). Arrows indicate a 38-kDa endogenous *Rae1* protein and the arrowheads indicate the 58-kDa transgenic NTAP-*Rae1* protein. Error bars denote s.e.m.

Figure 8.

Rae1-Hiw interaction prevents autophagy-mediated degradation of Hiw protein. **(a)** Representative confocal images of segment A3 muscle 6/7 synapses stained for both DVGLUT (green) and FasII (red), in wild-type, *Rae1^{EX28}*, *Rae1^{EX28}; atg1/+*, *Rae1^{EX28}; atg2/+* and *Rae1^{EX28}; atg18/+* wandering larvae. Scale bar represents 10 μ m. **(b)** Quantification of the number of boutons in wild-type, *atg1/+*, *atg2/+*, *atg18/+*, *Rae1^{EX28}*, *Rae1^{EX28}; atg1/+*, *Rae1^{EX28}; atg2/+* and *Rae1^{EX28}; atg18/+* larval NMJs (segment A3 muscle 6/7; $n = 12, 12, 12, 12, 16, 12$ and 21 , respectively). **(c)** Western blot analysis on total proteins extracted from wild-type, *Rae1^{EX28}*, *Rae1^{EX28}; atg1/+*, *Rae1^{EX28}; atg2/+* and *Rae1^{EX28}; atg18/+* larval brains. The neural form of β -catenin (82 kDa, see Online Methods) was used as an internal control of neuronal proteins. Full-length blots are presented in Supplementary Figure 13. **(d)** Quantification of Hiw protein levels in wild-type, *Rae1^{EX28}*, *Rae1^{EX28}; atg1/+*, *Rae1^{EX28}; atg2/+* and *Rae1^{EX28}; atg18/+* larval brains (based on six independent experiments). **(e)** Representative confocal images of muscle 4 synapses stained for both DVGLUT (green) and FasII (red) in wandering larvae of wild-type (*C155-Gal4/+*), Atg1 Gap-GFP (*C155-Gal4/+; UAS-Gap-GFP/+; UAS-Atg1^{6B}/+*), and Atg1 NTAP-Rae1 (*C155-Gal4/+; UAS-NTAP-Rae1/+; UAS-Atg1/+*). Scale bar represents 10 μ m. **(f)** Quantification of the number of boutons at muscle 4 synapses in wild-type, *Rae1* expression (*C155-Gal4/+; UAS-NTAP-Rae1/+*), Atg1 Gap-GFP and Atg1 NTAP-Rae1 wandering larvae (segment A2–4, $n = 28, 27, 33$ and 34 , respectively). Error bars denote s.e.m. * $P < 0.001$, ** $P < 0.05$.