

# The non-muscle actinopathy-associated mutation E334Q in cytoskeletal $\gamma$ -actin perturbs interaction of actin filaments with myosin and ADF/cofilin family proteins

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## Abstract

Various heterozygous cytoskeletal  $\gamma$ -actin mutations have been shown to cause Baraitser–Winter cerebrofrontofacial syndrome, non-syndromic hearing loss, or isolated eye coloboma. Here, we report the biochemical characterization of human cytoskeletal  $\gamma$ -actin carrying mutation E334Q, a mutation that leads to a hitherto unspecified non-muscle actinopathy. Following expression, purification, and removal of linker and thymosin  $\beta$ 4 tag sequences, the p.E334Q monomers show normal integration into linear and branched actin filaments. The mutation does not affect thermal stability, actin filament nucleation, elongation and turnover. Model building and normal mode analysis predict significant differences in the interaction of p.E334Q-filaments with myosin motors and members of the ADF/cofilin family of actin-binding proteins. Assays probing the interactions of p.E334Q-filaments with human class 2 and class 5 myosin motor constructs show significant reductions in sliding velocity and actin-affinity. E334Q differentially affects cofilin-mediated actin dynamics by increasing the rate of cofilin-mediated *de novo* nucleation of actin filaments and decreasing the efficiency of cofilin-mediated filament severing. Thus, it is likely that p.E334Q-mediated changes in myosin motor activity, as well as filament turnover contribute to the observed disease phenotype.

### eLife assessment

This study presents a **useful** characterization of the biochemical consequences of a disease-associated point mutation in a nonmuscle actin. The study uses **solid** and well-characterized *in vitro* assays to explore function. In some cases the statistical analyses are **inadequate** and several key *in vitro* assays are not employed.

## Introduction

Cytoskeletal  $\beta$ - and  $\gamma$ -actin are the basic building blocks of the actin cytoskeleton in human non-muscle cells. The spatio-temporal regulation of these two actin isoforms, mediated by distinct actin-binding proteins (ABPs), drives the establishment and maintenance of specialized cytoskeletal compartments that are defined by their network architecture and the presence of specific filamentous actin-associated proteins (Pollard, 2016 [↗](#); Plastino & Blanchoin, 2019 [↗](#)). The regulated ATP-dependent turnover of these actin networks as well as the coordinated interplay of actin with network-specific ABPs contribute to essential cellular processes like directed migration, adhesion, division and mechanotransduction (Ponti *et al*, 2004 [↗](#); Abercrombie *et al*, 1970 [↗](#), 1971 [↗](#); Ridley *et al*, 2003 [↗](#); Pollard *et al*, 2002 [↗](#); Lauffenburger & Horwitz, 1996 [↗](#)).

$\beta$ - and  $\gamma$ -actin differ by only four amino acids in the N-terminal region of the molecule but show isoform-specific spatial organization in studies performed with monoclonal antibodies. In motile and stationary cells,  $\beta$ -actin is found in contractile stress fibers and the cell periphery (Dugina *et al*, 2009 [↗](#); Latham *et al*, 2013 [↗](#); Dugina *et al*, 2015 [↗](#)). In contrast,  $\gamma$ -actin localization is less static. During cell movement,  $\gamma$ -actin is recruited to the leading edge of the cell, resulting in an enrichment of the isoform in the lamellipodium together with  $\beta$ -actin (Dugina *et al*, 2009 [↗](#)). In non-moving cell states,  $\gamma$ -actin is co-localized with  $\beta$ -actin in stress fibers (Dugina *et al*, 2009 [↗](#)). In order to reconcile the highly similar protein sequences with the varying localization, several hypotheses on isoform regulation were put forward. These include the preferential interaction of ABPs with a particular isoform depending on the minor differences in the protein sequence, post-translational modifications as a means of isoform-specific regulation, and differences in the translation speed of *ACTB* and *ACTG1*, the genes encoding cytoskeletal  $\beta$ -actin and  $\gamma$ -actin (Zhang *et al*, 2010 [↗](#); Vedula *et al*, 2017 [↗](#); Pavlyk *et al*, 2018 [↗](#); Müller *et al*, 2013 [↗](#)).

Diseases associated with heterozygous pathogenic variants in *ACTB* and *ACTG1* are typically referred to as non-muscle actinopathies. These include a spectrum of rare syndromes that manifest themselves as more or less pronounced developmental disorders in patients (Verloes *et al*, 2015 [↗](#); Di Donato *et al*, 2014 [↗](#); Latham *et al*, 2018 [↗](#); Rivière *et al*, 2012 [↗](#); Baraitser & Winter, 1988 [↗](#); Cuvertino *et al*, 2017 [↗](#)). Baraitser–Winter–Cerebrorotofacial–Syndrome (BWCF, (Baraitser & Winter, 1988 [↗](#))) is a condition that affects the development of multiple organs, but particularly results in facial anomalies and brain malformations. BWCF occurs due to various missense mutations in *ACTB* or *ACTG1* (Verloes *et al*, 2015 [↗](#); Di Donato *et al*, 2014 [↗](#); Rivière *et al*, 2012 [↗](#); Di Donato *et al*, 2016 [↗](#)). BWCF-patients show a recognizable facial gestalt, with variants in *ACTB* generally resulting in a stronger facial phenotype than mutations in *ACTG1* (Di Donato *et al*, 2014 [↗](#)). BWCF-patients regularly suffer from cortical malformations that lead to severe developmental and mental impairments. In addition, symptoms such as hearing loss, dystonia, hypotonia and polydactyly occur with increased frequency (Di Donato *et al*, 2014 [↗](#); Rivière *et al*, 2012 [↗](#)). Variants in *ACTB* alone are responsible for two other well-defined syndromes. *ACTB*-associated pleiotropic malformation syndrome and *ACTB*-associated syndromic thrombocytopenia can be caused by single-point missense mutations, truncating mutations, frameshift mutations or deletion (Latham *et al*, 2018 [↗](#); Cuvertino *et al*, 2017 [↗](#)). These *ACTB*-associated syndromes differ from BWCF in that they cause only mild craniofacial malformations and developmental disorders (Cuvertino *et al*, 2017 [↗](#); Latham *et al*, 2018 [↗](#)). Selected single-point missense mutations in *ACTG1* lead to a non-syndromic form of hearing loss (Rendtorff *et al*, 2006 [↗](#); van Wijk *et al*, 2003 [↗](#); Yuan *et al*, 2016 [↗](#)).

How exactly mutations in the ubiquitously produced actin isoforms of the cytoskeleton lead to various syndromes and mutation-specific symptoms remains unclear. Currently, four main disease mechanisms are under discussion (Greve *et al*, 2022 [↗](#); Costa *et al*, 2004 [↗](#)). First, mutations in the actin isoform can lead to functional haploinsufficiency, where the produced molecule is either

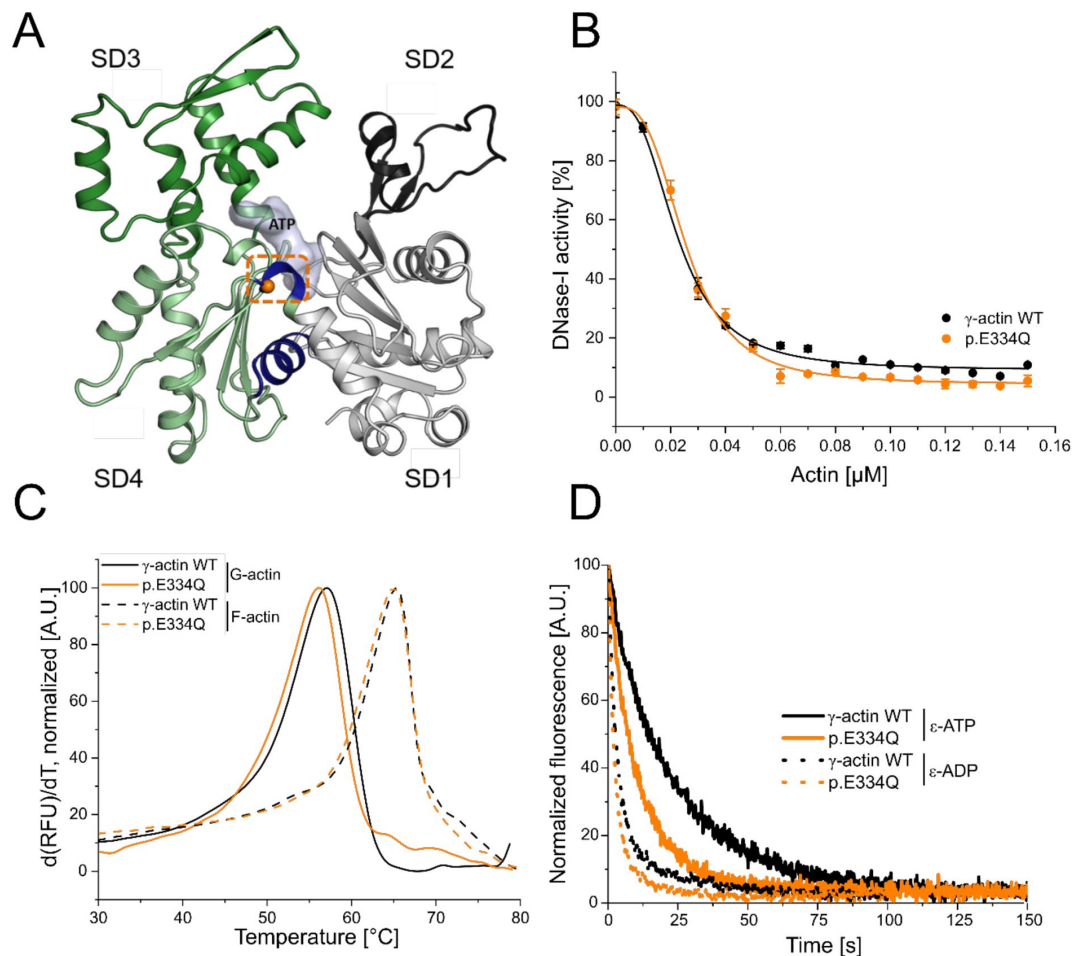
intrinsically unstable and therefore degraded by the proteasome or unable to associate with other actin molecules and thus unable to partake in the necessary steps involved in the dynamic remodeling of the actin cytoskeleton. This results in a reduced amount of “active” actin molecules, which cannot fully be compensated for by the remaining allele (Vang *et al*, 2005 [↗](#)). Second, the mutant protein is produced in normal quantities and integrated into filaments but interferes with actin filament turnover (Costa *et al*, 2004 [↗](#)). Third, the mutation perturbs interactions with actin binding proteins, either by direct or allosteric disturbance of actin–actin or actin–ABP interaction interfaces (Greve *et al*, 2022 [↗](#); Hundt *et al*, 2014 [↗](#); Bryan *et al*, 2006 [↗](#); Müller *et al*, 2012 [↗](#)). Fourth, the mutant protein shows an increased propensity for forming toxic rod-like oligomeric structures, which can potentially harm the cell (Ilkovski *et al*, 2004 [↗](#); Schröder *et al*, 2004 [↗](#)).

Therefore, one of the significant challenges is elucidating the relationship between the disturbed molecular mechanisms of the cell’s actin dynamics and disease patterns observed in patients. In order to achieve this, it is necessary to resolve the structure–function relationship of the various actin mutations at the molecular level. Here, we report the biochemical characterization of p.E334Q, a cytoskeletal  $\gamma$ -actin variant, which causes a syndromic form of non-muscle actinopathy. Features associated with the production of p.E334Q include mild facial anomalies and an abnormal gyral pattern of the cerebral cortex (Di Donato *et al*, 2016 [↗](#)). At four years (age of diagnosis), a patient carrying this mutation showed developmental delay and severe muscle hypotonia (Di Donato *et al*, 2016 [↗](#)). Although the patient was initially reported within the *ACTG1*–associated BWCF cohort, careful reassessment of the phenotype revealed that the clinical phenotype cannot be classified as BWCF and is therefore assigned as an unclassified NMA. Structural analysis of the disease-causing mutation shows that residue E334 is located in the hinge region of the actin molecule, which connects the two subdomain pairs of the protein. The region’s flexibility and ability to act as a pivot point are essential for the subdomain rearrangement when actin monomers polymerize into filaments (**Figure 1A** [↗](#)). Furthermore, E334 is part of a contact region on the F-actin surface that interacts with the so-called cardiomyopathy-loop in the motor domain of cytoskeletal myosin isoforms and contributes to the formation of the actin–cofilin complex (**Figure 3** [↗](#)). We present experimental evidence for the impact of changes involving residue E334 with respect to productive interactions with cytoskeletal myosin isoforms, actin polymerization dynamics, and the formation of stable actin-cofilin complexes. We show that mutation E334Q does not adversely affect protein folding. The thermal stability of p.E334Q resembles that of the wild-type protein, both as a monomer and following integration into filaments. The mutant protein efficiently copolymerizes with wildtype (WT) monomers and integrates into linear and branched filament networks. In contrast, the interaction of mutant filaments with non-muscle myosin-2A/C and myosin-5A is strongly impaired, as shown by *in vitro* motility experiments and solution kinetics. Furthermore, p.E334Q filaments are less susceptible to cofilin-mediated disassembly, while monomers are more susceptible to cofilin-mediated nucleation.

## Results

### Production of cytoskeletal $\gamma$ -actin and its variant p.E334Q

We produced untagged cytoskeletal  $\gamma$ -actin and its variant p.E334Q in insect cells, free of contaminating endogenous *S. frugiperda* actin, using a method that was originally designed for the production of toxic actin mutants in *Dictyostelium discoideum* and later adapted for use with the baculovirus/Sf9 insect cell expression system (Noguchi *et al*, 2007 [↗](#); Lu *et al*, 2015 [↗](#)). The monomeric actin constructs were initially produced fused to the actin-monomer sequestering protein thymosin- $\beta$ 4. Purification of the fusion protein by immobilized metal affinity chromatography, followed by chymotrypsin-mediated cleavage of C-terminal linker and tag



**Figure 1**

### Analysis of actin folding, thermal stability and nucleotide exchange

**(A)** Homology model of human cytoskeletal  $\gamma$ -actin (based on PDB-ID: 2BTF). Subdomains (SDs) of actin are colored in grey (SD1), dark grey (SD2), light green (SD3) and green (SD4). The hinge region encompassing residues 137–145 and 333–338 is colored in blue. The mutant residue E334 is shown as an orange sphere. **(B)** Inhibition of DNase-I activity by monomeric  $\gamma$ -actin WT and p.E334Q. Data is the mean of three individual experiments  $\pm$  SEM. A Hill equation was fitted to the data, which yields the half-maximal inhibitory concentration ( $IC_{50}$ , **Table 1**). **(C)** The protein denaturation temperature of monomeric and filamentous p.E334Q and WT actin were determined by DSF. Representative experimental traces are shown. The protein denaturation temperature is derived from the peak of the melting curve (**Table 1**) (p.E334Q (G-actin), N=3; WT (G-actin), N=5; p.E334Q (F-actin), N=5; WT (F-actin), N=5). **(D)** Nucleotide exchange rates ( $k_T$ ,  $k_D$ ) were determined for monomeric p.E334Q and  $\gamma$ -actin WT using fluorescently labeled ATP ( $\epsilon$ -ATP) (p.E334Q, N=18; WT, N=21) and ADP ( $\epsilon$ -ADP) (p.E334Q, N=11; WT, N=11). Representative experimental traces are shown. Rates were determined by fitting a single exponential function to the data (**Table 1**).

sequences, results in homogeneous protein without non-native residues. The method worked equally well for both WT and mutant actin. Starting with  $2 \times 10^9$  cells, typical yields ranged from 5–7 mg of purified protein for cytoskeletal  $\gamma$ -actin and p.E334Q (N=3 for WT and N=4 for p.E334Q).

## Impact of mutation E334Q on protein folding and thermal stability

Mutation-induced misfolding of actin has been identified as one of the main causes for the development of actinopathies (Bryan *et al*, 2006 [↗](#); Vang *et al*, 2005 [↗](#)). Residue E334 is located in the hinge region, formed by residues 137–145 and 333–338 (Figure 1A [↗](#)), which is strongly involved in the structural reorganization of the monomer upon incorporation into a growing filament (Dominguez & Holmes, 2011 [↗](#)). Previous studies have shown that an intact hinge region is crucial for the interactions with the eukaryotic chaperonin CCT/TriC and myosin (Noguchi *et al*, 2012 [↗](#); McCormack *et al*, 2001b [↗](#), 2001a [↗](#)). We first performed a DNase-I inhibition assay to analyze the effect of mutation E334Q on the folding of the protein. Previous studies have shown that the activity of DNase I is only effectively inhibited by correctly folded actin (Schüler *et al*, 2000 [↗](#)). We observed a 10% increase in the apparent  $IC_{50}$ -value of p.E334Q ( $25.7 \pm 0.6$  nM) compared to the WT protein ( $22.7 \pm 0.7$  nM) (Figure 1B [↗](#)). This is in good agreement with CD-spectroscopy results showing nearly identical secondary structure compositions for WT and mutant proteins (Suppl. Figure 1 [↗](#)). Thermal denaturation temperatures ( $T_M$ ) analyzed by differential scanning fluorimetry (DSF) gave values of  $56.77 \pm 0.24^\circ\text{C}$  and  $56.19 \pm 0.30^\circ\text{C}$  for monomeric WT actin and p.E334Q. The values obtained for WT and E334Q filaments were  $65.00 \pm 0.76^\circ\text{C}$  and  $64.83 \pm 0.25^\circ\text{C}$ , respectively (Figure 1C [↗](#), Table 1 [↗](#)). Overall, these results show that the mutant actin is correctly folded and has comparable stability to the WT protein.

As the hinge region is in close proximity to the nucleotide-binding cleft, we examined a potential effect of the mutation on the interaction with nucleotides and the turnover of ATP. Initial tests gave near identical rates of  $0.00089 \pm 0.00005 \text{ s}^{-1}$  and  $0.00088 \pm 0.00004 \text{ s}^{-1}$  for the steady-state turnover of ATP by filaments formed from cytoskeletal  $\gamma$ -actin or the p.E334Q variant (Table 1 [↗](#)). Next, we determined the nucleotide dissociation rates  $k_T$  and  $k_D$  by monitoring the decrease in fluorescence amplitude associated with the displacement of  $\epsilon$ -ATP or  $\epsilon$ -ADP after rapid mixing with a large excess of unlabeled ATP. With p.E334Q-actin, we observed rates of  $k_T = 0.0972 \pm 0.0019 \text{ s}^{-1}$  and  $k_D = 0.3199 \pm 0.0027 \text{ s}^{-1}$ . These rates are about 2-fold and 1.5-fold faster than those observed in experiments performed with WT monomers ( $k_T = 0.0473 \pm 0.0013 \text{ s}^{-1}$ ,  $k_D = 0.2155 \pm 0.0017 \text{ s}^{-1}$ ) (Figure 1D [↗](#), Table 1 [↗](#)). The observed nucleotide dissociation rates for human cytoskeletal  $\gamma$ -actin are in good agreement with values obtained for a mixture of pyrene-labeled cytoskeletal  $\gamma$ -actin (15%) and  $\beta$ -actin (85%) purified from human platelets in experiments performed at the same ionic strength (De La Cruz & Sept, 2010 [↗](#)).

## p.E334Q shows normal polymerization behavior and is efficiently integrated into linear and branched actin structures

The regulated assembly and disassembly of filamentous actin drive various cellular processes, including directed cell migration, adhesion and endocytosis. A slight conformational change of the actin monomer accompanies the integration of a monomeric actin molecule into a growing filament. A small change in the hinge region leading to a  $12^\circ$  to  $13^\circ$  rotation of the inner subdomain pair relative to the outer subdomain pair results in a flatter actin molecule within the filament compared to the free actin monomer (Dominguez & Holmes, 2011 [↗](#); Oda *et al*, 2009 [↗](#)). Mutations in the hinge region can thus potentially affect filament assembly by impairing the required structural reorganization of the monomer, as it attaches to the growing filament end. TIRF microscopy-based tracking of the polymerization of monomeric Atto-655-labeled WT or mutant actin *in vitro* revealed a small but significant reduction in the elongation rate from  $12.6 \pm 0.3 \text{ nm s}^{-1}$  for WT to  $11.1 \pm 0.3 \text{ nm s}^{-1}$  for mutant filaments (Figure 2A, B [↗](#)). To detect any differences in nucleation efficiency, we determined the time-dependent increase in the number of

| Parameter                                                                                       | $\gamma$ -actin                              | p.E334Q                                                          | 1:1 Mixture                                                     |
|-------------------------------------------------------------------------------------------------|----------------------------------------------|------------------------------------------------------------------|-----------------------------------------------------------------|
| IC <sub>50</sub> (DNase-Inhibition) [nM]                                                        | 22.7 ± 0.7                                   | 25.7 ± 0.6<br>(* 1.1-fold ↑)                                     | –                                                               |
| T <sub>M</sub> (Mg <sup>2+</sup> –ATP–G–actin) [°C]                                             | 56.77 ± 0.24                                 | 56.19 ± 0.30<br>(ns)                                             | –                                                               |
| T <sub>M</sub> (F–actin) [°C]                                                                   | 65.00 ± 0.76                                 | 64.83 ± 0.25<br>(ns)                                             | –                                                               |
| k <sub>T</sub> (Mg <sup>2+</sup> –ε–ATP) [s <sup>–1</sup> ]                                     | 0.0473 ± 0.0013                              | 0.0972 ± 0.0019<br>(**** 2.1-fold ↑)                             | –                                                               |
| k <sub>D</sub> (Mg <sup>2+</sup> –ε–ADP) [s <sup>–1</sup> ]                                     | 0.2155 ± 0.0017                              | 0.3199 ± 0.0027<br>(**** 1.5-fold ↑)                             | –                                                               |
| F–actin ATPase [s <sup>–1</sup> ]                                                               | 0.00089 ± 0.00005                            | 0.00088 ± 0.00004<br>(ns)                                        | –                                                               |
| Bulk–polymerisation rate k <sub>obs</sub> (pyrene) [s <sup>–1</sup> ]                           | 0.0140 ± 0.0004                              | 0.0129 ± 0.0008<br>(*** 1.1-fold ↓)                              | 0.0134 ± 0.0006<br>(* 1.04-fold ↓)                              |
| Bulk–polymerisation rate k <sub>obs</sub> (pyrene) + 10 nM Arp2/3 [s <sup>–1</sup> ]            | 0.6441 ± 0.0163                              | 0.5458 ± 0.02256<br>(** 1.2-fold ↓)                              | 0.6162 ± 0.0776<br>(ns)                                         |
| Apparent rate of filament elongation (TIRF–M) [nm/s]                                            | 12.6 ± 0.3                                   | 11.1 ± 0.3<br>(**** 1.1-fold ↓)                                  | –                                                               |
| Rate of filament disassembly k <sub>obs</sub> (pyrene–actin) [s <sup>–1</sup> ]                 | 0.0022 ± 0.0002                              | 0.0029 ± 0.0003<br>(**** 1.3-fold ↑)                             | 0.0023 ± 0.0002<br>(ns)                                         |
| Rate of filament disassembly k <sub>obs</sub> (pyrene–actin) + 40 nM cofilin [s <sup>–1</sup> ] | 0.0163 ± 0.0028                              | 0.0059 ± 0.0008<br>(*** 2.8-fold ↓)                              | 0.0084 ± 0.0018<br>(** 1.9-fold ↓)                              |
| Sliding velocity on NM2A–HMM [nm/s]                                                             | 195.3 ± 5.0                                  | 39.1 ± 3.2<br>(**** 5.0-fold ↓)                                  | 131.2 ± 10.0<br>(*** 1.5-fold ↓)                                |
| Sliding velocity on Myo5A–HMM at optimal myosin density [nm/s]                                  | 315.1 ± 28.9                                 | 190.4 ± 46.9<br>(**** 1.7-fold ↓)                                | –                                                               |
| k <sub>A</sub> (NM2A–2R) [s <sup>–1</sup> ]                                                     | 8.0×10 <sup>–5</sup> ± 3.3 ×10 <sup>–6</sup> | 4.5×10 <sup>–4</sup> ± 6.7×10 <sup>–6</sup><br>(**** 5.6-fold ↑) | 2.3×10 <sup>–4</sup> ±1.3×10 <sup>–5</sup><br>(*** 2.9-fold ↑)  |
| k <sub>A</sub> (NM2C–2R) [s <sup>–1</sup> ]                                                     | 3.1×10 <sup>–4</sup> ± 5.0×10 <sup>–6</sup>  | 1.7×10 <sup>–3</sup> ± 5.8×10 <sup>–5</sup><br>(*** 5.6-fold ↑)  | 6.7×10 <sup>–4</sup> ± 1.5×10 <sup>–5</sup><br>(*** 2.2-fold ↑) |
| K <sub>A</sub> (NM2A–2R) [nM]                                                                   | 1.7 ± 0.3                                    | 16.9 ± 0.3<br>(** 9.9-fold ↑)                                    | –                                                               |
| K <sub>DA</sub> (NM2A–2R) [nM]                                                                  | 122.6 ± 11.4                                 | 353.0 ± 20.6<br>(** 2.9-fold ↑)                                  | –                                                               |
| Coupling (K <sub>DA</sub> / K <sub>A</sub> ) (NM2A–2R)                                          | 72.1                                         | 20.9<br>(**** 3.5-fold ↓)                                        | –                                                               |
| 1/K <sub>1</sub> (NM2A–2R) [μM]                                                                 | 812.1 ± 82.7                                 | 863.8 ± 91.6<br>(ns)                                             | –                                                               |

**Table 1**

**Comparison of biochemical parameters, velocities, rate and equilibrium constants measured in experiments with human cytoskeletal  $\gamma$ -actin and variant p.E334Q.**

filaments (**Figure 2C** [↗](#)). Aside from short-term fluctuations, the linear increase in the number of filaments recorded for WT ( $0.039 \pm 0.002 \text{ s}^{-1}$ ) and p.E334Q ( $0.041 \pm 0.004 \text{ s}^{-1}$ ) was similar over the full 1000 s duration of the experiment. Overall, p.E334Q shows only a mild polymerization defect.

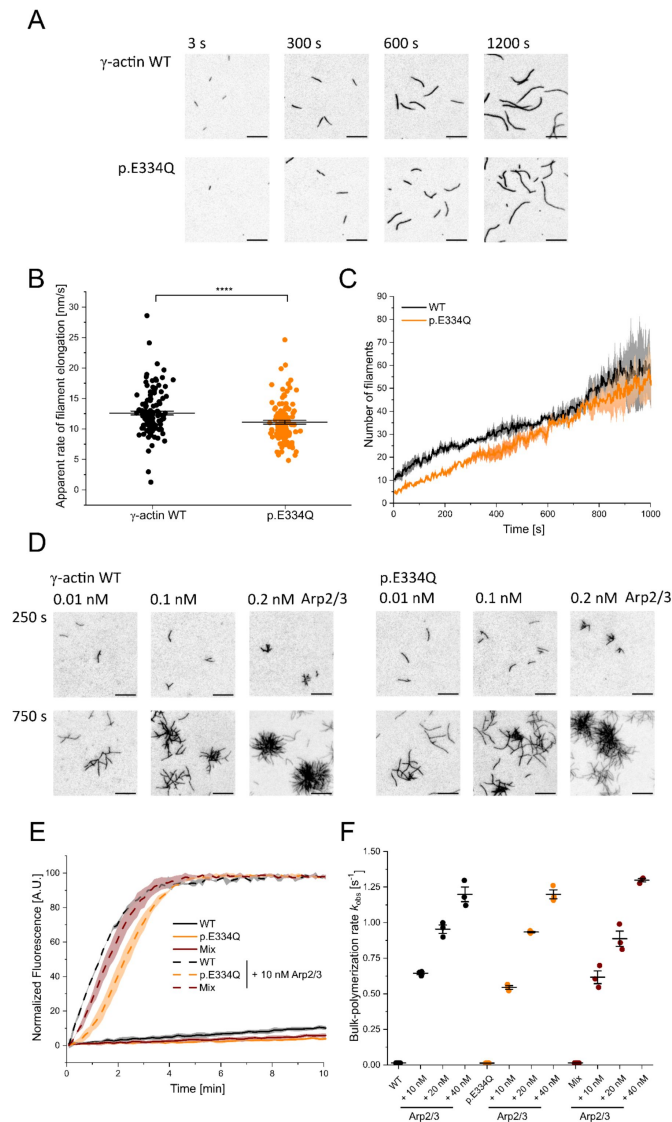
Since actin polymerization *in vivo* is tightly regulated in a spatio-temporal manner by different ABPs, we analyzed the interaction of p.E334Q with one of the key-regulators of actin polymerization, the Arp2/3 complex (Goley & Welch, 2006 [↗](#)). The Arp2/3 complex promotes the nucleation of new actin filaments from the sides of existing filaments and caps their pointed ends. Thereby, it promotes the formation of branched actin networks and contributes to essential processes that depend on the dynamic remodeling of the actin cytoskeleton, such as cell migration, endocytosis, and organelle positioning (Nicholson-Dykstra *et al.*, 2005 [↗](#); Mullins *et al.*, 1998 [↗](#); Blanchoin *et al.*, 2000 [↗](#); Loisel *et al.*, 1999 [↗](#)). The ability of p.E334Q to form branched actin networks was probed in TIRF microscopy-based experiments in the presence of increasing concentrations of VCA-activated human Arp2/3 complex (**Figure 2D** [↗](#)). At the lowest Arp2/3 concentration used (0.01 nM), we observed the appearance of the first branches after approximately 250 s. In the presence of 0.1 nM and 0.2 nM of the VCA-activated Arp2/3 complex, mutant and WT actin continued forming branched networks with comparable branch formation and branch elongation rates. Similar results were obtained with WT actin, mutant actin or with 1:1 mixtures of WT and mutant actin, when we analyzed the effect of adding increasing concentrations of VCA-activated human Arp2/3 in pyrene-actin based bulk-polymerization assays (**Figure 2E, F** [↗](#)). The results are summarized in **Table 1** [↗](#).

## Impact of E334Q mutation on interactions with ADF/cofilin family proteins

Homology models of the complexes formed by human cofilin-1 with filamentous cytoskeletal  $\gamma$ -actin and p.E334Q were generated to evaluate the functional impact of mutation E334Q. Cofilin-1 binds to actin filaments by making simultaneous contact with two adjacent monomers. Actin residue E334 is located halfway between two adjacent cofilin molecules, but only makes direct contact with residue L111 of the cofilin-1 molecule on the pointed end side (**Figure 3A** [↗](#)). Cofilin-1 residue L111 is located in an  $\alpha$ -helix (L111-L128) that is essential for actin-binding (Pope *et al.*, 2000 [↗](#)). Mutation E334Q maintains the van der Waals interaction with L111, but reduces the electrostatic attraction between the interacting surfaces, with likely effects on the stability and allosteric coupling of the complex (**Figure 3B, C** [↗](#)).

To evaluate the impact of mutation E334Q on the elastic properties of F-actin in the presence and absence of cofilin-1 binding, we performed normal mode analysis using bare, partially decorated and fully decorated filaments (**Suppl. Figure 2A** [↗](#)). Since residue E334 is located on the outer surface of the actin filament, the mutation produces no significant change in the normal mode frequencies of bare filaments. Similarly, fully decorated filaments show only minor differences. Substantial changes in the low-frequency modes associated with global conformational changes were observed when comparing the partially cofilin-decorated filaments of WT and mutant filaments, where the bare and cofilin-decorated regions move as rigid bodies and the interface between the two regions acts as a hinge (modes 7, 8) or fulcrum (mode 9) (**Suppl. Figure 2B** [↗](#)).

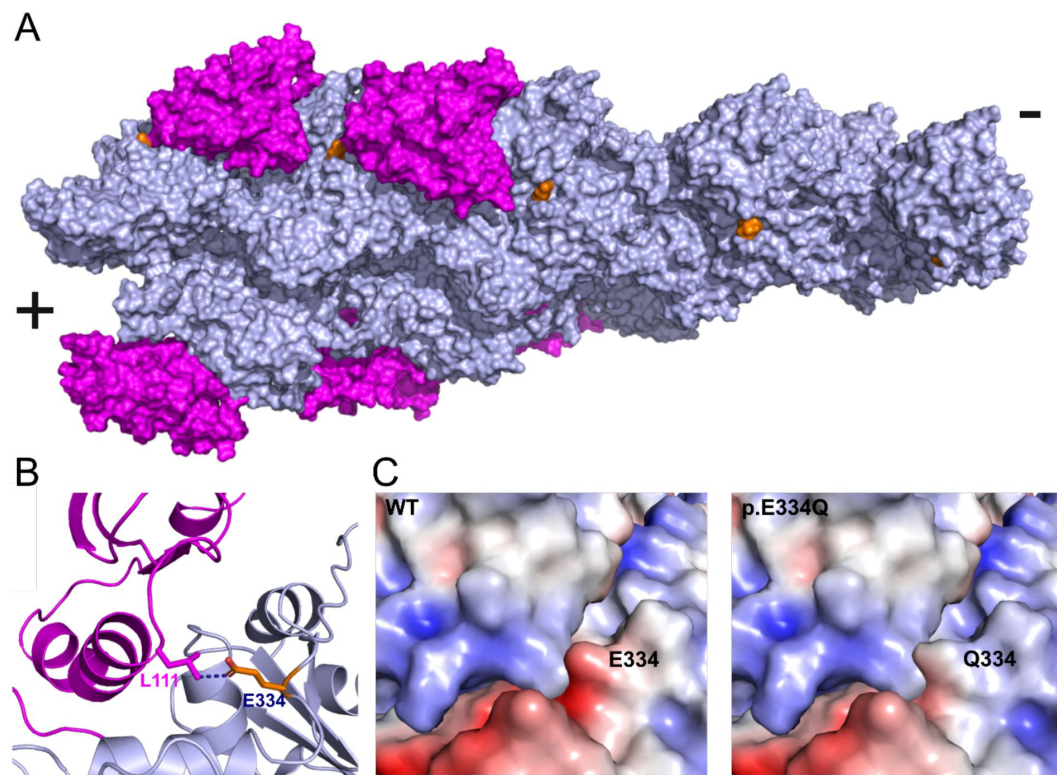
To test the validity of our modeling results, we performed biochemical and functional assays probing the interactions of human cofilin-1 with monomeric and filamentous cytoskeletal  $\gamma$ -actin and p.E334Q. The effect of cofilin-1 on filament assembly was assessed by TIRF microscopy experiments. The polymerization of 1  $\mu\text{M}$  actin was induced in the presence of 0, 50 or 100 nM cofilin-1. Following an initial lag phase of 200 to 300 seconds, the filament formation rate increased with the cofilin-1 concentration (**Figure 4** [↗](#)). In experiments with p.E334Q-actin, *de novo* filament nucleation proceeded faster (**Figure 4A, B** [↗](#)). Movies showing filament formation by *de novo* nucleation in the presence of cofilin-1 are available in the supplement (**Suppl. Video 1–6**).



**Figure 2**

### Analysis of the polymerization capacity of WT and p.E334Q $\gamma$ -actin in the absence and presence of human Arp2/3 complex.

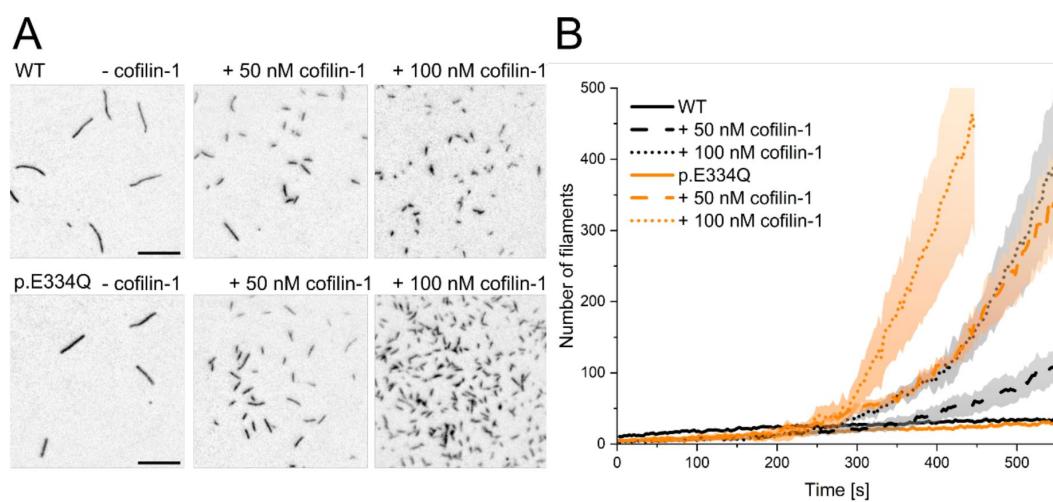
(A) Polymerization of p.E334Q and WT  $\gamma$ -actin (1  $\mu$ M, 10% Atto-655 labeled) was induced by salt-shift and the progression of the reaction was tracked by TIRF microscopy. Shown are representative micrographs at the indicated points in time. Scale bar corresponds to 10  $\mu$ m. (B) The elongation rates of individual filaments were determined by manual tracking of the elongating barbed ends of the filaments. Every data point represents an individual filament (WT:  $n = 128$  filaments, p.E334Q:  $n = 114$  filaments). Data is shown as the mean  $\pm$  SEM from 4 individual experiments. (C) Nucleation efficiencies were determined by monitoring changes in the number of filaments over the time of the polymerization experiments. The solid lines and shades represent the mean  $\pm$  SEM of four individual experiments. (D) TIRF microscopy-based observation of human Arp2/3 complex mediated differences in the salt-induced polymerization of p.E334Q and WT  $\gamma$ -actin (1  $\mu$ M, 10% Atto-655 labeled). Shown are representative micrographs at the indicated points in time. For both WT and p.E334Q  $\gamma$ -actin, the formation of the first branch points was observed after 250 s in the presence of 0.01 nM Arp2/3. Scale bar corresponds to 10  $\mu$ m.  $N=3$  for each Arp2/3 concentration (E) Pyrene-based polymerization experiment of 2  $\mu$ M  $\gamma$ -actin WT, p.E334Q actin, or a 1:1 mixture of both (5% pyrene-labeled) in the absence and presence of Arp2/3. Shown are representative traces. The solid lines/shades represent the mean  $\pm$  SEM of at least three individual experiments. (F) Bulk-polymerization rates determined from the experiments shown in (E). Every data point corresponds to an individual polymerization experiment, as shown in (E). Data is shown as the mean  $\pm$  SEM. Individual values and differences with statistical significance are summarized in **Table 1**.



**Figure 3**

***In silico* analysis of the effects of the E334Q mutation on the actin-cofilin interaction.**

(A) Human homology model of a cytoskeletal  $\gamma$ -actin filament partially decorated with cofilin-1 (model based on PDB-ID:6UC4). Cofilin-1 is colored magenta, actin monomers are depicted in light blue and the actin residue E334 is colored orange. (B) Close up showing the details of the Van der Waals interaction between E334 and cofilin residue L111 on the pointed end side. The length of the dotted line between the side chains of actin-E334 and cofilin-L111 corresponds to 3.1 Å in our model. (C) The change in surface charge at the actin-cofilin interaction interface introduced by mutation E334Q is visualized by coulombic surface coloring.



**Figure 4**

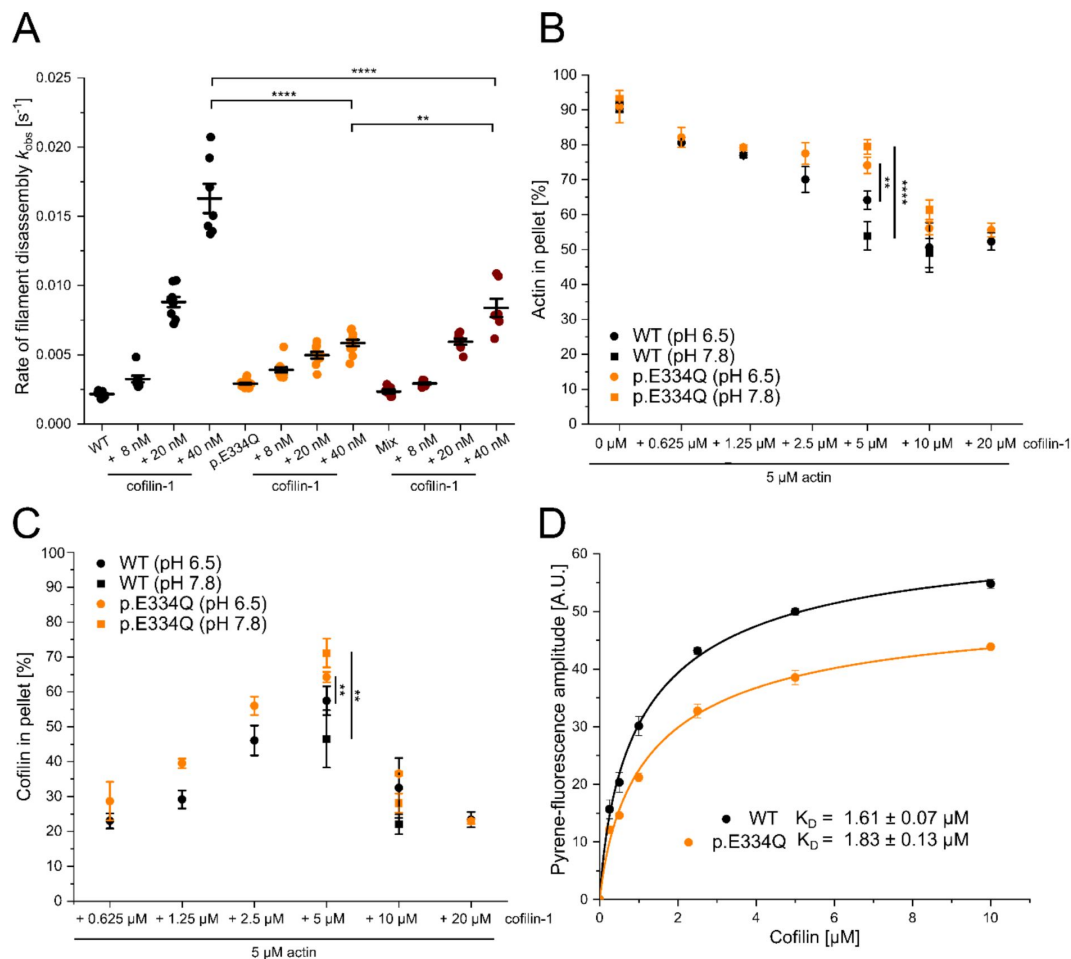
**Analysis of the effect of cofilin-1 on the spontaneous polymerization of WT and p.E334Q  $\gamma$ -actin.**

**(A)** TIRF microscopy-based observation of cofilin-1 mediated differences in the salt-induced polymerization of p.E334Q and WT  $\gamma$ -actin (1  $\mu$ M, 10% Atto-655 labeled). Shown are representative micrographs 450 s after induction of polymerization. Scale bar corresponds to 10  $\mu$ m. **(B)** Time dependence of the increase in filaments observed by TIRF microscopy. The solid and dotted lines and shades represent the mean  $\pm$  SEM of 3 individual experiments.

Next, we investigated the effect of cofilin-1 on the disassembly of preformed cytoskeletal  $\gamma$ -actin and p.E334Q filaments. The effect of cofilin-1 on existing WT and mutant filaments was initially tested by performing pyrene-based dilution-induced disassembly experiments with actin filaments polymerized for 16 hours and containing predominately ADP-actin. Cofilin-1 has previously been shown to increase the bulk-disassembly rate of actin in this experimental setup (Breitsprecher *et al*, 2011 [↗](#); Greve *et al*, 2022 [↗](#)). In the absence of cofilin, the changes in pyrene-fluorescence are best described by single exponential functions with  $k_{\text{obs}}$  values of  $0.0022 \pm 0.0002 \text{ s}^{-1}$  and  $0.0029 \pm 0.0003 \text{ s}^{-1}$  for WT and p.E334Q filaments, respectively (Figure 5A [↗](#), Suppl. Figure 3 [↗](#)). The addition of cofilin-1 increased the observed rate of the dilution-induced disassembly in a concentration-dependent manner for both mutant and WT filaments. At the highest cofilin-1 concentration used (40 nM), the change in pyrene-fluorescence with p.E334Q filaments is still best described by a single exponential function with a  $k_{\text{obs}}$  value of  $0.0059 \pm 0.0008 \text{ s}^{-1}$ . In contrast, the change for WT filaments is best-fitted to a bi-exponential function. Here, the  $k_{\text{obs}}$  values for the fast and slow phases are  $0.0163 \pm 0.0028 \text{ s}^{-1}$  and  $0.0002 \pm 0.00003 \text{ s}^{-1}$ , respectively (Figure 5A [↗](#), Suppl. Figure 3 [↗](#)). Additional experiments with heterofilaments showed milder but significant differences (Table 1 [↗](#)).

The pyrene-based bulk experiments show an evident change in the p.E334Q-cofilin interaction. However, they cannot distinguish between changes affecting filament binding and filament disassembly. To analyze the perturbed interaction of p.E334Q with cofilin-1 in more detail, we first performed high-speed co-sedimentation assays with constant concentrations of F-actin (5  $\mu\text{M}$ ) and increasing concentrations of cofilin-1 (0.0625 – 20  $\mu\text{M}$ ) at pH 6.5 and pH 7.8. Cofilin-1 binds faster but severs filaments less efficiently under slightly acidic pH conditions, whereas a weakly basic environment favors cofilin-mediated severing (Wioland *et al*, 2019 [↗](#)). We first performed experiments at pH 6.5. This endpoint experiment showed that cofilin-1 binds to and disassembles p.E334Q and WT filaments (Figure 5B, C [↗](#)). Most pellet fractions from the experiments conducted with WT filaments were found to contain less cofilin-1, as higher disassembly efficiency results in more cofilin-1 being bound to actin monomers and retained in the supernatant fraction. We repeated the experiment at pH 7.8 with an equimolar and a 2-fold excess of cofilin, (Figure 5B, C [↗](#)). In experiments with equimolar cofilin-1, we found significantly less WT actin in the pellet fraction than at pH 6.5, consistent with a higher disassembly-efficiency at pH 7.8. The shift to pH 7.8 did not have the same enhancing effect on the disassembly of p.E334Q filaments, as indicated by an increase of p.E334Q actin in the pellet fraction. The endpoint measurements show that cofilin-1 binds to p.E334Q filaments but severs them less efficiently. Next, we performed fluorescence quench experiments with pyrene-labeled WT and p.E334Q filaments, as it was previously shown that cofilin-1 efficiently quenches the fluorescence of pyrene-labeled actin filaments (Blanchoin & Pollard, 1999 [↗](#); De La Cruz, 2005 [↗](#)). We determined the change in fluorescence amplitude when 2  $\mu\text{M}$  pyrene-labeled p.E334Q or WT filaments were mixed with increasing concentrations of cofilin-1. Fitting a quadratic equation to the obtained experimental values gave only slightly different dissociation equilibrium constants of  $K_{\text{D}}$ ,  $K_{\text{D, p.E334Q}} = 1.83 \pm 0.13 \text{ }\mu\text{M}$  and  $K_{\text{D, WT}} = 1.61 \pm 0.07 \text{ }\mu\text{M}$  (Figure 5D [↗](#)). These  $K_{\text{D}}$  values are in the range of values previously determined for muscle  $\alpha$ -actin filaments (Blanchoin & Pollard, 1999 [↗](#); De La Cruz, 2005 [↗](#)), actin filaments from *Acanthamoeba castellanii* (Blanchoin & Pollard, 1999 [↗](#)) and filaments containing a mixture of human  $\psi$ - and  $\beta$ -actin (De La Cruz, 2005 [↗](#)) using the same method.

To better understand the underlying changes leading to the differences in cofilin-mediated filament disassembly efficiency, we performed two-color TIRF microscopy experiments using Atto-655 labeled WT or mutant filaments and eGFP-cofilin-1. This setup allows us to observe the binding of eGFP-cofilin-1 to individual filaments and the resulting disassembly of the filaments in real-time. eGFP-cofilin-1 was shown to bind to actin filaments and disassemble actin as efficiently as WT cofilin-1 (Wioland *et al*, 2019 [↗](#), 2017 [↗](#); Breitsprecher *et al*, 2011 [↗](#)). Immediately after mixing 300 nM “aged” actin filaments, capped at their barbed end by human capping protein (CP), with 100 nM eGFP-cofilin-1, the reaction mixture was flushed into a flow-chamber and



**Figure 5**

### Analysis of the interaction of filamentous WT and p.E334Q $\gamma$ -actin with cofilin-1.

**(A)** Pyrene-based dilution-induced depolymerization experiments (5 % pyrene-labeled actin) were used to quantify the impact of cofilin-1 on the disassembly of filaments formed by p.E334Q, WT and a 1:1 mixture of p.E334Q and WT  $\gamma$ -actin. Depolymerization was induced by adding G-buffer (pH 7.8). Every data point represents an individual experiment. Data is shown as the mean  $\pm$  SEM. **(B, C)** In addition, cofilin-1 mediated filament disassembly at different pH values (pH 6.5, pH 7.8) was investigated in co-sedimentation experiments. Shown are the means  $\pm$  SEM of at least three individual experiments. **(D)** The dissociation equilibrium constant  $K_D$  for binding of cofilin-1 to pyrene-labeled WT or p.E334Q filaments was determined by monitoring the change in fluorescence amplitude upon cofilin-1 binding. Shown are the means  $\pm$  SEM of at least three individual experiments.

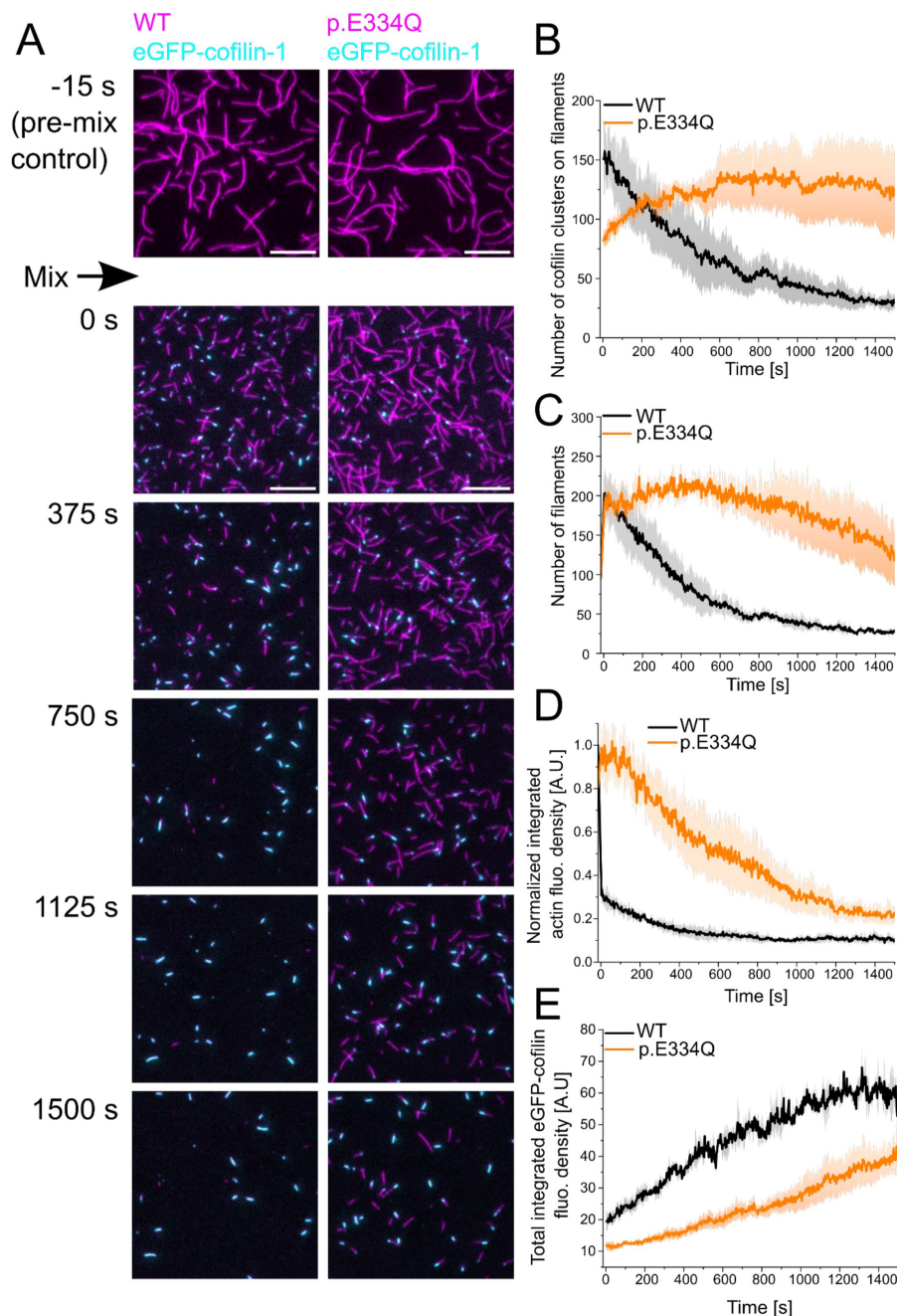
imaging was started. The delay between mixing and the start of imaging was approximately 15 seconds for each sample. At the end of the experimental dead time, almost twice as many cofilin clusters had formed in the field of view on WT filaments ( $151 \pm 29$ ) as on p.E334Q filaments ( $82 \pm 6$ ) (**Figure 6A** [↗](#)), the number of both WT and mutant actin filaments had doubled from initially about 95 to about 200 filaments (**Figure 6B** [↗](#)), and the Atto-655 fluorescence of the WT filaments had decreased to 30% of the starting value, whereas the Atto-655 fluorescence of p.E334Q filaments remained unchanged (**Figure 6C** [↗](#)). During the following 500 s, total eGFP-cofilin-1 fluorescence increased linearly, with the rate of increase being approximately twice as fast for WT as for p.E334Q filaments (**Figure 6D** [↗](#)). While the total eGFP-cofilin-1 fluorescence in the presence of p.E334Q filaments continued to increase linearly over the next 1000 seconds, the signal for WT filaments began to saturate and reached a plateau value (**Figure 6D** [↗](#)). The number of cofilin clusters on WT filaments decreased steadily during the observation period of 1500 s. In contrast, their number increased in the presence of p.E334Q filaments, reaching a maximum after 500 s, followed by a plateau of 400 s and a subsequent slow decrease (**Figure 6A** [↗](#)). The changes in the number of actin filaments showed similar time dependencies, a steady decrease for WT filaments and an initial increase for p.E334Q filaments followed immediately by a linear decrease without an intervening plateau phase (**Figure 6B** [↗](#)). For WT and p.E334Q filaments, the size of eGFP-cofilin-1 clusters on the steadily shrinking filaments expanded continuously until either severing occurred or the actin filament was fully decorated. No severing events were observed with fully decorated filaments.

## Impact of E334Q mutation on interactions with myosin family proteins

The precise nature of the six actin-binding elements in the myosin motor domain varies considerably among myosin isoforms (Sellers, 2000 [↗](#); Cope *et al*, 1996 [↗](#)). The associated sequences have conserved hydrophobic and electrostatic properties, variations of which contribute to the tuning of kinetic and functional properties. Actin residue E334 interacts with a positively charged patch in the cardiomyopathy loop (CM-loop) of the myosin upper 50 kDa domain of NM2C and Myo5A (Pospich *et al*, 2021 [↗](#); Von Der Ecken *et al*, 2016). In the case of NM2C, the electrostatic interaction of E334 with K429 in the CM-loop is further stabilized by van der Waals interactions with I420 and V427 (**Figure 7** [↗](#)). A lysine residue corresponding to K429 in NM2C is present in all human myosin-2 and myosin-5 isoforms. During force-generating interactions with mutant actin filaments, the weakening of complementary electrostatic interactions in this region of the actomyosin interface is likely to attenuate the binding affinity of class 2 and 5 myosins as well as the coupling between their actin and nucleotide binding sites.

Cytoskeletal  $\gamma$ -actin filaments move with an average sliding velocity of  $195.3 \pm 5.0 \text{ nm s}^{-1}$  on lawns of surface immobilized NM2A-HMM molecules (**Figure 8A, B** [↗](#)). Filaments formed by p.E334Q actin move 5-fold slower, resulting in an observed average sliding velocity of  $39.1 \pm 3.2 \text{ nm/s}$ . Filaments copolymerized from a 1:1 mixture of WT and p.E334Q actin move with an average sliding velocity of  $131.2 \pm 10 \text{ nm s}^{-1}$  (**Figure 8A, B** [↗](#)).

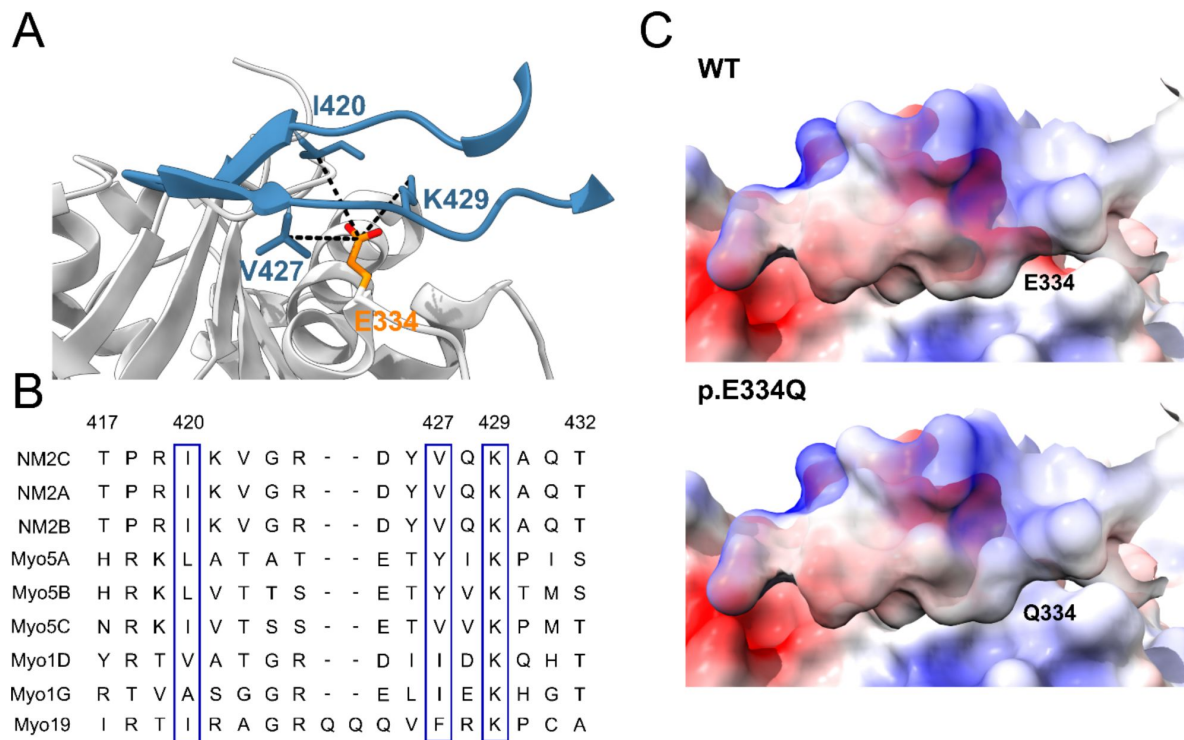
While we observed similar densities of surface-attached WT and mutant filaments, we noticed increased rapid dissociation and association events for p.E334Q filaments (**Suppl. Video 7-9**). This prompted us to investigate the impact of the mutation on actomyosin-affinity using transient-kinetic approaches. We determined the dissociation rate constants using a single-headed NM2A-2R construct (**Figure 8C** [↗](#)). NM2A-2R is a constitutively active motor-domain construct generated by fusing the motor-domain of NM2A to two spectrin-like repeats serving as a rigid lever arm extension (Anson *et al*, 1996 [↗](#)). The construct is ideally suited for the kinetic characterization of the NM2-isoforms (Heissler & Manstein, 2011 [↗](#)). The dissociation rate constant  $k_A$  is 5.6-fold increased from  $0.08 \times 10^{-3} \text{ s}^{-1}$  to  $0.45 \times 10^{-3} \text{ s}^{-1}$  for NM2A-2R when p.E334Q filaments are used. A similar change from  $0.31 \times 10^{-3} \text{ s}^{-1}$  to  $1.70 \times 10^{-3} \text{ s}^{-1}$  was observed for the analogous NM2C-2R



**Figure 6**

### Analysis of the interaction of WT and p.E334Q $\gamma$ -actin with eGFP-cofilin-1

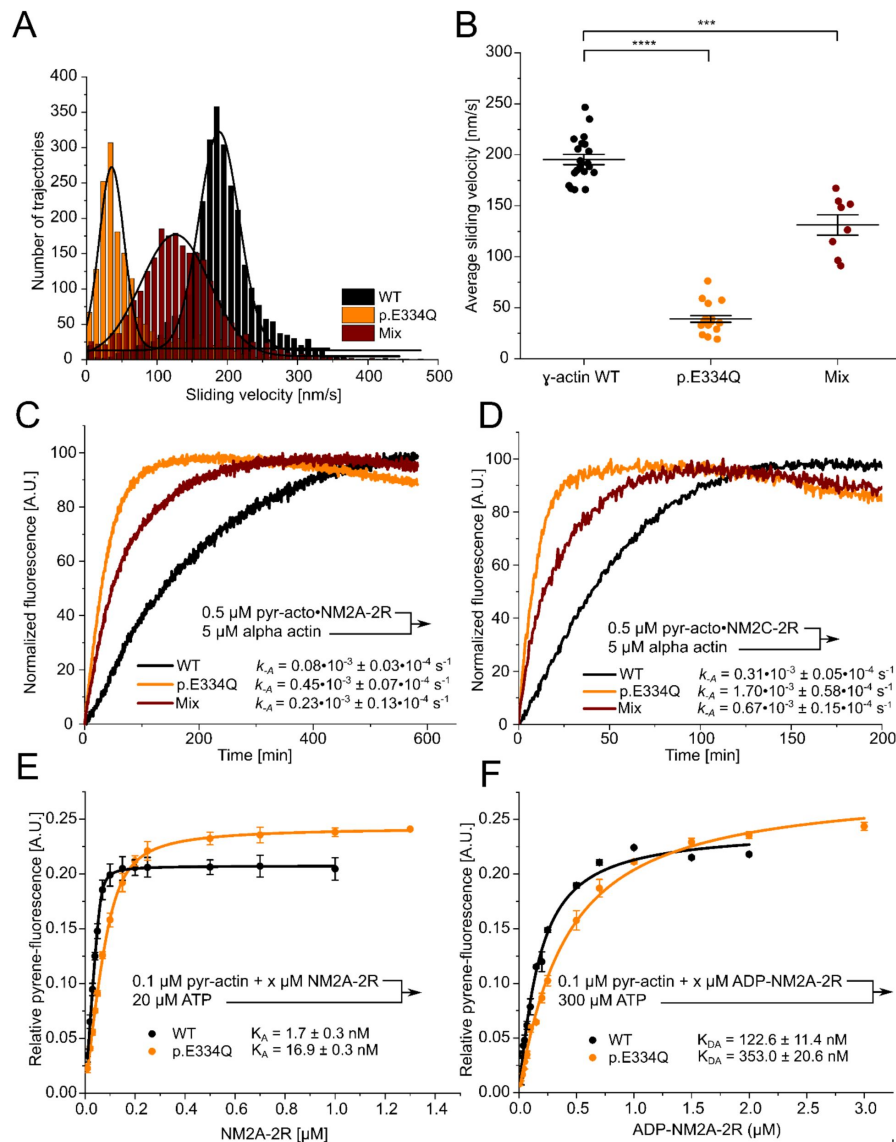
**(A)** The disassembly of 300 nM “aged” WT and p.E334Q F-actin (10% Atto-655 labeled, capped by CP) in the presence of 100 nM eGFP-cofilin-1 was visualized using two-color TIRF microscopy. F-actin is shown in magenta, eGFP-cofilin in cyan. Shown are representative micrographs at the indicated time points. Scale bar corresponds to 10  $\mu$ m. **(B)** Time dependence of the change in the number of eGFP-cofilin-1 clusters on actin filaments. The solid lines and shades represent the mean  $\pm$  SEM of three individual experiments. **(C)** Time dependence of the decrease in filament count. The solid lines and shades represent the mean  $\pm$  SEM of three individual experiments. **(D)** Temporal tracking of actin filament disassembly by monitoring changes in total actin fluorescence per image frame. The solid lines and shades represent the mean  $\pm$  SEM of three individual experiments. **(E)** Visualization of temporal changes in the abundance of eGFP-cofilin-1 clusters based on monitoring total eGFP-cofilin-1 fluorescence per image frame. The solid lines and shades represent the mean  $\pm$  SEM of three individual experiments.



**Figure 7**

***In silico* analysis of the effects of the E334Q mutation on the actin–myosin interaction.**

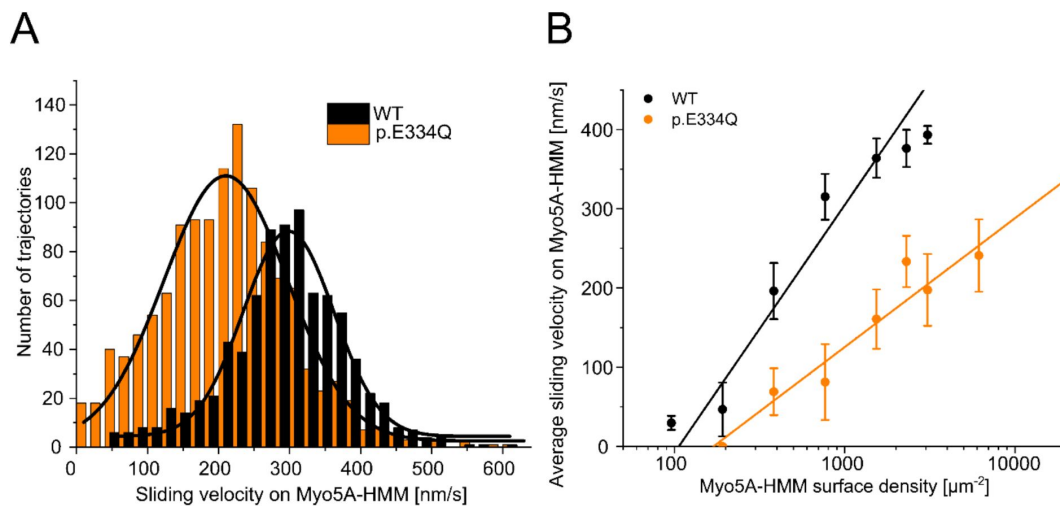
**(A)** Contact region between the CM-loop of NM2C (blue) and residue E334 (orange) in cytoskeletal  $\gamma$ -actin (PDB-ID: 5JLH) (Von Der Ecken *et al.*, 2016). Actin interacts with a core contact triad in the myosin CM-loop, which in the case of NM2C consists of lysine K429 and hydrophobic residues I420 and V427. **(B)** Multiple sequence alignment of the CM-loop of selected cytoskeletal myosin isoforms. The alignment shows complete sequence identity for NM2C, NM2A and conservation of residues contributing to the core contact triad in class 5, class 19 and selected members of class 1 myosins (blue boxes). Residue numbering refers to the NM2C primary structure. **(C)** Mutation E334Q leads to a weakening of complementary electrostatic interactions. The interaction interface involving the CM-loop of NM2C and the region affected by the mutation on cytoskeletal  $\gamma$ -actin is visualized by coulombic surface coloring. Positive potentials are shown in blue, and negative potentials in red.



**Figure 8**

### Analysis of the interaction of human NM2A with WT and p.E334Q γ-actin.

**(A)** The interaction of surface-immobilized NM2A-HMM with WT γ-actin filaments, p.E334Q γ-actin filaments and heterofilaments (1:1 mixture) was analyzed using the unloaded *in vitro* motility assay. Representative velocity distributions are obtained from recorded trajectories of WT and mutant filaments in a single experiment. The average sliding velocity of the filaments in each experiment was determined by applying a Gaussian fit (black line) to the obtained velocity distributions. **(B)** Secondary plot of all measured sliding velocities. Each data point represents a single experiment (N=20 for WT filaments, N=18 for p.E334Q filaments, N=8 for heterofilaments) in which the sliding velocities of a minimum of 600 filaments have been analyzed. The average sliding velocity in each experiment was determined as described in (A). **(C, D)** Determination of the dissociation rate constant ( $k_A$ ) for the interaction of NM2A-2R (C) or NM2C-2R (D) with p.E334Q or WT γ-actin in the absence of nucleotide.  $k_A$  was determined in displacement experiments where unlabeled α-skeletal actin was added to pyrene-labeled actin•NM2A-2R/NM2C-2R. Shown are representative traces. The shown  $k_A$  values are the results of 5–11 individual experiments using single-exponential fit functions. **(E, F)** The affinity of WT and p.E334Q γ-actin for NM2A-2R in the absence ( $K_A$ ) and in the presence of saturating ADP concentrations ( $K_{DA}$ ) was determined using the method developed by Kurzwaga and Geeves (Kurzwaga & Geeves, 1996). Increasing concentrations of myosin were dissociated from pyrene-labeled WT or p.E334Q F-actin by mixing the complex with excess ATP. The obtained fluorescence amplitudes of the individual transients were plotted against the corresponding myosin concentrations. A quadratic equation (see Material and Methods) was fitted to the data, which yields the respective affinity. Data is shown as the mean ± SD of three individual experiments.



**Figure 9**

**Analysis of the interaction of human Myo5A-HMM with WT and p.E334Q  $\gamma$ -actin in the unloaded *in vitro* motility assay at different Myo5A surface densities.**

(A) The interaction of surface-immobilized Myo5A-HMM with WT  $\gamma$ -actin and p.E334Q  $\gamma$ -actin filaments was analyzed using the unloaded *in vitro* motility assay. Representative velocity distributions were obtained from one individual experiment at the optimal Myo5A-HMM surface density for WT ( $770 \mu\text{m}^{-2}$ ) and p.E334Q filaments ( $3100 \mu\text{m}^{-2}$ ). The average sliding velocity of the filaments in each experiment was determined by applying a Gaussian fit (black line) to the obtained velocity distributions. (B) Semi-logarithmic plot summarizing all measured sliding velocities at different Myo5A-HMM surface densities. Each data point is the result of 3–10 individual experiments. The dependence of the average sliding velocity on surface motor density is best-described in the semi-logarithmic graph by lines up to approximately  $1000 \text{ motors } \mu\text{m}^{-2}$  for WT filaments and  $7000 \text{ motors } \mu\text{m}^{-2}$  for p.E334Q filaments.

construct (**Figure 8D** [↗](#)). Next, we determined the affinities of NM2A-2R for WT and p.E334Q filaments in the absence of nucleotide ( $K_A$ ) and the presence of saturating concentrations of ADP ( $K_{DA}$ ). We performed affinity–titration experiments with pyrene–labeled, phalloidin–stabilized filaments and NM2A-2R (Kurzawa & Geeves, 1996 [↗](#)). The values measured under rigor conditions show a 9.9-fold reduced affinity of NM2A-2R for p.E334Q filaments ( $K_A = 16.9 \pm 1.3$  nM) compared to WT filaments ( $K_A = 1.7 \pm 0.3$  nM) (**Figure 8E** [↗](#)). When the experiment was performed in the presence of saturating concentrations of ADP, we obtained values for  $K_{DA}$  of  $353.0 \pm 20.6$  nM in the presence of p.E334Q filaments and  $122.6 \pm 11.4$  nM with WT filaments (**Figure 8F** [↗](#)). The apparent ATP–affinity ( $1/K_1$ ) of NM2A in complex with WT and p.E334Q filaments showed no significant differences (**Table 1** [↗](#)).

Next, we investigated the effects of the E334Q mutation on the motile activity of the processive motor Myo5A. Since the lysine residue corresponding to K429 in the CM loop of NM2C is also present in all human myosin 5 isoforms, the resulting changes in binding strength and thermodynamic coupling are expected to reduce the motor activity of Myo5A upon interactions with p.E334Q filaments. Structural studies have shown that the interaction between actin residue E334 and the CM-loop in the Myo5A motor domain is maintained from the rigor to the ADP-bound state (Pospich *et al*, 2021 [↗](#)). We performed unloaded *in vitro* motility assays to evaluate the effect of the mutation on the interaction with Myo5A–HMM. The rapid transition from weak to strong binding with a slow and rate-limiting ADP release leading to a high working ratio (De La Cruz *et al*, 1999 [↗](#)) allows the *in vitro* motility assay to be performed on surfaces with a low motor density. In initial experiments, we found a Myo5A–HMM surface density of 770 molecules  $\mu\text{m}^{-2}$  to be optimal for experiments with WT filaments. Movement was steady and filament breaks were practically non-existent at 770 molecules  $\mu\text{m}^{-2}$ , while higher surface densities led to increased filament breaks, resulting in rapid fragmentation. At all Myo5A–HMM surface densities used, the sliding velocity was significantly reduced for p.E334Q compared to WT filaments. In experiments with p.E334Q filaments, a 4-fold higher Myo5A–HMM surface density was required to achieve a similar actin surface decoration and optimal motile activity. Furthermore, filament breaks occurred less frequently, indicating a less efficient actomyosin interaction. The number of moving filaments and their sliding velocity decreased sharply below surface densities of 770 molecules/ $\mu\text{m}^2$  for WT filaments and 3100 molecules  $\mu\text{m}^{-2}$  for p.E334Q filaments. Below a surface density of 380 Myo5A–HMM molecules  $\mu\text{m}^{-2}$ , no productive attachment events were observed with p.E334Q filaments. Thus, mutation E334Q in cytoskeletal  $\psi$ -actin leads to an impairment of the interaction of the actin filament with Myo5A, similar to the changes observed with NM2A and NM2C, but with additional manifestations specific to processive motors.

## Discussion

At present, the exact pathogenesis of non-muscular actinopathies remains poorly understood. A rigorous molecular characterization of the contribution of disturbances in actin dynamics, contractility and transport processes is necessary to develop an understanding of the diverse disease patterns.

In the past, the functional characterization of disease-associated mutations in cytoskeletal  $\psi$ -actin focused on mutations p.T89I, p.K118N/M, p.E241K, p.T278I, p.P264L, p.P332A, and p.V370A, which all cause non-syndromic hearing loss. Two mutants showed abnormal polymerization behavior, indicated by a reduced polymerization rate (p.V370A) (Bryan *et al*, 2006 [↗](#)) or an increased propensity for bundle formation (p.E241K) (Morín *et al*, 2009 [↗](#)). The mutants p.K118N/M showed a perturbed interaction with the Arp2/3 complex, reducing branching efficiency (Kruth & Rubenstein, 2012 [↗](#); Jepsen *et al*, 2016 [↗](#)). A significantly reduced protein stability was observed for two mutant proteins (p.P264L, p.P332A) (Bryan *et al*, 2006 [↗](#)). Perturbed interactions with cofilin were observed for a range of mutations. p.T89I, p.E241K, p.T278I, and p.V370A are characterized by an increased sensitivity to cofilin-mediated filament severing (Bryan &

Rubenstein, 2009 [↗](#)). Interestingly, mutation p.P332A, close to the analyzed mutation p.E334Q, enhances resistance towards cofilin-mediated severing (Bryan & Rubenstein, 2009 [↗](#)). The perturbed cofilin interaction is the only overlap between the spectrum of molecular phenotypes observed for syndromic deafness-associated mutations and mutation p.E334Q, which results in a non-syndromic actinopathy. It has to be noted that all cited studies relied on the use of actin purified from budding yeast for their biochemical studies, which shows 91% sequence identity to human cytoskeletal  $\psi$ -actin (Ng & Abelson, 1980 [↗](#)).

Here, we describe mutation E334Q in cytoskeletal  $\gamma$ -actin that leads to a syndromic actinopathy with hypotonia and is not associated with non-syndromic hearing loss. Our biochemical analysis shows that the mutation does not affect the folding of the actin monomer or the stability of the monomer and the resulting filaments, demonstrating that mutations in the hinge region do not necessarily affect protein stability. The mutant protein displays a robust interaction with the VCA-activated Arp2/3 complex. Its polymerization kinetics are equivalent to those of the WT protein, with which it effectively copolymerizes *in vitro*. Based on these observations, we exclude functional haploinsufficiency as a potential disease mechanism and favor a mechanism in which p.E334Q integrates into the cytoskeletal actin networks of the cell but shows defects in the interaction with key binding partners. Consistent with this hypothesis and based on several structural studies (Pospich *et al*, 2021 [↗](#); Behrmann *et al*, 2012 [↗](#); Robert-Paganin *et al*, 2021 [↗](#); Risi *et al*, 2021 [↗](#); Fujii & Namba, 2017 [↗](#); Gyimesi *et al*, 2020 [↗](#); Gong *et al*, 2022 [↗](#); Gurel *et al*, 2017 [↗](#); Von Der Ecken *et al*, 2016) as well as our initial modeling of complexes formed by p.E334Q-filaments, we observed perturbed interactions with cofilin-1 and human cytoskeletal myosin isoforms NM2A, NM2C and Myo5A (Table 1 [↗](#)).

We interpret the observed changes in the interaction with NM2A, NM2C and Myo5A to primarily reflect the weakening of complementary interactions between actin residue E334 and a core contact triad in the myosin CM-loop, which in the case of NM2C consists of K429 and hydrophobic residues I420 and V427 (Figure 7 [↗](#)). Analogous changes in the interaction of mutant filaments with Myo1D, Myo1G, NM2B, Myo5B, Myo5C and Myo19 are expected due to the presence of the core contact triad residues in their CM-loops (Figure 7B [↗](#)). In contrast, the absence of a basic residue corresponding to K429 in NM2C and additional changes affecting their CM loop structure make it difficult to predict the consequences for the interaction of p.E334Q-filaments with the cytoskeletal myosin isoforms Myo1A, Myo1C, Myo1E, Myo1F, Myo3A, Myo3B, Myo15, Myo18A, Myo18B and Myo19.

In addition to primary consequences, reflected in the observed changes in the binding interactions with NM2A and cofilin-1, our results point to significant changes that result from allosteric perturbations. The results from our transient-kinetic analysis of the complexes formed by p.E334Q with NM2A-2R indicate that reduced allosteric coupling between the actin and nucleotide binding sites within the myosin motor domain contributes to the observed decrease in motor activity. The coupling efficiency is given by the ratio of the dissociation equilibrium constants for actin binding in the absence ( $K_A$ ) and presence of ADP ( $K_{DA}$ ). The value for  $K_{DA}/K_D$  obtained for the interaction of NM2A-2R in the presence of p.E334Q (20.9) is 3.5-fold lower than the value measured in the presence of WT actin (Table 1 [↗](#)). Allosteric perturbations are also major contributors mediating the impact of mutation E334Q to the changes in elastic properties and the rate of actin filament disassembly in the presence of cofilin-1. Our results are consistent with the E334Q mutation slowing the initial complex formation due to less favorable charge interactions and allosteric perturbations having a major impact on subsequent steps.

Patients carrying mutations in *ACTB* or *ACTG1* predominately suffer from developmental disabilities, which can be directly linked to cortical malformations of varying severity. Therefore, neuronal cells and/or neuronal progenitor cells are assumed to be the most affected cell population in the patients (Verloes *et al*, 2015 [↗](#); Di Donato *et al*, 2016 [↗](#); Rivière *et al*, 2012 [↗](#)). The actin composition of the neuron is thought to reach ratios between 1:1 and 1:2 for  $\psi$ -actin to

$\beta$ -actin in the mature state after fluctuations during neuronal development (Cheever & Ervasti, 2013). Studies of the spatial localization of the cytoskeletal actin isoforms in the mature neuron are contradictory. A study showing that  $\beta$ - and  $\psi$ -actin strongly colocalize in hippocampal and cerebral neurons (Cheever *et al.*, 2012) appears to contradict previous studies, which reported spatial enrichment of  $\beta$ -actin in dynamic structures such as the dendritic spine and exclusion from less dynamic structures such as the axon shaft, where  $\psi$ -actin was found to be enriched (Weinberger *et al.*, 1996; Micheva *et al.*, 1998; Bassell *et al.*, 1998). Based on our results, we conclude that p.E334Q actin is readily produced and integrated into the cytoskeletal actin networks of neuronal cells, where it perturbs interactions with cytoskeletal myosin isoforms that play essential roles in the function and maintenance of healthy neurons. The functions of myosin-5 isoforms in neuronal cells are all linked to their role as actin barbed-end directed processive cargo transporters (Hammer & Wagner, 2013). Several of the myosin-5 driven transport-processes are important contributors to synaptic plasticity, i.e. the ability of the neurons to change synaptic strength based on previous activities (Hammer & Wagner, 2013).

The different NM2 isoforms are found in a wide range of cell types in the brain, but they show varying spatial expressions (Murakami & Elzinga, 1992; Golomb *et al.*, 2004; Rochlin *et al.*, 1995; Kawamoto & Adelstein, 1991). NM2A is predominately enriched in the vasculature of the brain, consistent with the importance of NM2A for angiogenesis (Ma *et al.*, 2020). NM2B and NM2C are mainly found in growth cones and dendritic spines of neuronal cells (Murakami & Elzinga, 1992; Golomb *et al.*, 2004; Rochlin *et al.*, 1995). NM2 isoforms are known to contribute to various neuronal processes, but unlike for Myo5A, their exact role is less well understood. NM2B was shown to determine the maturation and morphology of the dendritic spines in hippocampal neurons and was additionally linked to basal synaptic function and plasticity (Hodges *et al.*, 2011; Ma *et al.*, 2004; Rex *et al.*, 2010). NM2C and NM2B contribute to the differentiation of oligodendrocytes (Domingues *et al.*, 2020). The NM2 isoforms compete with proteins of the ADF/cofilin family for binding to F-actin and this competition is essential for stable actomyosin contractility and an intact cell cortex (Wiggin *et al.*, 2012).

In line with this, ADF/cofilin family members are highly abundant in growth cones and dendritic spines and essential for spine morphology and determination of spine length by modulating actin turnover (Star *et al.*, 2002; Hotulainen *et al.*, 2009; Namme *et al.*, 2021). Cofilin-mediated severing and nucleation were previously proposed to synergistically contribute to global actin turnover in cells (Andrianantoandro & Pollard, 2006; Du & Frieden, 1998). Our results show that the mutation affects these different cofilin functions in actin dynamics in opposite ways. Cofilin-mediated filament nucleation is more efficient for p.E334Q monomers, while cofilin-mediated severing of filaments containing p.E334Q is significantly reduced. The interaction of both actin monomers and actin filaments with ADF/cofilin proteins involves several distinct overlapping reactions. In the case of actin filaments, cofilin binding is followed by structural modification of the filament, severing and depolymerizing the filament (De La Cruz & Sept, 2010). Cofilin binding to monomeric actin is followed by the closure of the nucleotide cleft and the formation of stabilized “long-pitch” actin dimers, which stimulate nucleation (Andrianantoandro & Pollard, 2006).

While the number of disease-associated mutations reported for *ACTB* and *ACTG1* is steadily increasing, the total number of patients affected remains low compared to patients with disease-associated mutations in muscle actin isoforms (Parker *et al.*, 2020). High sequence and structure conservation across all actin isoforms enable us to extrapolate our findings obtained with cytoskeletal  $\gamma$ -actin to diseases associated with mutations of E334 in other actin isoforms. Mutations of residue E334 in  $\alpha$ -skeletal actin (encoded by *ACTA1*) are reported to result in nemaline myopathy (E334K, (Laing *et al.*, 2009)) or core myopathy (E334A, (Kaindle *et al.*, 2004)) (reviewed in (Parker *et al.*, 2020)). The mutations either lead to the inversion of the charge at residue 334 (E334K) or the complete loss of charge (E334A). Our study shows that loss of charge at actin residue 334 leads to the loss of a conserved core contact between actin and the myosin motor

domain of cytoskeletal class 2 myosin. Skeletal muscle myosin-2 is a central component of the sarcomere. The myosin forms the thick filaments that interact with the thin filaments formed by actin and regulatory proteins, generating force that powers muscle contraction (Sweeney & Hammers, 2018 [DOI](#)). Because the core contact triad that interacts with actin residue E334 is present in all class 2 myosin isoforms, it is reasonable to assume that mutations of residue E334 in  $\alpha$ -skeletal actin affect the interaction with skeletal muscle myosin 2 to a similar extent, resulting in less efficient force generation and thereby contributing to the development of myopathy in patients.

Interestingly, a recent study analyzing the prevalence of cytoskeletal actin mutations in cancer using the cBioPortal database found that mutations in *ACTB* and *ACTG1* are associated with lymphoid cancers, specifically mutations in *ACTB* with diffuse large B-cell lymphoma and mutations in *ACTG1* with multiple myeloma. When the authors extended the analysis to all cancer types, they found that the observed mutations in the two genes were scattered across the entire actin sequence, with no apparent preference for a particular region. Nevertheless, the group identified residue E334 in *ACTB* as a potential hotspot, as the missense variants p.E334Q/p.E334K are more frequent in all analyzed datasets (Witjes *et al*, 2020 [DOI](#)). In this paper, we investigated the impact of the E334Q exchange on the function of cytoskeletal  $\gamma$ -actin in the context of developmental diseases. However, the high sequence similarity between the cytoskeletal actin isoforms makes it highly likely that the E334Q mutation in cytoskeletal  $\beta$ -actin results in similar molecular perturbations of actin dynamics but with potentially different consequences for the organism due to varying enrichment of the two isoforms in functional compartments of the actin cytoskeleton. Although future studies are needed to determine the role of *ACTB* and *ACTG1* mutations as driver or passenger mutations in different cancers, our data show a possible link between impaired cytoskeletal actomyosin dynamics, cofilin-mediated actin turnover and cancer development.

## Material and Methods

### Plasmids

The coding sequence of human cytoskeletal  $\gamma$ -actin (Uniprot-ID: P63261) was fused via a C-terminal linker to a His<sub>6</sub>-tagged thymosin  $\beta$ 4 moiety (Uniprot-ID: P62328), as previously described for yeast actin (Uniprot-ID: P60010) (Noguchi *et al*, 2007 [DOI](#)). The fusion protein was cloned into the multiple cloning site of the pFastBac-Dual vector under the control of the polyhedrin promoter. The plasmid encoding the mutant protein p.E334Q was generated by performing site-directed mutagenesis on the WT plasmid. Motor domain carrying fragments of human NM2A, NM2C, and Myo5A were produced using MYH9, MYH14, and MYO5A heavy chain sequences, respectively (Uniprot-IDs: P35579, Q7Z406, Q9Y4I1). For the production of HMM-like constructs of human NM2A and human Myo5A, expression vectors derived from pFastBac were used, as described previously (Hundt *et al*, 2016 [DOI](#); Reindl *et al*, 2022 [DOI](#)). NM2A-HMM was co-produced with human light chains MYL6a (Uniprot-ID: P60660) and MYL12b (Uniprot-ID: O14950). Myo5A HMM was co-produced with human calmodulin (Uniprot-ID: P0DP23). NM2A-2R and NM2C-2R, single-headed, constitutively active motor domain constructs of human NM2A and NM2C-2C0 with an artificial lever arm region, were produced using pFastBac-derived expression vectors, as described previously (Heissler & Manstein, 2011 [DOI](#); Müller *et al*, 2013 [DOI](#)). Human cofilin-1 (Uniprot-ID: P23528) was cloned into the expression vector pGEX-6P-2 (Greve *et al*, 2022 [DOI](#)). The human Arp2/3 complex consisting of subunits Arp2 (Uniprot-ID: P61160), Arp3 (Uniprot-ID: P61158), ArpC1B (Uniprot-ID: O15143), ArpC2 (Uniprot-ID: O15144), ArpC3A (Uniprot-ID: O15145), ArpC4 (Uniprot-ID: P59998), ArpC5 (Uniprot-ID: O15511) and a C-terminal FLAG tag on ArpC3A was produced using the expression vector pBIG2abc, provided by Roberto Dominguez (University of Pennsylvania, Philadelphia, USA). The VCA domain of human N-WASP (Uniprot-ID: O00401) was cloned into pGEX-6P-2. Human capping protein (subunits  $\alpha$ 1/ $\beta$ 2, Uniprot-ID: P52907/P47756) in pET-Duet-1

and eGFP-tagged human cofilin-1 (Uniprot-ID: P23528) in pGEX-6P-2 vector were provided by Jan Faix (Medizinische Hochschule Hannover, Hannover, Germany). Recombinant bacmids and viruses for the production of proteins in the Sf9 system were produced according to the *Bac-to-Bac* baculovirus expression system protocol (Thermo Fisher Scientific, Waltham, MA, USA).

## Protein production and purification

We produced recombinant human cytoskeletal  $\gamma$ -actin and p.E334Q mutant protein as actin-thymosin  $\beta$ 4-His<sub>6</sub> fusion protein using the baculovirus/*S. frugiperda* insect cell expression system. The C-terminal native actin residue is a phenylalanine, which provides a cleavage site for chymotrypsin to remove downstream tag sequences completely. The tag sequence employed consists of a 14-amino acid linker (ASSGSGSGSGGA), 43 thymosin- $\beta$ 4 residues, followed by 6 histidine residues. To produce WT and mutant fusion proteins,  $2 \times 10^9$  cells were harvested 72 hours after infection. Cells were pelleted, washed with PBS, shock frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Cell pellets from two liters of Sf-9 culture were thawed for a typical preparation. For lysis, cells were resuspended in lysis buffer-A (10 mM Tris pH 7.8, 5 mM  $\text{CaCl}_2$ , 10 mM imidazole, 1.25% Triton X-100, 100 mM KCl, 7 mM  $\beta$ -mercaptoethanol, 1 mM ATP, 1 mM PMSF, 100  $\mu\text{g/mL}$  TAME, 80  $\mu\text{g/mL}$  TPCK, 2  $\mu\text{g/mL}$  pepstatin, 5  $\mu\text{g/mL}$  leupeptin) and sonicated. The lysate was cleared by centrifugation at  $30,000 \times g$  (30 min,  $4^\circ\text{C}$ ). The cleared lysate was loaded onto 3 mL equilibrated PureCube 100 NiNTA agarose (Cube Biotech, Mannheim am Rhein, Germany) and incubated for two hours under constant rotation. Afterwards, the material was washed extensively with 10 mM Tris pH 7.8, 0.2 mM  $\text{CaCl}_2$ , 10 mM imidazole, 50 mM KCl, 1 mM ATP and the actin-thymosin  $\beta$ 4 fusion protein was eluted with 10 mM Tris pH 7.8, 250 mM imidazole, 0.2 mM  $\text{CaCl}_2$ , 1 mM ATP. Peak fractions were pooled and dialyzed overnight against G- buffer (10 mM Tris pH 7.8, 0.2 mM  $\text{CaCl}_2$ , 0.1 mM ATP, 0.5 mM DTT). The next day, protein aggregates were removed by centrifugation at  $15,000 \times g$  (20 min,  $4^\circ\text{C}$ ). The supernatant was pooled and  $\alpha$ -chymotrypsin (Sigma-Aldrich, Schnelldorf, Germany) was added at a w/w ratio of 1:300 (chymotrypsin:actin) to remove the tag-sequences. The optimal digestion time varied from 45 to 80 min and needs to be determined for each batch of  $\alpha$ -chymotrypsin. PMSF was added to a final concentration of 0.2 mM to stop the digestion. Actin was separated from the thymosin-His tag using polymerization-depolymerization steps. Unless otherwise stated, all protein purification steps were carried out at  $4^\circ\text{C}$ . F-actin was stored on ice. G-actin was concentrated to approximately 150  $\mu\text{M}$ , flash frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  in G-buffer. Clarified G-actin, free of aggregates and oligomers, was always prepared fresh. G-actin stocks were thawed, diluted in G-buffer to the appropriate concentration, and centrifuged at  $136,000 \times g$  (15 min,  $4^\circ\text{C}$ ). The top 2/3 of the centrifuged sample was carefully removed for use in further experiments. All myosin constructs were produced in the baculovirus/*S. frugiperda* insect cell expression system and purified as previously described for NM2A-HMM (Greve *et al.*, 2022 [DOI](#); Hundt *et al.*, 2016 [DOI](#); Pathan-Chhatbar *et al.*, 2018 [DOI](#)), Myo5A-HMM (Reindl *et al.*, 2022 [DOI](#)), the single-headed, constitutively active constructs NM2A-2R (Heissler & Manstein, 2011 [DOI](#); Müller *et al.*, 2013 [DOI](#)) and NM2C-2R (Heissler & Manstein, 2011 [DOI](#); Müller *et al.*, 2013 [DOI](#)). Production of the human Arp2/3 complex in insect cells followed the procedure described in Zimmet *et al.* (Zimmet *et al.*, 2020 [DOI](#)). Human cofilin-1 and human eGFP-cofilin-1 were produced as GST-fusion proteins in Rosetta2 (DE3) cells. Purification was carried out as previously described for human cofilin-1 (Greve *et al.*, 2022 [DOI](#)).

Human GST-N-WASP-VCA was produced in ArcticExpress (DE3) cells. The cells were lysed by treatment with lysozyme followed by sonification in lysis buffer-W (25 mM Tris pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 1 mM PMSF). The cleared cell lysate was loaded onto a self-packed GSH-Sepharose column. The column was then washed with five column volumes of wash buffer-W (25 mM TRIS pH 7.4, 50 mM NaCl, 1 mM EDTA, 1 mM DTT). GST-N-WASP-VCA was eluted from the column by applying 10 mM glutathione in wash buffer-W. The eluted protein was concentrated and loaded onto a S75 16/600 size exclusion chromatography column (GE Healthcare, Chicago, USA). After chromatography, fractions containing pure protein were pooled, concentrated and the concentration was determined via absorbance at 280 nm. The GST-N-WASP-VCA protein was frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ .

Human capping protein ( $\alpha 1/\beta 2$ ) carrying a 6×His-tag was produced in Rosetta2 (DE3) cells. For purification, cells were lysed by lysozyme followed by sonification in lysis buffer-CP (25 mM Tris pH 7.8, 250 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1.5 % Triton X-100, 10 mM imidazole, 7 mM beta-mercaptoethanol). The cleared lysate was loaded onto PureCube 100 NiNTA agarose (Cube Biotech, Monheim am Rhein, Germany) in a gravity-flow column. The agarose was washed with wash buffer-CP (25 mM Tris pH 7.8, 250 mM NaCl, 30 mM imidazole) and the protein was then eluted with elution buffer-CP (25 mM Tris pH 7.8, 50 mM NaCl, 250 mM imidazole). Fractions with pure capping protein were pooled, concentrated and loaded onto a S200 16/600 size exclusion chromatography column (GE Healthcare, Chicago, USA). The purified protein was frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

$\alpha$ -skeletal actin (Uniprot-ID: P68139) was prepared from chicken pectoralis major muscle, as previously described for rabbit  $\alpha$ -skeletal actin (Lehrer & Kerwar, 1972 [\[1\]](#)).

Unless otherwise stated, all purification steps were performed at  $4^{\circ}\text{C}$ .

## DNase-I inhibition assay, DSF and nucleotide exchange assay

The DNase-I inhibition assay and DSF to study actin folding and thermal stability were performed as previously described (Greve *et al*, 2022 [\[2\]](#)).

Nucleotide exchange experiments were performed with freshly clarified G-actin as follows. The G-actin solution was passed through a Zeba™ spin desalting column (Thermo Fisher Scientific, Waltham, MA, USA) equilibrated with G-buffer to remove excess nucleotide. The sample was then incubated with a 2-fold excess of  $\epsilon$ -ATP or  $\epsilon$ -ADP (Jena Bioscience, Jena, Germany) for two hours on ice. After incubation, excess nucleotide was removed by passing the sample through a Zeba™ spin desalting column equilibrated with 10 mM TRIS pH 8.0 and 10  $\mu\text{M}$   $\epsilon$ -ATP/  $\epsilon$ -ADP. The concentration of the  $\text{Ca}^{2+}$ -  $\epsilon$ -ATP/ $\epsilon$ -ADP-G-actin solution was determined using the Coomassie protein assay (Thermo Fisher Scientific, Waltham, MA, USA). Prior to the nucleotide exchange experiment the  $\text{Ca}^{2+}$ - $\epsilon$ -ATP/ $\epsilon$ -ADP- G-actin solution was supplemented with 10×  $\text{Mg}^{2+}$ -exchange buffer (1 mM  $\text{MgCl}_2$ , 10 mM EGTA) to a final concentration of 0.1 mM  $\text{MgCl}_2$  and 1 mM EGTA. The resulting solution was rapidly mixed 1:1 with assay buffer-SF containing excess unlabeled ATP (10 mM TRIS pH 7.8, 0.2 mM  $\text{MgCl}_2$ , 0.2 mM ATP) using a HiTech Scientific SF61 stopped-flow system (TgK Scientific Limited, Bradford on Avon, UK). The  $\epsilon$ -ATP/ $\epsilon$ -ADP fluorescence was excited at 335 nm and monitored through a KV389 cut-off filter. The displacement of the fluorescent nucleotide analogs by ATP results in a decrease in the fluorescence amplitude, which is best described by a single exponential fit that yields the apparent rate of nucleotide dissociation

## CD spectroscopy

Directly prior to the measurement, the freshly clarified G-actin was diluted to 0.05 mg/mL in 10 mM phosphate buffer pH 7.4. A buffer reference was prepared by mixing G buffer with 10 mM phosphate buffer pH 7.4 in the same ratio. CD spectroscopy was performed with a PiStar 180 spectrometer (Applied Photophysics, Leatherhead, UK). Measurements were performed at  $25^{\circ}\text{C}$  using quartz cuvettes (0.3 cm path length) and nitrogen purging. CD spectra were recorded from 176 nm to 280 nm in steps of 1 nm using a bandwidth of 4 nm. For every sample, three spectra were recorded and subsequently averaged. The averaged buffer spectrum was subtracted, and the resulting protein spectrum smoothed using a Savitsky-Golay filter (Savitzky & Golay, 1964) in two consecutive iterations. The DichroWeb online platform was used to determine the secondary structure composition (Whitmore & Wallace, 2004 [\[3\]](#)). Data were analyzed based on the mean residue ellipticity  $[\theta]_{\text{MRE}}$  of the sample using the CDSSTR algorithm and reference set 3 (optimized for 185 nm–240 nm).

## Steady-state ATPase assay

The steady-state ATPase activity of F-actin was measured using a NADH-coupled enzymatic assay (Furch *et al*, 1998 [↗](#)). Experiments were performed with 10  $\mu$ M F-actin at 37 °C in assay buffer-A (25 mM HEPES pH 7.3, 5 mM  $MgCl_2$ , 50 mM KCl, 0.5 mM DTT) supplemented with the enzymatic system, which generates the quantifiable readout (1 mM ATP, 0.5 mM NADH, 0.5 mM 2-phosphoenolpyruvate, 0.02 mg/mL lactate dehydrogenase from rabbit muscle (Sigma-Aldrich, Schnelldorf, Germany), 0.05 mg/mL pyruvate kinase from rabbit muscle (Sigma-Aldrich, Schnelldorf, Germany), 0.5 mM NADH). The change in absorbance at 340 nm due to oxidation of NADH was recorded in a Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Waltham, MA, USA).

## TIRF microscopy-based assays

To distinguish between the impact of the mutation on filament nucleation and elongation, we performed TIRF microscopy-based assays using freshly clarified protein. Unlabeled WT or mutant G-actin was supplemented with 10% Atto-655 labeled WT or mutant G-actin for visualization of the filament assembly process. Glass coverslips were cleaned, surfaces chemically treated and flow cells assembled as described previously (Greve *et al*, 2022 [↗](#)). To induce polymerization, the G-actin solutions were diluted to a final concentration of 1  $\mu$ M with 2 $\times$  TIRF-buffer (20 mM imidazole, 50 mM KCl, 1 mM  $MgCl_2$ , 1 mM EGTA, 0.2 mM ATP, 15 mM glucose, 20 mM  $\beta$ -ME, 0.25 % methylcellulose, 0.1 mg/mL glucose oxidase and 0.02 mg/mL catalase. The solutions were mixed, immediately flushed into flow cells and image acquisition was started.

To follow filament disassembly, two-color TIRF experiments with Atto-655 labeled actin and eGFP-cofilin-1 were performed with preformed filaments that were “aged” and capped at their barbed end by human capping protein. WT or mutant actin was polymerized for 3 hours at room temperature and then moved to 4 °C overnight. “Aged” F-actin and human capping protein were diluted into TIRF- buffer to generate capped actin filaments. eGFP-cofilin-1 was added to final concentrations of 300 nM F-actin, 25 nM capping protein and 100 nM eGFP-cofilin-1. Immediately adding eGFP-cofilin-1, the reaction mixture was flushed into the flow cell and image acquisition was started. Images were captured using a Nikon Eclipse TI-E inverted microscope equipped with a 100 $\times$ /1.49 NA Apo oil immersion objective and Ixon3 897 EMCCD cameras (Andor, Belfast, UK) under control of the NIS software (Nikon, Minato, Japan). Images were taken every 3 seconds with an exposure time of 60 ms.

To analyze actin polymerization in the presence of ABPs, the ABPs were first pre-diluted in KMEI buffer and diluted to their final concentration in TIRF buffer just before adding actin. Image acquisition was performed as described for experiments with actin only. Image analysis for all TIRF microscopy experiments was performed using ImageJ (Rueden *et al*, 2017 [↗](#)). The elongation rate of actin filaments was determined by manually tracking individual filaments. Only filaments that could be tracked for at least four consecutive minutes were used to determine the elongation rate. Using these criteria, 20 to 30 filaments were tracked in each individual experiment (in a microscopic field of view of 81.41  $\times$  81.41  $\mu$ m). For quantification of two-color TIRFM-experiments, 52  $\times$  52  $\mu$ m ROIs from the entire microscopic field of view were used. The number of filaments observed throughout an experiment was determined using the *Analyze Particle* tool in ImageJ after background-subtraction (sliding paraboloid, rolling ball radius: 25 pixels).

## Unloaded *in vitro* motility assay

The unloaded *in vitro* motility assay to study the interaction between WT and mutant actin and NM2A- HMM or Myo5A-HMM was essentially performed as previously described (Greve *et al*, 2022 [↗](#); Reindl *et al*, 2022 [↗](#); Kron & Spudich, 1986 [↗](#)). Flow cells used in the *in vitro* motility assay were constructed using nitrocellulose-coated coverslips. NM2A-HMM requires phosphorylation of the associated regulatory light chain for full enzymatic activity. Purified NM2A-HMM was incubated with the GST-tagged kinase domain (residues 1425-1776) of human smooth muscle

myosin light chain kinase (Uniprot-ID: A0A8I5KU53) at a molar ratio of 10:1 in 25 mM MOPS, pH 7.3, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM CaCl<sub>2</sub>, 0.2 μM calmodulin, 3 μM regulatory light chain (MLC12b), 3 μM essential light chain (MLC6a), 1 mM DTT and 1 mM ATP for 30 minutes at 30 °C. In experiments with Myo5A–HMM, each buffer and actin solution was supplemented with 2 μM calmodulin to prevent calmodulin dissociation from Myo5A–HMM. Olympus IX70 or Olympus IX83 inverted fluorescence microscopes (Olympus, Hamburg, Germany) equipped with a 60×/1.49 NA PlanApo oil immersion objective and an Orca Flash 4.0 CMOS camera (Hamamatsu Photonics Deutschland GmbH, Herrsching, Germany) were used for image acquisition. All *in vitro* motility experiments were performed at a 37°C. Analysis of the recorded image series was performed in ImageJ (Rueden *et al*, 2017 [DOI](#)). The Plugin wrmTrck (Nussbaum-Krammer *et al*, 2015 [DOI](#)), developed by Jesper S. Pedersen, was used to determine the trajectories and corresponding velocities of the individual actin filaments. Only filaments that showed movement for at least 30 consecutive seconds were tracked and used to determine the average sliding velocity.

## Pyrene-actin based bulk-polymerization assays

The polymerization and depolymerization of pyrene-labeled recombinant WT or mutant actin (5% pyrene-labeled WT or mutant actin) was monitored as a function of increasing or decreasing pyrene-fluorescence using a Synergy 4™ microplate reader (BioTek Instruments, Winooski, VT, USA) and the built-in filter set (Excitation: 340/30 nm, Emission: 400/30 nm) at 25 °C. For polymerization experiments, 20 μL of a 10 μM solution of pyrene-labeled Mg<sup>2+</sup>-ATP-G-actin (WT or mutant) was placed in a black flat bottom 96-well plate (BrandTech Scientific, USA). 80 μL of 1.25 × polymerization buffer (10 mM TRIS, pH 7.8, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 0.1 mM DTT and 0.1 mM ATP) was added to the wells using the built-in pipetting function of the plate reader and the polymerization of actin was followed as an increase in pyrene-fluorescence. For depolymerization experiments, 3 μL of a 20 μM F-actin solution was placed in one well of the plate. The solution was rapidly diluted by the addition of 297 μL G-buffer. The depolymerization of the filaments was monitored as a decrease in pyrene-fluorescence.

The effects of Arp2/3 (+ GST-N-WASP-VCA) and cofilin-1 on filament assembly or disassembly were analyzed by dispensing the actin solution and the ABP solution into the well by hand without mixing.

Polymerization buffer or G-buffer was added, the reaction components were thereby mixed and recording of the ensuing changes in fluorescence intensity was started.

The bulk-polymerization rate was determined according to Doolittle *et al*. (Doolittle *et al*, 2013 [DOI](#)). A linear regression was fitted to the linear region around the time-point of half-maximal fluorescence. The depolymerization experiments were evaluated by fitting a single exponential function to the data, including a linear component that accounts for bleaching.

## Co-sedimentation experiments

Actin was polymerized at 20 μM for 3 hours at room temperature before being moved to 4 °C overnight. For co-sedimentation experiments at pH 7.8, 5 μM of “aged” F-actin and varying concentrations of human cofilin-1 were mixed in assay buffer-C (10 mM TRIS, pH 7.8, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM EGTA, 0.1 mM DTT and 0.1 mM ATP) to a final volume of 50 μL in 1.5 mL polypropylene centrifuge tubes (Beckman Coulter, Brea, CA, USA) suited for high-speed centrifugation. For experiments performed at pH 6.5, assay buffer-C was supplemented with piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES, pH 6.5) to a final concentration of 100 mM to induce a pH shift from 7.8 to 6.5. Actin and cofilin-1 were added after the pH shift. Samples were incubated for 10 minutes at 23°C and then centrifuged at 136,000 × g (15 min, 4°C). The pellet and supernatant fractions were subjected to SDS-PAGE and the gel was imaged at a ChemiDoc-MP-gel

documentation system (Bio–Rad Laboratories, Hercules, CA, USA). The amount of actin and cofilin–1 in the pellet and supernatant was determined via densitometry using the Image Lab software (Bio–Rad Laboratories, Hercules, CA, USA).

## Transient kinetic analysis of the actomyosin complex

Myosin binding to pyrene–labeled actin leads to strong pyrene–fluorescence quenching. Mixing of the rigor–pyrene–acto–NM2A–2R/NM2C–2R complex with an excess of unlabeled actin allows the determination of  $k_A$  (dissociation rate of myosin from F–actin in the absence of nucleotide). Prior to the experiment, pyrene–labeled phalloidin–stabilized WT or mutant F–actin and the myosin solutions were treated with apyrase (Merck KGaA, Darmstadt, Germany) to remove residual nucleotide. Pyrene–labeled actin was mixed with a 1.5–fold excess of NM2A–2R/NM2C–2R in assay buffer–P (20 mM MOPS pH 7.0, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 5 mM DTT) and incubated for 5 minutes on ice. The solution was mixed with an excess of chicken  $\alpha$ –skeletal actin in assay buffer–P to final concentrations of 0.5  $\mu$ M pyrene–labeled actin, 0.75  $\mu$ M NM2A–2R and 5  $\mu$ M  $\alpha$ –skeletal actin. The solution was immediately transferred into the well of a black 96–well microplate with a transparent bottom and a lid (Corning, Corning, NY, USA). Fluorescence was excited at 365 nm and measured at 407 nm in a CLARIOstar Plus microplate reader (BMG LABTECH, Offenburg, Germany) using bottom optics over 9 hours. The dissociation rate ( $k_A$ ) of myosin from actin was determined by applying a single–exponential fit to the data.

The apparent affinity of NM2A–2R to WT or mutant F–actin in the absence ( $K_A$ ) or presence ( $K_{DA}$ ) of nucleotide was determined using the method developed by Kurzawa and Geeves (Kurzawa & Geeves, 1996 [\[10\]](#)). To determine  $K_A$ , all protein samples were treated with apyrase before the experiment. 100 nM pyrene–labeled phalloidin–stabilized F–actin was incubated with increasing concentrations of NM2A–2R (10–1,300 nM) for 5 minutes on ice in assay buffer–P without DTT. The solutions were shot against 20  $\mu$ M ATP at a HiTech Scientific SF61 stopped–flow system (TgK Scientific Limited, Bradford on Avon, UK) in a 1:1 ratio at 20 °C. Fluorescence was excited at 365 nm and recorded using a KV389 cut–off filter. At least five transients were averaged for each concentration of NM2A–2R and fitted using a single–exponential function. The relative fluorescence amplitudes were plotted against the pre–mix myosin concentrations. The affinity was determined by applying a quadratic equation to the dataset, where  $A$  is the amplitude,  $A_{\max}$  the amplitude under saturation,  $[F\text{–actin}]$  the actin concentration prior mix,  $[NM2A\text{–}2R]$  the variable NM2A–2R concentration and  $K$  the dissociation constant in the absence or presence of nucleotide.

$$A = A_{\max} \times ([F\text{–actin}] + [NM2A\text{–}2R] + K) - \sqrt{\frac{([F\text{–actin}] + [NM2A\text{–}2R] + K)^2 - 4 \times [F\text{–actin}] \times [NM2A\text{–}2R]}{2 \times [F\text{–actin}]}}$$

Slight modifications of the experimental protocol were necessary to determine  $K_{DA}$ . Incubation with apyrase was omitted, instead the pyrene–actomyosin complex was incubated with 30  $\mu$ M ADP (Merck KGaA, Darmstadt, Germany) for 20 minutes on ice prior to measurement. To account for the lower affinity in the presence of ADP, the range of used myosin concentrations was extended up to 3,000 nM. Additionally, the concentration of ATP in the mixing experiment was increased to 300  $\mu$ M pre–mix. Data was recorded and evaluated as done for experiments without nucleotide.

The apparent ATP–affinity of the actomyosin complex was determined by mixing the apyrase–treated, pyrene–labeled, phalloidin–stabilized actomyosin complex with increasing concentrations of ATP at the stopped–flow system. Fitting an exponential function to the individual transients yields the ATP–dependent dissociation rate of NM2A–2R from F–actin ( $k_{\text{obs}}$ ). The  $k_{\text{obs}}$ –values were plotted against the corresponding ATP concentrations and a hyperbola was fitted to the data. The fit yields the apparent ATP affinity ( $1/K_1$ ) of the actomyosin complex and the maximal dissociation rate.

## Molecular modelling

Homology models of human cytoskeletal  $\gamma$ -actin, human cofilin-1 and the human NM2A motor domain were generated with MODELLER (Sali & Blundell, 1993 [↗](#)). Models of filamentous human cytoskeletal  $\gamma$ -actin with bound cofilin-1 or NM2A motor domain were generated based on cryo-EM structures obtained from the Protein Data Bank of the barbed end side of a cofilactin cluster (pdb: 6UC4, 6UBY, 6VAO) (Huehn *et al*, 2020 [↗](#)) and a human cytoplasmic actomyosin complex (pdb: 5JLH) (Von Der Ecken *et al*, 2016). The structures were accessed on May 25, 2023. Mutation E334Q was introduced using the mutagenesis tool in PyMOL (Schrödinger, LLC, 2015). PyMOL and ChimeraX (Goddard *et al*, 2018 [↗](#); Pettersen *et al*, 2021 [↗](#)) were used for distance measurements and visualization of Coulomb surfaces.

Identification and alignment of the CM-loop region of cytoskeletal myosin isoforms were performed using the match function of ChimeraX, by superimposing and aligning cytoskeletal myosin isoform structures on NM2C (pdb-NM2C: 5JLH, pdb-Myo5A: 7PM5, pdb-Myo5C: 5HMP, pdb-Myo1D: AF-O94832-F1, pdb-Myo15: 7UDT, pdb-Myo19: AF-Q96H55-F1). Where appropriate, the corresponding human sequences were retrieved from UniProt and structurally aligned.

Normal mode analyses (NMA) were performed using the Elastic Network Model (ENM) eINémo (Suhre & Sanejouand, 2004 [↗](#)) and an nrbl value of 5.

## Data Analysis

Data analysis and graph plotting were performed with Origin 2023 (OriginLab Corporation, Massachusetts, USA). Errors are given as standard error of the mean (SEM) based on three independent experiments if not otherwise specified. The significance of the data was evaluated in Origin 2023 using a two-sample t-test ( $p > 0.05 \triangleq$  ns,  $p \leq 0.05 \triangleq$  \*,  $p \leq 0.01 \triangleq$  \*\*,  $p \leq 0.001 \triangleq$  \*\*\*,  $p \leq 0.0001 \triangleq$  \*\*\*\*).

## Author Contributions

All authors discussed the results and contributed to the final manuscript; J.N.G., T.R., C.T. purified proteins; J.N.G. performed experiments; A.M. and R.H. performed *in silico* studies; J.N.G. and D.J.M. analyzed the data; J.N.G., M.H.T., N.D.D. and D.J.M. contributed to the experimental design; J.N.G., A.M., R.H. and D.J.M. designed figures; J.N.G. and D.J.M. conceived and coordinated the study, and wrote the manuscript; D.J.M. was responsible for funding acquisition, supervision and project administration.

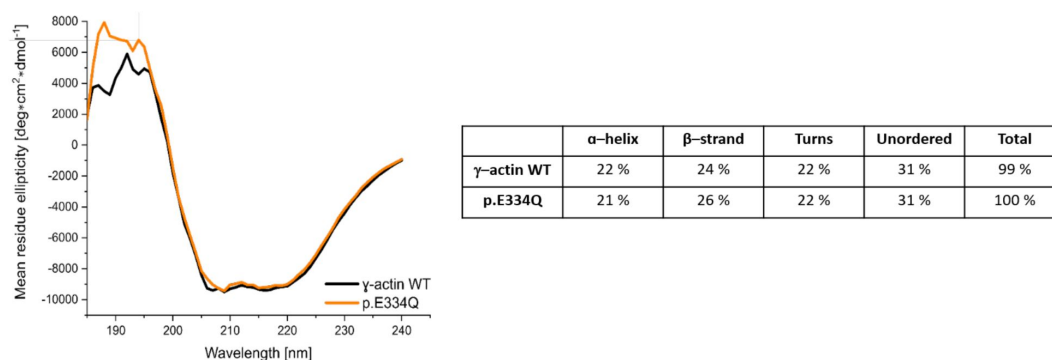
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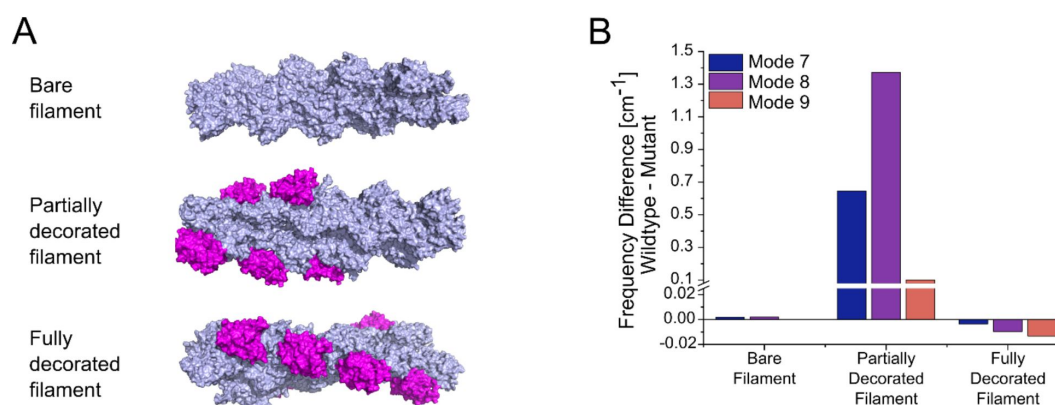
National High Performance Computing (NHR) infrastructure. The calculations for this research were conducted with computing resources under the project ID nib00018. T.R. was enrolled in the PhD program Molecular Medicine of Hannover Biomedical Research School (HBRS).

## Supplementary Figures



## Supplementary Figure 1

CD-Spectra of γ-actin WT and p.E334Q protein. Shown are the smoothed (Savitsky-Golay) and averaged spectra with the averaged buffer spectrum subtracted. The depicted secondary structure composition of γ-actin WT and p.E334Q protein was determined by using the DichroWeb online platform.

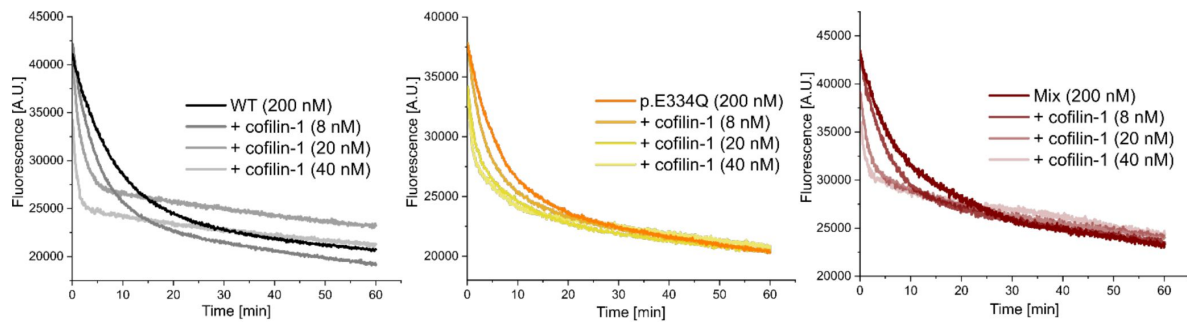


## Supplementary Figure 2

### Normal mode analysis of changes induced by mutation E334Q in the global mobility of bare, partially and a fully cofilin-1 decorated γ-actin mini-filaments

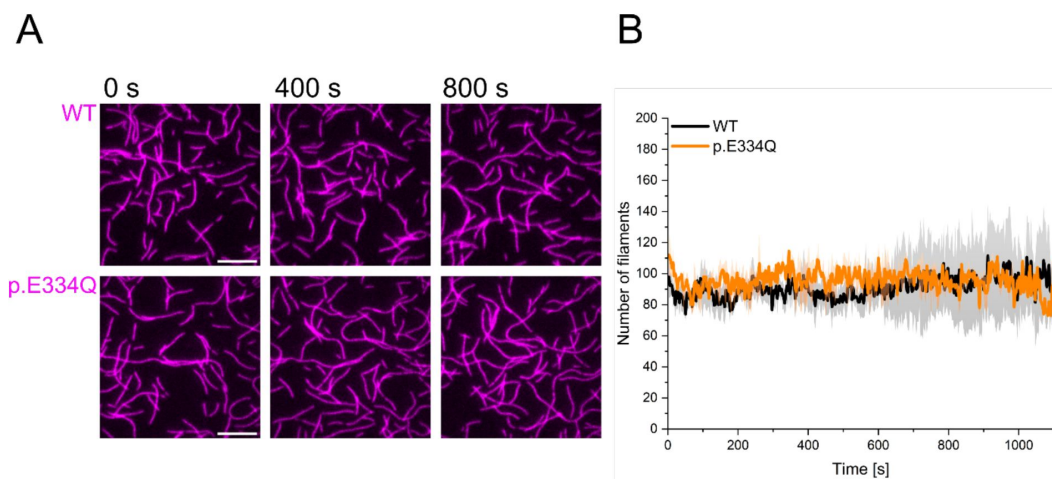
**(A)** Models of a bare-γ-actin mini-filament, a partially and a fully cofilin-1 decorated γ-actin mini-filament were generated for normal mode analysis. Each mini-filament consists of nine actin monomers. Actin is colored light blue and cofilin-1 magenta.

**(B)** Significant differences in the global mobility of the different types of mini-filaments shown in panel (A), containing either WT or mutant cytoskeletal γ-actin, were only observed in the case of partial cofilin-1 decoration. Large differences are observed for partially decorated filaments in modes 7, 8 and 9, which are the global motions with the lowest frequencies.



### Supplementary Figure 3

Exemplary traces of dilution-induced depolymerization experiments in the absence and presence of increasing human cofilin-1 concentrations.



### Supplementary Figure 4

**(A)** 300 nM “aged” WT and p.E334Q F-actin (10% Atto-655 labeled, capped by CP) was visualized using TIRF microscopy. Shown are representative micrographs at the indicated time points. Scale bar corresponds to 10  $\mu\text{m}$ . **(B)** Quantification of the number of actin filaments over the time of the experiment. The solid lines and shades represent the mean  $\pm$  SEM of 2–3 individual experiments.

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**Reviewer #1 (Public Review):****Strengths:**

The authors first perform several important controls to show that the expressed mutant actin is properly folded, and then show that the Arp2/3 complex behaves similarly with WT and mutant actin via a TIRF microscopy assay as well as a bulk pyrene-actin assay. A TIRF assay showed a small but significant reduction in the rate of elongation of the mutant actin suggesting only a mild polymerization defect.

Based on in silico analysis of the close location of the actin point mutation and bound cofilin, cofilin was chosen for further investigation. Faster de novo nucleation by cofilin was observed with mutant actin. In contrast, the mutant actin was more slowly severed. Both effects favor the retention of filamentous mutant actin. In solution, the effect of cofilin concentration and pH was assessed for both WT and mutant actin filaments, with a more limited repertoire of conditions in a TIRF assay that directly showed slower severing of mutant actin.

Lastly, the mutated residue in actin is predicted to interact with the cardiomyopathy loop in myosin and thus a standard in vitro motility assay with immobilized motors was used to show that non-muscle myosin 2A moved mutant actin more slowly, explained in part by a reduced affinity for the filament deduced from transient kinetic assays. By the same motility assay, myosin 5A also showed impaired interaction with the mutant filaments.

The Discussion is interesting and concludes that the mutant actin will co-exist with WT actin in filaments, and will contribute to altered actin dynamics and poor interaction with relevant myosin motors in the cellular context. While not an exhaustive list of possible defects, this is a solid start to understanding how this mutation might trigger a disease phenotype.

#### Weaknesses:

Potential assembly defects of the mutant actin could be more thoroughly investigated if the same experiment shown in Fig. 2 was repeated as a function of actin concentration, which would allow the rate of disassembly and the critical concentration to also be determined.

The more direct TIRF assay for cofilin severing was only performed at high cofilin concentration (100 nM). Lower concentrations of cofilin would also be informative, as well as directly examining by the TIRF assay the effect of cofilin on filaments composed of a 50:50 mixture of WT:mutant actin, the more relevant case for the cell.

The more appropriate assay to determine the effect of the actin point mutation on class 5 myosin would be the inverted assay where myosin walks along single actin filaments adhered to a coverslip. This would allow an evaluation of class 5 myosin processivity on WT versus mutant actin that more closely reflects how Myo5 acts in cells, instead of the ensemble assay used appropriately for myosin 2.

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#### Reviewer #2 (Public Review):

Greve et al. investigated the effects of a disease-associated gamma-actin mutation (E334Q) on actin filament polymerization, association of selected actin-binding proteins, and myosin activity. Recombinant wildtype and mutant proteins expressed in sf9 cells were found to be folded and stable, and the presence of the mutation altered a number of activities. Given the location of the mutation, it is not surprising that there are changes in polymerization and interactions with actin binding proteins. Nevertheless, it is important to quantify the effects of the mutation to better understand disease etiology. Some weaknesses were identified in the paper as discussed below.

Throughout the paper, the authors report average values and the standard-error-of-the-mean (SEM) for groups of three experiments. Reporting the SEM is not appropriate or useful for so few points, as it does not reflect the distribution of the data points. When only three points are available, it would be better to just show the three different points. Otherwise, plot the average and the range of the three points.

The description and characterization of the recombinant actin is incomplete. Please show gels of purified proteins. This is especially important with this preparation since the chymotrypsin step could result in internally cleaved proteins and altered properties, as shown by Ceron et al (2022). The authors should also comment on N-terminal acetylation of actin.

The authors do not use the best technique to assess actin polymerization parameters. Although the TIRF assay is excellent for some measurements, it is not as good as the standard pyrene-actin assays that provide critical concentration, nucleation, and polymerization

parameters. The authors use pyrene-actin in other parts of the paper, so it is not clear why they don't do the assays that are the standard in the actin field.

The authors' data suggest that, while the binding of cofilin-1 to both the WT and mutant actins remains similar, the major defect of the E334Q actin is that it is not as readily severed/disassembled by cofilin. What is missing is a direct measurement of the severing rate (number of breaks per second) as measured in TIRF.

Figure 4 shows that the E334Q mutation increases rather than decreases the number of filaments that spontaneously assemble in the TIRF assay, but it is unclear how reduced severing would lead to increased filament numbers, rather, the opposite would be expected. A more straightforward approach would be to perform experiments where severing leads to more nuclei and therefore enhances the net bulk assembly rate.

Figure 5 A: in the pyrene disassembly assay, where actin is diluted below its critical concentration, cofilin enhances the rate of depolymerization by generating more free ends. The E334Q mutation leads to decreased cofilin-induced severing and therefore lower depolymerization. While these data seem convincing, it would be better to present them as an XY plot and fit the data to lines for comparison of the slopes.

Figure 5 B and C: the cosedimentation data do not seem to help elucidate the underlying mechanism. While the authors report statistical significance, differences are small, especially for gel densitometry measurements where the error is high, which suggests that there may be little biological significance. Importantly, example gels from these experiments should be shown, if not the complete set included in the supplement. In B, the higher cofilin concentrations would be expected to stabilize the filaments and thus the curve should be U-shaped.

Figure 5 D: these data show that the binding of cofilin to WT and E334Q actin is approximately the same, with the mutant binding slightly more weakly. It would be clearer if the two plots were normalized to their respective plateaus since the difference in arbitrary units distracts from the conclusion of the figure. If the difference in the plateaus is meaningful, please explain.

Figure 6: It is assumed that the authors are trying to show in this figure that cofilin binds both actins approximately the same but does not sever as readily for E334Q actin. The numerous parameters measured do not directly address what the authors are actually trying to show, which presumably is that the rate of severing is lower for E334Q than WT. It is therefore puzzling why no measurement of severing events per second per micron of actin in TIRF is made, which would give a more precise account of the underlying mechanism.

Actin-activated steady-state ATPase data of the NM2A with mutant and WT actin would have been extremely useful and informative. The authors show the ability to make these types of measurements in the paper (NADH assay), and it is surprising that they are not included for assessing the myosin activity. It may be because of limited actin quantities. If this is the case, it should be indicated.

(line 310) The authors state that they "noticed increased rapid dissociation and association events for E334Q filaments" in the motility assay. This observation motivates the authors to assess actin affinities of NM2A-HMM. Although differences in rigor and AM.ADP affinities are found between mutant and wt actins, the actin attachment lifetimes (many minutes) are unlikely to be related to the rapid association and dissociation event seen in the motility assay. Rather, this jiggling is more likely to be related to a lower duty ratio of the myosins, which appears to be the conclusion reached for the myosin-V data. These points should be clarified in the text.

(line 327) The authors report that the  $1/K_1$  value is unchanged. There are no descriptions of this experiment in the paper. I am assuming the authors measured the ATP-induced dissociation of actomyosin and determined ATP affinity ( $K_1$ ) from this experiment. If this is the case, they should describe the experiment and show the data, provide a second-order rate constant for ATP binding, and report the max rate of dissociation ( $k_2$ ). This is a kinetic experiment done frequently by this group, so the absence of these details is surprising.

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