An Endocytic Capture Model for Skeletal Muscle T-tubule Formation

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Abstract

The skeletal muscle T-tubule is a specialized membrane domain essential for coordinated muscle contraction that shows dysmorphology in a number of genetically inherited muscle diseases. However, in the absence of genetically tractable systems the mechanisms involved in T-tubule formation are unknown. Here, we have used the optically transparent and genetically tractable zebrafish system to probe T-tubule development *in vivo*. By combining live imaging with three-dimensional electron microscopy we derived a four-dimensional quantitative model for T-tubule formation. To elucidate the mechanisms involved in T-tubule formation *in vivo* we developed a quantitative screen for proteins that associate with and modulate early T-tubule formation including an overexpression screen of the entire zebrafish Rab protein family. We present a new endocytic capture model involving i) formation of dynamic endocytic tubules at transient nucleation sites on the sarcolemma ii) stabilization by myofibrils/sarcoplasmic reticulum and iii) delivery of membrane from the recycling endosome and Golgi complex.

Keywords

T-tubule, zebrafish, skeletal, muscle, imaging, Rab, endocytosis

The transverse tubule (T-tubule) system of skeletal muscle is an extensive membrane domain that allows action potentials from innervating neurons to reach the depths of muscle fibres thereby triggering calcium release in a synchronized fashion. The T-tubule system is a vital component of the skeletal muscle, frequently disrupted in muscle disease. This is exemplified by aberrant T-tubules and mutations in key T-tubule components in many human myopathies[1-6]. An understanding of the biogenesis of this enigmatic organelle is necessary for further insight into these diseases, however progress has been compromised by the absence of adequate experimental models.

The T-tubule system develops within a defined, but extended period, in mammalian systems. In the mouse, the T-tubules are first observed as longitudinal elements at E16[7] which gradually become organized into transverse elements from around birth to post-natal day 10[8]. The mature T-tubule system is an elaborate system of surface-connected tubules permeating the entirety of the muscle fibre, and occupying a volume greater than that of the sarcolemma from which is it is thought to be derived[9]. The mature T-tubule system has a protein and lipid composition which is distinct from the sarcolemma, and an extremely precise organization and morphology which includes linear elements and triad junctions at specific points, making contact with the sarcoplasmic reticulum. No cell culture systems faithfully reproduce the development of the highly organized T-tubule system characteristic of vertebrate muscle, and a tractable vertebrate animal model has been lacking until now.

Here, we establish the first *in vivo* model to follow formation of T-tubules in real time. We combine live confocal imaging with state-of-the-art electron microscopic approaches, genome editing and quantitative overexpression screening to define the membrane pathways and molecular processes orchestrating T-tubule development. We derive a new model for T-tubule

formation involving the co-option of existing endocytic pathways and spatially-restricted tubule capture to generate the organized T-tubule system.

Results

Development of an *in vivo* system for real-time visualization of T-tubule development

In order to provide robust visualization of the developing T-tubule system, we first tested several transgenic markers and vital dyes for co-labeling with the T-tubule specific protein Bin1 (Fig. 1a-e, Supplementary Fig. 1 a & b)[10]. We found that fluorescent proteins tagged with the CaaX domains from either H-ras or K-ras were robustly localized to the T-tubules, as well as to the sarcolemma at 48hpf (Fig. 1a & b). For this reason we obtained an existing transgenic line which strongly and robustly expressed GFP-CaaX in all cells from a ubiquitous promoter to use in our analyses[11]. This general membrane marker allowed us to visualize development of the T-tubule membrane system at an early stage without bias caused by focusing on specific proteins. To investigate the development of the T-system during the transition from nascent myotube to muscle fibre, we imaged transgenic embryos from immediately post-fusion of the fast muscle (16hpf), until 48hpf when the T-tubules were observed to penetrate the cell all the way from the sarcolemma to the midline of the fibre (Fig. 1f, Supplementary Videos 1 and 2). Direct measurements from still images showed that the T-tubules adopted a regular spacing at 1.9µm intervals.

A quantitative model for formation and expansion of the T-tubule system

In order to understand the development of the T-system in a quantitative manner, and to probe more deeply our observations from light microscopy, we turned to electron microscopy. We used a modified version of a potassium ferricyanide stain[8] on thin transmission electron

microscopy (TEM) sections to specifically label the T-tubules and distinguish them from sarcoplasmic reticulum (SR). Sections taken at 16hpf showed electron dense, transverse oriented tubular membranes associated with the forming Z-lines (arrows Fig. 2a). By contrast, transverse sections taken towards the centre of the sarcomere some distance away from the Z-lines showed association of longitudinal sarcoplasmic reticulum (identifiable by its beaded appearance and lower electron density) with the early myofibrillar material (arrowheads, Fig. 2c). By 48hpf, the myofibrillar bundles, analogous to previously described "nascent myofibrils"[12] developed longitudinal furrows as they increased in diameter consistent with the Goldspink model of myofibrillar splitting during growth[13] (Fig. 2b, d-f). Thin sections and serial blockface scanning electron microscopy with 3D reconstruction showed that these furrows contained SR and, at the level of the Z-line, T-tubules (Fig. 2b, d-j, Supplementary Video 3). Tracing of thin sections taken between 16hpf and 10dpf showed that furrows develop in a spatially predictable manner, such that each contractile filament maintains close proximity to a furrow, and consequently a T-tubule (Fig. 2k). We reasoned that these reiterated spatial relationships would be amenable to mathematical modeling.

To produce a quantitative mathematical model of membrane sequestration into the forming tubules, we required more accurate empirical data from larger numbers of developing muscle fibres than is possible using serial blockface methods. Instead, we assembled large mosaics of tiled TEM micrographs covering entire transverse sections through the trunk musculature at 48hpf, 5dpf and 10dpf. Using custom written image analysis macros we were able to produce masks defining muscle fibres, myofibrils and furrows within a half-section through an entire animal (Fig. 3a-c), and extract morphometric data. Regression analyses of these data gave predictive functions for myofibril cross sectional area (Fig. 3d-h, Supplementary Fig. 3a-c) and linear transverse furrow distance, (the length of the "splits" in transverse orientation which is

directly equivalent to T-tubule length, Fig. 3e). Together with measurements of fibre length from confocal microscopy (Fig. 3g) precise measurements of tubule morphometrics obtained from focused ion beam electron microscopy (Fig 3h), we were able to generate a predictive mathematical model for the surface areas of membranes present in both T-tubule and sarcolemmal compartments for fibres of given sizes (Fig. 3g, Supplementary Fig. 2a-c).

We used this model to calculate surface areas for the plasma membrane of individual muscle fibres and their T-tubule system using the input parameters from the empirical measurements shown in Fig. 3f (somite width as a proxy for fibre length) and Supplementary Fig. 3a-c. These calculations were based on maximum fibre size (rather than the mean size or animal age) since new fibres are continuously added to the myotome and existing fibres grow at different rates (see cellularity profile histograms in Supplementary Fig. 3a-c). The T-tubule system itself is undetectable at 16h, but has developed into a complex organelle with a surface area comprising 35% of the total surface area of the cell (2579 : 4817 μm^2; T-system : sarcolemma) by 48hpf. By 5dpf the relative surface area of the T-tubule domain gradually increases to 40% (4737 : 7092 μm^2) and after 10dpf will eclipse the surface area of the sarcolemma (89082 : 28151 μm^2; 49%). For the largest fibres in adult fish, we applied our model to fibres from a 12 month old male from the zebrafish atlas (http://bio-atlas.psu.edu/zf/view.php?s=220&atlas=18; http://bio-atlas.psu.edu/zf/view.php?s=275&atlas=17). In these fibres, our model predicts that the T-tubule system represents approximately 60% of the total surface area of the cell (Fig. 3h). The increase in surface area of the two membrane systems during the two early time periods correspond to an average of ~1.03 μm^2 /minute of new membrane addition for the entire 48h-5dpf period and ~2.27 μm^2/minute for the 5dpf to 10dpf period. These results emphasize the magnitude of new membrane synthesis and, necessarily, flow through and delivery from the Golgi complex to allow this membrane expansion. Previous studies have

suggested a maximal rate of approximately 2.7μm^2 of membrane per minute for passage through the Golgi complex in rapidly dividing transformed cell lines[14] which would be adequate for the generation of the T-tubule system and for the expansion of the sarcolemma based on our estimates. However, additional membrane synthesis would obviously be required for the generation and expansion of the other crucial membrane systems during this period such as the SR. This implies that specific cellular machinery must be upregulated at the onset of T-tubule formation, including components required for membrane synthesis, for sorting, and for targeted delivery to specific domains including the T-tubule system during this growth period.

Transverse tubule stability is dependent upon sarcomere formation and integrity

The strict association of the T-tubules with the sarcomeric Z-lines led us to question whether the contractile apparatus was involved in the scaffolding of T-tubules during development. To examine further the relationship of sarcomerogenesis to T-tubule development, we made a transgenic line expressing a red fluorescent protein fused to the lifeact tag[15], which labels F-actin, including sarcomeric actin. Live imaging of fish carrying both red Lifeact and green CaaX transgenes revealed that the distance to which transverse tubules extended inwards towards the midline of the cell was spatially associated with the development of the actin-containing myofibrils (Fig. 4a & b, Supplementary Video 4), in agreement with our EM data. Examination of the sarcoplasmic reticulum, marked by another stable transgenic line expressing a red fluorescent protein tagged with an N-terminal calreticulin signal sequence and a C-terminal (KDEL) ER retention sequence, showed that the SR also adopts a regular, Z-line associated arrangement upon sarcomerogenesis (Fig. 4c-d). Titin cap (Supplementary Fig. 4a-b), alpha-actinin (c-d) and junctophilin 1a (e-f) were also spatially associated with T-tubule development, whereas SERCA1, a marker of the non-junctional SR was specifically excluded from the CaaX positive T-domain (g-h). Finally, in order to test directly whether tubule

formation is directly dependent upon the sarcomere, we employed a loss of function approach using CRISPR-Cas9[16]. We generated embryos possessing null alleles of slow myosin heavy chain (the only myosin expressed in zebrafish slow muscle at early stages[17]). All slow muscle cells in these embryos showed a reduction in the distance between T-tubules, reflecting a "concertina effect" on the sarcomeres, where the Z-lines became closer together (Fig. 4e, g). Next, we generated a null allele of titin, the "molecular scaffold" which links myosin filaments to the Z-line[18]. In these mutants, the T-tubules completely lost their transverse structure and instead became a disorganized meshwork (Fig. 4f). TEM images showed unstructured myosin and actin filaments and narrow unstructured electron dense tubules (Fig. 4h-j). We noted however, the stochastic coalescence of junctional SR and electron dense tubule (arrows, Fig. 4i & j). Quantitation of the diameters of SR-associated vs non-SR associated T-tubule in titin mutants showed a 90% increase in average diameter (43.5 vs 22.8µm) in tubules associated with SR, suggesting that tubule morphology is influenced by interactions with SR or thin filament components (Fig. 4k).

Surface connected tubules are stabilised by "endocytic capture" to form transverse orientated tubules

The tight association of the forming T-tubules with the Z-line and junctional SR led us to investigate further the first interactions of the tubules with the forming contractile apparatus. Using live imaging, we observed at the earliest stages, both tubular and vesicular CaaX positive structures beneath the plasma membrane (Fig 5a-c). These tubular structures appeared to then become stabilized into the familiar transverse-orientated tubules regularly spaced at 1.9µm intervals. We next asked whether, during formation, the T-tubules are in fact surface-connected, or whether the nascent tubule begins as an internal compartment which opens to the extracellular milieu later in development. To test this, we performed intramuscular injection of

Alexa-647 labelled UTP, which has been used as a low molecular weight fluorescent tracer that is unable to cross an intact plasma membrane[19]. Injection of the tracer into 16, 24 or 48 hpf GFP-CaaX zebrafish resulted in immediate infiltration into the tubules, confirming surface connectivity (Figs. 5d-f). In contrast, uptake into putative intracellular vesicles only occurred after several minutes (Supplementary Video 5). Similarly, serial blockface scanning electron microscopy and 3D reconstruction of 48hpf fibres at high resolution showed narrower tubular connections between the plasma membrane and the stabilized, sarcomere associated tubules (Fig 5g). In areas where sarcomeres were separated from the plasma membrane by cytoplasm, these connecting tubules frequently looped and formed connections to the sarcolemma some distance from their origin (Supplementary Fig. 5a). Injection of free HRP into the circulation at 48hpf was also shown to penetrate into the tubules (Supplementary Fig. 5b & c). Taken together with our analyses of smyhc1 and titin mutants, these data suggest that the earliest detectable tubules are surface-connected and are therefore analogous to endocytic tubules. In this model, tubules derived from the sarcolemma would be stabilized when they interact with the contractile apparatus either directly, or through specification of the junctional sarcoplasmic reticulum, a process which we term “endocytic capture”.

Molecular composition of the early T-tubules

In order to probe the composition of the early tubules in zebrafish muscle, and to gain insight into their biogenesis, we sought to examine the localization of the zebrafish orthologues of a suite of trafficking and T-tubule associated markers previously defined in mammalian systems. We developed a set of computational tools which allowed us to perform quantitative analyses of the association of transiently-expressed fluorescently-tagged proteins with the T-tubules in an unbiased fashion with reasonable throughput. Using this pipeline, we were able to calculate localization values for defined domains, in this case T-tubule and sarcolemma (Fig. 6i & j)

from large numbers of replicated images using ImageJ (full code is given in Supplementary Macros). We were also able to calculate the extent of T-tubule perturbation, by comparing the amplitude of CaaX intensity along the longitudinal axes of the cells (Fig. 6k-m). These analyses showed a striking and selective enrichment of a subset of these proteins; Bin1b, Cavin4a and Cavin4b, EHD1a, Cav3, Dynamin2b and CD44b to the area of the Z-line and junctional SR (Fig. 6a-j, Supplementary Fig. 6a-g). Localisation of these factors to this intracellular domain suggested an endocytic tubule formation mechanism with similarities to those described in non-muscle cells[20, 21]. To test a functional role for these proteins, we generated dominant negative forms of these proteins where possible, and compared the amplitude plots of the CaaX marker as a measure of the extent of perturbation (Fig. 6k & l). We found a marked reduction in amplitude with expression of some of the dominant negative forms, in particular, EHD1a and 1b-dEH and Dynamin2a and 2b-K44A (Fig. 6d, g, m, Supplementary Fig. 6a - f). Notably, in the cases of EHD1a and EHD1b, tubule intensity was also increased when the wild-type proteins were expressed. These analyses demonstrate that endocytic protein function is necessary for the normal development of the T-system and suggests a model where formation occurs by co-option of existing endocytic machinery.

Next, we used a set of well-established probes to examine the phosphoinositide composition of the T-tubules, since different membrane compartments have particularly distinct phosphoinositide signatures. T-tubule phosphoinositide composition has not been directly studied *in vivo*, although enrichment of PtdIns(4,5)P_2 on early tubules in C2C12 cells, and a tenfold increase in total phosphoinositides upon differentiation into myotubes have been demonstrated[10]. Of all probes tested, LactC2/PS, had the highest T-tubule signal and was elevated compared to the CaaX control (Fig. 7a, h) consistent with a previous biochemical study[22]. We also found substantial levels of BTK/PtdIns(3,4,5)P_3 (Fig. 7b, h) and PLC-

D/PtdIns(4,5)P_2 (Fig. 7c, h) in early T-tubule membranes. Of the other probes used, ING/ PtdIns(5)P was not specifically localized to the tubules (Fig. 7d, h), FAPP1/ PtdIns(4)P was largely confined to the Golgi complex as expected (Fig. 7e), ATG18/ PtdIns(3,5)P_2 labelled an internal compartment consistent with lysosomes (Fig. 7f) and 2xFYVE^{hrs}/ PtdIns(3)P gave a punctate pattern consistent with early endosomes (Fig. 7g, see also GalT, lamp1 and EEA1, Supplementary Fig. 7a-c, respectively). These data demonstrate that a defining feature of the early tubules is a distinct lipid composition, which may form a platform on which other proteins are recruited to establish the distinct protein composition, analogous to apical and basolateral domains in epithelia[23, 24].

An overexpression screen of all zebrafish Rab proteins implicates a subset of endosomal Rabs in T-tubule development

In order to identify specific cellular machinery upregulated or deployed during formation of the T-tubule/SR junction, we performed an overexpression screen of the entire zebrafish Rab family. Rab proteins are key regulators of membrane trafficking, controlling almost every step of vesicle trafficking including formation, transport and fusion. Approximately 60 Rab proteins are involved in different aspects of membrane regulation in mammalian cells and the functions of many of these proteins are well described in non-muscle cells. Indeed, Rab protein overexpression has been shown to have specific functional consequences upon specific membrane trafficking processes, presumably by sequestering key regulators[25], a strategy validated in other *in vivo* screening systems[26]. We generated a muscle-specific expression library by cloning each Rab open reading frame behind a muscle specific promoter and a red fluorescent protein tag. Identified Rab proteins were further tested using specific dominant-acting mutants.

Using this systematic quantitative analysis, we found a subset of Rab proteins with specific localization to the T-tubule/SR junction (Fig. 8a-e, i). This included Rab33bb, Rab32b and Rab40b which all showed strong and specific localization to this region (Fig. 8a-c). Closely related paralogues frequently had markedly different profiles confirming the specificity of the screen (e.g. compare Rab33bb to Rab33ba). Rab8b specifically localized to the tubules (ranked 10th) but also frequently produced ectopic "knots" of CaaX-negative tubules extending beyond the leading front of tubule formation (Supplementary Fig. 8b). In this case, overexpression was also associated with a decrease, rather than an increase in T-tubule intensity. In contrast, Rab32b, ranked second for localization, resulted in an approximately 80% increase in CaaX intensity, strongly suggestive of positive regulation of T-tubule formation. We found that the most perturbing construct was Rab6a (Fig. 8f, i). Rab6b was not localized to the T-tubules consistent with its role in vesicle fission from the Trans–Golgi Network (TGN)[27]. Strikingly, the next most perturbing group of Rab proteins all belong to a subset of endosomal Rabs, including Rab11ba, Rab4a, Rab22a, Rab5c which are all present on early (Rab22a, 5c) or recycling (Rab11ba, 4a, 22a) endosomes[28-31] (Fig. 8f-i, Supplementary Fig. 8a). These effects were confirmed using the lipid marker BODIPY-FLC_5-Ceramide (Supplementary Figure 8c-h) to avoid confounding effects specific to the Hras CaaX domain (note that this analysis showed that Rab23 caused redistribution of the CaaX signal, rather than a specific effect on the T-tubules themselves [Supplementary Fig. 8g] and was therefore omitted from further analysis). The specific role of a subset of Rab proteins (Rab6a, 11ba, 4a, 22a and 5c) on T-tubule development were confirmed by over-expression of the dominant negative and constitutively active variants (Supplementary Fig. 8i). Combined with the *in vivo* screen, we can conclude that a subset of Rabs associate with the developing T-tubule/SR junction, and that the majority of these are recruited to the minimal Bin1 tubule in a model system.

An *in vitro* model of T-tubule formation for validation of novel factors in T-tubule development

Our *in vivo* approach allowed identification of novel rab proteins which localise to the triadic compartment of the sarcomere, where the T-tubules form at the interface of the junctional SR and Z-line. In order to probe further these molecules in a systematic manner, and validate their tubule association, we developed a non-muscle *in vitro* system based on the readily transfectable baby hamster kidney (BHK) cell line. Upon transfection with zebrafish Bin1b, BHK cells were able to form an extensive plasma membrane-connected tubule system which was able to recruit both the CaaX surface marker, and caveolin3, but not the early endosomal marker snx8[32] (Supplementary Fig. 9e-g).

We took the top ten hits for localisation from our *in vivo* screen, and expressed them in this system. Remarkably, six out of the ten candidates (rab13, 8a, 8b, 33bb, 40b, 26; Fig. 9a-f) co-localised to the induced tubules suggesting that, even in the absence of both sarcomere and junctional SR they are able to be recruited to Bin1b-induced membrane tubules. Two factors (rab32b and 37; Supplementary Fig. 9a & b) marked puncta independent from the Bin1b positive tubules, and a further two did not localise to any discernible structure (rab38a and 19; Supplementary Fig. 9c & d).

Discussion

In this study, we have defined the molecular mechanisms underpinning vertebrate T-tubule development. Our unique system, utilizing live imaging of transgenic zebrafish embryos, 3-dimensional electron microscopy, mathematical modeling and semi-automated overexpression screening has allowed dissection of T-tubule formation *in vivo*, a process which has been refractory to study for decades. We propose that initial tubule formation, immediately following muscle fibre fusion, utilizes the machinery of endocytosis. The T-tubule system

further develops to become an extensive domain permeating the entire cell and eventually surpasses the sarcolemma in terms of surface area. Mathematical modeling of this process shows that its continuous growth requires rates of membrane synthesis comparable with those of rapidly dividing transformed cell lines. By combining overexpression screening of the entire zebrafish rab protein family with quantitative microscopic analysis we show a role for a Golgi complex small GTPase and a subset of rab proteins specific to the early and recycling endosomes in the expansion of the T-tubule system.

Based on our quantitative 4D analyses, utilizing advanced light and electron microscopy, we present a three-step framework for skeletal muscle T-tubule formation. Firstly, tubules derived from the sarcolemma nucleate on the inner leaflet of the plasma membrane. These early tubules possess markers of clathrin independent endocytosis, in particular CD44, EHD1 and dynamin2[33, 34]. Furthermore, we were able to reciprocally regulate T-tubule integrity when we over-expressed either Dnm2 or EHD1 in wildtype or dominant negative forms confirming that these proteins play a key role in this process. Secondly, these tubules are stabilized by interactions with the sarcomere, either directly or indirectly via the sarcoplasmic reticulum, a process which we have termed 'endocytic capture'. Our three-dimensional electron microscopy revealed that the spatial positioning of the tubules was dependent upon the splitting of myofibrils to form 'furrows' during growth, a phenomenon described over 30 years ago but which has received little attention since[13]. We present evidence that the sarcomere forms the scaffold for the early T-system, since genetic disruption of the sarcomere has a downstream effect on T-tubule morphology. Scaffolding appears to occur independently of the thin filament, since genetic perturbation of the thick filament by deletion of myosin results only in a change in tubule spacing. Genetic perturbation of titin however, results in dysregulation of T-tubule development such that tubules lose completely any ordered orientation. They are narrower, and form only stochastic junctions with SR, where they are also significantly wider

in diameter. This raises the intriguing possibility that T-tubule recruitment might actually be mediated through maturation of SR, and only indirectly though scaffolding from the sarcomere. In differentiating mouse fibres, the junctional SR appears to form absolutely concomitantly with the transversely oriented tubules[8], and non-transverse tubules, such as longitudinal elements, are not associated with SR. Our data also shows that the tubules extending from the sarcomere to the sarcolemma, remain narrow and tortuous, even after the mature architecture is fully developed, raising the possibility that they may be temporally and spatially dynamic, perhaps even remodeling between contraction cycles. Thirdly, because of the prodigious surface area described by our mathematical model, T-tubule development is sensitive to perturbation of trafficking pathways. We have shown that vesicles containing fluid phase dextran interact with the forming tubules, and that perturbation by overexpression of the endosomal rabs rab11ba, 4a, 22a or 5c, results in significant tubule loss, while the integrity of the sarcomere is preserved. Exactly how this directional membrane traffic into growing tubules is achieved is currently unclear, but a recent study using cultured fibroblasts from centronuclear myopathy patients carrying mutations in Myotubularin, demonstrated that the Myotubularin dependent conversion of endosomal PI3P into PI4P is necessary for tethering and fusion of exocytic carriers at the plasma membrane[35]. A similar mechanism regulating vesicle fusion at the T-tubule would provide a common axis for the pathobiology of Bin1, Dynamin2 or Myotubularin related centronuclear myopathies.

This study provides a new paradigm for the formation and stabilization of the T-tubule system during muscle development and establishes a framework in which to probe specific molecular events, such as causative mutations in human disease etiology.

Author Contributions

Conceptualization, T.E.H. and R.G.P.; Methodology, T.E.H., R.G.P, Z.X., N.A., C.F.; Y.L. Validation, T.E.H.; Formal Analysis, T.E.H.; Investigation, T.E.H., N.M., A.X., N.A., J.R. and C.F.; Resources, T.E.H., N.M., H.P.L., Z.X.; Writing, T.E.H.; Visualization, T.E.H.; Supervision T.E.H. and R.G.P.; Project Administration T.E.H. and R.G.P.; Funding Acquisition T.E.H. and R.G.P.

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Competing Financial Interests

None declared

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Figure Legends

Figure 1

A novel *in vivo* system for T-tubule development.

(**a-e**) The CaaX domain from Hras marks both sarcolemma and T-tubule. Markers were screened at 48hpf for T-tubule specificity on a stable transgenic background expressing Bin1b-Citrine from the endogenous locus. (**a**) CaaX domain from Hras (tH). (**b**) CaaX domain from Kras (tK). (**c**) Myristoylation domain from ARF5 (Millar et al., 1999). (**d**) BODIPY-TR vital dye (Cooper et al., 2005). (**e**) GPI anchor from zebrafish folate receptor gamma. (**f**) Time course of tubule formation using stable EGFP-CaaX background. See also Supplementary Videos 1 and 2. Scale bars; **a-e**, 10µm, **f**, 50 µm.

Figure 2

A quantitative model of T-tubule development.

(**a-f**) Thin transverse transmission electron microscope (TEM) sections showing early tubules and sarcoplasmic reticulum. (**a, b**) Transverse sections through the Z line (Z) at 16 and 48hpf. Electron dense, ferricyanide stained tubules are denoted by arrows. (**c, d**) Equivalent transverse sections taken through the centre of the sarcomere. Arrowheads denote sarcoplasmic reticulum, which has an unstained lumen and "beaded" appearance. (**e, f**) Oblique sections at 5 and 10dpf show both ferricyanide stained, electron dense tubules (arrows) and sarcoplasmic reticulum (arrowheads). M, M-line, Z, Z-line. See also Supplementary Video 3 (**g-i**) Serial blockface

electron microscopy and 3D reconstruction shows association of T-tubules with myofibril furrows at the Z-line (see also Supplementary Video 3). (**j**) Single planes from focussed ion beam volume enables precise measurements of tubule morphometrics (quantitative data shown in Fig. 3g). (**k**) Tracing of thin TEM images illustrates the architecture of myofibril furrows and associated tubules. Red, tubules/furrows; pink, myofibrils; green, sarcolemma. Full dataset is shown in Fig. 3. Scale bars; **a-f**, 1µm. **g-h**, 10µm. **j**, 200nm. **k**, 10µm.

Figure 3

(**a-c**) Tracing of tiled TEM montages allows extraction of quantitative data. (**d**) Relationship between fibre cross sectional area and myofibril cross-sectional area. (**e**) Relationship of myofibril cross sectional area to linear furrow distance. (**f**) Somite width during the first 10 days post fertilization. (**g**) Precise measurements from focussed ion beam volumes shows that tubules are elliptical (see also Fig. 2j). (**h**) Application of the mathematical model over a range of fibre sizes from the smallest to the largest. The surface area of membrane in the T-tubule system rapidly eclipses that of the sarcolemma. Scale bars; **a-c**, 50µm.

Figure 4

Markers of sarcomere formation and organized sarcoplasmic reticulum are associated spatially with forming T-tubules.

(**a, b**) Lifeact (marks actin). (**c, d**) KDEL (marks endoplasmic/sarcoplasmic reticulum). (**e**) *smyhc1*$^{-/-}$ embryos show intact tubules with reduced but regular spacing in slow muscle fibres. (**f**) *titin*$^{-/-}$ embryos show dysregulated tubule structure. (**g**) Quantification of inter T-tubule distance in fast and slow *smyhc1*$^{-/-}$ mutant embryos. (**h**) Thin transverse TEM section showing WT tubules (arrows). (**i, j**) Thin transverse TEM sections from *titin*$^{-/-}$ mutants showing difference in morphology between tubules associated with sarcoplasmic reticulum (arrows)

and tubules not associated with sarcoplasmic reticulum (arrowheads). (**k**) Quantification of sarcoplasmic reticulum associated and non-associated tubules in *titin*$^{-/-}$ mutant embryos. **** $p < 0.0001$. (**a**, **c)** 24hpf; (**b**, **d**, **e**,), 48hpf (**g**, **h**, **i**), 72hpf (**j**) 3dpf. Scale bars **a-f**, 5µm. **h-j**, 1µm.

Figure 5

T-tubules are surface connected, and dynamically interact with endocytic vesicles during formation.

(**a-c**) Different tubule morphologies by confocal microscopy. (**a**) Longitudinal elements are frequently seen between forming tubules (arrows) and additional elements appear to connect to the sarcolemma (arrowheads). (**b**) Glancing optical sections across the sarcolemma at 16hpf immediately before "stabilized" transverse orientated tubules appear, show tubules immediately beneath. (**c**) At 48hpf apparently surface connected elements (arrows) are frequently seen in glancing optical sections across the surface of the sarcolemma.

(**d-f**) Intramuscular injection of alexa-647 into EGFP-CaaX fish results in immediate penetration into the developing T-system. (**d)** 16hpf, (**e)** 24hpf, (**f)** 48hpf. (**g**) Serial blockface electron microscopy and 3D reconstruction shows tubules connecting stabilized, sarcomere associated tubules to the sarcolemma. See also Supplementary Fig. 5. Individual Z planes are shown in **g1-g6**. (**f**) Timelapse microscopy of 10,000MW-Dextran-alexa-647 injected EGFP-CaaX fish shows uptake of labelled dextran within 10 mins, followed by dynamic interaction between dextran-positive vesicles and T-tubules (see also Supplementary Video 5). Scale bars; **a-c**, 5µm. **d-f**, 10µm. **g-h** 5µm.

Figure 6

Molecular composition of the early T-tubules

Images of the top seven markers for T-tubule localization, compared to a non-tubule marker, ARF6. (**a**) Bin1b. (**b**) Cavin4a. (**c**) Cavin4b. (**d**) EHD1a. (**e**) Exoc6. (**f**) Caveolin3. (**g**) Dynamin2b (**h**) ARF6. Scale bar, 5µm. (**i**) Schematic showing domains used for localization analysis. TT, T-tubule domain; SL, sarcolemma; IT, inter-tubule domain. (**j**) Markers ranked for T-tubule localization. (**k**) Schematic showing the information captured by lateral optical sectioning of T-tubules by confocal microscopy. Although the average diameter of the T-tubules in the transverse plane was 96nm, when measured precisely by electron microscopy, these structures measured up to 380nm in confocal microscope images due to light scatter. Furthermore, this was expanded up to 800nm in the Z-plane. In this schematic, the T-tubules are scaled to a 380nm diameter, and the optical section is scaled to 800nm. (**l**) Schematic showing analysis strategy for assessment of perturbation. A boxed region of interest was placed over an array of eight T-tubules to give the average pixel intensity in the y dimension. This method ensured that quantitative assessment of signal was possible even where T-tubules were fragmented or only partially present. For each sample, amplitude was expressed as a percentage of that of a wildtype (non-expressing) cell from within the same image. (**m**) Comparison of constitutively active (CA) and dominant negative (DN) variants of the top eight hits for perturbation. (**e**) Comparison of constitutively active and dominant negative variants.

Figure 7

Phosphoinositide signature of the developing T-tubules.

(**a**) LactC2 probe for phosphatidylserine (PS). (**b**) BTK probe for PtdIns(3,4,5)P_2. (**c**) PLC-D probe for PtdIns(4,5)P_2. (**d**) ING probe for PtdIns(5)P. (**e**) FAPP1 probe for PtdIns(4)P. (**f**) ATG18 probe for PtdIns (3,5)P_2. (**g**) 2xFYVEhrs probe for PtdIns(3)P. (**h**) Ranked heatmap comparing average pixel intensity across each domain (T-tubule, sarcolemma and inter-T-

tubule domain), for markers of interest and EGFP-CaaX. Six fibres were analyzed per marker. Scale bars, 5µm. See also Supplementary Fig. 7.

Figure 8

An over-expression screen of the entire zebrafish Rab family identifies distinct Rab proteins with T-tubule specific localization, and Rab proteins with the ability to perturb T-tubule formation. (**a-e**) Top five hits for T-tubule localization. (**a**) Rab33bb. (**b**) Rab32b. (**c**) Rab40b. (**d**) Rab13. (**e**) Rab37. (**f-h**) Top three hits for perturbation of fluorescence amplitude. (**f**) Rab6a. (**g**) Rab23. (**h**) Rab4a. (**i**) Ranked heatmap showing specific localization to the T-tubule domain, and the capacity of Rab overexpression to perturb T-tubule formation. Asterisks indicate the level of statistical significance between cells over-expressing Rab proteins and wildtype cells within the same image. Six fibres were analyzed per marker. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bars, 5µm. See also Supplementary Fig. 8.

Figure 9

An *in vitro* model of T-tubule formation for validation of novel factors in T-tubule development. Tubules are induced in BHK cells by transfection of Bin1b, then assessed for colocalization with specific markers. (**a**) Rab13 (**b**) Rab8a, (**c**) Rab8b, (**d**) Rab33bb (**e**) Rab40b (**f**) Rab26 (**g**) summary graphic showing cells that possess tubules with Bin1b only (magenta), marker only (green) and tubule where bin1b colocalizes with the marker (dual color; (see also Supplementary Fig 9).

Figure 10

An endocytic capture model for T-tubule formation.

(**a**) Dynamic surface connected tubules nucleate on the inner leaflet of the plasma-membrane.

(**b**) Surface connected tubules are "captured" and stabilized by the contractile apparatus/sarcoplasmic reticulum.

(**c**) Additional tubule growth occurs through membrane delivery which is dependent upon rab6a Golgi trafficking, and the early and recycling endosomes.

Supplementary Figure 1

Myotubularin and Dysferlin mark the early, CaaX-positive T-tubules.

(**a**) Myotubularin. (**b**) Dysferlin. Scale bar, 10µm.

Supplementary Figure 3

A mathematical model for T-tubule formation

(**a-c**) Muscle fibre cellularity profile at (**a**), 48hpf (**b**) 5dpf (**c**) 10dpf. (**d**) Schematic of a "typical" muscle fibre illustrating the parameters used to model T-tubule and sarcolemmal surface area. (**e**) Equation for T-tubule surface area based on simple formulas for the area and perimeter of a circle, the perimeter of an ellipse and constants from the regression equations shown in Fig 3d-e. (**f**) Simplified version of the formula shown in **e**.

Supplementary Figure 4

Markers of sarcomere formation and organized sarcoplasmic reticulum correlate spatially with T-tubule formation.

(**a, b**) Titin cap (TCAP/telethonin). (**c, d**) Alpha-actinin2 (actn2). (**e, f**) Junctophilin 1a (Jph1a). (**g-h**) sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (SERCA1). (**a**, **c**, **e**, **g)** 24hpf; (**b**, **d**, **f**, **h)**, 48hpf. Scale bars, 5µm.

Supplementary Figure 5

(**a**) Serial blockface electron microscopy and 3D reconstruction shows tubules connecting stabilized, sarcomere associated tubules to the sarcolemma. Where the sarcomere is some distance from the sarcolemma, connecting tubules are often unstructured and looping (arrows). Individual Z planes are shown in **a1-a6**.

Supplementary Figure 6

Selected endocytic markers localize to the T-tubules and dominant negative forms perturb T-tubule formation.

(**a**) EHD1b (**b**) EHD1b-dEH (**c**) Dnm2a (**d**) Dnm2a-DN (**e**) Dnm2b-DN (compare with Fig. 2g) (**f**) EHD1a-dEH (compare with Fig 6d). (**g**) ARF6-DN (compare with Fig 6h). Scale bar, 5µm.

Supplementary Figure 7

Non-phosphoinositide binding markers of intracellular domains.

(**a**) 1,4-galactosyltransferase (GalT) marks the Golgi complex (compare with FAPP1, Figure 7e). (**b**) Lysosomal associated membrane protein 1 (LAMP1) marks the lysosomal compartment (compare with ATG18 Supplementary Figure 7f). (**c**) Early endosome associated 1 (EEA) marks the early endosomal compartment (compare with 2xFYVEhrs, Supplementary Figure 7g). Scale bars, 5µm.

Supplementary Figure 8

(**a**) Rab22a, fourth hit for T-tubule perturbation from Rab screen (continued from Fig 8). (**b**) Rab8b ectopic tubular "knots". (**c-h**) Validation of top hits for perturbation of the CaaX signal by BODIPY-FLC$_5$ staining. (**c**) Rab6a. (**d**) Rab23. (**e**) Rab11ba. (**f**) Rab4a. (**g**) Rab22a. (**h**) Rab5c. Note the lack of perturbation by overexpression of rab23 (compare **d** to Fig. 8g). (**i**)

Quantitative analysis for perturbation and localization using dominant negative and constitutively active forms of the top hits from the Rab overexpression screen. Scale bars, 5µm.

Supplementary Figure 9

An *in vitro* model of T-tubule formation for validation of novel factors in T-tubule development.

(**a**) Rab32b. (**b**) Rab3.7 (**c**) Rab38a. (**d**) Rab19. (**e**) Caveolin3. (**f**) CaaX. (**g**) Snx8.

Supplementary Video 1

Confocal timelapse series showing initial formation of the skeletal muscle T-tubule system from 18hpf to 31hpf. At 18hpf unstructured tubules can be seen adjacent to the sarcolemma (red arrows). The first tubules adopting regular, reiterated spacing are marked by blue arrows.

Supplementary Video 2

Confocal timelapse series showing initial formation of the skeletal muscle T-tubule system from 30hpf to 39hpf. Transient longitudinal elements can be seen between transverse tubules (blue arrows).

Supplementary Video 3

Serial blockface scanning electron microscope volume in transverse orientation at 48hpf. The sequence pans through serial sections showing heavily stained T-tubules at the Z-lines. It then returns back through the volume displaying a thresholded surface which distinguishes T-tubules, sarcolemma and mitochondria.

Supplementary Video 4

Confocal timelapse series showing a lateral view of a double transgenic zebrafish expressing Lifeact-mKate2 (magenta) and EGFP-CaaX (Green). As the actin-rich myofibrils develop, T-tubules develop at regular intervals along their length. Structured tubules are invariably associated with myofibrils (blue arrows).

Supplementary Video 5

24hpf EGFP-CaaX zebrafish injected with the low molecular weight tracer Alexa-647 labelled UTP (magenta). At the start of the sequence, tracer already present on the sarcolemma and in the T-tubules. After several minutes, tracer can be seen in intracellular vesicles (blue arrows).

Methods

Zebrafish husbandry

Fish were maintained according to standard procedures[36] by University of Queensland Biological Resources. All animals were maintained on the Tuebingen/AB (TAB) background.

Stable transgenic fish lines

The new transgenic lines Bact2-Lifeact-mKate2 and Bact2-mKate2-KDEL were made using the Tol2 system according to established methods[37]. However, in order to make our transposase RNA, we generated a new Gateway compatible backbone for in vitro transcription of RNA (pT3TS-Dest_R1-R3) and a new entry clone containing a codon optimized transposase sequence fused to an SV40 nuclear localization sequence on each end (pME-nls-transposase). These were recombined in an LR reaction with p3E-pA[38] to make pT3TS-nls-transposase. This plasmid was linearized with KpnI and XbaI before in vitro transcription with T3 polymerase as previously described[38, 39]. All transgenics were crossed until Mendelian frequencies were reached, indicative of single genomic integrations. Stable lines were maintained as

heterozygotes. Existing lines used in this study were acta1-EGFP-CaaX (Hras)[11], acta1-Lifeact-EGFP[40], acta1-mCherry-CaaX[40] (Table 1).

BODIPY staining

BODIPY-FLC$_5$ vital staining was carried out as previously described[19]. Briefly, embryos were incubated overnight in 5µg/ml BODIPY-FLC$_5$ (Molecular Probes, catalogue number D3521). Embryos were washed for 5min in standard embryo media before being mounted for microscopy.

Confocal Imaging

Confocal imaging of zebrafish was performed on Ziess 710 meta confocal microscopes as previously described[41]. Briefly, zebrafish were mounted in 1% low melting point agarose in embryo media[36], in either a MatTek dish (for inverted microscopes) or a standard 8cm petri dish for upright microscopes. Objectives used were Zeiss water immersion x40 N/A 1.0 (catalogue number 420762) on upright microscopes or oil immersion x 40 N/A 1.3 (catalogue number 421462) on inverted microscopes. Embryos were anaesthetized in 2.5mM tricaine prior to imaging. Confocal imaging of BHK cells was carried out at 37 °C on a Zeiss Inverted LSM 880 with Airyscan using a x 40 Plan Apochromat objective (catalogue number 420762-9800-799). At 18 h post transfection, cell culture medium was replaced with phenol red free DMEM/F12 medium (Invitrogen Australia, 11039-021) containing 10% FBS one hour prior to microscope imaging.

Tissue Culture

BHK cells from a baby hamster kidney fibroblast cell line (ATCC CCL-10), were cultured in DMEM (Life Technologies Australia Pty Ltd Invitrogen, 11995073) supplemented with 10%

(vol/vol) FBS (GE Healthcare Australia, SH30084.03) and 1% (vol/vol) L-glutamine (Life Technologies Australia, 25030-081) as previously described[42]. Cells were seeded onto glass bottom dishes and at 70% confluence were transfected with DNA constructs using Lipofectamine 3000 reagents (Life Technologies Australia Pty Ltd Invitrogen, L3000015) according to the manufacturer's instructions. All cells used were between passage 9 and 13 and all cell lines in use in our laboratory are subject to a quarterly mycoplasma testing regime (Sapphire Bioscience, B39032).

Mosaic transgenesis

Plasmids were delivered to recently laid embryos by microinjection of approximately 4nl into the yolk at 15ug/ml without transposase RNA. Embryos were sorted for transgenesis prior to imaging.

Constructs and cloning

New ORFs were generated from cDNA and first cloned into pGemTeasy (Promega). Primers were designed using primer3[43] to the boundary between the ORF and the UTR. Expression plasmids were constructed using the Tol2 kit[38]. New entry clones were made by PCR subcloning from existing plasmids using primers containing gateway compatible sites. The multisite gateway system is designed so as to be able to stitch together three cassettes in the correct order and orientation within a backbone. In order to streamline the production of large numbers of constructs using many of the same cassettes, we used a strategy in which we were able to perform the gateway reaction in reverse. That is, to take one of our final expression clones and replace either the middle cassette, 3' cassette, or both cassettes with the *ccdb* gene. This has the advantage that reactions are able to be performed with a smaller number of component vectors increasing the frequency of correct recombination, and that the largest and

most difficult cassettes to recombine (such as promoters) do not need to be recombined in every reaction. In order to do this, we set up gateway reactions with standard volumes and vector concentrations, but only two components; the final expression clone, and the appropriate entry clone containing the ccdb/chloramphenicol cassette. In order to perform the LR reaction in reverse, we used BP clonase, transformed into *ccdb* resistant cells, and spread onto plates containing both ampicillin and chloramphicol. In order to substitute both middle and 3' cassettes at the same time, used our new entry vector pDONR_p1-p3 (synthesized by Genscript, U.S.A.). All new constructs have been made available from Addgene and are listed in Supplementary Table 1.

Image processing

In order to avoid bias and ensure reproducibility, we developed a suite of custom macros for ImageJ which we were able to use for the majority of our image processing tasks (Table 2). Full code for all macros is given in Supplementary Macros. For all analysis, a minimum of six cells from a minimum of two fish were imaged per condition. For all data sets, we first used Macro 1 to produce merged .tif files from the native Zeiss .lsm or .czi formats that could be quickly browsed. Where single images are shown, a representative image was selected and then the original confocal file was opened in ImageJ, and the raw 16-bit data from each channel was copied and pasted directly into the RGB channels of Adobe Photoshop. Color-blind compatible images were made by duplicating the red channel into the blue to produce magenta. Brightness and contrast were adjusted as necessary.

Fibre morphometrics from tiled transmission electron microscope images were captured by manual tracing into regions of interest (ROIs) using Macro 2. This macro prevents ROIs from overlapping in the case of fibres by trimming the edges of new ROIs to abut existing ROIs. Myofibrils are trimmed to be within existing fibres, and are automatically named after the

parent fibre. Similarly myofibrillar furrow ROIs are trimmed to fall within myofibril ROIs and are named after the parent ROI. Data can then be quickly extracted from the ROI set using the standard measuring tools.

Analysis of perturbation in T-tubule intensity by amplitude was performed in a semi-automated manner. *In vivo* samples and images can be heterogeneous in intensity and quality. However, our mosaic analysis of single over-expressing fibres in an otherwise wildtype provided a robust an internal control, and allowed us to compare domains within individual images, before comparing between images. First, Macro 3 was used to generate .tif images from the original data with enhanced brightness and contrast, and any saturated pixels highlighted. Next, Macro 4 was used to generate sets of "box ROIs" covering arrays of eight T-tubules from adjacent expressing and non-expressing cells. This part of the analysis could not be automated, since the selection of equivalent areas through the midline of each cell was required. Next, Macro 5 was used to extract the amplitude data from the original, raw confocal files using the ROI sets. Similarly, localization of markers to the T-tubule domain was achieved using Macros 6 and 7 to define each localization domain, and Macro 8 to extract the intensity data using the ROI sets. Macros 5 and 8, apply a median filter with a pixel radius of 1 to the raw images before data extraction, to prevent potential noise generated by pixel spikes.

ROI sets were able to be exported to vector based .svg files using the python script "Export ROIs in RoiManager to a SVG file"
from the "Python + ImageJ, Fiji Cookbook" at the BioImage Analysis Wiki http://wiki.cmci.info/start

Transmission electron microscopy

Zebrafish were anesthetized with tricaine (Sigma) in E3 and subsequently fixed with 2.5% glutaraldehyde in a Pelco BioWave microwave. The head and tail-tips of the zebrafish embryos were removed and the remaining trunk was re-fixed in 2.5% glutaraldehyde in the microwave. Zebrafish were then processed as described previously[44]. Ultrathin sections were cut on a Leica UC6 ultramicrotome and imaged at 80kV on a JEOL-1011 transmission electron microscope fitted with an Morada 2k x 2k CCD camera under the control of Olympus software.

Large area tiled transmission electron microscopy

Large-area imaging was performed on a Tecnai T12 G2 (FEI) transmission electron microscope at 120kV fitted with a Direct Electron LC-1100 4k x 4k CCD camera using the Navigator program in SerialEM (Mastronade). Images were captured with a pixel size of 8nm and montages were aligned using the blendmont program in IMOD[45].

Serial blockface scanning electron microscopy

Samples for serial blockface sectioning scanning electron microscopy were processed as for transmission electron microscopy. Imaging was performed on a Zeiss Gemini FE-SEM fitted with a Gatan 3view. Image stacks were aligned using the xfalign program in IMOD and automated segmentation was performed by density-based thresholding in the Isosurface render program in IMOD.

Focussed ion beam electron microscopy

Following glutaraldehyde fixation and washing samples were placed in 2% osmium tetroxide in 0.1M cacodylate buffer and incubated in a Pelco Biowave at 80W for 6 min under vacuum in a 2min on, 2min off, 2min on, sequence. The osmium solution was removed without rinsing and a solution of 2.5% potassium ferricyanide was added and placed into the Pelco BioWave

for a further 6 minutes, at 80W, under vacuum on a 2min on, 2min off 2min on, sequence. Samples were then removed and washed in distilled water 6 times for 2mins. Samples were further incubated in an aqueous filtered solution of 1% thiocarbohydrazide, (6 min under vacuum) in a 2min off 2min on sequence. After rinsing in distilled water in a 6 x 2 min sequence, samples were placed in an aqueous 2% osmium tetroxide for 6 min under vacuum in a 2min on, 2min off, 2min on, sequence. After rinsing in distilled water in a 6 x 2 min sequence, samples were placed in 1% uranyl acetate solution and again irradiated at 80W for 3min. Samples were removed and washed in distilled water in a 6 x 2 min sequence, and dehydrated through an ethanol series (30%, 50%, 70%, 90% and 100% ethanol for 40s at 250W) in the Pelco BioWave, prior to embedding in Durcupan resin. Samples were imaged on an FEI Scios FIB Dual Beam SEM.

Statistical Analyses

Linear, second and third degree polynomial regression equations were generated using the LINEST function in Microsoft excel. Two-tailed T-tests on perturbation data (Fig. 8 and Supplementary Fig. 8) were performed using the T.TEST function. One-way ANOVA (Fig. 4k)and the one-tailed T-test (Fig. 4g) was carried out in GraphPad Prism.

CRISPR/Cas9

Guide RNAs were designed using CRISPRscan software[46]. To generate vectors for sgRNA production we modified the pT7-gRNA vector[47] to include a ccdb cassette vector (pT7-gRNA-ccdb) for negative selection to improve cloning efficiency, which in hands, was low with the original. sgRNA sequences were incorporated by one-step oligo annealing and ligation using BsmBI as described[47]. DNA templates were generated from these plasmids using a 100µl PCR (98° for 5s followed by 35 cycles of 98° for 5s, 55° for 5s, 72° for 20s) and the generic primers

sgRNA_F1 (5'-gcagctggcacgacaggtttcc-3') sgRNA_R1 (5'-aaaaaaaaagcaccgactcggtgccactttttcaagtt-3'). sgRNAs were generated from the purified amplicons using the Ambion MegaShortScript kit (quarter size reactions). sgRNAs were complexed with EnGen Spy Cas9 NLS protein (New England Biolabs M0646T) for 5 minutes at 37°C before injection of approximately 4nl into the yolk at the one cell stage. Gene-specific sgRNAs were co-injected with an sgRNA targeting tyrosinase[47], to allow selection of founders possessing the highest cutting frequencies. The molar ratio of Cas9 to total sgRNA was 1:1.5. Founders were outcrossed and F1 animals were genotyped by sequencing to identify null mutations and mutant lines were maintained as heterozygotes. sgRNA sequences used were tyr_80[47] (5'-GGACTGGAGGACTTCTGGGG-3'), smyhc1_977 (5'-CAGTGGCAATCAACTCTTCGG-3') and ttn.1_C3[48] (5'-TACTGGGGAATTCAGTGAAGG-3').

Knockout fish lines

The slow myosin heavy chain mutant generated in this study contains a 2bp deletion in the slow myosin heavy chain open reading frame (AACGTCAGTGGCAATCAACT[ct]TCGGCATCATCAATCGATGC). The titin mutant *ttn.1* generated in this study contains a 2bp deletion/7bp insertion in the titin open reading frame (TGGGTACTGGGGAATTCAGT[agtatgg]AGGCAGTATGGAAACTGCAC)

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Figure 1

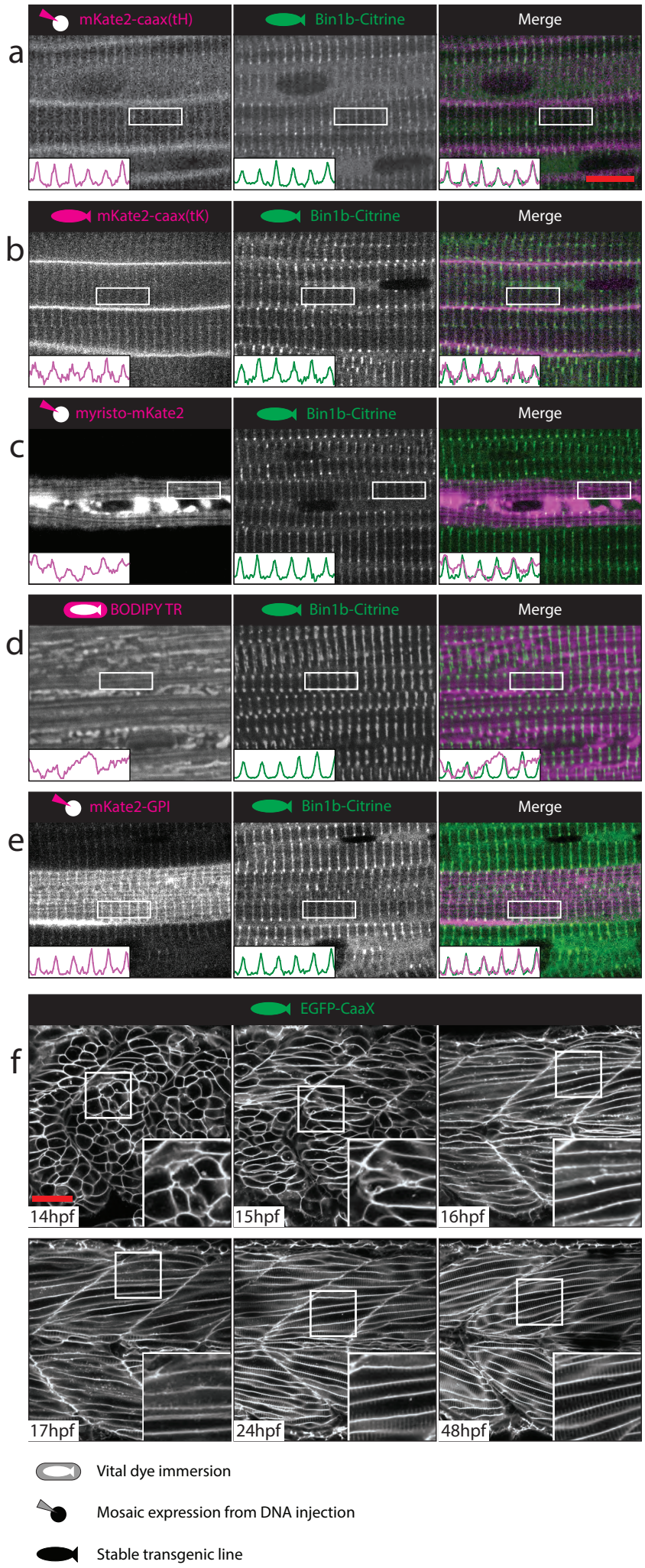
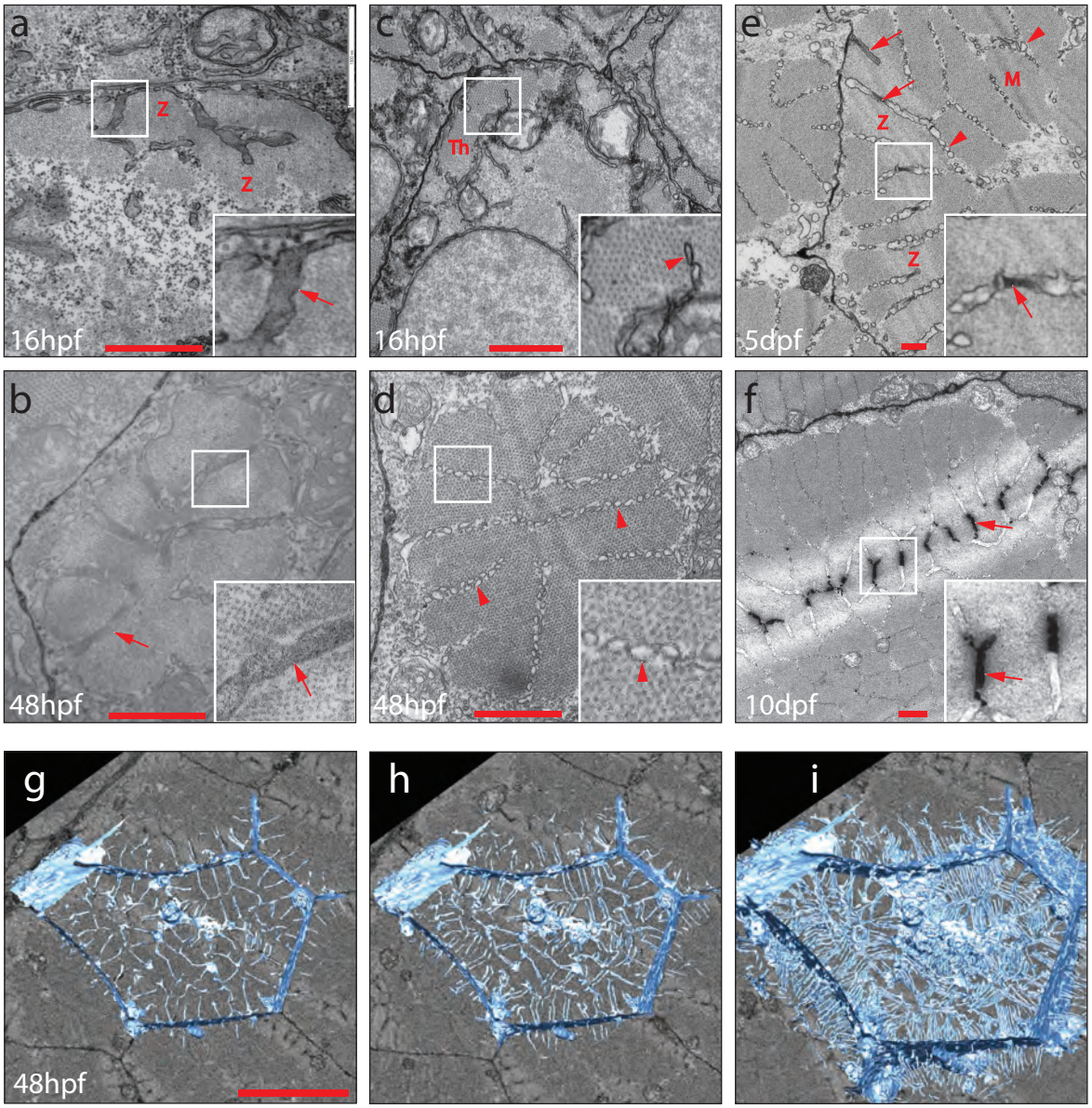
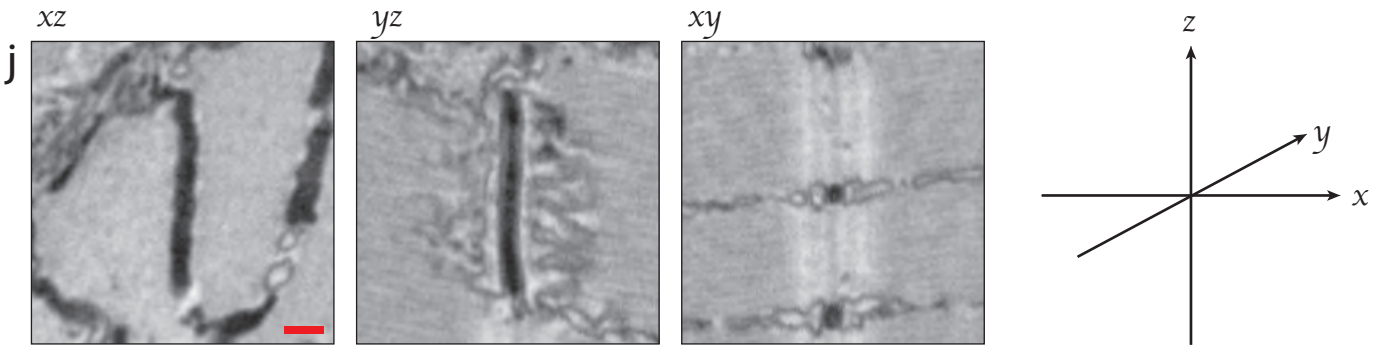

a
mKate2-caax(tH)
Bin1b-Citrine
Merge
b
mKate2-caax(tK)
Bin1b-Citrine
Merge
c
myristo-mKate2
Bin1b-Citrine
Merge
d
BODIPY TR
Bin1b-Citrine
Merge
e
mKate2-GPI
Bin1b-Citrine
Merge
f
EGFP-CaaX
14hpf
15hpf
16hpf
17hpf
24hpf
48hpf
Vital dye immersion
Mosaic expression from DNA injection
Stable transgenic line

Figure 2


a
Z
Z
16hpf
c
Th
16hpf
e
M
Z
Z
5dpf
b
48hpf
d
48hpf
f
10dpf
g
48hpf
h
i


j
xz
yz
xy
z
y
x

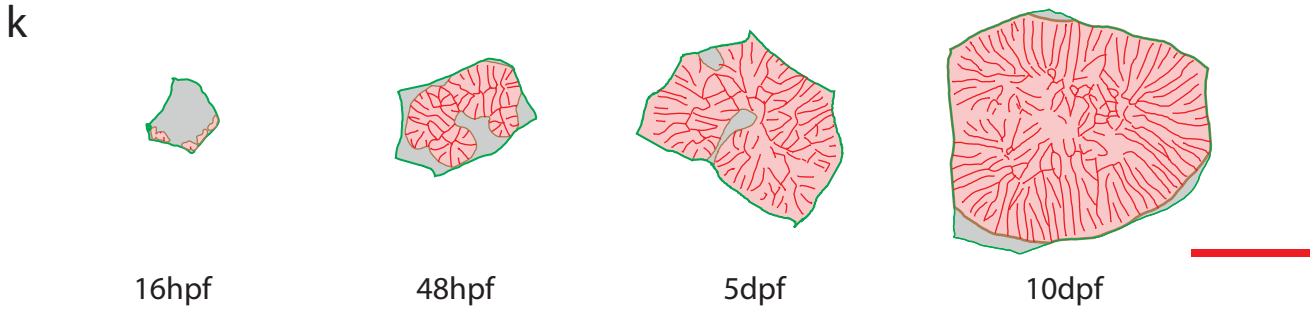

k
16hpf
48hpf
5dpf
10dpf

Figure 3

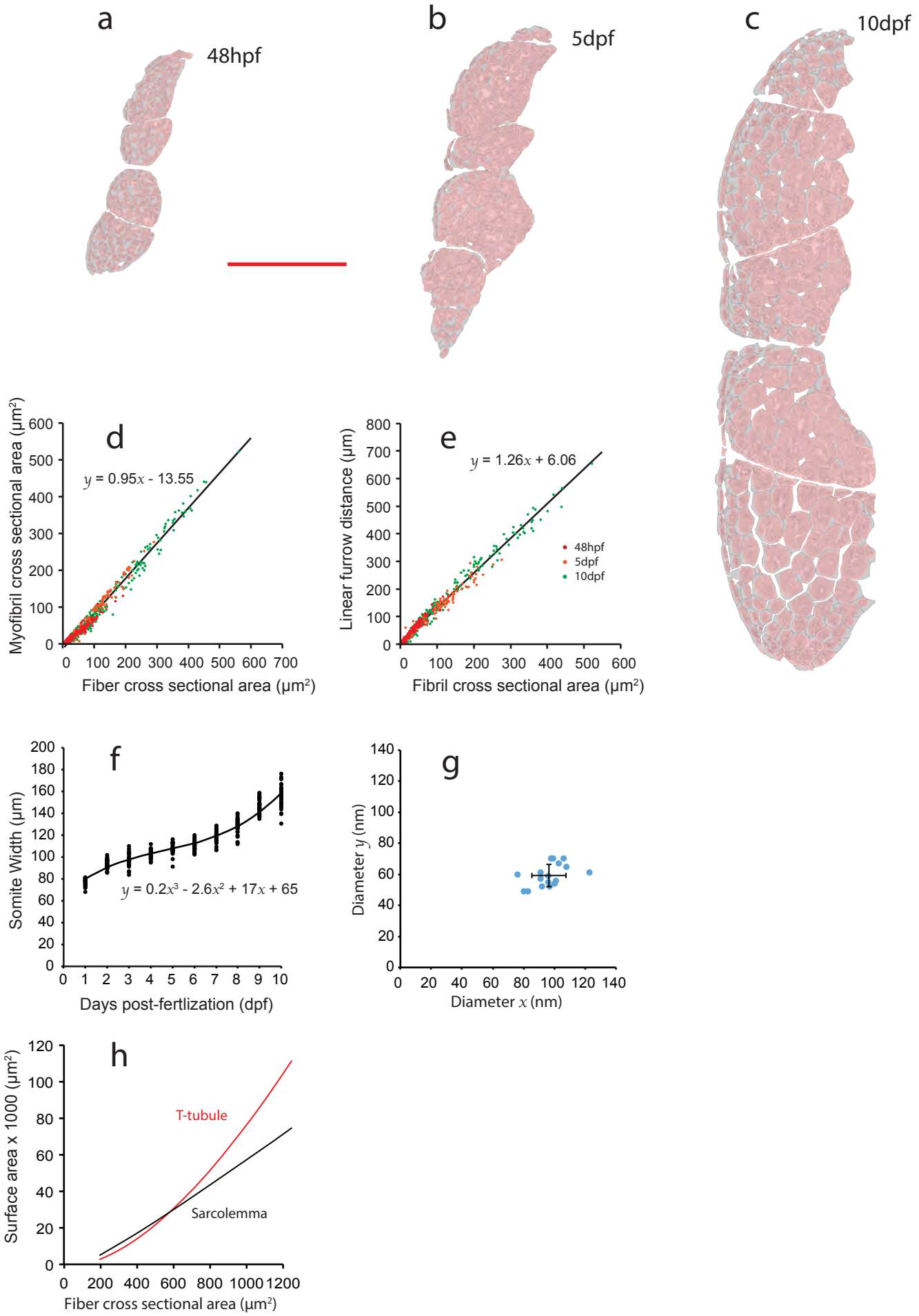
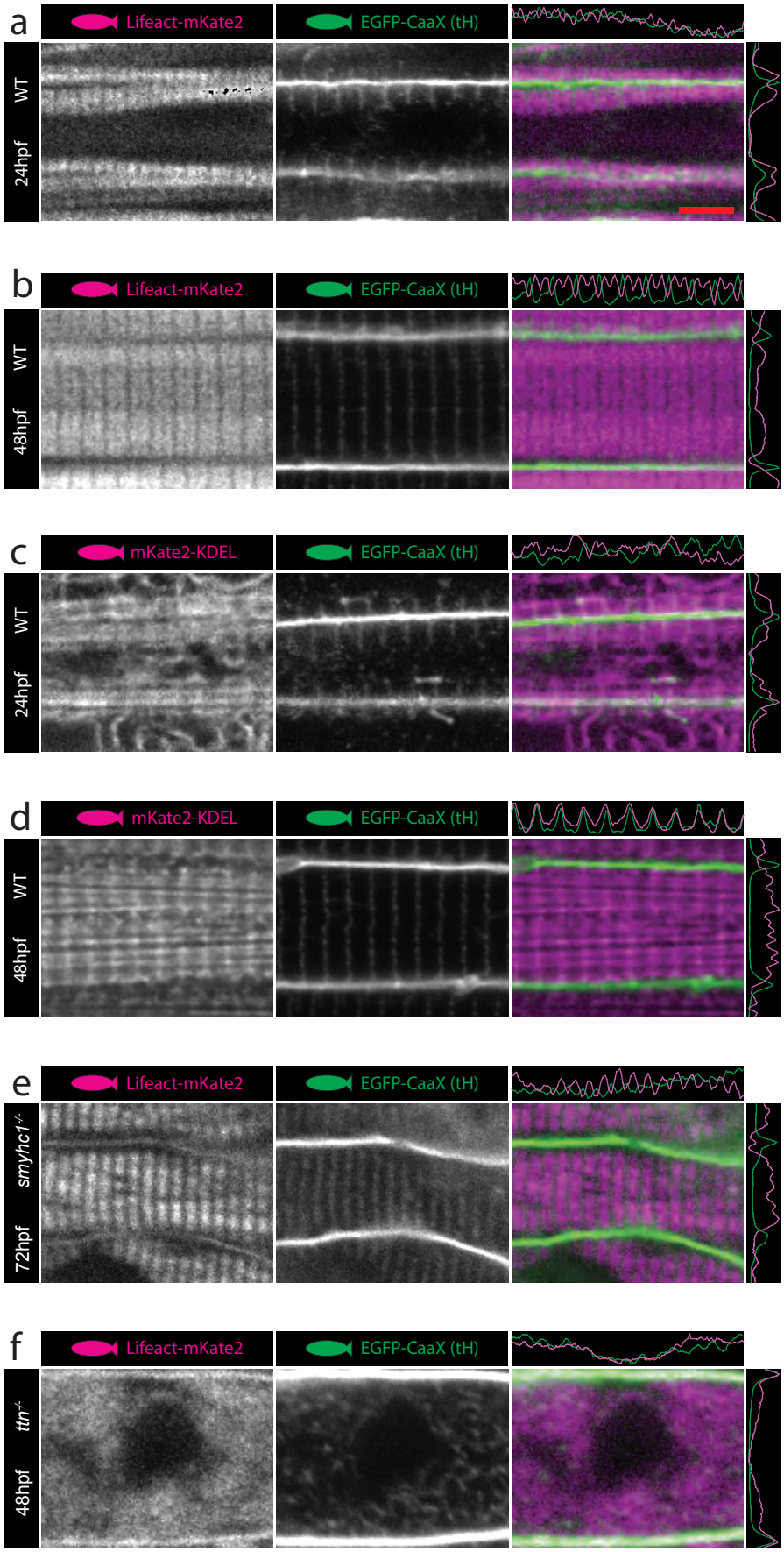
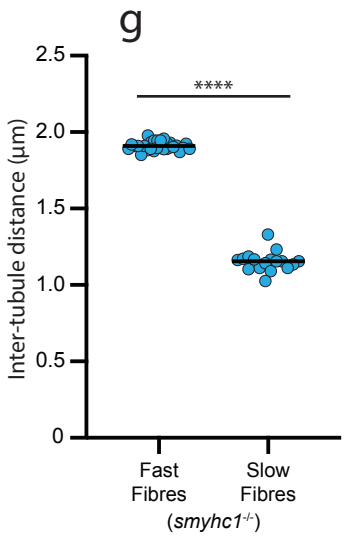
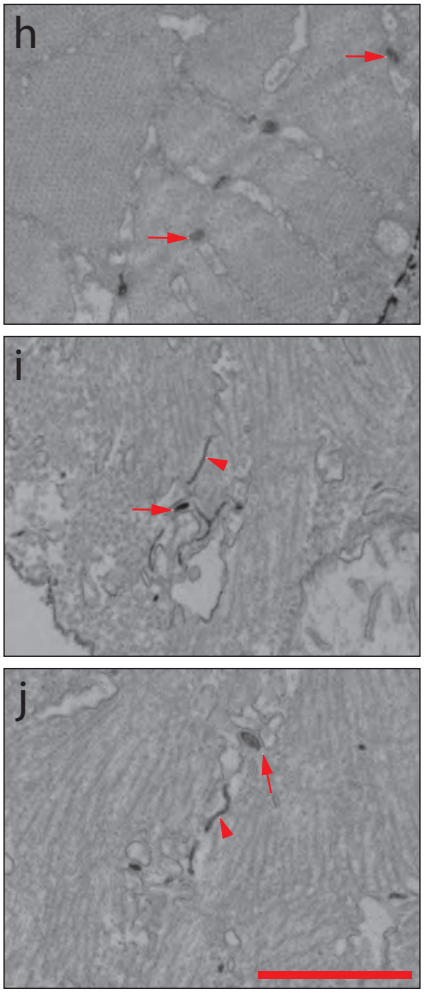

a
48hpf
b
5dpf
c
10dpf
d
y = 0.95x - 13.55
Myofibril cross sectional area (µm²)
Fiber cross sectional area (µm²)
e
y = 1.26x + 6.06
Linear furrow distance (µm)
Fibril cross sectional area (µm²)
48hpf
5dpf
10dpf
f
y = 0.2x³ - 2.6x² + 17x + 65
Somite Width (µm)
Days post-fertlization (dpf)
g
Diameter y (nm)
Diameter x (nm)
h
T-tubule
Sarcolemma
Surface area x 1000 (µm²)
Fiber cross sectional area (µm²)

Figure 4


a
Lifeact-mKate2
EGFP-CaaX (tH)
WT
24hpf
b
Lifeact-mKate2
EGFP-CaaX (tH)
WT
48hpf
c
mKate2-KDEL
EGFP-CaaX (tH)
WT
24hpf
d
mKate2-KDEL
EGFP-CaaX (tH)
WT
48hpf
e
Lifeact-mKate2
EGFP-CaaX (tH)
smyhc1-/-
72hpf
f
Lifeact-mKate2
EGFP-CaaX (tH)
ttn-/-
48hpf
Mosaic expression from DNA injection
Stable transgenic line


g
Inter-tubule distance (µm)
2.5
2.0
1.5
1.0
0.5
0
****
Fast Fibres
Slow Fibres
(smyhc1-/-)


h
i
j

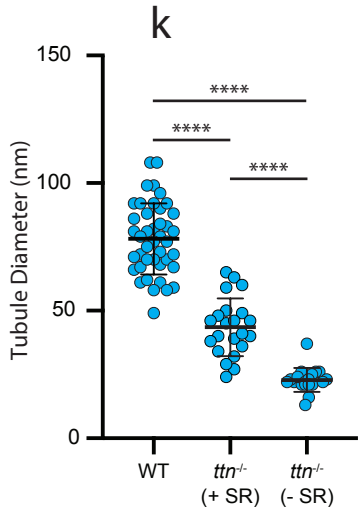

k
Tubule Diameter (nm)
150
100
50
0
****
****
****
WT
ttn-/- (+ SR)
ttn-/- (- SR)

Figure 5

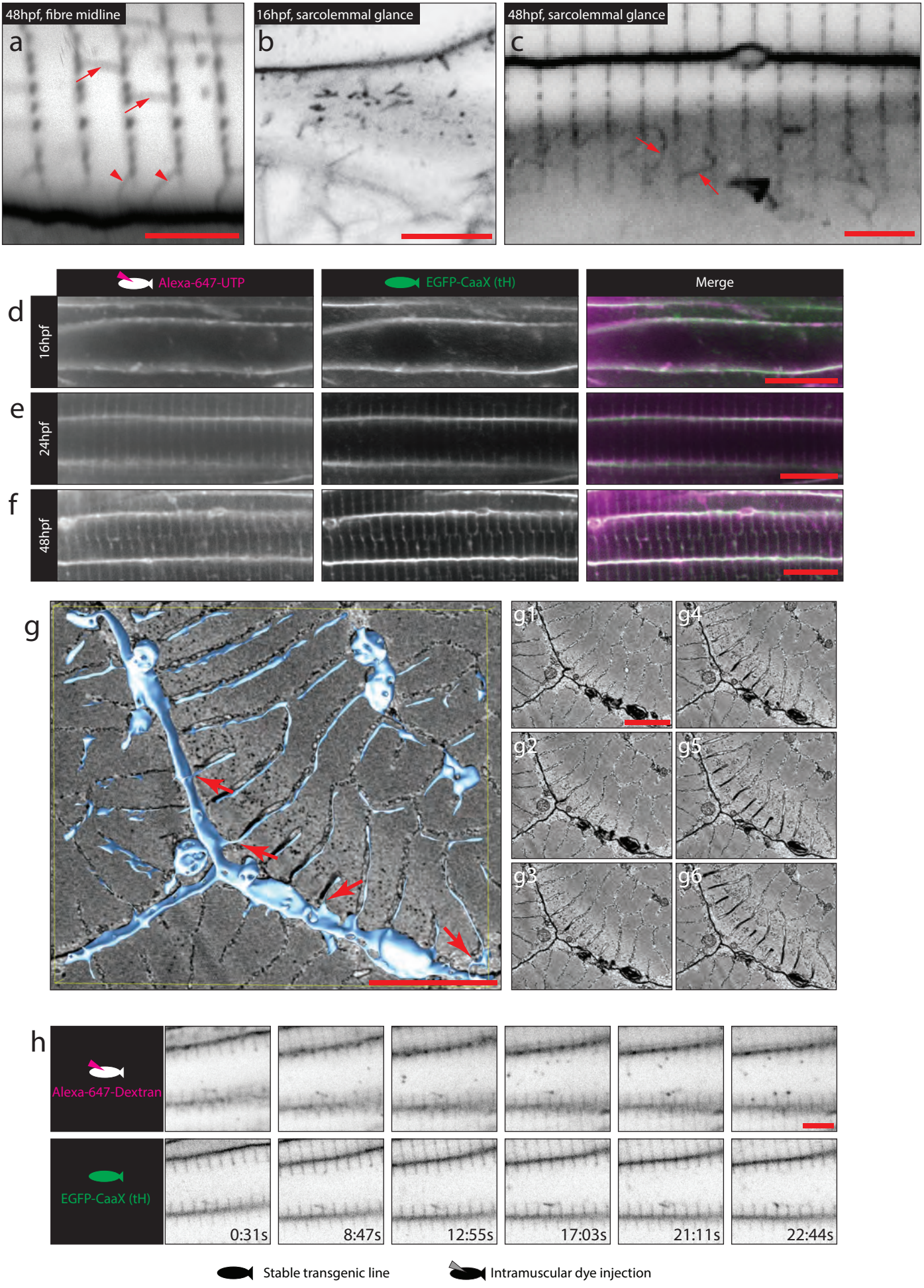
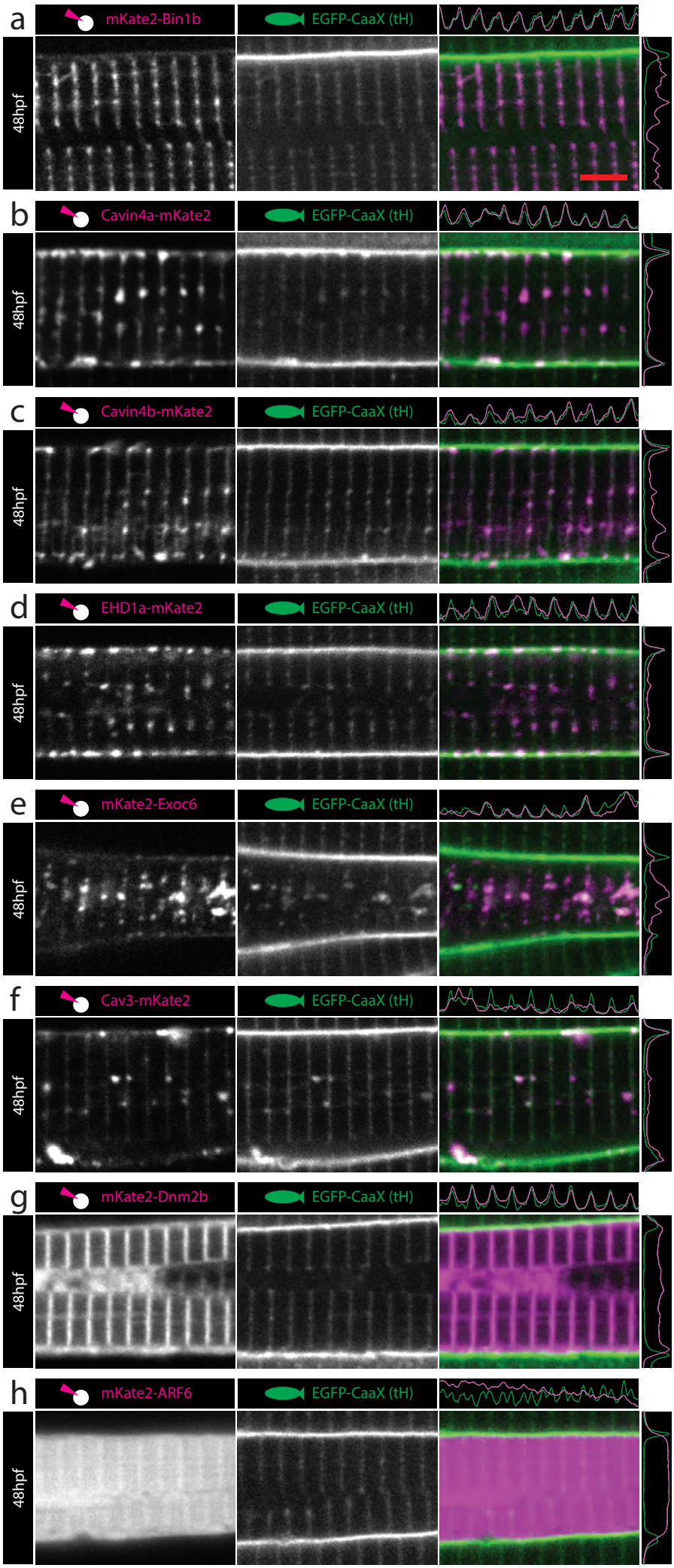
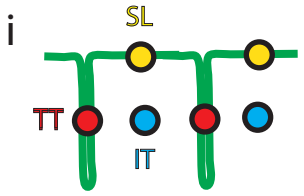

48hpf, fibre midline
a
16hpf, sarcolemmal glance
b
48hpf, sarcolemmal glance
c
Alexa-647-UTP
EGFP-CaaX (tH)
Merge
d
16hpf
e
24hpf
f
48hpf
g
g1
g2
g3
g4
g5
g6
h
Alexa-647-Dextran
EGFP-CaaX (tH)
0:31s
8:47s
12:55s
17:03s
21:11s
22:44s
Stable transgenic line
Intramuscular dye injection

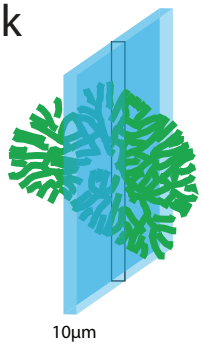
Figure 6

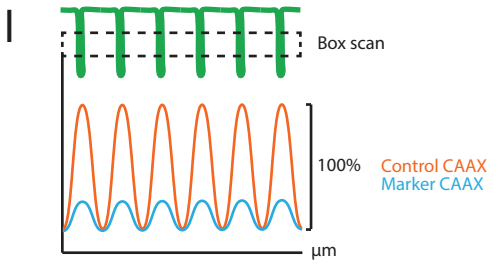


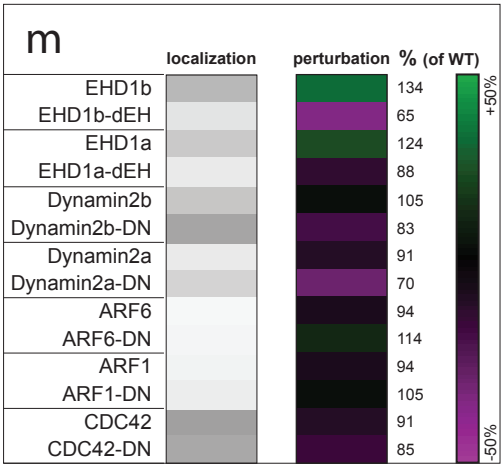


j

	CAAX SL	CAAX TT	CAAX IT	MARKER SL	MARKER TT	MARKER IT	Ratio TT:IT
Bin1b	68	20	12	29	54	16	3.28
Cavin4a	73	17	10	53	34	13	2.62
Cavin4b	73	18	9	35	46	19	2.41
EHD1a	74	17	9	55	31	14	2.31
Exoc6 (Sec15)	73	16	11	32	46	22	2.11
Caveolin3	69	18	13	47	35	19	1.82
Dynamin2b	70	19	11	34	43	24	1.81
CD44b	77	15	8	60	26	15	1.74
Dysferlin	76	15	9	67	21	12	1.66
EHD1b	62	23	15	54	28	18	1.6
CaveolinY	55	25	20	52	30	19	1.59
EHD2b	70	17	13	38	38	24	1.59
Myotubularin	76	16	8	55	27	18	1.54
Pacsin2	75	16	9	76	14	10	1.47
CDC42	61	22	17	58	25	17	1.45
Clathrin b	70	19	12	60	23	17	1.34
Exoc7 (Exo70)	63	21	16	43	32	25	1.31
Bin1a	72	18	10	43	32	25	1.24
Dynamin2a	70	18	11	46	29	25	1.15
EHD2a	78	13	9	74	14	12	1.12
ARF1	66	21	13	27	38	35	1.1
Clathrin a	69	19	12	37	33	30	1.08
ARF6	69	18	13	25	38	37	1.04
Exoc1 (Sec3)	69	19	12	37	31	32	0.96



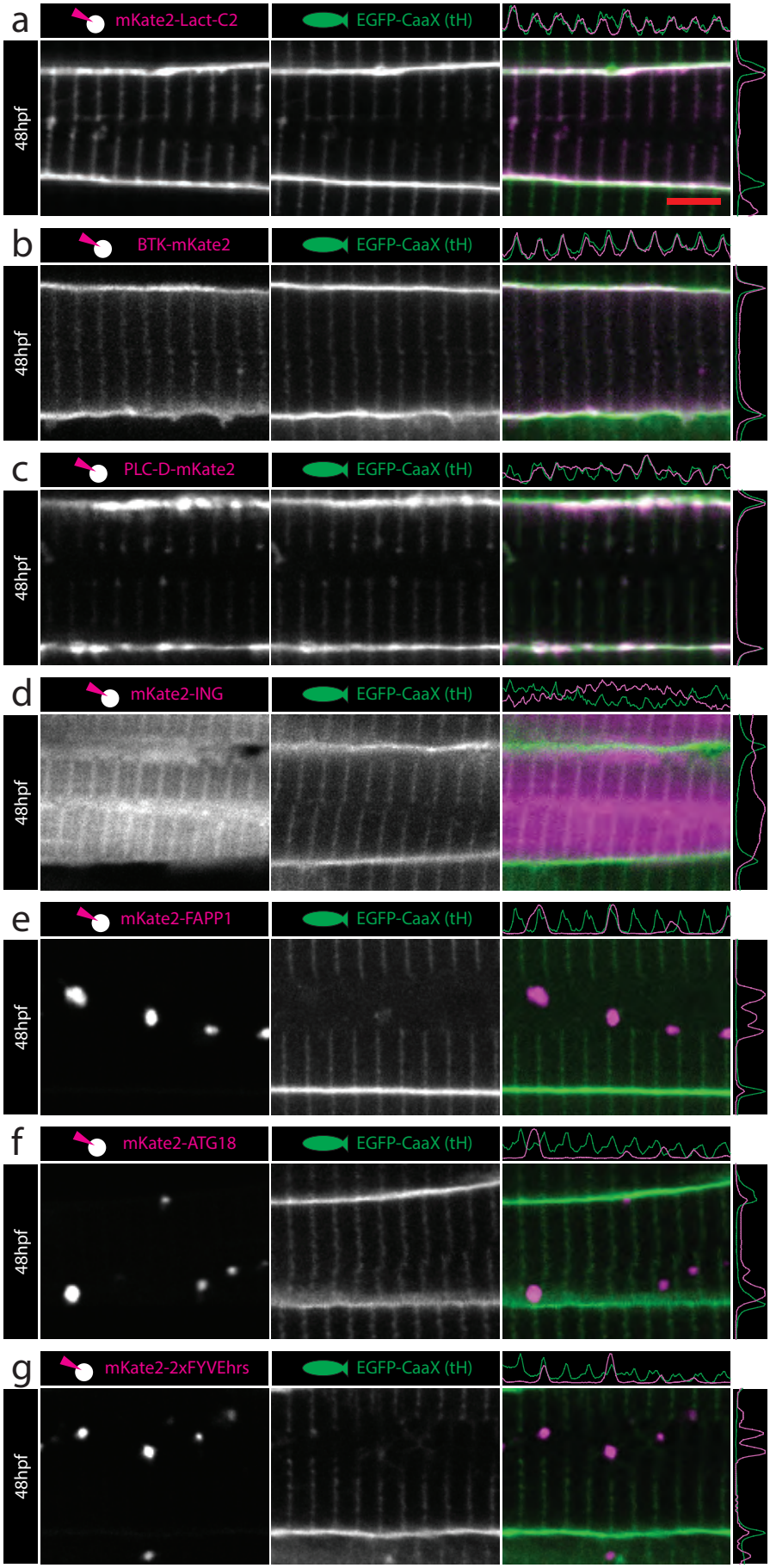




Mosaic expression from DNA injection

Stable transgenic line

Figure 7



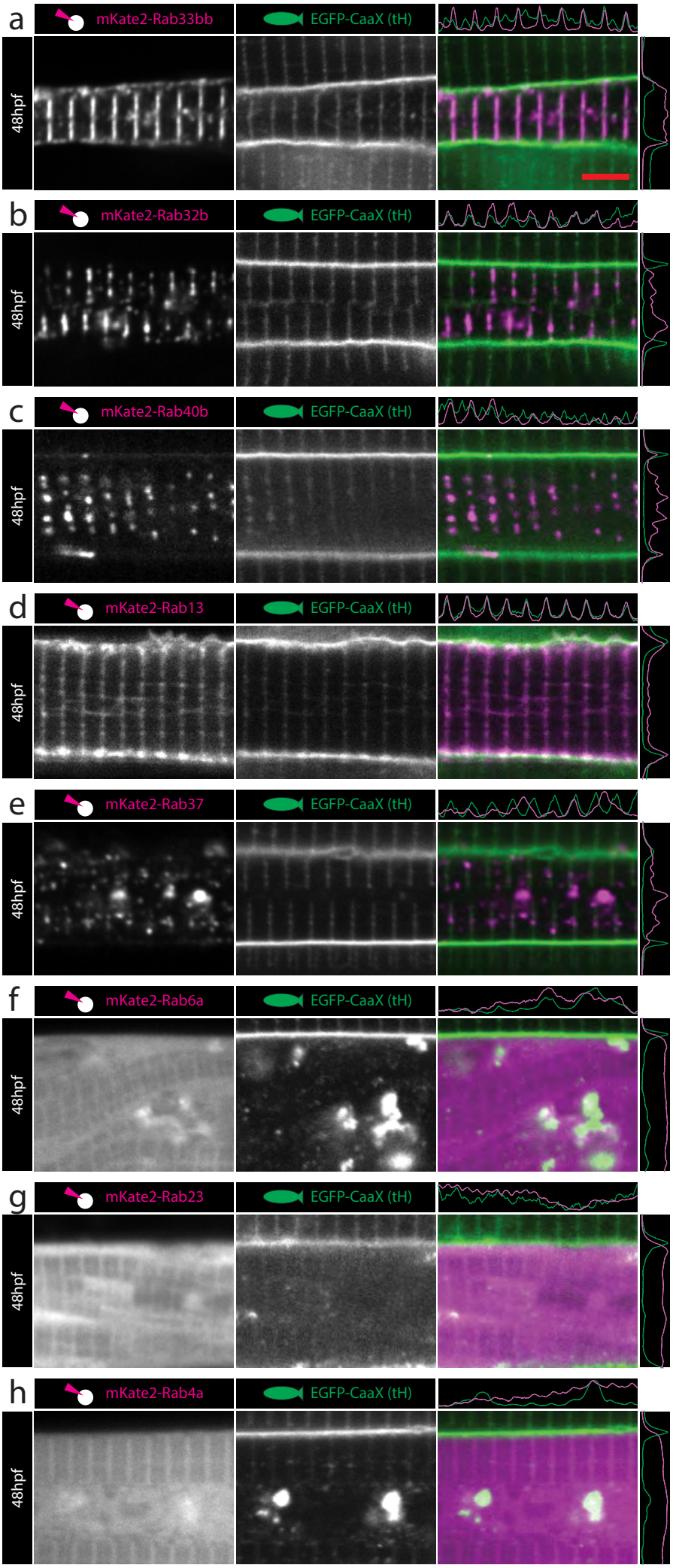
h

	CAAX			MARKER			Ratio
	SL	TT	IT	SL	TT	IT	TT : IT
LactC2 (PS)	66	20	15	62	25	13	1.92
BTK (PtdIns[3,4,5]P_3)	71	18	11	64	21	14	1.5
PLC-D (PtdIns[4,5]P_2)	70	17	12	66	20	14	1.43
INGdomain (PtsIns[5]P)	72	18	10	33	36	32	1.13
FAPP1 (PtsIns[4]P)	69	18	13	51	25	24	1.04

Mosaic expression from DNA injection

Stable transgenic line

Figure 8


a
mKate2-Rab33bb
EGFP-CaaX (tH)
48hpf
b
mKate2-Rab32b
EGFP-CaaX (tH)
48hpf
c
mKate2-Rab40b
EGFP-CaaX (tH)
48hpf
d
mKate2-Rab13
EGFP-CaaX (tH)
48hpf
e
mKate2-Rab37
EGFP-CaaX (tH)
48hpf
f
mKate2-Rab6a
EGFP-CaaX (tH)
48hpf
g
mKate2-Rab23
EGFP-CaaX (tH)
48hpf
h
mKate2-Rab4a
EGFP-CaaX (tH)
48hpf

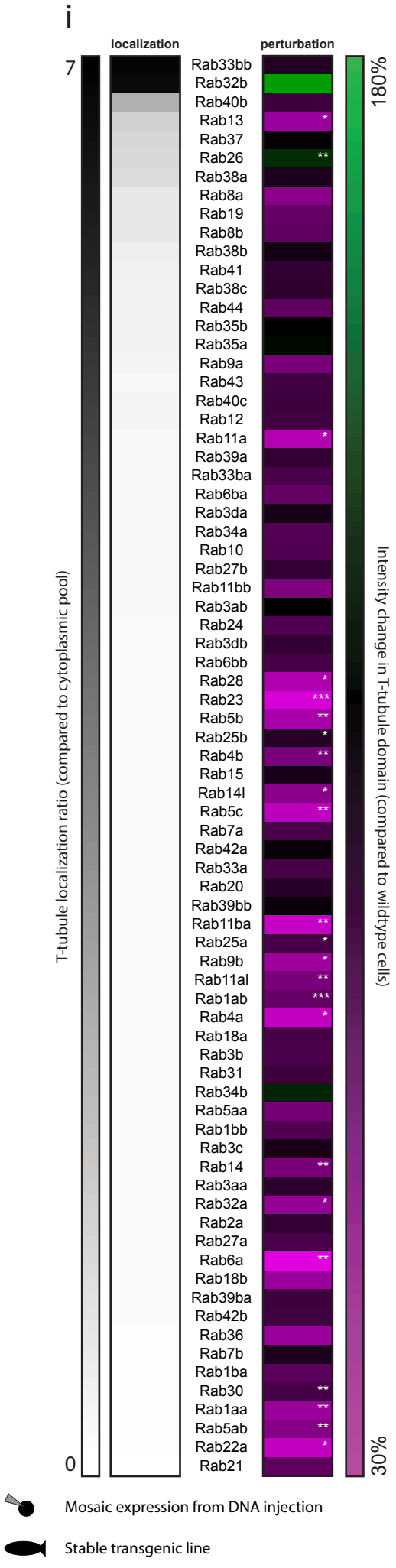
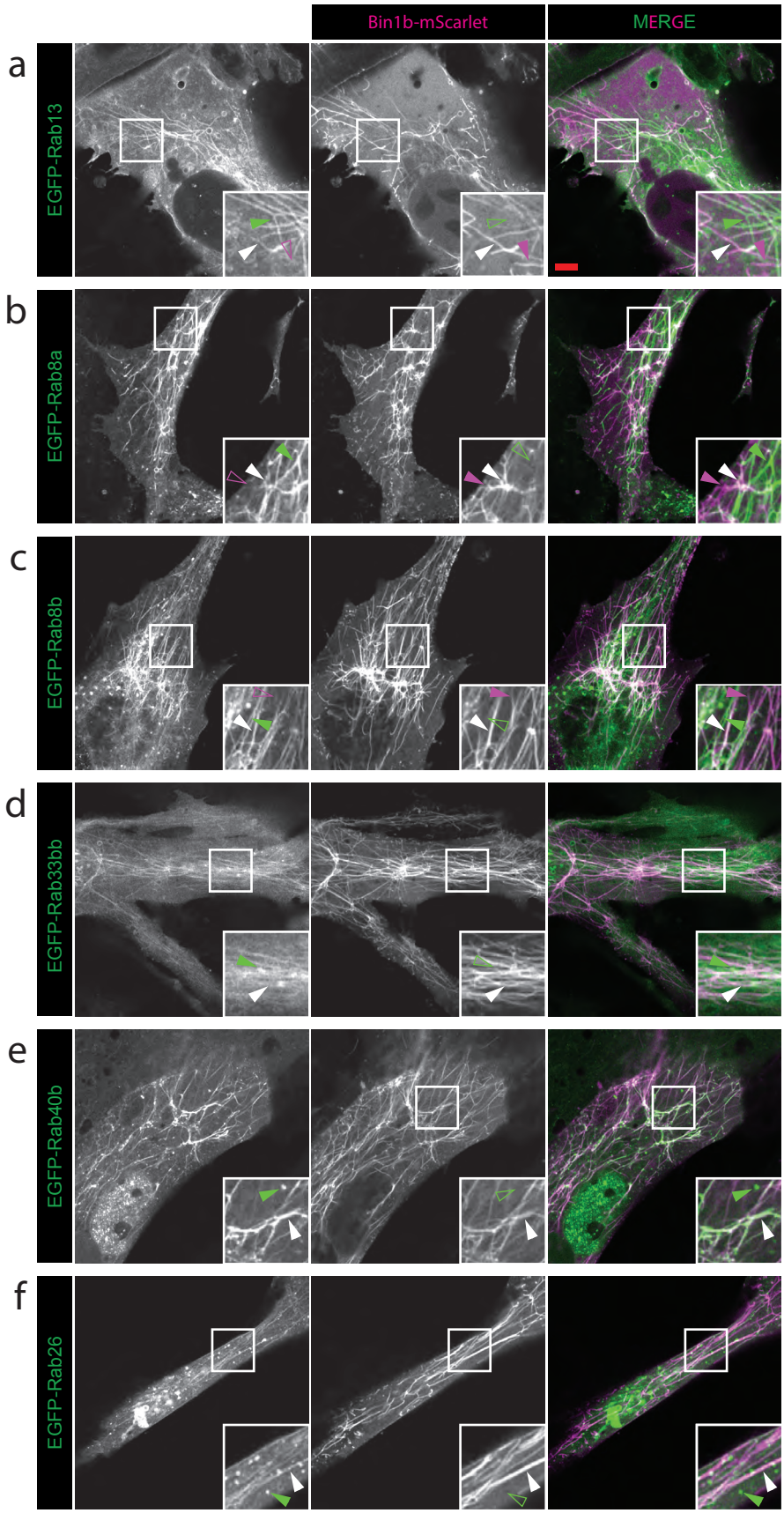

i
localization
perturbation
7
0
T-tubule localization ratio (compared to cytoplasmic pool)
Rab33bb
Rab32b
Rab40b
Rab13 *
Rab37
Rab26 **
Rab38a
Rab8a
Rab19
Rab8b
Rab38b
Rab41
Rab38c
Rab44
Rab35b
Rab35a
Rab9a
Rab43
Rab40c
Rab12
Rab11a *
Rab39a
Rab33ba
Rab6ba
Rab3da
Rab34a
Rab10
Rab27b
Rab11bb
Rab3ab
Rab24
Rab3db
Rab6bb
Rab28 *
Rab23 ***
Rab5b **
Rab25b *
Rab4b **
Rab15
Rab14l *
Rab5c **
Rab7a
Rab42a
Rab33a
Rab20
Rab39bb
Rab11ba **
Rab25a *
Rab9b *
Rab11al **
Rab1ab ***
Rab4a *
Rab18a
Rab3b
Rab31
Rab34b
Rab5aa
Rab1bb
Rab3c
Rab14 **
Rab3aa
Rab32a *
Rab2a
Rab27a
Rab6a **
Rab18b
Rab39ba
Rab42b
Rab36
Rab7b
Rab1ba
Rab30 **
Rab1aa **
Rab5ab **
Rab22a *
Rab21
180%
30%
Intensity change in T-tubule domain (compared to wildtype cells)
Mosaic expression from DNA injection
Stable transgenic line

Figure 9


Bin1b-mScarlet
MERGE
a
EGFP-Rab13
b
EGFP-Rab8a
c
EGFP-Rab8b
d
EGFP-Rab33bb
e
EGFP-Rab40b
f
EGFP-Rab26

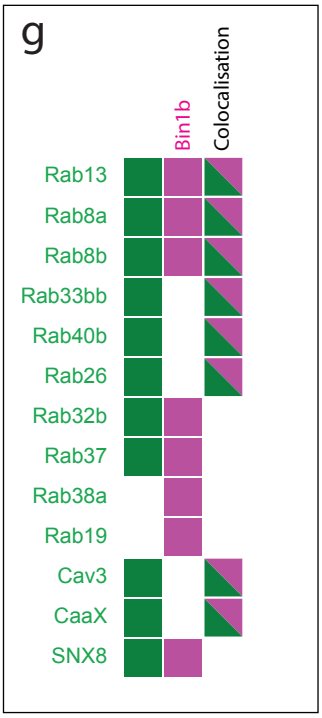

g
Bin1b
Colocalisation
Rab13
Rab8a
Rab8b
Rab33bb
Rab40b
Rab26
Rab32b
Rab37
Rab38a
Rab19
Cav3
CaaX
SNX8

Table 1. Transgenic zebrafish lines used in this study

Vector name	**Reference**	**Allele number**
bact2-mKate2-KDEL	This study	uq11rp
bact2-Lifeact-mKate2	This study	uq12rp
acta1-EGFP-CaaX (Hras)	Williams et al 2011	pc10
acta1-mCherry-CaaX (Hras)	Berger et al 2014	pc22
acta1-Lifeact-EGFP	Berger et al 2014	pc21

Table 2. Software and algorithms used in this study

Macro or Algorithm	**Reference**
GEN_Split_to_tif_CZI_AND_LSM.ijm	This study
GEN_Morphometrics.ijm	This study
GEN_Split_to_green_and_blue_highlight_saturated.ijm	This study
TT_GET_box_ROIs_for_amplitude_comparison.ijm	This study
TT_perturbation_process.ijm	This study
TT_GET_circle_ROIs_for_localisation_comparison.ijm	This study
GEN_Put_circle.ijm	This study
TT_localisation_comparison_process.ijm	This study
ROI to .svg (Python script)	BioImage Analysis Wiki