relationship to the kinesin-1 tetramer and examine the importance of cargo-induced TPR conformational change in motor activation.

**References and Notes**


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**Supplementary Materials**

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**Materials and Methods**

Supplementary Text

Figs. S1 to S7

Table S1

References (22–33)

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**Actin-Propelled Invasive Membrane Protrusions Promote Fusogenic Protein Engagement During Cell-Cell Fusion**

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Cell-cell fusion is critical for the conception, development, and physiology of multicellular organisms. Although cellular fusogenic proteins and the actin cytoskeleton are implicated in cell-cell fusion, it remains unclear whether and how they coordinate to promote plasma membrane fusion. We reconstituted a high-efficiency, inducible cell fusion culture system in the normally nonfusing *Drosophila* S2R+ cells. Both fusogenic proteins and actin cytoskeletal rearrangements were necessary for cell fusion, and in combination they were sufficient to impart fusion competence. Localized actin polymerization triggered by specific cell-cell or cell-matrix adhesion molecules propelled invasive cell membrane protrusions, which in turn promoted fusogenic protein engagement and plasma membrane fusion. This de novo cell fusion culture system reveals a general role for actin-propelled invasive membrane protrusions in driving fusogenic protein engagement during cell-cell fusion.

Cell-cell fusion occurs in many biological processes such as fertilization, myogenesis, placenta formation, bone remodeling, and immune response (1–3). Transmembrane fusogenic proteins are implicated in the fusion of multiple cell types in *Caenorhabditis elegans* (4), whereas actin polymerization is implicated in the fusion of muscle cells in *Drosophila*, zebrafish, and mice (5–7). It remains unknown whether fusogenic proteins and the actin cytoskeleton coordinate during cell-cell fusion, and if so, how this is accomplished. We addressed these questions by reconstituting cell fusion de novo in the otherwise nonfusing S2R+ cells, a hemocyte-like cell line derived from *Drosophila* embryos (8).

Transfection of known components of *Drosophila* myoblast fusion, including cell adhesion molecules (9, 10) and actin cytoskeletal regulators (11–14), failed to induce S2R+ cell fusion, despite causing extensive cell adhesion and F-actin enrichment at cell-cell contact sites (fig. S1, A to C). Expression of the C. elegans fusogenic protein Eff-1 (15, 16) induced low-level S2R+ cell fusion (Fig. 1, A and F). Multinucleate syncytia were observed 24 hours after Eff-1 transfection, and by 72 hours after transfection, ~12% (12.1 ± 1.1%) of Eff-1–positive cells were in multinucleate syncytia, with each syncytium containing a median number of eight nuclei (Fig. 1, F and G). These Eff-1–induced multinucleate syncytia resulted from cell fusion (fig. S2, A to B’’), and Eff-1 was required in both fusion partners (fig. S2C), similar to findings reported for moth SF9 cells (16).

Because close membrane apposition is a prerequisite for membrane fusion, we asked whether Eff-1–induced fusion could be enhanced by coexpressing cell adhesion molecules. Dumbfounded (Duf) and Sticks and stones (Sns) are immunoglobulin (Ig) domain–containing transmembrane proteins that are required for *Drosophila* myoblast fusion (9, 10) but are not normally expressed in S2R+ cells (fig. S1D). Exogenous Duf, but not Sns, promotes homophilic cell adhesion in cultured *Drosophila* cells (17–19), as does Echinoid (Ed), an Ig domain–containing transmembrane protein not implicated in myoblast fusion (20, 21). Among the three proteins, only Sns enhanced Eff-1–mediated fusion (Fig. 1, B, C, D, and F), which suggests that membrane apposition mediated by cell adhesion per se is not sufficient to promote Eff-1–mediated fusion. Nearly 90% (86.3 ± 2.9%) of the Sns-Eff-1–coexpressing cells were in multinucleate syncytia (Fig. 1C), representing a factor of 7 increase over Eff-1–induced fusion (Fig. 1F). These large syncytia contained up to 220 nuclei, with a median number of 44 nuclei per cell (Fig. 1G). Live imaging confirmed that Sns-Eff-1–induced syncytial formation resulted from cell fusion (fig. S3, A and B, and movies S1 and S2). Besides Sns, overexpression of an α subunit (orPS2) of the cell-matrix adhesion molecule integrin (22), which has been implicated in multiple types of cell fusion events (23–26), enhanced Eff-1–mediated fusion by a factor of 5 (63.9 ± 4.3%) with a median number of 20 nuclei per cell (Fig. 1, E to G). The marked enhancement of Eff-1–mediated cell fusion by Sns and integrin, neither of which facilitated homophilic cell adhesion nor interacted with Eff-1 more strongly than did Duf (fig. S4), prompted us to examine the cellular mechanisms underlying their fusion-enhancing activity.

In *Drosophila*, Sns and Duf trigger distinct actin cytoskeletal changes during myoblast fusion. Sns organizes an F-actin–enriched invasive podosome-like structure (PLS) in the fusion competent myoblast (27, 28), whereas Duf promotes the formation of a thin sheath of actin underlying the apposing founder cell membrane (27). Their differential activity in remodeling the actin cytoskeleton was recapitulated in S2R+ cells, as F-actin–enriched foci were observed at cell-cell contact sites marked by the accumulation of Eff-1 and the cell adhesion molecule in Sns-Eff-1–expressing cells (Fig. 1H and fig. S5A) but not in Duf-Eff-1–expressing cells (Fig. 1I and fig. S5B). Live imaging of Sns-Eff-1–expressing cells using green fluorescent protein (GFP) fused to the F-actin–
binding C-terminal tail of moesin (GFP-moesin) (29) revealed that these F-actin foci corresponded to sites of fusion (Fig. 2A, fig. S6, A and B, and movies S3 to S5). Using a cell-mixing fusion assay, we showed that Sns was required in only one of the two fusion partners to promote efficient cell fusion (fig. S7).

Because the actin nucleation-promoting factors (NPFs) of the Arp2/3 complex, WASP (11–13) and Scar/WAVE (14), are required for Sns-induced PLS formation in Drosophila myoblast fusion (27), we investigated whether WASP and Scar are required for Sns-Eff-1–induced cell fusion. RNA interference (RNAi) knockdown of WASP, its binding partner WASP-interacting protein (WIP) (11, 12), or Scar abolished Sns-induced F-actin foci (fig. S8) and eliminated Sns-enhanced cell fusion (Fig. 2B). At sites of fusion, fluorescence recovery after photobleaching (FRAP) analysis revealed more dynamic exchanges of WASP and Scar than of Sns (Fig. 2, C and D, fig. S9, and movies S6 and S7), which suggests that Sns provides a relatively stable organizing center at these sites to recruit WASP and Scar. These findings indicate that dynamic actin cytoskeletal rearrangement is required for Sns-Eff-1–induced cell fusion. RNAi knockdown of the P40 subunit of the Arp2/3 complex in moth S9 cells also decreased Eff-1–induced fusion (7.2 ± 1.2% versus 16.7 ± 6.1%, fig. S10), indicating that Arp2/3-mediated actin polymerization is generally required for fusion in different cell types.

To examine whether Arp2/3-mediated actin polymerization is sufficient to enhance Eff-1–mediated fusion, we fused WIP, WASP, or Scar to the C terminus of Duf or Sns and coexpressed each chimeric protein with Eff-1 in S2R+ cells. Attaching WIP to Sns did not affect Sns’s ability to organize actin polymerization at cell-cell contact sites (fig. S11A) or to enhance fusion (Fig. 2E). Attaching WIP to Duf induced the formation of F-actin–enriched hairlike protrusions at cell-cell contact sites (fig. S11B) and converted Duf into a fusion-promoting molecule (Fig. 2F). This result suggests that WIP-mediated actin cytoskeletal rearrangement is sufficient to enhance Eff-1–mediated cell fusion. Unexpectedly, attaching WASP or Scar directly to Duf did not enhance Eff-1–mediated fusion (Fig. 2F), and attaching these NPFs to Sns abolished Sns’s fusion-enhancing activity (Fig. 2E) because of mislocalization of the chimeric proteins. Unlike Duf-WIP or Sns-WIP, which were correctly targeted to the cell membrane (fig. S11, A and B), Duf-WASP, Duf-Scar, Sns-WASp, and Sns-Scar were localized in the cytoplasm (fig. S11, C and D), where they induced actin comet tails propelling rapid movement of vesicles containing Eff-1 and the corresponding chimeric protein (fig. S11, C and D, and movies S8 and S9). Thus, localized Arp2/3-mediated actin polymerization at cell-cell contact sites promotes Eff-1–mediated fusion.

Because Arp2/3 nucleates a branched actin network that drives membrane protrusions, we predicted that the F-actin foci at cell-cell contact sites may be invasive. To test this, we conducted super-resolution total internal reflection fluorescence (TIRF)–based stochastic optical reconstruction microscopy (STORM) (30) and ultrastuctural electron microscopy (EM). Both analyses revealed a tightly packed group of fingerlike protrusions extending from one cell into its fusion partner in Sns-Eff-1–expressing cells but not in Duf-Eff-1–expressing cells (Fig. 2, G and H, b and c; Fig. 3, A and A´; and fig. S12), consistent with the reported ultrastructure of the invasive PLS in Drosophila myoblast fusion (27, 31, 32). These fingers contained distinct clusters of Sns and Eff-1 (Fig. 2G, d to d´) and frequently overlapping Sns and actin signals (Fig. 2H, d to d´). Strikingly, segments of electron-dense “ladders” were present between the apposing membranes along the invasive fingers (Fig. 3, A, A´ and A´´). These electron-dense ladders grossly resembled the electron-dense spikes formed by the virus-packaged Eff-1 on the viral envelope (33) (albeit thinner than the latter) and spatially corresponded to clusters of Eff-1 on the invasive fingers observed by STORM (Fig. 2G, d´). Immunogold labeling confirmed the presence of Eff-1 on the membranes.
along the invasive fingers (fig. S13, B and B’). Thus, in Sns-Eff-1–induced cell fusion, Arp2/3-mediated actin polymerization generates finger-like membrane protrusions that promote Eff-1 engagement across the apposing cell membranes.

To determine whether actin-propelled membrane protrusions are generally involved in cell-cell fusion, we investigated how integrin modulated Eff-1–mediated fusion. Loss or gain of αPS2 function abolished or enhanced Eff-1–mediated cell fusion commensurate with the strength of cell-matrix adhesion (Fig. 1, E and F, and Fig. 4, A to C). Unlike Sns-Eff-1–induced fusion, αPS2-Eff-1–induced fusion only required Scar but not the WASP-WIP complex (Fig. 4A and fig. S14A), the latter of which is normally recruited to sites of myoblast fusion by Sns in Drosophila (11, 12).

As a consequence, αPS2-Eff-1–expressing cells formed numerous F-actin–containing hairlike projections, instead of dense F-actin foci, along the broad cell-cell contact zone (Fig. 4D and movie S10). Notably, cells expressing Eff-1 alone also occasionally formed multiple hairlike projections along cell-cell contact zones (Fig. 4E), and RNAi of Scar, but not of WASP or WIP, abolished the basal level of Eff-1–mediated cell fusion (Fig. 4B and fig. S14B). STORM and EM analyses revealed individual fingerlike protrusions sparsely localized at the cell periphery and along the cell-cell contact zone of αPS2-Eff-1–expressing cells (Fig. 3B and Fig. 4, F and G, b and c), corresponding to the hairlike projections visualized by confocal microscopy (Fig. 4D).

**Fig. 2.** Actin-propelled invasive fingerlike protrusions promote high-efficiency cell fusion in Sns-Eff-1–expressing 2SR+ cells. (A) A dynamic F-actin focus mediating cell fusion. Cells coexpressing Eff-1, Sns, and GFP-moesin were subjected to time-lapse imaging. Stills from representative movie S3 are shown; 0 min corresponds to 3:00:51 in movie S3. Arrow indicates the F-actin focus at the protruding tip of cell a, which was about to fuse with cell b. Scale bar, 10 μm. (B) Arp2/3-mediated actin polymerization is required for Sns-enhanced cell fusion. Data are fusion indices (%SD) resulting from RNAi knockdown of Sns, WASP, WIP, or Scar in cells coexpressing Sns and Eff-1 treated with corresponding double-stranded RNA (dsRNA). Statistical significance was determined using the two-tailed Student t test (**P < 0.001) by comparing control and dsRNA samples. (C and D) FRAP assays of WASP, Scar, and Sns at sites of fusion. Sns-mCherry and WASP-GFP (or Scar-GFP) foci were photobleached and monitored with respect to recovery percentage (C) and recovery half-time (D) in cells coexpressing Eff-1, Sns-mCherry, and WASP-GFP (or Scar-GFP). N = 4; error bars denote SD. (E and F) Effects of chimeric proteins of cell adhesion molecules and Arp2/3 regulators on cell fusion. Cells were transfected with Eff-1 alone or cotransfected with Eff-1 and indicated plasmids. Fusion indices (%SD) were quantified as described. (G and H) Sns and Eff-1 accumulate in distinct clusters on the cell membrane along the fingerlike protrusions. Cells cotransfected with Sns-V5 and Eff-1–HA were stained with antibodies to V5 (red), HA [green in (G)], and actin [green in (H)], and subjected to STORM analysis. Images were acquired by either wide-field microscopy (a) or STORM (b to d’). Boxed area in (a) and (b) is enlarged in (c); boxed area in (c) is enlarged in (d) to (d’). Arrow in (a) and (b) indicates fingerlike protrusions extending from a cell to its fusion partner. In (G), distinct clusters of Sns and Eff-1 are indicated by arrows in (d) and arrowheads in (d’). Boxed area in (a) and (b) is enlarged in (c); boxed area in (c) is enlarged in (d) to (d’). Arrow in (a) and (b) indicates fingerlike protrusions extending from a cell to its fusion partner. In (H), arrowheads in (d) to (d’) indicate the frequently overlapping signals of Sns and actin. Scale bars, 20 μm [(a) and (b)], 500 nm [(c), 100 nm [(d) to (d’)].
**Fig. 3.** Invasive fingerlike membrane protrusions promote fusogenic protein engagement. (A) Electron micrographs of Sns-Eff-1–expressing cells. Boxed area in (A) is enlarged in (A’); boxed area in (A’) is enlarged in (A’’). (A) Low-magnification view of two adherent cells. (A’) Cell on the right (pseudo-colored purple) extended a group of fingerlike protrusions (black arrows) to invade the cell on the left. Segments of electron-dense materials were present on the membranes along the protrusive fingers but were absent elsewhere on the cell membrane (magenta arrows). (A’’) At higher magnification, the electron-dense materials showed a ladderlike pattern between the two apposing cell membranes (red arrowheads). (B) Electron micrographs of αPS2-Eff-1–expressing cells. Boxed area in (B) is enlarged in (B’); boxed area in (B’) is enlarged in (B’’). (B) Low-magnification view of two adherent cells. (B’) Cell at the top (pseudo-colored purple) extended individual fingerlike protrusions to invade the cell at the bottom. The invasive fingers (black arrows) were scattered along the broad cell-cell contact zone and were associated with segments of electron-dense materials, which were absent elsewhere on the cell membrane (magenta arrows). (B’’) At higher magnification, the electron-dense materials also showed a ladderlike appearance (red arrowheads) between the two apposing membranes, as in Sns-Eff-1–expressing cells (A’’). Scale bars, 500 nm [(A), (A’), (B), and (B’)], 100 nm [(A’’) and (B’’)].

**Fig. 4.** F-actin–enriched hairlike projections promote cell fusion in αPS2-Eff-1–expressing S2R+ cells. (A) Scar, but not WASP-WIP, is required for αPS2-Eff-1–induced cell fusion. Fusion indices resulting from RNAi knockdown of Sns, WASP, WIP, Scar, or αPS2 in αPS2-Eff-1–expressing cells are shown. (B) Cell fusion induced by Eff-1 alone is mediated by endogenous Scar and αPS2. Fusion indices resulting from RNAi knockdown of indicated genes in Eff-1–expressing cells are shown. (C) Cells overexpressing αPS2 show stronger adhesion to the culture dish. Cells expressing indicated proteins were subjected to adhesion assays (see supplementary materials). αPS2 expression reduced the percentage of transfected cells in the detached population. Error bars in (A) to (C) denote SD. (D) and (E) F-actin–enriched hairlike projections along the cell-cell contact zone of αPS2-Eff-1– and Eff-1–expressing cells. Cells were transfected with GFP-moesin and αPS2-Eff-1 (D) or Eff-1 alone (E), and stained with antibodies to V5 (red; αPS2-V5) and HA (cyan; Eff-1–HA). Boxed areas are magnified in insets; schematic drawings of F-actin are at the right. Inset in (D) shows F-actin–enriched hairlike projections at the cell-cell contact zone of αPS2-Eff-1–expressing cells. Arrow in (D) to (D’’) indicates a small punctum of αPS2 within a hairlike projection. Inset in (E) shows the occasionally observed F-actin–enriched projections (one indicated by arrow) in Eff-1–expressing cells. Scale bars, 10 μm. (F and G) Distinct distribution of αPS2 and Eff-1 on the membrane along the F-actin–enriched fingerlike protrusions. Cells cotransfected with αPS2-V5 and Eff-1–HA were stained with antibodies to V5 (red), HA [green in (F)], and actin [green in (G)] and subjected to STORM analysis. Images were acquired by either wide-field microscopy (a) or STORM [(b) to (d’’)]. Boxed area in (a) and (b) is enlarged in (c); boxed area in (c) is enlarged in (d) to (d’’). Arrows in (c) indicate individual fingerlike protrusions. Arrow in (d) and (d’’) and arrowheads in (d’) and (d’’’) indicate distinct domains of αPS2 and Eff-1 (or actin). Scale bars, 20 μm [(a) and (b)], 500 nm [(c), 100 nm [(d) to (d’’)].
Bat and Rat Neurons Differ in Theta-Frequency Resonance Despite Similar Coding of Space

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Both bats and rats exhibit grid cells in medial entorhinal cortex that fire as they visit a regular array of spatial locations. In rats, grid-cell firing field properties correlate with theta-frequency rhythmicity of spiking and membrane-potential resonance; however, bat grid cells do not exhibit theta rhythmic spiking, generating controversy over the role of theta rhythm. To test whether this discrepancy reflects differences in rhythmicity at a cellular level, we performed whole-cell patch recordings from entorhinal neurons in both species to record theta-frequency resonance. Bat neurons showed no theta-frequency resonance, suggesting grid-cell coding via different mechanisms in bats and rats or lack of theta rhythmic contributions to grid-cell firing in either species.

The medial entorhinal cortex (mEC) of rodents, humans, nonhuman primates, and bats encodes space by using similar neural firing patterns (1–5). Unit recordings from awake-behaving rodents and bats demonstrate that single neurons in the mEC, termed grid cells, fire when an animal traverses spatial locations on a periodic triangular array, tiling the environment. In rodents, grid-cell coding correlates with rhythmicity at theta frequency (4 to 10 Hz). Subthreshold membrane-potential resonance (sMPR) at theta frequency matches the anatomical distribution of grid cells (6) and differs along the dorsal-to-ventral axis of rodent mEC (7–11), in proportion with grid-cell firing field properties (2). Rodent grid-cell spiking shows preccession in theta phase relative to location (12), theta rhythmicity correlated with grid field properties and running velocity (13, 14), and loss of spatial periodicity in the absence of network theta rhythm (15, 16).

However, bat recordings demonstrate grid cells in mEC and place cells in the hippocampus in the absence of continuous theta-frequency rhythmicity in field potentials or spike-train autocorrelograms (5, 17), arguing against theta-rhythmic mechanisms for grid-cell firing, but controversy remains that theta rhythmicity could be masked by low firing rates in bat grid cells (18). To test for intrinsic theta-rhythmic mechanisms in the bat species Eptesicus fuscus and Rousettus aegyptiacus, we made in vitro whole-cell patch recordings in brain slices, looking for theta-frequency sMPR. In contrast to layer II stellate cells in rodent mEC, layer II neurons of bat mEC do not show theta-frequency sMPR. These data corroborate earlier bat unit recording data (5, 17) showing that continuous theta rhythmicity during navigation is not present in all mammalian species.

Previous studies demonstrated that parahippocampal areas in bat and rodent brains show similar anatomical organization (5, 19, 20). Neurons loaded with biocytin during whole-cell patch clamp recordings in horizontal brain slices were stained to reveal position and morphology (Fig. 1). Rat neurons were layer II stellate cells on the

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