RESEARCH NOTE

Study of bantam miRNA expression in brain tumour resulted due to loss of polarity modules in *Drosophila melanogaster*

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Introduction:

Tumorigenesisinvolves a series of events at cellular level leading acquisition of morphological and physiological changes of the tissue/organ.In humans, evidences have suggested that irregularities inexpression of endogenous, 22 nucleotides long non-coding microRNA (miRNA/s)directly or indirectlyinvolved indifferentdisorders including cancer. ManymiRNAs not only behaveas oncogenes and tumour-suppressors but alsoregulate key signaling pathways critical for normal cell cycle progression (Jansson andLund 2012). For centuries, *Drosophila*whose genome sharesstriking similarities with the mammalian genome played a pioneer role in understanding the complex molecular pathways of mammalian system (Pandey and Nichols 2011). *Drosophila* has several tumour-suppressor genes which show structural and functional homology with their mammalian counterpart. Among many, the polarity modulators, viz.,*brain tumor* (*brat*), *scribble* (*scrib*)and *lethal (2) giant larvae*(*lgl*)are three well studied tumour-suppressorsin *Drosophila*(Albertson and Doe 2003, Humbert*et al*. 2008). Mutation ineither of these genes leads to neoplasia or overproliferation of neuroblasts (neural stem cells) along withtumours in different tissues and other associated anomalies (Wirtz-Peitz andKnoblich 2006;Bowman*et al*. 2008). Loss of function of Brat homologue, Mei-P26, a critical regulator of germline stem cell division leads to a higher expression of bantam in ovary (Neumuller*et al*. 2008). bantam, isa well characterized miRNA in *Drosophila*whichis a positive regulator of tissue growth (Hipfner*et al*. 2002). Here, we report an elevated expression of bantam miRNA in the mutant background ofthree tumour-suppressor, *brat*, *scrib*and *lgl*.

Materials and Methods:

Drosophila stocks:

Unless otherwise stated, wildtype (*Oregon* R^{+}) and mutant flies were reared on standard yeast-cornmeal-agar medium and all experiments were performed at 24±1ºC. The following fly strains were used in this study: $brat^{11}$and $brat^{14}$ (D J Frank, Washington, USA), $bantam^{12}$(null allele) and $bantam^{20}$(hypomorphic allele) (P Jin, Atlanta, USA), *bantam sensor* (G Halder, University of Texas, USA). All the other stocks mentioned in the manuscript were obtained from Bloomington Stock Centre.

Immunofluoresence: Late third instar larval brain were dissected in PBS, fixed in 4% paraformaldehyde for 20min at RT, and preceded as previously described (Singh and Roy, 2013). DNA was visualized using DAPI (1µg/ml) (Sigma-Aldrich).

Microscopy and image analysis:

All fluorescent images were captured using LSM 510 Meta confocal microscope with picture size of 1024×1024 pixel and processed in Photoshop 7 (Adobe, San Jose, CA). For measuring the intensity of fluorescence, projection of image was obtained from selected z sections and measured using LSM 510 Meta software.

miRNATaqMan Assay:

For quantification of bantam miRNA expression in the desired genetic background, we made use of ABI (Applied Biosystem) miRNA TaqMan assay. Late third instar larval brain was dissected in 1X PBS and kept in Tri Reagent (Sigma-Aldrich). RNA from 50 wandering third instar larval brains was isolated using Tri Reagent as per manufacturers instruction followed by DNase I (Thermo Scientific) treatment to rule out DNA contamination. Reagents of TaqMan microRNA assay were used to prepare cDNA from 10ng RNA. In a MicroAmp optical 96- well plate, Real-time PCR reactions were performed using 7500 Fast Real-Time PCR System (ABI) in triplicate with specific forward and reverse primers (ABI) following manufacturer's protocol. For normalization, 2S RNA was used which showed no change in different genetic backgrounds.

Results

Since bantam expresses in brain (Brennecke*et al*. 2003) andits level is reported to be elevatedin ovarian tumour caused in *mei-P26* mutants(Neumuller*et al*. 2008),we were inquisitive to investigateexpression of bantam in brain tumour in *brat* mutant background. To assess the levels of mature bantam miRNA,we performed miRNA TaqMan assay with 2S

RNA as an internal control using primers for bantam and 2S RNA (Applied Biosystems), in tumours brain of*brat*mutants. Results of TaqMan assay showed elevated level of bantam in the *brat*$^{11/14}$trans-heterozygotes compared to the wild type (Figure 1A) indicates antagonistic relationship between *brat* and *bantam*.

During asymmetric division of neuroblasts, proper segregation of apical and basal fate determining factors is a crucial event which establishes polarity and maintains cell fate of the progenitor cells after division (Homem andKnoblich 2012).Lgl, Scrib and Brat areimportant candidatesinvolved in asymmetric division of neuroblasts(Albertson and Doe 2003). Loss of function in anyof thesegenes lead to an inappropriate segregation of cell fate determinants, which cause formation of supernumerary self-renewing daughter cells instead of differentiated cells resulting in an uncontrolled proliferation, consequently giving rise to a tumour (Wirtz-Peitz andKnoblich 2006;Bowman*et al.* 2008). So, we repeated our experiment in *lgl*and *scrib*mutant background, to study whether change in bantam expression was *brat*mutant specific or due to loss of polarity in dividing neuroblast. In*lgl*^{4}and *scrib*^{M101968}loss of functionmutants, we observed significant elevation in bantam level as compared to control individuals (Figure 1A). To consolidate our findings, we induced expression of*bantam sensor* in *brat*$^{11/14}$trans-heterozygote background and examined the expression of bantam (Figure 1B, B'). *bantamsensor* flies have a transgene with aGFP protein coding region under the tubulin promoter and its3'UTR consisting of two perfect bantam miRNA binding sites. The cells expressing bantam miRNA are GFP negative, as bantam interacts with RNA Induced Silencing Complex (RISC) to inhibit GFP translation by binding to the 3'UTR region of this sensor transcript.The cell that does not have bantam miRNA expression has GFP due to the absence of a translational blockade (Brennecke*et al.* 2003).Hence, the expression of this GFP coding transgene is opposite to bantam activity in a particular cell type or tissue.Quantitative estimation of GFPfluorescence intensity in third instar larval brain, revealed that the mean intensity of GFP was significantly higher in wild type as compared to the *brat*mutants (Figure 1C) indicating greater number of cells expressing bantamin mutant brain. Since, it was reported that*bantam* mutant individuals have smaller tissue size including brain compared to normal(Brennecke*et al.* 2003)and from our findings that it has elevated expression in tumorous background, we brought*bantam*nulland hypomorphic allele in *brat* trans-heterozygote background, to confirm whether bringing down bantam level could rescue tumorous phenotype. Surprisingly, it could not rescue the tumorous phenotype due to *brat* mutation instead it led toan early larval lethality compared to *brat* trans-heterozygote. Above

results indicate, higher expression of bantam in tumourous background couldpossibly bea consequence of brain tumour resulting from *brat*, *scrib* and*lgl* mutation.

Discussion:

In *Drosophila*, stem cells are found in testis, ovary, midgut, Malpighian tubules, haemolymph and the neuroblast lineage of brain(Kohlmaier and Edgar 2008). In brain the neuroblasts,like other stem cells,undergo asymmetric divisionto produce a larger cell (neuroblast) which retains the regeneration capability and a smaller GMC (Ganglion Mother Cell). GMCs undergo one more round of division to generate target neurons or glia. Brat along with other associate proteins, Miranda and Prospero form the cell fate determination complex to mediate the asymmetric division of the neuroblast. Apical proteins, Par-3 and 6, atypical protein kinase C (aPKC) helps in a basal localization of cell fate determination complex which after asymmetric division destined to form the GMC.Phosphorylation of Lglby aPKC on apical side confines the Lgl activity to the baso-lateral domain of cell (Humbert*et al.* 2008,Homem andKnoblich 2012). Further, Disc Large (Dlg) serves as an additional regulator of proper localization of Scrib and Lgl in neuroblasts.Lgl and Scribble in turn functions in basal localization of cell fate determinants in metaphase neuroblast but not in telophase neuroblast (Albertson and Doe 2003).Brat along with Prospero after segregating in GMCs promotes cell cycle exit and ultimately leads to the formation of ganglion cells (Wirtz-Peitz andKnoblich2006;Bowman*et al.* 2008;Homem andKnoblich 2012).Mutation of *lgl* and *scrib*, perturbed the machinery regulating the neuroblast self-renewal,generating excesses number of neuroblasts and their progeny. The neuronal progenitor cells in the *brat* loss of function mutantsfail to escape the cell cycle leading to exponential proliferation of cells causing brain tumor (Bowman*et al.* 2008).

bantam miRNA,was firstreportedin *Drosophila*duringa gain-of-function screen, it governs tissue growth by regulating cell proliferation and apoptosis (Hipfner*et al.* 2002). In *Drosophila*, bantam plays a wide range of functions which includesmaintenance of germline and intestinal stem cell (Yang *et al.* 2009; Huang *et al.* 2014),polyQ and tau- mediated neurodegenaration and various others (Bilen*et al.* 2006).Furthermore, bantam was reported to involve in Hippo and EGFR mediated tumorigenesis (Nolo*et al.*2006;Herranz*et al.* 2008). All these facts made us curious to explore the expression of bantam in *Drosophila* brain tumour. We carried out miRNA TaqMan assay that revealed an increased expression of mature bantam miRNA in $brat^{11/14}$, $scrib^{M101968}$and lgl^{4} brain tumour backgrounds. Decrease in bantam sensor GFP expressionin the tumorous brain of $brat^{11/14}$transhetrozygousfurther

corroborates our findings from TaqMan assay. Results from our preliminary study indicates, bantam miRNA could probably be playing some role inbrain tumours caused due to mutation of molecular signatures involved in distribution of cell fate determinants during asymmetric division of neuroblasts. However, inability to rescue *brat*$^{11/14}$tumorous phenotype by bringing down *bantam*level using *bantam* null and hypomorphicallele could be because either increased number of neuroblast like cells in *brat* mutantplausibly leads to ahigher expression ofbantam in tumorous background orsincebantam expresses in different tissues in spatio-temporal manner during *Drosophila* development (Brenneck*et al*. 2003), its null and hypomorphic allele will affect its function in different tissues including brain leading to early larval lethality. Recently, in a parallel study, Cohen and Weng(2015) have shown higher expression of brat, prospero and numb transcripts in *bantam* mutant neuroblasts.

In *Drosophila*, bantam and its target SOCS36E (human ortholog of SOCS5)are reported to be cooperating factors in EGFR mediated tumorigenesis (Herranz*et al.* 2012). Further, bantam is knownto be an important target of the highly conserved Hippo tumour-suppressor pathway(Nolo*et al.* 2006).Accumulating evidences and our study suggest bantam to be an important candidate in *Drosophila* tumorigenesis. Intriguingly, Scrib, Lgl and Brat are involved in basal segregation and formation of cell fate determining complex and mutation of these polarity determining candidates leads to transformation of certain neuroblast population. Thus, future experiments exploring function of bantam during asymmetric division of these neuroblasts and its relationship with molecular machineries involved in brain tumour will help us to gain new insights about role of bantam during *Drosophila* neurogenesis and brain tumour progression. Mutation of *scrib* and*lgl* results in epithelial tumours (Humbert*et al.* 2008). It will be of interest to study expression and function of bantam in these epithelial tumours. Eventhough, bantam is not conserved in mammals, but its target and regulators are highly conserved along the evolutionary tree and thus, further studiespossibly could lead to find novel targets involved invertebrate brain tumours.

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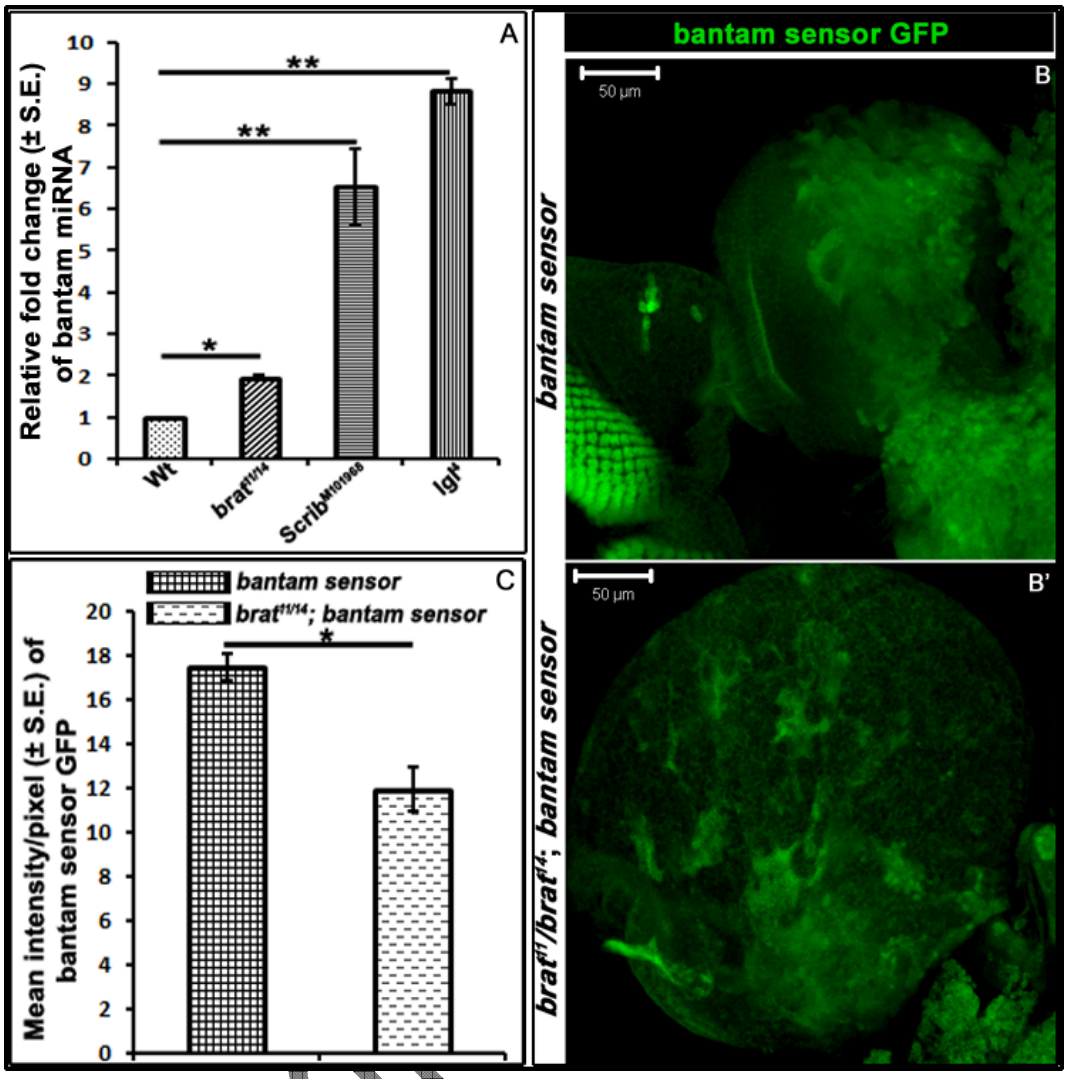
Figure 1.bantam is upregulated in tumorous background. A. miRNA TaqMan assay was performed with total RNA from third instar larval brain of different genotypes, namely, wild type (117 ±1h), $brat^{11/14}$(192 ±3h) and$scrib^{M101968}$(192 ±3h), lgl^{4}(192 ±3h) to compare the level of mature bantam miRNA. The Y-axis shows relative fold change of bantam expression in different genotypes mentioned along the X-axis. (B-B') Representative optical projection showing enrichment of bantam sensor GFP in mutant background (B') compared to bantam sensor used as control (B). (C) Graphical representation showing mean fluorescence intensity of bantam sensor GFP and third instar larval brain of mentioned genotypes. Statistical significance was calculated using One Way ANOVA (A) and Student's *t*-test (C). All the quantification data show mean ± S.E. ** $p< 0.001$ and * $p< 0.05$.


A
Relative fold change (± S.E.) of bantam miRNA
10
9
8
7
6
5
4
3
2
1
0
**
**
*
Wt
brat11/14
ScribH101965
lgl4
C
bantam sensor
brat11/14; bantam sensor
Mean intensity/pixel (± S.E.) of bantam sensor GFP
20
18
16
14
12
10
8
6
4
2
0
*
bantam sensor GFP
50 µm
B
bantam sensor
50 µm
B'
brat11/brat14; bantam sensor