**Mutations in repeating structural motifs of tropomyosin cause gain of function in skeletal muscle myopathy patients.**

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ABSTRACT

The congenital myopathies include a wide spectrum of clinically, histologically and genetically variable neuromuscular disorders many of which are cause by mutations in genes for sarcomeric proteins. Some congenital myopathy patients have a hypercontractile phenotype. Recent functional studies demonstrated that *ACTA1* K326N and *TPM2* ΔK7 mutations were associated with hypercontractility that could be explained by increased myofibrillar $Ca^{2+}$-sensitivity. A recent structure of the complex of actin and tropomyosin in the relaxed state showed that both these mutations are located in the actin-tropomyosin interface. Tropomyosin is an elongated molecule with a 7-fold repeated motif of around 40 amino acids corresponding to the 7 actin monomers it interacts with. Actin binds to tropomyosin electrostatically at two points, through Asp25 and through a cluster of amino acids that includes Lys326, mutated in the gain of function mutation. Asp25 interacts with tropomyosin K6, next to K7 that was mutated in the other gain of function mutation. We identified 4 tropomyosin motifs interacting with Asp25 (K6-K7, K48-K49, R90-R91, and R167-K168) and three E-E/D-K/R motifs interacting with Lys326 (E139, E181 and E218) and we predicted that the known skeletal myopathy mutations ΔK7, ΔK49, R91G, ΔE139, K168E and E181K would cause a gain of function. Tests by *in vitro* motility assay confirmed that these mutations increased $Ca^{2+}$-sensitivity, whilst mutations not in these motifs (R167H, R244G) decreased $Ca^{2+}$-sensitivity. The work reported here explains the molecular mechanism for 6 out of 49 known disease-causing mutations in the *TPM2* and *TPM3* genes, derived from structural data of the actin-tropomyosin interface.

INTRODUCTION

The congenital myopathies include a wide spectrum of clinically, histologically and genetically variable neuromuscular disorders, many of them caused by mutations in genes for sarcomeric proteins (1). These myopathies are generally defined on the basis of muscle weakness and histological abnormalities in the muscle fibres. Nemaline myopathy, characterized by nemaline (rod) bodies on muscle biopsy, is the most widely studied but congenital myopathy can also be associated with cap-like structures located under the sarcolemma (Cap disease) or with fibre-type disproportion (CFTD). Mutations in the skeletal muscle α-actin gene (*ACTA1*) account for about 20% of congenital myopathies and over 200 different mutations have been identified. Recently, disease-causing mutations have also been found in β- and γ- tropomyosins encoded by *TPM2* and *TPM3* genes respectively; 27 mutations have been reported in the *TPM2* gene and 22 mutations have been reported in the *TPM3* gene (1-14) see supplementary figures.

A small proportion of congenital myopathies are associated with a hypercontractile phenotype; this is a very heterogeneous disease category that includes distal arthrogryposis, trismus-pseudocamptodactyly syndrome and Escobar syndrome and some of these cases are reported to be due to mutations in *ACTA1*, *TPM2* or *TPM3* (12- 16). These apparent "gain of function" mutations are particularly interesting because of their parallels with hypertrophic cardiomyopathy that also presents as a hypercontractile phenotype and is associated with mutations in sarcomeric proteins including actin and tropomyosin (*ACTC* and *TPM1* genes (17)). Enhanced contractility indicates that the abnormality caused by the mutation is likely to be within the force-producing contractile machinery, whereas a loss of function could be due to defects in force production, force transmission, force sensing or sarcomere assembly.

Tropomyosin, together with actin and troponin, constitutes the basic thin filament structural and $Ca^{2+}$-regulatory machinery that interacts with myosin when muscle contracts. Tropomyosin forms a 40 nm long parallel coiled-coil dimer and is able to polymerise head-to-tail with other tropomyosin molecules into long strands spanning the whole thin filament length. Each tropomyosin molecule binds to 7 different actin monomers along the helical actin chain involving 7 quasi-repeats of about 40 amino acids each. The key function of tropomyosin is in cooperatively switching the location of the actin-tropomyosin interface between active and relaxed states under the control of troponin, $Ca^{2+}$ and myosin heads.

Previously, we studied the effect of actin and tropomyosin mutations on $Ca^{2+}$ regulation of muscle contractility at the single filament level in order to establish a genotype-phenotype relationship. In most biopsies from patients with *ACTA1* mutations it was not possible to establish any mechanism (18-20) but two mutations are of particular note, since they showed distinctive abnormalities in their regulatory interaction with tropomyosin. The *ACTA1* D292V mutation resulted in an actin with normal activity that was irreversibly switched off by tropomyosin (19), whilst the *ACTA1* K326N mutation from a patient with stiff muscles, was indistinguishable from wild-type actin on its own but, when incorporated into thin filaments, showed an increase in $Ca^{2+}$-sensitivity and crossbridge turnover rate consistent with the hypercontractile phenotype (21).

Since tropomyosin has a relatively simple structure and interacts only with itself, actin, tropomodulin and troponin, we expected that it would be easier to make a molecular explanation of the disease phenotype than for actin mutations. Most of the tropomyosin mutations reported to date are linked to different skeletal muscle myopathies characterized by generalised muscle weakness and, at the molecular level, a reduced cross-bridge cycling rate and $Ca^{2+}$-sensitivity. Examples of these mutations on β-tropomyosin are E41K and E117K linked to nemaline myopathy or cap disease (11, 22).

Some tropomyosin mutations have been associated with arthrogryposis and a gain of function (15). *TPM2* R91G was the first investigated at the molecular level and this showed a hypercontractile phenotype (23). Recently we have investigated in detail a common β-tropomyosin mutation, ΔK7 that has been described in a total of 9 families (13, 24). Histological inspection of biopsies taken from patients of all families showed nemaline bodies as a common feature. Most of the patients were therefore diagnosed with nemaline myopathy, although some were independently diagnosed with core-rod myopathy or distal arthrogryposis VII. Lysine 7 is placed in a region of crucial importance for tropomyosin, as it participates in the head-to-tail polymerisation of tropomyosin (25), is close to a residue binding to actin (26) and may also be involved in the binding of troponin T (27). Mokbel *et al.* showed that the mutation strongly impairs the ability of the protein to incorporate in the sarcomere and causes its accumulation in the nemaline bodies in transfected C2C cells. Nevertheless, the expressed mutant is incorporated into muscle thin filaments and it acts as a poison peptide *in vivo.* Studies with chemically skinned muscle from patient biopsies and isolated thin filaments using *in vitro* motility assay showed that this mutation increases the $Ca^{2+}$-sensitivity, the cross-bridge turnover rate and maximum force, producing a gain-of-function. In retrospect it

was noted that all the patients with this mutation presented a hypercontractile phenotype in childhood. Distal arthrogryposis, exhibited by some of the patients, is characterised by hypercontraction and most of the ΔK7 patients did not present evident myopathic symptoms until their teens, proving that their weak muscles were not congenital.

Recently a high resolution structure of α-tropomyosin bound to actin has been proposed, from a study involving electron microscopy and computational chemistry (26), that highlights the electrostatic interactions holding tropomyosin on F-actin. In this structure (corresponding to the Closed-state, roughly equivalent to the 'off' functional state(28)) tropomyosin blocks myosin's strong binding in accord with the well known observation that skeletal muscle tropomyosin on its own inhibits actin-activation of myosin ATPase. This structure provides the framework for understanding how the mutation could cause the gain of function.

In this manuscript we have examined the location of tropomyosin and actin mutations in the new structure of the actin-tropomyosin interface. The K7 residue is positioned just on the side of the K6 residue, which binds to Asp25 on the actin molecule, therefore we can propose that the mutation ΔK7 destabilises this inhibitory interaction.

Moreover, since tropomyosin is a modular protein, these binding motifs are repeated in some of the other actin-binding repeats, thus the structure gives us a unique opportunity to predict where other mutations might cause a similar phenotype. We found that four out of the seven quasi-repeats, present in all tropomyosin isoforms, share the feature of a second basic residue downstream of the basic residue binding to Asp25: K6-K7, K48-K49, R90-R91, R167-K168. It is interesting to note that, mutations linked to skeletal muscle myopathies have been reported in all four downstream basic amino acids: ΔK7, ΔK49 and R91G on β-tropomyosin and K168E on γ-tropomyosin and thus we predict they will cause a gain of function like ΔK7. A second actin-tropomyosin interface involves Lys326 of actin, the amino acids mutated in the 'stiff' patient and, interestingly, mutations have been reported in the acidic amino acids of tropomyosin (ΔE139, E181K, ΔE218) that interact with actin K326 so we predict these would lead to the same effect as the K326N gain of function mutation. In this study we have tested the effect of tropomyosin mutations on the actomyosin interaction by measuring $Ca^{2+}$-regulation of skeletal muscle thin filaments containing skeletal myopathy-causing tropomyosin mutations and have confirmed the molecular phenotype predicted.

RESULTS

***Structural analysis of actin-tropomyosin interface predicts gain of function mutations in tropomyosin***

Li *et al.* determined the structure of α-tropomyosin bound to actin, nevertheless tropomyosin isoforms are highly conserved and in fact the sequences around the actin contacts studied here are identical in the α-, β-, and γ-isoforms (see data supplement). The Li et al structure shows that tropomyosin is not closely bound to actin and makes electrostatic contact at just two points on every actin; at D25 and with a cluster of basic residues, K326, K328 and R147. This pattern is illustrated in Figure 1. The amino acids in tropomyosin that interact with actin are mapped in Figure 1B in which the sequence is divided into the seven actin-binding repeats proposed by McLachlan and Stewart (36), based on analysis of the amino acid sequence. They predicted that the alpha band sequences bound to actin when actin-tropomyosin was in the relaxed state. It will be seen that the basic amino acids interacting with D25 are found at or near the beginning of the alpha bands and the acidic amino acids interacting with the K326, K328, R147 cluster occur at two places near the end of the alpha band. In the latter case, the first site, 12 to17 amino acids into the alpha band showed interactions in every period of tropomyosin, whilst the second site, 16 to 20 amino acids into the alpha band showed interaction in only 6 of the 7 periods.

*Tropomyosin interaction with residue Aspartic acid 25 of actin*

We noted that the gain of function mutation ΔK7 was one amino acid down stream from K6 that interacts with actin D25 as described above. Figure 2A shows the interface at high resolution. Our initial hypothesis was that disruption of one of a cluster of basic amino acids (K5, K6, K7) would perturb the actin-tropomyosin interface, destabilising this structure but not have such a large effect that the mutation would be lethal. This structure corresponds to a functionally relaxed state of muscle, since it is well established that skeletal muscle tropomyosin inhibits actin activated ATPase and this position of tropomyosin relative to actin is present with troponin in the absence of myosin heads (37). Consequently destabilising the structure would shift the equilibrium towards the active state of actin-tropomyosin, thus accounting for the gain of function observed with the ΔK7 mutation.

The motif observed with K6 and K7 is repeated in four of the periods of tropomyosin: K6-K7, K48-K49, R90-R91, and R167-K168 and the structures of the interface for the four motifs is very similar (Fig 2 A-D). The other three periods have a single basic amino acid that interacts with D25 (see Figure 1). If our hypothesis about the mechanism of the gain of function due to the ΔK7 mutation is correct, we would expect charge change mutations at K49, R91 and K168 to also cause a gain of function. In fact the mutation R91G in period three of TPM2 is known to cause a hypercontractile phenotype, distal arthrogryposis and an *in vitro* investigation of the effect of this mutation did indeed find that it cause enhanced actomyosin ATPase and incomplete relaxation, although $Ca^{2+}$-sensitivity was not very different from wild-type (23) (Figure 2C). A survey of the published mutations in *TPM2* and *TPM3* reveals that mutations ΔK49 (period 2) and K168E (period 5) have been identified in single patients (Figure 2B, 2D) (7, 38), but not associated with a hypercontractile phenotype whereas no mutations have been reported in the equivalent positions of periods 4, 6, and 7. Mutations at the amino acid directly interacting with D25 were reported in skeletal myopathy patients: K128E in TPM2 and R90C, R167H and R244G in TPM3 and we would predict that these would have a different, perhaps opposite, effect on muscle function. In order to test our hypothesis, we have determined the effect of these mutations on $Ca^{2+}$-regulation of muscle thin filaments.

*Tropomyosin interaction with actin residues 326,328 and 147*

This cluster of amino acids on the surface of actin forms a second interface presenting basic amino acids to tropomyosin (Figure 3) with at least one of the three residues involved in interaction in every period of tropomyosin (Figure 1) . The actin (*ACTA1*) K326N mutation has been found in a baby suffering from a hypercontractile phenotype characterized by generalised stiffness (21). In that study, *in vitro* motility assay studies showed that the mutation was sufficient to generate an increased $Ca^{2+}$-sensitivity that explains the hypercontractile phenotype. We proposed that the loss of positive charge in the K326N mutant actin destabilized the inhibitory interaction with tropomyosin in the same way as the ΔK7 *TPM2* mutation. Consequently we would expect charge change mutations in the cognate amino acids of tropomyosin would have a similar effect. K326 binds to tropomyosin in 5 tropomyosin periods and one of these involves the residue E181 (Fig 1B). The mutation E181K in *TPM2* was reported in two cases of distal arthrogryposis and this mutation caused an increase in $Ca^{2+}$-sensitivity of force production in skinned fibres from the patient (12, 39). Figure 4 shows the interface between actin K326 and tropomyosin E181 as defined by the Li

et al structure. Thus destabilization of this interaction by loss of charge at either the actin or the tropomyosin residue produces the same increase in $Ca^{2+}$-sensitivity. There is an EEK (or EDK or EER) motif in tropomyosin homologous to the Actin K326-Tm 181 interface in just three of the seven periods (See Fig 1). We examined published myopathy mutations in these motifs in the other periods of the tropomyosin sequence and found ΔE139 and ΔE218 in *TPM2* (Figure 4) (8, 10). It is remarkable that there are three such motifs and three reported mutations whilst none have been reported at the equivalent position in other periods that do not have this motif. To test the predictions we investigated whether these mutations also caused a gain of function *in vitro.*

*Actin D292 mutation*

The mutation *ACTA1* D292V was found in a patient with weak muscles and congenital fibre-type disproportion (19, 40). *In vitro* studies of actin purified from this patient's skeletal muscle showed a very strong molecular phenotype. The patient sample contained about 45% mutant actin and with actin alone the interaction with myosin in the *in vitro* motility assay was the same as normal actin, however when tropomyosin was added, the filament motility appeared to be completely switched off. Li *et al'*s structure provides a possible reason for this effect. The acidic amino acid D292 is located on the surface of actin close to the K326,K328,R147 cluster (Figure 4); it does not participate in binding but is close to tropomyosin. It is possible that the acidic residue in this position may act to moderate the binding affinity of the K326, K328, R147 cluster so that the binding is not so tight that the transition of tropomyosin from OFF to ON state has a physiologically impossibly high energy barrier. If so, one could hypothesise that the loss of negative charge in the D292V mutation would strengthen binding and stabilise the off state. We have investigated this mutation further to test the hypothesis.

***Effect of mutations studied by in vitro motility assay***

*Tropomyosin mutations*

We expressed wild-type β-tropomyosin and the mutations ΔK49, R91G and ΔE139 and wild-type γ-tropomyosin and the mutations R167H, K168E and R244G in the baculovirus/*sf9* system that preserves native N-terminal acetylation. Thin filaments were reconstituted using the expressed tropomyosin with rabbit skeletal muscle actin and troponin and the regulation of their movement over immobilised HMM was studied in the *in vitro* motility assay. Thin filaments containing mutant tropomyosin species were fully functional in this assay. When

$Ca^{2+}$-regulation of mutant thin filaments was compared with thin filaments, containing the appropriate wild-type tropomyosin isoform, it was observed that all the predicted gain-of-function mutations produced a higher $Ca^{2+}$-sensitivity (curve shifted to the left for ΔK49, R91G, ΔE139 and K168E; Fig 5) and a slightly higher maximum sliding speed similar to the previously investigated gain of function mutations, ΔK7 and E181K (24, 39). In contrast the four skeletal muscle myopathy mutations that were expected to give a hypocontractile phenotype showed lower $Ca^{2+}$-sensitivity (Figure 5) and lower sliding speeds (β-tropomyosin E41K, E117K and γ-tropomyosin R167H and R245G; Table 1). This also corresponds to previously published data on E41K and R167H contraction in biopsy samples from Ochala's laboratory (22, 39). It is interesting to note that thin filaments containing 100% or 50% mutant tropomyosin gave similar alterations in thin filament function (Table 1).

*ACTA1 D292V mutation*

In a previous study, the *ACTA1* D292V mutation appeared to stabilise the inactive state of actin-tropomyosin. (19). We investigated the properties of the D292V mutation using actin extracted from a patient biopsy containing the mutation. Addition of tropomyosin caused the D292V actin filaments to stop moving, whilst it did not affect fraction motile and slightly increased the sliding speed of wild-type actin (Figure 7). In the presence of activating concentrations of $Ca^{2+}$, troponin did not re-activate the thin filaments containing D292V actin and tropomyosin. We then added N-ethylmaleimide-treated myosin subfragment 1 (NEM-S-1) to actin-tropomyosin; this modified S-1 forms rigor bonds with actin even in the presence of ATP, therefore a small quantity will cooperatively switch on thin filaments. However NEM-S-1 was incapable of switching on D292V actin- containing filaments at double the concentration that switched on native thin filaments in the absence of $Ca^{2+}$(41, 42). An actin mutation, E93K, that causes thin filaments to be inhibited by tropomyosin has been observed before, but this mutation was reactivated by either troponin or NEM-S-1 (41). Thus the D292V mutation seems to have induced a very profound change of the ON-OFF equilibrium towards the OFF state since neither of these treatments restored motility (Figure 7).

**DISCUSSION:**

*Structure of the actin-tropomyosin interface predicts gain of function mutations in skeletal muscle*

Our investigation of gain of function mutations was sparked by the observation that Lysine 326 of actin, mutated to asparagine in the muscles of the 'stiff' child, was in a critical position in the interface between actin and tropomyosin, according to the structure of Li *et al* (21, 26). The next observation was that, unexpectedly, the TPM2 ΔK7 mutation also caused a gain of function and that it was located near another critical part of the actin-tropomyosin interface close to Asp25 (24). The detailed information contained in Li *et al*'s structure combined with the repeating sequence of tropomyosin enabled us to identify a total of 4 similar motifs interacting with Asp25 and three motifs interacting with Lys326 and we anticipated that charge-change mutations at these sites would also cause a gain of function. Indeed, mutations at two of these sites, R91G and E181K have already been reported to cause distal arthrogryposis, a hypercontractile phenotype, and enhanced contractility, consistent with a gain of function phenotype (23, 39). In the current study, we have investigated the effect of the remaining predicted gain of function mutations and confirmed that they did indeed increase $Ca^{2+}$-sensitivity. The structure-function connection that we have observed is further supported by our observation that charge-change mutations at positions adjacent to the predicted gain-of-function sites (R167H and R244G) produced the opposite effect, namely a reduced $Ca^{2+}$-sensitivity.

The finding that the Li *et al* structure can accurately predict seven gain of function mutations in tropomyosin strongly supports the validity of actin-tropomyosin interface structure on which it is based.

*Implications for diagnosis of skeletal muscle disease- histopathology is an inappropriate criterion*

Skeletal muscle myopathies are historically defined by the histopathology of the muscle; however, the determination of the disease-causing mutations and the advent of functional studies suggest a different approach to diagnosis is needed(43).

No relationship between the conventional disease classification and the gain of function phenotype could be ascribed. Amongst the 7 mutations investigated, diagnoses include cap myopathy, nemaline myopathy, congenital fibre-type disproportion (CFTD), rod-core disease, distal arthrogryposis and trismus-pseudocamptodactyly In fact, the single mutation ΔK7 studied in 10 families included diagnoses of rod-core disease, CFTD and trismus-pseudocamptodactyly (13, 24). A similar range of diagnoses was given for the R167 mutations found in 14 families where nemaline myopathy, CFTD, cap disease and mixed

pathologies were reported (14).

Whilst muscle histology provides no clues about the basis of the myopathy, consideration of muscle contractility is more predictive, especially for the gain of function mutations. This aspect was thoroughly investigated for the ΔK7 mutation, where some patients were re-evaluated and re-diagnosed in the light of contractility measurements (24). Besides the overt hypercontractility seen in arthrogryposis (E181K, R91G, ΔK7) there was a tendency for the gain of function patients to have contractures in infancy and abnormal gait whilst major symptoms of muscle disorders were delayed; for instance the ΔE139 patient was first examined at 11 years for a muscular-skeletal disorder (10). A clearly hypocontractile phenotype from birth would rule out a gain of function type of mutation. These features may not be considered in most neuromuscular clinics: it will be noted that the patients with the three gain of function mutations investigated here (ΔK49, ΔE139 and K168E) were not diagnosed with a hypercontractile phenotype (10, 38, 44).

This situation somewhat parallels hypertrophic cardiomyopathy (HCM), which is also associated with increased $Ca^{2+}$-sensitivity, where symptoms frequently do not develop until an individuals's second and third decades. In both cases, an initially hypercontractile phenotype can trigger secondary muscle dysfunction leading to a hypocontractile phenotype mimicking the more common loss of function mutations as discussed by Mokbel et al. for the ΔK7 mutation (24). In HCM, enhanced $Ca^{2+}$-sensitivity is the primary defect that triggers hypertrophy but biopsies of interventricular septum from such patients invariably have a hypocontractile phenotype, indeed up to 30% of untreated patients subsequently develop symptoms of heart failure (17, 45)

*Implications for the structural analysis of tropomyosin regulation*

The control of striated muscle activity is mediated through $Ca^{2+}$-regulation of the thin filament and this has been shown to be due to a series of allosteric-cooperative transitions between activity states involving every protein component of the thin filament and myosin, largely defined by the position of tropomyosin on the surface of actin (49). Measurement of $Ca^{2+}$-sensitivity is therefore a very sensitive method to detect changes in equilibria between states. The Li *et al* structure of skeletal muscle tropomyosin on the surface of actin filaments indicates that, when freed of constraints imposed by troponin, myosin, or other actin-binding proteins, tropomyosin localizes to a favored position on F-actin defined primarily by electrostatic interactions that corresponds most closely to the blocked and closed states as

defined by Lehman et al. 2009 (26, 46). Recent energy landscape calculations confirm that tropomyosin is located in a shallow energy well (28) and that the "open" conformation is energetically unfavoured except in the presence of strong myosin binding to actin, as demonstrated in the recent determination of the actin-tropomyosin-S-1 structure (47).

It is logical to propose that the mutations in tropomyosin and actin that cause a gain of function by de-stabilising the actin-tropomyosin B/C-states and therefore shifting the equilibrium slightly in favour of the open state. The shift in equilibrium is detected as a higher $Ca^{2+}$-sensitivity since the change of activity state and $Ca^{2+}$ switching of the thin filament are linked equilibria (49). There is, in fact, direct evidence for this: firstly the affinity of tropomyosin for actin is reported to be reduced in the gain of function mutations ΔK7, ΔK49, R91G and ΔE139 (11, 23, 24) and secondly, energy landscape calculations of the effect of the actin K326N gain of function mutation actually show that the interaction energy (van der Waals + electrostatic) is reduced from 3286 to 2306 kcal/mol and the energetically most favoured position of tropomyosin is shifted in the direction of the open state (48).

A similar analysis may explain why the actin D292V mutation is so profoundly inhibitory; the location of negative charge relative to the three basic amino acids K326, K328 and R147 may be crucial in defining the shallow energy minimum of tropomyosin in the C state that is necessary to allow the regulatory transition to the other states with minimal energy expenditure. Preliminary energy landscape calculations indicate that actin-tropomyosin interaction energy is increased when D292 is mutated to valine from -3286 to -3442 kcal/mol based on the charge change alone. The mutation of the negatively charged amino acid to a hydrophobic amino acid could also allow a conformational change that makes the energy well deeper thus trapping tropomyosin in the C-state.

The information provided by these studies gives new insight about the structure-function properties at the actin-tropomyosin interface. One important implication of these results is the obligate requirement of charged residues in key positions to achieve normal regulation.

It is more difficult to explain why tropomyosin mutations causing a gain of function are located not in the amino acid that interacts directly with actin Asp25, but in the adjacent amino acid. There is no other pattern: two are deletions, one is a charge reversal and one is a charge loss. One might hypothesise that a positive charge-reducing mutation at the interaction site itself would have such a large effect as to be lethal and therefore not be seen

for that reason, however this argument fails since mutations of Arg167 to Cys, His or Gly are one of the commonest of skeletal myopathy-causing mutations, having been identified in 14 families.

Likewise there is no obvious explanation of why the gain of function mutations involved with interaction with Lys326 in actin are located at the amino acid in tropomyosin that interacts with actin.

On a more general point, these studies indicate that when the molecular structure and function of a protein-protein interface is well understood it is possible to make quite precise and testable predictions about the effect of disease-causing mutations, especially in a repeating structure like tropomyosin. The work reported here suggests a molecular mechanism for just 6 out of 49 known *TPM2* and *TPM3* mutations. It is interesting to note that none of the 26 mutations in *TPM1* identified as causes of cardiomyopathy are involved in the actin binding motifs described here (see supplementary data), indicating a different mechanism of modification of function for these phenotypes. The effect of mutations in tropomyosin and actin on the interface in the 'on' state as described by Behrman *et al.* (47) has not yet been investigated and the study of mutations in tropomyosin's interfaces with troponin and tropomodulin awaits further structural analysis. In the future such studies could define which other residues in the protein are key for optimal regulation and potentially highlight therapeutic targets or treatments.

## METHODS

### *Sources of contractile proteins*

Skeletal muscle myosin, heavy meromyosin, troponin and α-actin were prepared as described by Bing et al 1997. Tropomyosin was expressed in a baculovirus/*Sf9* system and purified with a protocol based on that of Akkari *et al*.(29) as described by Memo et al. (30). Age-matched normal and D292V human skeletal muscle actin was obtained from muscle biopsies as described by Clarke et al 2007 (19).

### *Quantitative in vitro motility assay*

Thin filaments were reconstituted with 10nM rabbit skeletal muscle α-actin (labelled with TRITC phalloidin) (31), tropomyosin (20-30nM) and troponin (10-30nM) to study $Ca^{2+}$-regulation of filament motility by the quantitative *in vitro* motility assay (32, 33). Thin filament movement over a bed of immobilised rabbit fast skeletal muscle heavy meromyosin (HMM) (100 μg/ml) was compared in paired motility cells in which troponin varied by a single factor (mutation or phosphorylation state). Filament movement was recorded and analysed as previously described (34), yielding two parameters, the fraction of filaments moving and the speed of moving filaments. In our motility system both these parameters are regulated by $Ca^{2+}$. The fraction motile changes from <0.1 to >0.8 in the range 1 nmol/L to 3.7 μmol/L free $Ca^{2+}$. The $Ca^{2+}$-dependent change of sliding speed was less and more variable (0-50% change), as previously noted Song 2010 (35).

The fraction motile and sliding speed was measured over a range of $Ca^{2+}$ concentrations to generate $Ca^{2+}$-activation curves. Each experiment involved the preparation of test and control troponins at the same time. To keep intra-experiment variability as low as possible, test and control thin filaments were reconstituted together and for each $Ca^{2+}$-concentration point they were pipetted into two channels of the dual chamber motility cell. Motility was then measured in each chamber within a couple of minutes of each other. The data was fitted to the 4-variable Hill equation to yield a value for $EC_{50}$. $EC_{50}$ values from replicate experiments were analysed by paired t-test since the distribution of $EC_{50}$ has been shown to be normal (32, 33). The absolute value of $EC_{50}$ was slightly variable between different troponin and myosin preparations, but the ratio of control to test was very consistent (Table 1).

*Molecular structures*

Structural analysis used a refined version of the structure of the actin tropomyosin interface published by Li et al. (26). PDB files describing the coordinates of 15 actins and one tropomyosin in this structure were rendered using MacPyMol. The pdb files are included in supplementary data.

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FIGURES

**Figure 1 Structure of the actin-tropomyosin interface**

A) Structure of one of the two tropomyosin molecules (cyan) bound to the actin double-helix (grey). Surface rendering using PyMol with coordinates from Li et al (2011). Actin Asp25 is coloured red and Lys 326 is coloured blue.

B) The β-tropomyosin sequence divided in the 7 quasi-repeating periods and α- and β-bands as defined by Mclachlan and Stewart (1976) . The purple circles highlight residues interacting with actin Asp25; the orange and red circles highlight the residues interacting with actin R147, K326 and K328 as defined by Li et al (2010). The β-tropomyosin (*TPM2*) mutations are indicated in blue boxes, the γ-tropomyosin (*TPM3*) mutations are in green boxes. The mutations increasing the $Ca^{2+}$-sensitivity are written in red, while those decreasing it are written in black.

**Figure 2 Details of the interactions of tropomyosin with actin Asp25**

Magnified views of the Li et al structure from Figure 2A show the locations of proposed gain of function mutations in tropomyosin relative to Actin Asp 25 for the first (K6,K7), second (K46,K47), third (R90,R91) and fifth (R167,K168) periods of tropomyosin.

**Figure 3 Location of actin residues Lys326, Lys328, Arg147 and Asp272**

Magnified views of the Li *et al* structure from Figure 2A. Left, actin alone is shown; right, tropomyosin is overlaid at 50% transparency. Amino acids are identified on the figure. The lower cluster is viewed face on whilst the upper cluster is rotated to the right.

**Figure 4 Details of the interactions of tropomyosin with Actin Lys326, Lys328 and Arg147**

Magnified views of the Li et al structure from Figure 2A show the locations of proposed gain of function mutations in tropomyosin relative to actin Lys326, Lys328 and Arg147 in the fourth (E139), fifth (E181) and sixth (E218,D219) periods.

**Figure 5 Comparison of $Ca^{2+}$ regulation of thin filaments containing wild-type and mutant tropomyosin.**

Thin filament motility was measured by *in vitro* motility assay over a range of $[Ca^{2+}]$ in paired cells. The fraction of filaments motile is plotted as a function of $[Ca^{2+}]$ for a typical

experiment. The points are the mean±SEM of four determinations of fraction motile measured in one motility cell. The curves are fits of the data to the Hill equation. Solid lines and points, wild-type thin filaments, dashed lines and open points, thin filaments with mutant tropomyosin. The mean values of $EC_{50}$ from replicate experiments is plotted in Figure 6 and summarised in Table 1.

**Figure 6 The effect of skeletal myopathy mutations in tropomyosin on thin filament $Ca^{2+}$-sensitivity.**

Data from this paper and published work is combined. Black, predicted gain of function mutations, white, predicted loss of function mutations.

Ratio of $EC_{50}$ of mutant thin filaments relative to $EC_{50}$ of wild-type troponin-tropomyosin is plotted. Error bars show SEM of up to 12 replicate comparative measurements (see table 1).

a - from Mokbel et al, 2013(24)

b – from Ochala et al 2012(39)

c – from Marttila et al 2012 (11)

d – from Ochala et al 2008 (22)

**Figure 7 The effect of the actin Asp292Val mutation on actin-tropomyosin interaction measured by the *in vitro* motility assay.**

Actin movement over immobilised HMM was recorded and analysed. Two parameters were calculated: fraction of filament motile and the sliding speed of motile filaments. Rhodamine-phalloidin labelled Actin concentration was 10nM. Solid lines: wild-type actin, dotted lines D292V actin.

A The effect of up to 30nM tropomyosin on wild-type and mutant actin motility

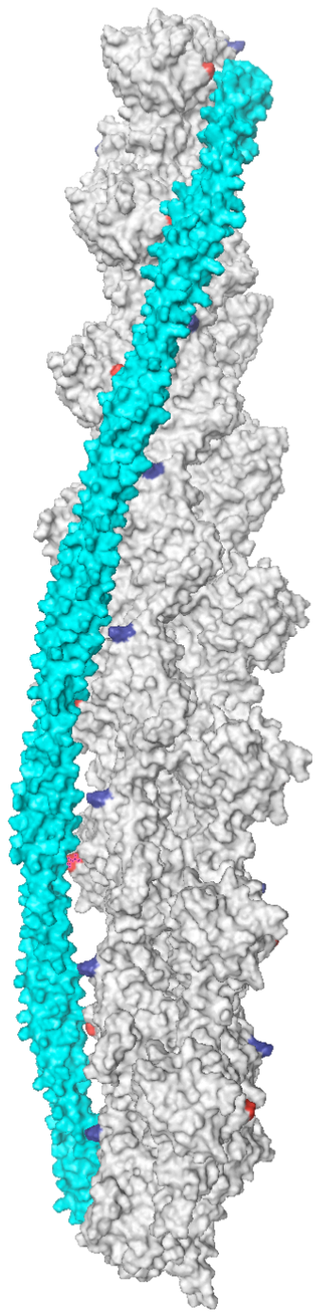
B The effect of adding 50nM troponin to actin-tropomyosin at $3.6x10^{-5}$ M $Ca^{2+}$

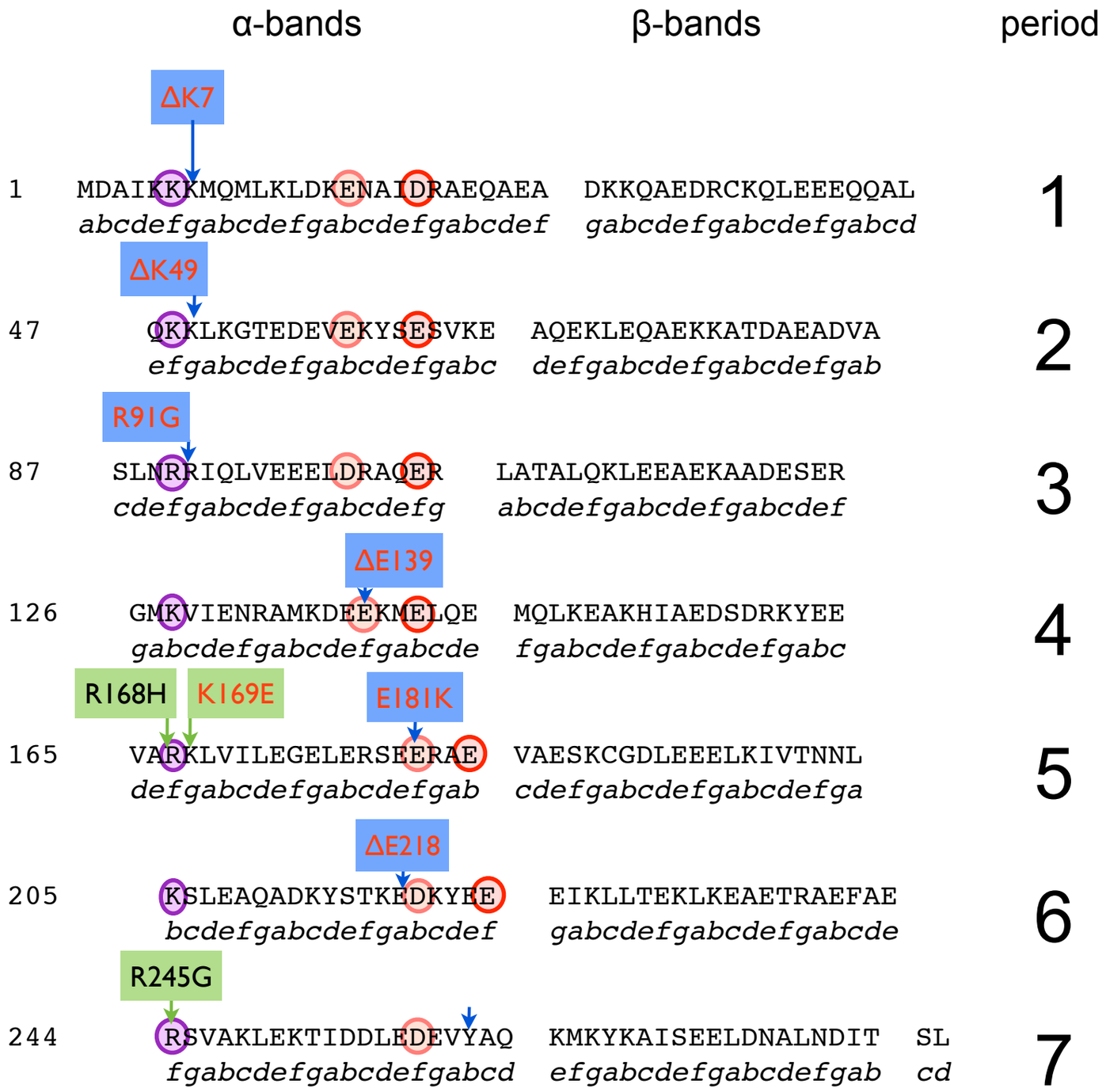
C The effect of adding 18nM NEM S-1 to actin-tropomyosin.

Table 1

The effect of tropomyosin mutations on $Ca^{2+}$-sensitivity and maximum sliding speed in reconstituted skeletal muscle thin filaments measured by in vitro motility assay. Combined data from n replicate $Ca^{2+}$-activation curve measurements like those shown in figure 5. The significance,p, of deviation of the$EC_{50}$ ratio from 1 was determined by a single value t-test.

| mutation | | $EC_{50}$ Mut, μM ± SEM, n,p | $EC_{50}$ WT,μM ± SEM, n,p | Ratio $EC_{50}$ WT/$EC_{50}$ Mut ± SEM, n,p | Max Sliding speed relative to WT, μm/s ± SEM, n,p |
|---|---|---|---|---|---|
| ΔK49 | 100% | 0.04 ± 0.002 | 0.08 ± 0.01 | **2.42 ± 0.25,** n=6, p=-.002 | 98.1±2.7%, n=3, p=0.555 |
| | 50% | 0.03 ± 0.002 | 0.09 ± 0.003 | **2.71 ± 0.06**, n=6, p<0.0001 | 101.0 ± 0.6% |
| R91G | 100% | 0.05 ± 0.01 | 0.09 ± 0.01 | **1.83 ± 0.18,** n=6, p=0.006 | 102.2 ± 1.0%, n=3, p=0.160 |
| | 50% | 0.06 ± 0.01 | 0.09 ± 0.004 | **1.55 ± 0.05**, n=6, p=0.0002 | 100.2 ± 0.6% |
| ΔE139 | 100% | 0.03 ± 0.004 | 0.12 ± 0.03 | **2.95 ± 0.43**, n=6, p=0.006 | 101.1 ± 2.4%, n=3, p=0.687 |
| | 50% | 0.03 ± 0.004 | 0.11 ± 0.01 | **2.58 ± 0.49**, n=6, p=0.003 | 104.2 ± 1.1% |
| R167H | 100% | 0.16 ± 0.02 | 0.07 ± 0.01 | **0.59 ± 0.08**, n=6, p=0.004 | 92.5 ± 2.6%, n=3, p=0.101 |
| | 50% | 0.13 ± 0.02 | 0.08 ± 0.01 | **0.59 ± 0.01**, n=2 | 95.0% |
| K168E | 100% | 0.05 ± 0.01 | 0.08 ± 0.01 | **2.17 ± 0.32**, n=8, p=0.008 | 107.2 ± 1.6% |
| | 50% | 0.05 ± 0.003 | 0.09 ± 0.001 | **1.84 ± 0.07**, n=12, p<0.0001 | 105.4 ± 2.7% |
| R245G | 100% | 0.12 ± 0.02 | 0.05 ± 0.02 | **0.46 ± 0.08,** n=6, p=0.001 | 97.1 ± 4.7%, n=3, p=0.598 |
| | 50% | 0.11 ± 0.003 | 0.08 ± 0.01 | **0.67 ± 0.07**, n=2 | 93.6% |

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β-bands
period
ΔK7
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1
ΔK49
47 QKKLKGTEDEVEKYSESVKE AQEKLEQAEKKATDAEADVA
efgabcdefgabcdefgabc defgabcdefgabcdefgab
2
R91G
87 SLNRRIQLVEEELDRAQER LATALQKLEEAEKAADESER
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3
ΔE139
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4
R168H
K169E
E181K
165 VARKLVILEGELERSEERAE VAESKCGDLEEELKIVTNNL
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5
ΔE218
205 KSLEAQADKYSTKEDKYEE EIKLLTEKLKEAETRAEFAE
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R245G
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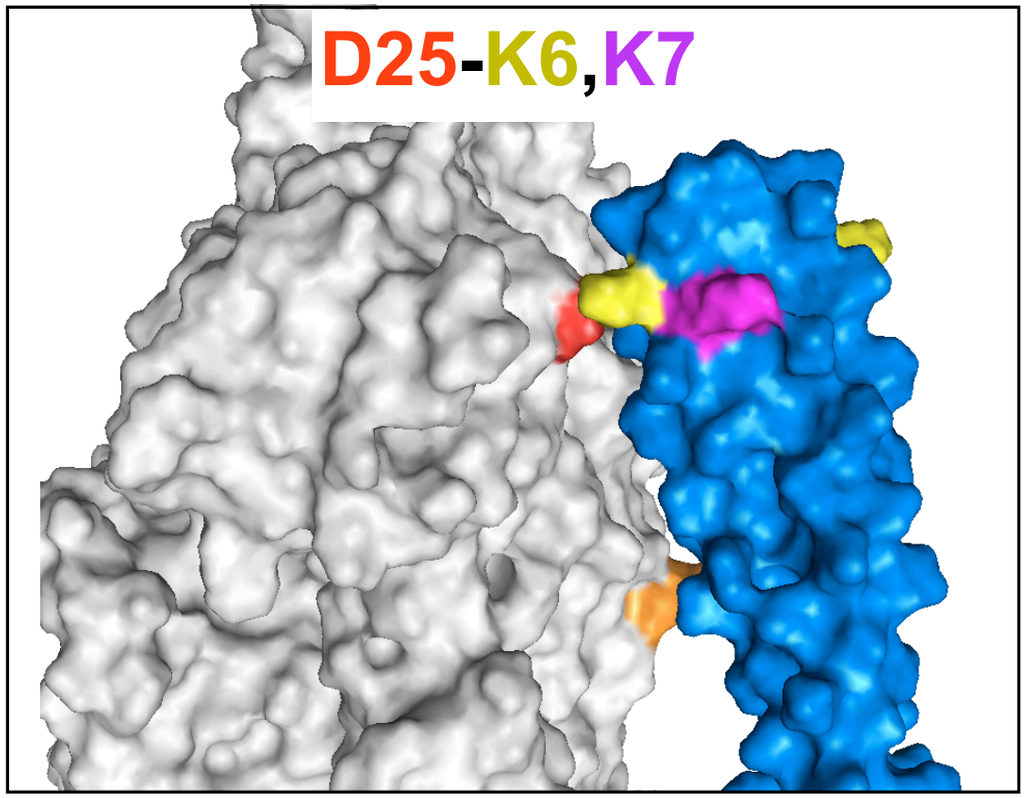
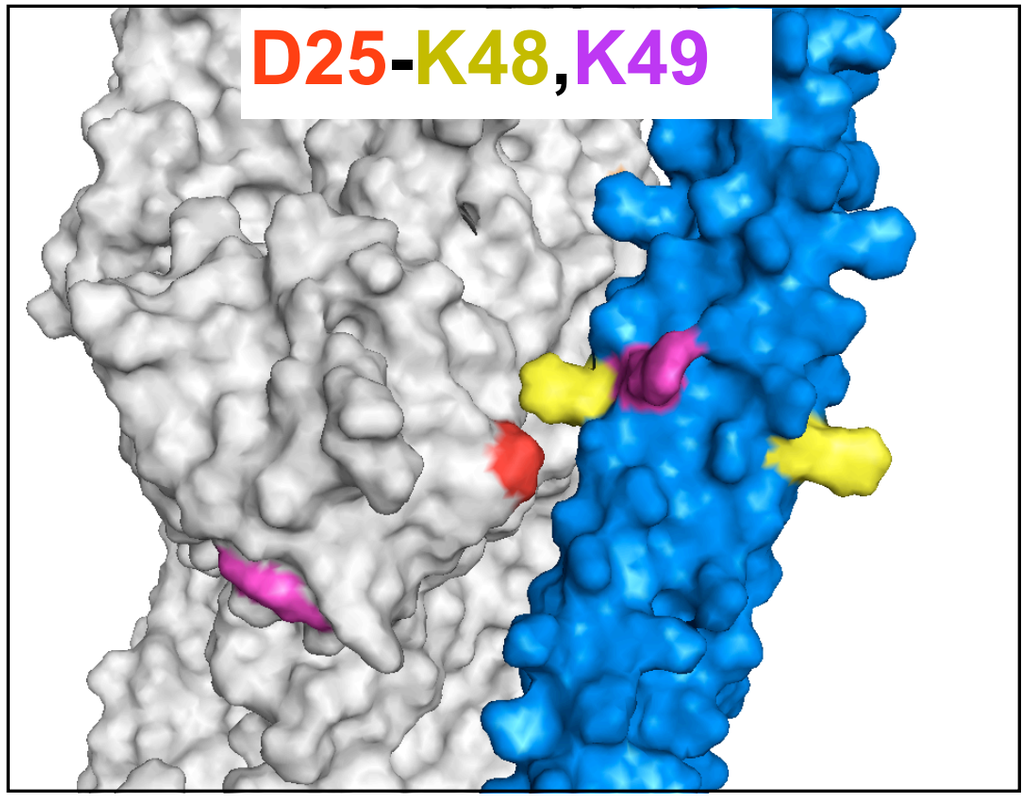
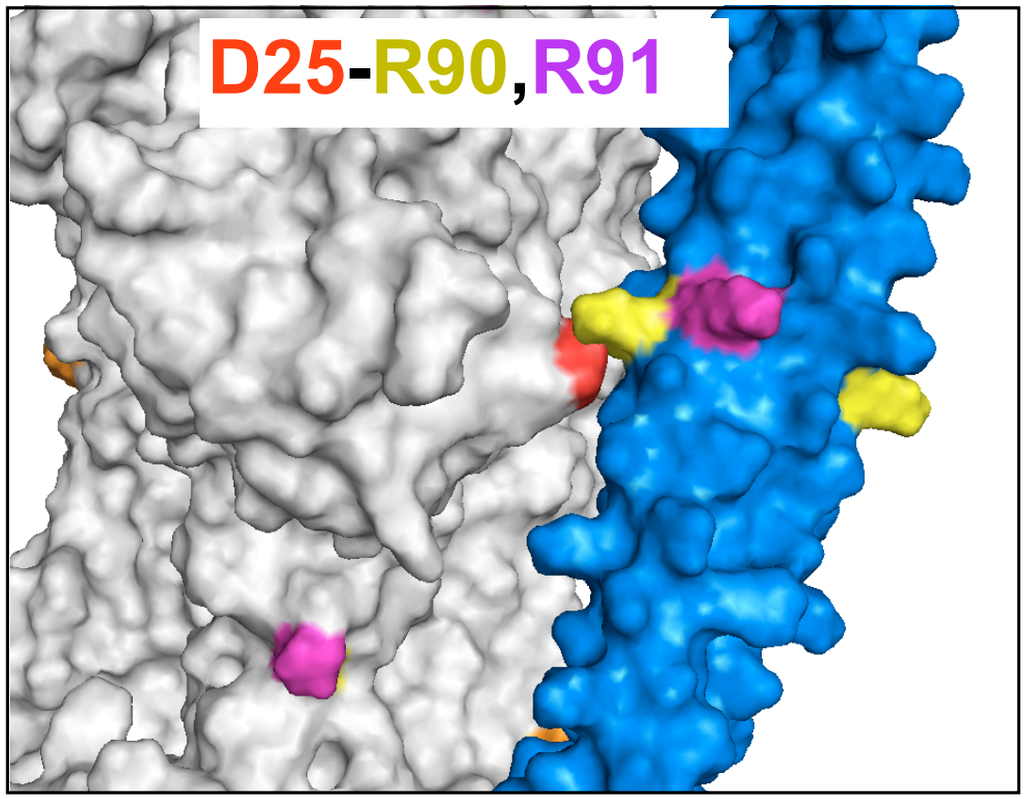
7

Fig 2



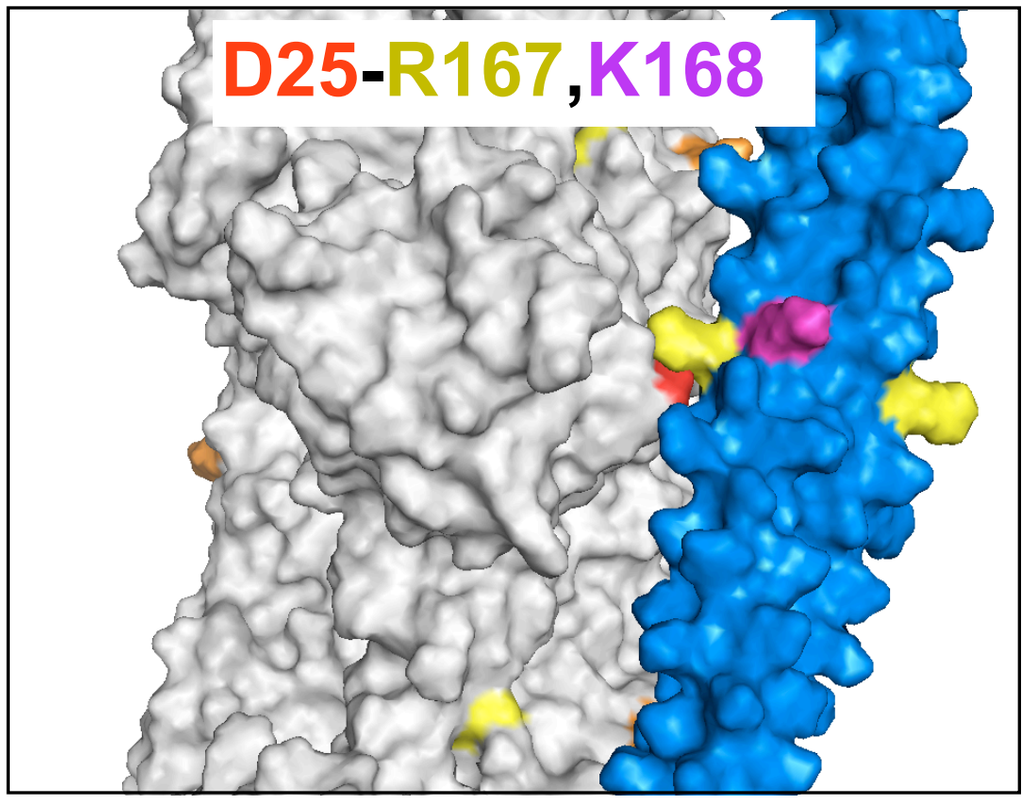
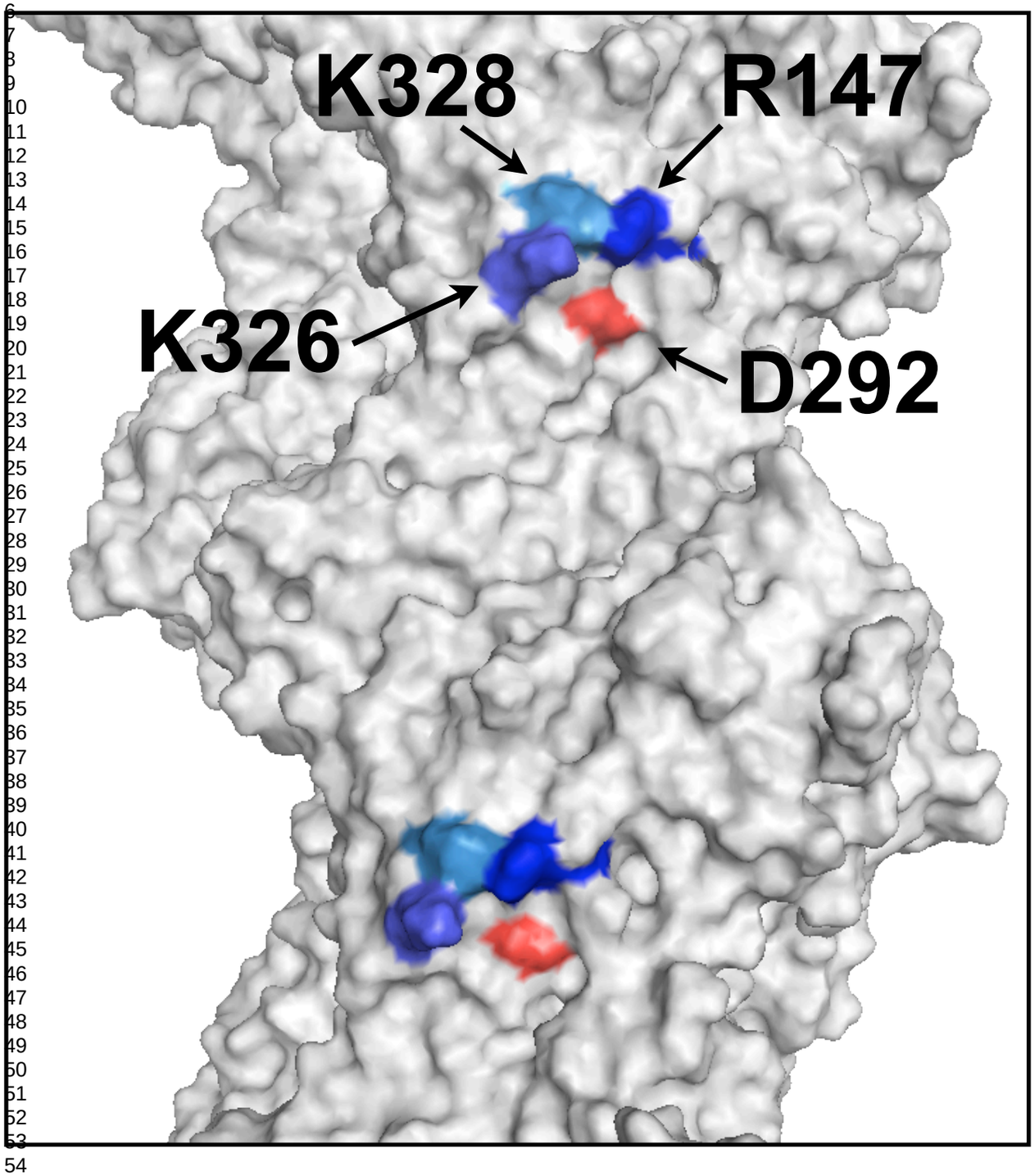




Fig 3



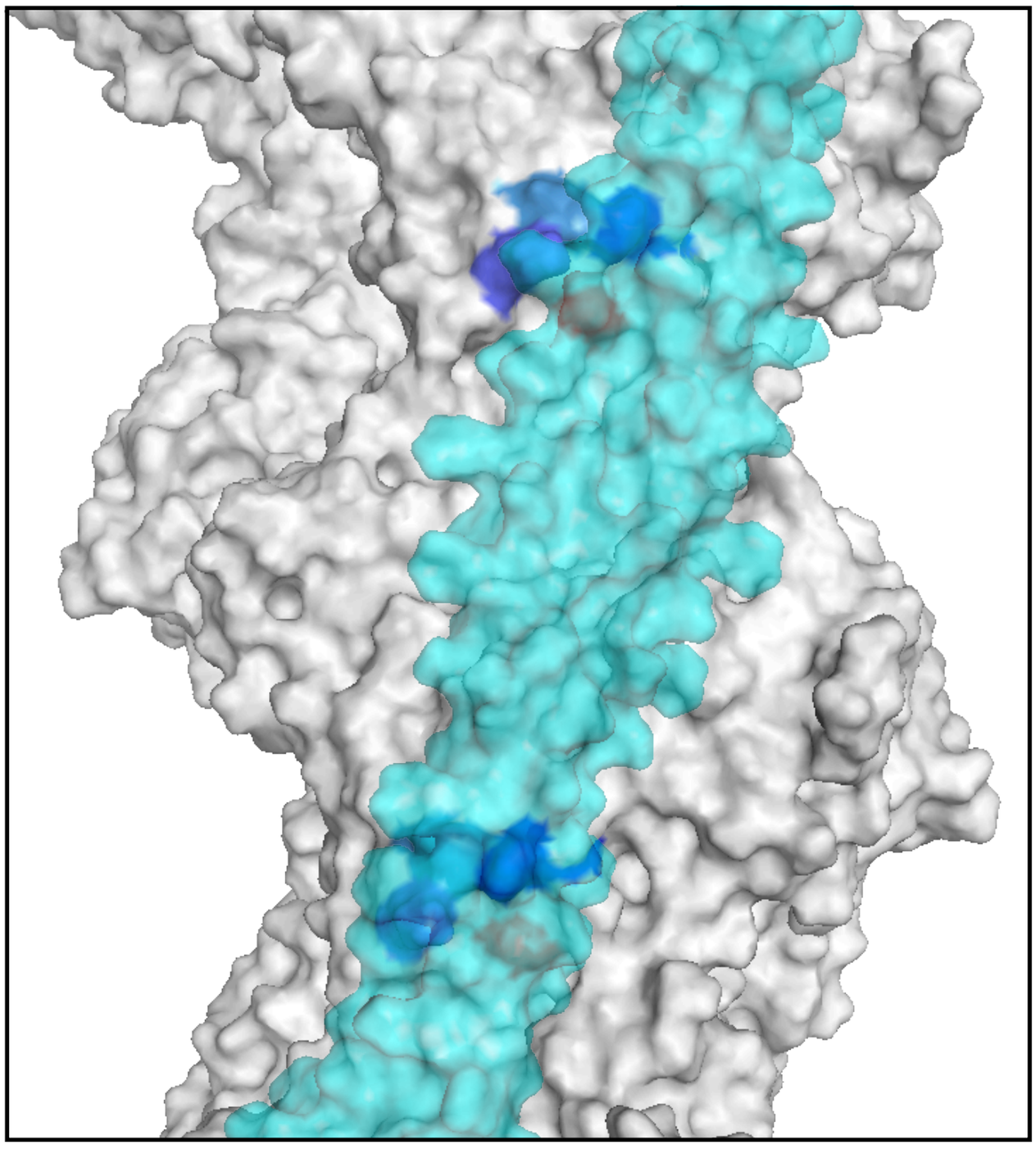
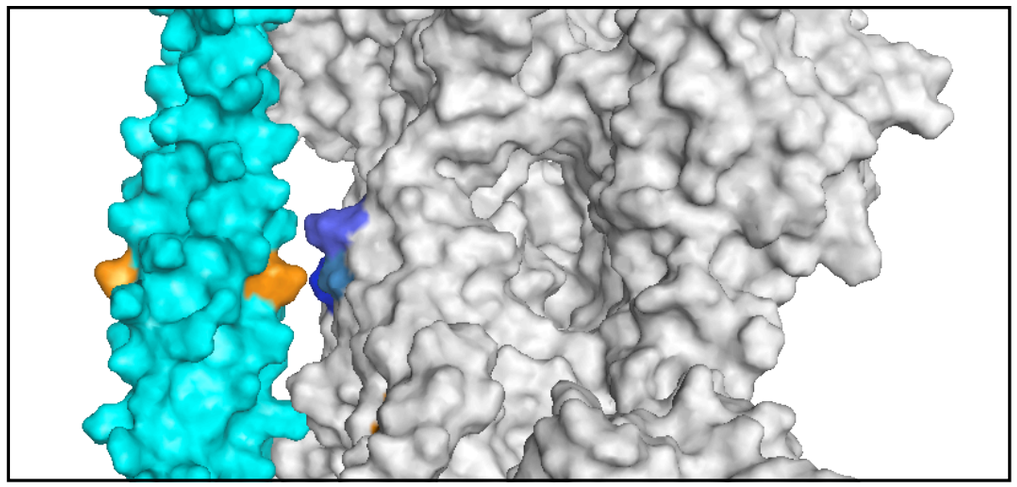



Fig 4



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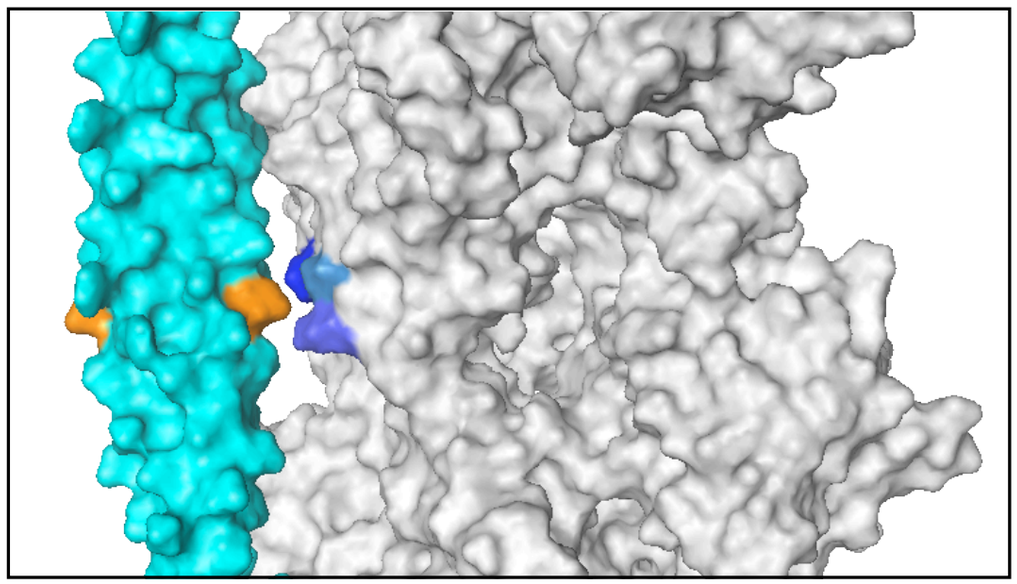

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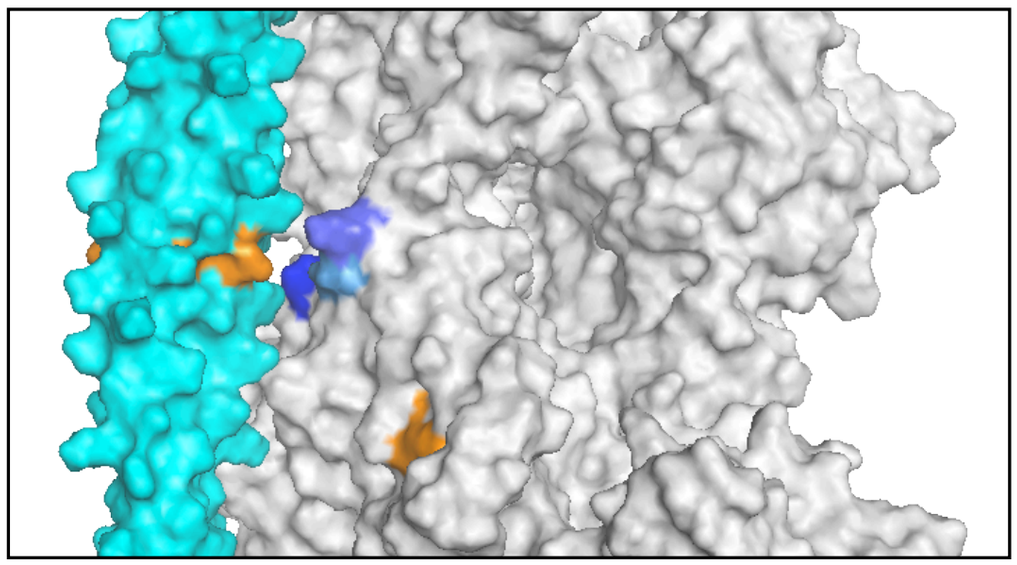
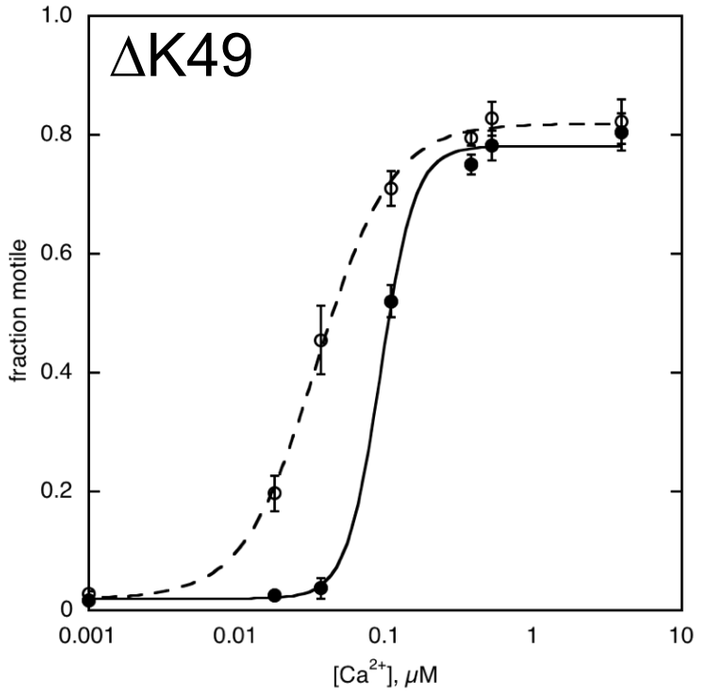


E218/219

Fig 5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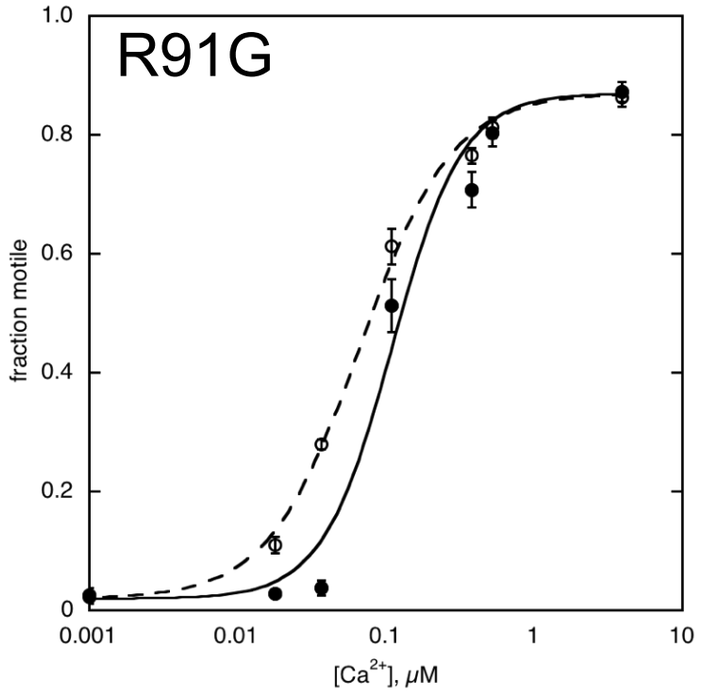
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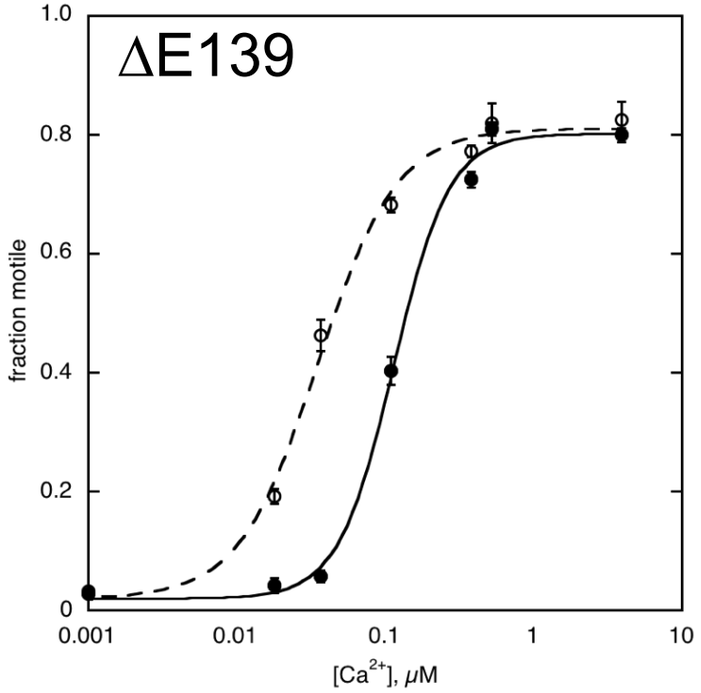
fraction motile
[Ca2+], μM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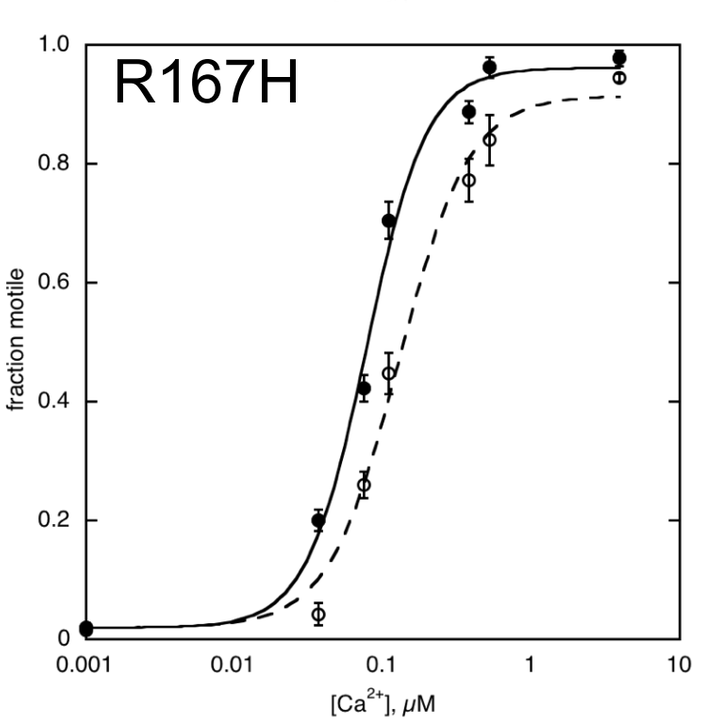
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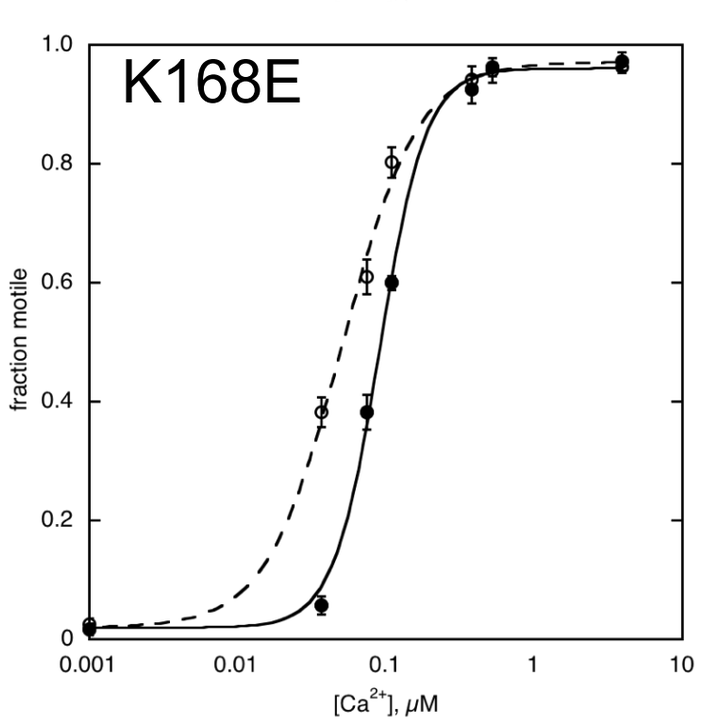
[Ca2+], μM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[Ca2+], μM

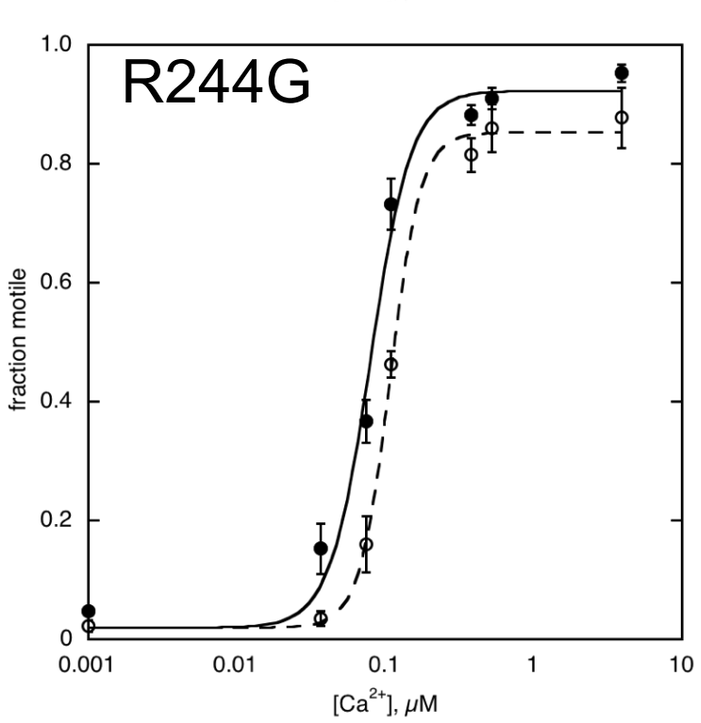
R244G
fraction motile
[Ca2+], μM

Figure 6

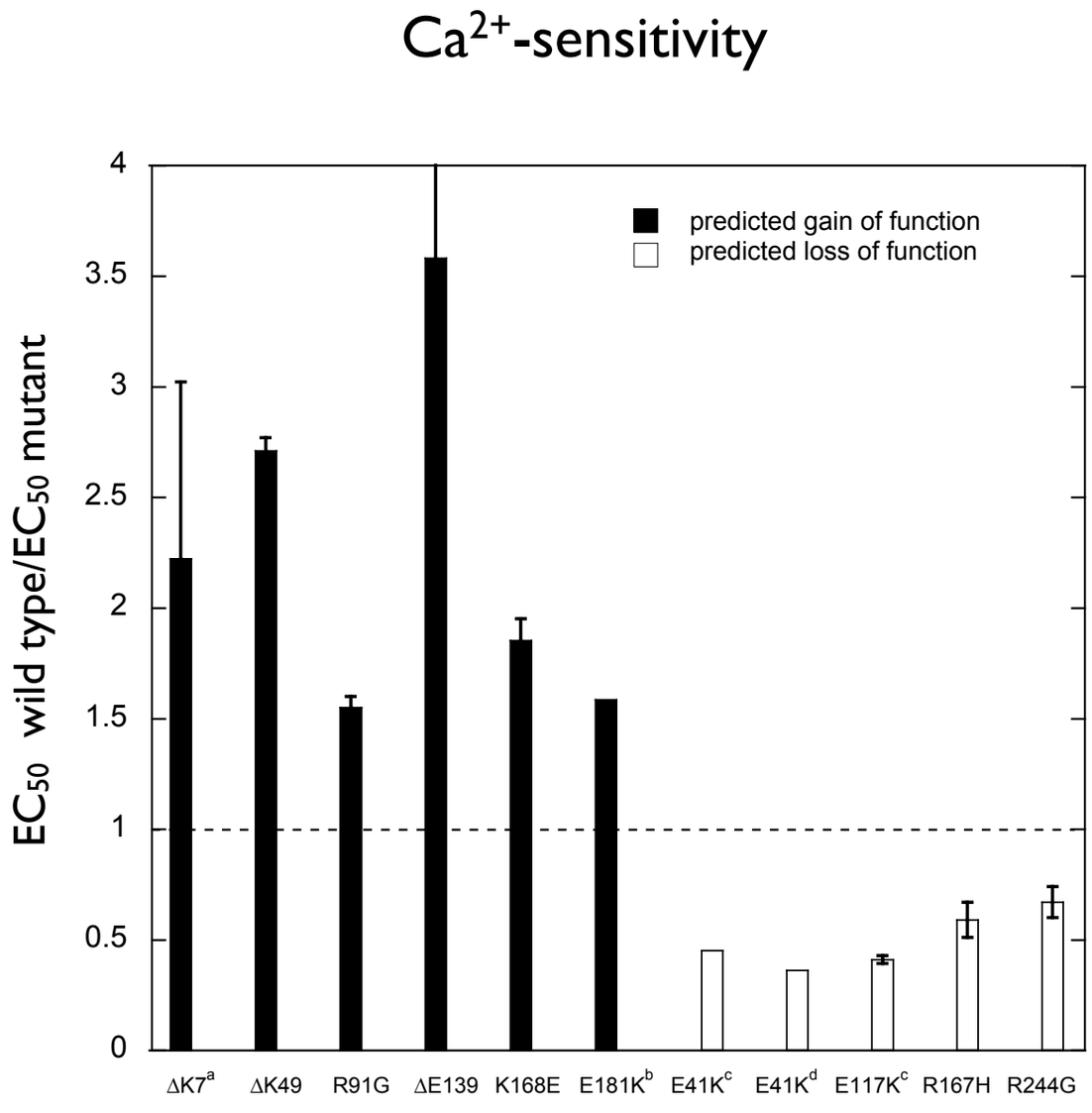
Ca2+-sensitivity
predicted gain of function
predicted loss of function
EC50 wild type/EC50 mutant
4
3.5
3
2.5
2
1.5
1
0.5
0
ΔK7a
ΔK49
R91G
ΔE139
K168E
E181Kb
E41Kc
E41Kd
E117Kc
R167H
R244G

Fig 6

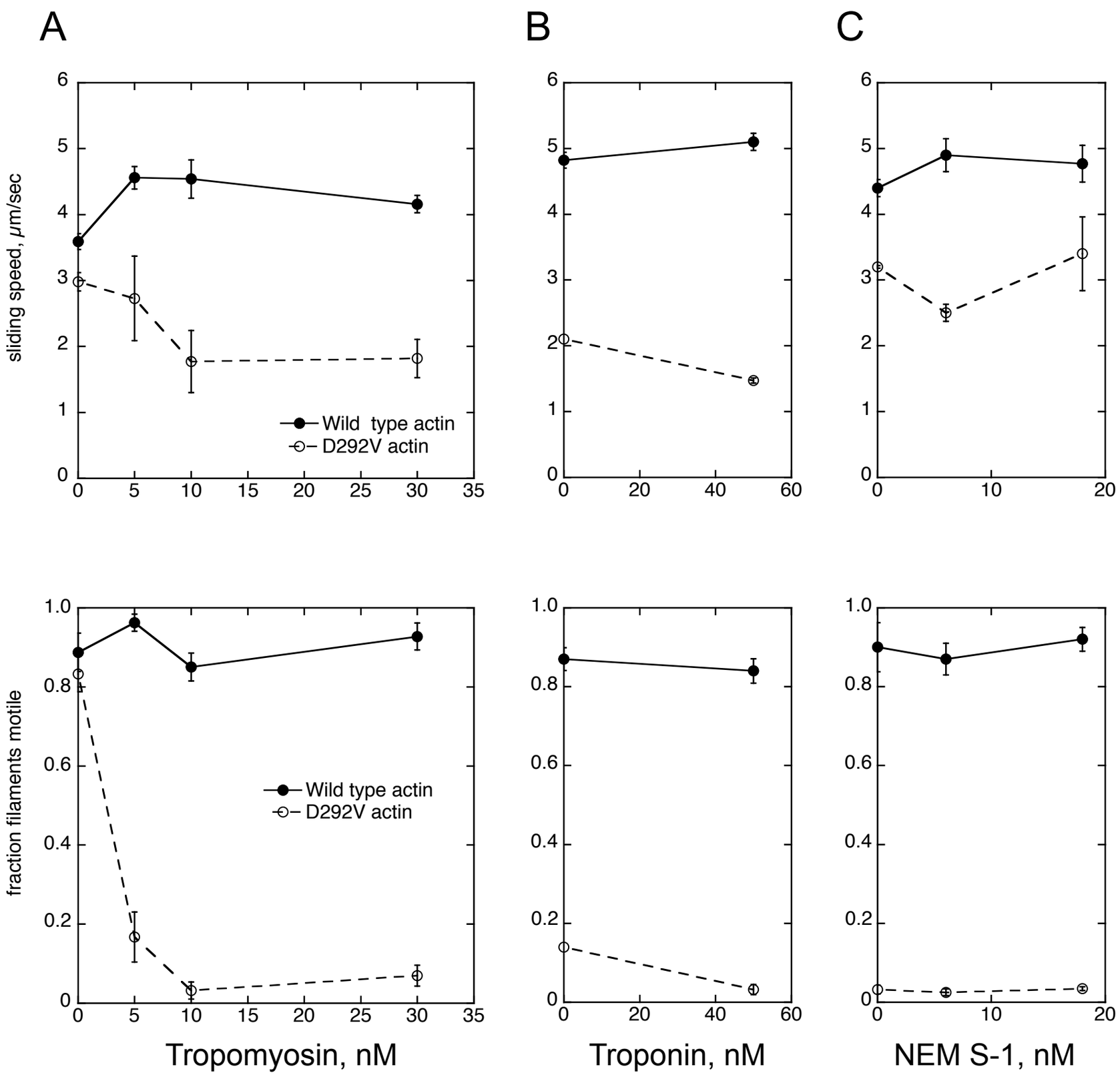
A
B
C
sliding speed, μm/sec
Wild type actin
D292V actin
fraction filaments motile
Wild type actin
D292V actin
Tropomyosin, nM
Troponin, nM
NEM S-1, nM

Fig 7