Molecular Genetic Analysis of a Schizophrenia Risk Gene, *Disrupted-in-Schizophrenia 1*, in *Drosophila melanogaster*

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Abbreviations

AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

ARC, Activity- regulated Cytoskeleton-associated protein

ASD, Autsm Spectrum Disorder

Brp, Brunchpliot

DGluRIIA, Drosophila Glutamate Receptor IIA

DISC1, Disrupted in Schizophrenia 1

$DISC1^{OE}$, DISC1 overexpression

DTNBP1, Dystrobrebin-Binding Protein 1

dysb, dysbindin

dfmr1, Drosophila fragile-X mental retardation 1

FMR1, Fragile-X Mental Retardation 1

FMRP, Fragile-X Mental Retardation 1 Protein

KAL7, Kalirin-7

MACF1, Microtubule-Actin Crosslinking Factor 1

MAP1B, microtubule-Associated protein 1B

NMJ, Neuromuscular Junction

PAK1, P21-activated kinase 1

PLA, Proximity ligation Assay

RAC1, Ras-related C3 botulinum toxin substrate 1

TNIK, Traf2 and Nck-interacting Kinase

VNC, Ventral nerve cord

Abstract

Dysregulated neurodevelopment with altered synaptic structure and function is believed to underlie diverse neuropsychiatric disorders, and the notion that mental disorders are diseases of synaptic functions is a major hypothesis for the biological basis of mental dysfunctions. *Disrupted-in-Schizophrenia-1* (*DISC1*) has been suggested to be a potent susceptibility gene for wide range of mental illnesses. Although recent studies have implicated the importance of *DISC1* in synapse functions and development, how *DISC1* plays a role in the pathogenesis of mental illness including in conjunction with other psychiatric risk genes remains elusive. Here, I investigated the molecular genetic functions of *DISC1* with the nervous system of the fruit fly, *Drosophila melanogaster*. Overexpression of *DISC1* in fly neurons caused suppression of glutamatergic synaptogenesis in developing neuromuscular junctions. To further understand the molecular mechanism of *DISC1* that controls synaptic development and function, I have examined genetic interactions between *DISC1* and a select set of risk genes for multiple psychiatric disorders using the fly glutamatergic synaptogenesis. Systematic genetic screen identified several interacting genes including *dysbindin* and *Drosophila fragile-X mental retardation 1* (*dfmr1*), both of which have crucial roles for synaptic development and plasticity and genetically modified the *DISC1* phenotype in the glutamatergic synaptogenesis. Further analyses demonstrated direct protein-protein interaction of the DISC1 and the Dysbindin proteins in the developing synapses. I also describe alterations of both pre- and postsynaptic alterations of the *DISC1* phenotypes by *dfmr1* mutations. These data implicate complex but pivotal roles of *DISC1* in the regulations of the molecular synaptic signatures that may cause functional abnormalities of the

glutamatergic synapses and may at least in part reflect a common molecular etiology for diverse psychiatric diseases.

Introduction

Schizophrenia is a debilitating psychiatric disorder that affects about 1% of the population worldwide (Weinberger, 2011, Insel, 2010). Although the etiology of schizophrenia has remained elusive, recent genome wide and familial lineage studies on patients with schizophrenia strongly suggest the presence of multiple genetic risk factors underlying the pathology. Indeed, numerous candidates for risk factor loci have been reported to date (Schizophrenia Psychiatric Genome-Wide Association Study, 2011, Ripke et al., 2013, Szatkiewicz et al., 2013, Kirov et al., 2012, Fromer et al., 2014, Purcell et al., 2014, Schizophrenia Working Group of the Psychiatric Genomics, 2014). One of the most supported hypotheses is that schizophrenia is a neurodevelopmental disease caused by disrupted synaptic formation and function (Mirnics et al., 2001, Ross et al., 2006, Insel, 2010). Several lines of evidence from recent epidemiological, neuroimaging and genetic studies support this hypothesis: (1) in postmortem brain of patients in schizophrenia, the density of dendritic spines, where most excitatory synapses in the brain are built on, were altered in multiple brain regions (Glausier and Lewis, 2013); (2) classical brain imaging techniques and the adaption of algorithms from network science revealed alterations in functional brain network connectivity between brain regions associated with higher cognitive functions such as prefrontal cortex, thalamus and hippocampus (Pratt et al., 2012); (3) a large number of risk genes for schizophrenia encode genes for synaptic proteins, which function in the pathways regulating synaptic development and plasticity (Kirov et al., 2012, Fromer et al., 2014);

(4) in good agreement with the genetic studies, molecular approaches with gene expression profiling of schizophrenia have demonstrated alterations in a group of transcripts encoding proteins that regulate synaptic functions (English et al., 2011, Horvath et al., 2011, Mirnics et al., 2006).

It is notable that dendritic spine pathology and altered functional brain dysconnectivity have also been observed in other neuropsychiatric disorders such as bipolar disorder, autism spectrum disorder (ASD) and Alzheimer's disease (Konopaske et al., 2014, Penzes et al., 2011, Diwadkar et al., 2014, Tao et al., 2013, Voineagu and Eapen, 2013, Minshew and Keller, 2010). On the other hands, genetic studies in diverse psychiatric disorders have suggested that many of the genetic risk loci found with schizophrenia are shared with other psychiatric diseases (Purcell et al., 2014, McCarthy et al., 2014, Kenny et al., 2014, Rauch et al., 2012, Pinto et al., 2010). It is also notable that a large number of such common risk loci encode genes for synaptic proteins, suggesting convergence in their biological functions to the pathways regulating synaptic development and plasticity (Ebert and Greenberg, 2013, Delorme et al., 2013, Hall et al., 2015, Schizophrenia Working Group of the Psychiatric Genomics, 2014, Kirov et al., 2012, Fromer et al., 2014), rendering further support to the synaptic hypothesis that disrupted neuronal connectivity and signaling might be one of the pivotal causes for brain dysfunctions in patients with diverse psychiatric diseases (Moghaddam and Javitt, 2012, Insel, 2010, Weinberger, 2011).

DISC1 was originally identified in a large Scottish family with a wide range of mental disorders including schizophrenia, major depression, bipolar disorder and

autism spectrum disorder (Soares et al., 2011, Porteous et al., 2011, Narayan et al., 2013, Hikida et al., 2012, Brandon and Sawa, 2011, Bradshaw and Porteous, 2012). Biological evidence has accumulated that *DISC1* encodes a multifunctional protein important for brain development and physiology. In particular, recent biochemical and animal model studies have suggested the important role of *DISC1* in synaptic development and plasticity (Wang et al., 2011, Hayashi-Takagi et al., 2010, Camargo et al., 2007). Nevertheless, except for the unique Scottish pedigree, how the *DISC1* locus is implicated at the genetic level in major mental disorders is still elusive with little success in genome-wide association studies to identify the locus as a prominent hit for schizophrenia (Kirov et al., 2012, Szatkiewicz et al., 2013). This enigma in turn might imply an intricate underlying mechanism that involves complex genetic interactions with other loci that exert functional modifications of neuronal DISC1 functions in the brain.

To further understand the molecular network of *DISC1* on synapse, genetically tractable model animals are challenging but necessary. The fruit fly (*Drosophila melanogaster*) has been used as a powerful model for understanding cellular and molecular mechanisms of diverse neurological disorders (Wangler et al., 2015, Lessing and Bonini, 2009, Bellen et al., 2010). In conjunction with the commonality of molecular genetic mechanisms in brain development (Wangler et al., 2015, Lessing and Bonini, 2009, Bellen et al., 2010), the vast array of transgenic techniques in fruit flies allows us to study human neurological disease genes either by loss-of-function or by gain-of-function approaches, involving overexpression of human

disease genes in the fly brain. Whereas phenocopying human psychiatric symptoms is a major challenge for animal models, particularly in evolutionary distant species such as fruit flies, recent efforts toward a framework for basic research on mental disorders highlight the importance of elucidating the underlying mechanisms of mental dysfunction (Morris and Cuthbert, 2012, Insel et al., 2010, Cuthbert, 2014). In such a framework, mental disorders can be addressed as disorders of brain circuits, which can be studied at multiple biological and genetic levels using non-human models. In line with this concept, several studies have utilized the fly model in the past few years to reveal the underlying mechanisms of diverse mental disorders at the molecular and genetic levels (van der Voet et al., 2014, van Alphen and van Swinderen, 2013, Sawamura et al., 2008, Furukubo-Tokunaga, 2009, Doll and Broadie, 2014, Androschuk et al., 2015). Although the *DISC1* gene is not conserved in the fly genome (Bord et al., 2006), fruit flies exhibit significant conservation (92%) of the genes that encode the DISC1 interacting proteins (Camargo et al., 2007) (Table 1), providing a platform for molecular genetic analyses of its functions and interactors in synaptogenesis.

The fly larval neuromuscular junction (NMJ) exhibits several important features in common with the excitatory synapses in the vertebrate brain (Collins and DiAntonio, 2007, Schwarz, 2006, DiAntonio, 2006, Menon et al., 2013, Charng et al., 2014). The fly NMJ utilizes glutamate as the major transmitter, and ionotrophic glutamate receptors that are homologous to human receptors are expresses at the NMJ (Schwarz, 2006, DiAntonio, 2006, Collins and DiAntonio, 2007). In addition, as with

the vertebrate central synapse, the synapses on the fruit fly NMJ exhibit dynamic plasticity with organized series of boutons that are formed auxiliary or eliminated during development and plasticity (Menon et al., 2013, Collins and DiAntonio, 2007, Charng et al., 2014). The fact that the fruit fly NMJ shows stereotypic synaptic connections with unique and identifiable presynaptic motoneurons and postsynaptic muscles make the system highly useful for studying the molecular genetic mechanisms of synaptogenesis and functions (Menon et al., 2013, Koles and Budnik, 2012, Collins and DiAntonio, 2007, Bayat et al., 2011).

In this study, as a way to analyze interactions between *DISC1* and diverse psychiatric risk factor genes involved in pathological processes, I expressed the human *DISC1* gene in the fly NMJ and investigated the molecular machinery of *DISC1* and the genetic interactors that controls glutamatergic synaptogenesis. Overexpression of *DISC1* (*DISC1*^{OE}) in fly neurons caused suppression of glutamatergic synaptogenesis in developing neuromuscular junctions. To further understand the molecular mechanism of *DISC1* that controls synaptic development and function, I have examined genetic interactions between *DISC1* and a select set of risk genes for multiple psychiatric disorders using the fly glutamatergic synaptogenesis. Systematic genetic screen identified several interacting genes including *dysbindin* (*dysb*) and *Drosophila fragile-X mental retardation 1* (*dfmr1*), both of which have crucial roles for synaptic development and plasticity and genetically modified the *DISC1* phenotype in the glutamatergic synaptogenesis. Further analyses demonstrated direct protein-protein interaction of the DISC1 and the Dysbindin proteins in the developing synapses. I will also describe

alterations of both pre- and postsynaptic alterations of the *DISC1* phenotypes by *dfmr1* mutations. These data implicate complex but pivotal roles of *DISC1* in the regulations of the molecular synaptic signatures that may cause functional abnormalities of the glutamatergic synapses and may at least in part reflect a common molecular etiology for diverse psychiatric diseases.

Results

Overexpression of *DISC1* suppresses synaptic development in fruit fly NMJ

To investigate molecular function of *DISC1* in synapse development, I performed anatomical analysis of developing NMJ overexpressing *DISC1*. I found that overexpression of *DISC1* (*DISC1*^{OE}) with a ubiquitous driver (*tubP-GAL*) caused mild but significant reduction in the total synaptic bouton area although the numbers of synaptic boutons and axonal branch points were not altered (Fig. 1).

Genetic screen of *DISC1* interactors in fruit fly synaptogenesis

To identify genetic factors that modify *DISC1*^{OE} phenotype in glutamatergic synaptogenesis, I set up a genetic crossing (Fig. 2) between the *DISC1*^{OE} flies and the fly mutants of diverse psychiatric risk genes. The mutations used in this study range from null to hypomorphic mutants, which could lead to ~50% reduction at most in the gene dosage under the heterozygous condition used in this screen, and only partially affect synaptic formations on their own. To evaluate genetic modifications, I compared NMJ synaptic anatomy with that of the phenotype caused by *DISC1*^{OE} alone, focusing on the three morphological parameters (bouton area, number of boutons, and number of axonal branch points), and compared them with the phenotype caused by *DISC1*^{OE} alone. I examined mutations found on the fly autosomes (second and third chromosomes) using the genetical scheme. Despite the small number of mutations analyzed, I found several

genes, including *dysbindin* (*dysb*) and *dfmr1*, the fruit fly homolog of the human *DTNBP1* and *FMR1* gene, respectively (Kurita et al., in preparation).

DISC1 directly interacts with Dysbindin in glutamatergic synaptogenesis

Recent protein–protein interaction (PPI) analysis of DISC1 suggested that DTNBP1 and DISC1 might share common protein interactions and affect common biological processes (Camargo et al., 2007). In addition, previous *in vitro* assay implicated direct interaction between DISC1 and DTNBT1 (Ottis et al., 2011). Because of this importance, among the candidate genes identified, firstly I focused on *dysbindin* (*dysb*), the fruit fly homolog of the human DTNBP1 gene. Mutation of *dysb* caused intriguing enhancement of the DISC1 phenotype (Fig. 3). DISC1 suppressed the synaptic area more profoundly in $dysb^{e01028}$/+ heterozygous background (Fig. 3C). Moreover, DISC1 caused significant reductions in the number of boutons and the individual bouton size in the $dysb^{e01028}$/+ heterozygous background (Fig. 3D and 3F). On the other hand, the number of axonal branch points of the motor neurons was not changed, arguing for specific suppression of synaptic bouton formation (Fig. 3E).

In order to determine whether DISC1 directly interacts with the fruit fly Dysbindin in synaptogenesis, we applied the proximity ligation assay (PLA) technique (Wang et al., 2015, Soderberg et al., 2006) in the larval NMJs. In this assay, the target proteins are localized with specific primary antibodies, which are then detected with secondary antibodies conjugated to PLA-PLUS or MINUS oligonucleotide probes. The

attached probes can be bridged through hybridization of connector oligonucleotides only when the two proteins are in close proximity to make direct contact. The annealed oligonucleotides are then closed by ligation into circular DNA molecules, which serve as templates for subsequent rolling circle amplification to be visualized by in situ hybridization with a fluorescently labeled probe. In negative control, I detected a few punctate PLA signals at the larval NMJ, which might be background signals due to non-specific binding of primary or secondary antibodies (Fig. 4A and 4B). On the other hand, prominent PLA signals were present at NMJ co-expressing both *UAS-DISC1* and *UAS-Venus::Drosophila Dysbindin* (Fig. 4C and 4D). Furthermore, statistical analysis suggested that PLA signals at NMJ co-expressing both *UAS-DISC1* and *UAS-Venus::Drosophila Dysbindin* were significantly higher than those at negative control NMJs (Fig. 4E). These results as a whole suggest an intriguing functional interaction between DISC1 and Dysbindin in the development of glutamatergic synapses.

Discussion

Synaptic development and plasticity have been hypothesized as crucial mechanisms of diverse mental disorders (Kirov et al., 2012, Purcell et al., 2014, Ebert and Greenberg, 2013, Delorme et al., 2013). In particular, it has been hypothesized that altered glutamate neurotransmission might be a critical cause for cognitive deficits in schizophrenia and other mental disorders (Kantrowitz and Javitt, 2012, Laruelle, 2014). Recent molecular studies have demonstrated the importance of *DISC1* for synaptic development and plasticity (Hikida et al., 2012, Porteous et al., 2011, Bradshaw and Porteous, 2012, Narayan et al., 2013, Soares et al., 2011, Brandon and Sawa, 2011, Camargo et al., 2007, Hayashi-Takagi et al., 2010, Wang et al., 2011). In this study, I expressed human DISC1 in developing fly glutamatergic NMJ and found that *DISC1*^{OE} causes mild but significant suppression in the total synaptic area. In addition, the *Drosophila* homolog of *DTNBP1* (*dysbindin*) and *FMR1* (*dfmr1*) exhibited functional interactions with *DISC1* in glutamatergic synaptogenesis. Furthermore, I found that *DISC1* functionally interacts with *dysbindin* in synaptic development via direct protein–protein interaction, and that mutations of *dfmr1* modify the *DISC1*^{OE} synaptic phenotypes at the morphological and molecular levels.

Overlapping regulatory networks of DISC1 and Dysbindin in synaptic development

In this study, I have shown that *DISC1* interacts with *dysbindin* in synaptic development. Initially found as a component of the dystrophin–dystroglycan complex (Benson et al.,

2001), Dysbindin was subsequently identified as a component of biogenesis of lysosome-related organelles complex that controls organelle biogenesis and intracellular membrane trafficking (Ghiani et al., 2010, Iizuka et al., 2007). Dysbindin is expressed in both pre- and post-synaptic cells to control dendritic spine formation through the association with WAVE-2 and Abi-1, key regulators of Ras-related C3 botulinum toxin substrate 1 (Rac1) that control actin cytoskeletal dynamics (Ito et al., 2010). Consistently, knockdown of dysbindin results in disorganization of actin cytoskeletons that accompanies neurite shortening and growth cone abnormality (Ma et al., 2011, Ghiani et al., 2010). Interestingly, it has been shown that Dysbindin controls synaptic development and functions in fruit flies (Dickman and Davis, 2009, Shao et al., 2011).

Studies of DISC1 interactome (Camargo et al., 2007) suggested that Dysbindin and DISC1 bind several proteins in common, such as microtubule crosslinking factors Dystonin (DST/BPAG1) and microtubule-actin crosslinking factor 1 (MACF1). Both DISC1 and Dysbindin interact with members of the exocyst complex (Camargo et al., 2007), which regulate protein trafficking to synaptic terminals in both vertebrates and fruit flies (Heider and Munson, 2012, Mehta et al., 2005, Jafar-Nejad et al., 2005). It is also noteworthy that DISC1 interacts directly with Traf2 and Nck-interacting kinase (TNIK), a psychiatric risk factor and an activator of Wnt target genes (Wang et al., 2011), which in turn interacts with DST (Camargo et al., 2007). Moreover, the fly homolog (*short stop*) of MACF1 and DST has critical roles in axonal, dendritic and synaptic development (Lee et al., 2007, Alves-Silva et al., 2012). These

lines of evidence suggest a converging mechanism of FMRP and DISC1 in the cytoskeletal regulation in the developing synapses.

Recently, Ottis et al. (Ottis et al., 2011) have suggested intriguing convergence of DISC1 and Dysbindin by showing that DISC1 recruits Dysbindin into protein aggresomes in mouse neuroblastoma cells via protein–protein interaction mediated by the amino acids 316–597, which is consistent with the domains involved for synaptic modulation in fruit fly NMJs. Physical interaction between Dysbindin and DISC1 is also critical for the stability of Dysbindin and for neurite out growth in cultured neuronal cells (Lee et al., 2015). Taken together, our results as a whole suggest a complex but intriguing converging mechanism controlled by DISC1 and Dysbindin in the developing glutamatergic synapses.

Conclusions

In this study, I have performed molecular genetic dissections of the DISC1 functions in the development of the fruit fly glutamatergic synapses. It is noteworthy that the behavioral and developmental alterations caused by the overexpression of DISC1 in the fruit fly cognitive centers correspond to the endophenotypes that have been observed in murine models and human patients. This cross-species compatibility is likely mediated by the interactions between the human DISC1 and the associating fly proteins that are conserved despite the evolutionary distance. Genetic studies addressing epistatic mechanisms in mental disorders are so far limited but warranted to understand the molecular mechanisms that may involve complex polygene interactions of diverse psychiatric risk factor genes. In this perspective, my study would provide a foundation for further studies on the molecular genetic mechanisms of DISC1 functions using fruit flies. Given the unparalleled power of the *Drosophila* genetics, it is feasible to systematically identify interacting genetic loci that collaboratively function *in vivo* through shared pathways. Combined with the recent advancement in human psychiatric genetics, the fruit fly provides insights relevant to the understanding of the etiology of mental disorders at the brain circuit level.

Materials and Methods

Fly stocks

A *white* (*w*) stock outcrossed with Canton S 10 times (*w* (CS10)) was used as the standard stock. Construction of transgenic flies carrying *UAS-DISC1* transgene has been described previously (Furukubo-Tokunaga et al., 2016, Sawamura et al., 2008). To ensure homogeneous genetic background, fly stocks were outcrossed to *w* (CS10) at least 5 times. The *tubP-GAL4* (O'Donnell et al., 1994) was obtained from the Bloomington Stock Center (Bloomington, IN, USA). All stocks were raised at 25 ˚C on a standard food consisting of 5.5 g/L agar, 40 g/L yeast extract, 90 g/L cornmeal, 100 g/L glucose. Propionic acid (3 ml/L) and n-butyl-p-hydroxybenzoate (0.7 g/L) were added as fungicides.

Immunohistochemistry

Immunological staining was performed as described previously (Kurusu et al., 2002). The following antibodies were used: sheep anti-DISC1 antibody diluted 1:50 (AF6699, R&D systems, Minneapolis, MN, USA), mouse anti-Synaptotagmin diluted 1:2 (3H2 2D7, Developmental Studies Hybridoma Bank (DSHB), University of Iowa, IA, USA) (Dubuque et al., 2001), anti-Horseradish peroxidase protein (HRP) conjugated with fluorescein-isothiocyanate diluted 1:100 (Jackson ImmunoResearch, West Grove, PA, USA), and Alexa-conjugated secondary antibodies diluted 1:200 (Molecular probes,

Eugene, OR, USA). Confocal images were captured with a Zeiss LSM510 or LSM710 microscope.

Genetic screen

For the screen, mutant lines were balanced with a double balancer stock (*w/w; Sp / CyO, Act-GFP; Pr Dr/ TM6B, ubi-GFP*). The resulting progeny carrying a mutation were then crossed either with control (*w; +; tubP-GAL4/TM6B, ubi-GFP*) or with $DISC1^{OE}$ (*w; UAS- DISC1(CS10)6-6(II); tubP-GAL4/ TM6B, ubi-GFP*) flies. Larvae were raised at 25 ˚C, and non-GFP animals were selected for dissection at 116-120 hours after egg collection.

Proximity Ligation Assay

To examine whether two proteins are in close proximity to make direct contact at the larval NMJ, *UAS-Venus::Drosophila-Dysbindin* (gift from Graeme Davis) (Dickman and Davis, 2009) and *UAS-DISC1* were co-expressed with *tubP-GAL4*. Proximity ligation assay (PLA) was performed as described previously (Wang et al., 2015). Briefly, NMJ of third instar larvae were dissected in ice-cold phosphate saline, fixed for 30 min with 4% paraformaldehyde, and incubated with 1% bovine serum albumin in PBT (phosphate buffered saline containing 0.1 % Triton X-100). Tissues were then incubated with 1:50 dilution of sheep anti-DISC1 (AF6699) and 1:500 dilution of rabbit anti-GFP (Molecular Probes, Eugene, OR) overnight at 4˚C. After washing with PBT, tissues were further incubated with anti-Horseradish peroxidase (HRP) conjugated with FITC for three hours. After washing with PBT, tissues were incubated with 1:15 dilution of

anti-rabbit PLUS (DUO092002, Sigma-Aldrich, St. Louis, MO) and anti-goat MINUS (DUO092006, Sigma-Aldrich, St. Louis, MO) PLA probes for two hours at 37˚C. After washing with wash buffer A (Duolink, Sigma-Aldrich, St. Louis, MO), ligation reaction was done for one hour at 37 ˚C followed by amplification for two hours at 37˚C. Tissues were then washed with wash buffer B (Duolink, Sigma-Aldrich, St. Louis, MO) and mounted on a slide. PLA signals were captured with confocal microscopy LSM510 or LSM700 and analyzed with Image J.

Quantification of NMJ structure and fluorescent intensity

For quantification of synaptic phenotypes, I used the larval longitudinal muscles 6/7 in the abdominal hemisegment A2. Anti-HRP and anti-Synaptotagmin were used to label neuronal terminal and synaptic boutons, respectively. Total bouton area was determined using Image-J (Schneider et al., 2012) based on anti-Synaptotagmin immunoreactivity. Protein expression levels were determined based on fluorescent intensities of the NMJ preparations using the control and test samples processed simultaneously in the same tube. Confocal images were captured using identical settings. Anti-HRP immuno-reactivity was used as an internal control.

Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, San Diego, CA). Experimental data of anatomical alterations, protein expression and PLA signal intensity in NMJ synapses were analyzed using parametric tests (Student's *t*-test

and one-way ANOVA) without randomization and blinding. For multiple comparisons among relevant groups, Dunnett's *post hoc* test was used in conjunction with one-way ANOVA. Significance levels in the figures are represented as $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) by Student's *t*-test or one-way ANOVA. Error bars represent standard errors of means.

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Tables

Table 1. *Drosophila* homologues of DISC1 interacting protein

chomosome	Interactors	HGNC	UniProt KB	Encoded Protein	Physical Interaction with DISC1			*Dorosophila* Homologues
					FL	N	TR	
1	GNB1	4396	P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	-	+	-	G protein β-subunit 13F
	KIAA0470	28920	Q5SW79	Centrosomal protein of 170 kDa	+	+	-	Mucin 68D
	KIFAP3	17060	Q92845	Kinesin-associated protein 3	-	+	-	Kinesin associated protein 3
	MGC45441	28688	Q8N4L8	Coiled-coil domain-containing protein 24	-	-	+	Cytoplasmic linker protein 190
	MACF1	13664	Q9UPN3	Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	+	+	+	short stop
	PDE4B	8781	Q07343	cAMP-specific 3',5'-cyclic phosphodiesterase 4B	+	-	-	dunce
2	C2orf4	14014	Q9Y316	Protein MEMO1	-	+	-	CG8031
	DCTN1	2711	Q14203	Dynactin subunit 1	-	-	+	Glued
	FBXO41	29409	Q8TF61	F-box only protein 41	+	-	-	CG9003
	IMMT	6047	Q16891	Mitochondrial inner membrane protein	-	+	+	CG6455
	KIAA1212	25523	Q3V6T2	Girdin	+	+	-	Girdin
	KIF3C	6321	O14782	Kinesin-like protein KIF3C	-	+	+	Kinesin-like protein at 68D
	MYT1L	7623	Q9UL68	Myelin transcription factor 1-like protein	+	-	-	CG43689
	SPTBN1	11275	Q01082	Spectrin beta chain, non-erythrocytic 1	+	+	+	β Spectrin
	TRAF3IP1	17861	Q8TDR0	TRAF3-interacting protein 1	+	+	+	CG3259
	YWHAQ	12854	P27348	14-3-3 protein theta	-	-	+	14-3-3ζ
3	ARIH2	690	O95376	E3 ubiquitin-protein ligase ARIH2	-	+	-	ariadne 2
	FLJ13386	25815	Q96MT8	Centrosomal protein of 63 kDa	+	-	-	zipper, sti
	KALRN	4814	O60229	Kalirin	-	-	+	trio
	SH3BP5	10827	O60239	SH3 domain-binding protein 5	+	+	-	parcas
	SRGAP2	19751	O75044	SLIT-ROBO Rho GTPase-activating protein 2	-	-	+	-
	TNIK	30765	Q9UKE5	TRAF2 and NCK-interacting protein kinase	+	+	-	misshapen
	ZNF197	12988	O14709	Zinc finger protein 197	-	+	-	crooked legs
4	KIAA0826	29127	O94915	Protein furry homolog-like	+	+	-	furry
	SEC3L1	30380	Q9NV70	Exocyst complex component 1	+	-	+	Sec3 ortholog
	SPARCL1	11220	Q14515	SPARC-like protein 1	-	-	+	BM-40-SPARC
	STX18	15942	Q9P2W9	Syntaxin-18	-	-	+	Syntaxin 18
5	DPYSL3	3015	Q14195	Dihydropyrimidinase-related protein 3	-	+	-	Collapsin Response Mediator Protein
	KIF3A		Q9Y496	Kinesin-like protein KIF3A	-	+	+	Kinesin-like protein at 64D
	MATR3	6912	P43243	Matrin-3	-	+	-	Sh3β
	TRIO	12303	O75962	Triple functional domain protein	+	+	+	trio
6	C6orf182	21561	Q8IYX8	Centrosomal protein CEP57L1	-	-	+	CENP-meta, rudhira
	CDC5L	1743	Q99459	Cell division cycle 5-like protein	+	+	-	Cell division cycle 5 ortholog
	DST	1090	Q03001	Dystonin	+	+	+	short stop
	TIAM2	11806	Q8IVF5	T-lymphoma invasion and metastasis-inducing protein 2	-	+	-	still life
	TUBB	20778	P07437	Tubulin beta chain	-	+	-	betaTub85D
	UTRN	12635	P46939	Utrophin	-	-	+	Dystrophin
7	AKAP9	379	Q99996	A-kinase anchor protein 9	+	+	-	Stretchin-Mlck
	DKFZP434G156	22225	Q96JN2	Coiled-coil domain-containing protein 136	-	-	+	Cytoplasmic linker protein 190
8	CLU	2095	P10909	Clusterin	+	+	+	-
	DPYSL2	3014	Q16555	Dihydropyrimidinase-related protein 2	-	+	-	Collapsin Response Mediator Protein
	EIF3S3	3273	O15372	Eukaryotic translation initiation factor 3 subunit H	+	+	-	Eukaryotic initiation factor 3 p40 subunit
	RAD21	9811	O60216	Double-strand-break repair protein rad21 homolog	+	+	-	verthandi
	TNKS	11941	O95271	Tankyrase-1	-	+	-	tankyrase
	YWHAZ	12855	P63104	14-3-3 protein zeta/delta	-	-	+	14-3-3ζ

9	AGTPBP1	17258	Q9UPW5	Cytosolic carboxypeptidase 1	-	+	-	Drosophila Nna1 ortholog
	OLFM1	17187	Q99784	Noelin	+	+	-	CG6867
	RABGAP1	17155	Q9Y3P9	Rab GTPase-activating protein 1	-	+	-	GTPase activating protein and centrosome-associated ortholog
	SMC2L1	14011	O95347	Structural maintenance of chromosomes protein 2	-	-	+	SMC2
	SPTAN1	11273	Q13813	Spectrin alpha chain, non-erythrocytic 1	-	+	-	α Spectrin
	TUBB2	12412	Q13885	Tubulin beta-2A chain	-	+	-	betaTub85D
10	XPNPEP1	12822	Q9NQW7	Xaa-Pro aminopeptidase 1	+	+	-	Aminopeptidase P
	ZNF365	18194	Q70YC5	Protein ZNF365	+	-	-	-
11	NUP160	18017	Q12769	Nuclear pore complex protein Nup160	-	+	+	Nucleoporin 160kD
12	BICD1	1049	Q96G01	Protein bicaudal D homolog 1	-	+	-	Bicaudal D
	DCTN2	2712	Q13561	Dynactin subunit 2	+	-	+	Dynamitin
	MGC4170	29670	Q3T906	N-acetylglucosamine-1-phosphotransferase subunits alpha/beta	-	-	+	CG8027
13	-	-	-	-	-	-	-	-
14	C14orf135	20349	Q63HM2	Pecanex-like protein 4	-	+	-	pecanex
	C14orf166	23169	Q9Y224	UPF0568 protein C14orf166	-	-	+	CG31249
	DNCH1	2961	Q14204	Cytoplasmic dynein 1 heavy chain 1	-	+	+	Dynein heavy chain 64C
	SNX6	14970	Q9UNH7	Sorting nexin-6	+	-	-	Sorting nexin 6
15	MN7	4870	-	hect domain and RLD 2 pseudogene 2	+	-	-	-
16	FLJ22386	29478	Q9GZN7	Protein rogdi homolog	+	-	-	rogdi
17	ACTG1	144	P63261	Actin, cytoplasmic 2	-	+	-	Actin 5C, Actin 42A
	CDK5RAP3	18673	Q96JB5	CDK5 regulatory subunit-associated protein 3	+	+	-	CG30291
	DNAJC7	12392	Q99615	DnaJ homolog subfamily C member 7	-	+	+	Tetratricopeptide repeat protein 2
	EXOC7	23214	Q9UPT5	Exocyst complex component 7	+	-	-	Exo70 ortholog
	NDEL1	17620	Q9GZM8	Nuclear distribution protein nudE-like 1	+	+	-	nudE
	PAFAH1B1	8574	P43034	Platelet-activating factor acetylhydrolase IB subunit alpha	+	+	-	Lissencephaly-1
	PPM1E	19322	Q8WY54	Protein phosphatase 1E	-	+	-	CG10376
	PPP4R1	9320	Q8TF05	Serine/threonine-protein phosphatase 4 regulatory subunit 1	-	+	-	-
	SMARCE1	11109	Q969G3	SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1	+	-	-	dalao
	YWHAE	12851	P62258	14-3-3 protein epsilon	-	+	+	14-3-3ε
18	-	-	-	-	-	-	-	-
19	EEF2	3214	P13639	Elongation factor 2	-	+	-	Elongation factor 2
	PPP5C	9322	P53041	Serine/threonine-protein phosphatase 5	-	-	+	Protein phosphatase D3
20	CRNKL1	15762	Q9BZJ0	Crooked neck-like protein 1	-	+	-	crooked neck
	XRN2	12836	Q9H0D6	5'-3' exoribonuclease 2	-	+	-	Rat1
21	-	-	-	-	-	-	-	-
22	C22orf1	1306	O15442	Metallophosphoesterase domain-containing protein 1	-	+	-	CG16717
	TFIP11	17165	Q9UBB9	Tuftelin-interacting protein 11	-	+	+	septin interacting protein 1
X	DMD	2928	P11532	Dystrophin	+	-	-	Dystrophin
	GPRASP2	25169	Q96D09	G-protein coupled receptor-associated sorting protein 2	-	+	-	CG3108 nahoda
	PGK1	8896	P00558	Phosphoglycerate kinase 1	+	-	-	Phosphoglycerate kinase
Y	-	-	-	-	-	-	-	-

Abbereviations; FL Full length, N N-terminus Region, TR Truncated form

HGNC : HUGO Gene Nomenclature Committee

UniProtKB : UniProt Protein knowledgebase

Interacting proteins and the DISC1 domains are according to Camargo et al., 2007.

Note that 92% (75/82) of the genes encoding the DISC1 interacting proteins have homologs in the fly genome.

Figures

Figure 1. Ubiquitous *DISC1*^{OE} in the fruit fly larval NMJ.

(A, B) Confocal images of third instar larval NMJs. (A) Control without *UAS-DISC1*. (B) *DISC1*^{OE} with *tubP-GAL4*. (C-E) Quantification of the NMJ morphology with (+) or without (-) ubiquitous *DISC1*^{OE}. (C) Total synaptic bouton area. (D) Number of synaptic boutons. (E) Number of axonal branch points. * $P < 0.05$ with Student's *t*-test. n.s., not significant. n = 11-15. NMJs of the muscle 6-7 in the second abdominal segment were stained with anti-HRP (green) and anti-Synaptotagmin (Syt) (magenta). NMJ, neuromuscular junction; *DISC1*^{OE}, *DISC1* overexpression; HRP, horseradish peroxidase. Bar, 20μm.

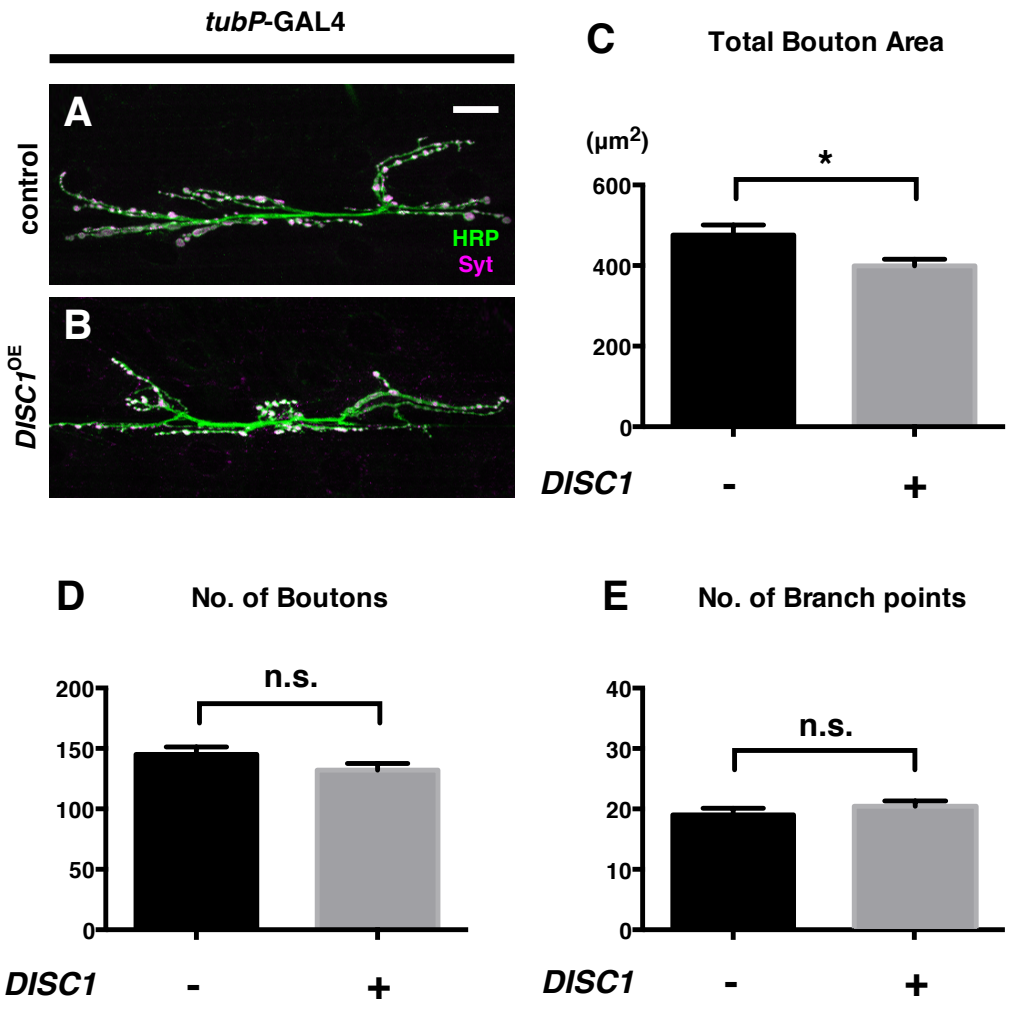

tubP-GAL4
A
control
HRP
Syt
B
DISC1OE
C
Total Bouton Area
(μm2)
600
400
200
0
*
DISC1
-
+
D
No. of Boutons
200
150
100
50
0
n.s.
DISC1
-
+
E
No. of Branch points
40
30
20
10
0
n.s.
DISC1
-
+

Figure 2. Genetic screen of *DISC1* interactors in the fruit fly synaptogenesis.

Mutant flies (+/*CyO-GFP*; *mutation*/TM6B-GFP) of the fruit fly homologue for a schizophrenia risk gene are crossed with the control (+/+; *tubP-GAL4*/*TM6B-GFP*) or the *DISC1*^{OE} (*UAS-DISC1*; *tubP-GAL4*/*TM6B-GFP*) flies. Larval NMJs of the control (+/+*; mutation*/*tubP-GAL4*) and *DISC1*^{OE} (+/*UAS-DISC1; mutation*/*tubP-GAL4*) progenies were compared. Note that while both progenies are heterozygous for the *mutation,* control flies carry the *tubP-GAL4* but not the *UAS-DISC1* transgene. Illustrated are the genetic crossings for a third chromosome mutation. Similar crossings were performed for second chromosome mutations. *DISC1*^{OE}, *DISC1* overexpression.

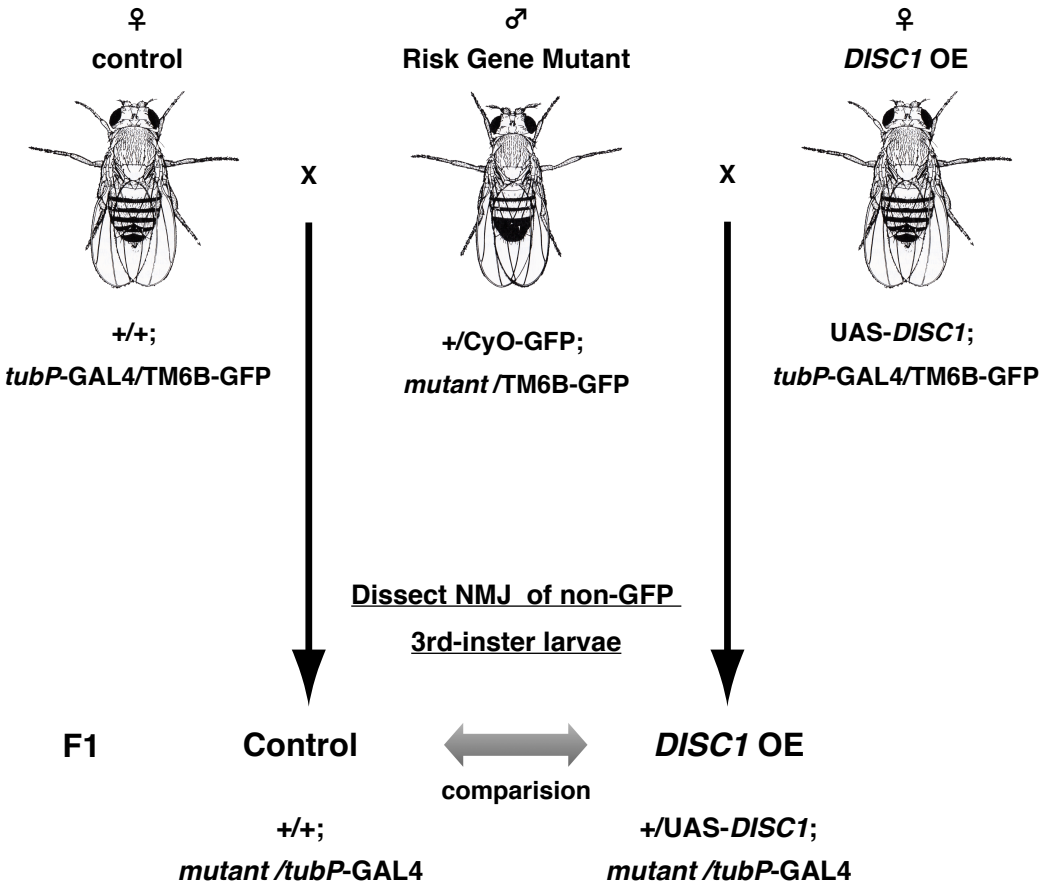

♀
control
♂
Risk Gene Mutant
♀
DISC1 OE
X
X
+/+;
tubP-GAL4/TM6B-GFP
+/CyO-GFP;
mutant /TM6B-GFP
UAS-DISC1;
tubP-GAL4/TM6B-GFP
Dissect NMJ of non-GFP
3rd-inster larvae
F1
Control
comparision
DISC1 OE
+/+;
mutant /tubP-GAL4
+/UAS-DISC1;
mutant /tubP-GAL4

Figure 3. DISC1 genetically interacts with dysbindin in the development of glutamatergic synapses.

(A, A', A", B, B', B") Synaptic terminal branches of *dysb*^{e01028} heterozygous NMJs without (A) or with (B) *DISC1*^{OE}. (A', B') Motor neuron termini labeled with an anti-HRP (Green in A and B). (A", B") Synaptic boutons labeled with anti-Synaptotagmin (Magenta in A and B). Scale bar, 40 μm. (C-F) Quantification of NMJ synaptogenesis in *w* (CS10) control and *dysb*^{e01028} /+ backgrounds with (+) or without (-) ubiquitous *DISC1*^{OE}. (C) Total synaptic bouton area. (D) Number of synaptic boutons. (E) Individual synaptic bouton size. (F) Number of axonal branch points. *$P <$ 0.05, **$P < 0.01$ and ***$P < 0.001$ with Student's *t*-test, $n = 10$ for each genotype. NMJ, neuromuscular junction; *DISC1*^{OE}, *DISC1* overexpression; HRP, horseradish peroxidase.

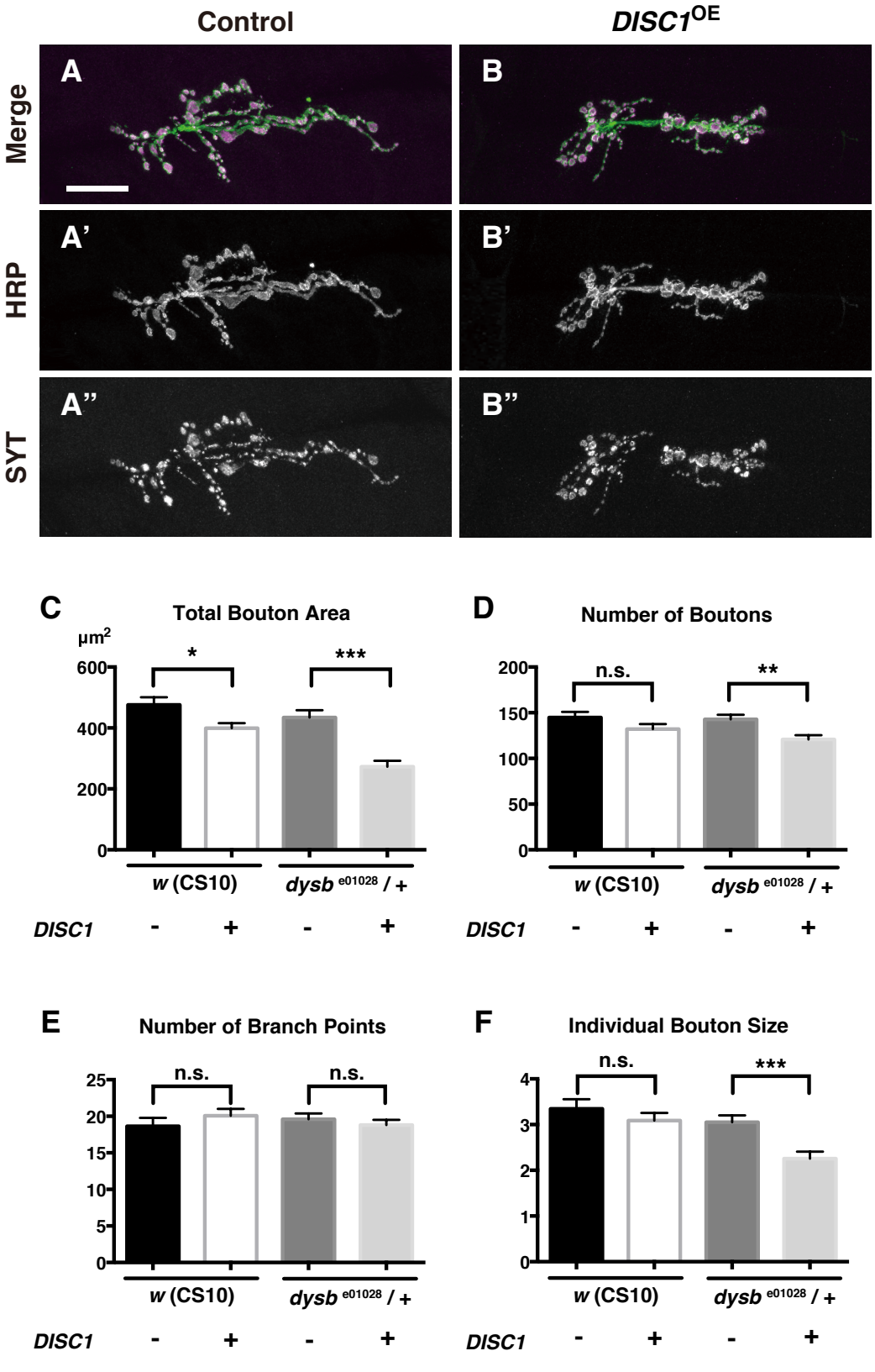
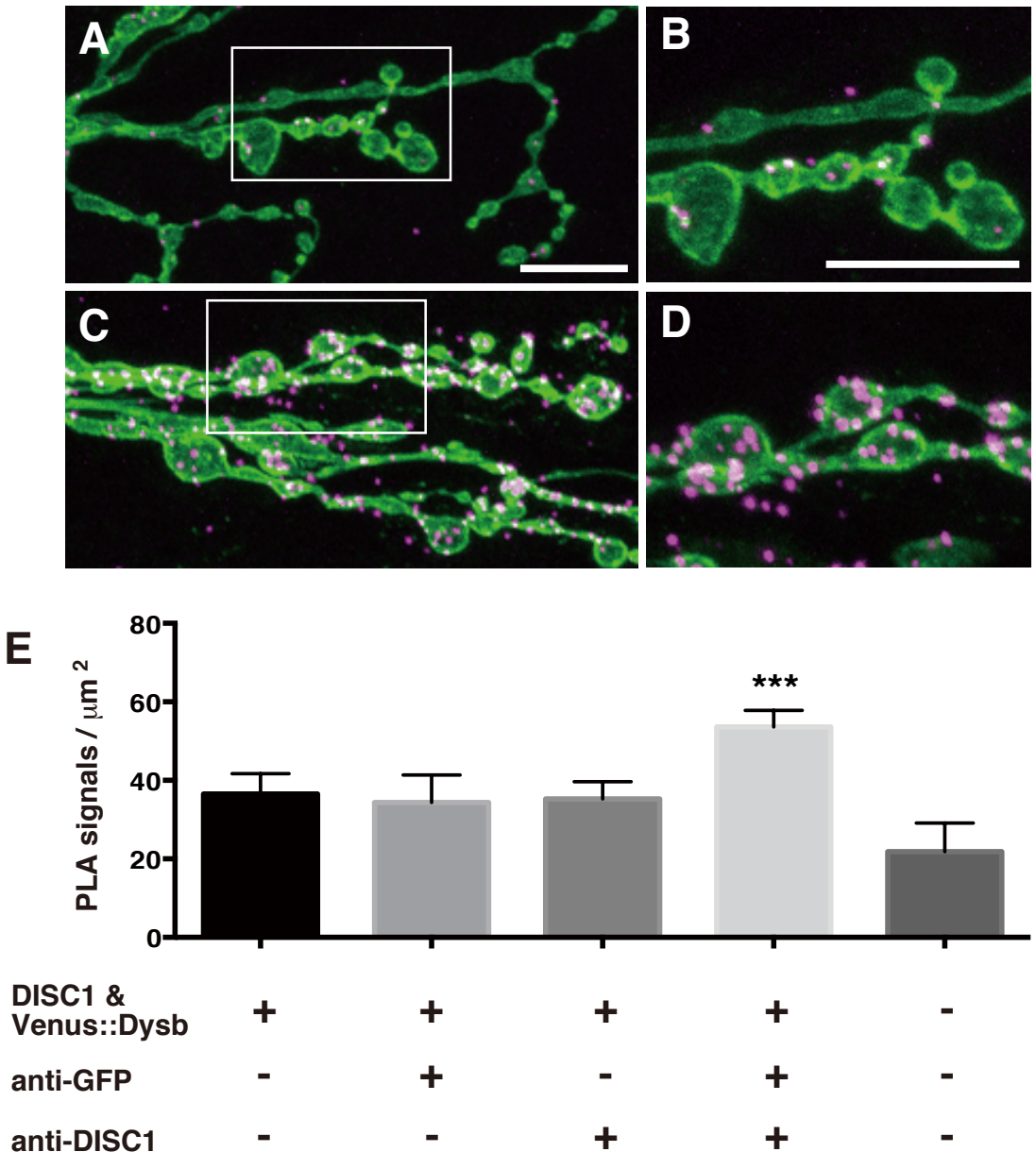

Control
DISC1OE
Merge
HRP
SYT
A
B
A'
B'
A''
B''
C
Total Bouton Area
µm2
600
400
200
0
*
***
w (CS10)
dysb e01028 / +
DISC1
- + - +
D
Number of Boutons
200
150
100
50
0
n.s.
**
w (CS10)
dysb e01028 / +
DISC1
- + - +
E
Number of Branch Points
25
20
15
10
5
0
n.s.
n.s.
w (CS10)
dysb e01028 / +
DISC1
- + - +
F
Individual Bouton Size
4
3
2
1
0
n.s.
***
w (CS10)
dysb e01028 / +
DISC1
- + - +

Figure 4. DISC1 directly interacts with the *Drosophila* Dysbindin in the larval NMJ.

(A–D) Confocal images of PLA signals in the third inster larval NMJ. (A and B) PLA in *w* (CS10) control NMJs. (C, D) PLA in NMJs expressing *Drosophila* Dysbindin and DISC1. UAS-Venus::*Drosophila* Dysbindin and UAS-DISC1 were co-expressed with *tubP*-GAL4. (B, D) Higher magnification of the area indicated in (A) and (C). Green, motor neuron termini labeled with anti-HRP. Magenta, PLA signals. Scale bars, 10 μm. (e). Quantification of PLA signals. Venus::Dysbindin was detected with anti-GFP. *** $P < 0.001$ by one-way ANOVA followed by Dunnett's post hoc test. $n = 9–19$. NMJ, neuromuscular junction; PLA, proximity ligation assay; GFP, green fluorescent protein.


A
B
C
D
E
PLA signals / µm 2
80
60
40
20
0
***
DISC1 &
Venus::Dysb
anti-GFP
anti-DISC1
+ + + + -
- + - + -
- - + + -