



Non-muscle actinopathy-associated loss-of-function actin variants modulate cytoskeletal reorganization

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ABSTRACT

Variants in ACTB gene encoding for cytoplasmic β -actin result in a group of rare disorders called non-muscle actinopathies (NMA). A subgroup of NMA patients with ACTB pLoF (predicted loss-of-function) disorder have various congenital malformations, such as microcephaly and intellectual disability. We investigated the cellular effects of an ACTB pLoF missense variant, G302A, and a four-amino-acid deletion, S338-I341 in cytoskeletal β -actin, in patient-derived fibroblast cells. We found that neither of the mutations affected the organization of actin or the width of the actin-filament bundles, while the mutation G302A reduced the stiffness of the cells as measured by using atomic force microscopy. The latter effect might be associated with the misorganization of tubulin and with the increased size and number of focal adhesions. When we challenged the cells by monolayer stretching and followed the reorganization of the actin cytoskeleton, we found that G302A mutant cells showed denser actin filament bundles within the cells compared to wild type cells. At the same time, the extent of cofilin reorganization from the cell periphery was increased upon stretch, and this correlated with an increased cofilin phosphorylation. In the case of deletion, while the extent of cofilin phosphorylation increased, the extent of reorganization was unaltered; rather, the phosphorylation of myosin light chain, important in counteracting external force, was reduced by 75%. We could partially rescue this effect by overexpressing the active form of the formin mDia1. Our findings open the possibility to verify the cellular phenotype in the most affected patients' cells, in neurons.

1. Introduction

Actin is a self-assembling biopolymer that plays a role in a wide array of physiological processes by forming dynamic networks: it contributes to maintaining the shape of cells, determines their mechanical properties and enables them to perform specific functions such as muscle contraction, cell motility and cell division (Schoenenberger et al., 2011). The diverse cellular functions of actin are regulated by interactions with specific actin-binding proteins (ABP-s) (Uyeda et al., 2025). Six actin isoforms are expressed in humans in a time- and tissue-specific manner. Four of them are muscle-specific (skeletal, cardiac, α - and γ -smooth) and two have cytoskeletal localization (β - and γ -cytoskeletal actin). The sequence identity of isoforms exceeds 93%, indicating that actin is a highly conserved intracellular protein (Vandekerckhove and Weber, 1978). The ubiquitously expressed β - and γ -cytoskeletal actin differ by

only four amino acids at their N-termini. This difference at the protein sequence level plays a role in only a few specific cell types, such as auditory hair cells (Patrinostro et al., 2018) or retinal cells (Vedula et al., 2023). Recent studies revealed that the two isoforms are largely interchangeable at protein level in terms of cell viability or migration (Patrinostro et al., 2018; Vedula et al., 2017), although their regulation at gene-level is different (Ross et al., 1997).

Mutations in genes ACTB and ACTG1 (encoding the β - and γ -cytoskeletal actin isoforms, respectively) cause a broad spectrum of rare disorders, called non-muscle actinopathies (NMAs). NMAs show high clinical variability, which are classified into several groups (Fig. 1A, (Dakos and Varga, 2026; Di Donato et al., 2026)). Baraitser-Winter cerebrofrontofacial syndrome (BWCFF) patients are characterized by facial dysmorphism. This group of patients has the most severe symptoms, such as developmental delay, intellectual disability and cortical malformation associated with

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epilepsy (Verloes et al., 2015). BWCFF-associated mutations change significantly the polymerization-depolymerization dynamics of actin (Di Donato et al., 2026; Greve and Manstein, 2025) and reduce the stability of F-actin (Grácz et al., 2026).

There is a group of patients with mutations in either cytoskeletal actin genes categorized as “unspecified NMA” (Fig. 1A). Among them, the effect of the cytoskeletal γ -actin carrying the mutation E334Q was shown to affect the interaction of actin with cofilin and myosin (Greve et al., 2024). There is also a group of patients with mutations in the ACTG1 gene, that seems to be unaffected by the mutations. Another group of patients with mutations in the ACTG1 gene has hearing loss (Bernardi et al., 2024; Miyajima et al., 2020). A specific mutation in the ACTB gene, R183W, is exclusively connected to dystonia-deafness syndrome (Procaccio et al., 2006).

The last group of individuals (previously categorized into different subgroups) is relatively mildly affected and classified into a group named ACTB pLoF (predicted loss-of-function) disorder (Fig. 1A). This group includes patients with syndromic thrombocytopenia and patients with ACTB-related pleiotropic developmental disorder (Baumann et al., 2020; Cuvertino et al., 2017; Latham et al., 2018). Most of the patients show mild intellectual disability, severe-to-mild microcephaly, brain anomalies such as heterotopia and variable thrombocytopenia.

Concerning the mutation landscape of the ACTB pLoF variants, point mutations, frameshift mutations or deletions affect the actin gene mostly in exons 5 and 6, although few frameshift or point mutations have also been found in exons 3 and 4 (Fig. 1A). Frameshift (fs) mutations in exons 3 (L110Rfs, (Cuvertino et al., 2017)) and 4 (V159Gfs, (Di Donato et al., 2026)) have been identified. Further mutations include point mutations

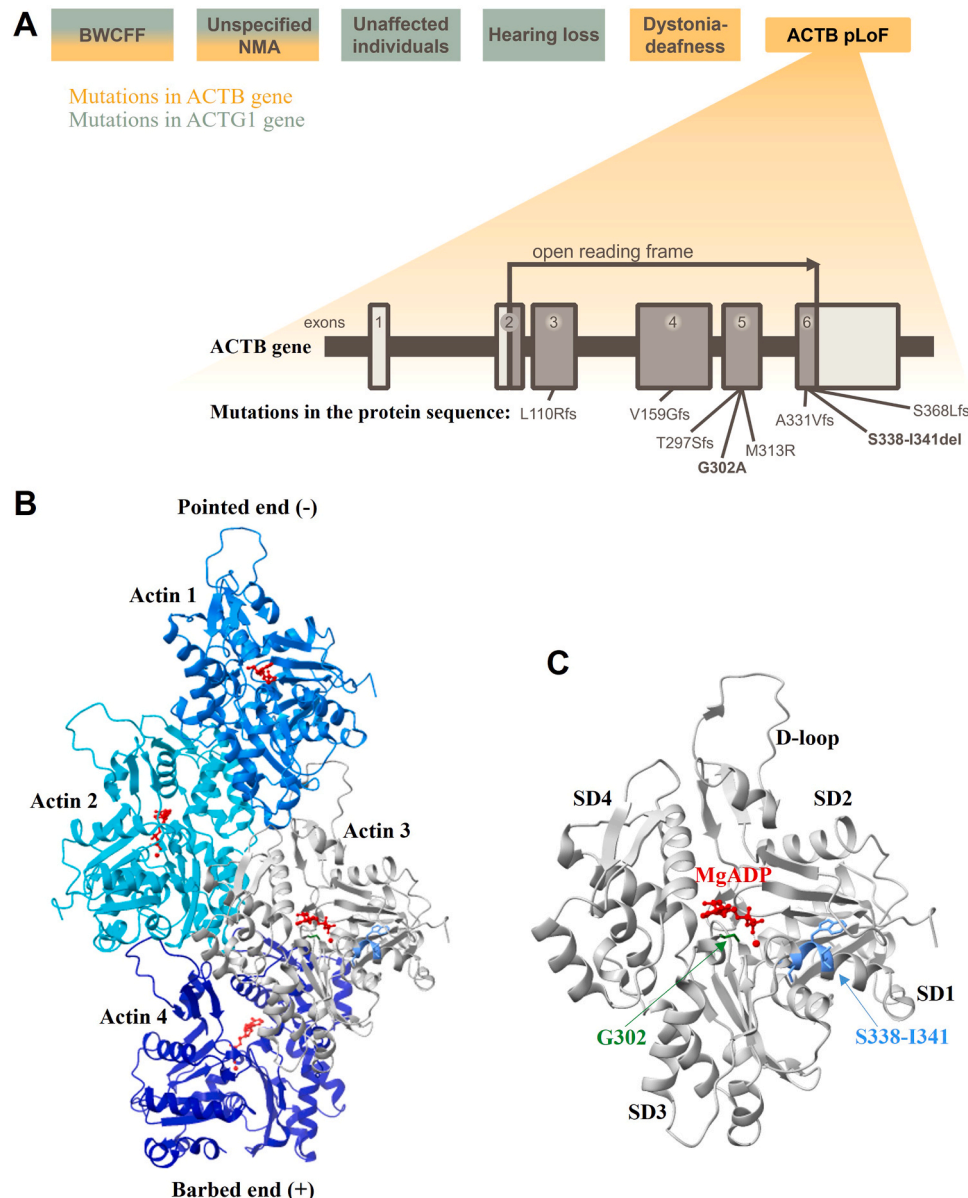


Fig. 1. Location of the residues associated with ACTB pLoF disorder (A) Classification of cytoskeletal actin mutations based on the clinical phenotype is shown together with the schematic representations of the location of mutations associated with ACTB pLoF disorder found in exons 3–6. The specific mutations at protein level, identified in patients with ACTB pLoF disorder, are shown below the gene structure. Color code: symptoms associated with mutations in β -actin or γ -actin are labeled orange and green, respectively. BWCFF and unspecified NMA can be associated with both β - and γ -actin mutations. (B) Structure of wild type cytoskeletal β -actin filament (PDB code: 8DNH, (Arora et al., 2023)) is illustrated as ribbon structure. Actin protomers are shown in different shades of blue and grey. The ball-and-stick model of MgADP is coloured red in all protomers, and the stick model of G302 is green, while the S338-I341 sequence (both the stick model of the residues and the corresponding ribbon structure) is blue in the “Actin 3” protomer. (C) The zoom-in view of an actin monomer (“Actin 3” – grey) is shown together with the labeling of subdomains, SD1-SD4 and the D-loop. For the visualization of structures represented in (B) and (C) ChimeraX program was used.

in exon 5 (G302A, (Baumann et al., 2020) and M313R, (Latham et al., 2018)), frameshift mutations in either exon 5 (T297Sfs, (Cuvertino et al., 2017)) or 6 (A331Vfs, S368Lfs, (Latham et al., 2018)) and a deletion in exon 6 (S338-I341del, (Latham et al., 2018)). Two patients (mother and son) harbored an intrachromosomal deletion (ACTB del), which completely removed one allele of the ACTB gene (Baumann et al., 2020).

Among the patients with ACTB pLoF disorder, S338-I341del mutation is associated with severe microcephaly and thrombocytopenia, while M313R, A331Vfs and S368Lfs mutations are linked to less severe microcephaly and thrombocytopenia. The mutation V159Gfs causes microcephaly, but not thrombocytopenia. In some cases, only mild-to-moderate intellectual disability can be observed, such as with the mutations L110Rfs or G302A. In few cases, only attention deficit can be seen (for T297Sfs and ACTB del) without microcephaly or thrombocytopenia (Baumann et al., 2020; Di Donato et al., 2026).

Among the ACTB pLoF variants the most severe microcephaly phenotype is connected to the S338-I341del variant, where reduced cell migration rate, detected in patient-derived fibroblasts, might be responsible for this phenotype (Latham et al., 2018). Furthermore, mutant cells showed a decreased amount of β -actin in the lysates, while the amount of γ - and smooth-muscle actin were increased as a compensation for β -actin loss. It has been shown that the S338-I341 deletion reduced the expression of recombinant β -actin in insect cells, providing a hint for a less stable protein (Di Donato et al., 2026). In contrast, the patient having the G302A mutation has no microcephaly, but a moderate intellectual disability (Di Donato et al., 2026). The recombinant G302A actin shows slightly impaired depolymerization-polymerization properties and decreased thermal stability. In blood mononuclear cells this variant disturbs the organization of microtubules (Baumann et al., 2020). In addition, from fractionation experiments the cells contained less β -actin in the detergent insoluble fraction, indicating that the mutant actin is less associated with the cytoskeleton.

Actin can transition from its monomeric, globular (G-actin) form to the polymeric, filamentous (F-actin) form by head-to-tail polymerization (Fig. 1B, (Oosterheert et al., 2025)). Polymerization occurs at the fast-growing barbed (+) end, while the pointed end (-) is considered as the primary site for filament disassembly. The actin monomer consists of four subdomains (SD): SD1 and SD3 facing towards the barbed end, while SD2 and SD4 are located at the pointed end (Fig. 1C). The nucleotide binding pocket is in the central cleft, surrounded by the four subdomains. Residue G302 is close to the nucleotide binding site of actin (Fig. 1C, green), while the residues of the S338-I341 deletion are positioned at the beginning of a helix in SD1 (Fig. 1C, blue). The binding sites of the actin-binding proteins (ABPs) cofilin and myosin are located on the outer surface of the actin filament, involving residues from two subdomains, SD1 and SD2. The positions of both mutations are far from the interfaces involved in the actin filament formation (Fig. 1B-C). Based on the known structural data one may predict that mutation of the G302 residue possibly influences the binding of the nucleotide, which might also be reflected in the binding of cofilin (Huehn et al., 2020; Tanaka et al., 2018), known to be sensitive to the nucleotide-binding state of actin (preference for ADP-bound actin). The deletion, based on its location, might affect both cofilin and myosin binding. According to these assumptions, actin reorganization might also be perturbed in the cells.

The question arises whether the severity of the clinical phenotypes can be connected to a specific actin-ABP interaction or lies in the perturbed dynamic organization of actin. Here, we investigated how the ACTB pLoF variants G302A and the S338-I341del mutants of β -actin affect the behavior and properties of patient-derived dermal fibroblasts. First, we compared the behavior of the cells in cell growth and migration assays. To uncover the molecular mechanism behind the cellular phenotype we quantified the amount of β -actin and the extent of its incorporation into F-actin. In addition, we analyzed actin organization

by confocal and superresolution (STED) microscopy and we compared cell stiffness with atomic force spectroscopy. Reorganization of actin was investigated by monolayer stretching. During actin reorganization we followed the localization of the actin depolymerizing factor cofilin. We also monitored the phosphorylation of cofilin (required for its inactivation and its dissociation from actin) and myosin light chain (MLC), as indicators of the efficiency of actin contraction upon external stretching forces. We found that both mutations affect actin reorganization upon applied force, but in different ways: while the G302A mutation affects the cofilin-mediated step, the deletion disturbs the contraction necessary to withstand the external force.

2. Materials and methods

Primary antibodies for western blotting (WB): β -actin antibody, Cat# MCA5775GA from Bio-Rad; pan-actin antibody, clone 2A3 Cat# MABT1333 from Sigma Aldrich; GAPDH antibody Cat# 97166; MLC antibody, Cat# 8505S; pMLC (Thr18/Ser19) antibody, Cat# 3674S; cofilin antibody, Cat# 5175S; p-cofilin antibody, Cat# 3313S; all from Cell Signaling Technology; tubulin antibody, Cat# PA5-58711, from Thermo Fisher Scientific; anti-GFP antibody, Cat# GFP-1020 from Aves Labs.

Primary antibodies for immunofluorescence (IF): cofilin, Cat# ab42824 from Abcam, tubulin antibody, Cat# PA5-58711, from Thermo Fisher Scientific. ZO-1, Cat# 33-9100 and pFAK (Tyr397), Cat# 44-624 G, both from Invitrogen.

Secondary antibodies for WB: Peroxidase AffiniPure Goat Anti-Mouse IgG (H+L), Cat# 115-035-003; Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L), Cat# 111-035-003, both from Jackson ImmunoResearch; bovine anti-Chicken IgY-HRP, Cat# sc-2917 from Santa Cruz Biotechnology.

Secondary antibodies for IF: Chicken anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Cat# A21441; Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546, Cat# A11003; all from ThermoFisher Scientific.

Actin stains: Alexa Fluor™ Plus 647 Phalloidin, Cat# A30107 used for confocal imaging was from ThermoFisher Scientific; Abberior STAR 635 P conjugated to phalloidin used for STED imaging, Cat# ST635P-0100-20UG was from Abberior.

Cell culture and cell treatment Patient-derived wild type and mutant fibroblast cells were isolated as described in (Di Donato et al., 2026) and cultured in MCDB medium supplemented with 5% fetal bovine serum, 1% Penicillin/Streptomycin, 1% Chemically Defined Lipid Concentrate, 1% HEPES, 1% GlutaMAX Supplement, 0.3% Insulin-Transferrin-Selenium, 1 ng/mL basic Fibroblast Growth Factor, 2 ng/mL Epidermal Growth Factor, 5 μ g/mL Vitamin C, 250 nM hydrocortisone and 7.5 U/mL heparin. HEK293T cells used for lentiviral production were cultured and transfected with the appropriate plasmids as described in (Hollosi et al., 2021). All cell culture was performed at 37 °C in a humidified atmosphere containing 5% CO₂. Wild type cells were treated with different concentrations of colchicine (Cat #C9754, Sigma) for 1 h at 37 °C as indicated in Fig. 5C. Colchicine was dissolved in 96% ethanol, and the same 96% ethanol was used as a vehicle control (0 nM colchicine). BMS-5 (Selleck Chemicals, Cat# S0185) was dissolved in DMSO (Sigma Aldrich, Cat# 276855-100 mL) and the cells were treated for 1 h at 37 °C at 25 μ M final concentration.

Cell growth assay 20 000 cells were seeded in Costar 24-well flat-bottom, tissue culture treated plastic plates (Corning, #3526) coated with 0.5% gelatine (2% gelatine from Sigma Aldrich, Cat# G1393, diluted in PBS). Cell numbers were determined each day up to 96 h by cell counting. Two technical replicates were counted each day, in a total of three independent experiments.

Wound closure assay 75 000 cells were plated in the same type of 24-well plates used for cell growth assay. 48 h later the cells were pre-treated with 10 μ g/mL mitomycin C (Cat# M5353, Sigma Aldrich) for 2 h to block proliferation and a cell-free area was created by scratching

the monolayer with a 10 μ l pipette tip. Subsequently, the cells were washed once with PBS. After that, MCDB medium, supplemented with 5% FBS and all the medium components listed above was placed on the top of the cells. Following scratching, images were recorded at several locations along the scratch line by using a ZOE Fluorescence Cell Imager (BioRad) with a 20x objective (N.A. 0.4). After 22 h, images were acquired at the same locations. The area devoid of cells was determined by using the Fiji plugin MRI Wound Healing tool (Montpellier Resources Imagerie, CNRS). The area in percentage was calculated where the cells migrated in related to the original scratch area. The individual values plotted represent 8–12 images of each biological replicate, having 4 technical replicates (2–3 images of each well was analyzed/technical replicate) in each experiment.

Live cell migration assay For individual cell tracking, 20 000 cells were seeded in 3.5 cm TPP tissue culture treated plastic plates coated with 0.5% gelatine the day before the experiment. Dishes were mounted onto a Nikon Ti2 inverted confocal microscope equipped with a DMK 33G274 camera (The Imaging Source, LLC, Germany) and an incubator (37°C, 5% CO₂). Live cell images were taken using a 10 $\times$ objective (N.A. 0.3) or a 4 $\times$ objective (N.A. 0.1). Images of the region of interest were obtained at 30-minute intervals for 6 h.

For analysis, cells were manually tracked with the MTrackJ plugin in ImageJ (Meijering et al., 2012). The speed, trajectories, and directional persistence (the latter was described by the slope (α value, Fig. 2D) of the log-log plot of the mean square displacement vs time for the individual cells (Fig. S1E–F)) of migrating cells were calculated by DiPer software (Gorelik and Gautreau, 2014). Cells that divided or got into contact with their neighbours were excluded from the analysis.

Determination of F-actin and G-actin content Samples of wild type, G302A and S338-I341del fibroblasts were lysed according to the protocol described in the G-actin / F-actin In Vivo Assay Kit (Cat# BK-037, Cytoskeleton Inc.). After a preclearing centrifugation step, G-actin and F-actin were separated by ultracentrifugation. The supernatant containing G-actin, and the pellet (resuspended in milliQ water supplemented with cytochalasin D) containing F-actin was loaded on an SDS-PAGE gel and the amount of actin was visualized by using pan-actin or β -actin antibody. After HRP-conjugated secondary antibody incubation, the membranes were incubated with chemiluminescence substrate and developed on Hyperfilms. Bands were quantified using ImageJ 1.53c.

Plasmid The active form (aa. 578–1198, i.e. the C-terminal part of the protein) of human mDia1 (DIAPH1 (NM_005219) Human Tagged ORF Clone, Origene, Cat# RC216318) was subcloned into a lentiviral destination vector (Addgene, Cat# 17454), already containing an EGFP coding sequence, using gateway cloning. Lentiviral supernatants were generated by co-transfection of HEK293T cells with a three-vector lentiviral system: using the EGFP-mDia1 expression vector combined with the lentiviral packaging and envelope plasmids pRSV-Rev, pMDLg/pRRE and pCMV-VSV-G (kind gift of Prof. Guillaume Charras).

Lenti-viral Packaging Lenti-viral packaging of EGFP-mDia1 was performed using HEK293T cells. Transfection was carried out using polyethylenimine, branched (PEI, Sigma, Cat# 408727) as a transfection reagent, which was prepared according to (Aricescu et al., 2006). 8.22 $\cdot 10^6$ HEK cells were seeded in T75 flask the day before transfection. The plasmid mixture contained 10 μ g construct plasmid, 3.75 μ g VSV-G, 1.875 μ g Rev and 1.875 μ g RRE virus plasmids. PEI was used in 1:2 ratio. HEK transfection medium was exchanged to cultured DMEM medium 3–4 h after transfection. Viral supernatant was harvested after a total of 48 h and filtered through 0.45 μ m filter. The virus was concentrated with the Lenti-X Concentrator of TakaraBio (Cat# 631232) according to the manufacturer's instruction. For transduction fibroblasts were trypsinized and 5 $\cdot 10^5$ cells were cultured in 50% MCDB and 50% viral supernatant for 24 h, supplemented with polybrene (Santa Cruz Biotechnology, Cat# sc-134220, 4 μ g/mL final concentration). 1.25 $\cdot 10^5$ cells were seeded on a gelatine (0.5% in PBS)-coated 12 mm $\times$ 12 mm cytostretchers chambers.

Monolayer stretching Fibroblast monolayers were cultured on special chambers (CuriBio Inc., Cat# CS-2 $\times$ 25-UPF for 5 mm $\times$ 5 mm chambers, and Cat# CS-0144-UPF for 12 mm $\times$ 12 mm chambers) used in the Cytostretcher LV instrument of CuriBio. The bottom of the chamber was treated with 0.2 mg/mL polydopamine solution (dissolved in 10 mM TRIS buffer, pH 8.5) for 2.5 h, washed 3 times with sterile water to create a hydrophilic surface for gelatin coating. Stretching was carried out with 0.5%/sec velocity and the monolayer was kept stretched for 15 min before fixation. 2 $\cdot 10^4$ cells and 1.25 $\cdot 10^5$ were seeded in 5 mm $\times$ 5 mm and 12 mm $\times$ 12 mm chambers, respectively and cells were grown for 36 h before stretch. Cells from 12 mm $\times$ 12 mm chambers were harvested at the indicated time points after stretch in 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 10% glycerol, supplemented with the following protease and phosphatase inhibitors: 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 5 mM sodium vanadate, 1 mM PMSF and cOmplete, EDTA-free protease inhibitor cocktail (Sigma, Tokyo, Japan). Lysates were centrifuged with 5000g for 5 min at 4°C and the supernatant was snap frozen for further immunoblotting.

Immunofluorescence staining of fixed monolayers Cells were fixed with Image-iT™ Fixative Solution (ThermoFisher Scientific, Cat# R37814) for 15 min. After that, cells were washed with HBSS, permeabilized (0.25% Triton X-100 in TBS-T, 10 min RT), blocked (1% BSA in TBS-T, 1 h RT), and incubated with the primary antibody (dilutions prepared in 1% BSA-TBS-T for cofilin – 1:200, pFAK – 1:150, tubulin – 1:100, ZO-1 – 1:100; incubation was done overnight at 4°C). After thorough washing in TBS-T, cells were stained simultaneously with the appropriate secondary antibodies (dilutions were prepared as 1:1000) and phalloidin (1:1000 in 1% BSA-TBS-T) for 1 h at RT, washed in TBS-T and PBS. The cell nuclei were labeled with Hoechst for 10 min (Cat# 62249, ThermoFisher Scientific) and finally washed in PBS prior to imaging. The imaging was carried out in an antifade solution, 1% DABCO 33-LV (Sigma, Cat# 290734), containing 50% glycerol in PBS.

Cell lysis and immunoblotting Wild type and mutant fibroblasts were harvested in 25 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1% NP-40, 10% glycerol, supplemented with the following protease and phosphatase inhibitors: 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 5 mM sodium vanadate, 1 mM PMSF and cOmplete, EDTA-free protease inhibitor cocktail (Sigma, Cat# 4693132001). Lysates were centrifuged with 5000g for 5 min at 4°C and the supernatant was snap frozen for further immunoblotting. Proteins were separated using standard SDS-PAGE gel electrophoresis with 12% SDS-PAGE gels, transferred to PVDF membranes (BioRad, Cat# 1620177) for immunoblot analysis using a wet blot transfer system. After blocking the membranes were stained with specific primary antibodies (overnight at 4°C) as indicated in each figure. Next morning, the membranes were incubated with the appropriate HRP-conjugated secondary antibodies (1.5 h at room temperature). Bands were visualized by using a chemiluminescence substrate (Thermo Fisher Scientific, Cat# 34580) and developed on Hyperfilms (VWR, Cat# 28-9068-37). Bands were quantified using ImageJ.

Confocal and STED microscopy Non-stretched and stretched samples were analyzed on a Nikon Ti2 confocal microscope. For confocal images of samples used in monolayer stretching pictures were taken by using a 20 $\times$ objective (numerical aperture: 0.75, field of view 140 μ m $\times$ 140 μ m, resolution: 1000 $\times$ 1000 pixels). All other confocal images were taken by using a 60 $\times$ objective (numerical aperture: 1.40, oil, field of view 120 μ m $\times$ 120 μ m, resolution: 1000 $\times$ 1000 pixels). STED images of fixed cells were taken by using a 100 $\times$ objective (numerical aperture: 1.45, oil) of the same Nikon Ti2 microscope. Depletion was carried out with a 775 nm laser, with 20% laser power. 2D STED images were taken from randomly selected areas of the cell monolayer.

Quantification of actin filament bundle width from the STED images STED images were deconvolved by using the Huygens Professional software 23.04, Scientific Volume Imaging BV. Quantification of actin width was carried out after the deconvolution by using a macro

plugin of ImageJ, which is using a Gaussian fit of the line profile of phalloidin fluorescence. The width is given as the fitted value corresponding to the 2x standard deviation (2xSD) of the Gaussian distribution. All analyses were performed blinded, such as data analyzers were unaware of the genotypes.

Quantification of perinuclear localization of tubulin Perinuclear region of fluorescence-labelled tubulin was determined by ImageJ. First, the nuclear area was manually outlined according to nuclear Hoechst-staining, then scaled with a proportion factor of 1.2 for both x and y axes. The perinuclear region was defined as the nuclear area subtracted from the upscaled area. In a few cases, the scale factor was 1.4 as the tubulin reorganization area was outside of the 1.2 scale. Then the perinuclear region was split in half (one half contained the disorganized tubulin and the other did not) by using an ImageJ macro. Calculations were carried out using the following formulas:

Fluorescence intensity (FI) = (Integrated Density of half of the ROI of a cell – Integrated Density of half of the selected cell nucleus) / (Area of half of the ROI of selected cell – Area of half of its nucleus). Then, the calculated fluorescence intensity normalized to the area that contained the disorganized tubulin was divided by the calculated fluorescence intensity of the other half of the same cell that did not contain the disorganized tubulin.

6–10 images were taken in each independent experiment, where 3–4 cells per image were analysed by using the provided formula.

Quantification of the number and size of focal adhesions from the confocal images The number of focal adhesions was determined in $120 \times 120 \mu\text{m}$ area. The threshold was set between 0.9% and 1.2% and the image was converted to a binary one. As a result, the focal adhesions appeared as white spots and the particles between sizes of $0.1 \mu\text{m}^2$ and infinity were counted using the Particle Analysis plugin in Fiji 1.53c software. The sum of the pFAK spots or areas was determined in each image covering the $120 \times 120 \mu\text{m}$ area, typically containing 5–7 cells. For one biological replicate 5–8 images were taken, meaning that one biological replicate is represented by 5–8 dots in the corresponding graph. Images were taken from randomly selected areas of the cell monolayer. All analyses were performed blinded, such as data analyzers were unaware of the genotypes.

Quantification of cofilin relocation upon stretch First, confocal images measured in the z direction were summed by using the ImageJ “Maximal Intensity Projection” function. After that, stacked images of both phalloidin- and cofilin-stained samples were converted to a binary image by using a threshold between 80% and 90% to identify the areas having a fluorescence signal. Then, the Particle analysis tool of ImageJ was applied on the masked images (to identify the areas having “no” fluorescence signal and a size limitation of $20\text{--}100 \mu\text{m}^2$ was used to exclude tiny and very large holes) to determine the size of each “empty” area. After that, the corresponding “empty” areas of phalloidin- and cofilin-stained images were used for further calculations. We subtracted the area of the phalloidin-stained “holes” from the area of the cofilin-stained “holes” and divided by the area of the phalloidin-stained “holes” to get the ratio of the area where cofilin staining cannot be detected with the used threshold. Then, the numbers were converted to percentage for each condition (wild type or mutant, stretched or non-stretched) were plotted and compared. All analyses were performed blinded, such as data analyzers were unaware of the genotypes and treatments.

AFM force spectroscopy of patient-derived fibroblasts AFM imaging (Igor Pro 6.37 software) was performed in contact mode with an MFP3D AFM (Kellermayer et al., 2006) using Bruker, MSCT-A probes (nominal typical spring constant = 70 pN/nm). Cantilevers were calibrated by the thermal method (Hutter and Bechhoefer, 1993). Cell monolayer samples were grown on circular microscope slides, which were mounted in the Bio-Heater module of the AFM. Imaging and force spectroscopy were carried out in a temperature-controlled liquid environment at 37°C . First an AFM image was taken, then *in situ* force spectroscopy was carried out collecting 100 force curves on selected

$3 \times 3 \mu\text{m}$ regions of cell surfaces (force mapping). The individual force curves were recorded with a vertical Z-piezo movement speed of $1 \mu\text{m/s}$, until the force set point (0.5 nN) was reached, then the tip was retracted. Elastic moduli were obtained by fitting the indentation curves with the blunted pyramidal model as described in Rico et al. (Rico et al., 2005). For the calculations (Kontomaris and Stylianou, 2017) the irregular pyramid shape with a semi-included angle of 20° , and with a spherical cap radius of 10 nm was used as described for the Bruker, MSCT-A probe. Poisson ratios of the tip and the sample were set to 0.2 and 0.5, respectively.

Statistical Analyses Statistical analyses were carried out in Graph-Pad Prism 4.01 and 8.0.1. Significance was determined by unpaired t-test as described in the specific Figure legend. Differences between groups were considered statistically significant if $p < 0.05$.

3. Results

3.1. Severity of the patient phenotype can be connected to the decreased migration of S338-I341del fibroblasts

First, we investigated if the G302A mutation, which has a milder phenotype in patients compared to S338-I341del patients, affected cell growth and migration of patient-derived fibroblast cells. Cell growth curves (Fig. 2A), as evaluated by cell counting, showed that the S338-I341del mutation reduced growth rate significantly (about 40% reduction 96 h after plating), while the growth rate of G302A cells was the same as wild type (control) cells.

We also assessed cell migration either by using a traditional wound healing assay (Fig. 2B and Fig. S1A-D) or by analyzing live cell migration (Fig. 2C-E). We found that migration of G302A cells was the same as wild type cells, while S338-I341del cells showed about 45% reduction in the extent of wound closure (Fig. 2B). Cell migration speed of live cells was reduced by 45% for the S338-I341del mutation (Fig. 2C). However, a few cells showed greater migration velocity comparable to those of the wild type cells. This might be attributed to the fact that the amount of β -actin is reduced in these individually tracked mutant cells, and the migration might be defined by the protein expressed from the residual wild type actin allele.

Directional persistence (Fig. 2D-E) was significantly reduced for S338-I341del, but not for the G302A mutant.

Thus, G302A mutation does not affect cell growth and migration, more specifically neither migration speed nor persistence changed. In the case of the S338-I341del variant, both cell growth and migration, including the speed of migration and the persistence were reduced.

3.2. S338-I341del, but not G302A mutation affects the stability of β -actin in patient-derived fibroblasts

As in the case of the S338-I341del mutant a reduced amount of β -actin was detected in cell lysates (Latham et al., 2018), we also wanted to explore whether the G302A mutation affects the amount (the stability) of β -actin. By comparing the amounts of β -actin and pan-actin of wild type and G302A fibroblasts (Fig. 3A-B), we found no significant differences between the two cohorts. We could, however, confirm the decreased amount of β -actin in S338-I341del cells. Thus, the effect of the two different mutations on the stability of β -actin is different, with the S338-I341del mutation having a more pronounced effect.

As it was shown recently that the G302A mutation at recombinant protein level slightly impairs actin (de)polymerization (Di Donato et al., 2026), we aimed to investigate whether G302A mutation affects the amount of F-actin in patient-derived fibroblast cells. Therefore, we separated F-actin from G-actin by ultracentrifugation and compared the amount of F-actin in wild type and G302A cells. The relative amount of F-actin showed no significant difference between the wild type and the G302A mutant cells as analyzed by either a β -actin or a pan-actin antibody (Fig. 3C-D). When we compared the F-actin content of wild type

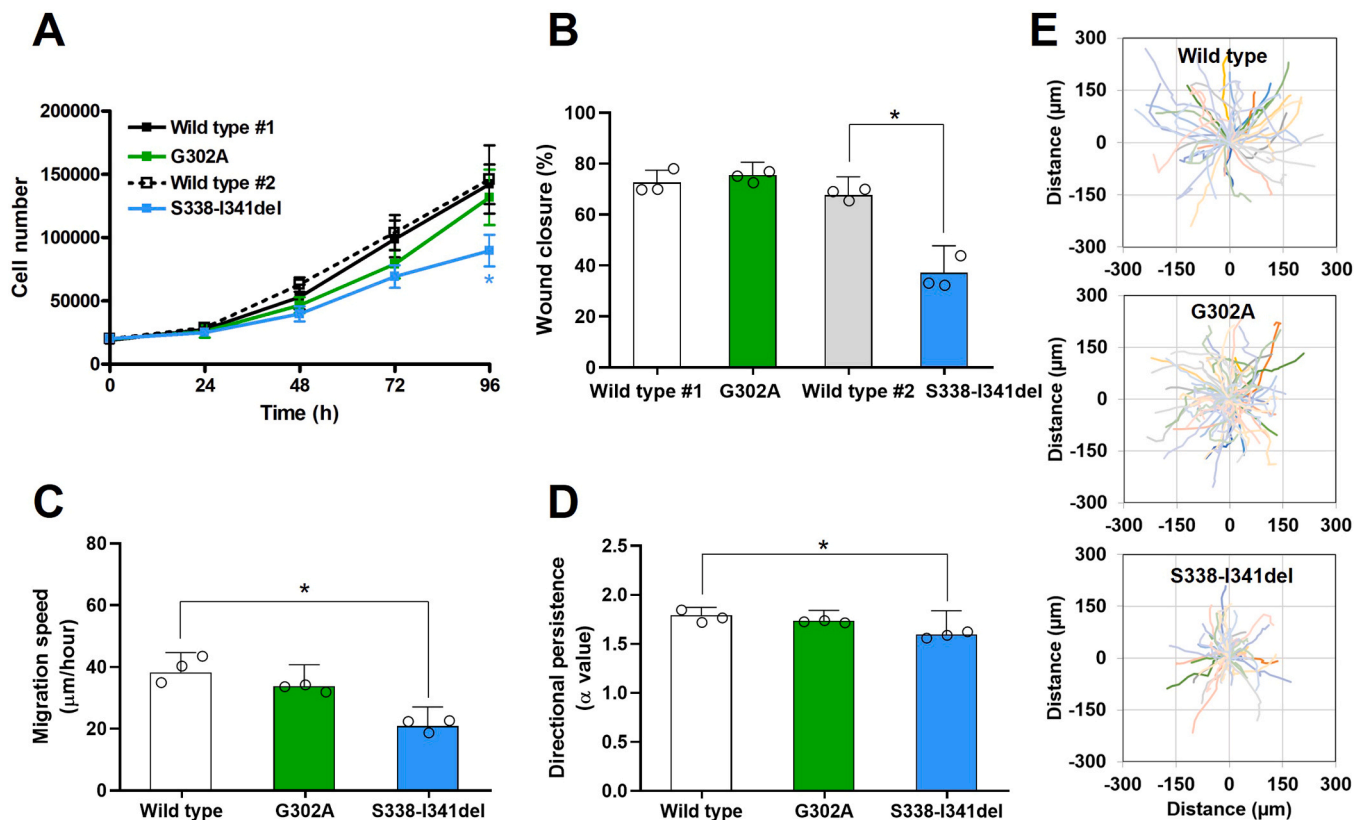


Fig. 2. S338-I341del, but not G302A mutation decreases the growth and migration of patient-derived fibroblasts (A) Cell growth curves of wild type (#1, black filled squares) and G302A (green filled squares) as well as of wild type (#2, black open squares) and S338-I341del (blue filled squares) cells are shown. Data are from three independent experiments and plotted as mean $\pm$ SD. (B) Cell migration was monitored in a wound closure assay (Fig. S1A-D). The mean values (open circles) of the percentage of wound closure in each independent experiment were plotted for wild type, G302A and S338-I341del cells. Mutants were measured separately, but always together with a corresponding wild type control (#1 with G302A and #2 with S338-I341del). The bars represent the mean $\pm$ SD values of three independent experiments. (C) Live cell migration speed and (D) directional persistence of wild type, G302A and S338-I341del cells were plotted as mean $\pm$ SD (bars) together with the mean value of each independent experiment (open circles). (E) Single cell trajectories of wild type and mutant cells. Data are from three independent experiments, altogether 17–20 cells were followed in each experiment for 6 h. Data were analyzed by unpaired *t*-test (**p* < 0.05).

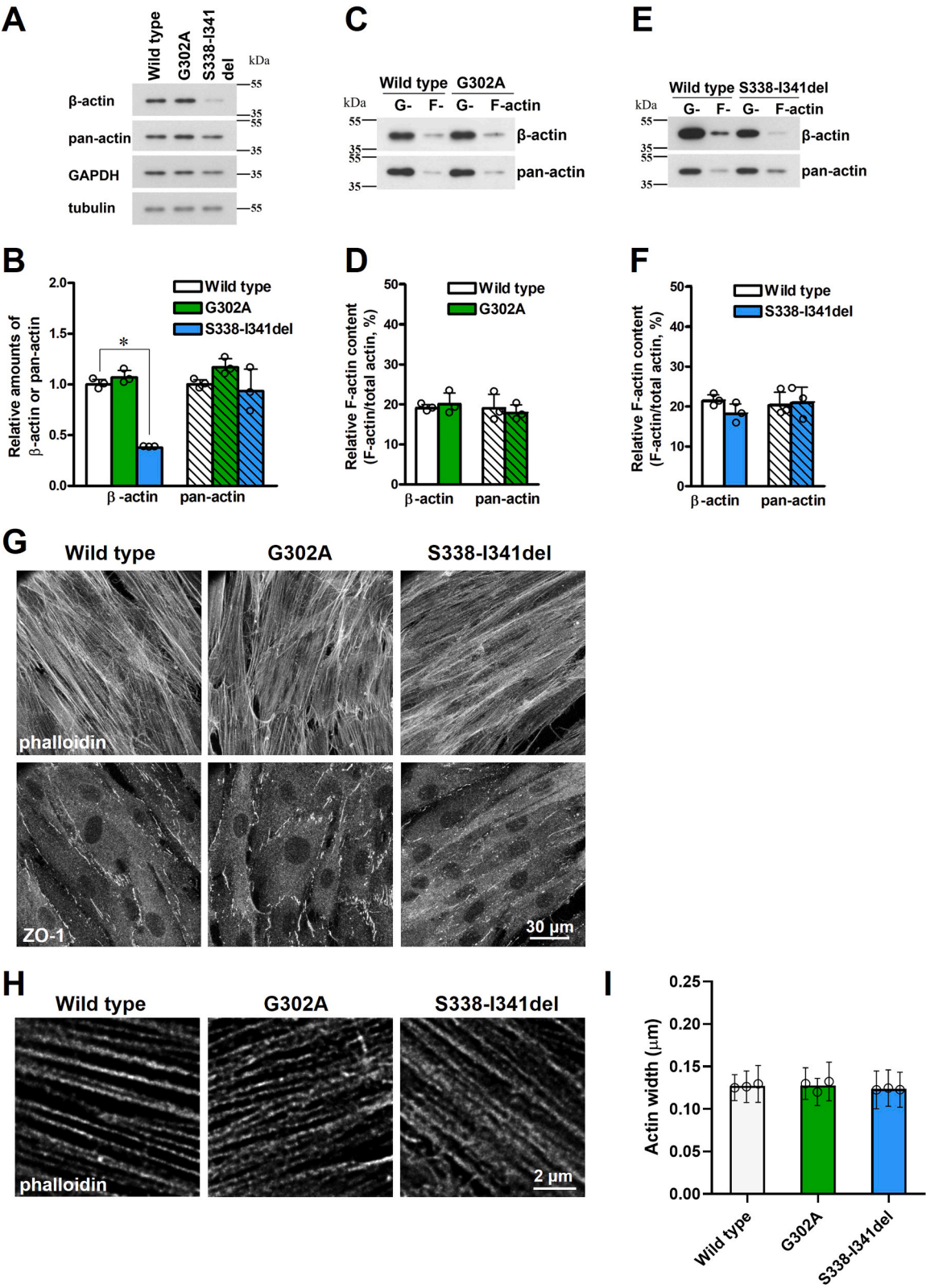
and S338-I341del cells we could not detect any significant changes in the amount of F-actin detected with the pan-actin antibody (Fig. 3E-F). As the amount of β -actin was reduced in the lysates of the S338-I341del cells (Fig. 3A-B), we wanted to analyze whether this is accompanied by the reduction of the F-actin amount containing β -actin in the S338-I341del cells. Although the total amount of β -actin was decreased in the S338-I341del cells, the relative amount of F-actin showed only a slight, but not significant decrease. Thus, although less β -actin is incorporated into the F-actin fibers (as less β -actin can be detected from the lysates), the transition from G-actin to F-actin is not affected significantly by the S338-I341del mutation.

Next, we wanted to investigate whether there is any difference in the F-actin organization within the patient-derived fibroblast cells. First, we compared the localization of actin by phalloidin staining in wild type, G302A and S338-I341del cells (Fig. 3G). We did not observe any striking differences in phalloidin staining, but the smaller cells obtained upon the mutation S338-I341del showed less pronounced zonula occludens (ZO-1) staining, indicating that tight junctions were less pronounced. Then, we also wanted to explore whether the mutations cause any changes in the structure of the actin filament bundles. Therefore, we applied superresolution (STED) microscopy (Fig. 3H) and quantified the filament width (Fig. 3I) from the fit of the Gaussian cross-sectional intensity profile (Fig. S2A-C). Although the width of the filament bundles was slightly reduced in the S338-I341del mutant, the differences were not significant (Fig. 3I). Based on the width of the actin double helix (7–9 nm) (Desroches and Harris, 2023), the estimated number of filaments per actin bundle were 14–18 in all the cases.

3.3. G302A mutation impairs cell stiffness and tubulin organization in patient-derived fibroblasts

The mechanical integrity of the actin cytoskeleton might be linked to the disease mechanism, as actin mutations might contribute to an improper assembly of specific actin networks. Therefore, we wanted to explore whether the actin mutations G302A and S338-I341del have any effect on the mechanical properties of patient-derived cells. We carried out atomic force microscopy on live fibroblasts and recorded force-distance curves. Typical force curves for wild type (WT), G302A and S338-I341del cells are shown in Fig. 4A. Distribution of the elastic moduli of wild type and S338-I341del mutant cells (Fig. 4B) revealed that there is a small difference in the full width at half-maximum (FWHM), but the mean value of the distribution (Fig. 4C) was not affected by the mutation. However, for G302A the mean value of the elastic modulus dropped to about one fourth of that in the wild type cells. This indicates that the G302A mutation reduces cell stiffness considerably, which cannot be accounted for any changes in actin bundle width. To uncover the mechanisms behind the reduced stiffness, we compared the amount of phosphorylated myosin light chain (pMLC) by western blotting in wild type and G302A cells (Fig. 4D-E). We found no significant differences in the extent of MLC phosphorylation. Thus, the reduced elastic modulus of the G302A mutant cells (Fig. 4C) cannot be explained by a decrease in contractility.

Since the organization of tubulin might also account for the changed stiffness, we compared the localization of tubulin both in wild type and G302A cells (Fig. 5A). We found an increased perinuclear localization of



(caption on next page)

Fig. 3. ACTB pLoF disorder - associated mutations do not impair actin filament organization in patient-derived fibroblasts (A) Total lysates of wild type, G302A and S338-I341del mutant fibroblasts were immunoblotted and labeled with the antibodies of β - and pan-actin. Tubulin and GAPDH were also labeled as loading controls. (B) Quantification shows the relative amount of β -actin or pan-actin normalized to the amount of tubulin and plotted below. Results of three independent experiments are shown. (C and E) F-actin (recovered from the pellet after ultracentrifugation) and G-actin (supernatant) content of wild type and G302A (C) or wild type and S338-I341del (E) mutant fibroblast cells were determined by western blotting using a β -actin or a pan-actin antibody. (D and F) The amount of F-actin was quantified for each condition as the ratio of F-actin and the total actin (the sum of F- and G-actin) for each sample separately and it is plotted below. Data are from three independent experiments and were analyzed by an unpaired *t*-test and expressed as mean $\pm$ SD (**p* < 0.05) and shown together with the individual data points. (G) Confocal images of fixed wild type, G302A and S338-I341del mutant cells stained with phalloidin and for zonula occludens (ZO-1). Maximum intensity projection of the confocal Z-stacks is shown. (H) 2D STED single-slice images of wild type, G302A mutant and S338-I341del cells stained with phalloidin. (I) Mean $\pm$ SD of the Gaussian fit of the distribution profile of actin width quantified from the line profile after image deconvolution is plotted for each independent experiment together with the mean value of the three independent experiments (bars). Examples of the histograms of wild type as well as of the mutants are shown in Fig. S2A-C.

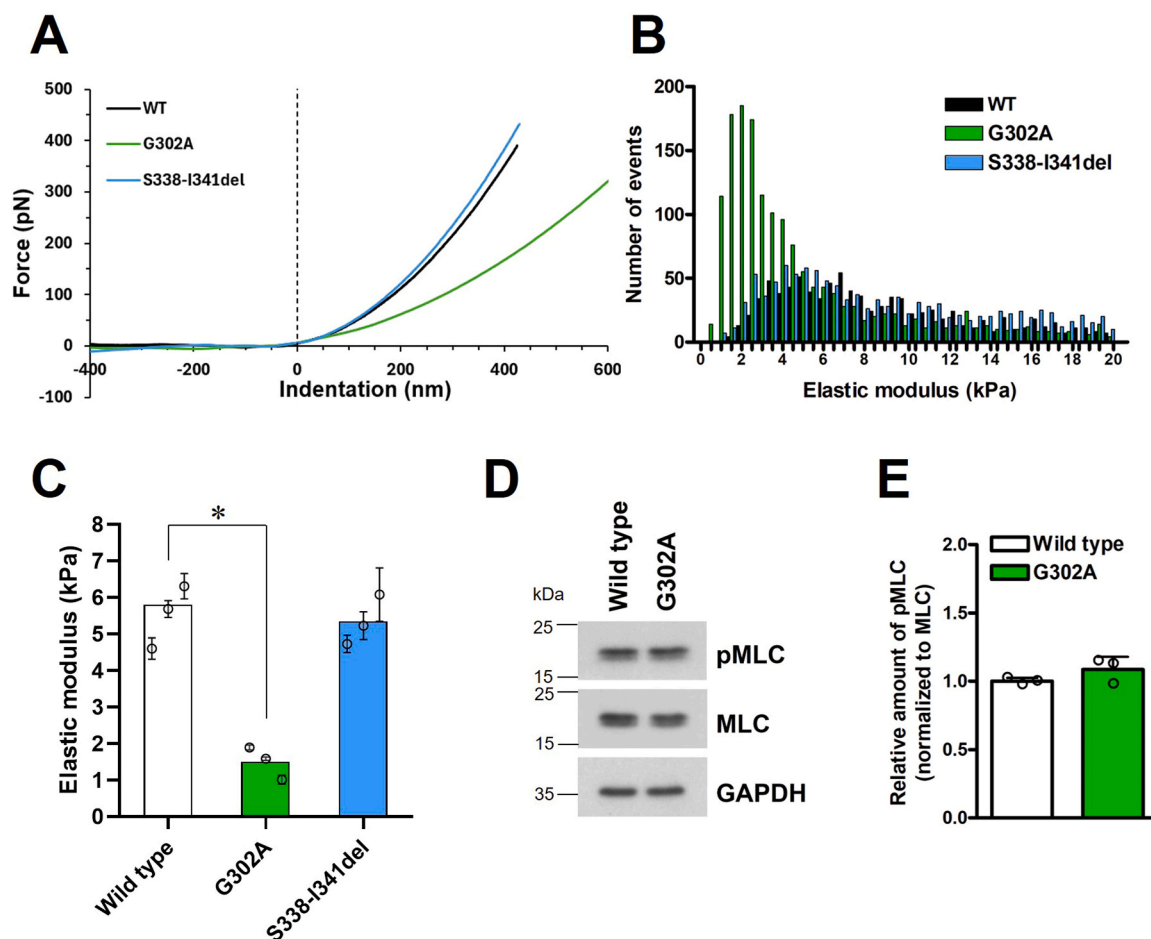


Fig. 4. G302A mutation decreases the cellular stiffness of patient-derived fibroblasts (A) Representative force-indentation curves of live wild type (WT, black), G302A mutant (green) and S338-I341del (blue) fibroblasts. (B) Distribution of the elastic moduli of wild type (WT, black), G302A (green) and S338-I341del cells (blue) calculated from the force-distance curves as described in the Materials and Methods section. (C) Mean values $\pm$ SD of the Gaussian fit of the histograms (calculated from the log-normal distribution) are shown for wild type, G302A and S338-I341del cells. The bars represent the mean values of the fit of the three independent experiments. (D) Phosphorylation of MLC was compared in wild type and G302A cells by western blotting. (E) Quantification of the amount of pMLC (normalized to MLC) is shown as bar graphs representing the mean $\pm$ SD together with the individual data points. Data are from three independent experiments and were analyzed by unpaired *t*-test (**p* < 0.05).

tubulin in the G302A mutant cells (Fig. 5A-B). The S338-I341del cells showed no difference in tubulin organization (Fig. 5A-B). To confirm that these changes are related to tubulin depolymerization, we treated wild type cells with different concentrations of colchicine (Fig. 5C). Upon increasing colchicine concentration, the fluorescence intensity of tubulin in the perinuclear region increased (Fig. 5C-D), while the microtubular network formation was reduced, which was the most obvious at the highest concentration of colchicine. This phenomenon observed at relatively low colchicine concentration (100 nM) was quite

similar to that in G302A mutant cells without colchicine treatment (Fig. 5C-D). To further explore a possible connection between the actin mutation and microtubule organization, we analyzed the focal adhesions in wild type and G302A mutant fibroblasts (Fig. 6A) by staining for focal adhesion kinase (FAK) phosphorylated on Tyr397 (pFAK). We found that the focal adhesion number (Fig. 6B) and size (Fig. 6C) was significantly increased by the G302A mutation. Compared to the wild type cells, S338-I341del cells showed no significant changes in either the number (Fig. 6B) or the size (Fig. 6C) of the focal adhesions.

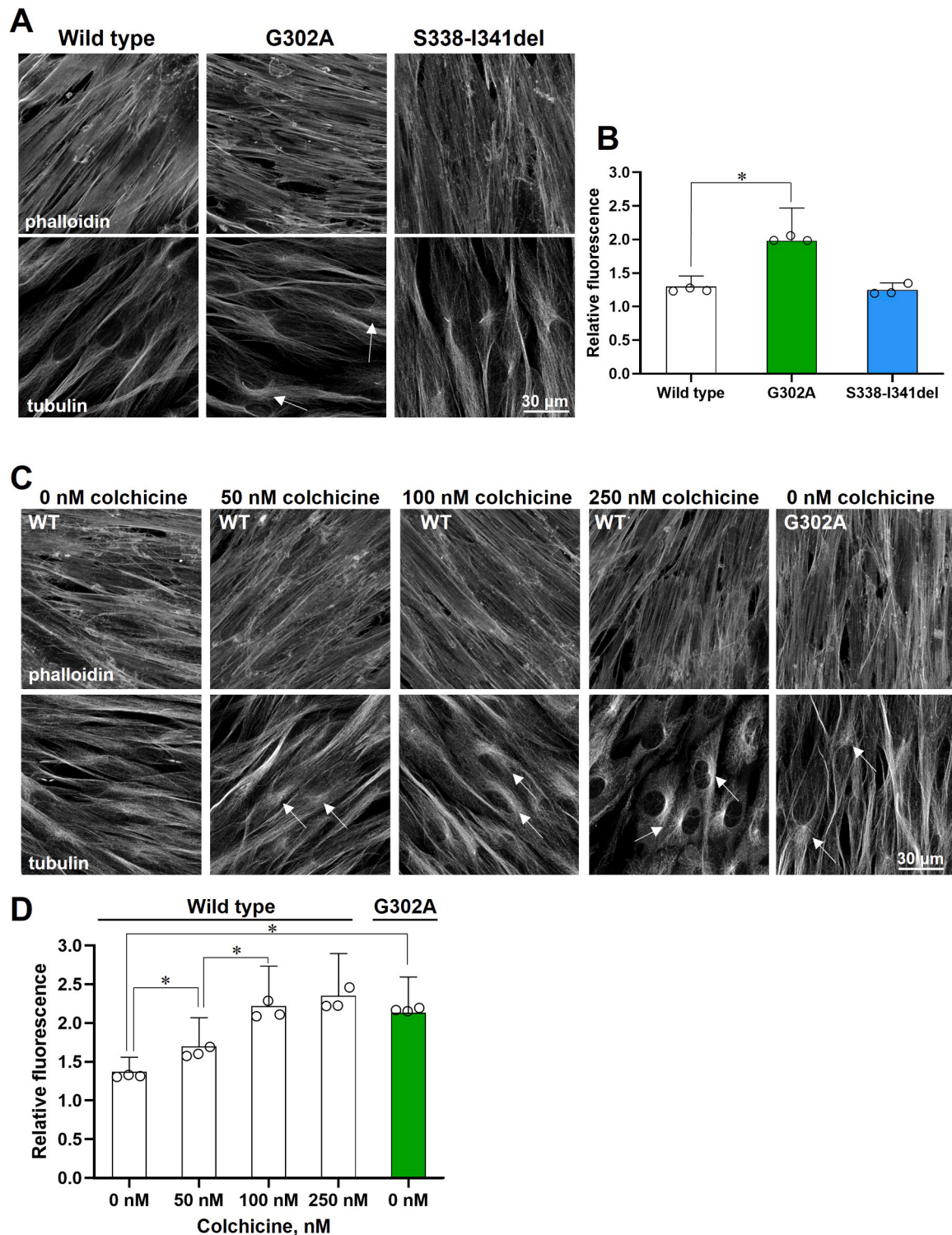


Fig. 5. G302A mutation affects tubulin organization in patient-derived fibroblasts (A) Phalloidin and tubulin stainings of wild type, G302A and S338-I341del cells. White arrows indicate examples of the increased perinuclear staining in G302A cells. Maximum intensity projection of the Z-stacks of microscope images is shown. (B) Quantification of perinuclear localization of tubulin in wild type (white), G302A (green) and S338-I341del (blue) cells. Mean values of each independent experiment are shown by open circles together with the mean $\pm$ SD (bars) of three independent experiments. (C) Confocal images of wild type (WT) or G302A cells treated with different concentrations of colchicine for 1 h. “No colchicine” stands for vehicle control. Maximum intensity projection of the Z-stacks of microscope images is shown. Fixed cells were stained with phalloidin and tubulin as indicated. White arrows show examples of the increased perinuclear staining. (D) Quantification of tubulin fluorescence intensities in the perinuclear region of colchicine-treated wild type or non-treated wild type and G302A mutant cells (vehicle control). Mean values of each independent experiment are shown by open circles together with the mean $\pm$ SD (bars) of three independent experiments. Data were analyzed by unpaired *t*-test (**p* < 0.05).

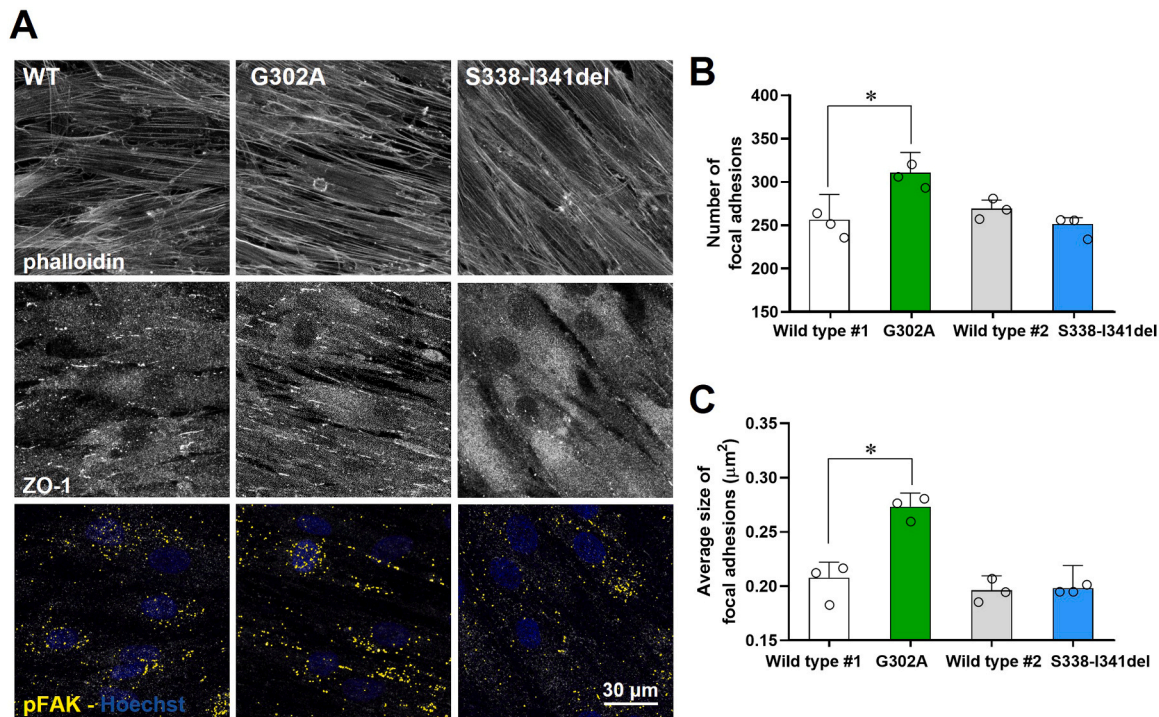


Fig. 6. G302A mutation perturbs focal adhesions of patient-derived fibroblasts (A) Wild type (WT), G302A and S338-I341del cells were fixed and stained with phalloidin, zonula occludens (ZO-1), pFAK antibodies, and Hoechst. Maximum intensity projection of the Z-stacks of microscope images is shown. The lowest panel shows the merged images of pFAK (yellow) and Hoechst (blue) stainings. (B) Quantification of the number of focal adhesions (pFAK-labeled spots) determined in a $120 \times 120 \mu\text{m}$ area of wild type and G302A as well as for wild type and S338-I341del cells are shown. Mean values of each independent experiment are shown by open circles together with the mean $\pm$ SD (bars) of three independent experiments. Mutants were measured separately, but always together with a corresponding wild type control (#1 with G302A and #2 with S338-I341del). (C) Quantification of the average size (area) of focal adhesions (pFAK-labeled spots) determined in a $120 \times 120 \mu\text{m}$ area of wild type, G302A and S338-I341del cells are shown. Mean values of each independent experiment are shown by open circles together with the mean $\pm$ SD (bars) of three independent experiments. Data were analyzed by unpaired *t*-test (* $p < 0.05$).

3.4. G302A mutation impairs cofilin reorganization, while S338-I341del mutation decreases myosin light chain phosphorylation upon stretch

To find out whether the investigated mutations affect the reorganization capacity of actin, we stretched wild type and mutant cells by 30% of their length, kept them stretched for 15 min, then fixed and stained for phalloidin and cofilin (Fig. 7A). By comparing phalloidin staining, we could observe more actin fibers within the cells in the direction of stretch in G302A mutant fibroblasts, whereas in the case of the S338-I341del mutation actin staining was stronger near the cell-cell junctions, but not in the cells' interior. Cofilin is required at the beginning of the stretch to sever/depolymerize actin, and this helps actin reorganization. In response to the external stress cells relocate their actin filaments to those junctions, which are parallel to the direction of stretch (stretched junctions). In response to stretch cofilin is phosphorylated by LIMK and dissociates from peripheral actin. Thus, the “relative movement” of cofilin is an indication of its disappearance from cell periphery. Indeed, when we inhibited LIMK by BMS-5 we did not observe any cofilin relocation from the cell periphery compared to non-stretched cells (Fig. S3B-E). The extent of the relative movement of cofilin from the cell periphery upon stretch might be changed by the investigated actin mutations. Therefore, we compared the extent of cofilin relocation from the cell periphery and found an increase in the case of the G302A mutant cells relative to its wild type counterpart (Fig. 7B), but not for the S338-I341del cells.

To correlate the extent of changes in cofilin reorganization with cofilin phosphorylation (which enhances its dissociation from actin), we terminated the stretch at different time points by cell lysis to reveal the kinetics of cofilin phosphorylation by western blotting (Fig. 8A-D). Cofilin phosphorylation was slightly increased already in the absence of

stretch and remained constant in the G302A mutant (Fig. 8A-B). This correlates with the increased extent of cofilin dissociation from actin (Fig. 7B). While in the case of the S338-I341del mutant we also observed an increased cofilin phosphorylation (Fig. 8C-D), it was not accompanied by an increased cofilin dissociation (Fig. 7B). As a result of the uniaxial stretch, parallel actin bundles are formed in the direction of stretch and those might be decorated by myosin so that the cells can counteract the external force in the direction of the stretch. By monitoring the extent of myosin light chain phosphorylation (Fig. 8E-F) we found no major difference between wild type and G302A mutant cells, while there was a drastic reduction of myosin light chain phosphorylation upon the S338-I341 deletion (Fig. 8G-H). This was already observed before stretch and although the extent of phosphorylation increased, it never reached the level of that in the wild type cells. It was shown that myosin II cooperates with formin (responsible for linear actin cable formation) in the regulation of force generation (Duda et al., 2019; Nishimura et al., 2021). Therefore, we attempted to rescue the myosin light chain phosphorylation defect observed in the S338-I341del cells by overexpressing the active form of the formin, mDia1 (Fig. S4A-D). Western blot analysis of the lysates (Fig. 9A-B) indicated a partial rescue of myosin light chain phosphorylation both in non-stretched (0') and stretched (20') cells.

4. Discussion

Very little is known about how the different ACTB pLoF variants impair the function of actin and how this affects cellular behavior. Here we compared the cellular mechanisms of two mutations giving a phenotype with different severity within the spectrum of the ACTB pLoF disorders. We show that indeed there is a difference in cell growth and

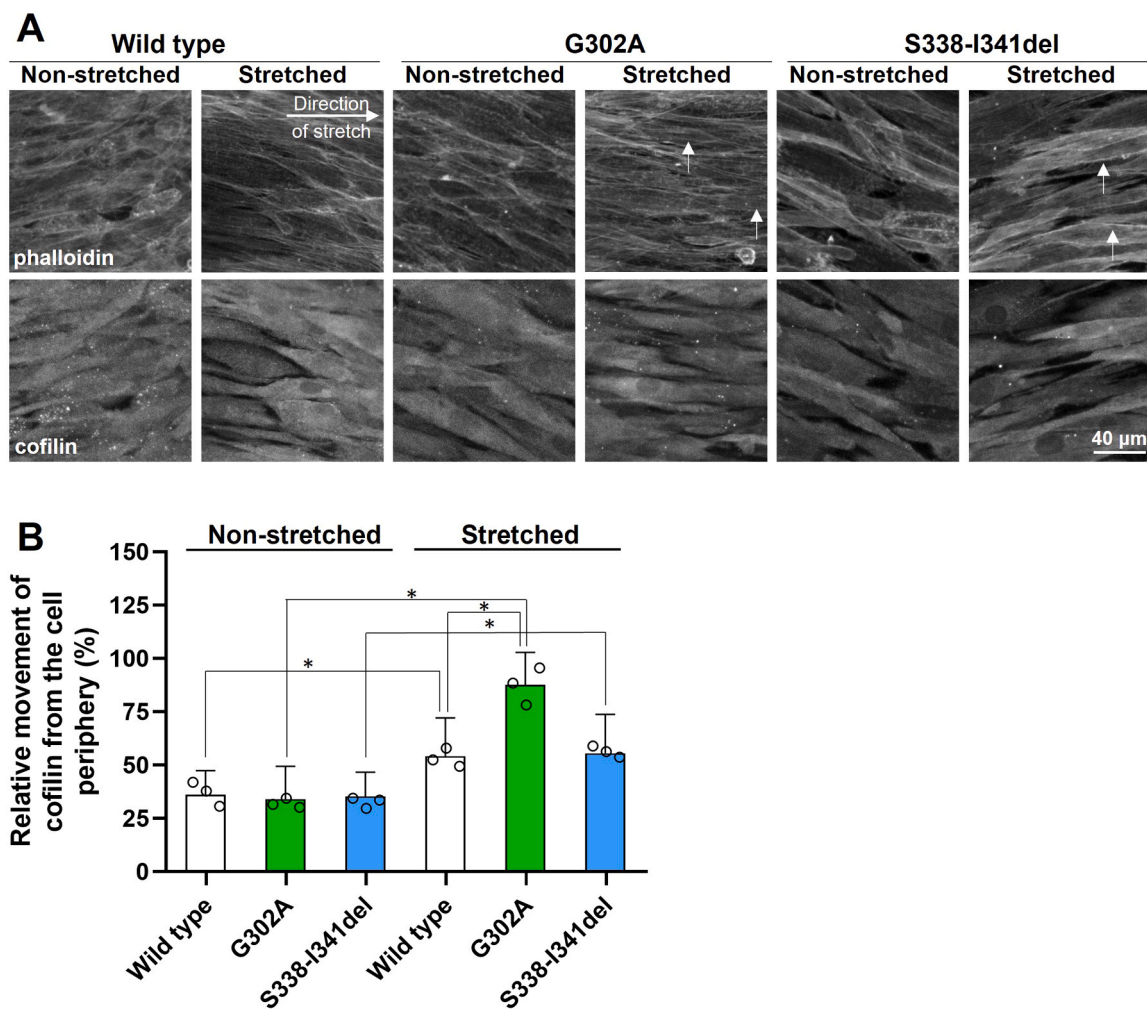


Fig. 7. G302A mutation increases the dynamic reorganization of actin by enhancing cofilin dissociation (A) Immunofluorescence analysis of wild type, G302A mutant and S338-I341del fibroblasts kept either non-stretched or stretched for 15 min. Samples were stained with either phalloidin or for cofilin. Maximum intensity projection of the Z-stacks of microscope images is shown. White arrows indicate the thicker internal stress fibers for the G302A mutants and the thicker peripheral actin bundles for the S338-I341del mutant. (B) Quantification of the relative movement of cofilin from the cell periphery. Mean values of each independent experiment are shown by open circles together with the mean $\pm$ SD (bars) of three independent experiments. Data were analyzed by unpaired t-test (* $p < 0.05$). The rationale of the quantification of the relative movement of cofilin is shown in Fig. S3A.

migration of the two mutants. Although both mutations perturb the reorganization properties of actin, the mechanisms behind are different.

It has been shown that at recombinant protein level some of the ACTB pLoF variants affect the stability of actin and slightly impair actin polymerization/depolymerization (Di Donato et al., 2026). Experiments comparing the expression of the G302A and the S338-I341del variants at recombinant protein level indicated that the effect of the S338-I341del mutation is more pronounced on the protein stability compared to the G302A mutation. Indeed, we found in patient-derived fibroblast cells that the amount of β -actin was reduced in S338-I341del, but not in G302A cells. Despite the reduced β -actin stability, S338-I341del cells showed no impairment in F-actin formation, although the amount of β -actin incorporated into F-actin was reduced. This might be explained by a compensation mechanism through the overexpression of γ -actin and/or α -SMA (Latham et al., 2018). We found no reduction in the amount of F-actin in the G302A cells compared to healthy (wild type) control cells. In mononuclear blood cells there was an indication that the G302A mutant actin has a decreased ability to form F-actin (Baumann et al., 2020). This discrepancy might be due to the fact that blood mononuclear cells and fibroblasts have different types of actin networks.

The filament formation in the different actin networks (such as cortical actin fibers or focal adhesions) might be differentially affected by the mutation.

Previously we have shown that the R196H actin mutation with impaired polymerization/depolymerization properties (Greve and Manstein, 2025) led to a decreased cellular stiffness of patient-derived fibroblasts (Grácz et al., 2026), showing that an appropriate cellular actin network is required to maintain the proper mechanical integrity of the cell. We observed a decreased cellular stiffness for the G302A cells compared to the wild type and S338-I341del cells, although there was no difference among the F-actin content of the same cell types. This decrease in stiffness is unlikely to be associated with changes in either the organization or the contractility of the actin cytoskeleton.

When we investigated the organization of the microtubule network, another critical determinant of the cellular stiffness, we found an increased perinuclear staining of tubulin, an indication for the perturbation of tubulin polymerization dynamics in G302A fibroblasts, but not in S338-I341del cells. Reduced cell stiffness might be linked to how actin regulates the localization and function of microtubules. Indeed, the application of the tubulin depolymerizing drug, colchicine (Laisne et al.,

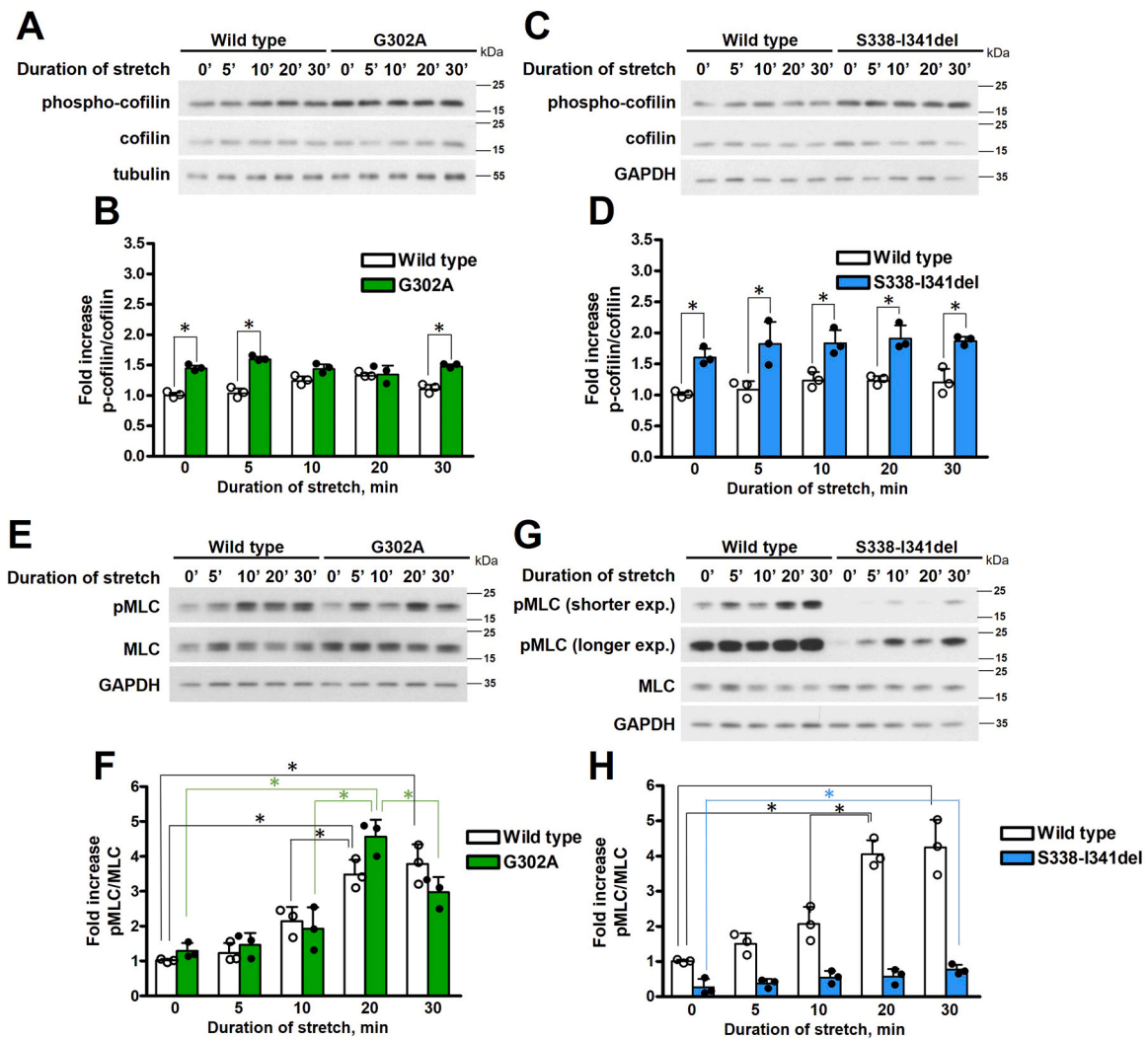


Fig. 8. S338-I341 deletion impairs myosin light chain phosphorylation (A) and (E) Wild type or G302A fibroblasts were stretched by 30% of their original length and were harvested at different time points. Phosphorylation of cofilin (A) and myosin light chain (MLC) (E) were analyzed by Western blotting. Quantification of the data is shown in (B) for p-cofilin and in (F) for pMLC. (C) and (G) Wild type or S338-I341del fibroblasts were stretched by 30% of their original length and were harvested at different time points. Quantification of the data is shown in (D) for p-cofilin and in (H) for pMLC. The amounts of pMLC and p-cofilin were normalized to the total MLC or cofilin amounts, respectively. Data are from three independent experiments. Bars represent mean $\pm$ SD and are plotted together with the individual data points (open circles for non-stretched, filled circles for stretched samples). Data were analyzed by unpaired *t*-test (**p* < 0.05).

2021) applied at relatively low concentration in wild type cells showed a similar reorganization of the tubulin cytoskeleton as it was observed in the G302A mutant ones without the drug. Thus, the G302A mutation might influence the stability of microtubules. It has been shown that focal adhesion disassembly is regulated by microtubule growth (Aureille et al., 2024) and, in turn, microtubule depolymerization promotes focal adhesion growth (Thapa et al., 2023). Indeed, we found that in the G302A fibroblasts both the size and the number of focal adhesions were increased, as identified by the autophosphorylated form (pTyr397) of focal adhesion kinase (FAK) (Le Coq et al., 2022). The observed slight, but significant increase in both the focal adhesion size and number does not result in a global increase in myosin light chain phosphorylation. We hypothesize that this might rather be a local effect. Hence, it might be possible that the G302A mutation weakens the interaction of actin with microtubules through an interacting protein and this locally uncouples the microtubules from actin, leading to the perturbation of microtubule dynamics.

Whether tubulin misorganization could play a role in patient's phenotype still needs to be addressed in neurons. It is clear, however, that the interplay between the actin cytoskeleton and the microtubules

is important for neurite outgrowth. The dynamic reorganization of actin is necessary for creating protrusions with stable microtubules to determine the polarity of the axon (Lasser et al., 2018). Dendrites are also developed from such protrusions, but dendrites harbor microtubules with mixed polarity. It was shown that inhibition of microtubule polymerization might affect dendritic branching (Ouzounidis et al., 2023), and the extent of branching correlates with the level of intelligence, i.e. lower branching might be associated with intellectual disability (Quach et al., 2021). This might be connected with the moderate phenotype of the ACTB pLoF patients (Hecquet et al., 2025).

Both mutations affect the reorganization properties of actin during uniaxial stretch. Actin reorganization is regulated by the actin depolymerizing factor cofilin (Duda et al., 2019). During stretch-induced actin reorganization cofilin is dissociated from peripheral actin. Therefore, a change in cofilin localization can be observed at the cell periphery upon stretch. This localization difference between non-stretched and stretched cells can be quantified in both wild type and mutant cells. We showed here that cofilin "removal" from the cell periphery is due to its phosphorylation by LIMK, and thus the dissociation of phosphorylated cofilin from actin. We found that the extent of cofilin "removal" from the

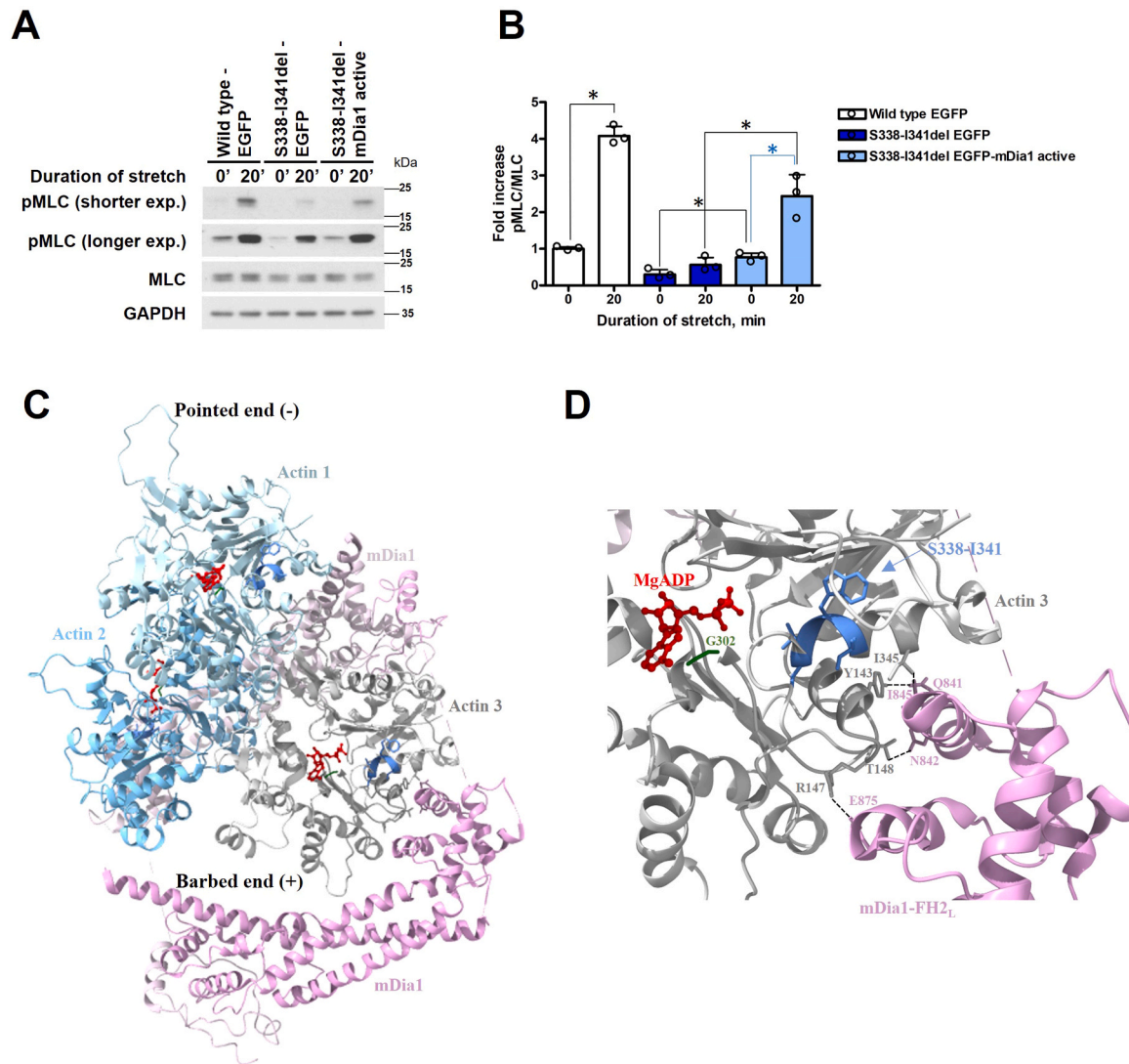


Fig. 9. Defect in myosin light chain phosphorylation in S338-I341del cells can be rescued partially by overexpressing mDia1 (A) Western blot analysis of wild type fibroblasts infected with EGFP, S338-I341del fibroblasts infected with either EGFP or active EGFP-mDia1 constructs, left either unstretched or stretched for 20 min before cell lysis. (B) Phosphorylation of myosin light chain was quantified and normalized to the total MLC amount. Data are from three independent experiments, mean $\pm$ SD is plotted together with the individual data points. Data were analyzed by unpaired *t*-test (**p* < 0.05). (C) mDia1 binding to cytoskeletal β -actin as represented by the recently published cryo-EM structure (PDB code: 8RU2, (Oosterheert et al., 2024)). Actin protomers are shown as ribbon in different shades of blue and grey, while mDia1 protomers in different shades of pink. The ball-and-stick model of MgADP is colored red, and the stick model of G302 is green, while the S338-I341 sequence (both the stick model of the residues and the corresponding ribbon structure) is blue. (D) The zoom-in view of an actin monomer ("Actin 3" – grey) is shown together with the interacting surface of mDia1 leading FH2 (mDia-FH2_L) domain highlighting the possible interactions between the residues of actin and mDia as black dashed lines. The cut-off distance for H-bonds, hydrophobic interactions and salt bridges was 4 Å. For the visualization of structures represented in (C) and (D) ChimeraX program was used.

cell periphery was increased in the case of the G302A mutant compared to the wild type fibroblasts. This might indicate that the binding of cofilin to the G302A mutant actin is decreased during actin reorganization. In line with this observation, we found a slight increase in cofilin phosphorylation both in the absence and presence of stretch. This might result in the stabilization of the internal stress fibers running parallel with the direction of stretch.

The extent of myosin light chain (MLC) phosphorylation, however, decreased in the S338-I341del mutant, suggesting that this mutation affects the contraction efficiency of actin fibers upon mechanical stress. MLC phosphorylation has been shown to change the conformation of myosin (Brito and Sousa, 2020), promoting the assembly of myosin filaments and increasing its ATPase activity. The decreased MLC phosphorylation suggests that most probably there is a decrease in the

number and/or in the organization of myosin filaments, which is defined by the delicate balance between myosin phosphorylation and dephosphorylation exerted by myosin kinases and phosphatases, respectively (Javier-Torrent and Saura, 2020). Based on the available structural data (PDB code: 5JLH, (von der Ecken et al., 2016)), the myosin binding site is close to that helix of actin, which contains the S338-I341 segment (labeled blue in Fig. S5A,B). This segment in actin does not interact directly with myosin but might help the positioning of a loop consisting of the residues D24 and D25 (through the interaction of W340 and P27). This loop interacts with the cardiomyopathy (CM) loop of myosin (Fig. S5A-B (von der Ecken et al., 2016)). A molecular model of the S338-I341del mutant actin showed significant changes in the interaction of actin with the CM-loop of myosin (Latham et al., 2018). In addition, the loop following the helix with the deletion contains two residues,

S350 and T351, which interact with E556 of myosin. The mutation of the latter residue in *Dictyostelium discoideum* myosin (residue E531) has been shown to reduce the binding of myosin to F-actin to one tenth (Furch et al., 2000). Therefore, the deletion might also perturb the positioning of residues S350 and T351, which in turn could reduce myosin binding through the residue E556 to S338-I341del actin.

The S338-I341del mutant perturbs the interaction of actin with myosin II in resting cells. The level of MLC phosphorylation was reduced in response to stretch, but cofilin localization was not affected. This suggests that actin severing and/or depolymerization by cofilin is not affected by the mutation. After its depolymerization, actin gets reorganized to those cell junctions, which run parallel to the direction of stretch (stretched junctions). Linear actin cables will be formed by the formin Dia (Duda et al., 2019), to provide a filamentous actin structure for myosin II binding. A recent study raised the possibility of a crosstalk between myosin II and formin in the regulation of force generation (Nishimura et al., 2021). The authors found that traction forces exerted by the stress fibers depend not only on myosin II, but also on formin. We indeed rescued the cell-contraction defect by overexpressing the active form of the formin mDia1. The overexpression partially restored MLC phosphorylation in the mutant, which points at the crosstalk between formins and myosins, that results in adjusting the extent and duration of contraction.

We also checked the binding site of the FH2-domain of formins on actin based on the available structural data (PDB code: 8RU2, (Oosterheert et al., 2024)). Indeed, the FH2 domain interacts with the surrounding of the helix with the deletion (I345 from the helix having the deletion, Fig. 9C-D). The figure illustrates the “knob” region of the leading FH2 domain of mDia1. The same region in the trailing FH2 domain needs to dissociate from actin so that the following actin monomer can be incorporated into the filament (Oosterheert et al., 2024). It has been shown that stabilizing mutation introduced into the “knob” region of the FH2 domain of mDia1 (e.g. Q841K) decrease actin filament elongation. If the interaction between actin and the “knob” region of mDia1 FH2 domain is weakened by the S338-I341del mutation of actin, that might possibly enhance filament elongation. The possible cross-talk between myosin and formin in our “rescue” experiment might be explained by the fact that overexpressing the active form of mDia1 can simply create more actin filaments capable of binding to the myosin filaments and therefore the contraction increases.

The decreased myosin light chain phosphorylation observed in the S338-I341del patient-derived fibroblasts might be relevant in the phenotype of the patients, since it has been shown that mutations in myosin II are linked to neurodevelopmental and neurodegenerative disorders (Newell-Litwa et al., 2015). Mutations associated with non-muscle myosin IIA are specifically linked to autism, intellectual disability and schizophrenia (Javier-Torrent and Saura, 2020). Migration of neurons during brain development is a critical event to establish appropriate connections between them (Buchsbaum and Cappello, 2019). Pharmacological inhibition of myosin activity showed the essential function of myosin in neuronal migration as it disrupted the forward movement of cells, and the pulling forces necessary for translocating the soma during neuronal migration (Solecki et al., 2009). Thus, the migration defect found in the S338-I341del cells (Latham et al., 2018) might be explained by the defect in myosin phosphorylation and contraction.

Taken together, we found that two variants associated with ACTB pLoF disorder affect the reorganization properties of actin, which might be important for neuronal migration, necessary for creating a properly connected neural network (Minegishi and Inagaki, 2020). An interesting additional observation was in the case of the G302A mutant that the structural organization of tubulin was affected by the actin mutation. This might have also critical consequences in neurons (Ouzounidis et al., 2023), affecting the branching ability of the cells. As no treatment is available for these patients so far, it would be critical to determine to what extent these defects appear in neurons and whether the activation

of mDia1 to rescue the defect in cell contraction would be a possible treatment option for the patients having the S338-I341 deletion in β -actin.

CRediT authorship contribution statement

Éva Gráczner: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Investigation, Data curation, Funding acquisition. **Kira Dakos:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Data curation. **Tamás Bozó:** Writing – review & editing, Formal analysis, Data curation. **Katalin Pászty:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Nataliya Di Donato:** Writing – review & editing, Writing – original draft, Conceptualization. **Miklós Kellermayer:** Writing – review & editing, Writing – original draft, Conceptualization, Funding acquisition. **Andrea Varga:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejcb.2026.151577.

Data availability

Data will be made available on request.

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