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Dynamin 2 regulates T cell activation by controlling actin polymerization at the immunological synapse

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Actin reorganization at the immunological synapse is required for the amplification and generation of a functional immune response. Using small interfering RNA, we show here that dynamin 2 (Dyn2), a large GTPase involved in receptor-mediated internalization, did not alter antibody-mediated T cell receptor internalization but considerably affected T cell receptor–stimulated T cell activation by regulating multiple biochemical signaling pathways and the accumulation of F-actin at the immunological synapse. Moreover, Dyn2 interacted directly with the Rho family guanine nucleotide exchange factor Vav1, and this interaction was required for T cell activation. These data identify a functionally important interaction between Dyn2 and Vav1 that regulates actin reorganization and multiple signaling pathways in T lymphocytes.

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Reorganization of the actin cytoskeleton is pivotal in influencing cellular responses to extracellular stimuli. The interaction of a T cell with an antigen-presenting cell (APC) induces reorganization of the cortical actin cytoskeleton, resulting in the polymerization of F-actin at the cell-cell contact site, thereby anchoring the interaction between the T cell and APC through integrin-dependent adhesion. In addition, reorganization of the actin cytoskeleton is required for T cell activation and is important in forming the immunological synapse and assembly of the 'signalosome' at the cell-cell contact site. Treatment of T cells with the actin-destabilizing agent cytochalasin D inhibits T cell receptor (TCR)–mediated interleukin 2 (IL-2) gene transcription[1], demonstrating that reorganization of the actin cytoskeleton is a requisite event controlling T cell activation.

Over the past decade, biochemical and genetic approaches have identified several signaling proteins activated by TCR engagement that are critical regulators of the actin cytoskeleton. Not unexpectedly, the TCR-proximal tyrosine kinases Lck and Zap70 are required for induction of normal cortical actin reorganization[2]. In addition, Jurkat T cells that overexpress a mutant form of the adaptor molecule protein SLP76 (SLP76-3YF) show defective actin capping after TCR engagement[3]. Moreover, Jurkat T cells deficient in the membrane adaptor Lat demonstrate abnormal actin dynamics when plated on coverslips coated with antibody to TCR (anti-TCR)[4]. Expression of either dominant negative or dominant active Cdc42, a Rho family GTP-binding protein, abrogates cell polarity, indicating that molecular pathways controlling Cdc42 activity are involved in T cell activation[5,6]. In fact, SLP76 and the Rho family guanine nucleotide exchange factor (GEF) Vav1 are required for localized activation of Cdc42 and its target effector protein Wiskott-Aldrich syndrome protein (WASP) at the cell-cell contact site[7]. Consistent with involvement of Vav1 in TCR-initiated actin cytoskeletal dynamics, T cells from Vav1-deficient mice show defects in immunological synapse formation and integrin-mediated adhesion[8]. T cells from WASP-deficient animals and from patients with Wiskott-Aldrich syndrome show defects in TCR capping and actin reorganization in response to TCR crosslinking[9]. Finally, T cells from mice deficient in the kinase Itk have considerable defects in actin rearrangement at the cell-cell contact site, which are at least partially attributable to the failure to recruit Vav1 to the immunological synapse[10]. These data collectively indicate that many signaling proteins that function 'downstream' of the TCR are involved in reorganization of the actin cytoskeleton, yet the mechanism by which these proteins are recruited and how they exert their actin-regulating activities are mostly unresolved. The identification of the molecular pathways that link multisubunit immune recognition receptors to reorganization of the actin cytoskeleton will help elucidate the mechanisms by which immune cells are regulated by this dynamic process.

Members of the dynamin superfamily of large GTPases are involved in reshaping the cortical actin meshwork at the sites of receptor activation[11]. The three conventional dynamin isoforms (Dyn1, Dyn2 and Dyn3) share at least 70% homology and show discrete patterns of gene expression[12]. Structurally, these proteins contain N-terminal GTP-binding, middle and GTPase effector domains that are involved in oligomerization and regulation of GTPase activity; a pleckstrin homology domain for interaction with membrane phosphoinositides;

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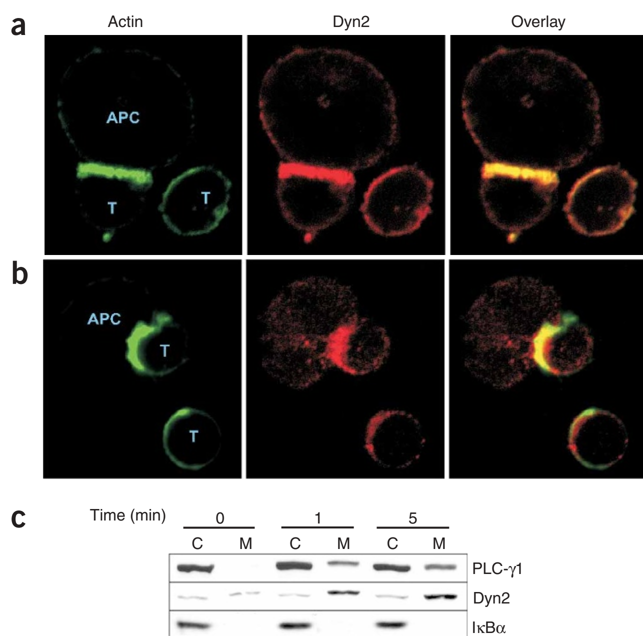

Figure 1 Dyn2 is recruited to the T cell–APC contact site. (**a**) Ovalbumin peptide–pulsed A20 B cells were incubated with secondary DO11.10 transgenic T cells. The conjugated cells were bound to poly-L-lysine-treated coverslips, fixed, and stained with anti-Dyn2 and fluorescein isothiocyanate–phalloidin to label F-actin. (**b**) Raji B cells were pulsed with a 'cocktail' of superantigens, incubated with primary human $CD4^+$ T cells and stained as described in **a**. 'T' indicates DO11.10 and human $CD4^+$ T cells. For **a** and **b**, APCs were distinguished from T cells with CMAC-7 tracker (data not shown). (**c**) Jurkat T cells were stimulated (times, above blot) with mAb to CD3 and mAb to CD28 and were subjected to a serial membrane (M)–cytosol (C) extraction protocol. The extracts were analyzed by immunoblot (antibodies, right margin). IκBα, inhibitor of κBα. Data are representative of three independent experiments.

and a C-terminal proline and/or arginine–rich domain that directly binds to the Src homology 3 (SH3) domains of multiple actin-regulating proteins, including profilin, Nck, Grb2, cortactin, syndapin and intersectin (a Cdc42-specific GEF)[11]. It is through these interactions that dynamins are thought to link the plasma membrane to actin, thus modulating cell shape, podosomal adhesion, phagocytosis and vesicle movement. Because of dynamin's potential function as part of a multisubunit signaling scaffold at the membrane-cytoskeleton interface[11], we investigated the function of Dyn2, the only dynamin found in the hematopoietic system, in T cell signaling. Here we show that Dyn2 controls actin cytoskeletal changes and distal TCR signaling events during T cell activation.

RESULTS

Dynamin2 is recruited to the immunological synapse

To investigate the function of Dyn2 in T cell activation, we first examined whether Dyn2 localizes to the T cell–B cell interface in DO11.10 T cells interacting with ovalbumin peptide–pulsed A20 B cells. Although the isolated T cells had a cortical actin ring and diffuse Dyn2 staining, T cells interacting with APCs demonstrated increased F-actin staining and recruitment of Dyn2 to the cell-cell contact site (**Fig. 1a**). We obtained similar results in primary human $CD4^+$ T cells interacting with Raji B cells pulsed with a 'superantigen cocktail' (**Fig. 1b**). We did not find recruitment of Dyn2 and actin reorganization in T cell–B cell conjugates formed in the absence of ovalbumin peptide or superantigen (data not shown). As a biochemical means of determining whether Dyn2 is recruited to the membrane after T cell activation, we prepared cytosolic and membrane fractions from Jurkat T cells stimulated with monoclonal anti-CD3 and monoclonal anti-CD28. Before stimulation, Dyn2 was present in equivalent amounts in the membrane and cytosolic fractions (**Fig. 1c**). However, within 1 min after CD3 and CD28 crosslinking, Dyn2 began accumulating in the membrane fraction (**Fig. 1c**). The movement from the cytosol to the membrane fraction mirrored that for phospholipase C-γ1 (PLC-γ1), a protein recruited to the membrane during T cell activation[13]. This was not because of contamination with cytosolic proteins, as there was no evidence for IκBα in the membrane fraction. These results suggested a potential function for Dyn2 in T cell activation.

Dyn2 regulates T cell activation responses

To examine whether Dyn2 has a function in regulating T cell activation, we used small interfering RNA–mediated gene silencing to acutely silence Dyn2 in Jurkat T cells and then measured IL-2 promoter (IL-2p) activity. Dyn2 short hairpin RNA (shRNA), but not a Dyn2 mutant targeting construct, effectively decreased Dyn2 protein by 48 h after transfection and maintained suppression for nearly 96 h (**Fig. 2a**). Consistent with a function for Dyn2 in T cell activation, Dyn2 'knockdown' induced a substantial defect in IL-2p activation after crosslinking with TCR or TCR plus CD28 or after incubation with superantigen-pulsed NALM6 B cells (**Fig. 2b** and data not shown). Pharmacological stimulation of IL-2p by the addition of phorbol 12-myristate 13-acetate plus ionomycin was not affected by the suppression of Dyn2, indicating that Dyn2-depleted cells were functional but were merely incapable of linking TCR-stimulated signaling events to the activation of the IL-2p (**Fig. 2b**). Using a mouse Dyn2 suppression vector we were able to effectively deplete Dyn2 from the mouse thymoma EL-4 (**Fig. 2c**, inset). Consistent with the data in human T cells, we noted similar effects in DO11.10 T cells transfected with the mouse Dyn2 suppression construct and stimulated with A20 B cells pulsed with ovalbumin peptide (**Fig. 2c**). These experiments suggest that Dyn2 can couple TCR-mediated signaling pathways to those regulating IL-2p.

To further characterize the T cell activation defect in Dyn2 suppressed cells, we analyzed the upregulation of the activation marker CD69 after TCR crosslinking in the Jurkat T cell line. Because the Rho GEF Vav1 regulates CD69 expression in T cells[13], we used a published Vav1 suppression vector as a positive control[14]. The vector control–transfected cell population showed increased CD69 expression after TCR-CD28 crosslinking (**Fig. 2d**). In contrast, cells transfected with the Dyn2 suppression vector showed a reproducible defect in CD69 expression similar to that achieved by suppression of Vav1 (**Fig. 2d**, left). We obtained similar CD69 expression in the control and shRNA-transfected cell populations when TCR signals were bypassed using phorbol 12-myristate 13-acetate and ionomycin (**Fig. 2d**, right). These data collectively showed that Dyn2 functions in TCR-mediated signaling leading to both IL-2p activity and CD69 upregulation.

Dyn2 does not affect TCR endocytosis

One function of dynamin proteins is the regulation of endocytosis of activated cell surface receptors. We therefore sought to determine if Dyn2 suppression could affect either the amount of TCR on the cell surface or the endocytosis of activated antigen receptors after receptor crosslinking. At 48 h after transfection, we measured cell surface TCR expression of Jurkat T cells transfected with control shRNA and Dyn2 knockdown Jurkat T cells by flow cytometry. Dyn2 knockdown did

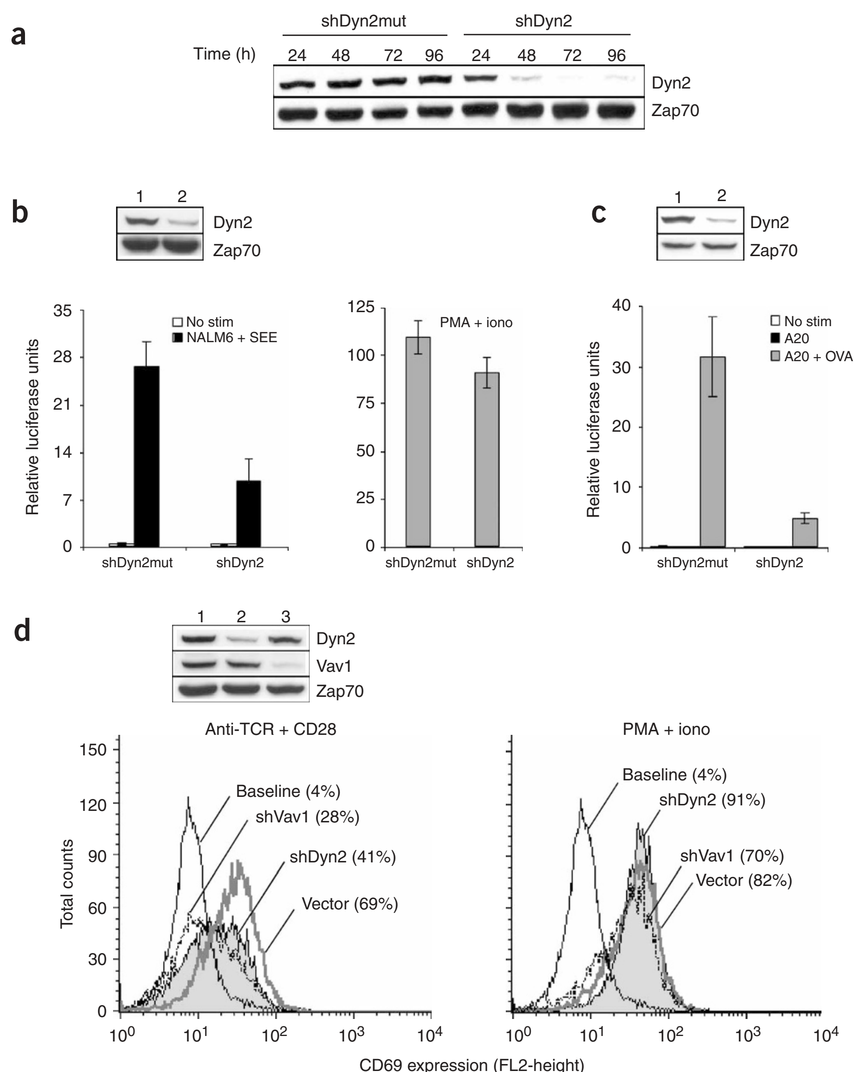

Figure 2 Dyn2 regulates T cell activation. (**a**) shRNA suppression time course for Dyn2. Jurkat T cells were transfected with mutant Dyn2 shRNA (shDyn2mut) or Dyn2 shRNA (shDyn2) targeting vectors. Cell lysates were prepared after transfection (time, above lanes) and equivalent amounts of protein were separated by SDS-PAGE, transferred to nylon membrane and analyzed for Dyn2 and Zap70 by immunoblot. (**b**) Jurkat cells were cotransfected with IL-2p.luc luciferase reporter and either shDyn2mut (lane 1) or shDyn2 (lane 2) suppression plasmid; inset, immunoblot. After 48 h, cells were left unstimulated (No stim) or were stimulated for 5 h with SEE-pulsed NALM6 B cells (NAML6 + SEE), or were stimulated with phorbol 12-myristate 13-acetate plus ionomycin (PMA + iono), and luciferase activity was measured and normalized. (**c**) Inset, EL-4 mouse thymoma cells were transfected with shDyn2mut (lane 1) or shDyn2 (lane 2) targeting plasmid. Cell lysates were prepared at 48 h after transfection and were analyzed for Dyn2 protein expression as described in **a**. Bottom, secondary DO11.10 T cells were cotransfected with IL-2p.luc luciferase reporter and either shDyn2mut or shDyn2. After 18 h of incubation, an equal number of cells were left unstimulated (No stim) or were incubated with A20 B cells pulsed with ovalbumin peptide (A20 + OVA) or were left unpulsed (A20). After 16 h of stimulation, luciferase activity was measured and normalized. Bars (**b**,**c**) represent the mean ± s.d. from triplicate samples. (**d**) Jurkat T cells were transfected with control vector (lane 1), Dyn2 shRNA (lane 2) or shVav1 shRNA (lane 3) targeting plasmids; inset, immunoblot. After 48 h, each cell population was left unstimulated or was stimulated as indicated above plots, was stained with anti-CD69–phycoerythrin and was analyzed by flow cytometry for CD69 expression. The baseline histogram is representative of basal CD69 expression by all cell populations. Percents (in parentheses) indicate the percentage of cells positive for CD69 expression.

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not affect cell surface TCR expression (**Fig. 3a**). To investigate whether Dyn2 suppression affected TCR internalization, we analyzed the internalization of the TCR after crosslinking in control and Dyn2 knockdown Jurkat T cells labeled with anti-CD3ε–phycoerythrin. At various time points, we removed all surface-labeled antibody by acid stripping and then analyzed the cells by flow cytometry to measure internalized antibody. Dyn2 knockdown and control shRNA–transfected cells showed similar kinetics of TCR internalization, indicating that the absence of Dyn2 did not affect TCR internalization rates after crosslinking (**Fig. 3b**). We obtained similar results using phycoerythrin-labeled anti-TCRα/β to measure the rates of TCR internalization and surface re-expression after anti-CD3 crosslinking (data not shown). These data collectively indicate that Dyn2 does not regulate T cell activation by altering TCR cell surface expression or internalization rates after antibody crosslinking.

Dyn2 does not affect proximal TCR signaling

After TCR crosslinking, Lck phosphorylates the immunoreceptor tyrosine-based activation motifs in the TCR ζ chains, providing docking sites for Zap70. To determine whether TCR ζ-chain phosphorylation (an indicator of Lck activity) or Zap70 phosphorylation was affected by Dyn2 suppression, we immunoprecipitated Zap70 from control or Dyn2-depleted Jurkat T cells after stimulation with fixed NALM6 B cells labeled with superantigen. Both control and Dyn2-depleted cells show similar kinetics of Zap70 tyrosine phosphorylation (**Supplementary Fig. 1** online). Moreover, we noted similar kinetics and amounts of tyrosine phosphorylation of the Zap70-coimmunoprecipitated TCR ζ chain in these cell populations, indicating that TCR-mediated Lck activation is intact in the absence of Dyn2 (**Supplementary Fig. 1** online). Therefore, these data indicate that the inability of Dyn2-suppressed cells to activate either IL-2- or CD69-mediated gene transcription after TCR engagement is not due to defects in the activation of either Lck or Zap70. Thus, Dyn2 must be functioning in an alternate capacity downstream of these proximal tyrosine kinases during T cell activation.

Dyn2 regulates actin reorganization at the immunological synapse

Dynamins can link activated membrane receptors with actin cytoskeletal changes through their interactions with multiple actin-regulating proteins[11]. Because TCR-induced cytoskeletal changes are a requisite

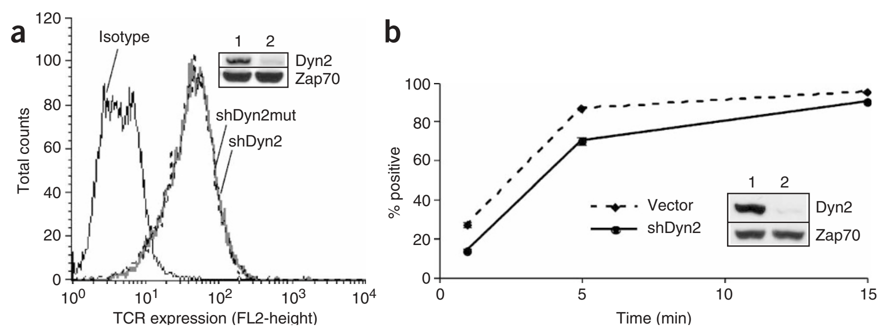

Figure 3 TCR expression and antibody-mediated internalization are not altered in Dyn2 suppressed cells. (**a**) Jurkat T cells were transfected with either shDyn2mut control (lane 1) or shDyn2 (lane 2); inset, immunoblot. After 48 h, transfected cells were stained on ice with phycoerythrin-labeled mAb to TCRα/β and cell surface expression of the TCR was analyzed by flow cytometry. (**b**) Jurkat T cells were transfected with either vector control (lane 1) or shDyn2 (lane 2); suppression of Dyn2 was analyzed by immunoblot (inset; antibodies, right margin). After 48 h, the cells were stained on ice with mAb to CD3, crosslinked with goat anti-mouse IgG at 37 °C (time, horizontal axis), incubated immediately in ice-cold stripping buffer to remove surface-bound antibodies and analyzed by flow cytometry for percent cells positive for TCR internalization. The average rate of TCR internalization between 1 and 5 min (vector, 14.84 ± 1.31; shDyn2, 13.98 ± 2.22) and 10 and 15 min (vector, 0.86 ± 0.132; shDyn2, 2.02 ± 0.093) was similar for the two cell populations. Data are a representative example of two experiments done in triplicate.

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event controlling T cell activation, we investigated the function of Dyn2 in controlling actin polymerization at the T cell–APC contact site. To examine this, we transfected Jurkat T cells with Dyn2 or vector control shRNA vectors that contained a separate green fluorescent protein (GFP) transcriptional cassette (shRNA-GFP) to identify transfected T cells. We allowed the transfected cells to form conjugates with superantigen-pulsed NALM6 B cells. We analyzed conjugates formed with GFP$^+$ T cells for Dyn2 and F-actin at the T cell–B cell contact site by immunofluorescence microscopy. In contrast to the vector control–transfected GFP$^+$ population, T cells transfected with the Dyn2 shRNA–GFP vector failed to reorganize F-actin at the cell-cell contact site (**Fig. 4a**). Analysis of the frequency of conjugates with actin polarization at the T cell–APC contact site showed that Dyn2 suppression decreased actin reorganization to background amounts, as defined by conjugates formed in the absence of superantigen (**Fig. 4b**). These defects in the actin cytoskeleton are not attributable to inefficient conjugate formation by the Dyn2-suppressed cells, because T cells with Dyn2 knockdown were as efficient in forming conjugates as the control transfectants (**Fig. 4c**). These data identify Dyn2 as a critical regulator of the actin cytoskeleton in response to TCR engagement.

The Dyn2 proline-rich domain regulates T cell activation

Dynamins regulate multiple cellular functions through self-assembly, GTPase activity, localization and protein-protein interaction. In fact, the proline-rich domains (PRDs) of dynamins can associate with multiple SH3 domain–containing signaling molecules, including proteins involved in actin cytoskeletal dynamics[11,15]. To investigate whether GTP binding or the PRD of Dyn2 is required for T cell activation, we analyzed IL-2p activity in Jurkat T cells overexpressing wild-type Dyn2, a Dyn2 mutant in which the PRD domain had been deleted (Dyn2ΔPRD) or a GTP-binding-deficient version of Dyn2 (K44A). In cells stimulated by TCR or TCR and CD28 crosslinking, overexpression of wild-type Dyn2 reproducibly increased IL-2p activity (**Fig. 5a** and data not shown). Expression of the K44A mutant, which functions as a dominant negative in other experimental systems[16], acted like wild-type Dyn2 in TCR-CD28–stimulated IL-2p activity (T.S.G. and D.D.B., data not shown). In contrast, the PRD deletion mutant (Dyn2ΔPRD) did not stimulate IL-2p activity and functioned as a dominant negative protein, inhibiting both TCR and TCR-CD28–stimulated IL-2p activity (**Fig. 5a** and data not shown). Thus, these data identify a functional requirement for the Dyn2 PRD in linking TCR-CD28–mediated signals to IL-2p activation.

Among the SH3 domain containing proteins that bind the dynamin PRD are actin regulatory proteins and other proteins that have key functions in T cell activation[11,15,17]. However, the biological relevance of these interactions has not been completely addressed. To identify whether these known or other previously unknown interactions occur in T cells, we made glutathione *S*-transferase (GST) fusion proteins

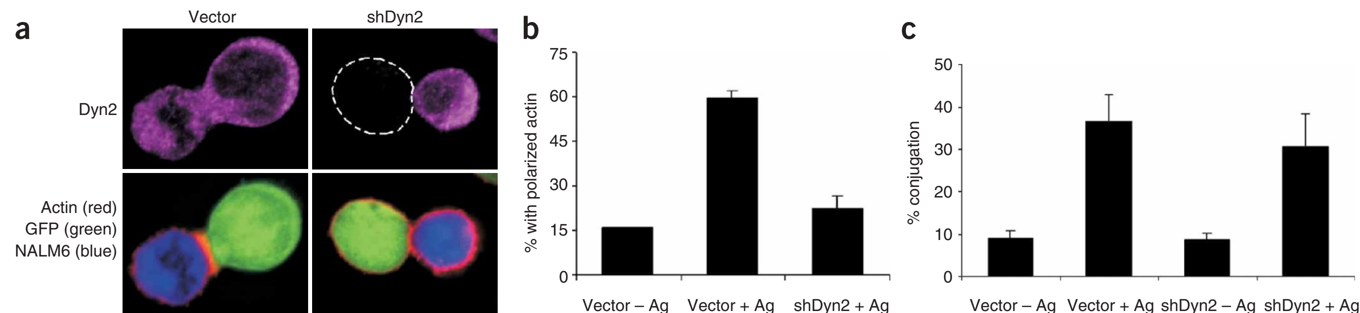

Figure 4 Dynamin2 suppression inhibits actin polarization at the T cell–APC contact site. (**a**) Jurkat T cells were transfected with control or Dyn2 shRNA targeting vectors containing a separate GFP transcriptional cassette. After 72 h of incubation, the transfected cells were incubated with SEE-pulsed NALM6 B cells (blue). The Jurkat-NALM6 conjugates were bound to poly-L-lysine-treated coverslips, fixed and stained with rhodamine-phalloidin for visualization of polymerized F-actin (red). Dyn2 was detected with anti-Dyn2 followed by indodicarbocyanine–goat anti-rabbit (violet). White outline indicates the Dyn2-suppressed Jurkat T cell. (**b**) Conjugates from **a** were counted and assigned scores for actin polymerization. – Ag, no antigen; + Ag, with antigen. (**c**) Jurkat T cells were transfected as described in **a** and were incubated with EBV-B cells that had been stained red with PKH26 and were pulsed with SEE. Percent conjugation was determined by two-color flow cytometry. Bars (**b**,**c**) represent the mean ± s.d. from three independent experiments.

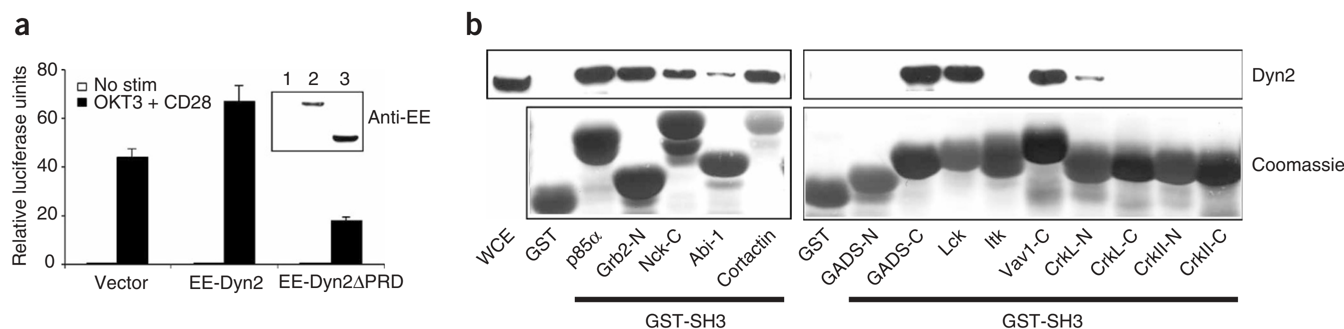

Figure 5 The Dyn2 PRD is required for T cell activation. (**a**) Jurkat cells were cotransfected with IL-2p.luc luciferase reporter and either vector control (lane 1), EE-Dyn2 (lane 2) or EE-Dyn2ΔPRD (lane 3) expression plasmids; EE.Dyn2 expression was verified by immunoblot. After 48 h, cells were left unstimulated or were stimulated with anti-CD3 and anti-CD28, and luciferase activity was measured. Bars represent the mean ± s.d. from triplicate samples. (**b**) Equal volumes of Jurkat whole-cell lysates were incubated with GSH-agarose–bound GST fusion proteins of various SH3 domains from either known (left) or potential (right) Dyn2-interacting proteins. Dyn2 was detected by immunoblot with anti-Dyn2, and loading of GST fusion protein was visualized by Coomassie blue staining. WCE, 100 μg of whole-cell extract presented for Dyn2 input. P58α, regulatory subunit of phosphatidylinositol-3 kinase; Grb2, growth factor receptor–bound protein 2; Abi-1, abl-interacting protein 1; GADS, Grb2-related adaptor downstream of Shc; CrkII, CT10 regulator of kinase; CrkL, Crk-like; -N, N-terminal SH3; -C, C-terminal SH3. (**c**) Cells were left unstimulated (US) or were stimulated with plate-bound mAb to TCR (TCR) or mAb to TCR plus mAb to CD28 (TCR + CD28). Anti-Vav1 or normal rabbit serum (NRS) was used to immunoprecipitate (IP) equal amounts of clarified whole-cell lysates, and bound proteins were detected by immunoblot. 2°, secondary; pTyr, phosphorylated Tyr. Data are a representative example of three independent experiments.

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containing SH3 domains from several intracellular signaling molecules involved in T cell activation. We prepared cell lysates from Jurkat T cells and determined if Dyn2 could coprecipitate with various GST-SH3 domains. The SH3 domains of several Dyn2-interacting proteins, including p85α, Grb2, Nck, cortactin and Abi-1 (refs. 11,15,17,18) were able to coprecipitate endogenous Dyn2 (**Fig. 5b**). In addition, we identified several other interactions with Dyn2, including an interaction with the SH3 domains from Lck, Gads and CrkL and, most notably, the C-terminal SH3 domain of the Rho family GEF Vav1 (**Fig. 5b**). Thus, Dyn2 has the potential to interact with several intracellular signaling molecules that are intimately involved in regulating T cell activation.

Because Vav1 is a phosphoprotein whose GEF activity is regulated by tyrosine phosphorylation downstream of the TCR[19] and Vav1 is a critical regulator of the actin cytoskeleton in T cells by regulating Rho family GTP-binding proteins, we investigated this interaction in more depth. We determined if Vav1 and Dyn2 interact endogenously and established whether the interaction was constitutive or induced by TCR crosslinking. To investigate this, we immunoprecipitated endogenous Vav1 from DO11.10 thymocytes (**Fig. 5c**, left) or DO11.10 secondary T cells (**Fig. 5c**, right) that were left unstimulated or were stimulated through the TCR or TCR-CD28 over various time courses. Vav1 and Dyn2 were coimmunoprecipitated from T cell lysates (**Fig. 5c**). As expected, Vav1 underwent TCR-CD28–induced tyrosine phosphorylation (**Fig. 5c**, top) in both thymocytes and peripheral T cells, yet there was no detectable change in its association with Dyn2 after TCR crosslinking (**Fig. 5c**, middle). We were unable to coimmunoprecipitate Vav1 and Dyn2 from J.Vav1 cells, which lack Vav1 (ref. 13) expression, but did effectively coimmunoprecipitate Vav1 and Dyn2 when Vav1 was re-expressed in these cells (**Supplementary Fig. 2** online). Although Dyn2 can undergo tyrosine phosphorylation during caveolae-mediated endocytosis in a Src-dependent way[20], we did not detect basal or TCR-mediated tyrosine phosphorylation of Dyn2 in either mouse T cells or Jurkat T cells (data not shown). These data demonstrate that endogenous Vav1 and Dyn2 interact in a constitutive way.

The Dyn2 interaction is specific to Vav1

As all three Vav isoforms can be expressed in T cells, we tested whether the C-terminal SH3 domains of Vav2 and Vav3 also interact with Dyn2. Using GST-SH3 fusion proteins of the various Vav family members, we found only the Vav1 C-terminal SH3 domain was able to coprecipitate dynamin from Jurkat T cell lysates (**Fig. 6a**). The interaction between the Vav1 SH3 domain and Dyn2 requires a functional SH3 domain, as a GST fusion protein containing a mutant Vav1 SH3 domain (W/Y) was unable to coprecipitate Dyn2 (**Fig. 6b**). To determine if the Dyn2-Vav1 interaction was direct or was mediated by another protein, we did a blot overlay assay. We transfected Jurkat T cells with a version of Dyn2 containing an EE epitope tag and then immunoprecipitated EE-Dyn2, separated it by SDS-PAGE and transferred it to a membrane. We then probed the blots with either GST or the GST-SH3 domain fusion protein of Vav1. We detected bound protein using polyclonal anti-GST. The GST-Vav1-SH3 fusion protein bound the immobilized Dyn2, indicating that the interaction between Vav1 and Dyn2 was direct (**Fig. 6c**).

Because SH3 domains interact with polyproline stretches, we sought to determine if the Dyn2-Vav1 interaction required the Dyn2 PRD. The GST-SH3 domain fusion protein of Vav1 coprecipitated EE-tagged full-length Dyn2 from transfected Jurkat T cell lysates but not the deletion mutant of Dyn2 lacking the PRD (**Fig. 6d**, bottom; Dyn2

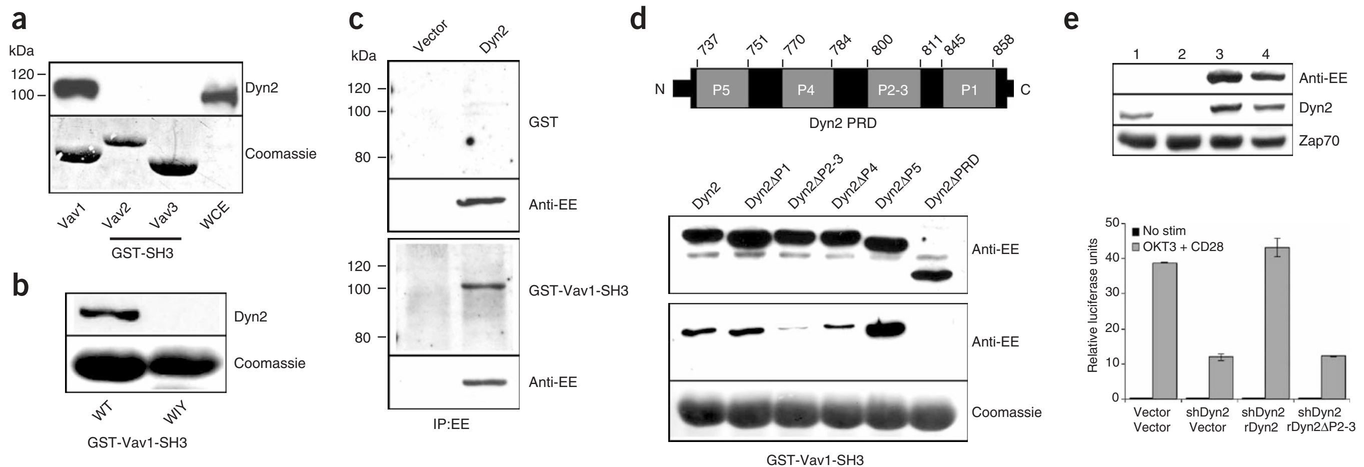

Figure 6 The Dyn2 PRD specifically, directly and functionally interacts with the C-terminal SH3 domain of Vav1. (**a**,**b**) Equivalent amounts of Jurkat whole-cell lysate were incubated with GST or GST-SH3 fusion proteins (below lanes) bound to GSH-agarose, and bound Dyn2 was detected by immunoblot. (**c**) EE-Dyn2 was immunoprecipitated from control or EE-Dyn2-expressing Jurkat cells, resolved by SDS-PAGE and then probed with GST or GST-Vav1-SH3 fusion proteins followed by rabbit anti-GST and protein A–coupled horseradish peroxidase. (**d**) Top, Dyn2 PRD, which contains four distinct proline-rich areas, P1, P2-3, P4 and P5. Below, Jurkat T cells were transfected with EE-tagged Dyn2 or various PRD deletion mutants and were immunoprecipitated with anti-EE (top) or Vav1 GST-SH3 (bottom), and bound proteins were detected by immunoblot. GST fusion protein in each coprecipitation was assessed by Coomassie blue staining. (**e**) Jurkat T cells were cotransfected with IL-2p-luc reporter plasmid along with either the appropriate control vector (lane 1) or shDyn2 RNA targeting vector, or control (lane 2), EE-tagged Dyn2 (lane 3) or EE-Dyn2ΔP2-3 (lane 4) expression plasmids containing shRNA-resistant cDNAs of Dyn2 (rDyn2). Whole-cell lysates prepared from each of the transfected cell populations were immunoblotted with anti-EE, showing Dyn2 re-expression and with anti-Dyn2 verifying suppression and re-expression. Cells were stimulated and luciferase activity was measured. Bars represent the mean $\pm$ s.d. from triplicate samples.

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versus Dyn2ΔPRD). To further characterize the interaction between Vav1 and Dyn2, we made several deletions within the Dyn2 PRD based on the identified proline-rich regions, P1–P5 (ref. 21; **Fig. 6d**) and examined their binding to the Vav1 GST-SH3 domain fusion protein. The Vav1 SH3 domain GST-fusion protein was as efficient in coprecipitating the EE-tagged ΔP1 and ΔP5 mutants as the wild-type protein, but was less efficient in coprecipitating the ΔP4 mutant, and was far less efficient in coprecipitating the ΔP2-3 mutant (**Fig. 6d**). These data suggest that the interaction of Dyn2 and Vav1 is mediated through a proline-rich motif within the P2-3 region but may also require some structural elements provided by the P4 region of the Dyn2 PRD.

The data above showed a direct link between Dyn2 and Vav1, but it was unclear if the physical association of Dyn2 and Vav1 was required for T cell activation. We therefore sought to determine whether the 12–amino acid P2-3 region of the Dyn2 PRD was required for linking TCR crosslinking to IL-2p. Using Dyn2 knockdown Jurkat T cells, we found that expression of a wild-type RNA interference–resistant version of Dyn2 'rescued' IL-2p activity, whereas the resistant version of Dyn2ΔP2-3 was unable to restore IL-2p activity (**Fig. 6e**). These data emphasize an important, functional interaction between Vav1 and Dyn2 that is required for TCR-mediated IL-2p activation.

Dyn2 regulates distal TCR signaling events

Overexpression studies and reconstitution of a Vav1-deficient Jurkat T cell line, J.Vav1, showed a critical function for the C-terminal SH3 domain of Vav1 in TCR-induced responses, including gene transcription mediated by the transcription factors NFAT and AP-1 and activation by the kinase Jnk[22,23] (D.N.S. and D.D.B., data not shown). In addition, Vav1 can participate in TCR-mediated signaling cascades controlling phosphorylation of the kinase Erk and CD69 upregulation[24,25]. Because our data (**Fig. 6e**) suggested that T cell activation requires a functional interaction between Vav1 and Dyn2, we investigated whether Dyn2 was involved in modulating Vav1-mediated signaling events. We initially assessed the affect of Dyn2 suppression on Erk and Jnk activation using phosphorylation-specific antibodies on lysates from Jurkat T cells stimulated with superantigen-pulsed NALM6 B cells. Consistently, Jurkat T cells transfected with the Dyn2 mutant control vector reproducibly demonstrated a bi-phasic Erk activation, with the initial peak at 5 min followed by another peak at 60 min. In contrast, Dyn2-suppressed cells showed a small but reproducible decrease in the initial Erk activation (**Fig. 7a**, 1 versus 5 min) and an absence of Erk activation at the 60-minute time point (**Fig. 7a**). In contrast to the modest effect on Erk activation, ablation of Dyn2 had a substantial effect on both the intensity and duration of Jnk activation after T cell activation (**Fig. 7a**). Thus, Dyn2, like Vav1, is required for efficient activation of Erk and Jnk downstream of the TCR inositol-4,5-trisphosphate.

In addition to Erk and Jnk activation, Vav1 participates in signaling pathways that regulate calcium mobilization, an event involved in both CD69 upregulation and TCR-mediated IL-2p activity. We therefore investigated whether Dyn2 regulates calcium mobilization after TCR crosslinking. One main phosphoprotein contributing to calcium mobilization in T cells is PLC-γ1, through its cleavage of phosphatidylinositol-4,5-bisphosphate into diacylglycerol and the second messenger molecule. Tyrosine phosphorylation of PLC-γ1 on Y783 correlates with activation of its lipase activity. Using a site-specific antibody to PLC-γ1, we found that after stimulation, in contrast to control-transfected cells, Dyn2 suppressed cells showed diminished tyrosine phosphorylation of PLC-γ1 at Y783 (**Fig. 7a**). Consistent with the defect in the biochemical modification of PLC-γ1 in Dyn2 suppressed cells, Jurkat T cells depleted of Dyn2 demonstrated an attenuated calcium mobilization profile after TCR crosslinking similar to that noted when Vav1 was depleted by small interfering RNA (**Fig. 7b**). These findings collectively

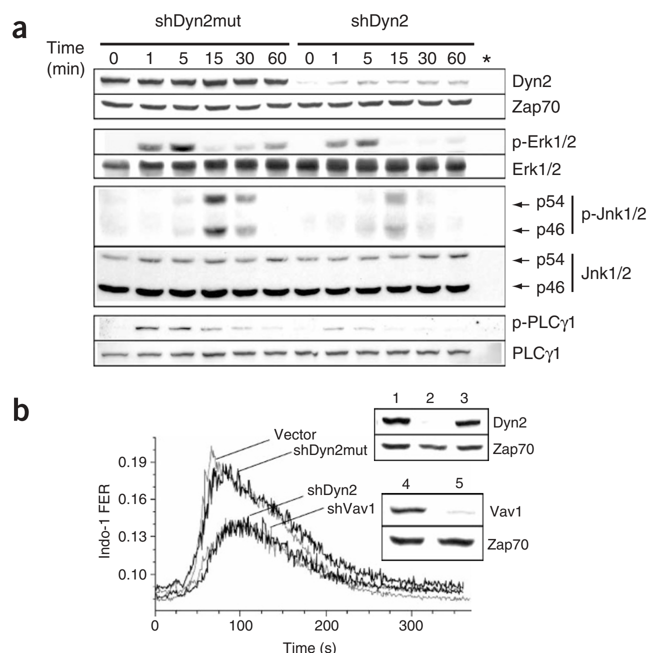

Figure 7 Dyn2 couples TCR signaling to the activation of Erk, Jnk and PLC-γ1. (**a**) Jurkat T cells were transfected with shDyn2mut or shDyn2 RNA targeting vector. After 72 h, the cells were left unstimulated (time 0) or were stimulated over time (above lanes) with SEE-pulsed NALM6 B cells. Lysates were immunoblotted with primary antibodies (left margin) and were developed with appropriate secondary reagents coupled to horseradish peroxidase. *, fixed NALM6 B cells added to the stimulation. p-, phosphorylated. (**b**) Jurkat T cells were transfected with vector control (lanes 1 and 4) or shVav1 (lane 5), shDyn2mut (lane 3) or shDyn2 (lane 2) RNA targeting vectors; inset, protein expression determined by immunoblot. After 48 h, calcium mobilization in response to TCR crosslinking was measured. Data are a representative example of three independent experiments.

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suggest that Dyn2 is capable of modulating several Vav1-mediated signaling pathways downstream of the TCR to regulate gene transcription (IL-2 and CD69) in part through its effect on Erk-, Jnk- and PLC-γ1-regulated signaling pathways.

Dyn2 does not regulate Lat complex formation

Vav1 can participate in the formation and/or stabilization of multi-molecule complexes with the transmembrane adaptor protein Lat after TCR receptor crosslinking[26,27]. In particular, thymocytes from Vav1-deficient mice have defects in the stable recruitment and formation of PLC-γ1–Lat, Gads-SLP76-Lat and Grb2-Sos1-Lat complexes after TCR-CD28 crosslinking. Because PLC-γ1, Jnk and Erk activation was defective, we investigated whether Dyn2 knockdown altered the formation of these multimolecular complexes with Lat. We immunoprecipitated Lat from control and Dyn2-suppressed Jurkat T cells stimulated with staphylococcal enterotoxin E (SEE)–pulsed NALM6 B cells and analyzed associated proteins (**Supplementary Fig. 3** online). Whether Dyn2 was present, several tyrosine-phosphorylated proteins coimmunoprecipitated with Lat, including SLP76, PLC-γ1 and several other unidentified proteins (**Supplementary Fig. 3** online). Consistent with previous data (**Fig. 7a**), PLC-γ1 total tyrosine phosphorylation was diminished in the absence of Dyn2 (**Supplementary Fig. 3** online). These data suggest that the formation of multimolecular complexes with Lat after T cell activation is not grossly altered, with the exception of PLC-γ1 phosphorylation.

Immunological synapse localization of Dyn2 requires Vav1

Because several Vav1-mediated signaling pathways were altered in the absence of Dyn2, we investigated whether Dyn2 was required for Vav1 recruitment to the immunological synapse. In Dyn2-depleted cells, recruitment of Vav1 to the T cell–APC contact site was not defective (data not shown). This observation was consistent with the data showing efficient recruitment and complex formation with Lat after TCR crosslinking (**Supplementary Fig. 3** online). In contrast, Vav1 depletion from Jurkat T cells diminished Dyn2 localization at the immunological synapse (**Supplementary Fig. 4** online). These data suggest that Vav1 is required for the recruitment of Dyn2 to the immunological synapse during T cell activation.

DISCUSSION

A function for the dynamin family as cytoskeletal regulators has become well established and extends the function of these proteins beyond that of a vesicular 'pinchase' essential for receptor-mediated endocytosis. Certainly, the putative functions and diverse locations of dynamins are consistent with a potentially similar function at the immune synapse, facilitating the substantial membrane and actin dynamics during cell-cell interactions. Because T cell activation requires actin reorganization at the immunological synapse, we investigated the function of Dyn2 in regulating this process in T cells. Here, Dyn2 was not required for TCR internalization after antibody crosslinking but participated mainly in remodeling of the actin cytoskeletal network at the T cell–APC contact site. Moreover, Dyn2 interacted constitutively with the Rho family GEF Vav1 and modulated intracellular signaling cascades leading to Erk, Jnk and PLC-γ1 activation. In addition, Dyn2 required its PRD to regulate TCR-mediated gene transcription and interacted with several intracellular signaling molecules involved in T cell activation. Thus, we propose that Dyn2 functions as a molecular scaffold in T cells, interacting with multiple signaling proteins to coordinate actin reorganization and the subsequent regulation of signal transduction cascades controlling TCR-mediated gene regulation and activation.

Dyn2 interacted with the Rho family diffuse B cell lymphoma GEF Vav1. Vav1 is a well characterized mediator of T cell development and activation through its ability to regulate multiple intracellular signaling pathways downstream of the TCR, including reorganization of the actin cytoskeleton[19]. The C-terminal SH3 domain of Vav1 is required for normal Vav1 function[22]. In fact, mutational inactivation of this domain results in defective activation of NFAT-AP-1–mediated gene transcription and Jnk activation, and deletion of this domain results in nuclear translocation of Vav1 (ref. 22). It is therefore of interest that TCR-activated Jurkat T cells lacking Dyn2 resemble *Vav1*$^{-/-}$ T cells in that they fail to stimulate a robust activation of Jnk and phosphorylation of PLC-γ1. A Dyn2 mutant lacking the Vav1-interacting P2-3 region was incapable of rescuing TCR-CD28–mediated IL-2p activity in Dyn2 knockdown cells. Thus, Dyn2 is likely to exert its effects on the actin cytoskeleton, kinase cascades and calcium mobilization mainly through its interaction with Vav1. Indeed, T cells lacking Dyn2 demonstrate a phenotype similar to that of T cells lacking Vav1, including Erk, Jnk and PLC-γ1 activation defects.

An important issue to emerge from these studies is how does the Vav1-Dyn2 axis affect TCR-stimulated actin cytoskeletal changes and the activation of kinase cascades. Although it is possible that Dyn2 affects Vav1 GEF activity, net TCR-stimulated tyrosine phosphorylation of Vav1 in the absence of Dyn2 is unaffected (T.S.G. and D.D.B., data not shown). However, it remains possible that specific tyrosines (in particular Y174) in the acidic regulatory domain are not fully phosphorylated, resulting in diminished Vav1 GEF activity. Another

possibility is that GTP-bound Rac1, a Rho family GTP-binding protein, does not accumulate at the immunological synapse in the absence of Dyn2. In fact, a report has suggested that in the absence of Dyn2, Rac1 is not localized to the membrane ruffles after the addition of platelet-derived growth factor[28]. Therefore, it is possible that the overall amount of Rac1 at the immunological synapse is decreased substantially in the absence of Dyn2. The development of reagents that specifically detect GTP-bound Rac1 at the immunological synapse will help in determining if this is the mechanism by which Dyn2 regulates actin reorganization at the immunological synapse. Finally, Dyn2 may regulate actin reorganization by recruiting proteins involved in actin cytoskeletal reorganization to the immunological synapse. Indeed, Dyn2 can interact with Abi-1, a component of the WAVE2 complex, which is involved in *de novo* actin polymerization. Further studies will be needed to determine how Dyn2 regulates T cell activation.

In addition to Vav1 and its effectors, other interacting partners of Dyn2 may also be important for T cell activation. Dyn2 interacts with the SH3 domain of PLC-γ1 (refs. 17,29). Indeed, the interaction of PLC-γ1 with Dyn2 is localized to a region that encompasses our P2-3 deletion[17,29]. Whether or not the Dyn2-PLC-γ1 interaction is required for Dyn2 function or for efficient activation of PLC-γ1 lipase activity or localization is not yet known. Regardless of the mechanism, in the absence of Dyn2, there is a considerable defect in the tyrosine phosphorylation of PLC-γ1 at Y783, a known site of phosphorylation that correlates with its lipase activity. *Vav1*$^{-/-}$ thymocytes demonstrate a similar defect in PLC-γ1 phosphorylation and complex formation with SLP76 (ref. 26). However, we have found that Dyn2 does not greatly affect the recruitment of PLC-γ1, Gads-SLP76 and Grb2-Sos1 complexes to Lat after TCR crosslinking, which is consistent with the observation that Vav1 is recruited to the immunological synapse in the absence of Dyn2. It remains possible that the interaction of PLC-γ1 with Dyn2 may be required for the proper activation of PLC-γ1 after its recruitment to Lat. T cells from mice lacking Itk, a Tec family kinase that can phosphorylate and activate PLC-γ1, have cytoskeletal defects similar to those of Vav1- and Dyn2-depleted cells[30,31]. Whether the recruitment and activation of Tec family kinases is impaired in Dyn2-depleted cells remains to be determined.

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In many receptor systems, dominant negative versions of dynamins, which cannot bind GTP, adversely affect receptor-mediated endocytosis and subsequent signaling events such as activation of the MAP kinase pathway[32–34]. Studies using one of these dominant negative forms (Dyn2K44A) have shown that pre-TCR endocytosis in mouse double-negative thymocytes is regulated by Dyn2 (ref. 35). However, in mature T cells, Dyn2 is the only dynamin isoform present (T.S.G. and D.D.B., data not shown), yet we found that suppression of Dyn2 did not affect either TCR cell surface expression or endocytosis after antibody crosslinking. One explanation for these disparate findings is that different mechanisms may be controlling pre-TCR and mature TCR receptor endocytosis. However, it is possible that endocytosis of the TCR by a more physiological stimuli (MHC-antigen complexes) may be affected in the absence of Dyn2. However, the observed defect in pre-TCR endocytosis could also be due to the use of a dominant negative Dyn2 construct, which may have pleiotropic effects not associated with its normal function. Additional studies will be required to 'dissect' the relationship between the function of Dyn2 in endocytosis and signaling. However, Dyn2-suppressed T cells have substantial defects in several aspects of T cell activation, in conditions in which alteration in TCR endocytosis are undetectable.

The actin cytoskeleton provides the meshwork for the generation and stabilization of signaling complexes that allows optimal transduction of signals after cell surface receptor engagement. Indeed, the Dyn2 PRD interacted with Lck, Gads and CrkL. Although Lck and Gads bind to the P1 and P4 region of Dyn2, respectively (T.S.G. and D.D.B., data not shown), whether proteins other than Vav1 interact with the P2-3 domain of Dyn2 to control T cell activation is unclear. However, the exact mechanism by which these interactions participate in Dyn2-mediated signaling in T cells will require further analysis and fine mapping of the Dyn2 PRD. WASP and WIP[36,37], two proteins involved in actin cytoskeletal dynamics in T cells, could be recruited to Dyn2 through interaction with the adaptor protein Nck. However, the interaction of Nck with Dyn2 occurs through its C-terminal SH3 domain, the same domain that it uses to interact with WASP. In addition to interacting with Nck, Dyn2 interacts with the actin-binding protein cortactin in 3T3 cells after the addition of platelet-derived growth factor[21,38]. Cortactin is not expressed in hematopoietic cells, but HCLS1 (also called HS1), a cortactin homolog, is present. *Hcls1*$^{-/-}$ mice demonstrate a defect in clonal expansion and negative selection[39]. However, whether HCLS1 interacts with Dyn2 through its SH3 domain in a way similar to that of cortactin remains to be established. Finally, the interaction of Dyn2 and the Abl tyrosine kinase interactor Abi-1 may provide a link between Rac1 and actin reorganization by recruiting the Rac1-regulated WAVE2 protein complex that is involved in actin reorganization[18,40]. Thus, failure to accumulate active Rac1 at the immunological synapse in the absence of Dyn2, could alter both Jnk activation and actin cytoskeletal changes after TCR crosslinkage.

In conclusion, we have identified a previously unknown function for the large GTPase Dyn2 in T cell activation and have demonstrated a newly identified, functional interaction between Dyn2 and the Rho family GEF Vav1. The observation that Dyn2 regulates actin reorganization and Rac1 activation at the immunological synapse after engagement of the TCR provides a link between Dyn2, Vav1 and their downstream target molecules. In addition, the failure to recruit Dyn2 to the cell-cell contact site in the absence of Vav1 may support a model in which Dyn2, through its PRD, recruits multiple regulators of *de novo* actin polymerization to the immunological synapse to promote T cell activation.

METHODS

Reagents and plasmids. All reagents were from Sigma unless otherwise specified. Antisera to PLC-γ1, Zap70, Vav1, Dyn2, Lat and 'pan-dynamin' (MC-63) have been described[41–46]. The EE monoclonal antibody (mAb) was purified from ascites fluid. We also used antibodies to Erk1/2, phosphorylated Erk1/2 (pErk1/2; T202/Y204), pJnk1/2 (T183/Y185) and pPLC-γ1 (Y783) from Cell Signaling Technology, as well as to Jnk (C-17) and Sos1 from Santa Cruz. The OKT3 antibody was obtained from the Mayo Pharmacy; the mAb to CD28 was purchased from BD Biosciences; and the mAb to phosphotyrosine (4G10) was purchased from Upstate Biotechnology. Antibodies used for flow cyometery included immunoglobulin G_1 (IgG_1)–phycoerythrin isotype control, anti-CD69–phycoerythrin, anti-TCRα/β–phycoerythrin and anti-CD3ε–phycoerythrin (all obtained from Becton Dickinson, Immunocytometry Systems).

The sequence encoding Dyn2 was amplified from a cDNA library with the oligonucleotides 5′-AGCAGCCGCCGGTACCATGGGCAACCGCGGGATGGAAGA-3′ and 5′-GCACGCCCCCGCGGCCGCCTAGTCGAGCAGGGATGGCTC-3′. The Dyn2ΔPRD was amplified from the full-length Dyn2 described above with the same 5′ primer along with the 3′ oligonucleotide 5′-ACCGATGATGGCGGCCGCCTACTTGAGGGCATGGTACAT-3′. Underlining indicates unique restriction sites used for subcloning the amplified cDNA. The Dyn2 deletion mutants ΔP1, ΔP2-3, ΔP4 and ΔP5 were generated, based on published regions[21], using a Quik Change Site-Directed Mutagenesis kit purchased from Stratagene. The oligonucleotide sequence 5′-GTCTACCCCGAGAAGGAtCAaGCcGAAAACGAGGATGGG-3′ and its complement were used to generate shRNA-resistant cDNAs of Dyn2 and Dyn2ΔP2-3, also using

the Quik Change Mutagenesis Kit. The IL-2p.luc luciferase reporter construct has been described[13].

The 'parental' vector (pFRT-H1P) used for shRNA silencing has been described[47]. A modified version of pFRT-H1P, containing a separate transcriptional cassette for GFP, was also used. The 19-nucleotide sequence used to target human Dyn2 mRNA is 5′-GGACCAGGCAGAAAACGAG-3′; the sequence used to target mouse Dyn2 mRNA is 5′-GGACCAGGCAGAGAATG AG-3′; and the sequence used to target Vav1 mRNA has been described[14]. A mutant-targeting construct was also generated to human Dyn2 (5′-GGAgCA GGCtGAAAACtAG-3′) as a control. Lower case letters indicate the nucleotides changed in the Dyn2 targeting sequence.

Cell culture, infection, transfection and stimulation. Primary human $CD4^+$, EL-4 and Jurkat T cells were grown in RPMI 1640 medium supplemented with 5% fetal bovine serum, 5% fetal calf serum and 4 mM L-glutamine. A20, Raji, EBV, J.Vav1 and NALM6 B cells were grown in that media as well. DO11.10 secondary T cells and thymocytes were obtained and cultured as described[48]. In some experiments, J.Vav1 cells were infected with a Vav1 recombinant vaccinia virus as described[45]. Transient transfections in Jurkat used 1×10^7 cells per sample along with 30–40 μg of plasmid DNA as described[13]. Transfected Jurkat cells were used after 18–24 h for expression and after 48–72 h for suppression. Transient transfections in DO11.10 secondary T cells used 3×10^7 cells per sample along with 100 μg of plasmid DNA as described[49].

For luciferase reporter assays, Jurkat cells (1×10^6 cells) were distributed in triplicate into 24-well plates and were stimulated as indicated in **Figures 2** and **6**. DO11.10 cells (3×10^6 cells) were distributed in triplicate in 12-well plates 18 h after transfection and were stimulated for 16 h with 1×10^6 A20 B cells pulsed with ovalbumin amino acids 323–339 (10 μg/ml) or were left unpulsed. All samples were collected and luciferase activity was measured with the Dual Luciferase Assay kit (Promega).

DO11.10 thymocytes and secondary cells were activated with plate-bound anti-CD3 and anti-CD28 as described[48]. For the stimulation time course studies in Jurkat T cells, Jurkat cells were activated with NALM6 B cells that were fixed in 2% paraformaldehyde, were washed three times in PBS and were pulsed for 30 min with 5 μg/ml of staphylococcal enterotoxin E (SEE; Toxin Technologies) in RPMI medium. Jurkat cells (2×10^6) transfected with shRNA were then stimulated with 1×10^6 fixed and pulsed NALM6 B cells at 37 °C for various times in RPMI medium (100 μl total volume). After each time point, the cells were immediately washed in ice-cold PBS and were lysed.

GST fusion protein coprecipitation, immunoprecipitation and immunoblots. All lysates were prepared in Nonidet-P40 (NP-40) lysis buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 5 mM EDTA, 0.5 mM CaCl, 1% NP-40, 1 mM phenylmethyl sulfonyl fluoride, 10 μg/ml of leupeptin, 5 μg/ml of aprotinin and 1 mM Na_3VO_4). For GST fusion protein coprecipitations, 30 μg of GST fusion protein was bound to glutathione (GSH)–agarose slurry in NP-40 lysis buffer for 30 min at 4 °C. The slurry was then washed once in NP-40 buffer. Jurkat T cells were lysed and approximately 0.75–1.0 mg of clarified whole-cell lysate was rotated at 4 °C for 45 min with the GST fusion protein–GSH agarose complex. The protein complexes were then washed twice with NP-40 lysis buffer, eluted in 40 μl of SDS-sample buffer, resolved by SDS-PAGE and transferred to Immobilon-P membranes (Millipore). Coimmunoprecipitation experiments were done with essentially those conditions except that monoclonal antibody (anti-EE) or rabbit polyclonal antibodies (anti-Vav1 or anti-ZAP70) were bound to either anti-mouse IgG agarose or protein A sepharose, respectively. In cases in which whole-cell lysates were prepared, 100 μg of protein was resolved by SDS-PAGE. For immunoblots, mAbs were followed by goat anti-mouse IgG coupled to horseradish peroxidase (Santa Cruz), and polyclonal rabbit antisera was detected by protein A–linked to horseradish peroxidase (Amersham) and SuperSignal (Pierce). For the 'far western' blot, GST fusion proteins containing the SH3 domain of Vav1 or GST alone were used to blot membranes (2 μg/ml), followed by anti-GST and finally protein A linked to horseradish peroxidase followed by SuperSignal.

TCR and CD69 expression by flow cytometry. Jurkat T cells were transfected with various shRNA targeting vectors and cell surface receptor expression was analyzed with CELLQUEST software on a FACScan apparatus (Becton Dickinson). TCR expression was determined by incubation of cells on ice for 30 min with IgG_1-phycoerythrin isotype control or anti-TCRα/β–phycoerythrin. The cells were then fixed in 4% paraformaldehyde and were analyzed by flow cytometry. For analysis of TCR internalization, 1×10^6 cells were incubated on ice with 0.5 μg/ml anti-CD3εphycoerythrin and were subsequently crosslinked with goat anti-mouse for various times at 37 °C. After each time point, cells were immediately incubated for 10 min with ice-cold surface antibody–stripping buffer (0.5 M NaCl and 0.2 M acetic acid), washed with the same stripping buffer and then finally washed in ice-cold PBS. Cells were then fixed with 4% paraformaldehyde and were analyzed by flow cytometry for phycoerythrin-positive cells. The average rate of TCR internalization (change in percent cells staining positive for TCR internalization over the change in time) was determined in triplicate for each transfected cell population. For CD69 expression, cells were treated for 5 h with anti-CD3 plus anti-CD28 or with phorbol 12-myristate 13-acetate plus ionomycin and were subsequently incubated on ice for 30 min with anti-CD69–phycoerythrin, washed with PBS, fixed in 4% paraformaldehyde and analyzed by flow cytometry.

Calcium mobilization. Cells were loaded with Indo-1 acetoxy-methyl ester (Molecular Probes) and were stimulated with 30 ng/ml of OKT3 mAb crosslinked with goat anti-mouse. Changes in intracellular calcium concentration were determined by flow cytometry as described[45]. Cells were transfected with various shRNA suppression vectors 48 h before being loaded with Indo-1, followed by analysis.

Cell fractionation. Jurkat cells (1×10^7 cells per sample) were stimulated with OKT3 and anti-CD28 (5 μg/ml) at 37 °C for various times and were subjected to a published serial extraction protocol[13].

Flow cytometry–based conjugate assay. Conjugate assays were done essentially as described[2]. EBV-B cells were stained with PKH26 according to the manufacturer's directions (Sigma), were incubated in the presence or absence of 2 μg/ml of SEE for 1.5 h, were washed and were resuspended at a density of 1×10^6 cells/ml in RPMI medium. Jurkat T cells expressing GFP alone or together with Dyn2 shRNA were also resuspended at a density of 1×10^6 cells/ml in RPMI medium. For conjugation, equal volumes of B and T cells were pelleted together and then were incubated at 37 °C for 10–15 min. The relative proportion of red, green and red-green events in each tube was determined by two-color flow cytometry with a FACSCalibur (BD Biosciences). The number of gated events counted per sample was at least 15,000.

Immunofluorescence microscopy. Cell conjugates were formed essentially as described for NALM6-Jurkat[50]. NALM6, A20 or Raji B cells were stained with CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin, Molecular Probes) and pulsed with or without 2 μg/ml of SEE, 10 μg/ml of ovalbumin amino acids 323–339, or 2 μg/ml of a 'cocktail' of staphylococcal superantigens (SEA, SEB, SEC3 and SEE; Toxin Technologies), respectively. B cells were centrifuged together with the same number of T cells, were incubated at 37 °C for 15 min, were plated onto poly-L-lysine–coated coverslips and were fixed for 20 min at 25 °C with 3% paraformaldehyde in PBS. Slides were labeled with rabbit anti-Dyn2 followed by goat anti-rabbit–indodicarbocyanine (Jackson Immunoresearch) or goat anti-rabbit–tetramethylrhodamine isothiocyanate (Molecular Probes). F-actin was visualized with fluorescein or rhodamine phalloidin (Molecular Probes). Coverslips were mounted in Mowiol 4-88 (Hoeschst Celanese) containing 10% 1,4-diazobicyclo [2.2.2] octane. Actin polarization was quantified by random selection of 50 conjugates containing a green T cell (transfected with the GFP-expressing suppression vector) contacting a blue B cell. Those conjugates showing a distinct polymerized actin band at the T cell–B cell contact site were considered polarized. Statistical significance was determined with the paired Student's *t*-test.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT
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