Visualization of Endogenous Type I TGF-β Receptor Baboon in the Drosophila Brain

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The transforming growth factor β (TGF-β) signaling pathway is evolutionarily conserved and widely used in the animal kingdom to regulate diverse developmental processes. Prior studies have shown that Baboon (Babo), a Drosophila type I TGF-β receptor, plays essential roles in brain development and neural circuit formation. However, the expression pattern for Babo in the developing brain has not been previously reported. We generated a knock-in fly with a human influenza hemagglutinin (HA) tag at the C-terminus of Babo and assessed its localization. Babo::HA was primarily expressed in brain structures enriched with neurites, including the mushroom body lobe and neuropils of the optic lobe, where Babo has been shown to instruct neuronal morphogenesis. Since the babo 3' untranslated region contains a predicted microRNA-34 (miR-34) target sequence, we further tested whether Babo::HA expression was affected by modulating the level of miR-34. We found that Babo was upregulated by mir-34 deletion and downregulated by miR-34 overexpression, confirming that it is indeed a miR-34 target gene. Taken together, our results demonstrate that the baboHA fly permits accurate visualization of endogenous Babo expression during brain development and the construction of functional neural circuits.
Results

Commercially available Babo antibodies fail to faithfully detect Babo expression. We first collected and characterized commercially available Babo antibodies with the hope that one could be used to track the expression of endogenous Babo during brain development. Two commercial antibodies (Abcam #14681 and #14682) were tested to determine if either can be used in a western blot to detect Babo protein isoforms with predicted molecular weights of 66–69 kD (Fig. 1a,b). The #14682 antibody detected a protein band at 75 kD, while the #14681 antibody did not recognize any protein near the 66–69 kD range (Fig. 1a), suggesting that of these two antibodies, only the #14682 antibody has potential to be a useful Babo antibody. However, we quickly ruled out the #14682 antibody as a useful Babo antibody when we found it could not faithfully detect increased or decreased Babo expression in the late third instar larval brain following overexpression or knockdown of Babo with the elav-GAL4 pan-neural driver (Fig. 1b). We then evaluated three additional commercial antibodies (MyBioSource #540193, #540486 and #610062) by western blotting. Similar to the previous antibodies, these three also failed to detect either endogenous Babo expression (#610062 antibody; right panel of Fig. 1c) or increased Babo expression (#540193 and #540486 antibodies; left and middle panels of Fig. 1c) when Babo was overexpressed by two ubiquitously expressed drivers, da-GAL4 (weakly expressed) and tub-GAL4 (strongly expressed). Taken together, these results suggested that direct immunodetection of Babo may be a suboptimal strategy for tracking its endogenous expression in the brain.

Visualization of endogenous Babo expression using a baboHA knock-in fly. Since we could not unambiguously detect Babo with commercially available antibodies, we used a CRISPR-Cas9-based strategy to generate a knock-in fly with a DNA fragment encoding the human influenza hemagglutinin (HA) tag inserted into the babo locus (Fig. 2a)10,11. We reasoned that this strategy would permit us to visualize the pattern of endogenous Babo expression by labeling Babo::HA with HA immunostaining. Based on previous studies and Flybase annotation, the babo gene is known to encode three Babo isoforms, Babo-A, Babo-B and Babo-C 12,13, which all have the same C-terminus (Fig. 2a). Therefore, we generated a baboHA fly by knocking-in the HA tag at the C-terminus of babo, immediately preceding the translational stop codon (Fig. 2a). We then inspected whether the baboHA fly can be used to faithfully detect endogenous Babo expression. First, we detected Babo::HA expression in the baboHA fly but not in the wild-type wt (Fig. 2b). We further found that Babo::HA expression level was significantly reduced in the late third instar larval brain following overexpression or knockdown of Babo with the elav-GAL4 pan-neural driver (Fig. 1b). We then evaluated three additional commercial antibodies (MyBioSource #540193, #540486 and #610062) by western blotting. Similar to the previous antibodies, these three also failed to detect either endogenous Babo expression (#610062 antibody; right panel of Fig. 1c) or increased Babo expression (#540193 and #540486 antibodies; left and middle panels of Fig. 1c) when Babo was overexpressed by two ubiquitously expressed drivers, da-GAL4 (weakly expressed) and tub-GAL4 (strongly expressed). Taken together, these results suggested that direct immunodetection of Babo may be a suboptimal strategy for tracking its endogenous expression in the brain.
Babo:HA is primarily expressed in neuropils and nerve fibers of the brain. After validating the babo$^{HA}$ knock-in fly, we then determined the Babo expression pattern in the developing brain. In general, we found that Babo was expressed in neuropils and nerve fibers at different developmental stages, ranging from the early larval stage to adulthood (Fig. 3a–f, Supplemental Figs. 1, 2). Since Babo is known to play important roles in the axonal and dendritic morphogenesis of MB neurons in the central brain and Tm neurons in the medulla of the optic lobe3,5, we specifically examined Babo::HA expression in MB neurons and neurons within the developing optic lobe. Babo::HA was clearly expressed in the MB lobe from larvae to adults (Fig. 3g–n). We then carefully profiled Babo::HA localization throughout developmental stages. At the late third instar larval stage, we found that the Babo signal appeared in most of the MB lobe with a concentrated signal in the center of the peduncle (Fig. 3g,k), suggesting that Babo may be expressed not only generally in MB $\gamma$ and $\alpha'/\beta'$ neurons but also highly in the newly generated MB neurons. Notably, the Babo::HA expression in MB $\gamma$ neurons and newly generated MB neurons persisted until the mid-late pupal stage, whereas the Babo::HA expression in MB $\alpha'/\beta'$ neurons was greatly reduced at the mid pupal stage (Fig. 3g–i,k–m). At the adult stage, the Babo::HA expression was only observed in MB $\gamma$ neurons (Fig. 3j,n). In the optic lobe, we found that Babo::HA was initially expressed in inner and outer proliferation centers of the developing optic lobe from early- to mid-larval stages (Supplemental Fig. 1). Correspondingly, Babo::HA expression was later seen in the lamina and medulla neuropils and in the structure that develops into the lobule neuropil at the late third instar larval stage (Fig. 3d–f, Supplemental Fig. 2a–d). Since Babo acts together with type II TGF-$\beta$ receptors to transduce TGF-$\beta$ signaling4, we wondered whether co-localization of Babo and type II TGF-$\beta$ receptors can be visualized in the brain. Interestingly, we found that Wit, a type II TGF-$\beta$ receptor, was generally expressed in neuropils and nerve fibers just like Babo, and Babo:HA and Wit were strongly co-expressed in the developing optic lobe (Supplemental Fig. 3a–f). In addition to its neuronal expression, Babo::HA signal was found to tightly surround glial cell bodies, especially in the optic lobe region (Supplemental Fig. 3g–l), implying the possibility that Babo may also be expressed in glia cells. Taken
together, our data using Babo::HA immunostaining to reveal endogenous Babo expression patterns lead us to conclude that Babo is expressed in brain regions enriched with neurites, including the MB, optic lobes and many other neuropils and nerve fibers.

**Utilization of the baboHA fly to validate babo as a miR-34 target gene.** Our previous study showed that overexpression of miR-34 impairs axon pruning in MB neurons, and interestingly, this axon pruning defect can be rescued by overexpressing Babo. Based on the microRNA target gene prediction algorithm, TargetScan, the 3’ untranslated region (UTR) of the babo gene contains a putative miR-34 target site (Fig. 4a). However, the expression level of Babo mRNA was only mildly downregulated (91% remaining) in S2 cells when miR-34 was overexpressed. Therefore, it has been unclear whether babo is a true miR-34 target gene in the Drosophila brain. To address this question, we quantified Babo::HA expression after modulating miR-34 in the baboHA fly. We found that the Babo::HA expression was upregulated (207 ± 34% of control levels, p < 0.01) in the mir-34-deficient adult.
head and downregulated (30 ± 17% remaining, p < 0.05) in miR-34 overexpressing animals, according to western blot analysis (Fig. 4b,c). Thus, we conclude that miR-34 negatively regulates Babo expression. Notably, we further observed that Babo::HA expression was significantly reduced in the MB lobe when miR-34 was overexpressed using GAL-OK107 (Fig. 4d,e), and the knockdown efficiency was comparable to that produced by expression of babo-a RNAi under control of the same driver (Supplemental Fig. 4). These findings support the idea that the miR-34-induced MB axon pruning defect in our previous study was probably due to downregulation of the Babo expression. Taken together, our data suggest that babo is a miR-34 target gene, which participates in crucial biological processes such as the axon pruning of MB neurons. Furthermore, our results demonstrate the utility of the baboHA fly for monitoring Babo expression in the brain.

**Discussion**

Babo acts as an important regulator of diverse and complicated developmental processes, such as neuroblast proliferation in brain development and neuronal morphogenesis in neural circuit formation. However, the Babo expression pattern in brain has remained enigmatic due to difficulties in detecting the endogenous protein. In this study, we generated a baboHA knock-in fly to visualize the expression pattern of endogenous Babo protein. Babo::HA was expressed in brain structures enriched with neurites, including the MB and optic lobes (Fig. 2, 3). Interestingly, Babo::HA and Wit (a type II TGF-β receptor) displayed similar neuropil expression patterns in the late third instar larval brain, especially in the optic lobe region (Supplemental Fig. 3a–f). These results correspond nicely to previous functional studies that suggested Babo and Wit can form a regulatory complex, which is crucial for axon pruning of MB neurons and dendritic patterning of Tm neurons in the optic lobe. We also utilized the baboHA fly to solve an existing puzzle of whether babo is truly a miR-34 target gene. We found that mir-34 depletion upregulated Babo::HA, while miR-34 overexpression downregulated its expression (Fig. 4). Therefore, we are confident in our conclusion that babo is indeed a miR-34 target gene. These results suggest that the baboHA fly can serve as an excellent reagent in studies that seek to clarify the participation of Babo in the brain development and formation of functional neural circuits. Despite our elucidation of the endogenous Babo expression pattern in the brain, at least three issues remain to be addressed. First, we cannot distinguish the expression patterns of each Babo protein isoform since the HA tag was inserted immediately upstream of the common amino acid stop.
codon for Babo-A, -B and -C. Second, despite our finding that Babo tightly encases glia cell bodies (Supplemental Fig. 3g–l), we still cannot be entirely sure whether Babo is expressed in glia. Third, we also did not specifically assess whether Babo expression is present in tissues other than the brain. In the future, RNAi reagents specific to certain Babo variants may be used to knock down expression in neurons and/or glia, followed by assessment of Babo expression patterns. Additionally, expression in other tissues of the babo^{HA} fly can be tracked to provide a complete description of the distribution of endogenous Babo during development. Such studies may hint at unexplored territories and functions of Babo in the animal.

Methods

generation of the babo^{HA} knock-in fly. Two PCR DNA fragments carrying a HA-tag in frame with the C-terminus of Babo immediately upstream of the translational stop codon were generated with two pairs of primers (Fig. 2a): (L-arm primers) attcactagtgtacctcgaaagggcatttcggtcgc and taatgctgacagtctgattgag; (R-arm primers) tgtttgcgtacagttgctggtctgcc and tagatgcatgcggacaggttagttataagggagtctgacgaat. L-arm and R-arm PCR products were cloned into the Spel/Xhol sites of pCR2-TOPO vector with an In-Fusion HD cloning Kit (Clontech) to generate the pCR2-TOPO-Babo-1xHA-stop HR donor vector. The CRISPR target site of the babo gene was selected (acaaggtcaagaactgattg; Fig. 2a). The target site (guide RNA) fragment was generated by annealing primers with the sequences: cttcgacaaggtcaagaactgattg and aaaccaatcagttcttgaccttgtc. The annealed target site fragment was cloned into the BbsI site of pBFv-U6.3-Babo-stop gRNA plasmid. The annealed target site fragment was cloned into the BbsI site of pBFv-U6.3 to generate the pBFv-U6.3-Babo-stop gRNA plasmid. The pCR2-TOPO-Babo-1xHA-stop HR donor vector and the pBFv-U6.3-Babo-stop gRNA plasmid were injected into a Cas9 founder strain by WellGenetics Inc. to generate the babo^{HA} knock-in fly.

Fly strains. The following fly strains were used in this study: (1) UAS-mCD8::GFP^{15}; (2) da-GAL4 (Bloomington stock [BL] 55850); (3) tub-GAL4^{14}; (4) elav-GAL4^{15}; (5) GAL4-OK107^{16}; (6) UAS-babo-a RNAi (BL 44400^{17}); (7) UAS-mir-34^{17}; (8) UAS-Babo-A^{18}; (9) tub-GAL80^{19} (BL7017); (10) w^{118} (BL5905); (11) mir-34^{17–19}.

Fly brain preparation, image processing and western blot analysis. Dissection, immunostaining and mounting of fly brains were performed as described previously^{20}. Primary antibodies used in immunostaining were rabbit anti-GFP (1:800, Life Technologies), rat anti-HA (1:500, Roche), mouse anti-Fasciculin II (1:104), anti-Repo (8D12), and anti-Wit (23C7) (1:50, Developmental Studies Hybridoma Bank). Secondary antibodies conjugated to Alexa 488, 546 or 647 (1:800) were purchased from Life Technologies. Immunofluorescence images were captured using a Zeiss LSM 700 confocal microscope and were processed using the Zeiss LSM image browser to project images from confocal stacks and Photoshop (Adobe) to adjust image intensity. No other image processing was performed. The western blots were conducted using Bio-Rad mini-PROTEAN tetra and Sema-dry transfer systems. Primary antibodies used for western blotting were rabbit anti-A (1:500; Abdcam #14681 and #14682; MyBioSource #540193, #540486 and #610062), rabbit anti-HA (1:1,000, Cell Signaling), rat anti-HA (1:5,000, Roche), mouse anti-β-Actin (1:5,000, Millipore), mouse anti-α-Tubulin (1:5,000, GeneTec). Secondary antibodies conjugated to Horseradish peroxidase (1:100,00) were purchased from The Jackson Laboratory. The western blot signals were detected using GE Healthcare ECL reagent and Fuji X-ray films. The quantitation of protein bands was performed with ImageJ, and student's t-test was used for statistical analysis. The quantitation of immunostaining was also performed with ImageJ, and one-way ANOVA with post hoc Tukey test was used for statistical analysis.

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Y.W.L., C.H.C. and H.H.Y. conceived the experiments, Y.W.L., S.Y.C., P.L.C. and J.C.L. conducted the experiments, Y.W.L., S.Y.C., C.H.C. and H.H.Y. analyzed the results, C.H.C. and H.H.Y. composed the manuscript. All authors have reviewed the final version of the manuscript.

Competing interests
The authors declare no competing interests.

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