SCIENCE CHINA
Life Sciences

Tsinghua Special Issue
• RESEARCH PAPER •

January 2014 Vol.57 No.1: 46–51
doi: 10.1007/s11427-013-4595-9

The β-alanyl-monoamine synthase ebony is regulated by schizophrenia susceptibility gene *dysbindin* in *Drosophila*

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Received October 15, 2013; Accepted November 20, 2013

The *Drosophila* homolog of schizophrenia susceptibility gene *dysbindin* (*Ddysb*) affects a range of behaviors through regulation of multiple neurotransmitter signals, including dopamine activity. To gain insights into mechanisms underlying Ddysb-dependent regulation of dopamine signal, we investigated interaction between Ddysb and Ebony, the *Drosophila* β-alanyl-monoamine synthase involved in dopamine recycling. We found that Ddysb was capable of regulating expression of Ebony in a bi-directional manner and its subcellular distribution. Such regulation is confined to glial cells. The expression level of *ebony* and its accumulation in glial soma depend positively on Ddysb activity, whereas its distribution in glial processes is bound to be reduced in response to any alterations of Ddysb from the normal control level, either an increase or decrease. An optimal binding ratio between Dysb and Ebony might contribute to such non-linear effects. Thus, Ddysb-dependent regulation of Ebony could be one of the mechanisms that mediate dopamine signal.

Dysbindin, ebony, glial cell, subcellular distribution, dopamine

Citation: Lu BY, Shao LS, Feng SX, Wang T, Zhong Y. The β-alanyl-monoamine synthase ebony is regulated by schizophrenia susceptibility gene *dysbindin* in *Drosophila*. Sci China Life Sci, 2014, 57: 46−51, doi: 10.1007/s11427-013-4595-9

Schizophrenia is a complex neuropsychiatric disorder demonstrating considerable heritability. In linkage and association studies, the gene *dystrobrevin binding protein* (*DTNBP1*, also known as "*dysbindin*") is identified as a major schizophrenia susceptibility gene [1,2], of which a reduced expression level is reported in the prefrontal cortex and hippocampus of schizophrenia patients [3,4]. Dysbindin is known to interact with α- and β-dystrobrevin (DTNA and DTNB), which are members of the dystrophin-associated protein complex (DPC) [5]. DPC proteins link the cytoskeleton to the extracellular matrix and serve as a scaffold for signaling proteins [5,6]. Dysbindin is also an essential subunit of the octameric biogenesis of lysosome-related organelles complex 1 (BLOC-1 complex) [7–9], which interacts with different adaptor proteins, mediating a common endosome-trafficking route for different cargoes. Thus, Dysbindin is critical in regulation of expression and localization of an array of proteins.

Accumulated evidence suggests that Dysbindin is involved in regulation of the dopamine synthesis and transportation [7,10]. The naturally occurred *dysbindin* mutation, DTNBP1, in mice (*sandy*) shows an increased dopamine turnover in specific brain regions [11]. In a previous study, we revealed that the *Drosophila* homolog of *dysbindin* (*Ddysb*) regulates the brain dopamine level and related behaviors, locomotor activity and mating orientation [12]. Thus, abnormal dopamine signal could be a general feature underlying behavioral impact caused by altered *dysbindin* functions.

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Effects of Ddysb are associated with altered β-alanyl-monoamine synthase, Ebony, which is a glia-specific non-ribosomal peptide synthetase (NRPS) involved in neurotransmitter recycling via conjugating β-alanine to biogenic amines, including dopamine, histamine and serotonin [13]. The physiological functions of ebony in *Drosophila* are multi-dimensional, ranging from cuticle pigmentation [14], mating [15,16], circadian rhythm [17,18], to stimulated activity [12]. Although the mammalian sequence homolog of *ebony* has yet to be identified, an isoenzyme of Ebony, carnosine synthetase, is described in chicks and mammals. Resembling to Ebony, carnosine synthetase is expressed exclusively in glial cells in chicks, and is localized primarily to glia in the mammalian brain [19]. Interestingly, carnosine plays an essential role in neuron-glia interaction and is also involved in locomotor activity in chicks [20]. In all these cases, how Ebony and its iso-enzyme are regulated remains to be elucidated.

In the current study, we examined interaction between Ebony and Ddysb through genetic manipulation in *Drosophila*. We found that Ddysb regulates the protein level of Ebony specifically in glial cells in a bi-directional manner. Evidence also indicated that Ddysb contributed to the subcellular distribution of Ebony via direct physical binding. This study provides new clues as to how ebony is regulated in glial cells and suggests a potential role of dysb in protein transportation in glial cells.

1 Materials and methods

1.1 Fly stocks

The *Drosophila dysbindin* mutant $dysb^1$ (#17918) was obtained from the Bloomington Stock Center. The *Drosophila dysbindin* mutant $dysb^2$ (#141729) was requested from the Drosophila Genetic Resource Center (DGRC; Kyoto, Japan). The RNAi lines (*DdysbIR-1*, *DdysbIR-2*) were from the fly stocks of National Institute of Genetics (NIGFly; Shizuoka, Japan). *UAS-Ddysb*, *embryonic lethal abnormal vision* (*elav*)-*Gal4* (pan-neuron Gal4), *Reversed polarity* (*Repo*)-*Gal4/TM6*, and *Tb* (glial Gal4) were extant stocks in the laboratory. All lines were back-crossed with the *w1118* (*isoCJ1*) control flies to equilibrate the genetic backgrounds.

1.2 Western blot analysis

The Western blot was performed the same as previously described [12]. Briefly, all the flies used in this research were raised at 24°C, and after eclosion the flies kept at the same temperature for 3 d were then prepared for Western blot analysis. The adult flies' heads were firstly isolated and homogenized. The samples were then centrifuged at 12000×*g* for 12 min at 4°C to discard large cuticular debris. The head extracts were subsequently electrophoresed on SDS/polyacrylamide gels and blotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). Blots were incubated with antibodies against β-actin (1∶8000) (Zhongshan Gold Bridge Biotech, Beijing, China), Ddysb (1∶1000; extant stocks in the laboratory), or ebony (1∶1000; provided by Dr. Sean Carroll, University of Wisconsin-Madison, Madison, WI, USA [21]) overnight at 4°C and with HRP-conjugated goat anti-rabbit IgG (Beyotime, Jiangsu, China) for 2 h at room temperature and were visualized with BeyoECL Plus (Beyotime, Jiangsu, China).

1.3 Co-immune-precipitation (Co-IP)

The Co-IP assay was performed as described previously [22]. The Protein G-Agarose (Roche Diagnostics GmbH, German) (30 μL per well) was firstly incubated with antibody against Ddysb (1∶200) for 3 h at 4°C, and then incubated with protein lysis from adult flies' heads (0.1 g adult fly heads per well) overnight at 4°C. After centrifugation, the Protein G-Agarose was dissolved with 1×SDS-PAGE sample loading buffer (Beyotime, Jiangsu, China) (15 μL per well) and incubated at 100°C for 5 min to release the bound proteins. Then, the sample could be continued with normal Western blot procedures.

1.4 Immunohistochemistry and confocal microscopy

Whole-mount immune-staining of 5-day-old adult brains was performed as described previously [23]. Briefly, dissected brains were fixed in 4% paraformaldehyde at room temperature, vacuumed for 30 min, washed in PBT (PBS with 2% Triton X-100, Sigma Aldrich, St. Louis, USA) three times each for 15 min, and then were blocked in PNT (PBT with 10% normal goat serum, Zhongshan Gold Bridge Biotech) at room temperature for 30 min. Then, the tissue was incubated successively in the primary antibody (rabbit anti-ebony, 1∶400; mouse anti-nc82, 1∶20) and the secondary antibody (fluorescence-conjugated goat anti-rabbit or anti-mouse IgG, 1∶200, Jackson ImmunoResearch, West Grove, PA, USA) at 4°C overnight, with PBT washing in between. Then, the brain was cleared and mounted in Vectashield Mounting Medium (Vector, CA, USA) and imaged with a Zeiss LSM 710 confocal microscope.

1.5 Statistical analysis

All data are shown as mean±SEM and analyzed by Student's *t*-test or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using Origin 8.0 (OriginLab Corporation). * indicates $P<0.05$; ** indicates $P<0.01$; *** indicates $P<0.001$; n.s. represents nonsignificance ($P>0.05$).

2 Results

We started our inquiry to the interaction between Ddysb and

Ebony from an analysis of two *dysb* mutant alleles. One was the hypomorphic mutant, dysb1, used in our previous study [8], with a piggyBac transposon inserted at the 3′-untranslated region (UTR) of the *Ddysb* gene [24], whereas the other was a newly identified mutant, harboring a piggyBac transposon at the 5′-UTR of the *Ddysb* gene (Figure 1).

2.1 Positive correlation between expression levels of Ddysb and Ebony

Our previous publication indicated an altered expression of ebony in the dysb1 mutant [12]. Here, our Western data confirmed the previous observation, showing diminished protein levels of both Ddysb and Ebony in the heads of dysb1 mutant (Figure 1B and C). To our surprise, the protein level of Ddysb was dramatically increased in the second mutant in which *Ddysb* was interrupted at the 5′-UTR (Figure 1B and C, left panels). Therefore, we termed this hypermorphic *Ddysb* mutant the dysb2 mutant. Interestingly, we found that, in correlation with the increased Ddysb level, the protein level of ebony was also significantly higher than that of the control flies (Figure 1B and C, right panels). This observation suggests that Ddysb and Ebony are positively correlated. In other words, Ddysb regulates expression of Ebony in a bidirectional manner.

2.2 Ddysb-dependent regulation of Ebony is confined within glial cells

Since *ebony* is specifically expressed in glial cells, it raised a question as to whether Ddysb-dependent regulation happens in glial cells or in neurons. To address this concern, we determined effects of either knocking-down or overexpression of *Ddysb* in neurons versus in glial cells, respectively, via the Gal4-UAS binary expression system. We found that neither overexpression of the *Ddysb* transgene (*elav-Gal4/+*; *UAS-Ddysb/+*) nor knocking-down of Ddysb via expression of interferent RNAs (*elav-Gal4/+*; *DdysbIR-1*, *DdysbIR-2/+*) in neurons induced detectable changes in the protein level of Ebony (Figure 2A). In contrast, Ebony was significantly reduced, resembling the Ebony decrement in the $dysb^1$ mutant, when the Ddysb-RNAis were specifically expressed in glial cells (*DdysbIR-1*, *DdysbIR-2/+*; *Repo-Gal4/+*). We failed to observe an Ebony increment in flies with overexpression of the *Ddysb* transgene in glial cells though (Figure 2B). This might result from an insufficient overexpression of *Ddysb* (as our observation indicated that *UAS-Ddysb* was weak and phenotypes usually could only be induced by double copies of Gal4 and UAS transgenes [12]). The result presented suggests that Ddysb regulates *ebony* expression within glial cells.

2.3 Ddysb regulates distribution of Ebony protein in glial cells

To gain further insights into mechanisms of Ddysb-dependent regulation of Ebony function, we investigated the expression pattern of *ebony* in glial cells. Results from immunohistochemistry staining revealed that reduction of Eb-

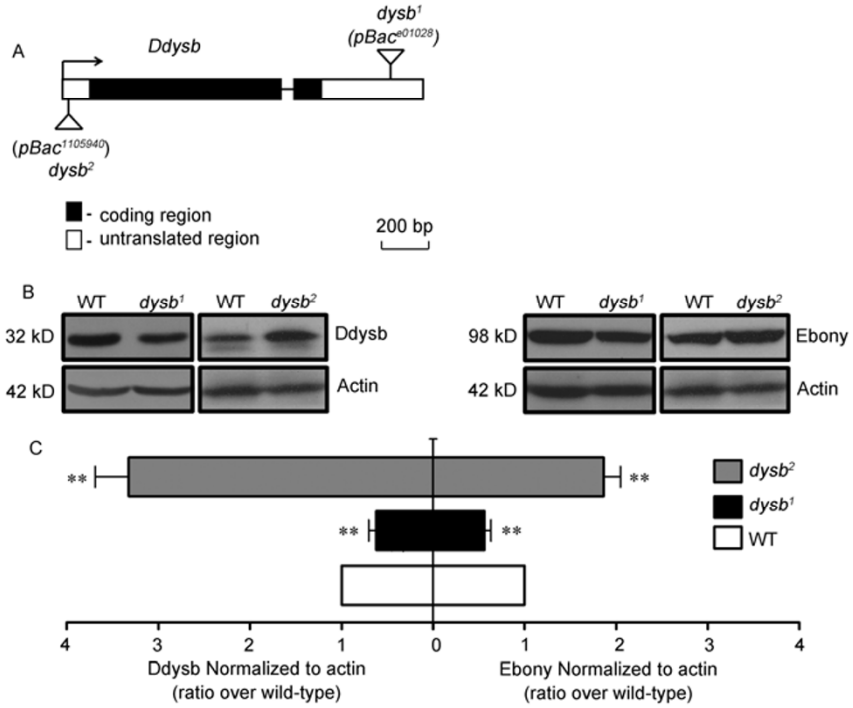

Figure 1 Expression of Ddysb and Ebony is positively correlated. A, Transposon insertion sites in $dysb^1$ and *dysb2* mutants. B, Representative Western blots show that Ddysb and Ebony are both decreased in the head of $dysb^1$ mutant (left) and increased in the head of $dysb^2$ mutant (right). C, Statistic analysis of Western data. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

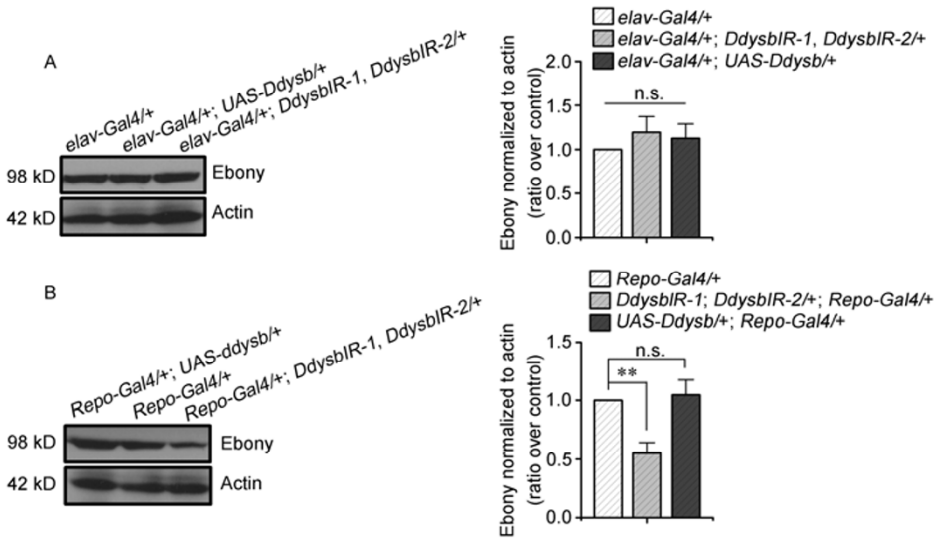

Figure 2 Confined regulation of ebony expression within glial cells. A, Ddysb-dependent regulation of ebony expression in neuronal cells. Left panel, representative Western blots indicated that similar levels of Ebony in flies of control (*elav-Gal4/+*), pan-neuronal knockdown (*elav/+*; *DdysbIR-1*, *DdysbIR-2/+*) and over-expression of *Ddysb* (*elav-Gal4/+*; *UAS-Ddysb/+*); right panel, statistical analysis. B, Ddysb-dependent regulation of *ebony* expression in glial cells. Left panel, representative Western blots of flies in control (*Repo-Gal4/+*), overexpression (*UAS-Ddysb/+*; *Repo-Gal4/+*) and knockdown (*DdysbIR-1*, *DdysbIR-2/+*; *Repo-Gal4/+*) of *Ddysb* in glial cells; right panel, statistical analysis of Western data. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

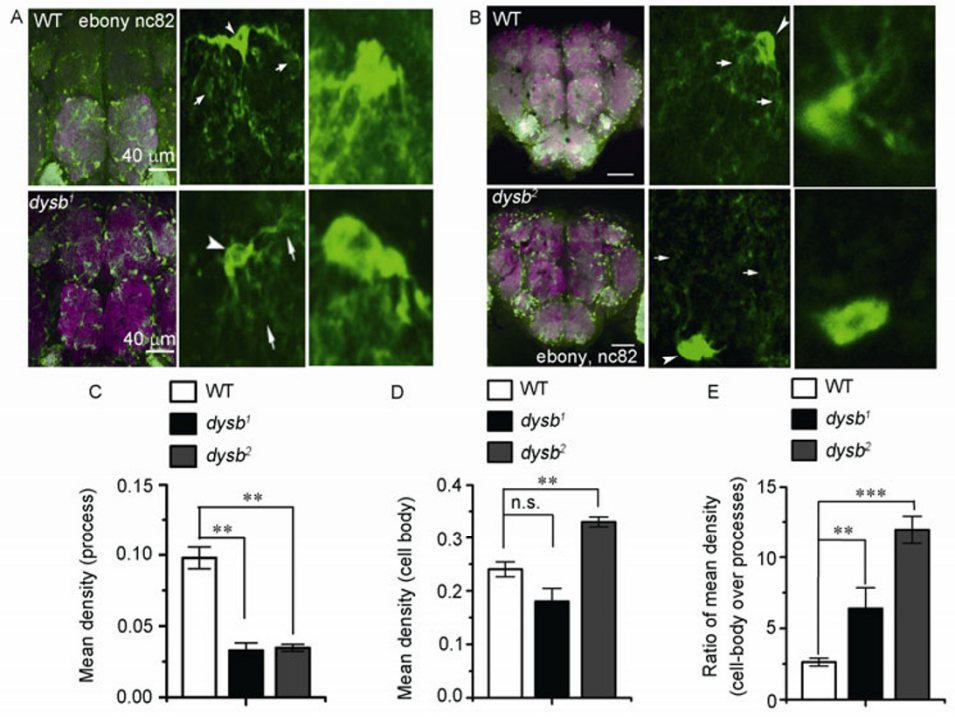

Figure 3 Distribution of Ebony in glial cells. A and B, The endogenous expression of *ebony* in glial cells in *dysb*^{1} and *dysb*^{2} mutants. The brain was immune-labeled with mAb nc82 (magenta). Ebony protein was stained with rabbit Anti-ebony (green). Ebony immune-signal was weaker in the *dysb*^{1} and stronger in *dysb*^{2} as compared to the wild-type control. Scale bar, 40 μm. C–E, Statistical analyses of immune-signals of Ebony at the processes and cell body of glial cells. Altered expression of Ebony in mutants is more pronounced in processes than in soma. *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$.

ony signals was more severe at the processes of glial cells than at the cell body region (Figure 3C and D). Although the overall immune-signal of Ebony was reduced in the *dysb*^{1} mutant in comparison with the wild-type control, an increase in the Ebony signal is indeed observed in the glial cell-body region (Figure 3A and D) but with a decreased ebony signal in the processes of glia (Figure 3A and E). It is unexpected for us to see that Ebony signal in glial processes was also significantly reduced in the *dysb*^{2} mutant (Figure 3B and C), even though the expression level of ebony is

increased as shown in Western blots (Figure 1). Thus, Ebony expressed in processes of glial cells is reduced in both *dysb*^{1}, with a decrease in total Ebony, and *dysb*^{2}, with an increase in total Ebony. To address such discrepancy, we quantified ratios of the Ebony signal in soma over the signal in processes. Analysis revealed that the ratio was elevated significantly, but in both *dysb*^{1} and *dysb*^{2} mutant alleles (Figure 3E). We concluded that not only the level of expression but also subcellular distribution of Ebony could be regulated by expression of *Ddysb*. In a genetic background with altered expression in *Ddysb*, accumulation of Ebony in glial soma is positively associated with total Ebony while accumulation of Ebony in glial processes is reduced, no matter whether total Ebony is increased or decreased.

2.4 Physical interaction between Ddysb and Ebony

To gain insights into mechanisms by which Ddysb regulates subcellular distribution of Ebony, we examined the possibility of direct interaction between these two proteins. We performed a co-immune-precipitation assay and found that the Ebony protein was pulled down together with Ddysb that bound to the beads coated with Protein G-Agarose and Anti-Ddysb antibody in wild-type and both *Ddysb* mutants (Figure 4A; Figure S1 in Supporting Information). We excluded possibility of non-specific binding of ebony with Protein G-Agarose coated beads. This observation suggests that Ebony and Ddysb do physically interact with each other. To ensure the similar pull-down efficiency, the Protein G-Agarose coated beads were saturated with the antigen in the brain lysate. This was verified in Figure 4B, in which the pull-down Ddysb level was similar in all genotypes. Quantitative analysis revealed that the possibility of binding of Ebony with Ddysb is noticeably reduced in both *dysb*^{1}, with lower Ddysb and Ebony, and *dysb*^{2}, with higher Ddysb and Ebony (Figure 4C). In other words, either an abnormal decrease or increase in expression of both proteins, Ddysb and Ebony, all lead to decreased binding.

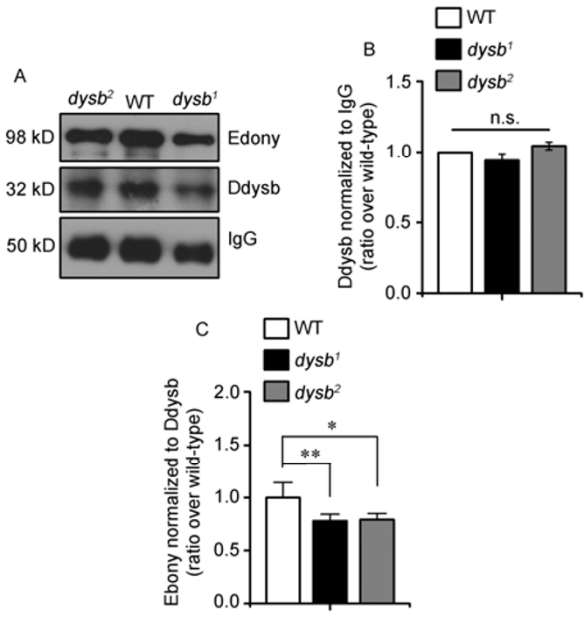

Figure 4 Physical interaction between Ddysb and Ebony. A, Representative image of Western blots. Ebony was pulled down with Ddysb in the co-immune-precipitation assay in *Ddysb* mutants and in wild-type control. B, Quantitative analysis of similar pull-down efficiency in different genotypes. C, Decrease in the ratio of Ebony over Ddysb in both *dysb*^{1} and *dysb*^{2} mutants. *, $P<0.05$; **, $P<0.01$.

3 Discussion

The current study investigated Ddysb-dependent regulation of expression of *ebony* as well as its subcellular localization. In two independent *Ddysb* mutants, with expression of *Ddysb* decreased in one and increased in the other, our study showed that *Ddysb* regulated the expression level of *ebony* in a bi-directional way in glial cells (Figures 1 and 2). Expression of *ebony* is suppressed in *dysb*^{1}, with reduced *Ddysb* expression, and elevated in *dysb*^{2}, with increased *Ddysb* expression. Altered functions of Ddysb in neurons had no effects on Ebony (Figure 2). Ddysb was also capable of affecting subcellular localization of Ebony (Figure 3), but in a complex manner. In glial soma, accumulation of Ebony is positively related to the *Ddysb* expression level. More Ddysb leads to a higher level in total amount of Ebony and accumulation of more Ebony in glial cell-body region. In glial processes, however, the effects are non-linear. Either more or less abnormal *Ddysb* expression leads to less accumulation of Ebony in the processes. This reduction correlates well with a decrease in the binding ratio of Ddysb with Ebony (Figure 4). It is possible that the binding ratio is optimal in the control and such optimal binding ratio could be critical for subcellular distribution of Ebony. Thus, Ddysb is capable of regulating expression and subcellular localization of Ebony.

The current study did not provide clues on how expression of *ebony* is regulated via Ddysb in glial cells. Since Dysbindin is a member of BLOC1 [7,25], one possibility might be protein degradation in the Ddysb/BLOC1-dependent pathway. Also, we cannot exclude the possibility of Dysbindin-dependent regulation of gene expression at the translation or transcription level either [10–12], although there is no such direct evidence reported. For subcellular localization of Ebony, direct binding between Ddysb and Ebony could be critical. In particular, Dysbindin is known to interact with members of DPC involved in protein transportation to special subcellular location [5–9]. The current study reveals Ddysb-dependent regulation of ebony expression and subcellular distribution in glial cells. This mechanism may contribute to Dysbindin-dependent regulation of dopamine signal and related behaviors.

This work was supported by the National Basic Research Program of Ministry of Science and Technology of China (2009CB941301 and 2013CB835102), the Tsinghua University Initiative Scientific Research

Program (20111080956, all to Zhong Yi), and the China Postdoctoral Science Foundation (2012M510412). Shao LiSha was supported by the Postdoctoral Fellowship at Tsinghua-Peking Center for Life Sciences.

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Supporting Information

Figure S1 Ddysb and Ebony have physical interaction. Representative image of Western blots. Ebony and Dysb were not pulled down without antibody against Ddysb in the co-immune-precipitation incubation in wild-type control (right panel).

The supporting information is available online at life.scichina.com and link.springer.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.