Spectrin is a mechanoresponsive protein shaping fusogenic synapse architecture during myoblast fusion

Rui Duan1,2,8, Ji Hoon Kim1,8, Khursht Shilagardi1,8, Eric S. Schiffhauer2, Donghoon M. Lee3, Sungmin Son4, Shuo Li5, Claire Thomas5, Tianzhi Luo6, Daniel A. Fletcher4, Douglas N. Robinson2 and Elizabeth H. Chen1,3,8*

Spectrin is a membrane skeletal protein best known for its structural role in maintaining cell shape and protecting cells from mechanical damage. Here, we report that α/βH-spectrin (βH is also called karst) dynamically accumulates and dissolves at the fusogenic synapse between fusing Drosophila muscle cells, where an attacking fusion partner invades its receiving partner with actin-propelled protrusions to promote cell fusion. Using genetics, cell biology, biophysics and mathematical modelling, we demonstrate that spectrin exhibits a mechanosensitive accumulation in response to shear deformation, which is highly elevated at the fusogenic synapse. The transiently accumulated spectrin network functions as a cellular fence to restrict the diffusion of cell-adhesion molecules and a cellular sieve to constrict the invasive protrusions, thereby increasing the mechanical tension of the fusogenic synapse to promote cell membrane fusion. Our study reveals a function of spectrin as a mechanoresponsive protein and has general implications for understanding spectrin function in dynamic cellular processes.

The mechanical properties of cells are dynamically controlled in many cellular processes, such as cell division, fusion, migration, invasion and shape change. Spectrin is best known as a membrane skeletal protein that is critical for maintaining cell shape and providing mechanical support for the plasma membrane1–3. The functional unit of spectrin is a flexible, chain-like heterotetramer composed of two antiparallel heterodimers of α-spectrin and β-spectrin that interact head to head to form a tetramer4–7. Whereas vertebrates have two α-spectrins (αI and αII) and five β-spectrins (βI–βV), invertebrates encode one α-spectrin and two β-spectrins (β and βH, also known as karst or kst)8,9,10. In erythrocytes and neurons, spectrins, together with actin, ankyrin and associated proteins, form either a static polygonal lattice structure4–6 or an ordered periodic longitudinal array9,10 underneath the plasma membrane to protect cells from mechanical damage11. Such a mechanoprotective function of spectrin is made possible by holding the spectrin network under constitutive tension12. However, in many cellular processes, mechanical tension is generated upon transient cell–cell interactions. How spectrins, which are expressed in most eukaryotic cells, respond to transient mechanical stimuli in dynamic cellular processes remains largely unknown.

Cell–cell fusion is a dynamic process that occurs in fertilization, immune response, bone resorption, placenta formation and skeletal muscle development and regeneration12–14. Studies in various cell fusion events from Drosophila to mammals have demonstrated that cell fusion is an asymmetric process12–17. At the site of fusion, known as the fusogenic synapse, an attacking fusion partner invades its receiving partner with actin-propelled membrane protrusions12–14,16,17, whereas the receiving fusion partner mounts a myosin II (MyoII)-mediated mechanosensory response18. The pushing and resisting forces from the two fusion partners bring the two cell membranes into close proximity and put the fusogenic synapse under high mechanical tension to promote fusogen engagement and cell membrane merger12,14. Although multiple long and narrow invasive protrusions from the attacking fusion partner are known to be required for cell–cell fusion12,13,16,17, it is unclear how these protrusions are spatially constricted and shaped to generate high mechanical tension at the fusogenic synapse.

Results

α/βH-Spectrin is required for Drosophila myoblast fusion. In a deficiency screen for genes required for myoblast fusion, we uncovered Df(3L)1226. Genetic analyses of candidate genes within this deficiency led to the identification of βH-spectrin (also known as karst or kst)19,20. Zygotic null mutants of α-spectrin or βH-spectrin exhibited minor myoblast fusion defects (Fig. 1Aa–Ad, B), which suggests that α/βH-spectrin heterotetramer formation was significantly compromised when the concentrations of both α-spectrin and βH-spectrin were low. The functional specificity of α/βH-spectrin in myoblast fusion was demonstrated by a genetic rescue experiment, in which full-length βH-spectrin expressed in all muscle cells rescued the fusion defect in the βH-spectrin mutant (Fig. 1Ag, B). By contrast, overexpressing dominant-negative βH-spectrin (mini-βH-spectrin; deleting 15 of the 29 spectrin repeats)19 or β-spectrin containing 17...
**Fig. 1 | α/β₅-Spectrin is required for myoblast fusion and is enriched at the fusogenic synapse in founder cells.** A, Myoblast fusion phenotype in the α/β₅-spectrin mutant. Stage 15 embryos immunolabelled with anti-muscle MHC. Ventral lateral muscles of three hemisegments are shown in each panel. Unfused myoblasts are indicated by arrowheads. A wild-type (WT) embryo is shown (Aa). A minor fusion defect is demonstrated in the α-spectrin (α-spec–/–) (Ab), β₅-spectrin (β₅-spec–/–) (Ac) and transheterozygous β₅-spec/Df(3L)1266 (Ad) mutants. A severe fusion defect is shown in the α/β₅-spectrin (α-spec–/– β₅-spec–/–) double mutant (Ae). Expressing mini-β₅-spectrin (mini-β₅-spec) in all muscle cells with twi-GAL4 exacerbated the fusion defect in the β₅-spec–/– mutant was rescued by expressing β₅-spectrin in all muscle cells with twi-GAL4 (Ag), in founder cells with sns-GAL4 (Ah), but not in FCMs with sns-GAL4 (Ai). For each genotype, 10 embryos (biologically independent samples) were imaged with similar results. Scale bar, 20 μm. B, Quantification of the fusion index. The number of Eve-positive nuclei in the dorsal acute muscle 1 (DA1) was counted for each genotype in A. The number of DA1 analysed for each genotype: n = 42, 17, 45, 45, 55, 45, 42 and 42 (left to right). The red horizontal bars indicate the mean values. Significance was determined by the two-tailed Student’s t-test. C, Localization of α/β₅-spectrin at the fusogenic synapse. Confocal images of side-by-side pairs of FCM (outlined with dashed lines in the merge panels) and the founder cell in stage 13–14 embryos triple labelled with phalloidin (F-actin), anti-Flag (GFP/Flag trap line; Ae), or anti-V5 (V5-β₅-spectrin) expressed in founder cells with rP298-GAL4 (Ac) or FCMs with sns-GAL4 (Af)). The expression of β₅-spectrin in FCMs was visualized in the fusion-defective sltr mutant without β₅-spectrin diffusion from FCMs to founder cells. Note the enrichment of α-spectrin (Ca) and β₅-spectrin (Cb–Cd) at the fusogenic synapse (arrowheads) and specifically in founder cells (Ce), but not in FCMs (Cf). For each genotype, 20 fusogenic synapses (biologically independent samples) were imaged with similar results. Scale bar, 5 μm.

spectrin repeats in muscle cells exacerbated the fusion defect of the β₅-spectrin mutant (Fig. 1Af,B and Supplementary Fig. 1a) and caused a minor fusion defect in wild-type embryos (Supplementary Fig. 1a). Thus, both mini-β₅-spectrin and β₅-spectrin interfere with α/β₅-spectrin heterotetramer formation and disrupt the α/β₅-spectrin network. Moreover, β₅-spectrin expression specifically in the receiving fusion partners (muscle founder cells), but not in the attacking cells (fusion-competent myoblasts (FCMs)), rescued the fusion defect (Fig. 1Ah,Ai), demonstrating that α/β₅-spectrin functions specifically in founder cells.
α/β₃-Spectrin enrichment at the fusogenic synapse in founder cells. To determine the subcellular localization of α/β₃-spectrin, we performed antibody-labeling experiments using anti-α-spectrin and anti-β₃-spectrin in wild-type embryos (Fig. 1Ca,Cb), and anti-Flag and anti-green fluorescent protein (GFP) in two protein trap lines, kst₄MID134 (Fig. 1Cc) and kst₄P99266 (Fig. 1Cd). Both α-spectrin and β₃-spectrin were enriched at the fusogenic synapse, largely colocalizing with Dumbfounded (Duf), an immunoglobulin domain-containing founder cell-adhesion molecule (CAM)⁴⁴, and closely associating with the FCM-specific F-actin focus, which is part of an invasive podosome-like structure (PLS). By contrast, β-spectrin was not detected in muscle cells, despite its high expression in epithelial cells (Supplementary Fig. 1b). Ectopically expressed β-spectrin in muscle cells did not enrich at the fusogenic synapse as did α/β₃-spectrin and mini-β₃-spectrin (Fig. 1C and Supplementary Fig. 1c,f). In addition, two of the major accessory proteins that are known to stabilize spectrin–actin interactions, adducin²³ and protein 4.1 (refs ⁴⁶,⁴⁷), were also absent at the fusogenic synapse (Supplementary Fig. 1c,d). An amino-terminal-tagged, functional β₃-spectrin (V5-β₃-spectrin) that was specifically expressed in founder cells, but not in FCMs, was enriched at the fusogenic synapse (Fig. 1Ce,Cf), supporting the functional requirement for α/β₃-spectrin in founder cells.

Dynamic accumulation of α/β₃-spectrin at the fusogenic synapse. To investigate whether α/β₃-spectrin forms a stable membrane skeletal network at the fusogenic synapse, we performed live-imaging experiments in Drosophila embryos. Surprisingly, instead of forming a static network, mCherry-β₃-spectrin exhibited dynamic accumulation and dissolution at the fusogenic synapse accompanying the appearance and disappearance of the FCM-specific F-actin focus (lifespan: 6–30 min, average: ~12 min)²⁸ (Fig. 2a,b and Supplementary Video 1). The amount of β₃-spectrin accumulation correlated with the density and invasiveness of the
β/ not restricted to muscle cells and is a general feature of this protein. CAMs, Duf and its functionally redundant paralogue Roughest and are outlined (dashed lines) in the merge panels. Note the β synapse (arrowheads) in the absence of Duf intracellular signalling. Scale bar, 5 μm.

H-spectrin expressed in epithelial cells

FRAP analysis of mCherry-β results. Scale bar, 5 (arrow) from the attacking cell (II; outlined in the right panel; co-expressing Sns and Eff-1). Thirty-five fusogenic synapses were examined with similar ΔΔ of Duf fusogenic synapses in stage 13–14 embryos triple labelled with anti-V5 (β-spec), phalloidin (F-actin) and anti-Ants or anti-Duf (DufIntra-Flag). FCMs are outlined (arrow) in the left panel; co-expressing GFP-β-spec and Eff-1) accumulated at the fusogenic synapse (arrowhead) in response to the F-actin-propelled invasive protrusions (arrow) from the attacking cell (II; outlined in the right panel; co-expressing Sns and Eff-1). Thirty-five fusogenic synapses were examined with similar results. Scale bar, 5 μm.

H-spectrin accumulation at the fusogenic synapse is triggered by given the correlation between spectrin accumulation and PLS invasiveness, we tested whether β-spectrin accumulation at the fusogenic synapse is triggered by the protrusive force from FCMs or recruited by the founder-cell CAMs, Duf and its functionally redundant paralogue Roughest (Rst)12,29. Remarkably, β-spectrin still accumulated at fusogenic synapses in the duf, rst double mutant expressing Duf that lacks its entire intracellular domain (DufIntra) (Fig. 3A,Ba,Bb,C). DufΔ intra does not transduce chemical signals but functions normally to attract the FCM-specific immunoglobulin domain-containing CAM, Sticks and stones (Sns)13–25. The overall weaker accumulation of β-spectrin in these mutant embryos corresponds to a partial rescue of myoblast fusion (Fig. 3C). By contrast, Antisomal (Ants; also known as Rols7), a founder cell-specific adaptor protein that binds to the intracellular domain of Duf12–17, did not accumulate at the fusogenic synapse (Fig. 3Ba,Bc,C). Thus, β-spectrin accumulation in founder cells can be triggered by invasive forces from the PLS, independent of chemical signalling from CAMs. Furthermore, β-spectrin accumulated at the fusogenic synapse in cultured Drosophila S2R+ cells that were induced to fuse by co-expressing Sns and the Caenorhabditis elegans fusogen Eff-1 (refs 13,29). Specifically, the F-actin foci in the attacking cells were always associated with β-spectrin accumulation in the receiving cells, despite the lack of endogenous Duf and Rst in these cells13 (Fig. 3D).

α/β-spectrin exhibits mechanosensitive accumulation. To test directly whether β-spectrin exhibits mechanosensitive accumulation, we performed micropipette aspiration (MPA) assays, in which a pulling force is applied to Drosophila S2 cells by a micropipette. GFP-β-spectrin showed rapid mechanosensitive accumulation at the base area of the aspirated portion of the cell (Fig. 4A,D), in contrast to the previously demonstrated tip accumulation of the mechanosensory protein MyoII12. This effect was not due to an increased amount of membranous materials, F-actin or adaptor proteins at the base area, as a red fluorescent protein (RFP)-tagged PtdIns(4,5)P2-interacting pleckstrin homology (PH) domain34, GFP-actin or Ants did not accumulate at this area (Fig. 4Ba,Bb,D and Supplementary Fig. 3a,b). In addition, no accumulation was observed for GFP-β-spectrin-ΔC, which deleted a carboxy-terminal fragment containing the tetramerization domain29 (Fig. 4Bc,D and Supplementary Fig. 4a), or GFP-β-spectrin-ΔN, which deleted an amino-terminal fragment containing the
The distinct domains of mechanosensitive accumulation of MyoII and spectrin induced by pulling forces prompted us to ask whether they exhibit a similar response to pushing forces. Coarse-grained modelling of cells invaded by protrusions with a 5-μm diameter predicted clear separation of dilation versus shear domains along the invasive protrusion, with maximal dilation corresponding to the tip and maximal shear deformation corresponding to the base (Fig. 4F). However, when the invasive protrusions became narrower (~400-nm diameter), there was a gradual increase of shear deformation at the tip, where the dilation deformation remained largely the same (Fig. 4G–J). This model predicted that the mechanosensitive accumulations of βH-spectrin and MyoII induced by narrow protrusions may no longer be clearly separated. To test this directly, we performed atomic force microscopy (AFM) experiments, in which a pushing force was applied to cells expressing GFP-βH-spectrin and RFP-MyoII by a cantilever with a tip diameter of ~200 nm, which mimics the length scale of the invasion protrusions at a mature fusogenic synapse (Fig. 4K). When indented at a depth of 2–5 μm, βH-spectrin and MyoII exhibited rapid and largely overlapping domains of accumulation to the indented area (Fig. 4L and Supplementary Video 5), consistent with the pattern of mechanosensitive response predicted by the course-grained model and the enrichment of both βH-spectrin and MyoII at the fusogenic synapse in Drosophila embryos (Fig. 1C).

α/βH-Spectrin responds to shear deformation. It is intriguing that α/βH-spectrin and MyoII show distinct patterns of mechanosensitive accumulation revealed by MPA. Previous coarse-grained modelling suggests that the tip of an aspirated cell corresponds to an area of maximal actin network dilation (or radial expansion), whereas the base area corresponds to the maximal shear deformation (or shape change)52. MPA analyses suggest that MyoII is a mechano-sensory protein for actin network dilation, whereas α/βH-spectrin responds specifically to shear deformation. Consistent with the distinct areas of mechanosensitive accumulation of MyoII and spectrin, βH-spectrin remained at the base area in cells treated with Y27632, a small molecule that decreases MyoII activity by inhibiting Rho-associated protein kinase (ROCK), the upstream activator of MyoII (compare Fig. 4Ca and Fig. 4Cb; Fig. 4D), and MyoII (RFP-Zip43) remained at the tip of βH-spectrin knockdown cells (compare Fig. 4Ca and Fig. 4Cc; Fig. 4E). At late time points, weak βH-spectrin accumulation was observed at the neck and tip areas of aspirated cells in a MyoII-dependent manner (Fig. 4A, 85 s; Supplementary Fig. 3aii), suggesting that MyoII-mediated cortical contraction at the tip may gradually create shear deformation along the aspirated portion of the cell.

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α/βH-Spectrin restricts CAMs at the fusogenic synapse. What are the biological functions of spectrin accumulation at the fusogenic synapse? In the α/βH-spectrin double mutant, the founder-cell CAM Duf and its interacting protein Ants were both dispersed at the fusogenic synapse, instead of forming a tight aggregate as in wild-type cells (Fig. 5A,B). Time-lapse imaging revealed the dynamic dispersion of Duf in these mutant embryos (Supplementary Video 6), compared to the tight Duf cluster associated with dense F-actin foci in wild-type embryos (Fig. 5C and Supplementary Video 7). Occasional Duf aggregates in mutant embryos gradually diffused over time, suggesting that α/βH-spectrin is required for the maintenance, but not the initiation, of the Duf clusters (Fig. 5D and Supplementary Video 6).

As Duf and Sns interact in trans41, we tested whether Duf dispersal in founder cells of the α/βH-spectrin mutant affects Sns distribution in FCMs. Indeed, Sns was also dispersed at the fusogenic synapse in these embryos (Fig. 5E), and so did the actin nucleation-promoting factors and their interacting proteins, such as WASP-interacting protein (WIP; also known as Solitary (Sltr)), which is recruited to Sns at the fusogenic synapse42,43 (Fig. 5F). The diffusion of actin nucleation-promoting factors resulted in a fuzzy F-actin structure in the FCM (Fig. 5A,B,D–F), with an average fluorescence intensity of 61 ± 19 per focus on a 0–255 scale (n = 35), compared to 170 ± 15 per focus (n = 28) in wild-type embryos. The low intensity of F-actin indicates a low filament density, which generated stubby and closely abutting toe-like protrusions, instead of the long, narrow and well-separated finger-like protrusions in wild-type embryos44,45,46 (Fig. 5H). Thus, Duf restriction by α/βH-spectrin in founder cells regulates Sns localization and the distribution of actin filaments at the fusogenic synapse in FCMs.
α/β_H-Spectrin maintains Duf enrichment at the fusogenic synapse via biochemical interactions. To investigate how spectrin restricts Duf at the fusogenic synapse, we performed co-immunoprecipitation experiments using *Drosophila* embryos expressing Flag-β_H-spectrin and Duf-GFP in muscle cells. An antibody against Flag, but not a control antibody, co-precipitated α-spectrin and Duf-GFP, suggesting that the α/β_H-spectrin heterotetramers interact with Duf (Fig. 5I and Supplementary Fig. 5a). Moreover, DufΔintra, which can no longer interact with α/β_H-spectrin, appeared to be diffused at many fusogenic synapses in the *duf* rst mutant, similar to Duf diffusion in the α/β_H-spectrin mutant (Fig. 5G). As a consequence, the F-actin foci that formed initially due to the trans-interactions between DufΔintra and Sns also gradually dispersed at the fusogenic synapse (Supplementary Video 8), as in the α/β_H-spectrin

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Fig. 5 | α/βin-Spectrin restricts CAMs at the fusogenic synapse. A–B, Duf and Ants in founder cells are dispersed at the fusogenic synapse in the α/βin-spectrin mutant. Stage 14 embryos immunolabelled with anti-Duf or anti-Ants, phalloidin (F-actin) and anti-βin-spectrin. Duf (and Ants) was concentrated in a tight cluster associated with each F-actin focus (arrowheads) in WT (Aa,Ba) but dispersed along with F-actin (arrowheads) in the α/βin-spectrin mutant (Ab,Bb). C–D, Time-lapse stills of WT (C) or the α/βin-spectrin mutant (D) expressing Duf-mCherry and GFP-actin. Duf remained in tight clusters associated with F-actin foci in WT (C), but diffused over time with F-actin in the α/βin-spectrin mutant (D). E–F, Sns and Sltr in FCMs are dispersed in the α/βin-spectrin mutant. Stage 14 embryos immunolabelled with anti-Sns or anti-Sltr and phalloidin (F-actin) are shown. G, Localization of DufΔintra expressed in dufΔintra mutant embryos. Stage 14 dufΔintra mutant embryos expressing DufΔintra-Flag with twi-GAL4 immunolabelled with anti-Duf and phalloidin (F-actin) are shown. Note the presence of both dispersed (arrowheads) and clustered (arrow) DufΔintra. In A–G, for each antibody (or fluorophore) combination, 20 (or 10) fusogenic synapses were imaged with similar results. Scale bars, 5 μm. FCMs are outlined (dashed lines) in the merge panels (A,B,E–G). H, Electron micrographs of the invasive PLS in WT (left panel) and the α/βin-spectrin mutant (right panel). FCMs are pseudo-coloured in purple. Dashed lines delineate the F-actin-enriched area of the PLS. Note the long, narrow and well-separated finger-like protrusions in WT (arrowheads) and the stubby and closely abutting toe-like protrusions in the α/βin-spectrin mutant (arrows). n, nucleus. For each genotype, 10 fusogenic synapses were analysed with similar results. Scale bars, 500 nm. I, Biochemical interaction between βin-spectrin and Duf. A co-immunoprecipitation (IP) experiment using extracts from embryos expressing Flag-βin-spectrin and Duf-GFP in muscle cells with twi-GAL4 is shown. Duf was pulled down with anti-Flag, but not with a control (ctrl) antibody (Ab; anti-V5), suggesting interactions between βin-spectrin and Duf. α-Spectrin was probed to indicate the presence of βin-spectrin, the latter of which was difficult to detect owing to its large size (~480 kDa). These experiments were repeated three times with similar results. Unprocessed original scans of blots are in Supplementary Fig. 5a. IB, immunoblotting.

mutant (Fig. 5A,B,D–F and Supplementary Video 7). Interestingly, time-lapse imaging revealed a gradual diffusion of accumulated βin-spectrin in DufΔintra-expressing dufΔintra mutant embryos (Supplementary Video 9), suggesting that the α/βin-spectrin–Duf interaction is also required for stabilizing the mechanoaccumulative α/βin-spectrin at the fusogenic synapse.

Despite the largely ‘co-localized’ α/βin-spectrin and Duf at the fusogenic synapse observed with confocal microscopy, structured
**Fig. 6 | The α/βH-spectrin network restricts the CAM Duf and constricts actin-propelled invasive protrusions.** SIM images of fusogenic synapses in stage 14 embryos of a βH-spectrin trap line. In A and the ‘top view’ panels in B, the imaging plane was perpendicular to the axis of FCM invasion. A, The α/βH-spectrin network restricts Duf. An early-stage (Aa) and a late-stage (Ab) fusogenic synapse labelled with βH-spectrin, Duf and phalloidin (F-actin) are shown. Note the distinct microdomains occupied by βH-spectrin (arrowhead) and Duf (arrow) at the early stage (Aa) and the ring-like structure formed by βH-spectrin and Duf at the late stage, where these two proteins were closely associated with each other (Ab). In addition, note that βH-spectrin was localized at the outer rim of the ring structure that kept most of the Duf clusters inside (Ab). Twenty-five fusogenic synapses were imaged with similar results. Scale bars, 1 μm. B, The α/βH-spectrin network constricts invasive protrusions. Side view (Ba,Bd,Be) and top view (Bb,Bc) of early-stage (Ba,Bb) and late-stage (Bc–Be) fusogenic synapses, labelled with βH-spectrin and phalloidin (F-actin). Accumulated βH-spectrin (arrowhead) locally blocked protrusions shown on this focal plane (Ba,Bd,Be). Actin-propelled protrusions triggered βH-spectrin accumulation at the base areas (Bb,Bc). These protrusions appeared wider at the early stage (Bb) than at the late stage (Bc). The dashed circle (Bc, middle panel) outlines the cross-section of a narrow protrusion. Two examples of late-stage fusogenic synapses, showing spectrin-enriched patches (arrowheads) blocking protrusions, as well as long and narrow protrusions from the FCM penetrating through spectrin-free microdomains (Bd,Be). Note that these protrusions triggered further accumulation of βH-spectrin at their tips and/or sides. Forty fusogenic synapses were imaged with similar results. Scale bars, 1 μm. C, Three-dimensional reconstruction of the fusogenic synapse shown in Bc. Note the actin-propelled protrusions penetrating through the spectrin-free microdomains. Ten fusogenic synapses were reconstructed with similar results.

illumination microscopy (SIM) revealed distinct microdomains occupied by these proteins at early stages of the fusogenic synapse marked by small actin foci (Fig. 6Ba), suggesting that Duf does not directly recruit βH-spectrin in founder cells. βH-Spectrin appeared to surround the actin focus, which is consistent with the mechanosensitive accumulation of α/βH-spectrin to the base areas of invasive protrusions. At late stages of the fusogenic synapse, characterized by large actin foci and a ring-like structure formed by βH-spectrin and Duf, these two proteins exhibited closer association, probably mediated by the α/βH-spectrin–Duf interaction (Fig. 6Ab). Strikingly, α/βH-spectrin was mostly seen at the outer rim of the ring (Fig. 6Ab), indicating that the spectrin network functions as a cellular fence to restrict Duf diffusion.

**α/βH-spectrin network functions as a cellular sieve to constrict the invasive protrusions.** The closely abutting morphology of the invasive protrusions in the α/βH-spectrin mutant prompted us to ask whether spectrin is involved in shaping the invasive structure to well-separated, long and narrow protrusions. At early stages of the fusogenic synapse, actin polymerization in the FCM propelled wide protrusions that triggered mechanosensitive accumulation of βH-spectrin at the base (Fig. 6Ba,Bb). As foci grew, more βH-spectrin accumulated at the fusogenic synapse, resulting in an uneven spectrin network with smaller spectrin-free domains (Fig. 6Bc). At the late stage, only narrow protrusions were seen penetrating through spectrin-free microdomains (Fig. 6Bc–Be and Supplementary Video 10). Thus, the spectrin network in the founder cell functions as a ‘cellular sieve’ to constrict the diameters of the invasive protrusions from the FCM. The resulting long and narrow protrusions put the fusogenic synapse under high mechanical tension to promote plasma membrane fusion12,14.

**βV-spectrin is required for mouse myoblast fusion.** The requirement for βV-spectrin in *Drosophila* myoblast fusion led us to test whether the mammalian orthologue of βV-spectrin, βV-spectrin (also known as Sptbn5), is involved in myoblast fusion. Knocking down βV-spectrin with two independent short interfering RNAs (siRNAs) in mouse C2C12 myoblasts significantly decreased C2C12 cell fusion (Fig. 7A–C). This was not due to a failure in muscle cell differentiation, as the expression level of myogenic regulatory factors—MyoD and myogenin—remained similar in knockdown versus control cells (Fig. 7D,E and Supplementary Fig. 5b). In addition, the expression of skeletal muscle myosin heavy chain (skMHC) was not affected by the knockdown (Fig. 7E). Consistent with the normal expression of these proteins, the βV-spectrin-knockdown cells had a normal, elongated morphology and were MHC positive.
Fig. 7 | βV-spectrin, the mammalian homologue of Drosophila β₅-spectrin, is required for C2C12 myoblast fusion. A, Confocal images of C2C12 cells treated with either transfection reagent alone (Aa) or two individual siRNAs against βV-spectrin (Ab,Ac). Cells were fixed on day 6 post-differentiation and stained with anti-MHC (green) and DAPI (red) to visualize differentiated muscle cells. Note the thinner myofibres in Ab and Ac than in Aa. These experiments were repeated three times with similar results. Scale bar, 50 μm.

B, siRNA knockdown (KD) of βV-spectrin analysed by qRT–PCR. The mRNA level of βV-spectrin in KD cells was normalized against control in n = 3 independent experiments. C, The fusion index was calculated as the percentage of nuclei in multinucleated syncytia versus the total number of nuclei per microscopic field. Each data point represents the fusion index of a random microscopic field; 34 fields pooled from three independent C2C12 cell differentiation experiments. Note that βV-spectrin KD significantly decreased the fusion index. D,E, βV-spectrin KD did not inhibit myoblast differentiation. Western blot analyses showed no significant difference in the expression levels of myogenic differentiation markers (MyoD, myogenin and skMHC) between control and βV-spectrin KD cells (D). The cell lysates used for SDS–PAGE were derived from the same experiment and the gels/blots were processed in parallel. β-Tubulin was used as a loading control. Unprocessed original scans of blots are in Supplementary Fig. 5b. The quantification of protein expression from panel D is displayed (E). In the graphs, the y axes indicate the measured band intensity ratio of each protein relative to the loading control (β-tubulin). n = 3 independent experiments. Each data point represents the relative protein expression level measured in a single experiment. In B, C, E, the red horizontal bars indicate the mean values, and significance was determined by the two-tailed Student’s t-test. GM, growth medium; DM, differentiation medium.

Discussion

This study has revealed a dynamic mechanoresponsive property of αβ₅-spectrin in response to invasive forces during cell–cell fusion. The mechanosensitive accumulation of αβ₅-spectrin in the receiving fusion partner establishes a transient and uneven spectrin-enriched network at the fusogenic synapse, which functions both as a cellular fence to restrict CAMs and a cellular sieve to constrict the invasive protrusions from the attacking cell. Through these actions, spectrin helps to build a fusogenic synapse under high mechanical tension to facilitate cell membrane fusion.

An intercellular mechanoresponsive feedback loop at the fusogenic synapse. The fusogenic synapse is established by trans-interactions between cell-type-specific CAMs, which initiate a series of downstream cellular events in both cell types. In FCMs, Sns recruits the Arp2/3 nucleation-promoting factors to activate actin polymerization and generate invasive protrusions, which triggers mechanosensitive accumulation of αβ₅-spectrin in the apposing founder cells. The accumulated αβ₅-spectrin keeps Duf at the fusogenic synapse, which recruits additional Duf by lateral diffusion.
and oligomerization. Newly recruited Duf and transiently stabilized Sns sets off additional rounds of protrusion formation, mechnanosenstive accumulation of α/β₃-spectrin and the recruitment of additional CAMs. Through such a positive-feedback loop, a mature fusogenic synapse forms with appropriate levels and localization of CAMs, actin and spectrin. The absence of α/β₃-spectrin in founder cells breaks the positive-feedback loop, such that Duf and Sns cannot maintain or increase their concentrations at the fusogenic synapse and the structure eventually falls apart. Thus, the intercellular mechanosensitive feedback loop is critical for the growth and stabilization of the fusogenic synapse.

α/β₃-Spectrin as a dynamic mechanoresponsive protein for shear deformation. Spectrin has long been thought as a scaffolding protein that stably links the plasma membrane and the actin cytoskeleton. Our study revealed a mechanosensitive behaviour of α/β₃-spectrin in response to shear stress (Supplementary Fig. 6a). Under shear stress, the actin network’s shape/angle change leads to changes in the distances between actin crosslinker-binding sites. Whereas shorter and stiffer crosslinkers are prone to dissociating from the network, α/β₃-spectrin heterotetramers, each with 29 spectrin repeats and flexible linker regions, can accommodate a range of angle/distance changes by folding or unfolding the spectrin repeats and stay bound to the shear-deformed actin network for an extended period of time. In this regard, it has been demonstrated that spectrin heterotetramers in red blood cells unfold their spectrin repeats under shear stress⁴⁸. FRAP analyses revealed a fraction of α/β₃-spectrin that remains associated with the actin network at the fusogenic synapse, consistent with the prolonged binding of some spectrin heterotetramers. We propose that the extensibility and flexibility of α/β₃-spectrin heterotetramers are the two major properties enabling its transient stable association with the shear-deformed actin network. In support of this, filamin, an actin crosslinker organized as flexible and extensible V-shaped dimers (having immunoglobulin-like folds⁴⁴ instead of spectrin repeats), also exhibited mechanosensitive accumulation under shear stress⁴⁴.

Once the shear stress is removed from the cell cortex, the actin network is no longer under strain and α/β₃-spectrin dissociates from the actin network, generating a pool of free α/β₃-spectrin heterotetramers that are available for future mechanosensitive responses. Two factors may influence the dynamic dissociation of spectrin from actin: accessory proteins and the actin-binding affinity of spectrin. The absence of adducin and protein 4.1 in embryonic muscle cells suggests that the α/β₃-spectrin–actin interaction is relatively unstable compared to that in erythrocytes and axons, such that α/β₃-spectrin is more likely to dissociate from the actin network in muscle cells. Although the actin-binding affinities of the structurally similar β₃-spectrin and β-spectrin are not known, the difference in their mechanoresponsive behaviours suggests that β-spectrin, similar in size to mini-β₃-spectrin, may bind to F-actin with a higher affinity than β₃-spectrin or mini-β₃-spectrin. Thus, most β-spectrin proteins are stably integrated into the α/β-spectrin heterotetramers at the cell cortex, leaving few free-β-spectrin available for transient mechanosensitive response at the fusogenic synapse. In this regard, α-actinin-1, which has a 90-fold higher actin-binding affinity than α-actinin-4, does not show mechanosensitive accumulation, whereas α-actinin-4 does¹⁰.

The α/β₃-spectrin network functions as a cellular fence and a cellular sieve. The mechanocumulative spectrin network serves at least two functions at the fusogenic synapse. In founder cells, the accumulated spectrin builds a cellular fence to restrict Duf diffusion, probably through two complementary mechanisms (Supplementary Fig. 6b). First, biochemical interactions between Duf and spectrin could prevent Duf clusters from lateral diffusion when they encounter spectrin-enriched patches. Second, the spectrin heterotetramers are linked to the plasma membrane via the PH domain of β₃-spectrin and may collide with the cytoplasmic domain of Duf to block Duf diffusion. A similar role for spectrin in restricting transmembrane protein diffusion has been demonstrated in mouse erythrocytes, in which the transmembrane protein band 3 diffuses faster in spectrin-deficient mutant erythrocytes than in normal cells⁵¹ and the cytoplasmic portion of band 3 slows down the diffusion of the protein⁵². Spectrin also functions as a cellular sieve to constrict the invasive protrusions from the FCM (Supplementary Fig. 6b). The build-up of the sieve is a dynamic process involving continuous mechanical stimulation and mechanosensitive accumulation. The early mechanosensitive accumulations of spectrin in founder cells locally block future protrusions from the FCM, forcing new protrusions to penetrate through neighbouring spectrin-free areas, thus triggering additional spectrin accumulation. Eventually, large areas of the fusogenic synapse will be populated by spectrin heterotetramers, forming an uneven spectrin network with a few spectrin-free microdomains. Only narrow protrusions that have sufficient mechanical stiffness can ‘squeeze’ through these microdomains to invade the founder cell deeply (Supplementary Fig. 6b). Thus, the dynamically accumulated spectrin network gradually constricts the invasive protrusions from the FCM and increases the mechanical tension at the fusogenic synapse to promote cell–cell fusion. Given the widespread expression of spectrin in most eukaryotic cell types, our characterization of α/β₃-spectrin as a dynamic mechanoresponsive protein in fusogenic cells has broad implications for understanding spectrin functions in many dynamic cellular processes beyond cell–cell fusion.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41556-018-0106-3.

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Methods

Fly stocks and genetics. The following strains were obtained from the Bloomington Drosophila Stock Center: fly stocks wild (white, the α-spectin gene, was amplified by PCR from the UAST-Myc-β-spectrin construct (from C.T.) by Gibson assembly is as follows:

(1) mCh-α-spectin (2:1,000), rabbit anti-β-spectrin (1:100), rabbit anti-β-spectrin (1:200), mouse anti-11122, Invitrogen), rabbit anti-GFP (1:1,000; A-11122, Invitrogen). The following secondary antibodies were used at 1:200: Alexa 488-, Alexa 568- and Alexa 647-conjugated phallodin (Invitrogen) were used at 1:200. Fluorescent images were obtained on a LSM 700 Meta confocal microscope (Zeiss), with LSM Image Browser software (Zeiss) and Zen software (Zeiss). For presentation by the ImageJ program (http://imagej.nih.gov/ij/).

Drosophila cell culture. S2 and S2R+ cells were cultured in Schneider’s medium (Gibco) supplemented with 10% FBS (Gibco) and penicillin/streptomycin (Sigma). Cells were transfected using Effectene (Qiagen) per the manufacturer’s instructions. For immunofluorescent staining, cells were fixed and stained with anti-skeletal muscle myosin antibody (1:100; F59, sc-32732, Santa Cruz Biotechnology). Protein quantification in muscle or epithelial cells were collected and dechorionated in 50% NaCl, 2 mM EDTA and 10 mM sodium phosphate, pH 7.2) containing protease inhibitors. The cell lysates were briefly sonicated, centrifuged and analysed by SDS–PAGE and western blotting with antibodies against MyoD (1:100; sc-377460), in multinucleated syncytia versus the total number of nuclei per

assembly. This created a de novo Agel site upstream of the Kozak sequence (GCC ACC) followed by the McR Cherry sequence, the flexible linker sequence and the full-length α-spectrin. The Agel–NotI piece containing mCherry and the full-length α-spectrin was subsequently subcloned into the fly expression vector pAc-V5-His (Invitrogen). The primer pair used to create the mCherry-linker tag for Gibson

immunoassay and imaging. Fly embryos were fixed and stained as described previously. The following primary antibodies were used: rabbit anti-α-tubulin (1:1,000), rabbit anti-β-spectrin (1:100), rabbit anti-β-spectrin (1:400), mouse anti-α-tubulin (1:1,349, Developmental Studies Hybridoma Bank (DSHB)), guinea pig anti-β-spectrin (1:500), guinea pig anti-α-tubulin (1:1,000), rat anti-Slr (1:30), rat anti-Sns (1:500), mouse anti-Eve (1:30; 31C0, DSHB), mouse anti-adenin (1:400; 1B1, DSHB), mouse anti-protin 4.1 (1:400; C566.9, DSHB), rabbit anti-GFP (1:500; A-11122, Invitrogen), mouse anti-Flag (1:200; F3165, Sigma), mouse anti-My-100; MA1-980, Thermo Fisher Scientific) and mouse anti-V5 (1:200; R906-25, Invitrogen). The following secondary antibodies were used at 1:200: Alexa 488-, Alexa 568- and Alexa 647-conjugated phallodin (Invitrogen) were used at 1:200. Fluorescent images were obtained on a LSM 700 Meta confocal microscope (Zeiss), acquired with LSM Image Browser software (Zeiss) and Zen software (Zeiss). For presentation by the ImageJ program (http://imagej.nih.gov/ij/).

Mouse G2C12 myoblast culture. A pair of predefined siRNAs against the mouse β-spectrin gene (siRNA1, β-spectrin-1: GAGATGCGCCCTGAAACTCA; siRNA2, β-spectrin-2: AAGAAGATTCTCAAGGCTCTAA) were obtained from Qigem. RNA interference was performed per the manufacturer’s instructions. Briefly, approximately 3 × 10^5 cells were seeded on each well of a 6-well tissue culture dish and transfected with the individual siRNAs against β-spectrin (10 μM final concentration) using HiPerFect transfection reagent (Qiagen). On day 2, the cells were transfected again and differentiated, and cells that were treated in parallel were subjected to qRT–PCR to access the knockdown level. Five days post-differentiation, cells were fixed and stained with anti-skeletal muscle myosin antibody (1:100; F59, sc-32732, Santa Cruz Biotechnology) to identify differentiated cells. Cells were cultured using Prolong Gold antifade reagent with 4,6-diamidino-2-phenylindole (DAPL; Molecular Probes, Invitrogen) to visualize the nuclei. The fusion index was calculated as the percentage of nuclei in multinucleated synctia versus the total number of nuclei per x20 microscopic fields under LSM 810 (Zeiss). Cells in at least 10 random fields were counted in each experiment and three independent experiments were performed.

Time-lapse imaging and FRAP. Time-lapse imaging of embryos was performed as previously described. Briefly, embryos expressing fluorescently tagged proteins in muscle or epithelial cells were collected and dechorionated in 50%
bleach. Subsequently, embryos were washed in water, placed onto a double-sided tape (3 m) and covered with a layer of Halocarbon oil 700/27 (2:1; Sigma). Time-lapse image acquisition was carried out on an LSM 700 Meta confocal microscope (Zeiss). The FRAP experiments were performed using the same conditions described previously, which allowed full fluorescence recovery of GFP-actin, GFP-WASP and Stb-Cheery. Specifically, the solid 488-nm laser output was set to 2% to avoid general photobleaching and phototoxicity. A region of interest was manually selected and imaged in 3–5 frames every 30 s to record the original fluorescent intensity (pre-bleach). Then, the region of interest was quickly photobleached to a level of ~20% of its original fluorescence intensity by 5–10 times of consecutive 3-s laser scans with 2% laser output and subsequently imaged every 30 s to record fluorescence recovery (post-bleach). The fluorescence intensities of the pre-bleached and post-bleached region of interest were measured using the ImageJ program. The Prism software was used to determine the maximal recovery level (that is, the percentage recovery compared to the pre-bleach level) and the half-time of recovery using a kinetic curve fit with an exponential decay equation.

**SIM.** Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy. Stage 13–14 embryos were fixed and stained as described above. The samples were then mounted in Prolong Gold (Molecular Probes) and imaged by Electron microscopy.

**Electron microscopy.** Embryos were fixed by the high-pressure freezing and freeze substitution method, as previously described. Briefly, a Bal-Tec device was used to freeze stage 12–14 embryos. Freeze substitution was performed with 1% osmium tetroxide, 0.1% uranyl acetate in 98% acetone and 2% methanol on dry ice. Fixed embryos were embedded in Epon (Sigma-Aldrich) and cut into thin sections with an ultramicrotome (Ultracut R; Leica). The sections were mounted on copper grids and post-stained with 2% uranyl acetate for 10 min and Sato’s lead solution for 1 min to improve image contrast. Images were acquired on a transmission electron microscope (CM120; Philips).

**Recombinant protein purification and F-actin co-sedimentation assay.** To purify GST-fused βp45-spectrin fragments from BL21-DE3 cells (NEB), protein expression was induced with 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) at room temperature for 12–15 h. Cells were harvested and lysed by sonication in the lysis buffer: PBS (pH 7.4), 1% Triton X-100, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and cOmplete Mini Protease Inhibitor Cocktail (Roche). After centrifugation, the supernatant was collected and incubated with pre-equilibrated glutathione agarose resin at 4°C for 2–3 h. After washing in the lysis buffer, βp45-spectrin protein was eluted with the elution buffer: 50 mM Tris (pH 7.5), 150 mM NaCl, 5 mM dithiothreitol and 10 mM glutathione (Sigma). The F-actin co-sedimentation assay was performed following the manufacturer’s protocol (Cytoskeleton). Briefly, 0.5–1 μM purified protein was incubated with 4 μM F-actin assembled from monomeric actin for 1 h in F buffer: 5 mM Tris-HCl (pH 8.0), 0.2 mM CaCl₂, 50 mM KCl, 2 mM MgCl₂ and 1 mM ATP. The F-actin–protein mixtures were centrifuged at 140,000g for 30 min, and supernatants and pellets were separated and analysed by SDS–PAGE and Coomassie Blue staining.

**Co-immunoprecipitation.** Embryos expressing GFP-tagged Duf and Flag-tagged βp45-spectrin (kstMI03134/twi-GAL4; UAS-Duf-GFP/+; UAS-βp45-spectrin-GFP/+; UAS-Flag-GFP) were co-expressed and dechorionated in 50% bleach. Embryos were frozen in liquid nitrogen and then dissociated in H-spectrin medium supplemented with 10% FBS on an Olympus IX81 microscope with a ×100 oil NA 1.49 CFI SR Apochromat TIRF objective lens and an ORCA-Flash 4.0 cMOS camera (Hamamatsu Photonics K.K.). The images were processed using Adobe Photoshop CS6.

**Eur Star.** Co-immunoprecipitation. Embryos expressing GFP-tagged Duf and Flag-tagged βp45-spectrin (kstMI03134/twi-GAL4; UAS-Duf-GFP/+; UAS-βp45-spectrin-GFP/+; UAS-Flag-GFP) were co-expressed and dechorionated in 50% bleach. Embryos were frozen in liquid nitrogen and then dissociated in H-spectrin medium supplemented with 10% FBS on an Olympus IX81 microscope with a ×100 oil NA 1.49 CFI SR Apochromat TIRF objective lens and an ORCA-Flash 4.0 cMOS camera (Hamamatsu Photonics K.K.). The images were processed using Adobe Photoshop CS6.

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of interest was associated. The shear deformation of each node was calculated in a similar way. The contour of the deformation on the protrusion was plotted by the software Tecplot (Supplementary Fig. 7). The deformations along the length direction of the protrusion were obtained by the extraction tool of the software.

**Statistics and reproducibility.** Statistical significance was assessed using two-tailed Student's t-test and ANOVA with Fisher's least significant difference. P values were obtained using the Microsoft Excel 2010, GraphPad Prism 5 and Kaleidagraph 4.1 softwares. The number of biological replicates for each experiment is indicated in the figure legends. Immunofluorescence images were representative of at least ten independent samples, MPA images of at least eight independent cells and western blots of three independent experiments.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Code availability.** The C programs for the computation of the energy of the cells with protrusions are available from: https://pan.baidu.com/s/1wjroHlyh7eXZQ3jHGfRUA.

**Data availability.** The main data supporting the findings of this study are available within the article and its Supplementary Information files. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.

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| ☐   | ☑ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐   | ☑ The statistical test(s) used AND whether they are one- or two-sided (Only common tests should be described solely by name; describe more complex techniques in the Methods section.) |
| ☐   | ☑ A description of all covariates tested |
| ☐   | ☑ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐   | ☑ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☐   | ☑ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever suitable. |
| ☐   | ☑ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☐   | ☑ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☐   | ☑ Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated |
| ☐   | ☑ Clearly defined error bars |
| ☐   | ☑ State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection: Fluorescent images were acquired with LSM Image Browser software (Zeiss) and Zen software (Zeiss).

Data analysis: For imaging analyses, Image J software from NIH and Adobe photoshop CS5 were used. For statistical analyses, Microsoft Excel 2010, GraphPad Prism 5 and KaleidaGraph 4.1 softwares were used.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

The main data supporting the findings of this study are available within the article and its Supplementary Information files. All other data supporting the findings of this study are available from the corresponding author upon reasonable request.
Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☑ Life sciences  ☐ Behavioural & social sciences  ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No statistical method was used to predetermine sample size. Sample sizes were determined based on previous studies in the field to enable statistical analyses and ensure reproducibility. |
|-------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | No data were excluded from this study. |
| Replication | Sample sizes have been increased wherever possible. Immunofluorescence images were representative of at least ten independent samples, MPA images of at least eight independent cells, and western blots of three independent experiments. All attempts for replication were successful. |
| Randomization | Experiments described here were not randomized. |
| Blinding | The investigators were not blinded to group allocation during experiments and outcome assessment, because all data were acquired from cell or tissue samples of specific genotypes or with designated genetic or chemical manipulations. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐ | Unique biological materials |
| ☑ | Antibodies |
| ☑ | Eukaryotic cell lines |
| ☐ | Palaeontology |
| ☐ | Animals and other organisms |
| ☐ | Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☐ | ChIP-seq |
| ☐ | Flow cytometry |
| ☐ | MRI-based neuroimaging |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials There is no restriction on availability of materials used in this study.

Antibodies

Antibodies used

All antibodies are listed in Methods under "Immunofluorescent staining and imaging" with species, company/catalog#, clone, dilution used per application, and published references if applicable.

The following primary antibodies were used:

- rabbit α-muscle myosin heavy chain (1:1000)
- rabbit anti-βH-spectrin (1:100)
- rabbit anti-βα-spectrin (1:400)
- mouse anti-α-spectrin (1:1; DSHB; 3A9)
- guinea pig anti-Duf (1:500)
- guinea pig anti-Ants (1:1000),
- rat anti-Str (1:30)
- rat anti-Sns (1:500)
- mouse anti-Eve (1:30; DSHB; 3C10)
- mouse anti-adducin (1:400; DSHB; 1B1)
- mouse anti-protein 4.1 (1:400; DSHB; C566.9)
The following primary antibodies were used at 1:500:
- rabbit anti-GFP (1:500; Invitrogen; A-11122)
- mouse anti-V5 (1:200; Invitrogen; R960-25)
- mouse anti-Flag (1:200; Sigma; F3165)
- mouse anti-Myc (1:100; Thermo Fisher Scientific; MA1-980)
- mouse anti-skeletal muscle myosin (1:100; Santa Cruz Biotechnology; sc-32732)
- mouse anti-MyoD (1:100; Santa Cruz Biotechnology; sc-377460),
- mouse anti-Myogenin (1:100; Santa Cruz Biotechnology; sc-52903),
- mouse anti-skeletal muscle myosin (1:100; Santa Cruz Biotechnology; sc-32732)
- mouse anti-alpha-tubulin (1:100; Santa Cruz Biotechnology; sc-58666) f

The following secondary antibodies were used at 1:200:
- Alexa488-, Alexa568-, and Alexa647-conjugated (Invitrogen) and biotinylated (Vector Laboratories) antibodies made in goats.

**Validation**

These antibodies have either been validated in published literatures, which are cited in the Methods, or are widely used for similar experiments by other researchers worldwide.

**Eukaryotic cell lines**

*Policy information about [cell lines](#)*

**Cell line source(s)**
- Drosophila S2R+ cell line from Dr. P. Beachy; Drosophila S2 cell line from Drosophila Genomics Resource Center;
- Mouse C2C12 cell line from ATCC.

**Authentication**
- The Drosophila cell line were authenticated based on their morphology, growth condition, and specific gene expression.
- The C2C12 cells were authenticated by their morphology and their ability to differentiate into multinucleated myotubes.

**Mycoplasma contamination**
- All three cell lines were tested with a PCR based kit from Sigma #MP0035, and no mycoplasma contamination was found.

**Commonly misidentified lines**
- No commonly misidentified cell lines were used.

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**Palaeontology**

*Specimen provenance* No animal-derived materials were used.

*Specimen deposition* N/A

*Dating methods* N/A

Tick this box to confirm that the raw and calibrated dates are available in the paper or in Supplementary Information.

**Animals and other organisms**

*Policy information about [studies involving animals](#) ARRIVE guidelines recommended for reporting animal research*

**Laboratory animals**
- No laboratory animals were used.

**Wild animals**
- N/A

**Field-collected samples**
- N/A

**Human research participants**

*Policy information about [studies involving human research participants](#)*

**Population characteristics**
- The study did not involve human research participants.

**Recruitment**
- N/A

**ChIP-seq**

*Data deposition*

- Confirm that both raw and final processed data have been deposited in a public database such as [GEO](#).
- Confirm that you have deposited or provided access to graph files (e.g. BED files) for the called peaks.

*Data access links*
- For "initial submission" or "revised version" documents, provide reviewer access links. For your "final submission" document, provide a link to the deposited data.

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May remain private before publication.
Files in database submission
Provide a list of all files available in the database submission.

Genome browser session (e.g. UCSC)
Provide a link to an anonymized genome browser session for "Initial submission" and "Revised version" documents only, to enable peer review. Write "no longer applicable" for "Final submission" documents.

Methodology

Replicates
Describe the experimental replicates, specifying number, type and replicate agreement.

Sequencing depth
Describe the sequencing depth for each experiment, providing the total number of reads, uniquely mapped reads, length of reads and whether they were paired- or single-end.

Antibodies
Describe the antibodies used for the ChIP-seq experiments; as applicable, provide supplier name, catalog number, clone name, and lot number.

Peak calling parameters
Specify the command line program and parameters used for read mapping and peak calling, including the ChIP, control and index files used.

Data quality
Describe the methods used to ensure data quality in full detail, including how many peaks are at FDR 5% and above 5-fold enrichment.

Software
Describe the software used to collect and analyze the ChIP-seq data. For custom code that has been deposited into a community repository, provide accession details.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Describe the sample preparation, detailing the biological source of the cells and any tissue processing steps used.

Instrument
Identify the instrument used for data collection, specifying make and model number.

Software
Describe the software used to collect and analyze the flow cytometry data. For custom code that has been deposited into a community repository, provide accession details.

Cell population abundance
Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.

Gating strategy
Describe the gating strategy used for all relevant experiments, specifying the preliminary FSC/SSC gates of the starting cell population, indicating where boundaries between "positive" and "negative" staining cell populations are defined.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

Magnetic resonance imaging

Experimental design

Design type
Indicate task or resting state; event-related or block design.

Design specifications
Specify the number of blocks, trials or experimental units per session and/or subject, and specify the length of each trial or block (if trials are blocked) and interval between trials.

Behavioral performance measures
State number and/or type of variables recorded (e.g. correct button press, response time) and what statistics were used to establish that the subjects were performing the task as expected (e.g. mean, range, and/or standard deviation across subjects).
### Acquisition

| Imaging type(s)            | Specify: functional, structural, diffusion, perfusion. |
| Field strength            | Specify in Tesla                                      |
| Sequence & imaging parameters | Specify the pulse sequence type (gradient echo, spin echo, etc.), imaging type (EPI, spiral, etc.), field of view, matrix size, slice thickness, orientation and TE/TR/flip angle. |
| Area of acquisition       | State whether a whole brain scan was used OR define the area of acquisition, describing how the region was determined. |
| Diffusion MRI             | [ ] Used [ ] Not used                                  |

### Preprocessing

| Preprocessing software    | Provide detail on software version and revision number and on specific parameters (model/functions, brain extraction, segmentation, smoothing kernel size, etc.). |
| Normalization             | If data were normalized/standardized, describe the approach(es): specify linear or non-linear and define image types used for transformation OR indicate that data were not normalized and explain rationale for lack of normalization. |
| Normalization template    | Describe the template used for normalization/transformation, specifying subject space or group standardized space (e.g. original Talairach, MNI305, ICBM152) OR indicate that the data were not normalized. |
| Noise and artifact removal | Describe your procedure(s) for artifact and structured noise removal, specifying motion parameters, tissue signals and physiological signals (heart rate, respiration). |
| Volume censoring          | Define your software and/or method and criteria for volume censoring, and state the extent of such censoring. |

### Statistical modeling & inference

| Model type and settings   | Specify type (mass univariate, multivariate, RSA, predictive, etc.) and describe essential details of the model at the first and second levels (e.g. fixed, random or mixed effects; drift or auto-correlation). |
| Effect(s) tested          | Define precise effect in terms of the task or stimulus conditions instead of psychological concepts and indicate whether ANOVA or factorial designs were used. |
| Specify type of analysis  | [ ] Whole brain [ ] ROI-based [ ] Both |
| Statistic type for inference | Specify voxel-wise or cluster-wise and report all relevant parameters for cluster-wise methods. |
| Correction                | Describe the type of correction and how it is obtained for multiple comparisons (e.g. FWE, FDR, permutation or Monte Carlo). |

### Models & analysis

| n/a Involved in the study | Functional and/or effective connectivity [ ] Graph analysis [ ] Multivariate modeling or predictive analysis |
| Functional and/or effective connectivity | Report the measures of dependence used and the model details (e.g. Pearson correlation, partial correlation, mutual information). |
| Graph analysis            | Report the dependent variable and connectivity measure, specifying weighted graph or binarized graph, subject- or group-level, and the global and/or node summaries used (e.g. clustering coefficient, efficiency, etc.). |
| Multivariate modeling and predictive analysis | Specify independent variables, features extraction and dimension reduction, model, training and evaluation metrics. |