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Mob as tumor suppressor is regulated by *bantam* microRNA through a feedback loop for tissue growth control

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ABSTRACT

The evolutionarily conserved Hippo signaling pathway plays an important role in regulating normal development as well as tumorigenesis in animals. How this growth-inhibitory signaling is maintained at an appropriate level through feedback mechanisms is less understood. In this report, we show that *bantam* microRNA functions to increase the level of the Mob as tumor suppressor protein Mats, a core component of the Hippo pathway, but does not regulate *mats* at the transcript level. Genetic analysis also supports that *bantam* plays a positive role in regulating *mats* function for tissue growth control. Our data support a model that *bantam* up-regulates Mats expression through an unidentified factor that may control Mats stability.

1. Introduction

Cell growth, proliferation and apoptosis are tightly regulated to allow proper tissue growth and organ formation in animal development. Cellular signaling pathways such as the Hippo pathway play an important role in these processes. While first discovered in *Drosophila*, Hippo (Hpo) signaling operates in both vertebrate and invertebrate animals to mediate growth inhibition (recently reviewed in [1–3]). A major downstream target of the Hpo pathway is Yki/Yap/Taz, which functions as a co-activator of a number of transcription factors to control tissue growth. Interestingly, Yki has been found to up-regulate transcription of a number of upstream genes such as *expanded* and *merlin* in *Drosophila*[1,2]. Such negative feedback provides an effective mechanism to maintain proper levels of Hippo signaling during animal development.

Bantam (*ban*) mircoRNA was first identified in a gain-of-function screen for genes affecting tissue growth in *Drosophila*[4–6]. As a downstream target of the Hpo signaling pathway, *ban* promotes tissue growth by stimulating cell proliferation and inhibiting cell apoptosis [7,8]. To better understand the growth-promoting effect of *ban*, it is important to identify its target genes. Bioinformatics tools are useful to predict the target sites of genes and an algorithm (MinoTar – miRNA ORF Targets) was developed to identify miRNA target sites in open reading frame (ORF) [9]. Accordingly, *ban* is predicted to regulate over 200 genes by targeting either at 3′ untranslated region (UTR) or ORF. However, it is not clear at this point how many of these sites are functionally relevant. An experimental approach is the best way to address this issue. Interestingly, two putative *ban* target sites are identified in the ORF of *mats*, which is a core component of Hpo signaling pathway. Because *ban* is a downstream gene in the Hpo pathway, the possible mutual regulation between *ban* and *mats* may form a feedback loop and provide a mechanism of growth regulation by *ban* and *mats*. In this study, we have tested the possibility that *ban* miRNA regulates *mats* expression by targeting its coding region. Our results show that *ban* indirectly regulates *mats* expression in developing tissues. This study also highlights the importance of experimental validation of bioinformatics predictions.

2. Materials and methods

2.1. DNA constructs and site-specific mutagenesis

In the *UAS-C* line, a 584-nucleotide *ban* genomic fragment has been cloned into the 3′ UTR of *gfp* gene [5]. We cloned this *gfp-ban* fusion construct in the *pAc5.1* vector for over-expression in cultured *Drosophila* S2 cells. The *pAc5.1-GFP* construct served as a control.

A full-length cDNA of *Drosophila mats* was tagged with an HA epitope and cloned into the *pAc5.1* vector [10,11]. Two *ban* miRNA seed regions in *mats* ORF were identified by TargetScan FlyORFs (http://www.targetscan.org/fly_52orfs/). We mutated both seed

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regions by synonymous point mutations. The sequences of two seed regions in *mats* and mutagenesis primers are shown below.

The 7-mer site in *mats* ORF: **GATCTCA** (Nucleotides 154–160 in *mats* ORF).

Mutagenesis primers used to generate $mats^{m1}$:

F: CTTCCAGATGGCGAGGACCTTAACGAGTGGGTGGCC
R: GGCCACCCACTCGTTAAGGTCCTCGCCATCTGGAAG

The 8-mer site in *mats* ORF: **TGATCTCA** (Nucleotides 78–85 in *mats* ORF).

Mutagenesis primers used to generate $mats^{m2}$:

F: GGCACACACCAATATGACCTTATGAAGCATGCGGCAG
R: CTGCCGCATGCTTCATAAGGTCATATTGGTGTGTGCC

2.2. Cell culture and transfection

Drosophila S2R + cells were cultured in the Schneider's Medium (Sigma–Aldrich, Inc.) with 10% fetal bovine serum in a 25 °C incubator. Cells were transfected in 12-well plates using Cellfectin II reagent (Invitrogen) according to manufacturer's instruction. Mats wild-type or mutant constructs were co-transfected with either *pAc5.1-GFP* (control) or *pAc5.1-GFP-ban*. Cells were cultured for 3 days before lysis and analysis.

2.3. Western blot analysis and quantitative RT-PCR

Western blot analysis was done by running 10% SDS–PAGE, followed by standard blotting and immune detection procedures. Antibodies used in this study include: anti-Mats mouse (1:500) [10], anti-HA mouse (1:2000) (Sigma–Aldrich, Inc.) and anti-α-Tubulin mouse (1:2000) (Sigma–Aldrich, Inc.) antibodies.

Total mRNA from *Drosophila* wing disc was isolated via Qiagen RNAeasy Kit and mRNA was reverse-transcribed using Quanta qScript cDNA superMix. Real-time PCR was performed using PerfeCTA SYBR Green FastMix and data was collected via Applied Biosystem Step-One Plus Real-Time PCR system. The relative amount of specific mRNAs under each condition was calculated after normalization to the histone H3 transcripts.

2.4. Drosophila stocks and adult wing analysis

All fruit flies were cultured under standard conditions. The following fly stocks were used in the experiments: $FRT80B\ ban^{\Delta 1}$/*TM6B*[4] and *UAS-C*[5] (gift from Dr. George Halder), *UAS-Myr-mats*[12], *MS1096-Gal4* and *C5-Gal4* (Bloomington *Drosophila* Stock Center). Analysis of adult fly wing was done as described in [13].

3. Results

To test a hypothesis that *ban* regulates *mats* expression, a transgenic line that carries a 584-base pair *ban* genomic DNA under the control of the *UAS* promoter, *UAS-C*[5], was crossed to *MS1096-Gal4* driver to allow wing-specific gene expression. At the late third-instar larval stage, wing imaginal discs were isolated for protein extract preparation and Western blot analysis. In a control experiment using wild-type tissues, the endogenous Mats protein was expressed at a low level (Fig. 1A). However, the level of Mats protein increased about threefold in transgenic tissues that overexpressed *ban* miRNA (Fig. 1A and, B). Thus, *ban* is sufficient to increase the level of Mats expression.

To have an impact on the expression level of Mats protein, *ban* may function to regulate *mats* transcription and/or mRNA stability. To find out whether *ban* regulates *mats* expression at the transcript level, Real-time Polymerase Chain Reaction (PCR) was carried out to determine the steady level of endogenous *mats* mRNA expressed

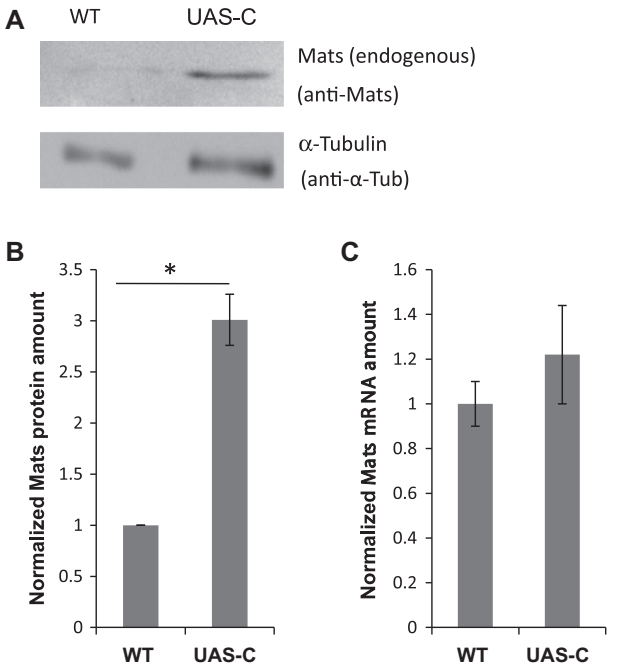

Fig. 1. Overexpression of *ban* miRNA increases the level of Mats protein in developing wing discs. (A) Western blot analysis for the endogenous Mats protein from *Drosophila* third-instar larval wing discs. Mats protein was increased in *ban* over-expressing wing discs compared with wild-type. Expression of *ban* in *UAS-C* was driven by *MS1096-Gal4*, a wing-specific Gal4 driver. α-Tubulin was used as a loading control. (B) WB data quantification of normalized Mats protein amount in wild-type and *UAS-C* samples driven by *MS1096-Gal4*. (C) Real-time PCR results indicate that *ban* does not regulate *mats* at the mRNA level. mRNA was extracted from third-instar larval wing discs. Both wild-type and *UAS-C* flies were crossed with the *MS1096-Gal4* driver line.

in larval tissues. Compared to the wild-type control, there was no significant change of *mats* mRNA level in larval wing discs that over-express *ban* (Fig. 1C). Thus, *ban* does not appear to regulate *mats* at the transcript level in developing tissues.

As a miRNA, *ban* is predicted to negatively regulate *mats* expression [9]. Therefore, the result shown in Fig. 1 is surprising. To further examine the relationship between *ban* and *mats*, we used cultured *Drosophila* S2 cells to test how *mats* expression might be influenced by *ban*. In S2 cells, *ban* was shown to be also sufficient to increase the Mats protein level (Fig. 2B and, C).

To test whether two predicted *ban* target sites in the *mats* coding region are indeed responsible for mediating the effect of *ban*, these two sites were altered to prevent *ban* recognition (Fig. 2A). The coding capacity of the *mats* mutants was still the same as wild-type *mats* gene. When one or both of these *ban* targets were mutated, *ban* miRNA was still able to increase the level of Mats protein (Fig. 2B, C). Therefore, *ban* appears to increase *mats* expression through a mechanism that is not based on its direct interaction with these two target sites in the *mats* coding region.

To further investigate the physiological relevance of *ban* regulation of *mats* in growth control, we performed a sensitized assay to test their genetic relationship. As a recessive mutation, heterozygous *ban* mutant does not show phenotypic difference in the wing compared with wild-type (compare Fig. 3B with 3A) [7]. Over-expression of a membrane targeted *mats* (*Myr-mats*) using wing-specific driver *C5-Gal4* resulted in a dramatic small wing (Fig. 3C) [12], while over-expression of *Myr-mats* in a *ban* heterozygous background could alleviate the wing phenotype (Fig. 3D). This genetic data indicate that *mats* function is sensitive to *ban* dosage *in vivo*, which is consistent with the fact that *ban* can increase Mats protein level. Thus, it further supports that *ban* plays a positive role in *mats* regulation for tissue growth control.

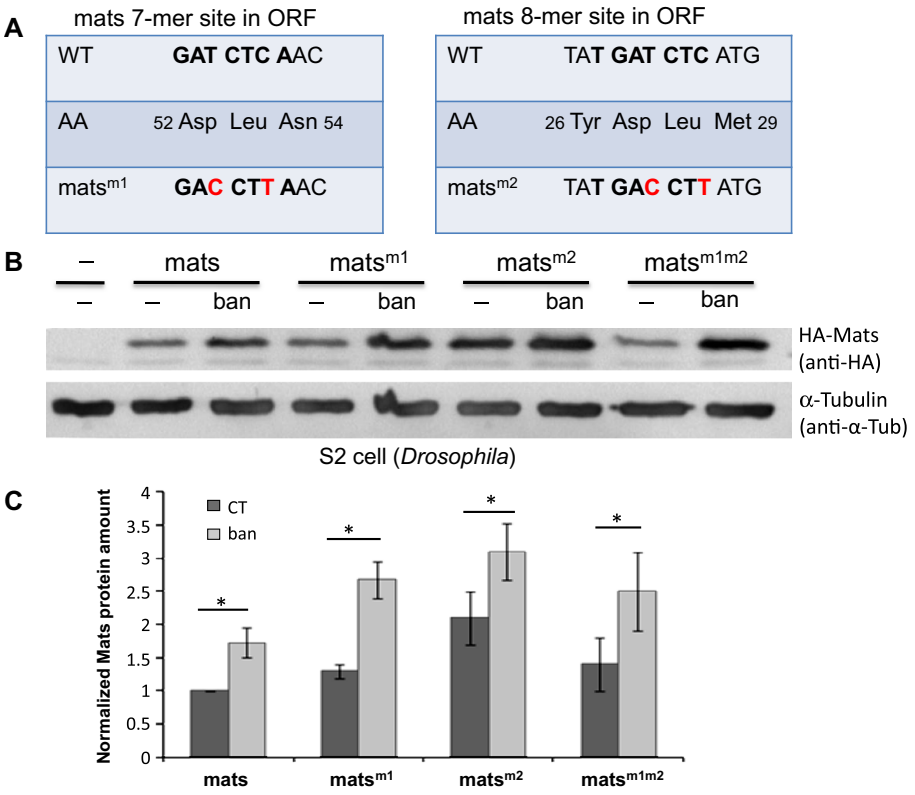

Fig. 2. The increase of Mats protein level is not related to the two predicted *ban* target sites in the *mats* coding region. (A) Two predicted *ban* target sites in *mats* ORF and mutagenesis for their disruptions. *mats*^{m1} is a mutant version of the 7-mer site and *mats*^{m2} is a mutant of the 8-mer site. Both m1 and m2 do not change the Mats coding capacity. (B) Levels of Mats, Mats^{m1}, Mats^{m2} and Mats^{m1m2} proteins are all increased by *ban* over-expression. (C) For Western blot data shown in (B), Mats protein levels have been normalized to protein input and relative Mats protein amounts are presented.

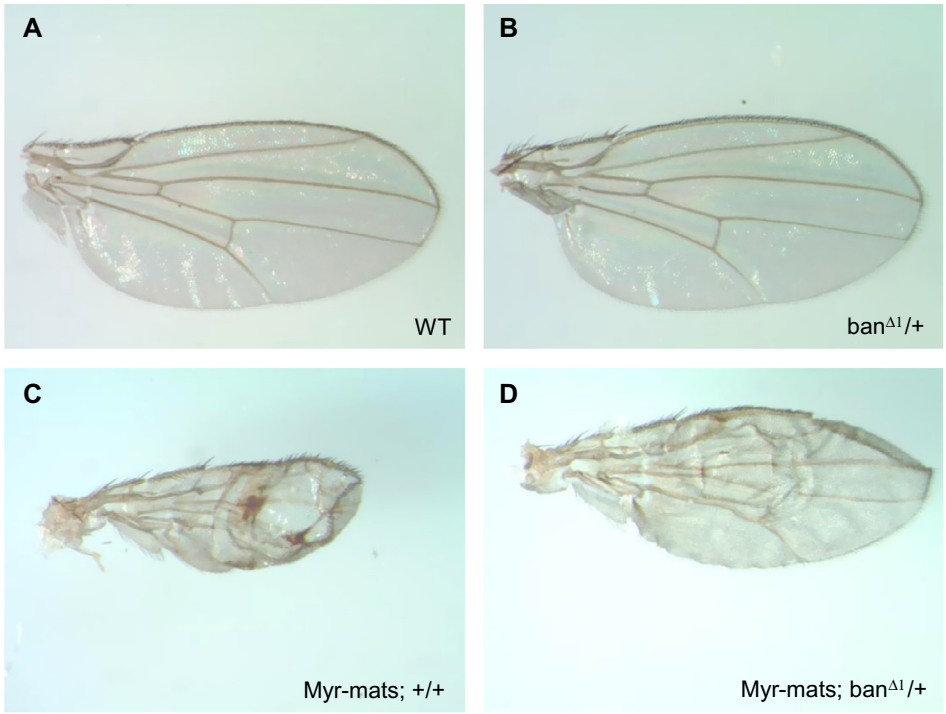

Fig. 3. Reduction of *ban* dosage suppresses *Myr-mats* induced small wing phenotype. (A) A wild-type adult wing. (B) A *ban*$^{\Delta 1}$ heterozygous wing appears normal in shape and size. *ban*$^{\Delta 1}$ is a null allele (Herranz et al. [14]). (C) Over-expression of Myr-mats reduced wing size. (D) In the *ban*$^{\Delta 1}$/+ heterozygous background, Myr-mats-induced small wing phenotype was less severe. All fly stocks were crossed with *C5-Gal4*, a wing-specific Gal4 driver.

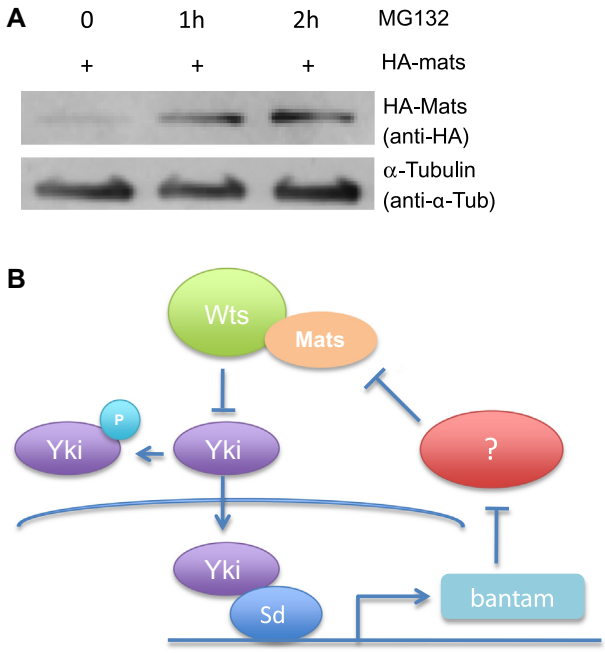

Fig. 4. Regulation of Mats protein via proteasome and a working model for a feedback loop involving *ban* miRNA and *mats* in the Hpo pathway. (A) The Mats protein is degraded through proteasome. MG132 treatment inhibits Mats protein degradation. S2 cells that express HA-tagged Mats were treated by MG132 for 1 or 2 h before sample preparation. (B) *ban* miRNA is proposed to regulate Mats protein level through an unknown factor. The regulation between *ban* and *mats* revealed the existence of a negative feedback loop in the Hpo signaling pathway.

To explore the possibility that stability of Mats protein could be regulated by the proteasome protein degradation machinery, we have treated S2 cells with MG132 to inhibit the function of proteasome. We found that Mats protein level was increased compared with the ones without MG132 treatment (Fig. 4A), indicating that protein stability is an effective mechanism for regulating the Mats protein level.

4. Discussion

Cellular signaling pathways are important in determining how cells behave in a multi-cellular organism. Regulatory mechanisms have been evolved to adjust the level of signaling activity of individual pathway. In recent years, miRNA has emerged as a unique class of regulators of gene expression and pathway activity. In *Drosophila*, miRNA *ban* functions to promote tissue growth and itself is up-regulated by the growth-promoting gene Yki [4–8]. As a miRNA, it decreases expression of genes such as Capiua, an HMG-box transcription factor in the epidermal growth factor receptor (EGFR) pathway [14]. Through a bioinformatic approach, *mats* was predicted to be a target gene of *ban* because two putative *ban* target sequences exist in the *mats* coding region. *mats* is the only core component of the Hpo pathway to be such a potential target of *ban* miRNA. Surprisingly, we found that *ban* increases *mats* expression and this is opposite to the prediction that *ban* is a direct negative regulator of *mats*.

Although generally miRNAs regulate gene expression in a negative manner, is it possible that *ban* miRNA somehow activates *mats* expression by binding to the target sites in *mats*? This appears unlikely because mutations of the two putative *ban* target sites in *mats* did not cause an obvious reduction of *mats* expression (Fig. 2). Interestingly, $mats^{m2}$ exhibited a moderate increase of its protein level in the absence of *ban* over-expression, suggesting that this *ban* target site might be indeed involved in negatively regulating *mats* expression. Even if this is true, the positive role of *ban* in regulating *mats* is still clearly stronger than its negative role because the final outcome is an increase of *mats* expression when *ban* is over-expressed (Fig. 1). In comparison, $Mats^{m1}$ did not show any significant change of its protein level. Also, it is not clear why there is no significant change of the level of $Mats^{m1m2}$ double mutant protein when *ban* was not over-expressed (Fig. 2). An analysis of the entire *mats* gene did not identify any additional *ban* target site in *mats*. As a take-home message, this study certainly highlights the importance of experimental validation of bioinformatics predictions.

While Mats protein stability is regulated through proteasome, *ban* may indirectly regulate *mats* expression through an unknown factor, which decreases the level of Mats protein via proteasome (Fig. 4). Molecules like E3 ubiquitin ligases could serve such a role through ubiquinylation and proteasome-mediated protein degradation. Because *Mei-P26* encodes an E3 ubiquitin ligase and is a target of *ban* miRNA [15], we designed two independent RNAi constructs to knockdown *Mei-P26* function in S2 cells. Our data indicate that the Mats protein level could not significantly changed by the reduction of *Mei-P26* function (data not shown). Therefore, Mei-P26 does not appear to be the factor that mediates the effect of *ban* on *mats*. Here we propose that *ban* may negatively control a factor that has the capacity to decrease Mats stability (Fig. 4B). Further investigation is needed to identify the factor responsible for down-regulation of Mats expression.

In summary, our data show that *ban* miRNA functions to increase the level of *mats* expression through a feedback loop. Similarly, the downstream target of the Hpo pathway, Yki, is also known to mediate transcriptional regulation of several upstream genes such as *ex* and *mer* in the Hpo pathway. Though such negative feedback mechanisms, Hpo signaling can be effectively maintained at appropriate levels to control tissue growth and organ size during animal development.

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