Troponin I and Tropomyosin regulate chromosomal stability and cell polarity

Virender Kumar Sahota*, Benjamin Filip Grau, Alicia Mansilla and Alberto Ferrús‡

Cajal Institute, CSIC, Dr Arce 37, Madrid 28002, Spain

*Present address: Peter MacCallum Cancer Institute, Research Division, St Andrews Place, East Melbourne, Victoria 3002, Australia

‡Author for correspondence (e-mail: aferrus@cajal.csic.es)

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Summary

The Troponin-Tropomyosin (Tn-Tm) complex regulates muscle contraction through a series of Ca^{2+}-dependent conformational changes that control actin-myosin interactions. Members of this complex in *Drosophila* include the actin-binding protein Troponin I (TnI), and two Tropomyosins (Tm1 and Tm2), which are thought to form heterodimers. We show here that pre-cellular embryos of TnI, Tm1 and Tm2 mutants exhibit abnormal nuclear divisions with frequent loss of chromosome fragments. During cellularization, apico-basal polarity is also disrupted as revealed by the defective location of Discs large (Dlg) and its ligand Rapsynoid (Raps; also known as Partner of Inscuteable, Pins). In agreement with these phenotypes in early development, on the basis of RT-PCR assays of unfertilized eggs and germ line mosaics of TnI mutants, we also show that TnI is part of the maternal deposit during oogenesis. In cultures of the S2 cell line, native TnI is immunodetected within the nucleus and immunoprecipitated from nuclear extracts. SUMOylation at an identified site is required for the nuclear translocation. These data illustrate, for the first time, a role for TnI in the nucleus and/or the cytoskeleton of non-muscle cells. We propose that the Tn-Tm complex plays a novel function as regulator of motor systems required to maintain nuclear integrity and apico-basal polarity during early *Drosophila* embryogenesis.

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Key words: Troponin-Tropomyosin complex, Muscle, SUMOylation, Aneuploidy, *Drosophila*

Introduction

Troponin I (TnI) and Tropomyosin (Tm) are actin-binding proteins that regulate muscle sarcomere contraction. The Tn-Tm complex contains three different Troponin polypeptides, C, T and I, and it regulates acto-myosin interactions in response to the rise of free calcium (Clark et al., 2002). Mammals have three genes expressing TnI known as slow twitch (*TNNI1*), fast twitch (*TNNI2*) and cardiac (*TNNI3*). In humans, mutations in *TNNI2* and *TNNI3* cause distal arthrogryposis type 2B (Sung et al., 2003) and familial hypertrophic cardiomyopathy (Kimura et al., 1997), respectively. In *Drosophila*, viable mutations in the single gene expressing TnI, *wings up A* (*wupA*) [also known as *held up* (*hdp*)], result in hypercontraction and degeneration of the indirect flight muscles of the thorax due to recessive hypomorphic point mutations (Prado et al., 1995). However, studies on lack of function mutations for this gene have been hampered by the fact that null alleles are dominant lethals (Prado et al., 1999). Mammals contain four tropomyosin genes, *TPM1-4*, while *Drosophila* has two, *Tm1* and *Tm2*. In humans, mutant *TPM1* is thought to be responsible for type 3 familial hypertrophic cardiomyopathy (Thierfelder et al., 1994), whereas *TPM2* is involved in nemaline myopathy (Donner et al., 2002) and *TPM3* has been linked to dominant nemaline myopathy (Laing et al., 1995). *TPM1* has also been identified as a suppressor of malignant transformation as it is downregulated in mammalian transformed cells (Shah et al., 2001), and its expression is abolished in human breast tumors (Raval et al., 2003). Indeed, it is widely accepted that actin regulation plays a crucial role in cell motility, which is a key feature in metastatic cancers (Olson and Sahai, 2008).

Although some of these pathological phenotypes appear unrelated to muscle biology, several lines of evidence indicate that these muscle-specific proteins could have a role in other cell types and processes. For instance, Tm1 is part of the maternal deposit during *Drosophila* oogenesis, it is required to localize the *oskar* mRNA at the posterior pole of the oocyte (Erdelyi et al., 1995), and later in development it localizes to various cell types including the gut, brain and epidermis (Hales et al., 1994). Also, as we demonstrate in this report, TnI RNA is detected in mature unfertilized eggs, which suggests a role in early embryogenesis. Thus, we set out to analyze early development phenotypes and their mechanisms in TnI and Tm mutants.

We show here a novel function for the Tn-Tm complex in regulating nuclear divisions during early embryogenesis in *Drosophila*. We provide evidence that TnI is required for maintaining stable chromosomal integrity, which we also show for Tm1 and Tm2. Importantly, the three genes seem required for correct epithelial apico-basal polarity as mutant phenotypes include cellularization defects that mislocalize the polarity markers Discs large (Dlg) and its ligand Rapsynoid (Raps) [also known as Partner of Inscuteable (Pins)] (Humbert et al., 2008). Consistent with the function of these genes in cellularization and spindle integrity, defects in mitosis and chromosome segregation are observed. In a stable cell line, S2, TnI can be detected within the nucleus. Furthermore, we show that the translocation of TnI to the nucleus is dependent upon a mechanism involving SUMOylation. Taken together, these data implicate the Tn-Tm complex in regulating nuclear functions. Moreover, our results suggest that the Tn-Tm complex is required to maintain correct segregation of chromosomes, as disruption of this complex leads to aberrations including chromosome fragment losses. This is the first evidence that the Tn-Tm complex can regulate both nuclear divisions and

cell polarity in *Drosophila*. This is likely to have important implications in cancer progression since chromosomal instability and the generation of aneuploidies are characteristic hallmarks of many cancers (Hanahan and Weinberg, 2000; Hariharan and Bilder, 2006).

Results

Lethal mutations in the TnI gene can be rescued using an early expressed isoform transgene

We obtained embryonic lethal insertion lines located near the *wupA* locus called *PL87* and *PG31*, which are *lacZ* reporter and *Gal4* lines, respectively (Bourbon et al., 2002). Both insertion sites were located upstream of the promoter region by means of plasmid rescue experiments (Marin et al., 2004). A third mutant, *Df(1)23437*, deletes 2 Kb of the promoter region and is also an embryonic lethal (Fig. 1A). Quantitative RT-PCR data had shown severely reduced levels of TnI RNA expression in these three mutants, with *23437* showing the most reduction, followed by *PL87* and then *PG31* (Marin et al., 2004). Here, we wanted to confirm that their lethal mutant phenotypes were caused by the TnI gene, as opposed to another gene putatively affected by these chromosomal rearrangements. To this end we generated transgenic lines using the embryonic L9/wupRA isoform of *wupA* cDNA (Fig. 1B), under the control of upstream activating sequences (UAS). This isoform was sufficient to completely rescue the embryonic lethality of all three alleles when driven by the general Gal4 driver LL7 [inserted at the α*tubulin84B* (*tub*) gene]. Thus, the *PL87* and *PG31* alleles represent bona fide mutants for TnI, and the TnI L9/wupRA isoform encodes all functions required for correct embryogenesis. Rescued adults were fertile and able to fly, although 40% of pupae failed to emerge and showed a cryptocephalic phenotype (Fig. 1C). This phenotype is consistent with the reported downregulation of the TnI gene during metamorphosis (Furlong et al., 2001). Given that there are adult isoforms of the TnI gene that contain an additional exon, it is unlikely that the L9/wupRA isoform is able to completely replace the functionality of the adult isoforms. This was confirmed by the failed attempt to rescue the *wings held up* phenotype of viable *wupA* alleles when driving the L9/wupRA isoform in the adult indirect flight muscles (not shown). Indeed, the *wupA* gene can produce a repertoire of cDNA isoforms, several of which are embryo-specific whereas others are adult-specific. Because we had previously detected embryonic TnI gene expression before the onset of myogenesis (Prado et al., 1999), we used the embryonic lethal mutants to look for defects during early embryogenesis. Embryos were obtained as described (see Materials and Methods) and classified as pre-cellular or cellular, and as normal or abnormal. The ratio of normal to abnormal agreed with the expected 25% ratio (Fig. 1D; Table 1). These data demonstrate that the embryonic

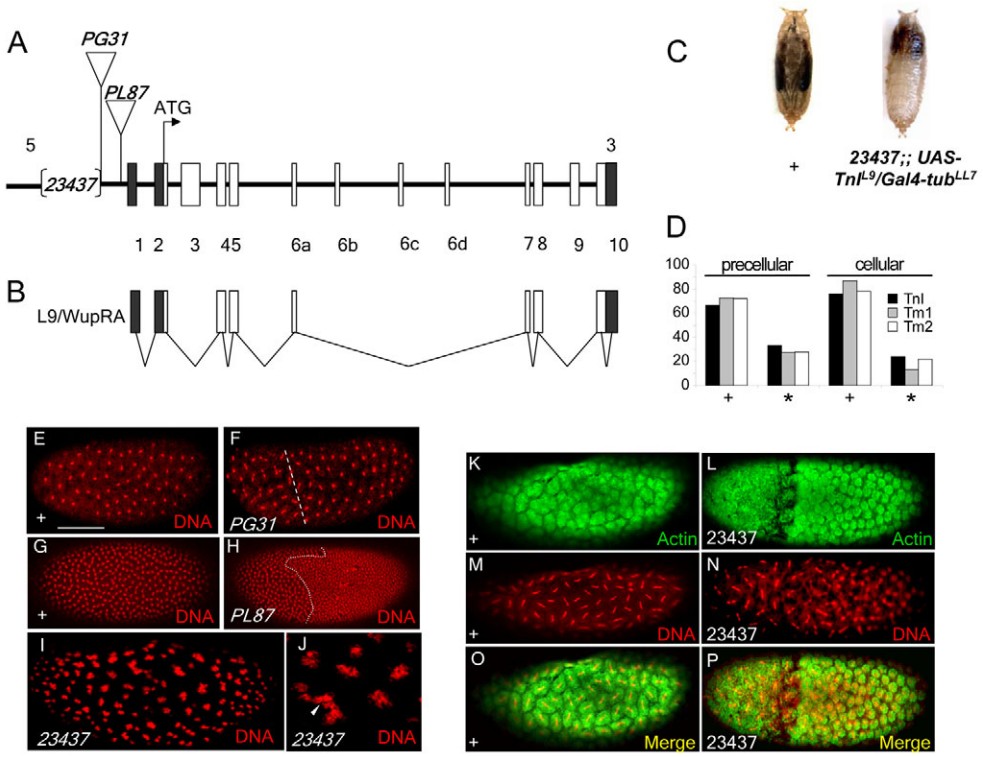

Fig. 1. Map of TnI locus and constructs. (A) TnI mutations are located within the promoter region of the gene. The ATG translational start site is within exon 2. Boxes in black indicate cDNA with untranslated regions. Triangles indicate P-elements, brackets indicate deletion. The TnI gene has ten alternatively spliced isoforms with one exon 6 being exclusively expressed in each isoform. (B) L9/wupRA isoform is among the earliest expressed isoform in the embryo. Exons 3, 6b, 6c, 6d and 9 are absent from this isoform. (C) The L9/wupRA isoform, driven by the *tub*Gal4, is able to completely rescue the embryonic lethality of *23437* mutants, although approximately 40% of pupae fail to eclose and display a cryptocephalic phenotype. (D) Percentage of normal (+) and abnormal (*) embryos at the pre-cellular or cellular stages from 2-hour egg-laying periods of heterozygous mutant females (see also Table 1). (E-H) Propidium iodide staining of wild-type embryos (E,G), *PG31* and *PL87* mutant alleles for TnI (F,H). Nuclei in the anterior region of the embryo have either stopped dividing or have undergone one division less than nuclei at the posterior end. Dotted line separates nuclei that are in a different phase of division. (I) TnI mutant allele *23437* embryos, enlarged in J. Arrowhead indicates clumped nuclei. (K-P) Actin and propidium iodide stainings in syncitial embryos. (K,M,O) Wild-type. (L,N,P) TnI allele *23437*. The abnormal staining for actin correlates with the abnormal nuclear divisions. Anterior is to the right, dorsal is up. Scale bar: 250 μm in E-H, 180 μm in I, 10 μm in J, and 200 μm in K-P.

Table 1. Morphological analysis of embryos

Pre-cellular embryos					
TnI		Tm1		Tm2	
+	*	+	*	+	*
66.6%	33.3%	72.5%	27.5%	72%	28%
n=156		*n*=167		*n*=196	
Cellularized embryos					
TnI		Tm1		Tm2	
+	*	+	*	+	*
75.9%	24.1%	86.8%	13.2%	78.3%	21.7%
n=62		*n*=144		*n*=138	

Numbers indicate the embryos obtained from 2-hour egg laying periods from fecundated females of genotypes: *23437/FM7i,GFP* or PG31/+ (TnI); Tm1-GFP$^{\text{Thor 325-2}}$/TM3 (Tm1); and Tm2-GFP$^{\text{TropIIG5}}$/TM3 (Tm2). Embryos were classified first according to their cellularization state and then each class was independently determined as normal (+) or abnormal (*). Note the expected Mendelian ratio of 25% abnormal embryos. Morphology was determined under Nomarski and phase contrast optics of dechorionated embryos. Those with abnormal aspect were further characterized as described.

Table 2. Germ line mosaics

Genotype	Number of clones	Average offspring per clone
♀ + / *ovo*^{D}	19	64±8
♀ *PL87* / *ovo*^{D}	14	41±5
♀ *23437* / *ovo*^{D}	3	5±4

Animals were treated with X-rays at 24-48 hours after egg laying. Mosaics were identified as egg-laying females under CO_2 anesthesia. The same number of females (200) was screened in each genotype. Note the reduced fertility of *23437* homozygous germ line clones as indicated by the number of average adult offspring per germ line clone.

lethality of the TnI mutants can be rescued to viability using the earliest expressed TnI isoform, and that the phenotypes associated with the three TnI alleles are due to the absence of the TnI gene products.

Troponin I mutants show nuclear defects in the syncitial embryo

The first 13 nuclear divisions within the *Drosophila* embryo occur in the absence of cytokinesis. Division cycles 1-10 are synchronized, after which rounds 11-13 become asynchronized and spread across the embryo as waves of nuclear division. The fourteenth division incorporates cytokinesis as the plasma membrane invaginates and furrow canals form, eventually surrounding the nuclei to become cells. We looked at nuclei of mutant TnI pre-cellular embryos, and all three mutant alleles showed nuclear defects (Fig. 1E-J). Mild defects were observed in *PG31* and *PL87* mutants, and consisted of embryos with nuclei dividing asynchronously (Fig. 1E-H). *23437* mutants showed the more severely affected embryos, with nuclear material clumped together (Fig. 1I-J). Both degrees of severity appeared equally frequent among abnormal pre-cellular embryos. All three alleles showed the same range of nuclear defects. Because TnI binds actin, we looked for a correlation between actin disruption and nuclear structure. All embryos showing severe defects in nuclei structure also showed the most altered localization of actin (Fig. 1K-P).

Troponin I is part of the maternal deposit during oogenesis

The early phenotypes observed here should originate from a depletion of a putative maternal component of TnI. First, we analyzed the presence of TnI mRNA in oocytes by means of RT-PCR assays in unfertilized eggs. The data show the presence of an mRNA band amplified with TnI-specific primers (supplementary material Fig. S1). To test for a functional requirement of TnI in oogenesis we generated germline mosaics for *PL87* and *23437* mutants. Allele *PL87* yielded a mild reduction in the number of clones and the average offspring per clone with respect to controls. Data from *23437* were more severe, yielding only 10% of the control values (Table 2). This allelic difference is consistent with the different effects of these mutants in reducing TnI transcription, as shown previously by QRT-PCR (Marin et al., 2004). For these reasons, we focused on the *23437* allele for most descriptions of

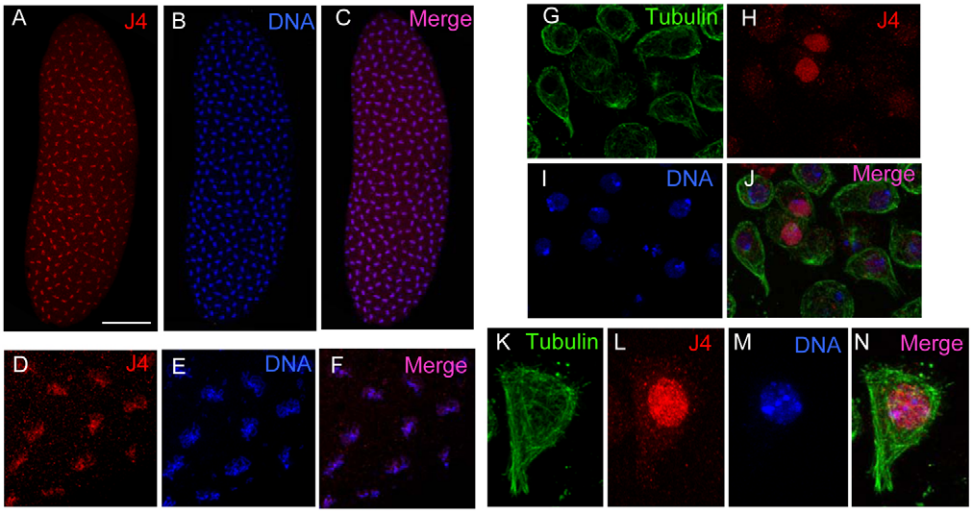

Fig. 2. TnI can localize to the nucleus. Wild-type embryos (A-F) and S2 cells (G-N) stained with the J4 antibody (red) using the nuclear staining with DAPI (blue) and the cytoplasmic staining with anti-tubulin (green) as references. In embryos undergoing chromosome condensation, TnI is closely associated with or near to chromosomes. In S2 cells, TnI can accumulate within the nucleus in some cells, while remaining diffusely distributed in the cytoplasm in others. Scale bar: 70 μm in A-C; 4 μm in D-F; 6 μm in G-J; 4 μm in K-N.

TnI mutant phenotypes. In addition to providing evidence for a maternal component for TnI, these data demonstrate a germ line autonomous requirement for TnI during oogenesis.

Native Troponin I can localize to the nucleus

To visualize the cellular localization of the native TnI, we used the previously described anti-TnI J3 serum (Barbas et al., 1993) and a newly generated antibody directed against a peptide sequence specific to embryonic TnI isoforms, anti-TnI J4 (see Materials and Methods). Immunostaining was done on wild-type embryos and cultures of the S2 cell line (Fig. 2). In pre-cellular embryos, the TnI immunosignal appears closely associated with chromosomes approaching metaphase (Fig. 2A-F; supplementary material Fig. S2A). In interphase, however, the signal is found in the periphery of the syncitial nuclei associated with the actin caps (supplementary material Fig. S2B-D). In S2 cells, which seem to express embryonic TnI isoforms, the signal can be found inside the nuclei (Fig. 2G-L). The nuclear localization was evident in about 15% of cells. In most cases, however, TnI was found as a diffuse immunosignal in the cytoplasm. Finally, in mitotic cells, in about 1% of cases, TnI appeared located around the metaphase plate. Thus, we concluded that TnI can translocate between the cytoplasm and the nucleus, perhaps according to the physiological state of the cell. Higher resolution studies are needed to specify the location and binding partners of TnI during the cell cycle.

SUMOylation is required for nuclear translocation of Troponin I

We reasoned that if these phenotypes were a direct effect of TnI depletion, and the protein shuttles between the cytoplasm and the nucleus, there should be a structural motif or mechanism for the nuclear import. Analysis of the TnI protein sequence revealed no obvious nuclear localization signal. We therefore looked for other types of sequences that could allow the expected nuclear translocation. The small ubiquitin-related modifier (SUMO) is highly conserved in eukaryotes. It is reversibly attached to lysine residues (Gill, 2004; Hay, 2005) and modifies protein functions, including nuclear transport. Putative SUMOylation target sequences were identified in TnI (supplementary material Fig. S3) and one of them was present in exon 10, which is included in all TnI isoforms. Further, we observed that TnI L9/wupRA does not rescue the TnI mutant alleles in an RNAi-attenuated SUMO expression background (data not shown). This result is compatible with the hypothesis that SUMOylation is be important for normal TnI function.

More directly, we tested whether the TnI protein was modified by SUMO in S2 cells. SUMO-specific isopeptidases catalyze the removal of SUMO conjugates from their substrates (Melchior et al., 2003). We immunoprecipitated TnI from cytosolic and nuclear extracts in the presence or absence of the SUMO isopeptidase inhibitor *N*-ethylmalemide (NEM). The resulting western blot was further incubated with an anti-SUMO antibody to demonstrate the presence of SUMOylated TnI in nuclear extracts (Fig. 3A). Finally, we tested the functional role of this SUMO labeling by mutating the putative target site in TnI exon 10 (Fig. 3B). *Drosophila* S2 cells were transfected with a TnI that incorporates a tetra-cysteine tag (TC) to allow visualization (Szecsi and Spindler-Barth, 2006) (see Materials and Methods). This tag was chosen because the more standard reporters (i.e. GFP, myc, HA) appeared to interfere with the functional properties of the chimeric TnI protein and render it unable to rescue TnI mutants. In agreement with the data on the localization of native TnI, the TC-tagged normal protein was identified in the cytoplasm and nucleus of transfected cells (Fig. 3C,D), while the mutated version was only found in the cytoplasm (Fig. 3E,F). For a quantification of the corresponding signals see supplementary material Fig. S4. Thus, we concluded that a sequence in exon 10 of TnI protein is SUMOylated and that mutation of this site abolishes the ability of TnI to translocate to the nucleus and achieve its normal function.

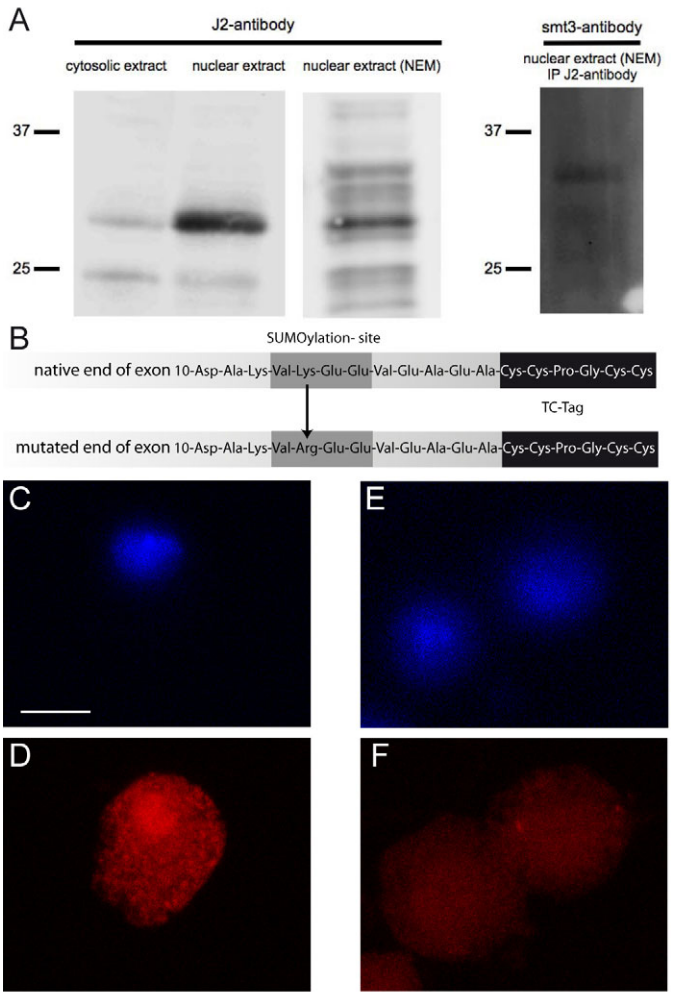

Fig. 3. SUMOylation of TnI is required for nuclear translocation. (A) Western blots from cytosolic and nuclear extracts in the absence and presence of NEM reveal the localization of a fraction of NEM-protected TnI isoforms (J2-antibody to identify TnI) in the nucleus. Immunoprecipitation using the J2-antibody show a SUMOylated (smt3-antibody) form of TnI present in the nucleus. (B) Sequence of exon 10 of the cloned TnI constructs (the latter with a mutated SUMOylation site) in frame with a tetra-cysteine tag for fluorescent detection. (C-F) S2 culture cells stained with DAPI and transfected with the normal (C,D) or mutated (E,F) TnI construct labeled with ReAsH. Note the colocalization of both signals and the accumulation of TnI in the nucleus. Cells transfected with the mutated TnI construct show the nuclei devoid of TnI signal. The full sets of confocal images were quantified (supplementary material Fig. S4). Scale bar: 8 μm.

Tropomyosin mutants show nuclear and cell-cycle defects similar to TnI mutants

We hypothesized that if the absence of TnI caused nuclear defects, then perhaps other actin-binding proteins associated with TnI might also be involved. TnI forms a complex with Tm1 and Tm2, both of which also bind actin. We compared wild-type nuclear divisions

(Fig. 4A-D) to those of *23437* mutants (Fig. 4E-H). We observed segregation and mitotic spindle defects as nuclei divided, and that adjacent nuclei formed clumps with tubulin intermingled with the nuclei. Also, we analyzed embryonic lethal mutant alleles for the Tm1 and Tm2 genes, looking for nuclear division defects in early embryogenesis. In Tm1 (Fig. 4I-K) and Tm2 (Fig. 4L-N) pre-cellular mutant embryos, nuclei divided asynchronously with more severely affected embryos showing clumped nuclei similar to those observed in *23437* mutants. As microtubules are key elements in nuclear divisions, we looked at tubulin localization in the Tm1 and Tm2 mutants. Close examination of the Tm1 mutant interphase nuclei revealed altered tubulin structure throughout the embryo, and fragmented chromosomes were often observed (supplementary material Fig. S5A-D). The Tm2 mutant showed more severe defects in nuclear distribution, with spindles and chromosomal alignment at metaphase appearing scrambled (supplementary material Fig. S5E-H). In a small number of cases (<5% of mutant embryos), there appeared to be supernumerary centrosomes with respect to nuclei, and where clumping of nuclei was observed, tubulin was also severely perturbed (supplementary material Fig. S5H1-10).

Surface analysis of embryos using scanning electron microscope (SEM) imaging revealed that compared to wild type (Fig. 5A; supplementary material Fig. S6A,E), TnI mutant embryos often failed to cellularize in certain areas (Fig. 5B), including the characteristic absence of pole cells at the posterior end of the embryo (see supplementary material Fig. S6A-D,F-H). Similar defects were also observed in Tm1 (supplementary material Fig. S6I-J) and Tm2 (supplementary material Fig. S6K-L) mutants.

In addition to nuclei clumping, milder defects of TnI mutants included abnormal spindle orientation (Fig. 5E) in about 22% of cases, while 28% showed disrupted nuclei and fragmented chromosomes (Fig. 5F). To further characterize the nuclear division defects, we looked for cell-cycle defects using a phosphohistone 3 antibody (PH3) as a mitotic marker (Fig. 5C-D; supplementary material Fig. S7A-F). PH3 staining was detected only in discrete patches, which demonstrates that mitotic activity was variable across each mutant embryo. We also looked at PH3 staining in Tm1 and Tm2 mutants. Both mutants showed defects in PH3 staining that correlated with altered chromosomal fragment segregations. Tm1 mutants showed asynchronized divisions, with some nuclei displaying a different stage of nuclear condensation (Fig. 5G-L), indicated by the absence of PH3 staining and a nuclear structure that differed from neighboring nuclei. Tm2 showed a more severe disruption of the cell cycle, with nuclei displaying various stages of condensation (Fig. 5M-R) and abnormal expression of PH3. Thus, abnormal PH3 expression and defective nuclear condensation are consistent phenotypic traits for the mutant alleles of the three genes. The similarity of phenotypes strongly suggests that the three proteins are involved in the same nuclear function.

TnI and Tm mutants alter the localization of cell polarity proteins

We also visualized actin in TnI mutants at the onset of cellularization. F-actin cytoskeleton was disrupted as well as cell shape, which became irregular (supplementary material Fig. S6M-N). Polarity defects are commonly associated with cellularization abnormalities (Bilder et al., 2000). In this context, we analyzed the location of two membrane-associated proteins, Dlg and Pins. Polarity within epithelia is maintained by three complexes that act antagonistically to restrict proteins to the apical or basolateral membranes. Dlg functions more basolaterally and associates with Scribble (Assemat et al., 2008). In cellularizing embryos, Pins is found associated to the periphery of actin caps (supplementary material Fig. S8). The most striking defect observed in TnI mutant embryos was a disruption of apico-basal polarity, with both Dlg

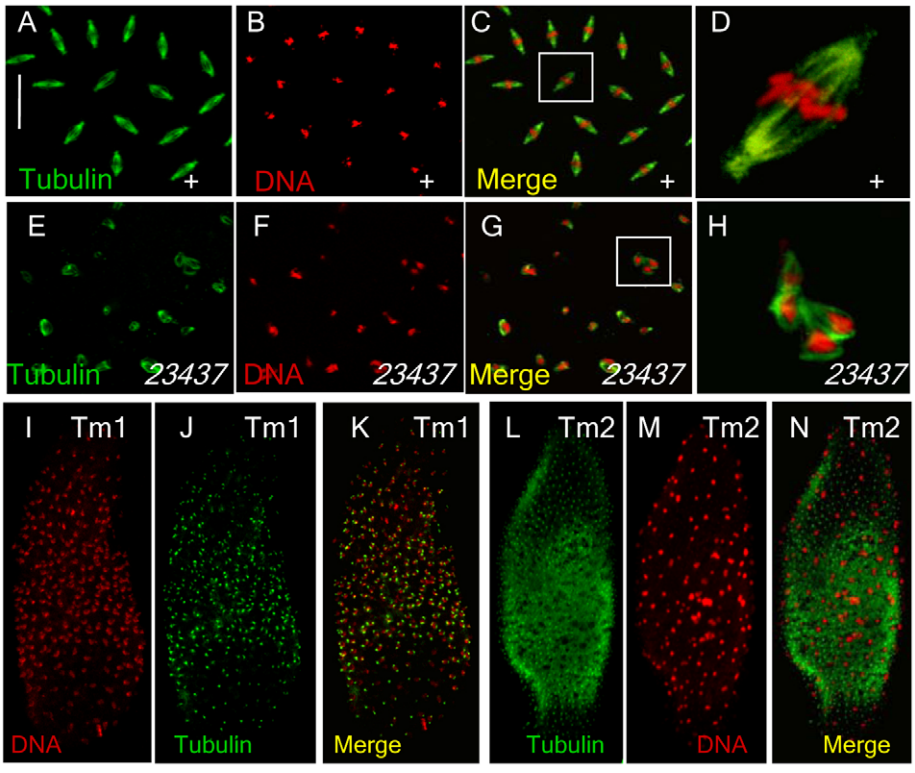

Fig. 4. Tubulin and propidium iodide staining of wild-type, TnI, Tm1 and Tm2 mutant embryos. (A-D) Wild-type nuclei. (D) Enlargement from box in C. (E-H) Nuclei from a *23437* TnI mutant embryo. (H) Enlargement from box in G. Tubulin is present and structured but nuclei are clumped. (I-K) Tm1 mutant embryos are abnormally shaped and typically show reduced tubulin content in anterior and posterior ends of the embryo. Nuclei are also clumped together. (L-N) Tm2 mutant embryos are abnormally shaped and nuclei are clumped together. Tubulin is also abnormally located throughout the embryo and is reduced at the anterior and posterior ends. Scale bar: 5 μm in A-C, E-G, 2 μm in D,H and 200 μm in I-N).

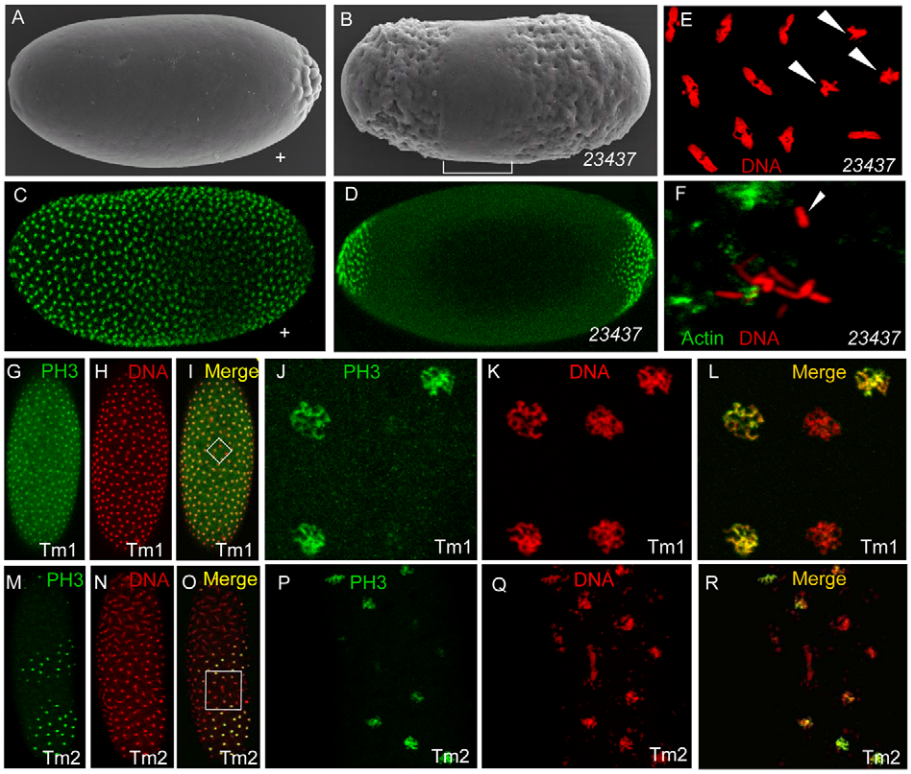

Fig. 5. TnI, Tm1 and Tm2 mutant embryos have defects in nuclear divisions. (A,B) SEM images of early wild type (A) and TnI mutant allele *23437* (B). Note the lack of cellularization in centre of embryo (bracket) and absence of pole cells. (C,D) Phosphohistone H3 (PH3) staining of wild-type embryos (C) indicate that nuclear divisions are synchronized as staining is homogeneous throughout the embryo. (D) In *23437* embryos, nuclear divisions only occur at the anterior and posterior ends in this image. (E,F) Nuclei from *23437* embryos. (E) Arrowheads indicate nuclei dividing in different orientation to the adjacent nuclei. (F) Chromosomes are often fragmented (arrowhead). (G-L) PH3 staining of Tm1 mutant embryos (G-I) show synchronization defects slightly less severe than in TnI. Box in I is enlarged in J-L. Note that chromatin structure appears more compact than in normal PH3 nuclei. (M-O) PH3 staining of Tm2 mutant embryos. Nuclei are asynchronized in the anterior end of the embryo and PH3 staining is not homogeneous throughout. Box in O is enlarged in P-R. Anterior is up. Scale bracket: 250 μm in A-E, 15 μm in E,F, J-L, P-R, and 300 μm in G-I, M-O.

and Pins being mislocalized (Fig. 6A-F) as compared to wild-type embryos. In contrast to the regular near-hexagonal shape of wild-type cell profiles, mutant embryos showed aberrant cell shapes in those patches of the embryo surface where cellularization had been initiated. Similar cell defects were also observed with Tm1 and Tm2 mutant embryos (Fig. 6G-L). Taken together, this repertoire of phenotypes and the similarities between the three mutant genes tested indicates that the Tm-TnI complex plays a hitherto unknown role in maintaining nuclear integrity and cell polarity.

Discussion

The Tn-Tm complex has been well studied in the context of muscle contraction (Boussouf and Geeves, 2007). We have shown here that members of the complex also play an earlier role in development to maintain nuclear integrity. The nuclear defects observed in mutants for the three proteins TnI, Tm1 and Tm2 suggest that the whole Tn-Tm complex is required to maintain nuclear integrity. Embryonic lethal mutants are currently not available for the remaining components, mainly TnC and TnT.

Several sarcomere proteins have been reported to play nuclear functions. Titin, a large protein spanning the sarcomere length between Z bands is required for chromosome integrity (Machado and Andrew, 2000) and control of gene expression through one of its kinase domains (Lange et al., 2005). However, the chromosomal effects are not likely to result from a direct binding of titin to chromosomes because a thorough search for proteins associated to metaphase chromosomes of HeLa cells failed to identify it (Takata et al., 2007). The issue, however, seems to be still controversial since a titin domain has been identified within the cell nucleus playing a role in proliferation (Qi et al., 2008). Zyxin, another actin-associated protein, acts as a tumor suppressor gene in Ewing tumor cells on the basis of its DNA-binding LIM domain (Amsellem et al., 2005) and localizes to the nucleus to regulate gene transcription (Fujita et al., 2009; Martynova et al., 2008).

We have immunolocalized TnI to the nucleus and shown nuclear phenotypes in the mutants. It should be noted, however, that the nuclear localization, either in the syncitial embryo or the regular S2 cells, seems dependent on the physiological state of the cell and nucleus. Also, with the techniques used here, we cannot determine whether TnI is bound directly to the chromosomes or through intervening proteins. Because the repertoire of HeLa metaphase chromosome-associated proteins (Takata et al., 2007) does not include TnI, nor other muscle proteins, the observed effects on chromosome integrity might be produced through indirect links. Nevertheless, one should realize that the referred repertoire is also subject to the technical constrains of the purification methods used in the study of HeLa cells.

We have also shown that the required nuclear translocation is achieved by SUMOylation, at least in the case of TnI. We demonstrated that the putative SUMOylation sequence in exon 10 is required for nuclear import. This site, VKEE, is found in the C-termini of all TnI isoforms because it can be incorporated into the protein sequence, either from exon 9 or exon 10. Thus, all TnI isoforms could be tagged for their function. Other putative SUMOylation sites (supplementary material Fig. S1), if actually used for SUMOylation, could provide further functional diversity for TnI. This mechanism for tagging TnI in *Drosophila* is likely to be conserved in mammals since the VKEE motif is present in the three TnI gene types (slow twitch, fast twitch and cardiac). Although not addressed here, it is possible that a similar mechanism might be used to import Tm1 and Tm2 into the nucleus as they contain suitable motifs in the three isoforms of Tm2 and in one of the two isoforms of Tm1.

This work on the Tn-Tm complex provides an insight into how DNA aberrations and cellularization defects can be linked, and how

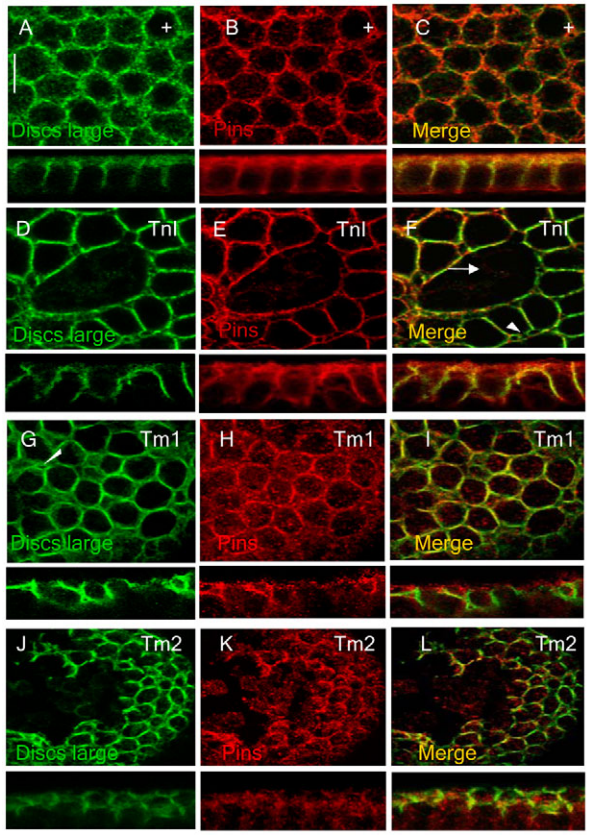

Fig. 6. TnI, Tm1 and Tm2 mutant phenotypes at cellularization. (A-C) Wild-type embryos show regular hexagonal cells, which are columnar in shape. Dlg and Pins signals largely colocalize. TnI (D-F), Tm1 (G-I) and Tm2 (J-L) mutants all show similar defects in apico-basal polarity. Small panels below each image, show apico-basal section through the embryo. In all three mutants, cells have become rounded and piled up upon each other. TnI mutants display abnormal membrane formation (arrowheads). Both Dlg (green) and Pins (red) expression is disrupted during cellularization. Arrow in F indicates that membranes are still present, but Dlg and Pins expression is no longer localized at the membrane. Pins expression in Tm1 and Tm2 mutants also appears more diffuse through the cell. Scale bar: 5 µm.

this complex is crucially required for both DNA and cellular stability. Given that the Tn-Tm complex is also involved in muscle contraction, it appears likely that there may be other processes where disruption of this complex may be detrimental to the development of the organism. In support of this, we have previously shown that mutant TnI allele *23437* displays severe defects in axon guidance and fasciculation and that the TnI L9/wupRA isoform rescues these defects (data not shown). Considering the role of the Tn-Tm complex in sarcomere contraction and the range of phenotypes we have described here, it seems reasonable to propose that TnI, Tm1 and Tm2 are components of a force-generating complex within the nucleus and in the cytoplasm. However, this remains to be determined since the TnI-associated partners have not being investigated here.

Being an actin-binding protein, TnI should perform its nuclear functions in association with actin. This protein is known to help RNA polymerase to move during gene transcription (Ye et al., 2008). It is currently a matter of debate whether this function requires actin in a globular or a filament structure (Pederson, 2008). However, a recent study reports the interaction of vertebrate fast skeletal TnI with the estrogen receptor during transcription (Li et al., 2008). By analogy to the role that TnI plays in the sarcomere, where the Tn-Tm complex interacts with the actin filaments, it seems likely that during transcription actin has a filament structure, as in the sarcomere thin filament. Actin is also important for morphogenesis of cells and organs in the early embryo, ranging from nuclear divisions (Gerisch et al., 2004) and chromosomal segregation in conjunction with myosin (Fabian and Forer, 2007), to the regulation of cell shape and movements. All these processes are also relevant to the formation and progression of tumors (Bissell and Radisky, 2001; Brumby and Richardson, 2005). In addition, chromosomal instability, mitotic defects and cell polarity defects are characteristic features of many cancers (Kops et al., 2005). The fact that TnI, Tm1 and Tm2 all regulate actin strengthens the argument that they execute this regulation as a complex. Defects in all three genes give rise to similar DNA defects, and also to similar defects in apico-basal cell polarity. These common features provide the basis for a mechanism leading to aneuploidy and aberrant cell signaling. That is, molecules that ensure proper actin function during nuclear divisions also ensure that actin correctly regulates cell polarity, which, in turn, is important in proliferation and growth (Wodarz, 2000). The tubulin spindle was also affected in the three mutants, indicating that the integrity of the cytoskeletal network may be compromised when any of these molecules are depleted.

In addition to the cytoskeletal network, the localization of Dlg and Pins were also shown to be disrupted in TnI-Tm mutants. Dlg has been described as a neoplastic tumor suppressor and disruption of polarity is a hallmark of cancer progression. The Pins protein is involved in orientation of asymmetric cell divisions (Schaefer et al., 2000), which is important for specifying cell fate. Consistent with the altered Pins expression, we observed spindle orientation defects in the three mutants. Also, spindle orientation is particularly important for specifying neuronal identity in *Drosophila* neuroblasts (Kaltschmidt et al., 2000). The recycling of molecules for distinct processes is a recurrent theme in development. Indeed, many actin-binding proteins were first identified for their effects on axon guidance and growth (Wills et al., 2002), and were subsequently shown to play important roles during cellularization. Also, Dlg was associated with synaptogenesis before its role in cellularization was determined (Masuko et al., 1999). The novel function for the Tn-Tm complex uncovered here might have opened the way to reveal requirements in other actin-associated events. We have observed that TnI, as well as Tm1 and Tm2, are crucial for the correct development of the central nervous system (our unpublished data). Further studies on the role of the Tn-Tm complex during nuclear divisions seem appropriate towards understanding how these proteins affect cell proliferation, and might provide novel targets for controlling cell divisions.

Materials and Methods

Fly stocks and genetic constructs

The following lines were used: *PL87* and *PG31* insertions (Bourbon et al., 2002), *f*^{5}*23437os* line was generated in our laboratory, Gal4 line *P{w[+mC]=tubP-Gal4LL7* was obtained from the Bloomington Stock Centre. For TnI overexpression, the L9/wupRA cDNA was cloned into the pUAST vector, transgenic lines were generated, and the expression was driven with the tubulin-Gal4^{LL7} (Brand and Perrimon, 1993). The construct contained 5′- and 3′-UTR sequences. The same fragment was also cloned in frame with a TC-tag (CCPGCC) into the CH2 vector for expression in *Drosophila* culture cells and detection with ReAsH labeling reagent (Invitrogen). The mutation of the putative SUMOylation site in exon 10 was introduced via directed mutagenesis on this vector. Mutant Tm1 and Tm2 were GFP lines obtained from Flytrap (http://flytrap.med.yale.edu/). They correspond to the P(w^{+})Thor-GFP/TM3 and P(w^{+})TropII-GFP/TM3 stocks, respectively (Morin et al., 2001).

Western blots, immunoprecipitations and cellular transfection

Nuclear extracts were prepared as described elsewhere (Gim et al., 2001) with and without the SUMO isopeptidase inhibitor *N*-ethylmalemide (NEM). For western blotting, protein samples were dissolved by SDS-gel electrophoresis, electroblotted on nitrocellulose membrane and incubated with the corresponding primary antibodies. Peroxidase-conjugated goat anti-rabbit immunoglobin (Ig) and rabbit anti-mouse (Sigma Aldrich) specific antibodies were used as secondary antibodies. The blots were developed using Super Signal West Pico chemiluminescent substrate (Pierce). Immunoprecipitation was performed on nuclear extracts after crosslinking the J2-antibody to Protein G agarose beads (Sigma Aldrich) using dimethyl pimelimidate (DMP; Sigma Aldrich). *Drosophila* S2 cells were grown in Scheinder's Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Gibco). S2 cells were transfected with lipofectamin (Qiagen) following the manufacturer's instructions. The SUMO tagging was identified with the *Drosophila* antibody smt3 that recognizes the C-terminus (AP1287b; Abgent, San Diego, CA). Transfected cells were treated with ReAsH labeling reagent, fixed using standard procedures, and prepared for microscopic analysis. Images for the localization analysis of the expressed constructs were acquired under constant conditions using a Leica TCS SP5 confocal microscope. Image analysis was performed with ImageJ (http://rsb.info.nih.gov/ij/).

Phenotype analysis and immunostaining

The total number of normal and mutant embryos analyzed in this study is shown in Table 1 and corresponds to TnI (n=218), Tm1 (n=311) and Tm2 (n=334). Each lethal mutant was maintained as a balanced stock from which 2-hour egg-laying samples were obtained. These embryos were classified as pre-cellular or cellular and each class was further classified as abnormal or wild type. Light (normal and UV) microscopy and phase-contrast optics were used. Each mutant yielded the expected 25% ratio of genetically mutant embryos (Table 1). For immunostaining of endogenous proteins in cells, exponentially growing S2 cells were fixed in 4% paraformaldehyde. Embryos were dechorionated using bleach and fixed using standard protocols. Embryos were prepared for SEM as previously described (Turner and Mahowald, 1979). Images were taken using a Hitachi S520 scanning electron microscope. For immunostaining, the following primary antibodies were used: J3 Anti-Troponin I (1:100; third bleed from the same J2 rabbit previously described) (Barbas et al., 1993), chick anti-actin (1:20; DSHB, Iowa City, IA), anti-Dlg (1:20; DSHB), anti-tubulin E7 (1:50; DSHB), rabbit phosphohistone H3 (Santa Cruz Biotechnology), rabbit anti-Pins (1:1000; a gift from Jürgen A. Knoblich). The J4 polyclonal antibody was generated against the peptide CKDYHSKILKLESE encoded in exon 6b. The corresponding RNA isoforms are expressed in embryo and larval stages but not in the adult (Barbas et al., 1993). Peptide synthesis, rabbit immunization and affinity purification were performed by Genscript (Piscataway, NJ). The following secondary antibodies were used: Cy2-conjugated goat anti-rabbit IgG (1:1000; Jackson ImmunoResearch) and Fluorolink Cy3-labeled goat anti-mouse IgG (1:1000; Amersham), and Alexa Fluor 488 Cy2-coupled goat anti-mouse and Alexa Fluor 568 Cy3-coupled goat anti-rabbit IgG antibodies (1:1000; Molecular Probes). DNA was visualized using propidium iodide (working dilution 2 μg/ml; Sigma) or DAPI contained in Vectashield (Vector Laboratories). Images were recorded on a Leica TLS4D confocal microscope equipped with a Leitz DMIRB microscope and processed with Q500 MC software (Leica, Germany), and Adobe Photoshop 5.0. All embryo images shown as figures are stacks of 4-5 μm confocal sections and 1 μm confocal sections for S2 cells figures. Three-dimensional reconstructions were made by taking 1.5 μm confocal sections and reconstructed using the Amira 3.1 program (http://www.amiravis.com).

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