

INF2 promotes the formation of detyrosinated microtubules necessary for centrosome reorientation in T cells

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T cell antigen receptor–proximal signaling components, Rho-family GTPases, and formin proteins DIA1 and FMNL1 have been implicated in centrosome reorientation to the immunological synapse of T lymphocytes. However, the role of these molecules in the reorientation process is not yet defined. Here we find that a subset of microtubules became rapidly stabilized and that their α -tubulin subunit posttranslationally detyrosinated after engagement of the T cell receptor. Formation of stabilized, detyrosinated microtubules required the formin INF2, which was also found to be essential for

centrosome reorientation, but it occurred independently of T cell receptor–induced massive tyrosine phosphorylation. The FH2 domain, which was mapped as the INF2 region involved in centrosome repositioning, was able to mediate the formation of stable, detyrosinated microtubules and to restore centrosome translocation in DIA1-, FMNL1-, Rac1-, and Cdc42-deficient cells. Further experiments indicated that microtubule stabilization was required for centrosome polarization. Our work identifies INF2 and stable, detyrosinated microtubules as central players in centrosome reorientation in T cells.

Introduction

T cells polarize in response to appropriate antigens presented by an antigen-presenting cell (APC), forming a surface subdomain at the cell-to-cell contact, referred to as the immunological synapse (IS), that is enriched in specific membrane receptors and signaling molecules (Fooksman et al., 2010). T cell polarization is accompanied by extensive accumulation of polymerized actin at the IS, reorganization of the microtubule (MT) cytoskeleton, and reorientation of the centrosome, the major MT-organizing center (MTOC), to face the IS. The reorientation of the MTOC to the IS, which is one of the hallmarks of T cell polarization, is required for normal signaling through the T cell antigen receptor (TCR) and polarization of the secretory apparatus to the IS for directed release of lymphokines in T helper cells or cytotoxins in cytolytic T cells (Kuhn and Poenie, 2002; Billadeau et al., 2007; Martin-Cófreces et al., 2008; Griffiths et al., 2010).

Molecules involved in signaling through the TCR, such as Lck, Fyn, ZAP-70, linker for activation of T cells (LAT), and SLP-76 (Lowin-Kropf et al., 1998; Kuhné et al., 2003), as well as the motor dynein–dynactin complex (Combs et al., 2006; Martin-Cófreces et al., 2008), are important in the process of MTOC reorientation. Formins are a widely expressed family of proteins that nucleate the formation of linear filaments of actin. The best studied of these are the diaphanous-related formins, such as mDia1, -2, and -3, which are direct effectors of Rho-family GTPases (Goode and Eck, 2007). The binding of the effector Rho GTPase regulates the actin polymerization activity of mDias by exposing the formin homology (FH) 1 and 2 domains, which are involved in profilin binding and actin nucleation, respectively. Two formin proteins, DIA1 (the human orthologue of mDia1) and FMNL1, have been shown to be required for MTOC reorientation in T cells (Gomez et al., 2007). Cdc42 was initially identified as the Rho-family

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Abbreviations used in this paper: APC, antigen-presenting cell; FH, formin homology; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IS, immunological synapse; KD, knockdown; MT, microtubule; MTOC, MT-organizing center; SEB, staphylococcal enterotoxin B; SEE, staphylococcal enterotoxin E; TCR, T cell antigen receptor.

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GTPase regulating MTOC repositioning in T cells (Stowers et al., 1995), but more recent work has also implicated Rac1 in this process (Gomez et al., 2007). Despite the considerable advances, we are largely ignorant of how the MTOC moves toward the IS, of the mechanism operating to remodel the MT cytoskeleton, and of the role played by formins in these two processes.

Similar to T cells during IS formation, migrating fibroblast cells as well as other cell types accumulate polymerized actin at the leading edge, reorient the MTOC to face the leading edge, and reorganize the MT cytoskeleton (Li and Gundersen, 2008; Insall and Machesky, 2009). In NIH 3T3 fibroblasts, MT reorganization involves the formation of a subset of stable MTs oriented toward the leading edge (Gundersen and Bulinski, 1988). Elevated levels of post-translationally detyrosinated α -tubulin, which arises through the removal of the carboxyl-terminal tyrosine residue and the subsequent exposure of the adjacent glutamate residue, characterize these stable MTs, referred to as Glu-MTs (Gundersen et al., 1984; Gundersen et al., 1987). Detyrosination renders MTs resistant to motor-driven depolymerization (Peris et al., 2009), abolishes the plus-end MT tracking of proteins containing cytoskeleton-associated protein glycine-rich (CAP-Gly) MT binding domains (Peris et al., 2006), and regulates kinesin-1 binding to MTs (Kreitzer et al., 1999; Dunn et al., 2008; Konishi and Setou, 2009). In fibroblasts, RhoA and its effector mDia1 control the formation of Glu-MTs (Palazzo et al., 2001a,b). Although the precise mechanism of Glu-MT formation is still poorly understood, the activity of mDia2 involved in this process maps to the FH2 domain but is independent of its actin nucleation activity (Bartolini et al., 2008). Despite the importance of MT detyrosination in the regulation of various cellular functions, such as axon outgrowth (Erck et al., 2005; Witte et al., 2008) and polarized recycling (Lin et al., 2002), the formation and function of Glu-MTs has not yet been investigated in T lymphocytes.

Inverted Formin 2 (INF2) was originally characterized as an atypical formin that, in addition to nucleate actin polymerization, has in vitro actin depolymerization activity (Chhabra and Higgs, 2006). Mutations in the *INF2* gene have been found to cause focal segmental glomerulosclerosis with or without associated Charcot-Marie-Tooth neuropathy (Brown et al., 2010; Boyer et al., 2011). Formin INF2 associates with Cdc42 and Rac1 (Madrid et al., 2010), and regulates specialized pathways of vesicular transport in hepatic cells and T lymphocytes by a process requiring both its actin polymerization and depolymerization activities (Andrés-Delgado et al., 2010; Madrid et al., 2010). Here we show that INF2, through its FH2 domain, mediates the formation of an array of stable Glu-MTs that is necessary for MTOC reorientation to the IS. The formation of this array was independent of the actin polymerization and depolymerization activities of INF2, and did not require TCR-induced tyrosine phosphorylation. Importantly, the expression of the FH2 domain, which was mapped as the INF2 domain involved in this process, restored MTOC reorientation and Glu-MT formation in DIA1-, FMNL1-, Rac1-, and Cdc42-deficient cells. Supporting the importance of stable Glu-MTs, we found

that MTOC reorientation was also restored by treatment with taxol, a tubulin-binding drug that induces MT detyrosination by increasing MT stability. Therefore, INF2 plays a crucial role in the process of MTOC reorientation in T cells through the formation of stable Glu-MTs.

Results

MTOC translocation is impaired in INF2 knockdown (KD) cells

In resting Jurkat cells, endogenous or exogenous INF2 distributes at the cell periphery, MT cytoskeleton, and pericentriolar region (Andrés-Delgado et al., 2010). In Jurkat cells, conjugated to staphylococcal enterotoxin E (SEE) superantigen-loaded Raji B cells, which act as APCs, endogenous INF2 maintained the distribution observed in resting cells but with its pericentriolar pool polarized to the IS (Fig. 1 A). A similar behavior was observed for exogenously expressed INF2-1 and INF2-2 (Fig. 1 A), two INF2 isoforms that differ in their carboxyl-terminal sequence (Madrid et al., 2010). To investigate whether INF2 is required for MTOC reorientation, we expressed two different shRNAs (shINF2a and shINF2b), which target both INF2-1 and INF2-2 isoforms, able to knock down total INF2 content to $\sim 20\%$ of its endogenous levels (Fig. 1 B). Similar to DIA1 and FMNL1 KD (Gomez et al., 2007), INF2 KD allowed actin polarization to the IS but greatly blocked MTOC relocation (Fig. 1 C and D; and [Videos 1](#) and [2](#)). Similarly, INF2 was required for MTOC translocation in primary T cells (Fig. 1 E).

Mutation of Lys¹⁶⁰¹ in the FH2 domain of the yeast formin Bni1p or in the equivalent position of mDia2 impairs the actin nucleation activity of these formins (Xu et al., 2004; Bartolini et al., 2008). However, mutation of three critical leucine residues in the DAD of mouse INF2 abrogates its in vitro depolymerization activity (Chhabra and Higgs, 2006). We expressed intact INF2 or INF2 proteins with equivalent mutations in either actin polymerization (INF2-K/A) or depolymerization (INF2-3L/A) activities, or in both simultaneously (INF2-K/A-3L/A), and used them to determine whether they are able to replace the function of endogenous INF2 in MTOC translocation. To this end, the transcripts encoding the exogenous INF2 proteins were designed to resist shINF2b expression. Notably, expression of wild type or the three mutant INF2 proteins corrected the defect on MTOC reorientation (Fig. 1, F–H). Deletion analysis showed that the expression of INF2 fragments containing the FH2 domain was able to correct MTOC polarization in INF2 KD cells (Fig. 1, F and G). The possibility that the effect of INF2 KD on MTOC reorientation was caused by low levels of Lck at the plasma membrane (Andrés-Delgado et al., 2010) was ruled out by forcing the presence of Lck at the plasma membrane using a chimera (Krummel et al., 2000) consisting of the ectodomain and transmembrane segment of mouse CD4 appended to Lck ([Fig. S1](#)). In summary, MTOC reorientation in T cells requires INF2 expression but not its actin polymerization or depolymerization activities, and takes place by a process dependent on its FH2 domain.

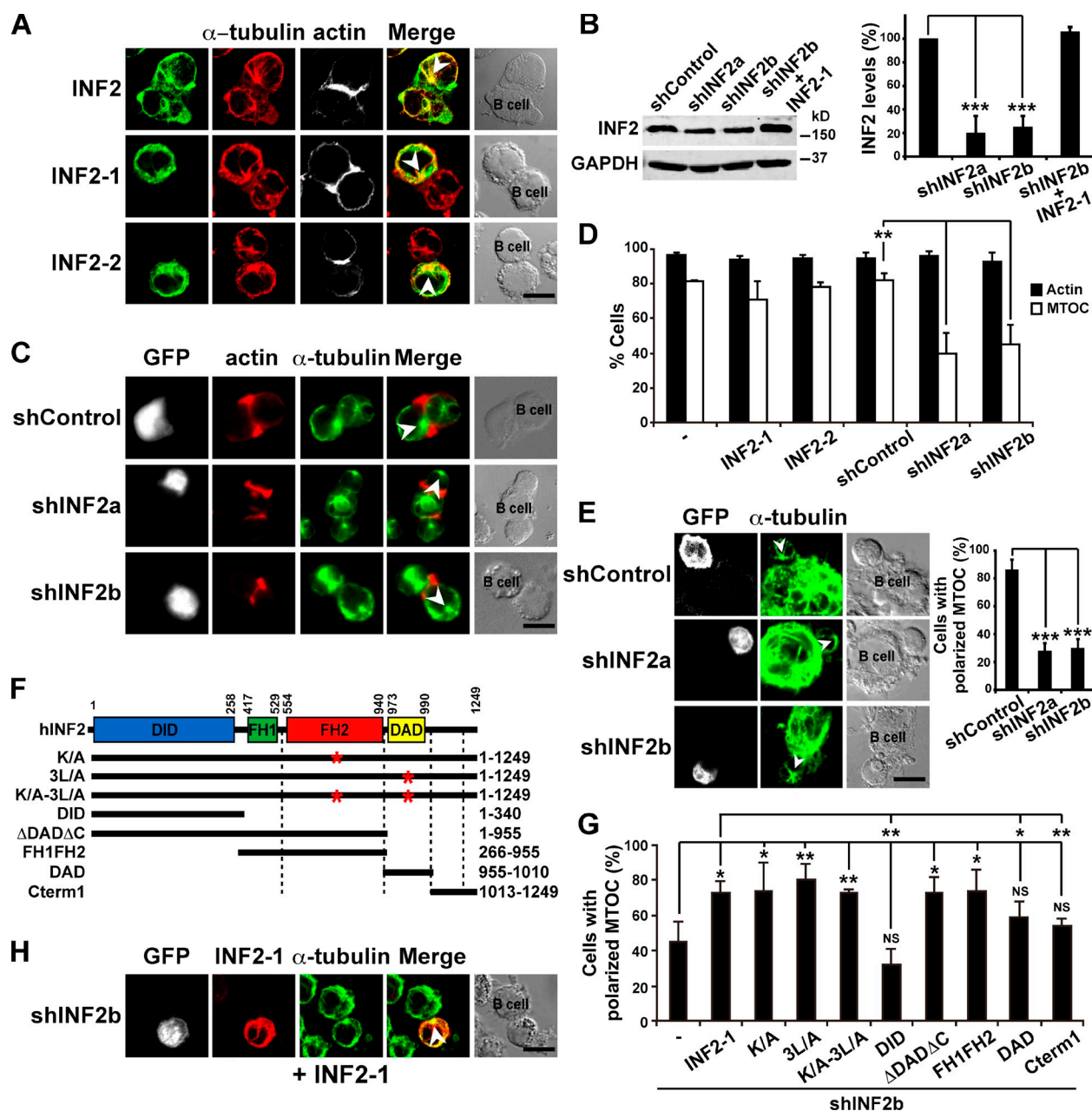


Figure 1. INF2 is required for MTOC reorientation in Jurkat cells and primary T cells. (A) Control Jurkat cells (top) and Jurkat cells transiently expressing exogenous, tagged INF2-1 (middle) or INF2-2 (bottom) were conjugated to SEE-loaded APCs. The distribution of α -tubulin, F-actin, and endogenous (top) or exogenous INF2 (middle and bottom) was analyzed. (B–D) Jurkat cells were transfected with DNA constructs coexpressing GFP and shControl, shINF2a, or shINF2b. Cells were processed for immunoblotting with anti-INF2 or anti-GAPDH (GAPDH) antibodies. The histogram represents the percentage of INF2 content (B). Cells were conjugated to SEE-loaded APCs and stained for F-actin and α -tubulin (C). The histogram represents the percentage of T cells with F-actin or MTOC polarized to the IS (D). (E) Peripheral blood lymphocytes transfected with a DNA construct coexpressing GFP and shControl, shINF2a, or shINF2b were conjugated to SEE-loaded APCs and stained for α -tubulin. The histogram represents the percentage of T cells with MTOC polarized to the IS. (F and G) Jurkat cells transiently expressing intact INF2 or the indicated INF2 mutants (F) were transfected with a DNA construct coexpressing GFP and shINF2b. Cells were conjugated to SEE-loaded APCs and the position of the MTOC was analyzed. The histogram represents the percentage of T cells with MTOC polarized to the IS (G). (H) Jurkat cells transiently expressing INF2-1 were transfected with a DNA construct coexpressing GFP and shINF2b. Cells were conjugated to SEE-loaded APCs and stained for INF2-1 and α -tubulin. The arrowheads indicate the position of the MTOC of the T cells. The p-value for the results of DID, DAD, and Cterm1 expression in INF2 KD cells relative to that of INF2 KD cells was of 0.102, 0.086, and 0.138, respectively (G). At least 40 T cells were analyzed in B, D, E, and G. Quantitative data in B, D, E, and G are summarized as means \pm SEM from three independent experiments (error bars; NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Bars, 5 μ m.

Glu-MT formation is rapidly induced upon TCR stimulation

In addition to nucleating actin polymerization, it is becoming apparent that some formins induce the formation of stable,

detyrosinated MTs (Bartolini and Gundersen, 2010). Because INF2-mediated MTOC reorientation was independent of the INF2 activities involved in actin dynamics, we investigated the levels and distribution of Glu-MTs in T cells forming

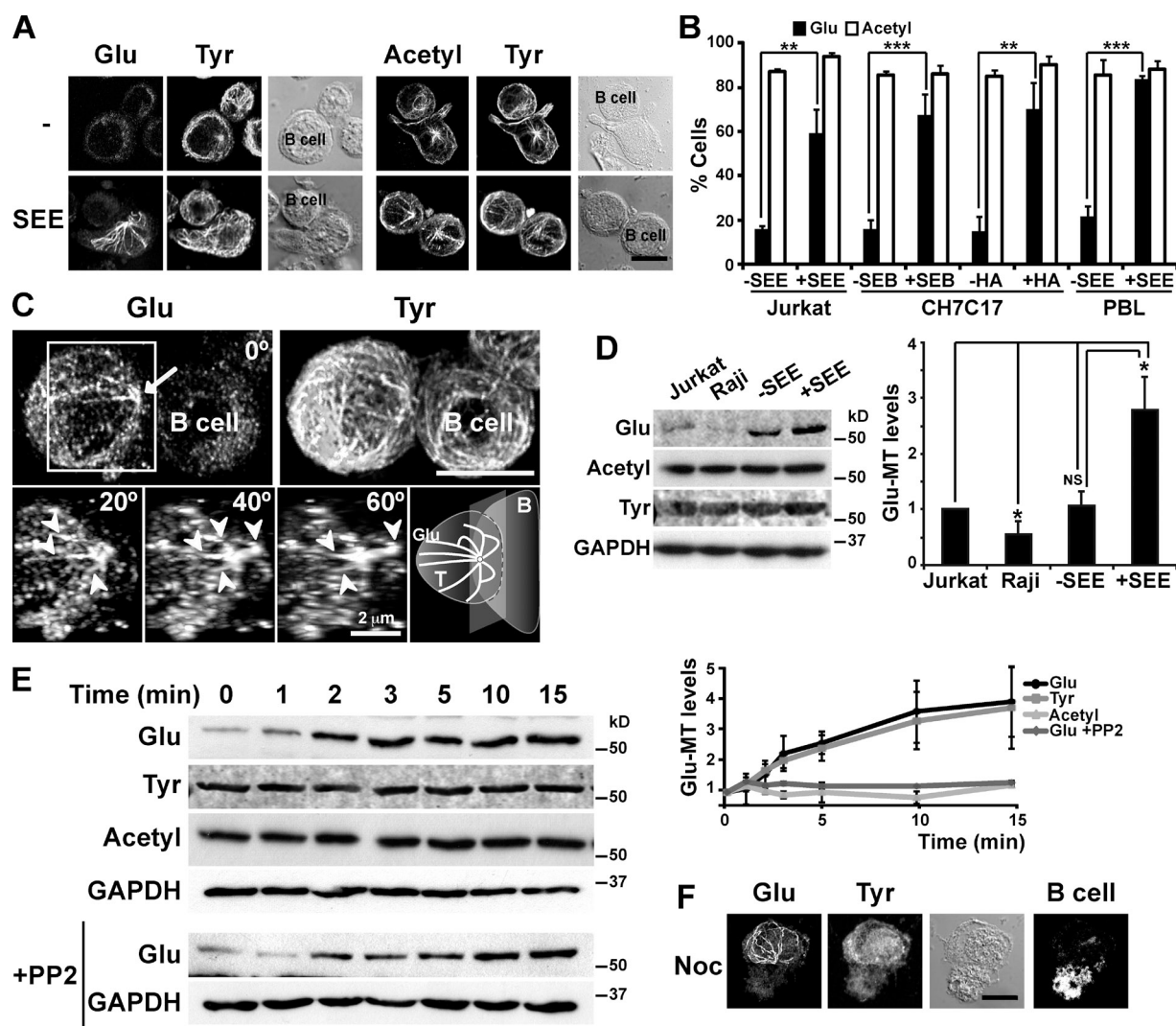


Figure 2. Glu-MTs are rapidly induced after T cell stimulation. (A and B) Jurkat cells (A and B), CH7C17 Jurkat cells (B), or peripheral blood lymphocytes (PBL; B) were conjugated to APCs loaded or not with SEE, SEB, or hemagglutinin (HA) peptide as indicated. The distribution of Glu-, Tyr-, and acetyl-MTs was analyzed with specific antibodies (A). The histogram represents the percentage of T cells with Glu- or acetyl-MTs (B). (C) Conjugates formed by Jurkat cells with SEE-loaded Raji cells were stained for Glu- and Tyr-MTs. The images were analyzed under a confocal microscope and the resulting stacks were deconvolved and subsequently reconstructed in 3D. Views of the cell-to-cell contact rotated 0°, 20°, 40°, and 60° are shown for Glu-MT-stained cells in one conjugate. The arrow indicates the position of the MTOC and the arrowheads point to Glu-MTs that bend away from the IS following the T cell curvature. (D) Equal numbers of Jurkat and Raji cells or Jurkat cells conjugated to Raji cells loaded or not loaded with SEE as indicated were processed for immunoblotting for Glu-, acetyl-, and Tyr-tubulin, or for GAPDH as a loading control. The histogram represents Glu-MT levels relative to control Jurkat cells. (E) Jurkat cells were conjugated to latex beads coated with anti-TCR antibodies for the indicated times in the presence or absence of 2.5 μM PP2. Cells were analyzed by immunoblotting for Glu-, acetyl-, and Tyr-tubulin or for GAPDH. The graphics represents the levels of Glu-, Tyr-, and acetyl-tubulin at different times of conjugation relative to those at zero time. (F) Distribution of Glu- and Tyr-MTs in Jurkat cells conjugated to SEE-loaded APCs for 15 min and then treated with 2 μM nocodazole for 30 min. At least 40 T cells were analyzed in B. Quantitative data in B, D, and E are summarized as means ± SEM from three independent experiments (error bars; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Bars, 5 μm.

conjugates with APCs for 15 min. As controls, we analyzed in parallel the predominant population of MTs, which contains α -tubulin with an intact Tyr carboxyl terminal residue (Tyr-MTs), and also the pool of MTs that are acetylated (acetyl-MTs). Cells were scored as positive for Glu-MTs when they had three or more discernable Glu-MTs. Whereas <20% of T cells scored positive for Glu-MTs when conjugated to APC in the absence of antigenic stimulus, the percentage greatly increased in response to stimulation with SEE (Fig. 2, A and B). As a control, we observed that the percentage of T cells with acetyl-MTs was unaffected. The Glu-MTs that formed

in the T cells bent away from the cell-to-cell contact following the T cell curvature and extended toward different points of the cell periphery (Fig. 2 C and Video 3). Glu-MT formation was also induced in primary T cells activated with SEE-loaded APC (Fig. 2 B). It is apparent that Glu-MT formation was not restricted to the use of SEE, given that similar results were specifically observed in conjugates formed with Jurkat CH7C17 cells (Niedergang et al., 1997), which bear an influenza virus hemagglutinin peptide-specific TCR, in the presence of APCs loaded with hemagglutinin peptide or with staphylococcal enterotoxin B (SEB) superantigen

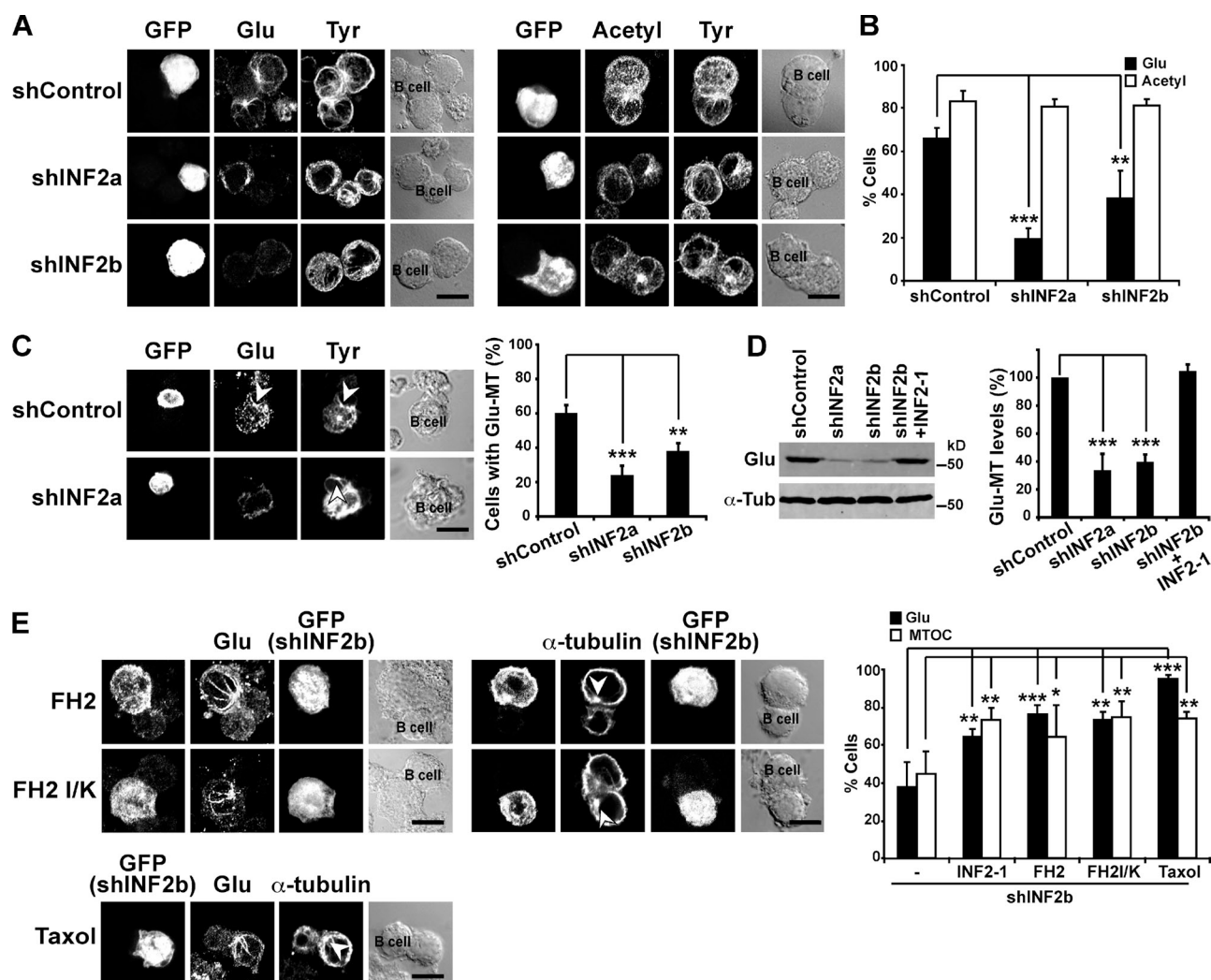


Figure 3. INF2 is required for Glu-MT formation in T cells. (A and B) Jurkat cells were transfected with DNA constructs coexpressing GFP and shControl, shINF2a, or shINF2b. Cells were conjugated to SEE-loaded APCs and stained for Glu- and Tyr-MTs or acetyl- and Tyr-MTs, as indicated (A). The histogram represents the percentage of T cells with Glu- or acetyl-MTs (B). (C) Primary T cells were transfected with DNA constructs coexpressing GFP and shControl, shINF2a, or shINF2b. Cells were then conjugated to SEE-loaded APCs and stained for Glu- and Tyr-MTs. The histogram represents the percentage of T cells with Glu-MTs. (D) Jurkat cells expressing shControl, shINF2a, shINF2b, or shINF2b and exogenous INF2-1 were conjugated to SEE-loaded APCs and immunoblotted for Glu- or total α -tubulin. The histogram represents the percentage of Glu-tubulin content. (E) Jurkat cells expressing GFP and shINF2b from the same plasmid were either cotransfected with DNA constructs expressing the intact FH2 domain of INF2 or the I/K mutant, or were treated with 3 nM taxol for 18 h. Cells were then conjugated with SEE-loaded APCs, fixed with methanol, and stained for the expressed INF2 FH2 fragment and for Glu- or total α -tubulin, as indicated. The histogram represents the percentage of T cells with Glu-MTs or with polarized MTOC. The arrowheads in C and E indicate the position of the MTOC of the T cells. At least 40 T cells were analyzed in B, C, and E. Quantitative data in B–E are summarized as means \pm SEM from three independent experiments (error bars; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Bars, 5 μ m.

(Fig. 2 B). The induction of Glu-MTs observed by confocal microscopy was confirmed by immunoblotting (Fig. 2 D), and it was also observed in Jurkat cells activated with latex beads coated with anti-TCR antibodies (Fig. 2 E). It is of note that treatment with PP2, an inhibitor of Src-family tyrosine kinases, did not affect Glu-MT formation (Fig. 2 E). The newly formed Glu-MTs were resistant to treatment with nocodazole, a MT-disrupting drug, at a concentration that depolymerized Tyr-MTs (Fig. 2 F), which is consistent with the higher stability of Glu-MTs (Cook et al., 1998). In conclusion, Fig. 2 shows that TCR engagement triggers the rapid formation of an array of stable Glu-MTs by a mechanism independent of massive tyrosine phosphorylation.

Formin INF2, through its FH2 domain, mediates Glu-MT formation and MTOC reorientation independently of its actin polymerization activity

Consistent with a role of INF2 in Glu-MT formation, the percentage of T cells with Glu-MTs was severely impaired in INF2 KD Jurkat cells, whereas no alterations were observed in the percentage of cells with acetyl-MTs (Fig. 3, A and B). Glu-MT formation was also reduced in primary T cells with silenced levels of INF2 (Fig. 3 C). The block in Glu-MT formation in INF2-KD Jurkat cells was confirmed by immunoblotting (Fig. 3 D). To confirm that the FH2 domain of INF2, even when deprived of its actin polymerization activity, is

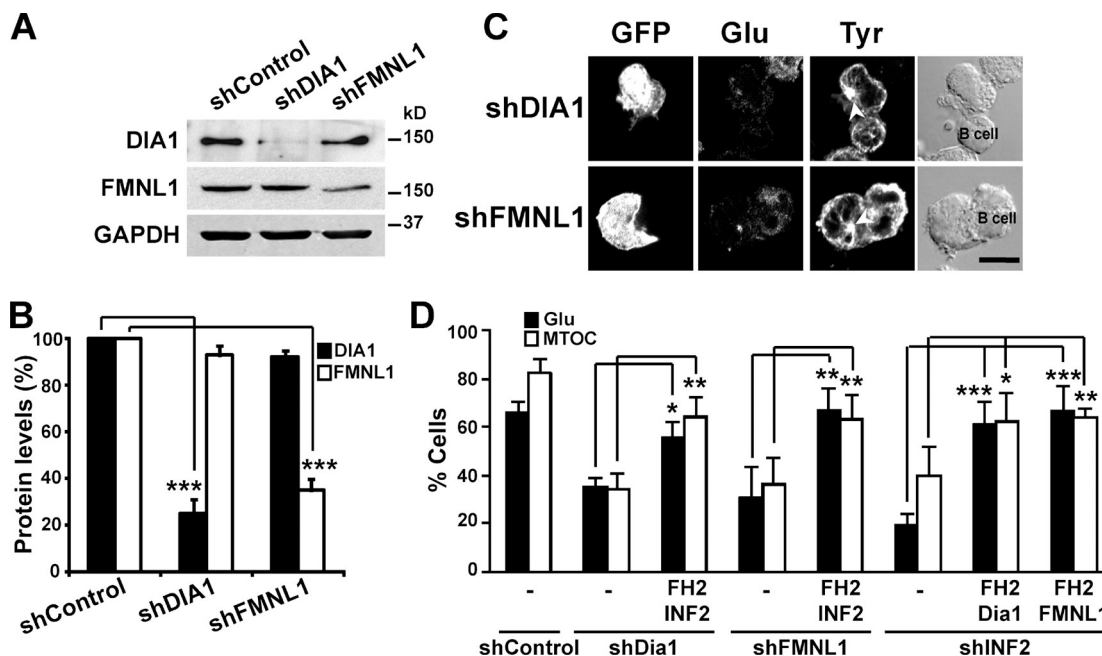


Figure 4. FH2 domain expression allows Glu-MT formation and MTOC polarization in DIA1, FMNL1, or INF2 KD cells. (A and B) Jurkat cells were transfected with DNA constructs coexpressing GFP and shDIA1 or shFMNL1. Cells were immunoblotted for Dia1, FMNL1, or GAPDH (A). The histogram represents the percentage of DIA1 or FMNL1 content (B). (C and D) DIA1-, FMNL1- (C), or INF2-KD cells were left untransfected or were transfected with constructs expressing the indicated FH2 domains (D). After conjugation to SEE-loaded APCs, cells were stained for Glu- and Tyr-MTs. The arrowheads indicate the position of the MTOC of the T cells. The histogram represents the percentage of T cells with Glu-MTs or polarized MTOC (D). Bars, 5 μ m. At least 40 T cells were analyzed in D. Data in B and D are summarized as means \pm SEM from three independent experiments (error bars; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

active in MTOC translocation, and to investigate whether it is also able to mediate Glu-MT formation, we expressed the intact FH2 domain of INF2 or a double mutant form (FH2 I-K) in which, in addition to the K/A mutation, the Ile⁶⁴³ residue was mutated to Ala. This residue of INF2 is equivalent to the Ile¹⁴³¹ of Bnp1 and the Ile⁷⁰⁴ of mDia2, which is required for actin polymerization by these formins (Xu et al., 2004; Harris et al., 2006). The expression of FH2 and FH2 I/K in resting Jurkat cells did not perturb F-actin distribution (Fig. S2 A). Remarkably, the individual expression of either the intact FH2 or the FH2 I-K mutant restored the formation of Glu-MTs and the translocation of MTOC in conjugates formed by INF2 KD Jurkat cells (Fig. 3 E). Glu-MT formation was observed even in resting Jurkat cells expressing INF2 FH2, which indicates that the expression of this INF2 domain is sufficient for Glu-MT formation (Fig. S2, B and C). Treatment with 3 nM taxol, an α -tubulin-binding drug known to stabilize MTs, induced the formation of Glu-MTs (Fig. S3 A) in resting Jurkat cells and did not affect the process of MTOC repositioning in normal Jurkat cells (Fig. S3 B). Importantly, taxol treatment restored MTOC polarization in INF2 KD cells (Fig. 3 E). This result strongly highlights the importance of Glu-MTs in the process of MTOC reorientation in T cells.

Formins Dia1 and FMNL1, which were previously involved in MTOC reorientation (Gomez et al., 2007), also appeared to be important for Glu-MT formation because the Glu-MT content was severely impaired in conjugates formed by Jurkat cells knocked down for either of these two formins,

paralleling the effect on MTOC translocation (Fig. 4, A–D). It is of note that expression of the isolated FH2 domain of INF2 restored to a large extent both formation of Glu-MTs and reorientation of MTOC in these cells (Fig. 4 D). A similar effect was observed by the expression of the isolated FH2 domains of mDia1 or FMNL1 in INF2 KD cells (Fig. 4 D).

In summary, the results illustrated in Figs. 1–4 indicate that INF2 is required for Glu-MT formation and MTOC translocation and that this ability resides in its FH2 domain and is independent of its actin polymerization activity. Dia1 and FMNL1 KD cells, which were previously found to be deficient in MTOC reorientation, are also defective in Glu-MT formation. Both processes can be restored by expression of the isolated FH2 domain of INF2, FMNL1, or mDia1.

Rac1 and Cdc42 regulate Glu-MT formation and MTOC reorientation upstream of INF2

Regulation of formins is primarily thought to occur through interactions with Rho-family GTPases. INF2 associates with Cdc42 and Rac1 (Madrid et al., 2010), FMNL1 with RhoA and Rac1 (Yayoshi-Yamamoto et al., 2000; Gomez et al., 2007), and mDia1 with Rho (Watanabe et al., 1997). Silencing of Cdc42 or Rac1 in Jurkat cells, but not that of RhoA, diminished Glu-MT formation and MTOC translocation (Fig. 5, A–D). It is of particular note that the expression of the FH2 domain of INF2 (Fig. 5 D) or taxol treatment of Cdc42 or Rac1 KD cells (Fig. 5 E) restored both processes. Together, the results in Fig. 5 implicate Rac1 and Cdc42 in the regulation

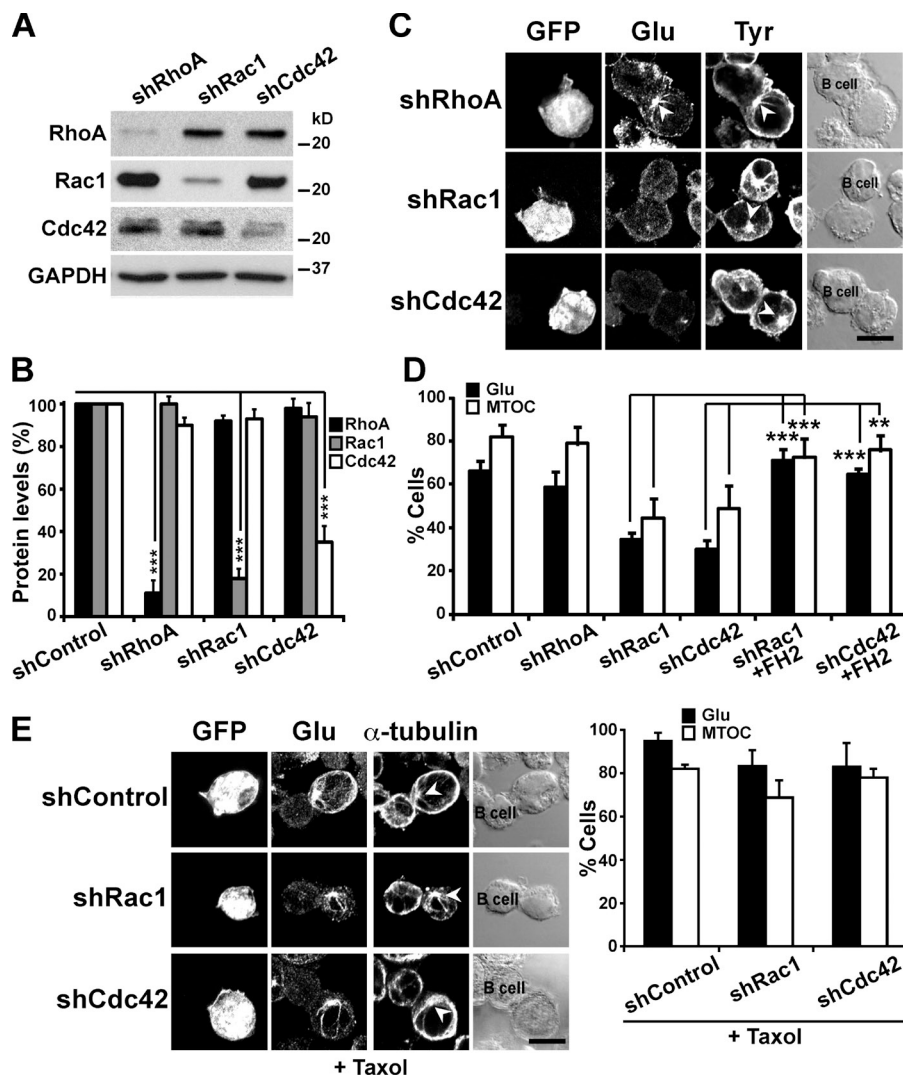


Figure 5. The expression of the FH2 domain of INF2 restores Glu-MT formation and MTOC polarization in Rac1- or Cdc42-KD cells. (A and B) Jurkat cells were transfected with DNA constructs coexpressing GFP and shRhoA, shRac1, or shCdc42, and were immunoblotted for RhoA, Rac1, Cdc42, or GAPDH (A). The histogram represents the percentage of RhoA, Rac1, and Cdc42 content (B). (C and D) RhoA-, Rac1-, or Cdc42-KD cells were left untransfected (C and D) or were transfected with the construct expressing the FH2 domain of INF2 as indicated (D). After conjugation to SEE-loaded APCs, cells were stained for Glu- and Tyr-MTs (C). The histogram represents the percentage of T cells with Glu-MTs or with polarized MTOC (D). (E) Control, Rac1-KD, or Cdc42-KD cells were treated with 3 nM taxol for 18 h. After conjugation to SEE-loaded APCs, cells were stained for Glu- and α -tubulin. The histogram represents the percentage of T cells with Glu-MTs or polarized MTOC (E). The arrowheads indicate the position of the MTOC of the T cells. At least 40 T cells were analyzed in D and E. Data in B, D, and E are summarized as means \pm SEM from three independent experiments (error bars; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Bars, 5 μ m.

of Glu-MT formation and MTOC reorientation in T cells and identify INF2 and Glu-MTs as central players in these processes downstream of Rac1 and Cdc42.

MT polarization to the cell-to-cell contact during IS formation is defective in INF2 KD T cells

In migrating 3T3 fibroblasts, MTOC reorientation takes place by maintaining the MTOC at the centroid in a process regulated by Cdc42, dynein/dynactin, and backward movement of the nucleus dependent on Cdc42 and actin dynamics (Gomes et al., 2005; Schmoranz et al., 2009). Unlike migrating 3T3 fibroblasts, it was clearly the MTOC and not the nucleus that moved during MTOC polarization in T cells (Fig. 6 A and Video 4). In these cells, when the MTOC moved toward the IS, most of the MTs became oriented toward the cell-to-cell contact. In contrast with control cells, the MTOC moved erratically and the MT array did not polarize toward the cell-to-cell contact in INF2 KD cells (Fig. 6 B and Video 5). MTOC reorientation was sensitive to nocodazole at a concentration that disrupts all types of MTs but, unlike the process in fibroblasts, was insensitive to treatment with inhibitors of actin dynamics (Fig. 6 C). Using latrunculin

A, we observed that Glu-MT formation took place normally in cells with impaired actin dynamics (Fig. 6 D). EB1, a MT plus end-binding protein, participates in MT stabilization through the formation of a complex with adenomatous polyposis protein and mDia. The carboxyl terminus of EB1 interacts with adenomatous polyposis protein and p150^{Glu}, and its expression interferes with the formation of Glu-MTs (Wen et al., 2004). Consistent with the requirement of Glu-MTs, MTOC reorientation did not take place in cells expressing EB1-C-GFP, whereas it occurred normally in cells expressing the EB1-C-KR-GFP mutant (Fig. 6 E), which does not affect Glu-MT formation (Wen et al., 2004). In conclusion, in T cells it is the MTOC and not the nucleus that moves for MTOC polarization; Glu-MT formation and MTOC reorientation are independent of actin dynamics; and the process of MTOC reorientation does not occur in the absence of Glu-MTs.

Glu-MTs are necessary for MTOC positioning at the IS

To investigate the processes of Glu MT formation and MTOC reorientation in T cells in a simple, controlled context, we used latex beads coated with anti-TCR antibodies as surrogate APCs. Glu-MT formation required TCR engagement, as it did

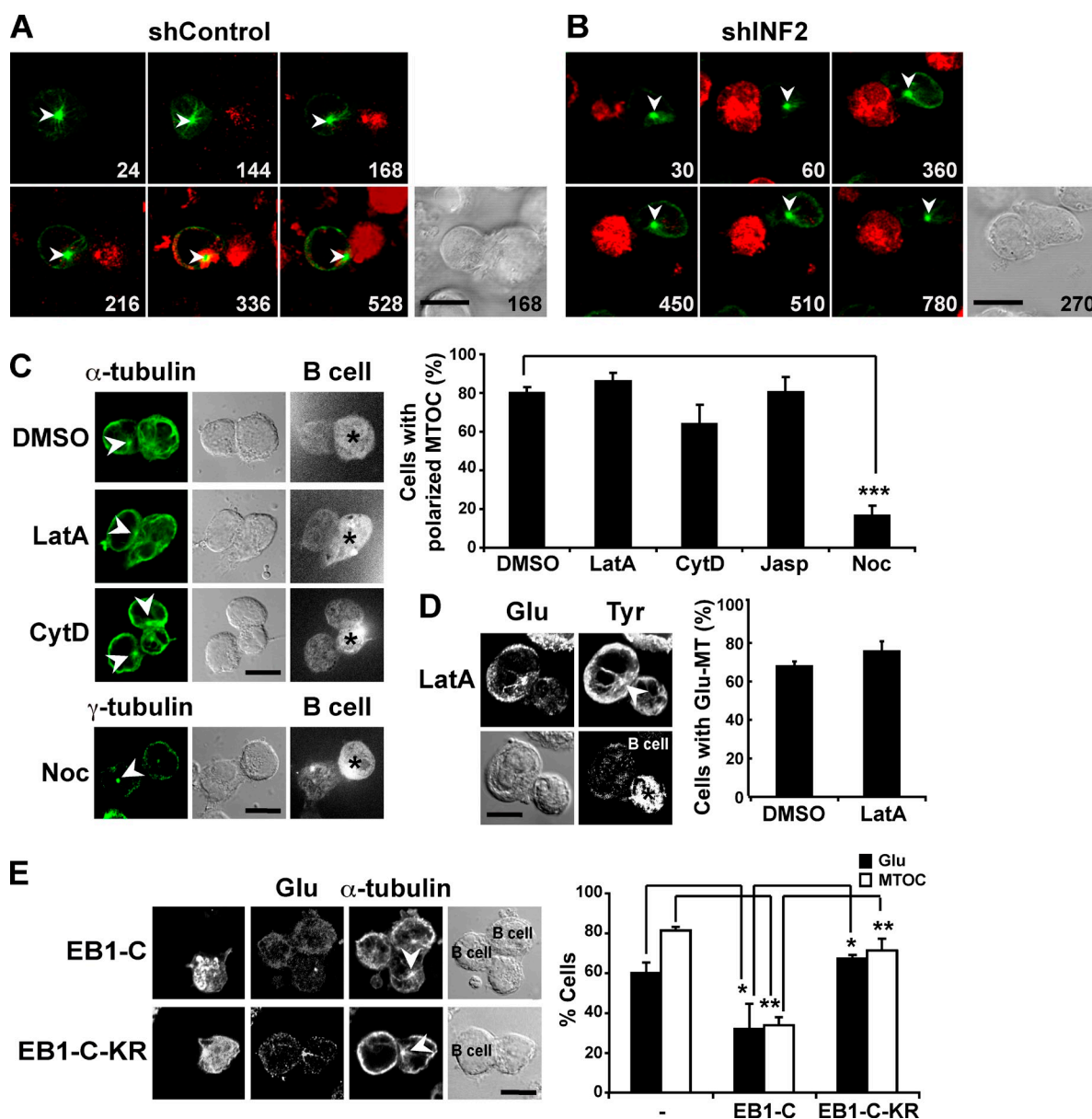


Figure 6. MTs do not polarize to the contact site in INF2 KD cells. (A and B) Control (A) or INF2 KD Jurkat cells (B) expressing GFP-tubulin were conjugated to SEE-loaded APCs and subjected to time-lapse video microscopy. Numbers indicate time in seconds. (C) Jurkat cells were treated with 0.1% DMSO, 1 μ M latrunculin A (LatA), 2.5 μ M cytochalasin D (CytD), 0.5 μ M jasplakinolide (Jasp), or 10 μ M nocodazole (Noc) for 1 h. Cells were conjugated in the presence of the drugs to SEE-loaded APCs and were stained for α - or γ -tubulin as indicated. APCs (B cells) were stained with CMTMR. Note that the CMTMR stain from B cells diffuses into the medium, resulting in some background staining of T cells. The CMTMR images were subjected to nonlinear adjustment to help B cell identification. CMTMR-positive cells are marked with an asterisk. The histogram represents the percentage of T cells with polarized MTOC. (D) Jurkat cells were treated with 1 μ M latrunculin A and conjugated in the presence of the drug to SEE-loaded APCs that were stained with CMTMR. Cells were finally stained for Glu- and Tyr-MTs. The histogram represents the percentage of T cells with Glu-MTs. (E) Control or Jurkat cells expressing EB1-C-GFP or EB1-C-KR-GFP were conjugated to SEE-loaded APCs. Cells were then stained for Glu-MT and α -tubulin. The histogram represents the percentage of transfected T cells with Glu-MTs or polarized MTOC. The arrowheads indicate the position of the MTOC of the T cell. At least 40 T cells were analyzed in C–E. Data in C–E are summarized as means \pm SEM from three (C and D) or two (E) independent experiments (error bars; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Bar, 5 μ m.

not occur in cells incubated with anti-transferrin receptor-coated beads, used as a control (Fig. 7, A and B). Remarkably, treatment with PP2 did not affect Glu-MT formation induced by TCR engagement (Figs. 2 E and 7, A and B), although, consistent with previous reports (Tsun et al., 2011), it did block MTOC reorientation (Fig. 7, A and B).

To investigate whether Glu-MTs are necessary for MTOC polarization, we took advantage of expression of the INF2 FH2

domain that produces constitutive expression of Glu-MTs (Fig. S2, B and C). To this end, we used INF2 KD Jurkat cells to prevent TCR-induced formation of Glu-MTs, and expressed or did not express the FH2 domain of INF2 to allow constitutive Glu-MT formation or to leave the cells without Glu-MTs, respectively. Cells were then incubated with beads coated with either anti-transferrin receptor or anti-TCR antibodies and examined for Glu-MT formation (Fig. 7, C and E)

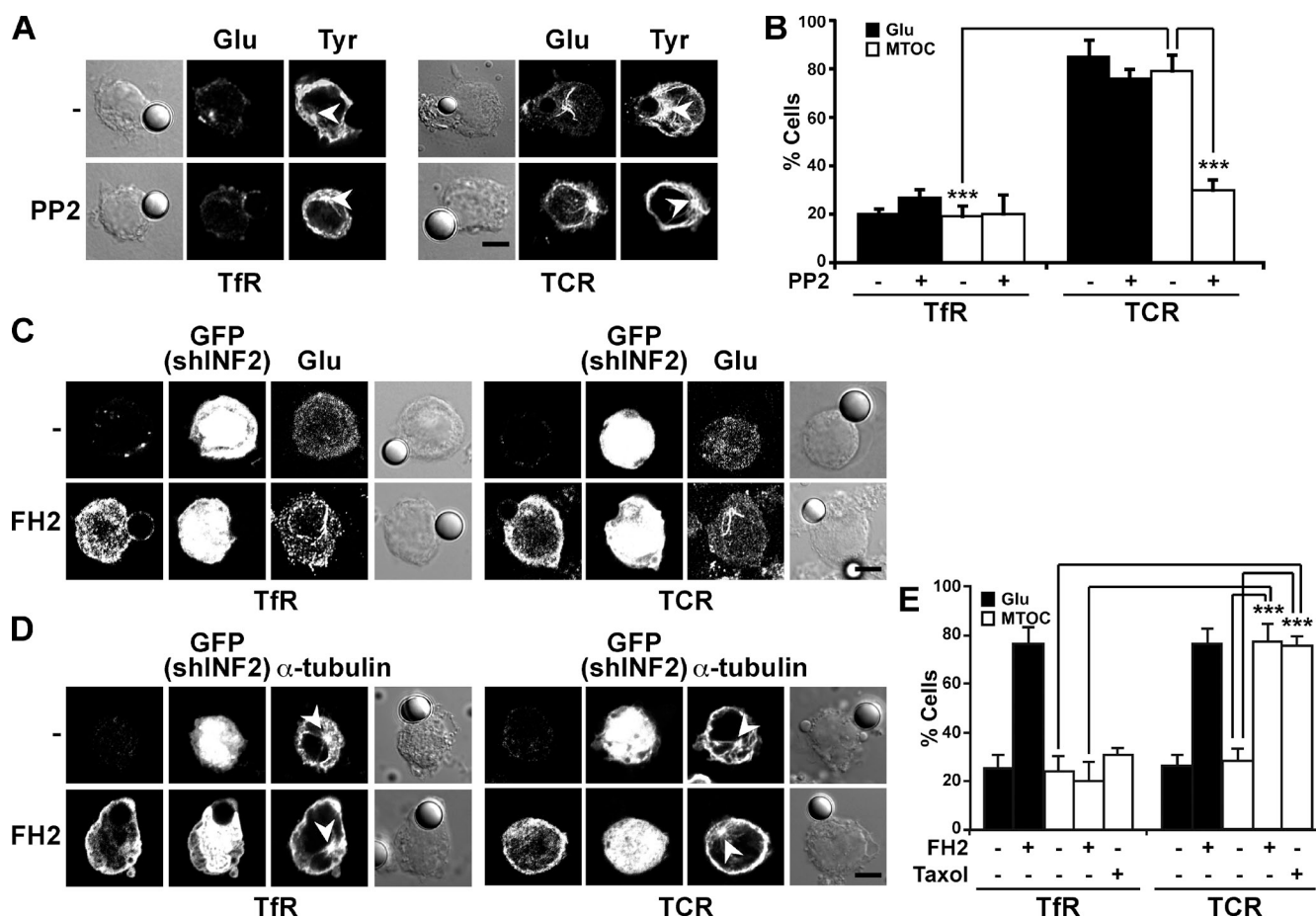


Figure 7. Glu-MTs are necessary for MTOC reorientation. (A and B) Glu-MT formation and MTOC distribution in Jurkat cells conjugated with latex beads coated with either anti-transferrin receptor (TfR) or anti-TCR antibodies in the presence or the absence of 2.5 μ M PP2 (A). The histogram represents the percentage of T cells with Glu-MTs or with polarized MTOC (B). (C–E) Jurkat cells coexpressing GFP and shINF2b were transfected or not transfected with a DNA construct expressing the intact FH2 domain of INF2, as indicated. Cells were then conjugated to latex beads coated with either anti-TfR or anti-TCR antibodies and were stained for the expressed INF2 FH2 fragment and Glu-MT (C) or α -tubulin (D), as indicated. INF2-KD cells treated with 3 nM taxol for 18 h were activated with anti-TfR or anti-TCR-coated beads, and analyzed for MTOC reorientation by staining for α -tubulin (E). The histogram represents the percentage of T cells with Glu-MTs or polarized MTOC (E). Bars, 5 μ m. The arrowheads indicate the position of the MTOC. At least 40 T cells were analyzed in B and E. Data in B and E are summarized as means \pm SEM from three independent experiments (error bars; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

and MTOC reorientation (Fig. 7, D and E). Remarkably, MTOC polarization was only observed in the case of TCR engagement of cells bearing Glu-MTs. TCR engagement also led to MTOC polarization in INF2-KD cells treated with taxol, confirming the importance of Glu-MTs in this process (Fig. 7 E). The results in Fig. 7 indicate that both Glu-MTs and TCR-induced tyrosine phosphorylation are simultaneously required for MTOC repositioning.

Discussion

A repositioned MTOC is necessary for polarizing the exocytic and endocytic compartments and the subsequent vectorial delivery of proteins (Kuhn and Poenie, 2002; Stinchcombe et al., 2006; Griffiths et al., 2010). In T cells that form an IS, MTOC positioning has been shown to rely on the expression of TCR-proximal signaling components that affect multiple pathways downstream of the TCR, and on other proteins, such as dynein and the formins DIA1 and FMNL1, whose exact role in the process is poorly understood (Billadeau et al., 2007). In this study,

we found that stable Glu-MTs rapidly form upon TCR engagement. This process and that of MTOC reorientation were dependent on the expression of formin INF2. We studied the role of INF2 and found that the FH2 is the region of INF2 necessary for both Glu-MT formation and MTOC reorientation. Experiments with T cells forced to display Glu-MTs constitutively, either by expressing FH2 domains or by pharmacological treatment with taxol, indicated that this subset of MTs is essential for MTOC polarization. INF2, therefore, plays a central role in T cells by mediating the formation of arrays of the Glu-MTs required for MTOC reorientation to the IS.

The INF2 isoforms 1 and 2, which differ in their carboxyl-terminal sequence, are differentially expressed depending on the cell type. NIH 3T3 fibroblasts express INF2-1 and have undetectable levels of INF2-2, whereas the opposite is true for Jurkat T cells and human osteosarcoma U2OS cells. Endogenous INF2 localizes mainly at the endoplasmic reticulum in NIH 3T3 cells but in a distinct, poorly defined, web-like pattern in U2OS cells (Ramabhadran et al., 2011). Consistent with cell type-specific differences that occur in INF2 localization,

endogenous INF2 distributed at the plasma membrane, the pericentrosomal region, and radial MT in resting T cells (Andrés-Delgado et al., 2010). INF2 translocated rapidly with the MTOC to the IS in cells conjugated with APCs in the presence of antigenic stimulation, as is the case with formins DIA1 and FMNL1 (Gomez et al., 2007), which have a similar distribution to that of INF2. As with Dia1 and FMNL1 KD (Gomez et al., 2007), INF2 KD greatly impaired MTOC reorientation without altering actin polymerization at the IS. MTOC reorientation took place efficiently when INF2 proteins with mutations in specific residues critical for its actin polymerization and depolymerization activities substituted endogenous INF2. This finding is consistent with previous results and our own observations showing that actin dynamics are not necessary in T cells for MTOC polarization, as this process takes place efficiently in the presence of actin polymerization inhibitors (Sedwick et al., 1999). Therefore, INF2 is required for MTOC reorientation independently of its actin polymerization and depolymerization activities.

A second, less well-known activity of some formins, such as mDia1-2, is to induce the formation of stable, detyrosinated MTs (Bartolini and Gundersen, 2010). Using different stimuli and cell model systems, we observed that Glu-MTs were rapidly and specifically formed in response to TCR engagement in T cells. Tyrosine phosphorylation of the CD3 subunits of the TCR-CD3 complex by Src-family kinases Lck and Fyn is the best characterized early TCR signaling event, and is followed by the tyrosine phosphorylation of other substrates (Smith-Garvin et al., 2009). TCR stimulation increases the levels of phosphotyrosinated α -tubulin, but the significance of this modification is unclear, as phosphorylated α -tubulin does not incorporate into MTs, but remains in the soluble, unpolymerized tubulin pool (Ley et al., 1994). Therefore, α -tubulin phosphorylation does not seem to play a role in Glu-MT formation in T cells. Importantly, Glu-MT formation in activated T cells was insensitive to Src-family tyrosine kinase inhibition, which is consistent with previous observations showing that formation of Glu-MTs is not inhibited in fibroblasts from triple Src, Yes, Fyn knockout mice (Palazzo et al., 2004). Although tyrosine phosphorylation occurs very early after TCR engagement, it is not the first event because it is preceded by a conformational change in the TCR-CD3 complex that occurs independently of tyrosine phosphorylation. This change involves the exposure of hidden sequences in the cytoplasmic tail of the CD3 ϵ subunit that allows binding of Nck, an adapter protein containing three SH3 domains and a carboxyl-terminal SH2 domain (Lettau et al., 2009), to a proline-rich motif (Gil et al., 2002). Therefore, it is conceivable that the TCR conformational change triggers, in a tyrosine phosphorylation-independent manner, the binding of proteins that mediate the formation of Glu-MTs to the cytoplasmic tail of CD3 ϵ .

INF2 silencing and reconstitution experiments in INF2 KD cells demonstrated that INF2 mediates Glu-MT formation and does so independently of its actin polymerization activity, as is the case of mDia2 (Bartolini et al., 2008). Also, as is seen with mDia2 (Bartolini et al., 2008), the FH2 was identified as the INF2 domain responsible for Glu-MT formation. It is of particular note that, in addition to restoring Glu-MT formation, INF2 corrected MTOC reorientation to the IS in INF2 KD T cells. It therefore

seems that INF2 mediates Glu-MT formation and MTOC polarization through its FH2 domain. The expression of the FH2 domain of FMNL1 and DIA1 cells also restored Glu-MT formation and MTOC reorientation in INF2 KD cells. This result indicates that a certain threshold of Glu-MTs is needed for MTOC reorientation and that, in their Glu-MT-stabilizing deregulated form, the three formins can substitute for one another. Importantly, when the cells were treated with concentrations of taxol that allow MT polymerization and stabilize MTs but do not completely block MT dynamics (Derry et al., 1995), the defect of MTOC repositioning found in INF2 KD cells was corrected. This result strongly indicates that stable Glu-MTs are crucial to MTOC polarization. This effect is reminiscent of the induction of axon growth and neuronal polarization in cells whose MTs were stabilized by low doses of taxol (Witte et al., 2008).

The involvement of several formins (at least INF2, DIA1, and FMNL1) calls into question how different formin proteins manage to act in coordination to control MTOC movement. Cross-talk between INF2 and mDia1-3 has recently been reported (Sun et al., 2011). The diaphanous inhibitory domain (DID) of INF2 binds the diaphanous autoregulatory domain (DAD) of these mDia proteins, and the interaction inhibits *in vitro* actin polymerization by mDia. Therefore, the interaction of INF2 with mDias may allow all these molecules to function as each other's regulators, and may be a subtle mechanism for controlling actin polymerization by different formins. We observed that Glu-MT formation was impaired not only in INF2 KD cells but also in DIA1 or FMNL1 KD cells. The FH2 domain of mDia1, mDia2, and INF2 has been identified as being necessary for these formins to interact with MTs *in vitro* (Bartolini et al., 2008; Gaillard et al., 2011). Therefore, it is possible that, in addition to regulating actin polymerization, the interaction between different formins might also regulate their association with MTs and their activity in Glu-MT formation.

Regulation of formin proteins is thought to occur primarily through interactions with Rho-family GTPases (Goode and Eck, 2007). The participation of Rho-family GTPases in the process of MTOC reorientation in T cells remains puzzling. Pioneer work using expression of a dominant-negative form of Cdc42 established a role for Cdc42 in the control of MTOC reorientation (Stowers et al., 1995). However, the effect of the expression of similar mutants of Rho or Rac1 has not been examined. It has also been reported that Rac1 KD resulted in diminished MTOC reorientation, whereas Cdc42 KD had, at most, a moderate effect (Gomez et al., 2007). The effect of Rho KD was not analyzed in that study. Consistent with the involvement of INF2, which binds Rac1 and Cdc42, the silencing of either of these two GTPases significantly impaired MTOC reorientation, whereas that of RhoA had no effect. The discrepancy between our results with Cdc42 KD cells and those previously published (Gomez et al., 2007) is probably caused by differences in the residual expression of Cdc42. It is worth noting that, in addition to MTOC translocation, Glu-MT formation was impaired in Rac1 or Cdc42 KD cells but not in RhoA KD cells. These results indicate that Rac1 and Cdc42 regulate both processes, although the contribution of Rho isoforms other than RhoA cannot be ruled out. Most importantly, the expression of the INF2 FH2 domain and the treatment with taxol

corrected the defects in Glu-MT formation and MTOC reorientation observed in Rac1 and Cdc42 KD cells. These results confirm the importance of INF2 and identify INF2 and Glu-MTs as key players acting downstream of Rac1 and Cdc42 in these processes.

Our results indicate that Glu-MTs are essential for MTOC polarization in T lymphocytes. This finding contrasts with what occurs in NIH 3T3 fibroblasts, in which Glu-MT formation and MTOC reorientation are independently regulated (Palazzo et al., 2001b). The question arises as to how Glu-MTs mediate MTOC translocation. In natural killer cells, loss of DIA1 perturbs the MT cytoskeleton, including the targeting of MTs to the lytic synapse (Butler and Cooper, 2009). Our analysis of MT movement in control T cells during MTOC repositioning revealed that the MTs orient toward the cell-to-cell contact and subsequently, once the MTOC has reoriented completely, attach to distant parts of the cell cortex as if they are maintaining the MTOC in its final position. This was also observed in cytotoxic T lymphocytes during the killing of their target cell (Kupfer and Dennert, 1984; Kuhn and Poenie, 2002; Stinchcombe and Griffiths, 2007). In contrast with control cells, the MTOC moved erratically and the MT array did not orient toward the cell-to-cell contact in INF2 KD cells, which lack Glu-MTs. However, the MTOC oriented efficiently when Glu-MT formation was forced by expression of the INF2 FH2 domain or by treatment with low doses of taxol in INF2 KD cells. In addition, MTOC polarization was blocked by expression of EB1-C-GFP, which interferes with Glu-MT formation (Wen et al., 2004). These observations indicate that Glu-MTs are necessary for MTOC polarization during IS formation. Tyrosination is crucial for MT interaction with plus end-tracking proteins containing CAP-Gly MT-binding domains (Peris et al., 2006; Galjart, 2010), which include proteins such as CLIP170, the large subunit of the dynactin complex p150^{glued}, and kinesin KIF13B (Galjart and Perez, 2003; Galjart, 2010). Glu-MTs could contribute to the MTOC reorientation process through their enhanced stability (although acetylated MTs are also stable and do not seem to be important for this process), by their ability to preferentially interact with kinesin-1 motor proteins (Kreitzer et al., 1999; Dunn et al., 2008; Konishi and Setou, 2009), or by delocalization of putative CAP-Gly domain-containing negative regulators. MTOC polarization in T cells is insensitive to treatment inhibitors that suppress the disassembly and reassembly of MTs, which indicates that the reorientation process relies on stable MTs (Knox et al., 1993; Baratt et al., 2008; Zyss et al., 2011). Supporting the requirement of stable MTs, it has recently been reported that casein kinase I δ phosphorylates the MT plus end-binding protein EB1 and contributes to the increase in the rate of MT growth, a mechanism that might serve to generate the stable MTs necessary for MTOC translocation (Zyss et al., 2011). Importantly, although formation of stable Glu-MTs does not require de novo tyrosine phosphorylation, MTOC reorientation toward the engaged TCR requires simultaneously stable Glu-MTs and, consistent with previous findings (Lowin-Kropf et al., 1998), TCR-induced tyrosine phosphorylation.

In addition to detyrosination, MTs are the subject of a large variety of posttranslational modifications, including phosphorylation, polyglutamylation, polyglycylation, and acetylation (Wloga and Gaertig, 2010). These modifications might provide

a mechanism for the functional specialization of MTs, although their role in T cells is not yet known. Nevertheless, subtle and transient deacetylation of MTs is known to accompany the process of MTOC reorientation (Serrador et al., 2004). Herein, we have shown that Glu-MTs form rapidly in response to TCR engagement and that the formation of this subset of MTs and the polarization of the MTOC to the IS are strictly dependent on the expression of formin INF2. Importantly, Glu-MT formation proves to be crucial to MTOC reorientation in T cells. Therefore, formin INF2 and Glu-MTs are key players in the process of MTOC reorientation to the IS in human T lymphocytes.

Materials and methods

Materials

The rabbit polyclonal antibodies to INF2 (Madrid et al., 2010) and those to Glu-MTs (Gundersen et al., 1984) have been described previously. The mouse mAb DM1A to total α -tubulin, the mAb to acetyl-tubulin, and the rat mAb YL1/2 to Tyr-tubulin were purchased from Sigma-Aldrich. The antibodies to RhoA, Rac1, Cdc42, and DIA1 were from BD, the antibody to FMNL1 was from Novus Biologicals, and the antibody to TIR was from Invitrogen. The anti-glyceraldehyde 3-phosphate dehydrogenase (anti-GAPDH) antibody was from Santa Cruz Biotechnology, Inc. The mAb to CD3 was provided by B. Alarcón (Centro de Biología Molecular "Severo Ochoa," Madrid, Spain). We obtained SEE and SEB superantigens from Toxin Technology. Taxol (paclitaxel) was from Sigma-Aldrich. HRP-conjugated secondary antibodies were obtained from Thermo Fisher Scientific. Fluorescent secondary anti-rabbit or anti-mouse IgG antibodies, TRITC-phalloidin, and cell tracker orange-fluorescent tetramethylrhodamine (CMTMR) were purchased from Molecular Probes. Secondary anti-rabbit or anti-mouse IgG antibodies coupled to HRP were from Jackson ImmunoResearch Laboratories.

Cell-culture conditions

Human T lymphoblastoid Jurkat cells were grown in RPMI 1640 supplemented with 10% FBS (Sigma-Aldrich), 50 U/ml penicillin, and 50 μ g/ml streptomycin at 37°C in an atmosphere of 5% CO₂/95% air. To distinguish Raji cells from Jurkat cells in the conjugates, Raji cells (3.0×10^6 cells/ml) were stained with CMTMR for 20 min at 37°C, washed, and resuspended in RPMI/5% FBS. For formation of T cell-APC conjugates, Raji B cells (3.0×10^6 cells/ml) were incubated for 20 min in the presence or absence of 4 μ g/ml SEE (Toxin Technology) and mixed with an equal number of Jurkat cells (5.0×10^5 cells/well) in a final volume of 50 μ l, incubated at 37°C for 15 min, and plated onto poly-L-lysine-coated slides. Jurkat CH7C17 cells expressing exogenous TCR α and TCR β (V β 3) chains specific for hemagglutinin (Niedergang et al., 1997) were conjugated to HOM2 cells in the presence of 200 μ g/ml of hemagglutinin peptide 307–319 (PKYVKQNTLKLAT), a control inactive peptide (PKYVKQNTLELAT), or 4 μ g/ml SEB. For conjugation of primary T cells, freshly isolated T lymphocytes from healthy donors were incubated with SEE-pulsed Raji cells and processed as described for the Jurkat-APC conjugates. Primary T cell isolation was done according to the guidelines of the Bioethics Committee of the Spanish Research Council and with the approval of the institutional management committee of the Centro de Biología Molecular "Severo Ochoa" (Madrid, Spain).

DNA constructs and transfection conditions

The plasmids coexpressing GFP and shRNAa or shRNAb specific to human INF2 were made in the pSR-GFP/neo vector (Oligoengine) as described previously (Andrés-Delgado et al., 2010; Madrid et al., 2010). The DNA constructs in the pGeneClip hMGFP vector coexpressing GFP and shRNA specific to human RhoA, Rac1, Cdc42, DIA1, or FMNL1 were from SABiosciences. The DNA constructs in the pEGFP-C1 or pmCherry-C1 vectors (BD) expressing GFP or Cherry fusions of wild-type INF2 or INF2 proteins with point mutations in sequences encoding the FH2 and/or the DAD (INF2-K/A; INF2-3L/A and INF2-K/A-3L/A mutants) as well as the constructs in the pCR3.1 expression vector (Invitrogen) expressing specific myc-tagged INF2 fragments have been described previously (Andrés-Delgado et al., 2010; Madrid et al., 2010). The FH2 domain of INF2, the FH2 I/A-K/A mutant, and the FH2 of mDia1 or FMNL1 were obtained by standard procedures using mDia1 (a gift from S. Narumiya, Kyoto University, Kyoto, Japan) or FMNL1 (Source Bioscience) cDNA, respectively, as templates, and were cloned in pCR3.1. The constructs used for expression of INF2 proteins

contain only the coding sequence of the INF2 mRNA, and therefore their expression products are resistant to knockdown by shINF2b, which targets the 3' untranslated region. The DNA constructs expressing the ectodomain and transmembrane region of murine CD4 fused to Lck (CD4/Lck; Krummel et al., 2000), and EB1-C-GFP and EB1-C-KR-GFP (Wen et al., 2004) have been described previously. Jurkat cells and primary human T cells were transfected by electroporation using the Gene Pulser system (Bio-Rad Laboratories). To evaluate the extent of protein knockdown using the shRNA-expressing constructs, GFP-expressing cells were separated in a cell sorter and analyzed by immunoblotting with the appropriate antibodies.

Confocal microscopic analysis

Cells were fixed in 4% paraformaldehyde for 15 min, rinsed, and treated with 10 mM glycine for 5 min to quench the aldehyde groups. The cells were permeabilized with 0.1% Triton X-100, rinsed, and incubated with 3% BSA in PBS for 15 min. In the case of Glu-MT or γ -tubulin staining, cells were fixed with methanol at -20°C . Cells were then incubated for 1 h with the appropriate primary antibodies, rinsed several times, and incubated for 30 min with the appropriate combination of secondary antibodies coupled to Alexa Fluor 488, Alexa Fluor 594, or Alexa Fluor 647. Actin filaments were detected with TRITC-phalloidin. Coverslips were mounted with Fluoromount (Sigma-Aldrich). Controls to assess labeling specificity included incubations with, or omitting, control primary antibodies. Images were obtained at room temperature using LSM510 META confocal microscope equipment (Carl Zeiss) coupled to an inverted Axiovert 200 microscope (Carl Zeiss). A 63 \times /1.4 NA oil Plan Apochromat objective lens was used. For time-lapse experiments, cells were maintained at 37°C in Hank's balanced salt solution supplemented with 5% FBS. Cell images were captured at 30-s intervals. Images were analyzed with MetaMorph imaging software (Molecular Devices). Some images were deconvoluted using Huygens 3.0 software (Scientific Volume Imaging). 3D reconstruction was done using ImageJ software. Images were exported in TIFF format, and their brightness and contrast were optimized with Photoshop (Adobe).

Statistical analysis

Data are expressed as mean \pm SEM. A paired Student's *t* test was used to establish the statistical significance of differences between the means.

Online supplemental material

Fig. S1 shows the effect of the expression of CD4/Lck on MTOC reorientation in INF2 KD cells. Fig. S2 shows that the expression of the FH2 domain of INF2 is sufficient for Glu-MT formation in resting Jurkat cells. Fig. S3 shows that taxol treatment induces Glu-MT formation in resting Jurkat cells. Videos 1 and 2 show the dynamics of actin in control and INF2 KD cells, respectively. Video 3 shows 3D views of Glu-MTs in a T cell-APC conjugate. Videos 4 and 5 show the dynamics of MTs in control and INF2 KD cells, respectively. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201202137/DC1>.

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