DISC1 is needed for the organization of cellular components at the immunological synapse

By

Adarsh Ramakrishnan

Abstract:

T-cells carry out their immune functions through receptor-based recognition and adhesion to target cells. The cell-cell junction that is created from this connection is known as the immunological synapse (IS); a cellular structure composed of organized receptor clusters. Polymerization of F-actin at the synapse has been cited as a necessary step in the continuous activation and flow of T-cell receptors (TCR) towards the center of the synapse and activation of the integrin, Lymphocyte Function Associated Antigen 1 (LFA-1). These processes help T-cells form stable and specific connections with their cognate Antigen Presenting Cells (APCs). It has been previously shown that Disrupted in Schizophrenia 1 (DISC1), a protein with connections to actin-based signaling in neurons, is expressed in T-cells. Through immunofluorescence techniques and CRISPR-Cas9 knockout experiments, we have established that knocking out DISC1 inhibits the polymerization of actin filaments at the synapse. We also identified the actin-binding protein Girdin as a potential effector to actin-based functions of DISC1 at the synapse. We establish that Girdin is expressed at the immunological synapse and that Girdin knockout cell lines showed no detectable actin at the synapse, similar to DISC1 knockouts. Using immunoprecipitation techniques, we also show that DISC1 forms a complex with Girdin in Jurkat cells. Together, these experiments suggest that DISC1 plays a role in actin signaling at the synapse through interactions with Girdin.

Introduction:

In order for T-cells to perform their immune function they need to form physical connections with their target cells. The antigen receptors on T-cells interact with peptides bound to major histocompatibility complex (MHC) molecules found on antigen presenting cells to form an interface that subsequently organizes into a structure called the immunological synapse. The

immunological synapse can be characterized by concentric rings called supramolecular activation clusters (SMACs) which contain receptors and associated signaling proteins (Alarcón, Mestre, & Martínez-Martín, 2011). The innermost ring is called the central supramolecular activation cluster (cSMAC) and mainly contains T-cell receptors, CD28 and PKC-theta. This central ring is surrounded by another SMAC called the peripheral supramolecular activation cluster (pSMAC) which primarily contains the integrin Lymphocyte Function Associated Antigen 1 (LFA-1) and its associated actin-linking adapter protein, Talin. Surrounding the pSMAC is the distal supramolecular activation cluster (dSMAC) which contains microclusters of T-cell receptors and CD45. When T-cell receptors in the cSMAC are activated and CD28 is stimulated, T-cells can bind effectively to their target cells. The integrin LFA-1 helps T-cells form stable connections with their cognate targets by stimulating adhesion between the T-cells and Antigen Presenting Cells (APC) (Bachmann, et al., 1997).

When a T-cell comes into contact with an APC there is a series of cytoskeletal arrangements that lead to the formation of the immunological synapse. This includes the polymerization of F-actin at the outermost edges of contact, coupled with depolymerization of F-actin near the center of the interface (Hammer & Burkhardt, 2013). This cycle of actin polymerization and depolymerization results in the centripetal flow of actin towards the cSMAC. The pushing force generated by actin flow is produced in a structurally distinct region called the lamellipodium, which consists of a branched actin network. Behind the lamellipodium region is the lamellum, which contains linear actin arcs. The lamellum generates a pulling force through myosin II dependent contractions of the actin arcs. This pulling force in the lamellum is coupled with the pushing force generated through actin polymerization-driven retrograde flow in the

lamellipodium to stimulate the flow TCR microclusters and associated signaling molecules to the cSMAC (Yu, Smoligovets, & Groves, 2013). The mechanical force created by F-actin flow also helps the integrin LFA-1 bind to Talin and change its conformation to a high affinity binding state (Cambi, et al., 2006). In this high affinity state, LFA-1 can help in the adhesion of T-cells to their cognate APCs.

Once the immunological synapse is formed, the T-cell microtubule organizing center (MTOC) is translocated to the synapse. This reorganization of the MTOC is achieved through the localization of dynein and associated adapter proteins, Nuclear distribution E homolog 1 (Nde1) and Lissencephaly 1 (Lis1) to the synapse (Nath, et al., 2016). Dynein is anchored to the synapse through Nde1/Lis1 and pulls on microtubules which causes the MTOC to be reeled in towards the synapse. Dynein also forms a secondary complex with Dynactin, which mediates T-cell activity through the transport of lytic granules to the synapse.

Previous studies in our lab have shown that Disrupted In Schizophrenia 1 (DISC1), a scaffolding protein with connections to cell signaling in neurons, is expressed in T-cells (Nath, et al., 2016). While DISC1 has been extensively studied in neurons, its functions related to T-cell signaling remain relatively unknown. Our lab established that DISC1 participates in T-cell activation signaling through independent association with the Nde1/Lis1 and Dynactin complexes. Through interaction with these complexes, DISC1 plays a role in MTOC translocation and transport of mitochondria to the immunological synapse. Our lab was further interested in studying DISC1 functions in T-cells and its potential connection to the actin network. In our study, we also identified the protein Girdin as a potential effector to DISC1

functions at the synapse. In neurons, Girdin has been identified as an actin binding substrate that is an integral component in various signaling pathways (Enomoto, Ping, & Takahashi, 2006).

In this study, we use Jurkat cells and immunostaining techniques to show the expression of Girdin at the immunological synapse. Using CRISPR/Cas9 knockouts, we show that removal of DISC1 in Jurkat cells results in the lack of detectable actin at the synapse. Furthermore, we show that reintroduction of DISC1 to the knockout cell line results in the recovery of actin at the synapse. Similarly, lack of detectable actin was also observed on removal of Girdin using CRISPR/Cas9. Finally, using immunoprecipitation techniques, we show that DISC1 forms a complex with Girdin in T-cells.

Materials and Methods:

Regents and cell lines

The Jurkat (E6.1) and Raji cells were obtained from the American Type Central Collection and the Gryphon cell line was obtained from Dr. Lauren Ehrlich. RPMI 1640 (Cat # 31800022), Opti-MEM (Cat # 31985062), and DMEM (Cat # 12100046) were obtained from Gibco Thermo-Fisher. The 4 mm gap transfection cuvettes were obtained from Fisher Scientific (Cat # FB104). Puromycin (Cat # P8833), goat serum (Cat # G2093) and poly-l-lysine (Cat # P2636) were obtained from Sigma-Aldrich. G418 Sulfate was obtained from Gold Biotechnology (Cat # G-418-45). Mini Plasmid and Midi Fast Ion Plasmid Kits were obtained from IBI Scientific (Cat # IB47111, IB47111). All restriction enzymes were obtained from New England Biolabs. Xfect transfection reagent was obtained from Clonetech (Cat # 631318). The Cas9, DISC1 and Girdin sgRNA plasmids were obtained from Genecopoeia (Cat# CP-LvC9NU-

02-B, HCP268459-LvSG03-1-B, HCP259879-LvSG03-1 respectively). Partially purified Staphylococcus Enterotoxin E (SEE) was obtained from Toxin Technologies (Cat # ET404).

Rabbit anti-DISC1 antibody (Cat # PA2023) was obtained from Boster Biological and CCDC88A polyclonal antibody (Cat # A16132) was obtained from Abclonal. Rabbit anti-GFP antibody (Cat # G1544) was obtained from Sigma-Aldrich. Cell Tracker Blue (Cat # C2110) and MitoTracker CMXRos (Cat # M7512) were obtained from Invitrogen. Rhodamine B Isothiocyanate (Cat# 283924) and TRITC conjugated Phalloidin (Cat # P-1951) were obtained from Sigma-Aldrich.

Cell preparation

Cells from the Jurkat E6.1 human T-cell line and Raji human Burkitt's lymphoma B-cell line were grown in RPMI 1640 (ACC growth media) supplemented with 24 mM sodium bicarbonate, 1 mM sodium pyruvate, 2 mM l-glutamine, 50 µM beta-mercaptoethanol, 10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 10% (v/v) FBS. Gryphon cells were grown in DMEM supplemented with 44.4 mM sodium bicarbonate, 1 mM sodium pyruvate, 2 mM 1-glutamine, 50 µM beta-mercaptoethanol, 10,000 U/mL penicillin, 10,000 µg/mL streptomycin, and 10% (v/v) FBS. The cell lines were cultured at 37°C and 5% CO_2.

DISC1 constructs

Full sized DNA fragments of DISC1 isoform L were synthesized through PCR of Jurkat cDNA. XhoI was inserted into the 5' end of the DISC1 DNA fragments and XmaI was inserted into the 3' end using primers containing the appropriate restriction sites. After finding that the presence of a GFP tag interfered with the function of exogenous DISC1, DNA fragments were

subcloned into a pEGFP-N1 vector that was modified through point directed mutagenesis to contain a premature stop codon inserted directly before the C-terminal EGFP tag.

The DNA constructs were introduced into Jurkat cells through electroporation. Jurkat cells at a concentration of 2 x 10^7 /mL were suspended in Opti-MEM reduced serum medium and incubated with 10 µg of plasmid DNA for 15 minutes at 37^o C. The cells were then transferred to 4 mm gap transfection cuvettes and pulsed at 250V. Following electroporation, the cells were resuspended in fresh ACC growth media. The cells containing the DISC1 constructs were grown under selection with 1 mg/mL G418 for two weeks.

Knockouts using CRISPR/Cas9

Viral particles to be used in Girdin and DISC1 knockouts were prepared through transfection of Gryphon viral packaging cells with Girdin or DISC1 sgRNA expressing lentiviral plasmids, in addition to a Cas9 expressing lentiviral plasmid. The transfections were performed using Xfect transfection reagent. 4 hours following transfection the cells were washed and given fresh media. After 48 hours, viral particle-containing supernatent was collected and directly used to transduce Jurkat cells or stored at -80^{o}C for future use.

2 x 10^6 Jurkat cells were added to six-well plates and spinfected at 500 x g and 30^o C for an hour with media that contained polybrene at 8 µg/mL and viral particles. The spinfection was performed three times for 12 hours each. After spinfection, the media was replaced with fresh growth media. The transduction was confirmed through fluorescence of Enhanced Green fluorescent protein (EGFP) expressed by Cas9 and mCherry protein expressed by Girdin and

DISC1 sgRNA in the plasmids. Complete knockout of Girdin and DISC1 was attained through FACSAria sorting of the GFP and mCherry expressing cells. The sorting was verified through Western blot of the sorted cells. The cells were then grown under selection with G418 Sulfate at a concentration of 1 mg/mL and puromycin at a concentration of 2 µg/mL. The selection process took place 36 hours after transduction for a period of two weeks. Following selection, the cells were sorted using a FACS-Aria cell sorter.

Immunoprecipitation and Western Blot

Jurkat cells were initially pelleted at 750 x g and resuspended in RPMI at a cellular concentration of 1×10^6 /mL. Cells were treated with X phorbol myristate acetate (PMA) or X Vβ8 anti-TCR antibody and incubated at 37°C for 30 minutes. The Jurkat cells were then pelleted at 750 x g and the pellet was resuspended in lysis buffer and homogenized using a 21-gauge needle to create a Jurkat cell lysate. To prepare beads for immunoprecipitation, 5 µg antibody was added to 300µL of Phosphate-buffered saline (PBS) and 80µL of a 50% slurry of Protein A Agarose beads. The solution was mixed using a rotator at 4^{o}C for 24 hours. Subsequently, the beads were washed with lysis buffer for 15 minutes and transferred to the Jurkat cell lysate. The cells were mixed in a rotator at 4^{o}C for 2 hours, after which they were washed 4 times with PBS. The cells were then mixed with SDS-PAGE loading buffer containing glycerol and beta-mercaptoethanol.

The samples prepared were boiled for 5 minutes to separate the protein from the agarose beads and loaded onto a 10% SDS-PAGE gel. The gel was run at 150 V for 1 hour and the samples were subsequently transferred to nitrocellulose paper for Western blotting. The

membrane was blocked with a solution containing 5% Bovine Serum Albumin (BSA) and TBST to prevent non selective binding of antibodies. The primary antibody, diluted to a concentration of 1 µg/mL, was added to the membrane which was then washed three times with TBST for 10 minutes. The membrane was then coated with 5% skim milk and TBST. This was followed by the addition of the secondary antibody at a 1:2000 ratio. The membrane was incubated for an hour at room temperature, followed by three more 10-minute washes with TBST. 500 µL of Enhanced Chemiluminescence (ECL) solution was prepared and then added in a dropwise manner to the membrane to trigger catalysis of the ECL substrate. The membrane was then incubated for 2 minutes at room temperature. The samples were visualized in the darkroom and the results were recorded.

Immunofluorescence

Coverslips prepared for immunostaining were first washed with a 9:1 mixture of 100% ethanol and 1M KOH for 1 hour. The coverslips were subsequently washed with dH_2O and coated with a solution of 0.1 µg/mL poly-l-lysine. These were washed again with dH_2O and left to dry at 25ºC. To prepare the Jurkat-Raji cell pairs, $1x10^6$ /mL of Raji cells were suspended in RPM1 1640 and treated with SEE at 1 µg/mL for 1 hour at 37ºC. These were then stained with 10µM of Cell Tracker Blue (CTB) for 15 minutes. The Jurkat and Raji cells were both washed with ACC media and paired at a ratio of 3 Jurkat cells to 2 Raji cells. The cells were then centrifuged at 500 x g for 5 minutes and washed again with ACC. Following this, the cells were added to the prepared coverslips at a concentration of $1x10^6$ /mL for 15 minutes.

For immunostaining, the cells were fixed with 3 ml of 1% paraformaldehyde for 30 minutes. The cells were washed 3 times with PBS and permeabilized with a 1:1 mixture of acetone and methanol for 15 minutes on ice. The cells were then washed twice in PBS for 5 minutes. A solution of 0.5% Tween was added to the cells for 10 minutes. The cells were then treated with a blocking buffer that consisted of PBS, 5% serum and 0.5% Tween for 30 minutes at room temperature. Following blocking, cells were incubated for 1 hour with primary antibody diluted in blocking buffer. The cells were then washed with PBS and treated with the secondary antibody (or conjugated phalloidin) for another hour before being washed a second time with PBS. Coverslips were mounted on slides with Prolong Gold mounting media and left to cure overnight.

Imaging and data processing

Images were viewed using a Nikon inverted microscope and captured using a CMOS camera. The images were processed and analyzed using the ImageJ processing software. In order to determine protein accumulation at the synapse, a line of length 230 pixels and width 70 pixels was drawn on the Jurkat-Raji cell pairs such that the mid-point of the line (pixel 115) was on the synapse. Background intensity was obtained from a region outside the cell pairs and subtracted from the fluorescence intensity. The fluorescence was normalized by dividing all the pixel measurements by the average intensity of the row furthest away from the synapse. Beginning at the synapse (row 115), intensity values for five-pixel groups were treated as one increment and used for statistical analysis (mean +- SE of the mean). The compound mean and standard error for the increments were plotted against mean intensity of fluorescence. A one-tailed T-test with independent variance was performed for the increments starting from the synapse and moving to the back of the Jurkat cell.

Results:

DISC1 knockouts do not contain actin at the synapse

Using the CRISPR/Cas9 system, a stable DISC1 knockout line was created. The loss of DISC1 expression was confirmed through Western blotting (Fig 1E) and immunostaining of wild type and knockout cells with DISC1 antibody. Wild type cells showed presence of DISC1 whereas the knockouts showed little to no fluorescence (Fig 1A, 1C). When paired with Raji cells, the wild type Jurkat-Raji cells pairs showed localization of DISC1 to the synapse and the knockouts showed little to no fluorescence at the synapse (Fig 1B, 1D).

F-actin was visualized through cytochemical labelling using phalloidin tetramethylrhodamine-isothiocyanate (TRITC) conjugate solution. In Jurkat-Raji cell pairs, the DISC1 knockouts showed significantly less accumulation of actin at the immunological synapse compared to wild type cells (Fig. 2A, 2B). The DISC1 CRISPR/Cas9 phenotype was shown to be not the result of an off-target effect by the reintroduction of the DISC1 isoform L into Jurkat cells. When DISC1 L was expressed, actin at the synapse was identical to that seen in normal Jurkat cells (Fig. 2C). When a student T-test was performed to compare fluorescence intensity between wild type and knockout cell pairs, the first 8 increments of 5-pixel groups starting from the synapse and moving towards the Jurkat cell had p-values lesser than 0.001. Hence, there was a statistically significant difference in mean fluorescence intensity near the synapse (Fig. 2D). These results indicate that DISC1 is required for the localization of actin at the synapse.

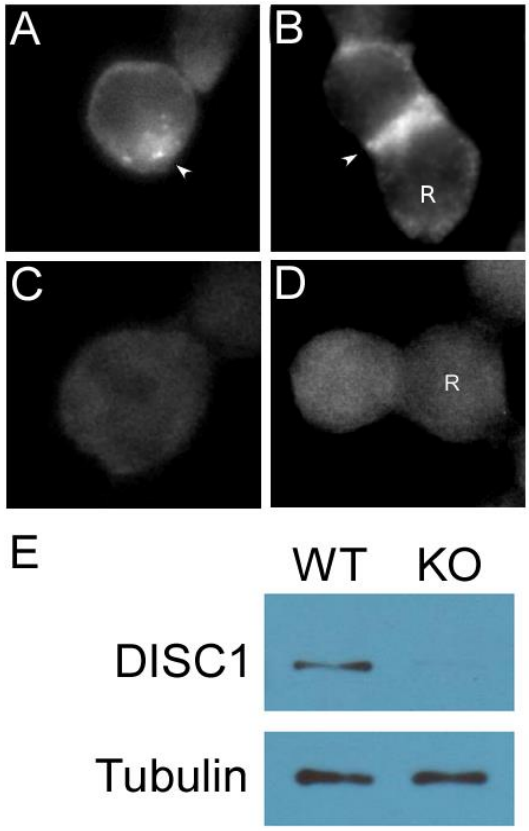

Figure 1. DISC1 in Jurkat cells

(**A**) Wild type Jurkat cells were fixed and immunostained for DISC1 (**B**) Jurkat cells were paired with SEE coated Raji cells, fixed and immunostained for DISC1 (**C**) DISC1 knockout cells were fixed and immunostained for DISC1 (**D**) DISC1 knockout cells were paired with SEE coated Raji cells, fixed and immunostained for DISC1 (**E**) The presence of DISC1 and β-tubulin in wild type Jurkat cells and DISC1 knockout cells was assayed through a Western blot

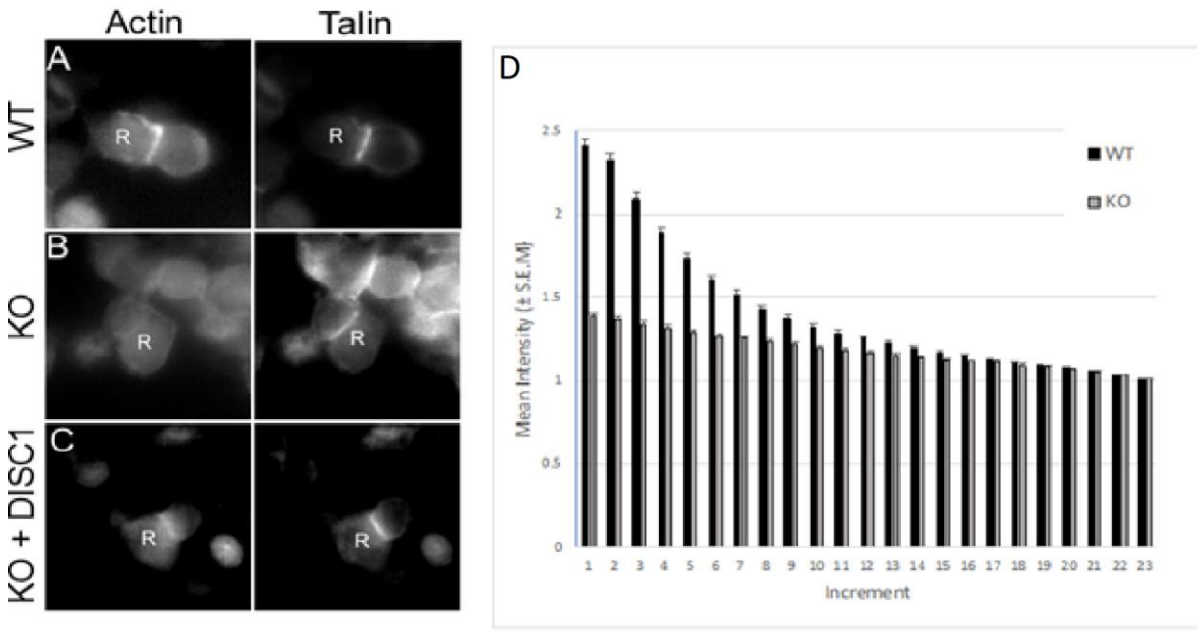

Figure 2. DISC1 plays a role in actin accumulation at the synapse

(**A**) Wild type Jurkat cells were paired with SEE coated Raji cells and immunostained for actin and Talin (**B**) DISC1 knockout cells were paired with SEE coated Raji cells and immunostained for actin (**C**) DISC1 L construct was introduced into the DISC1 knockouts and the resulting Jurkat/Raji conjugates were immunostained for actin (**D**) Mean actin fluorescence intensity +- SEM was plotted for five pixel-wide segments starting at the IS and moving to the back of the Jurkat cell. Data was obtained from 30 wild type or DISC1 knockout Jurkat/Raji cell pairs

Girdin forms a complex with DISC1 and is required for actin accumulation at the synapse.

In order to test for the presence of Girdin, wild type Jurkat cells and Jurkat-Raji conjugates were immunostained with CCDC88A anti-Girdin antibody. Wild type Jurkat cells showed the presence of Girdin which localizaed to the synapse in Jurkat-Raji conjugates (Fig. 3A, 3B). To determine if DISC1 forms a complex with Girdin**,** DISC1 was immunoprecipitated from Jurkat cell lysate and probed for Girdin on a Western blot. The results show that Girdin co-immunoprecipitated with DISC1 in Jurkat cells (Fig 4). This result suggests that DISC1 binds with Girdin to form a complex in Jurkat cells.

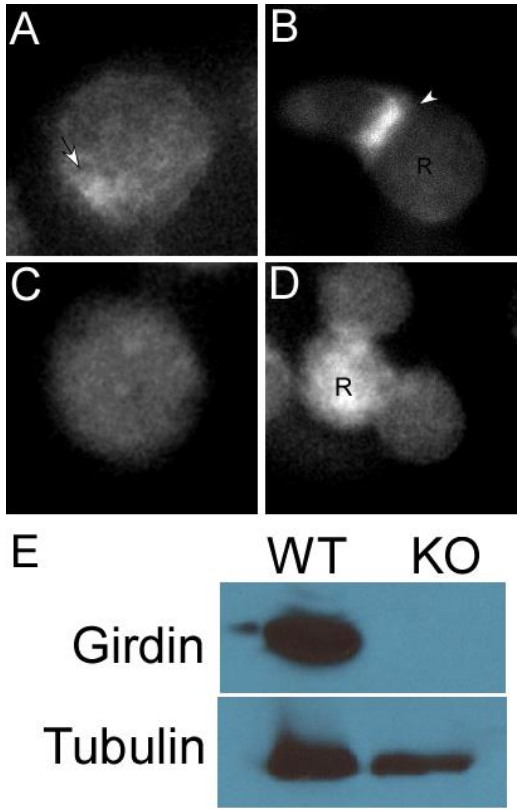

Figure 3. Girdin in Jurkat cells

(**A**) Wild type Jurkat cells were fixed and immunostained for Girdin (**B**) Jurkat cells were paired with SEE coated Raji cells, fixed and immunostained for Girdin (**C**) Girdin knockout cells were fixed and immunostained for Girdin (**D**) Girdin knockout cells were paired with SEE coated Raji cells, fixed and immunostained for Girdin (**E**) The presence of Girdin and β-tubulin in wild type Jurkat cells and DISC1 knockout cells was assayed through a Western blot

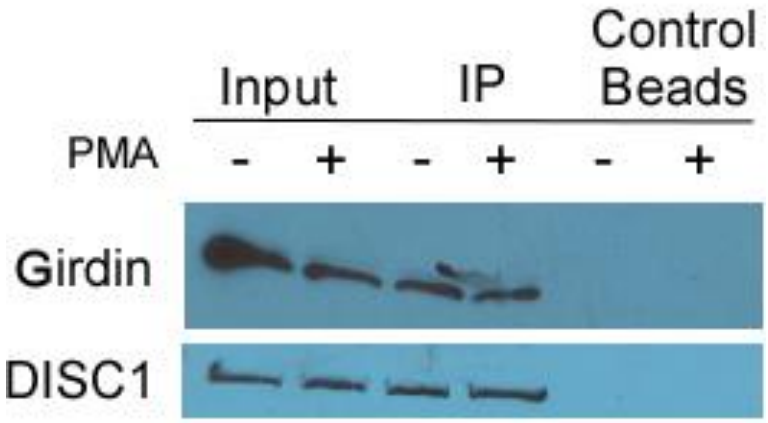

Figure 4. DISC1 forms a complex with Girdin in Jurkat cells

Jurkat cells were treated or untreated with PMA, lysed and immunoprecipitated for DISC1 using DISC1 antibody. Western blots were then probed for Girdin and DISC1.

To determine if Girdin was required for actin accumulation at the synapse a Girdin knockout line was generated using the CRISPR/Cas9 system. Loss of Girdin was confirmed through immunostaining for Girdin (Fig 3C, 3D) and a Western blot (Fig 3E). When the knockout line was paired to Raji cells and stained with phalloidin-TRITC, the knockout line showed no staining whereas the normal cells show a band of fluorescence at the synapse (Fig 5A, 5B). When a student T-test was performed to compare fluorescence intensity between wild type and knockout cell pairs, the first 6 increments of 5-pixel groups starting from the synapse and moving towards the Jurkat cell had p values lesser than 0.001. Hence, there was a statistically significant difference in mean fluorescence intensity near the synapse (Fig. 2D). These results suggest that a knockout of Girdin in Jurkat cells inhibits the localization of actin at the immunological synapse.

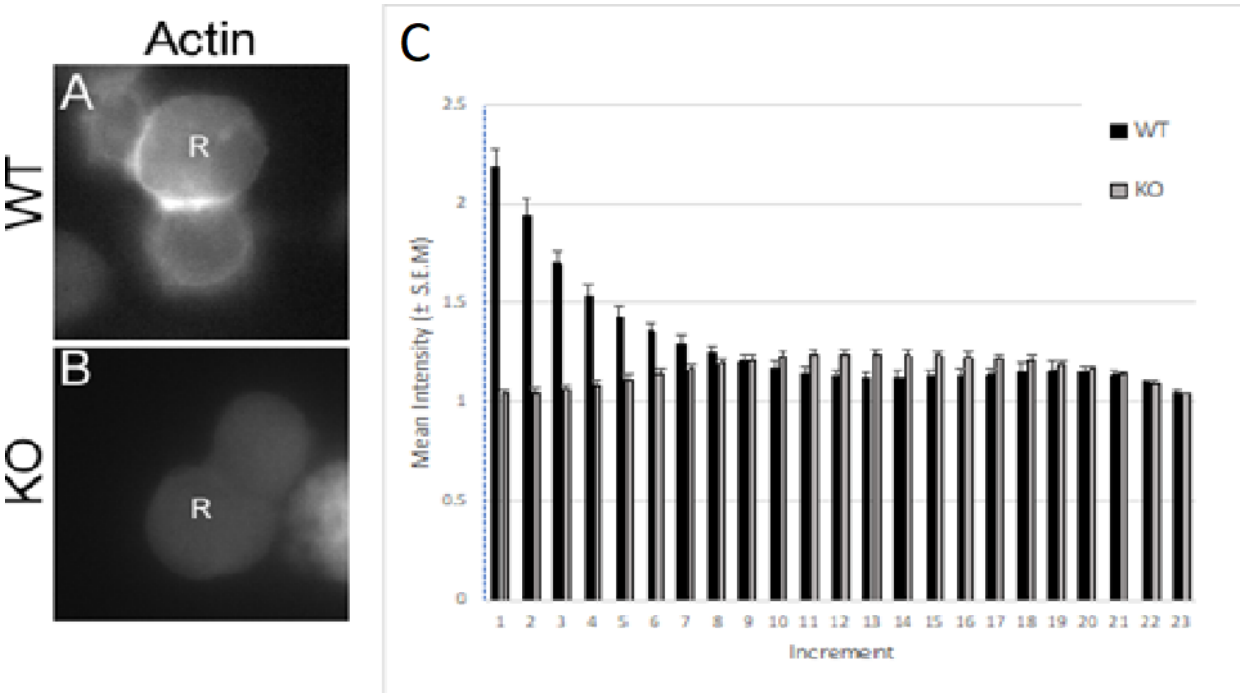

Figure 5. Girdin plays a role in actin accumulation at the synapse

(**A**) Wild type Jurkat cells were paired with SEE coated Raji cells and immunostained for actin (**B**) Girdin knockout cells were paired with SEE coated Raji cells and immunostained for actin (**C**) Mean actin fluorescence intensity +- SEM was plotted for five pixel-wide segments starting at the IS and moving to the back of the Jurkat cell. Data was obtained from 30 wild type or Girdin knockout Jurkat/Raji cell pairs

Discussion:

Previous work in our lab has shown that DISC1 is necessary for the recruitment of various organelles and protein complexes to the immunological synapse through the formation of a complex with the integrin LFA-1. The mechanical force generated by actin flow plays an important role in integrin regulation at the synapse. Hence, our lab was interested in studying the potential connection between DISC1 and actin flow at the synapse.

Through knockout experiments using the CRISPR/Cas9 system, we showed that Jurkat cells lacking DISC1 showed no detectable actin at the synapse when paired with Raji cells. Furthermore, reintroduction of DISC1 isoform L into the knockout cells restored actin at the

synapse. These results indicate that DISC1 is a key component in these actin related functions at the synapse. This has several implications since actin flow is responsible for the organization of various cellular processes at the synapse. The centripetal actin flow is needed for the clustering of TCR complexes at the center of the synapse and regulation of TCR signaling (Hammer & Burkhardt, 2013). Actin flow is also crucial in causing conformational changes in integrins such as LFA-1, which promotes the adhesion of T-cells to ligands on the surface of Antigen Presenting Cells (APC) (Cambi, et al., 2006). Hence, the dynamic actin network plays an important role in coordinating T-cell signaling events at the synapse and promoting the formation of stable connections between T-cells and target cells.

While studying the connection between DISC1 and actin, we investigated potential proteins that could serve as an effector to the actin-based functions of DISC1. Studies have shown that Girdin, an actin binding protein, performs several vital functions in various cellular processes. Girdin is known to bind to actin through its carboxyl terminal domain (Ito, et al., 2013). Glioblastoma LN229 cells with silenced Girdin expression were shown to have significantly reduced F-actin polymerization and decreased migration (Gu, et al., 2014). Silencing of Girdin expression was also shown to cause impaired phosphorylation in adhesion molecules such as focal adhesion kinase (FAK) which lead to defects in cell adhesion. Since T-cells rely on actin polymerization and focal adhesion complexes to form stable connections with their cognate targets, we wanted to test whether Girdin was expressed in T-cells and study its connection to the actin network in the immunological synapse.

Through immunofluorescence experiments, the presence of Girdin at the synapse was confirmed while observing Jurkat-Raji cell pairs stained with CCDC88A polyclonal antibody (figure). Previously established connections between Girdin and actin polymerization suggest that Girdin could potentially play a role in the centripetal flow of actin observed at the synapse and hence affect T-cell activation. When performing knockout experiments with Jurkat cells using the CRISPR/Cas9 system, it was shown that Jurkat cells lacking Girdin showed no detectable actin at the synapse when paired with Raji cells. These results suggest that Girdin interacts with the actin network at the immunological synapse and plays a role in F-actin polymerization.

Since both Girdin and DISC1 knockouts show inhibited actin localization at the synapse, it raises the possibility of connections between the two proteins and their actin-related functions. Results from a previously performed yeast two-hybrid screen have shown physiological interactions between DISC1 and Girdin (Porteous & Millar, 2009). DISC1 is also known to be involved in axonal development in the denate gyrus through interactions with Girdin (Enomoto, et al., 2009). Through immunoprecipitation we showed that DISC1 and Girdin directly interact in T-cells. Experiments previously conducted in our lab have indicated that DISC1 is part of the Nde1/Lis1 complex in T-cells. In neurons, DISC1 and Girdin are both known to dimerize and bind to NDEL1, which in turn binds to Nde1 and Lis1, key proteins in brain development (Porteous & Millar, 2009). This supports our findings that DISC1 and Girdin directly associate in T-cells, and suggests that Girdin could be part of the T-cell DISC1/Nde1/Lis1 complex. Work in our lab has demonstrated that N-terminal GFP/DISC1 fusion proteins do not have the same effect on actin at the synapse as endogenous or untagged exogenously expressed DISC1. Studies have

shown that the Girdin binding site of DISC1 is present at the N-terminal domain, which could explain the observed interference (Enomoto, et al., 2009).

Previous literature suggests that Girdin performs actin related functions through upstream signaling by DISC1. Previous studies have indicated that DISC1 inhibits neuronal migration in granule cells through localization of Girdin and subsequent stabilization of actin stress fibers (Namba & Kaibuchi, 2010). DISC1 is also known to regulate neuronal brain development by interacting with Girdin, which promotes the phosphorylation of the kinase Akt, and binding of Girdin to the actin cytoskeleton (Kim, et al., 2009). Several effects of Girdin appear to be related to its ability to increase Akt activity. Studies have also indicated that Girdin is required for integrin-FAK signaling in tumor cells which results in increased PI3K-Akt activity (Namba & Kaibuchi, 2010). This suggests that Girdin could potentially be involved in similar integrin outside-in signaling in T-cells.

The results of our experiments and previous research can be used to propose a model of DISC1 signaling where Girdin serves as an effector to actin related functions of DISC1 at the synapse. In this model, DISC1 signals Girdin which in turn causes the polymerization of F-actin, centripetal flow of T-cell receptors and activation of LFA-1 at the synapse. The signal ultimately results in the stable adhesion of T-cells to APCs. Girdin could potentially rely on phosphorylation of Akt in order to trigger its actin related functions.

The proposed model of DISC1 signaling at the synapse presents several potential avenues for future research into DISC1 and its connections to Girdin. While the Girdin knockout line

created using the CRISPR/Cas9 system exhibited lack of actin at the synapse, Girdin's role in this result could be confirmed by reintroducing Girdin into the knockout line through a cloned Girdin construct. While prior research has suggested that the amino-terminal globular domain of DISC1 (DISC1-NH) contains the Girdin binding site, further studies can help better elucidate the physical connection between DISC1 and Girdin (Enomoto, et al., 2009). Finally, experiments looking into phosphorylation levels of Akt in T-cells and the impact of Girdin on Akt phosphorylation can provide an insight into Girdin signaling at the immunological synapse.

References:

Yi, J., Wu, X. S., Crites, T., & Hammer, J. A. (2012). Actin retrograde flow and actomyosin II arc contraction drive receptor cluster dynamics at the immunological synapse in Jurkat cells. *Molecular Biology of the Cell, 23*(5), 834-852

Yokosuka, T., & Saito, T. (2009). The Immunological Synapse, TCR Microclusters, and T Cell Activation. *Current Topics in Microbiology and Immunology Immunological Synapse,* 81-107

Nath, S., Christian, L., Tan, S. Y., Ki, S., Ehrlich, L. I., & Poenie, M. (2016). Dynein Separately Partners with NDE1 and Dynactin To Orchestrate T Cell Focused Secretion. *The Journal of Immunology, 197*(6), 2090-2101

Comrie, W. A., Babich, A., & Burkhardt, J. K. (2015). F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse. *The Journal of Cell Biology, 208*(4), 475-491.

Jankowska, K. I., & Burkhardt, J. K. (2017). Analyzing Actin Dynamics at the Immunological Synapse. *The Immune Synapse Methods in Molecular Biology,* 7-29.

Namba, T., & Kaibuchi, K. (2010). Switching DISC1 Function in Neurogenesis: Dixdc1 Selects DISC1 Binding Partners. *Developmental Cell, 19*(1), 7-8.

Porteous, D., & Millar, K. (2009). How DISC1 Regulates Postnatal Brain Development: Girdin Gets In on the AKT. *Neuron, 63*(6), 711-713.

Ohara, K., Enomoto, A., Kato, T., Hashimoto, T., Isotani-Sakakibara, M., Asai, N., . . . Takahashi, M. (2012). Involvement of Girdin in the Determination of Cell Polarity during Cell Migration. *PLoS ONE, 7*(5).

Enomoto, A., Asai, N., Namba, T., Wang, Y., Kato, T., Tanaka, M., . . . Takahashi, M. (2009).

Roles of Disrupted-In-Schizophrenia 1-Interacting Protein Girdin in Postnatal Development of the Dentate Gyrus. *Neuron, 63*(6), 774-787.

Alarcón, B., Mestre, D., & Martínez-Martín, N. (2011). The immunological synapse: A cause or consequence of T-cell receptor triggering? *Immunology, 133*(4), 420-425

Bachmann, M. F., Mckall-Faienza, K., Schmits, R., Bouchard, D., Beach, J., Speiser, D. E., . . . Ohashi, P. S. (1997). Distinct Roles for LFA-1 and CD28 during Activation of Naive T Cells: Adhesion versus Costimulation. *Immunity, 7*(4), 549-557

Hammer, J. A., & Burkhardt, J. K. (2013). Controversy and consensus regarding myosin II function at the immunological synapse. *Current Opinion in Immunology*, *25*(3), 300-306.

Cambi, A., Joosten, B., Koopman, M., Lange, F. D., Beeren, I., Torensma, R., . . . Figdor, C. G. (2006). Organization of the Integrin LFA-1 in Nanoclusters Regulates Its Activity. *Molecular Biology of the Cell*, *17*(10), 4270-4281.

Yu, Y., Smoligovets, A. A., & Groves, J. T. (2013). Modulation of T cell signaling by the actin cytoskeleton. *Journal of Cell Science, 126*(5), 1049-1058.

Enomoto, A., Ping, J., & Takahashi, M. (2006). Girdin, a Novel Actin-Binding Protein, and Its Family of Proteins Possess Versatile Functions in the Akt and Wnt Signaling Pathways. *Annals of the New York Academy of Sciences, 1086*(1), 169-184

Gu, F., Wang, L., He, J., Liu, X., Zhang, H., Li, W., Fu, L. and Ma, Y. (2014). Girdin, an actin-binding protein, is critical for migration, adhesion, and invasion of human glioblastoma cells. *Journal of Neurochemistry*, 131(4), pp.457-469.