The basic unit of skeletal muscle in all metazoans is the multinucleate myofibre, within which individual nuclei are regularly positioned. The molecular machinery responsible for myonuclear positioning is not known. Improperly positioned nuclei are a hallmark of numerous diseases of muscle, including centronuclear myopathies, but it is unclear whether correct nuclear positioning is necessary for muscle function. Here we identify the microtubule-associated protein ensconsin (Ens)/microtubule-associated protein 7 (MAP7) and kinesin heavy chain (Khc)/Kif5b as essential, evolutionarily conserved regulators of myonuclear positioning in Drosophila and cultured mammalian myotubes. We find that these proteins interact physically and that expression of the Kif5b motor domain fused to the MAP7 microtubule-binding domain rescues nuclear positioning defects in MAP7-depleted cells. This suggests that MAP7 links Kif5b to the microtubule cytoskeleton to promote nuclear positioning. Finally, we show that myonuclear positioning is physiologically important. Drosophila ens mutant larvae have nuclear positioning defects in MAP7-depleted cells. This suggests that MAP7 is required for nuclear positioning processes in Drosophila. The only notable difference in these phenotypes is rescued by muscle-specific expression of the Kif5b motor domain.

MAP and kinesin-dependent nuclear positioning is required for skeletal muscle function

Thomas Metzger1,2*, Vincent Gache3*, Mu Xu1, Bruno Cadot1, Eric S. Folker1, Brian E. Richardson1, Edgar R. Gomes3,4* & Mary K. Baylies1,2*

To examine nuclear movement, we performed an F3 recessive ethylmethane sulphonate mutagenesis screen using the apterusME-NLS:dsRed transgenic line (apRed), which expresses dsRed in the nuclei of the four lateral transverse muscles of the Drosophila embryo. At the end of embryogenesis, the myonuclei are distributed throughout the muscles (Fig. 1a, b). One mutation resulted in clustered nuclei near the ventral end of each lateral transverse muscle. Within each hemisegment these clusters resembled the Nike trademark, so we named the mutant swoosh (swo) (Fig. 1a).

Further analysis of swo mutant embryos indicated that myoblast specification, fusion, muscle elongation and attachment occur normally. The only notable difference in swo mutant muscles is a ventral bulge in the cell body that correlates with the cluster of nuclei (Supplementary Figs 1 and 2). These data suggest that the clustering of nuclei in swo mutants is a genuine nuclear positioning defect.

To examine nuclear movement, we performed in vivo time-lapse imaging of swo,apRed mutant embryos demonstrated that the nuclei fail to undergo the initial separation into dorsal and ventral clusters during stage 15 and remain clustered at stage 17, indicating that swo does not simply cause a delay in myonuclear positioning (Supplementary Movie 2). In addition, the nuclei in every other somatic muscle examined in swo mutant embryos also failed to separate (Supplementary Fig. 1e).

We determined that the swo flies carry a nonsense mutation in ensconsin (ens), a gene encoding a MAP. Expression of haemagglutinin-tagged Ens specifically in the developing mesoderm and muscle rescued the nuclear positioning phenotype in swo embryos, confirming that the mutation in ens is responsible for the nuclear positioning defect. Depletion of ens solely in the developing mesoderm and muscle by RNA-mediated interference recapitulated the phenotype we observed in the swo mutant (renamed ens swo), and known ens mutant alleles failed to complement ens swo (Supplementary Figs 1c and 2a, and data not shown). These results indicate that ens is required autonomously within muscle for proper myonuclear positioning. We also found that nuclear position was disrupted in ens swo oocytes (Supplementary Fig. 3a, b), but not in ens swo photoreceptor cells (Supplementary Fig. 3c, and data not shown), indicating that Ens is necessary for some, but not all, nuclear positioning processes in Drosophila.

Four different genes in the mouse genome encode Ens orthologues: Map7 (E-MAP-115, Enscorsin), Map7D1, Map7D2 and Map7D3 (Supplementary Fig. 2e). Using cultures of C2C12 myoblasts and primary mouse myoblasts, we examined the effect on nuclear positioning in myotubes depleted of each Ens orthologue by short interfering RNA (siRNA) (Fig. 1 and Supplementary Fig. 4). MAP7 depletion caused a significant increase in the aggregation of nuclei within a myotube but did not affect myoblast fusion or myotube differentiation (Fig. 1c, d, Supplementary Fig. 4d, e and Supplementary Movies 3–6). Depletion of MAP7D1, MAP7D2 or MAP7D3 did not affect nuclear positioning (Supplementary Fig. 4b). Expression of full-length MAP7 in MAP7-depleted myotubes restored nuclear alignment (Fig. 1d and Supplementary Fig. 6h). Thus, MAP7 is required for nuclear positioning in both Drosophila muscles and cultured mammalian myotubes.

No major defects on the microtubule network were observed in ens swo mutant embryos or MAP7-depleted myotubes (Supplementary Figs 2g–h and 6g). Therefore, to gain mechanistic insight, we conducted a yeast two-hybrid screen to find Ens-binding proteins and identified kinesin heavy chain (Khc) (Supplementary Fig. 5).

Both a kinesin-null mutation (khc8) and a motor-dead mutation (khc11a) disrupted myonuclear positioning without affecting muscle elongation and attachment (Fig. 2a and Supplementary Fig. 5a). Likewise, siRNA-mediated depletion of the mammalian Khc orthologue Kif5b (ref. 11) in primary and C2C12 myotubes disrupted nuclear alignment similarly to MAP7 depletion without affecting myotube formation or differentiation (Fig. 2b, c, Supplementary Figs 4d–e).
Figure 1 | Myonuclear positioning requires ensconsin/MAP7.
a. Hemisegments from control and ens−/− embryos at the beginning (stage 14) and end (stage 17) of nuclear migration. Muscles are immunostained for tropomyosin (green); nuclei are immunostained for dSRed (red). Scale bar, 10 μm. b. Time-lapse images of nuclear migration in hemisegments of control and ens−/− embryos. Elapsed imaging times are shown along the top. Scale bar, 10 μm. c. Time-lapse images of nuclear migration in control and Map7-siRNA-depleted cells expressing short hairpin RNA (shRNA) against MAP7. Error bars indicate s.e.m. Asterisk, P < 0.05; two asterisks, P < 0.01; three asterisks, P < 0.001 (scrambled versus experimental condition).

Figure 2 | Kinesin is required for myonuclear positioning. a. Single hemisegments from stage 17 (22 h AEL) embryos of indicated genotypes. Scale bar, 10 μm. b. Representative immunofluorescence images of control and Kif5b-depleted C2C12 myotubes differentiated for 4 days and immunostained for myosin heavy chain (green) and 4′,6-diamidino-2-phenylindole (red). Scale bar, 15 μm. c. Histogram of nuclear distribution in C2C12 myotubes that were untreated (control) or treated with the indicated siRNA and Kif5b-siRNA-depleted cells expressing full-length FL-Kif5b. Error bars indicate s.e.m. Asterisk, P < 0.05; two asterisks, P < 0.01; three asterisks, P < 0.001 (scrambled versus experimental condition).

deprecated C2C12-H1B–GFP myotubes. Elapsed imaging times are shown along the top. Nuclei are stained red. Scale bar, 15 μm. d. Nuclear distribution in C2C12 myotubes that were untreated (control) or treated with the indicated siRNA and Map7-siRNA-depleted cells expressing full-length FL-MAP7 (no. 118 + FL-MAP7). Error bars indicate s.e.m. Asterisk, P < 0.05; two asterisks, P < 0.01; three asterisks, P < 0.001 (scrambled versus experimental condition).

To test whether the specific interaction identified above between Ens/MAP7 and Khc/Kif5b interact physically. Endogenous Kif5b co-immunoprecipitated with expressed full-length green fluorescent protein (GFP)-tagged MAP7, independently of microtubules (Fig. 3a, b). Using fragments of MAP7, we mapped the Kif5b interaction to the carboxy-terminal coiled-coil domain of MAP7 (CC2), which was confirmed by reciprocal co-immunoprecipitation (Fig. 3a–c). Similarly, fragments of Kif5b were used to identify the C-terminal region (Kif5b-motorless) as the MAP7-interacting domain (Fig. 3a, d, e). The C-terminal coiled-coil domain of MAP7 therefore interacts with the C-terminal region of Kif5b (Fig. 3).
in tracked larvae revealed that the myonuclei in Supplementary Fig. 7d). Moreover, analysis of the muscle structure larvae move significantly more slowly than controls (Fig. 4b and Asterisk indicates anti-Kif5b IgG.

immunoprecipitations from C2C12 cells expressing indicated constructs (top). These experiments define a subset of the nuclear behaviours that occur during muscle differentiation, and demonstrate conservation (Fig. 3f, g). These results indicate that the function of MAP7 in nuclear positioning is to link Kif5b to microtubules.

To investigate the physiological impact of mispositioned nuclei on muscle function in vivo, we examined larval motility14. ens mutant larvae move significantly more slowly than controls (Fig. 4b and Supplementary Fig. 7d). Moreover, analysis of the muscle structure in tracked larvae revealed that the myonuclei in ens mutant larvae are not required for fusion and myofibre formation. Furthermore, these results argue that the correct spacing of myonuclei is required for proper nuclear positioning. Adjacent nuclei could be positioned relative to each other by Ens/MAP7 and Khc/Kif5b interacting with and sliding antiparallel microtubules that have their minus ends anchored to the nuclear envelope, in a similar manner to the mechanism by which the Eg5 kinesin facilitates spindle elongation23 (Supplementary Fig. 10). This mechanism is distinct from the kinesin-1-dependent nuclear position in the Caenorhabditis elegans hypodermis, in which Khc is anchored to the nucleus by kinesin light

across species at both the cellular and molecular levels. We find that Ens/MAP7 and Khc/Kif5b are critical for myonuclear positioning and are not required for fusion and myofibre formation. Moreover, these proteins interact genetically and physically, and the physical interaction is necessary for proper nuclear positioning. Furthermore, our results argue that the correct spacing of myonuclei is required for proper muscle function.

Within a myotube, distinct microtubule networks emanate from each myonucleus, producing regions of overlapping antiparallel microtubules16–20. Furthermore, Ens/MAP7 functions to load Khc/Kif5b onto microtubules4. The results presented here therefore suggest a molecular mechanism for nuclear positioning. Adjacent nuclei could be positioned relative to each other by Ens/MAP7 and Khc/Kif5b interacting with and sliding antiparallel microtubules that have their minus ends anchored to the nuclear envelope, in a similar manner to the mechanism by which the Eg5 kinesin facilitates spindle elongation23 (Supplementary Fig. 10). This mechanism is distinct from the kinesin-1-dependent nuclear position in the Caenorhabditis elegans hypodermis, in which Khc is anchored to the nucleus by kinesin light

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function. Our results show that the ability to position myonuclei contribute to muscle weakness or are simply a result of impaired myofibre organization and efficient larval locomotion. a, Maximum-intensity xy projections of muscle VL3 from segment A3 of L3 larvae from the indicated genotypes stained for actin (red), nuclei (white) and Z-bands (green). Scale bar, 20 μm. b, Average velocity of migration for L3 larvae of the indicated genotypes. c, Nearest-neighbour analysis of nuclei within muscle VL3 from segment A3 from L3 larva of the indicated genotypes. Error bars indicate s.e.m. Asterisk, P < 0.05; three asterisks, P < 0.001. c, Nearest-neighbour analysis of nuclei within muscle VL3 from segment A3 from L3 larva of the indicated genotypes. Error bars indicate s.e.m. Asterisk, P < 0.05; three asterisks, P < 0.001.

Methods summary

Drosophila stocks used included apME-NLS:dsRed (ref. 4), Df(3L)GN34 (ref. 26), ens^{Hr6614} (Bloomington), enso10123 (Harvard), Df-ens^{Hr657}, ens^{Hr}, ens^{swo} (ref. 8), khc^{swo}, khc^{swo}, khc^{swo} (ref. 10), klc^{swo} (ref. 27), twi-Gal4 (ref. 4), alpha-Gal4, G7-Gal4 (ref. 28) (from K. Broadie), and UAS-ens-IR lines 106207 and 18491 (VDR). UAS-ensHA transgenic flies were generated by BestGene Inc. Mouse cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) or with DNA using Lipofectamine 2000 (Invitrogen). Primary antibodies used were rabbit anti-dsRed (Clontech), rat anti-tropomyosin (Abcam), mouse anti-myosin heavy chain (from S. Abmayr), rabbit anti-Zasp (from F. Schöck), chicken anti-β-gal (Abcam), mouse anti-α-tubulin (Sigma), rat anti-ensconsin (from P. Rorth), guinea pig anti-Krüppel (from J. Reinitz), rabbit anti-Eve (from M. Frasch), mouse anti-f-PS integrin (DSHB), rabbit anti-vestigial (from S. Carroll), fluorescein isothiocyanate-conjugated anti-horseradish peroxidase (Jackson ImmunoResearch), mouse anti-discs large (DSHB), rabbit anti-ATP synthase (from H. Duan), mouse anti-β-PS integrin (DSHB), mouse anti-α-PS integrin (DSHB), mouse anti-KHC (Santa Cruz), mouse anti-c-Myc (Roche) and rabbit anti-GFP (Invitrogen). Secondary antibodies were either biotinylated (Vector Laboratories and Jackson ImmunoResearch) or conjugated to Alexa Fluor 488, 555 or 647. The fusion index, sarcomere length, bouton number and larval velocity were quantified as described, with minor modifications (see Methods). The yeast two-hybrid screen was performed with full-length Ens by Hybrigenics SA Services using a 0–24-h Drosophila complementary DNA library. Standard protocols were used for immunoprecipitation, western blotting and quantitative polymerase chain reaction experiments and are described in Methods.

Full methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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METHODS

Flies and husbandry. Stocks were grown and maintained under standard conditions on standard cornmeal medium. Crosses were performed at 25°C. Drosophila stocks: apmE-NLS:dsRed (ref. 4), Df(3L)GN34 (ref. 26), en5Dp04680 (Blommington), en5Dp1211 (Harvard), en5N, en5C, Df-en4-1227 (ref. 8), UAS-enSHA (this study), kheC, kheL, kheR (ref. 10), kheX (ref. 27), and UAS-en-IR lines 106207 and 18491 (VDRC). Mutant alleles were balanced and identified using CyO P[w+en111AcZ] or CTG [CyO,twi-GAL4,UAS-2xCFP] (ref. 26), TM3 Sb;dlf-lacZ or TTG [TM3,twi,GAL4,UAS-2xCFP] (ref. 31). G4hl lines used in this work were twi-Gal4 (ref. 4), which is specifically expressed in the mesoderm and developing muscle (2.5–13 h AEL), and alpha-Gal4 and kif5b (ref. 4), which is specifically expressed in the larval muscles beginning at the late L1 and early L2 larval stages and perdures until pupation. Ethylmethane sulphonate mutagenesis and screening. A detailed description of the screen and its findings are available from the authors. In brief, males from an isogenized stock carrying the apmE-NLS:dsRed transgene on the third chromosome were mutagenized with 35 mM ethylmethane sulphonate dissolved in 1% sucrose in accordance with a standard protocol32. Individual mutant lines were established through the TTG balance. Live F2 progeny were screened under fluorescence, and embryos homozygous for the mutant chromosome were identified by the absence of the balancer GFP. Deficiency mapping, complementation and screening were used to identify the genetic lesion.

Phylogenetic analysis. Drosophila, human and mouse MAP7 orthologues were aligned using LALIGN (http://www.ch.embnet.org/software/LALIGN_form.html) and the phylogenetic tree was created using Vector NTI (Invitrogen).

Transfections. Myoblasts were transfected using Lipofectamine Transfections.

EMTB-Map7 fragment from pET17b-K560–GFP (gift from R. Vale) into KASH2ext were a gift from D. Hodzic35. from C. C. Hoogenraad) into pEGFP C1 construct. EGFP–KASH2 and EGFP–Kif5b fragments were cloned from a C2C12 cDNA library into the pEGFP-C1 vector.

Full-length Map7 (PM, 008635) was cloned into the Map7 fragments and was cloned into pEGFP-C1. EMTB-Map7 was made by cloning the fragment consisting of base pairs 1–947 from full-length Map7 into the pEGFP-C1 vector. K-term, K-EMTB and K-EMTB-M chimaera constructs were made by cloning the K690 fragment from pET17b-K690–GFP (gift from R. Vale) into N-Term, EMTB and EMTB-Map7 pEGFP constructs, respectively. Motor-Kif5b, S-Motorless-Kif5b and Motorless-Kif5b C-Myc were a gift from G. Kreitzer. FL-Kif5b was made by cloning the K690 fragment with a C-terminal region of full-length Kif5b (gift from C.C. Hoogenraad) into pEGFP C1 construct. EGF-P-KASH2 and EGF-P-KASH2ext were a gift from D. Hodzic35.

siRNA sequences (Ambion): Map7, 5’-CAGAAUAGAUGAUAGCAGATT-3’ (no. 118), 5’-CAUGCAUCAUUGUAAACACT-3’ (no. 119) and 5’-UCUACUGGUAUUGAAATTT-3’ (no. 120); Map7/D1, 5’-GGAGAAGAGGAGGAGCAGATT-3’ (no. 110), 5’-AGGUGCAUUCUAUAAUCAATT-3’ (no. 711) and 5’-GAAUCUGGCUUGUAUUGAATTT-3’ (no. 430); Map7/D3, 5’-GGAGCAGACUUCUUAUAAGT-3’ (no. 001) and 5’-CCAGCCUUCUUGUAUUGAATTT-3’ (no. 002); Kif5b-5’-GCAAGAAGAUGAGGCUUAGATT-3’ (no. 781), 5’-CCUGUUAUAUUAUGAUGACATT-3’ (no. 782) and 5’-GCAUGUGGCUUAGUAUAAATTT-3’ (no. 783).

Nuclear positioning was quantified in mammalian myotubes containing at least three nuclei, and myotubes were classified as follows: ‘aligned’, more than 70% of the nuclei did not align along the same axis; ‘aggregated’, more than 70% of the nuclei did not align along the same axis; ‘other’, nuclei in a myotube did not fall into either category. Fusion index was quantified as described36. A nearest-neighbour analysis in Drosophila was conducted to determine the average distance between a nucleus and its single closest neighbour within a muscle. To identify the closest neighbour for each nucleus in a muscle, the distance from the centre of a nucleus and the centre of the closest nucleus (usually four to eight) was measured. The shortest distance identified the closest nucleus or ‘nearest neighbour’ and this distance was recorded. A nearest neighbour was identified for each nucleus in every muscle analysed and the nearest neighbour distances were averaged to give an average ‘nearest-neighbour’ distance for the average space between one nucleus and the next closest nucleus in a muscle.
Sarcomere length was defined as the distance between the Z-lines labelled with the anti-ZASP antibody. Sarcomere length was measured at five separate regions per muscle and in the same five regions for each muscle examined.

Neuromuscular junctions were revealed by staining with horseradish peroxidase or Dlg, and quantified as previously described.

The position of the oocyte nucleus was determined by measuring the distance from the anterior border of the oocyte to the centre of the nucleus.

Time-lapse imaging. *Drosophila* embryos were prepared as described. Time-lapse sequences were acquired using a Zeiss Axio Imager.Z1 with a 20× 0.75 NA Plan-Apochromat dry objective. All time-lapse series were taken as a set of z-stacks over time (four-dimensional imaging) with optical sections every 3 μm. Only a single z-section with the greatest number of nuclei in focus was selected for each time point. Images were processed with ImageJ and compiled into movie by using Apple Quicktime. Single images of live embryos were acquired on a Zeiss Axiophot microscope.

Time-lapse imaging of mouse cells was done as reported.

**Yeast two-hybrid screen.** Yeast two-hybrid screening was based on a full-length Ens as bait and performed by Hybrigenics S.A Services using a 0–24-h embryonic *Drosophila* cDNA library; 99 million interactions were analysed, and 10% of the high-confidence clones that were recovered from the screen were Khc.

**Larval behaviour.** Larval behaviour was assessed as described previously, with minor modifications. In brief, embryos (stages 16 and 17) were selected for the presence of clustered apRed nuclei and/or the absence of the fluorescent balancer. Selected embryos were placed on a yeast-coated apple-juice plate overnight at 25°C, and L1 larvae were selected on the following day and placed into vials of standard food containing bromophenol blue. Larvae were picked from the vial 3 days later and tracked. Larvae were tracked individually as they migrated towards a single odour source (0.25 M ethyl butyrate; Sigma) and recorded for 5 min with a charge-coupled device camera until they reached the odour source or until they contacted any of the walls of the apparatus. Images were processed by Ethovision software (Noldus).

**Germline transformation and constructs.** UAS-ensHA DNA was constructed by PCR-amplifying the full-length cDNA from the ens RA transcript with primers to add a haemagglutinin tag to the C-terminal end of the protein followed by an EcoRI restriction site. Primers used were 5’-GCCGAATTCCACATGGGATGCTTCGGGGGCC-3’ (5’) and 5’-GCCGAATTCCTTATCAAGCGTGATCTGTGGA ACATGTATGGGTACAGCAGCGATATATCTTTATTTTGC-3’ (3’). Amplified cDNA was introduced into the Uni-ZAP XR vector (a derivative of pBlueScript SK-). This DNA was used by BestGene Inc. to generate transgenic flies.

**Statistics.** Statistical analysis was performed with Prism (GraphPad Software Inc.). Pairwise comparisons were made with Student’s t-test, and group comparisons were made by analysis of variance followed by Tukey’s posthoc analysis. In nuclear positioning analysis in C2C12 cells, Student’s t-tests were performed between scrambled siRNA and experimental condition for each category (aligned, aggregated and other).

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