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# *Drosophila* miR-932 modulates hedgehog signaling by targeting its co-receptor Brother of ihog

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ABSTRACT

Hedgehog (Hh) proteins act as morphogens in a variety of developmental contexts to control cell fates and growth in a concentration-dependent manner. Therefore, secretion, distribution, and reception of Hh proteins must be tightly regulated and deregulation of these processes contributes to numerous human diseases. Brother of ihog (Boi) and its close relative Ihog (Interference hedgehog) are cell surface proteins that act as Hh co-receptors required for Hh signaling response and cell-surface maintenance of Hh protein. MicroRNAs (miRNAs) are a group of widely expressed 21–23 nucleotides non-coding RNAs that repress gene function through interactions with target mRNAs. Here, we have identified a novel miRNA, miR-932, as an important regulator for Boi. We show that overexpression of miR-932 in the wing disc can enhance Hh signaling strength, but reduce its signaling range, a phenotype similar to that of *boi* knockdown. In both in vivo sensor assay and in vitro luciferase assay, miR-932 can suppress Boi by directly binding to its 3′UTR. Meanwhile, down-regulation of miR-932 by sponge elevates the protein level of Boi, confirming that miR-932 is an in vivo regulator of Boi expression. Further, we demonstrate that miR-932 can block Hh signaling when co-expressed with *ihog-RNAi*. Moreover, we find that other predicted miRNAs of Boi fail to suppress it as strong as miR-932. Taken together, our data demonstrate that miR-932 can modulate Hh activity by specifically targeting Boi in *Drosophila*, illustrating the important roles of miRNAs in fine regulation of the Hh signaling pathway.



## Introduction

Hedgehog (Hh) is a highly conserved secreted signaling protein that regulates the growth and patterning of many organs in fly and vertebrates (Ingham et al., 2011; Jiang and Hui, 2008; Strigini and Cohen, 1997). In the *Drosophila* wing imaginal disc, Hh is expressed in posterior (P) compartment cells and transported to anterior (A) compartment cells to transduce signaling. Hh binds to Patched (Ptc) and releases the inhibition on Smoothened (Smo). And then activated Smo forms a cytoplasmic signaling complex with two other components, Cos2 and Fu, to transduce signaling via the transcription factor Ci, which subsequently turns on the expression of target genes (Denef et al., 2000; Jia et al., 2003; Lum and Beachy, 2004; Lum et al., 2003b). Secretion, distribution and reception of Hh signals must be tightly regulated, and abnormal Hh signaling is associated with many developmental defects and cancers (Jiang and Hui, 2008; Taipale and Beachy, 2001). Understanding of precise mechanisms of Hh gradient formation will help us to find new strategies for the diagnosis and therapeutic treatment of cancers.

Ihog (Interference hedgehog) and Boi (Brother of ihog) are discovered as components of the Hh signaling (Lum et al., 2003a). As co-receptors of Ptc, they are redundantly required for high-affinity Hh binding and signaling response (Camp et al., 2010; Yan et al., 2010; Yao et al., 2006; Zheng et al., 2010). Ihog and Boi are single transmembrane proteins with four extracellular IgG domains, two extracellular fibronectin (Fn) domains and an intracellular tail. The first Fn domain is required and sufficient for direct binding to HhN (McLellan et al., 2006; Yao et al., 2006), while the second Fn domain is essential for physical interaction between Ihog/Boi and Ptc on the cell surface (Zheng et al., 2010). Cdo and Boc are two closest vertebrate homologs of Ihog and Boi

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which were reported to positively regulate myogenic differentiation (Kang et al., 1998,2002). Further studies demonstrated that they are Sonic Hh binding proteins essential for SHh signaling (Allen et al., 2011; Izzi et al., 2011; Okada et al., 2006; Tenzen et al., 2006).

microRNAs (miRNAs) are small noncoding RNAs that act as post-transcriptional repressors by base-pairing to the 3′ untranslated region (UTR) of their cognate mRNAs (Bushati and Cohen, 2007; Kloosterman and Plasterk, 2006). Their widespread and important roles in animals are highlighted by recent estimates that up to 30% of all genes are targets of miRNAs (Lewis et al., 2005; Stark et al., 2005; Xie et al., 2005). However, the physiological functions of many individual miRNAs remain largely unknown. Combined with computational methods, genetic approaches using model organisms such as *Drosophila* have been used to examine the biological roles of miRNAs at both the organismal and molecular levels (Smibert and Lai, 2008). The first identified miRNA in *Drosophila* is *bantam*, which controls cell proliferation and regulates the pro-apoptotic gene *hid* (Brennecke et al., 2003; Hipfner et al., 2002). Since then, hundreds of miRNAs have been identified, and their biological functions in multiple important signaling pathways have been characterized. For example, expression of miR-315 activates the Wingless pathway by targeting the negative regulators Axin and Notum (Silver et al., 2007). microRNA-9a ensures the precise specification of sensory organ precursors by regulating the pro-neural transcription factor Senseless (Li et al., 2006). miR-8 directly suppresses Notch ligand Serrate to inhibit Notch-induced overgrowth and tumor metastasis (Vallejo et al., 2011). miR-279 directly represses STAT to regulate border cell migration during oogenesis in *Drosophila* (Yoon et al., 2011).

Here, we report the identification of a novel microRNA (miR-932), which acts as a modulator of Hh signaling. Overexpression of miR-932 promotes Hh signaling strength while reducing signaling range. Importantly, we provide the evidence that miR-932 controls Hh signaling by regulating *boi*. Overexpression of miR-932 represses Boi protein level by directly binding to its 3′UTR, while knockdown miR-932 by sponge increases Boi expression. Moreover, we showed that miR-932 exhibited stronger activities in repressing *boi* than several other candidate miRNAs predicted by computational methods (Enright et al., 2003). Taken together, our findings suggest that miR-932 is required for the precise control of Hh signaling in *Drosophila* development.

## Material and methods

### *Drosophila genetics*

All stocks were maintained and crossed at 25 °C according to the standard procedures. The *en-Gal*4, *ap-Gal*4, *dpp-Gal*4 *and ywflp; act > y+ > Gal*4, *UAS-GFP* lines were obtained from Bloomington stock center. The *UAS-boi-RNAi and UAS-ihog-RNAi* lines were described in our previous paper (Yan et al., 2010). The *UAS-GFP-GPI-miR-932-sponge*, *UAS-GFP-GPI-miR-981*, *UAS-GFP-GPI-miR-314*, *UAS-GFP-GPI-miR-79*, *UAS-GFP-GPI-miR-4*, *UAS-GFP-GPI- miR-929*, *UAS-GFP-GPI-miR-1014* and *UAS-GFP-GPI-miR-956* transgenic flies were generated using the PhiC31 integrase-mediated site-specific transgenesis system. The *UAS-boi*, *UAS-DsRed-miR-932*, *miR-932-sensor*, *tub-EGFP* and *tub-EGFP-boi-3′UTR* flies were generated by P-element transformation. All transgenic flies were mapped using standard methods.

### *Plasmid construction*

To generate the pUAST-DsRed-miR-932 construct, 800 bp of genomic DNA surrounding miR-932 was amplified by PCR and cloned downstream of DsRed into the pUAST vector. PCR primers are:

forward, 5′-CGGGGTACCCATTTGATTGCGTTCCG-3′;
reverse, 5′-GCTCTAGAGGACAGTTTGGTCTTCG-3′.

A similar strategy was used to make the pWALIUM10-moe-GFP-GPI-miRNA constructs, PCR fragments containing miRNAs were cloned downstream of GFP-GPI in the XbaI site of the pWALIUM10-moe vector (Ni et al., 2011). PCR primers are:

miR-981 forward, 5′-GCTCTAGAGCTGCTGAGCACATTTCCGTTA-3′;
miR-981 reverse, 5′-GCTCTAGAGGTTTGGATTTACAAGCATGATC-3′.
miR-314 forward, 5′-GCTCTAGAGGCGAAACCTCCTACAACCCA-3′;
miR-314 reverse, 5′-GCTCTAGATGGTGGGGCCAAGTGGTAAAC-3′.
miR-79 forward, 5′-GCTCTAGAAGTCCTGGCACGCGTTTGACC-3′;
miR-79 reverse, 5′-GCTCTAGAGGCGAGCATATCTCCAGGGCAG-3′.
miR-4 forward, 5′-GCTCTAGAGATGCATCTTGTGCAGTTATGT-3′;
miR-4 reverse, 5′-GCTCTAGAACAGCCACTGTGATATAGATATG-3′.
miR-929 forward, 5′-GCTCTAGATACCTCGTCACTTACACAGG-3′;
miR-929 reverse, 5′-GCTCTAGAGCAGTATATAGATGCCACTA-3′.
miR-1014 forward, 5′-GCTCTAGATGTCTCCAATTGCTACGAGG-3′;
miR-1014 reverse, 5′-GCTCTAGAGCTGGCCATTCCACTGATGA-3′.
miR-956 forward, 5′-GCTCTAGATGACCAGCTAGGACTAGCAGC-3′;
miR-956 reverse, 5′-GCTCTAGACGTTGTCATGCGTATATGATTA-3′.

The tub-EGFP-boi-3′UTR was generated by cloning a 766 bp fragment of *boi* 3′UTR downstream of pCaSpeR-tub-EGFP (Pek et al., 2009) (a gift from T. Kai), through NotI and XhoI sites. PCR primers are:

forward, 5′-TAGCGGCCGCAGCACAACGAGGAGAAATAT-3′
reverse, 5′-GACTCGAGAGTTTAGGATGCTTCTCTCT-3′

To generate the miR-932-sensor construct, the following primers are annealed (95 °C for 5 min, then slowly cooled down to room temperature) in the annealing buffer (10 nM Tris-HCl, pH=7.5, 100 mM NaCl, 1 mM EDTA), and then directly cloned into the pCaSpeR-tub-EGFP vector through NotI and XhoI sites. PCR primers are:

forward, 5′-GGCCGCCTGCAATGCACTACGGAATTGAAATCA-CACCTGCAATGCACTA CGGAATTGAC-3′
reverse, 5′-TCGAGTCAATTCCGTAGTGCATTGCAGGTGTGATTT-CAATTCCGTAGTGC ATTGCAGGC-3′

To generate the miR-932-sponge construct, the following primers are annealed in the annealing buffer, and then directly cloned into the pWALIUM10-moe-GFP-GPI through NdeI and XbaI sites. PCR primers are:

forward, 5′-TATGCTGCAATGCACATGGGAATTGAGGCTAGCCCTG CAATGCACATGG GAATTGAGGCTAGCCCTGCAATGCACATGGGAATT GAGGCTAGCCCTGCAATGCACATGGGAATTGAGGCTAGCCCTGCAATGC ACATGGGAATTGAGGCTAGCCCTGCAATGCACATGGGAATTGAT-3′
reverse, 5′-CTAGATCAATTCCCATGTGCATTGCAGGGCTAGCCTCA ATTCCCATGTG CATTGCAGGGCTAGCCTCAATTCCCATGTGCATTGCAG GGCTAGCCTCAATTCCCATGTGCATTGCAGGGCTAGCCTCAATTCCCATG TGCATTGCAGGGCTAGCCTCAATTCCCATGTGCATTGCAGCA-3′

### *Luciferase assay*

For validation of miRNAs that targets *boi* 3′UTR, a 766-bp fragment was amplified by PCR from wild-type genomic DNA and cloned downstream of Renilla luciferase in the psiCheck-2 vector (Promega). PCR primers used are:

forward, 5′-GGTTTAAACAGCACAACGAGGAGAAATAT-3′;
reverse, 5′-TTGCGGCCGCAGTTTAGGATGCTTCTCTCT-3′.

*boi* 3′UTR mutants were generated by changing the seed-binding sites of different miRNAs to GGCGCGCC. Transfections were performed in 24-well plates by using Effectene transfection reagent in S2 cells. In each well, 1 ug of total DNA was added. After 48 h, cells were lysed in passive lysis buffer, dual luciferase assays were carried out (Promega), and the results were analyzed on the luminometer.