Figures and figure supplements

Mechanistic insights into the active site and allosteric communication pathways in human nonmuscle myosin-2C

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Figure 1. Myosin-2 phylogeny, overall topology, and active site characteristics of human NM2C. (A) Phylogenetic analysis divides human myosins-2 in the three subfamilies (i) nonmuscle and smooth muscle myosins-2, (ii) cardiac, and (iii) skeletal myosins-2. (B) Detailed view of the active site showing key residues. (C) Structural representation of the N-terminus (Nter) and C-terminus (Cter) domains.
(iii) and skeletal muscle myosins-2 (Föth et al., 2006). Nonmuscle myosin-2s are essential for the structural integrity of the cytoplasmic architecture during cell shape remodeling and motile events of eukaryotic cells, whereas all other myosins-2 play eminent roles in the contraction of smooth, cardiac and striated muscle cells (Sellers, 2000). Abbreviations used: NM2A: nonmuscle myosin-2A, NM2B: nonmuscle myosin-2B, NM2C: nonmuscle myosin-2C, SM: smooth muscle myosin-2, CardA: α-cardiac myosin-2, CardB: β-cardiac myosin-2, EO2: extraocular myosin-2, EMB: embryonic myosin-2, PERI: perinatal myosin-2, IIb: fast skeletal muscle myosin-2, IIx/d: skeletal muscle myosin-2, IIa: slow skeletal muscle myosin-2. (B) Architecture of the crystallized NM2C construct in the pre-powerstroke state. The myosin motor domain and the α-actinin repeats are shown in cartoon representation in green and grey color. The nucleotide is shown in spheres representation. Inset, Conserved key residues that interact with the nucleotide in the NM2C active site. The Fo-Fc omit map of Mg$^{2+}$-ADP-VO$_4$ is contoured at 4σ. The salt bridge between switch-1 R261 and switch-2 E483 is highlighted. (C) Subdomain architecture of NM2C. The U50 kDa is shown in green, the L50 kDa in purple, the converter in grey, and the Nter in blue. The region shown in orange corresponds to the active site and the junction of U50 kDa and L50 kDa. The bound nucleotide is shown in spheres representation. The location of the SH1-SH2 helix and the relay helix in the L50 kDa is highlighted.

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Figure 1—figure supplement 1. Myosin-2 ATPase cycle, expression constructs, and structural alignment of the NM2C Cα coordinates. (A) Consensus scheme of the myosin and actomyosin ATPase cycle. The upper part represents the myosin (M) ATPase cycle. The lower part represents the ATPase cycle in the presence of F-actin (A). The asterisk denotes enhanced states of the intrinsic myosin fluorescence, which are attributed to the nucleotide-induced changes in the microenvironment of the conserved relay loop W525. Lowercase $k$ denotes a rate constant. $k_{-D} = k_{+D}$, $k_{-6} = k_{+6}$, $k_{AD} = k_{A}$. An uppercase K denotes a dissociation equilibrium constant ($K = k_{-x}/k_{+x}$) throughout this work. Normal and bold face notation denote the respective kinetic constants in the absence and presence of F-actin. The main pathway of the actomyosin ATPase cycle is highlighted in grey. Strong and weak actin binding states are indicated. (B) Schematic representation of the expression constructs used in this study. Top, Nonmuscle myosin-2C
Figure 1—figure supplement 1 continued

contains a N-terminal motor domain (green) followed by a neck and a tail domain (dark grey). Middle, For kinetic studies of NM2C, R788K, and R788E, the motor domain was directly fused to spectrin repeats 1 and 2 from Dictyostelium α-actinin (light grey) which serves as an artificial lever arm. The concept of the artificial lever arm has been successfully used in structural and kinetic studies of myosins-2 (Heissler and Manstein, 2011; Furch et al., 1999; Kliche et al., 2001; Münich et al., 2014). Bottom: For structural studies, the N-terminal 45 amino acids were deleted. The numbering refers to the amino acid sequence of the full-length protein. (C) The NM2C Cα atoms (green) in ribbon representation superimpose with a root mean square deviation (r.m.s.d.) of 0.57 Å to chicken smooth muscle myosin-2 (grey, PDB entry 1BR2), with 0.78 Å to scallop striated muscle myosin-2 (orange, PDB entry 1QVI), and 0.73 Å to Dictyostelium nonmuscle myosin-2 (blue, PDB entry 2XEL), underlining a strong correlation between Cα geometry and overall motor domain fold. The JK-loop and the nucleotide are highlighted in color and spheres representation. (D) Relative orientation of converter and lever in NM2C (green), chicken smooth muscle myosin-2 (PDB entry 1BR2, grey), and scallop striated muscle myosin-2 (PDB entry 1QVI (orange), 1DFL (violet)) motor domain structures in cartoon representation. α-helices are depicted as cylinders.

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Figure 2. Conformational changes of the JK-loop in the myosin active site. (A) Top view on the NM2C active site in the pre-powerstroke state (green) superimposed on pre-powerstroke state structures from chicken smooth muscle myosin-2 (grey, PDB entry 1BR4), Dictyostelium nonmuscle myosin-2 (blue, PDB entry 2XEL), and scallop striated muscle myosin-2 (orange, PDB entry 1QVI). The ATP analog ADP·VO$_4^-$ is shown in spheres representation. (B) Conformation of the JK-loop in vicinity to the NM2C active site. The JK-loop flanks the active site and connects helices J and K. The distance between the residue Q340 of the JK-loop in the U50 kDa and the D257 of switch-1 of the active site is ~8.8 Å. The distance between residue S336 of the JK-loop and switch-1 D257 is ~7.4 Å. Switch-1 residue N256 interacts with α-phosphate (3.1 Å) and β-phosphate (3.5 Å) group of ADP·VO$_4^-$ in the active site. NM2C is colored in green/orange, the JK-loop is colored in brick red and ADP·VO$_4^-$ is shown in spheres. (C) Interactions between the JK-loop and the switch-1 region are compared between the NM2C (green) and scallop striated muscle myosin-2 (orange, PDB entry 1QVI). A-loop residue R128 is coordinating the interaction to the ADP adenosine in the active site of striated muscle myosin-2. The distance between the residues is 3.2 Å. R128 further forms a hydrogen bond (2.8 Å) with E184 of the P-loop. JK-loop N321 is in hydrogen bond interaction with switch-1 N238, located at a distance of 4.6 Å to the hydroxyl group of the C2’ of the ADP ribose. The connectivity between switch-1 and the nucleotide is further strengthened by a hydrogen bond between N237 and the ADP ribose. NM2C lacks all interactions described for scallop striated muscle myosin-2 due to the replacement of R128 with Q150 and JK-loop shortening which increases the distance to the adenosine in the active site to 5.8 Å and disrupts constrains between switch-1 and the JK-loop. All residues in the JK-loop region are labeled for legibility. (D) Superimposition of the NM2C pre-powerstroke state structure (green) and the actin-bound near-rigor actoNM2C complex (red) shows that the nucleotide-binding site does not undergo major structural changes. Actin subunits are colored in shades of grey and the nucleotide is shown in spheres representation. (E) Sequence alignment of select structural elements in the myosin motor domain that interact with the JK-loop. Interactions of A-loop R128 are highlighted with brackets for scallop striated muscle myosin-2 (PDB entry 1QVI). All highlighted interactions are absent in NM2C due to the presence of Q150 in the A-loop. Abbreviations used: Hs NM2C: human NM2C (NP_079005.3); Hs NM2A: human nonmuscle myosin-2A (NP_002464.1); NM2B: human nonmuscle myosin-2B (NP_005955.3); Gg SM: chicken smooth muscle myosin-2 (NP_990605.2); Hs CARD: human beta cardiac muscle myosin-2 (NP_000248.2); Ai ST: scallop striated muscle myosin-2 (P24733.1). PDB entries are indicated when available.

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Figure 2—figure supplement 1. Active site characteristics in myosin-2 motor domains. (A) Sequence alignment of myosin-2 JK-loops. The asterisk indicates the invariant, negatively charged residues corresponding to NM2C E341. The abbreviations used are as follows: Hs NM2C: human NM2C (NP_079005.3); Hs NM2A: human nonmuscle myosin-2A (NP_002464.1); NM2B: human nonmuscle myosin-2B (NP_005955.3); Gg SM: chicken smooth muscle myosin-2 (NP_990605.2); Hs CARD: human beta-cardiac muscle myosin-2 (NP_000248.2); Ai ST: scallop striated muscle myosin-2 (P24733.1); Dd NM2: Dictyostelium nonmuscle myosin-2 (XP_637740.1); Dm EMB: Drosophila embryonic body wall muscle myosin-2 (P05661 with spliced exon 7a); Dm IFI: Drosophila indirect flight muscle myosin-2 (P05661 with spliced exon 7d). PDB entries are indicated when available. Cardiomyopathy-associated mutations in beta-cardiac myosin-2 on positions F312 (F312C), V320 (V320M), A326 (A326P), and E328 (E328G) are highlighted in the boxed areas. (B) Close-up view of the active site region of pre-powerstroke state structures of NM2C (green), chicken smooth muscle myosin-2 (grey, PDB entry 1BR2), and Dictyostelium nonmuscle myosin-2 (blue, PDB entry 2XEL). The distance between the JK-loops of smooth muscle myosin-2 and Dictyostelium nonmuscle myosin-2 and switch-1 is ~2.8 Å and 8.8 Å for NM2C. The ADP-VO₄ complex in the active site is shown in spheres. (C) Close-up view of the active site region of pre-powerstroke state chicken smooth muscle myosin-2 (grey, PDB entry 1BR2). The distance between residue P324 of the JK-loop (yellow) in the US0 kDa and D243 of switch-1 is ~2.8 Å. Switch-1 residue N242 interacts with α-phosphate (2.8 Å) and β-phosphate (3.1 Å) group of ADP-ALF₄ (shown in spheres) in the active site. (D) The volume of the active site, indicated by the spheres, was determined to 3065 Å³ in NM2C by fitting a sphere with a radius (r) of 9.012 Å to the active site with UCSF Chimera (Pettersen et al., 2004). The volume of the sphere was calculated based on its radius using the equation volume = 4/3πr³. (E) The volume of the active site of scallop striated muscle myosin-2 (PDB entry 1QVI) was determined to 697 Å³ based on a radius of r = 5.5 Å as in (D). The structures shown in (D) and (E) are in the pre-powerstroke state.

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Figure 3. Interdomain connectivity at the converter/Nter/lever junction. (A) Interaction profile of R788 in the pre-powerstroke state. R788 is shown in orange colored sticks and the converter is colored in white, the SH1-SH2 helix in purple, and the lever arm in green colored cartoon representation. The inset shows a close-up view of the complete R788 interaction profile and is rotated 137° respective to the main panel. The guanidinium group of R788 forms hydrogen bonds (2.8 Å) with backbone oxygen atom of Q730 from the SH1 helix and the backbone oxygen atom of G731 (3.0 Å) of the converter. The $\text{d}$-nitrogen atom of R788 interacts (3.0 Å) with N776 backbone oxygen atom of N776. The R788 guanidinium group interacts (3.1 Å) with the hydroxyl group of N776 of the converter. The backbone nitrogen atom of R788 interacts (3.1 Å) with the carbonyl group of L777 of the converter. The backbone carbonyl group of R788 interacts (3.4 Å) with the backbone nitrogen of V791 of the lever as well as a water molecule (3.0 Å). The hydroxyl group from relay helix Y518 interacts with the backbone nitrogen atom of G731 (2.8 Å) and backbone oxygen atom of F732 (3.4 Å). F732 forms hydrophobic interactions with the methylene groups of R788 with the latter positioned in van der Waals distance to relay loop W525. All the amino acids involved in interactions with R788 are shown as sticks and water molecules as spheres. (B) Sequence alignment of selected regions from relay helix, SH1 helix, converter and lever arm shows the high sequence conservation within the myosin-2 motor domain. The asterisk indicates the invariant, positively charged residue corresponding to NM2C R788. The interactions of NM2C R788 with structural elements of the L50 kDa, the converter, and the lever are highlighted. Abbreviations used: Hs NM2C: human NM2C (NP_079005.3); Hs NM2A: human nonmuscle myosin-2A (NP_002464.1); NM2B: human nonmuscle myosin-2B (NP_005955.3); Gg SM: chicken smooth muscle myosin-2 (NP_990605.2); Hs CARD: human beta cardiac muscle myosin-2 (NP_000248.2); Ai ST: scallop striated muscle myosin-2 (P24733.1). PDB entries are indicated when available. Lysine residues that replace R788 in cardiac and striated muscle myosins-2 highlighted in the boxed area.

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Figure 3—figure supplement 1. Interdomain connectivity at the converter/Nter/lever junction in muscle myosins-2. In contrast to NM2C (Figure 3), the substitution of R788 with K763 in scallop striated muscle myosin-2 (PDB entry IQVI) (A) and K766 in human β-cardiac muscle myosin-2 (PDB entry 4DB1) (B) reduces the number of side chain and main chain interactions at the converter/Nter/lever junction. (C) In the conservative mutant R788K, the side chain: side chain and side chain: main chain interactions are reduced compared to NM2C (Figure 3). A further reduction in side chain: side chain and side chain: main chain interactions is achieved in the charge reversal mutant R788E (D). Coloring is according to Figure 3.

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Figure 4. Transient kinetic features of NM2C and R788E. (A) Interaction between NM2C/R788K/R788E with ATP under single-turnover conditions. Binding 0.375 μM d-mantATP to 0.5 μM myosin results in a transient fluorescence increase that is followed by a short plateau (hydrolysis) and a slow decrease in mantADP fluorescence that is associated with its release. All three phases are reduced in R788E (grey) compared to NM2C (green) and R788K (yellow). The very slow decrease of the fluorescence signal in R788E indicates that either the ATP hydrolysis rate or a subsequent release rate of the hydrolysis products are severely decreased when compared to NM2C and R788K. (B) Interaction between 0.25 μM pyrene-actoNM2C/R788K/R788E with 0.15 μM ATP under single-turnover conditions. Color coding is according to (A).

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Figure 4—figure supplement 1. Nucleotide binding characteristics of NM2C, R788K, and R788E and disease causing NM2C mutations. (A) Dependence of the observed rate constants ($k_{obs}$) upon d-mantATP binding to 0.25 μM NM2C/R788K/R788E on the nucleotide concentration. Linear. Figure 4—figure supplement 1 continued on next page
fits to the data result in second-order binding rate constants of $K_{1}k_{+2} = 0.48 \pm 0.01 \, \text{µM}^{-1} \text{s}^{-1}$ for NM2C (green), a two-fold reduced ATP binding constant $K_{1}k_{+2} = 0.22 \pm 0.01 \, \text{µM}^{-1} \text{s}^{-1}$ for R788K (yellow), and a five-fold reduced binding rate constant of $K_{1}k_{+2} = 0.09 \pm 0.005 \, \text{µM}^{-1} \text{s}^{-1}$ for R788E (grey) compared to NM2C. Inset, The rate constants ($k_{\Delta d}$) obtained from the binding reaction of ATP to NM2C/R788K dependent hyperbolically on the ATP concentration. The rate of ATP hydrolysis ($k_{+3} + k_{-3}$) and the second-order binding rate constants $K_{1}k_{+2}$ were determined from a fit to the data (NM2C: $k_{+3} + k_{-3} = 24.32 \pm 0.89 \, \text{s}^{-1}$; R788K: $k_{+3} + k_{-3} = 37.31 \pm 0.55 \, \text{s}^{-1}$). The respective parameters are not experimentally accessible for R788E. (B) The apparent second-order ATP binding rate constants, determined by the ATP-induced dissociation of the pyrene-labeled actoNM2C (green) or actoR788E (grey) complexes are with $K_{1}k_{+2} = 1.86 \pm 0.03 \, \text{µM}^{-1} \text{s}^{-1}$ and $K_{1}k_{+2} = 2.11 \pm 0.05 \, \text{µM}^{-1} \text{s}^{-1}$ similar. The respective constant is reduced to $K_{1}k_{+2} = 1.03 \pm 0.03 \, \text{µM}^{-1} \text{s}^{-1}$ for actoR788K (yellow). Inset, The maximum isomerization rate constants $k_{+2} = 643.06 \pm 22.6 \, \text{s}^{-1}$ (NM2C), $k_{+2} = 767.43 \pm 21.27 \, \text{s}^{-1}$ (R788K), and $k_{+2} = 579.27 \pm 12.81 \, \text{s}^{-1}$ was determined from a hyperbolic fit to the respective data. (C) The R788K (yellow) and the R788E (grey) mutation decrease the second-order ADP-binding rate constant two- to threefold to $k_{+D} = 0.21 \pm 0.02 \, \text{µM}^{-1} \text{s}^{-1}$ and $k_{+D} = 0.12 \pm 0.01 \, \text{µM}^{-1} \text{s}^{-1}$ when compared to $k_{+D} = 0.39 \pm 0.01 \, \text{µM}^{-1} \text{s}^{-1}$ for NM2C (green). (D) The ADP-binding rate constant $k_{+AD} = 0.03 \pm 0.001 \, \text{µM}^{-1} \text{s}^{-1}$, as calculated from the slope of the $k_{+d}$ versus [d-mantADP] plot, is 85-fold decreased for R788E (grey) when compared to $k_{+AD} = 2.54 \pm 0.18 \, \text{µM}^{-1} \text{s}^{-1}$ for NM2C (green). (E) Time-dependent change in the intrinsic tryptophan signal upon mixing 0.5 mM ATP with 0.25 mM NM2C (grey) or R778E (grey) in the presence of 5 µM ADP. ATP binding increases the fluorescence signal in NM2C but not R778E under identical conditions in a stopped-flow spectrophotometer. (F) Missense mutations G376C and R726S are associated with autosomal dominant hearing impairment (DFNA4) and their location in NM2C is shown in spheres representation. G376C is in proximity to the JK-loop, R726S is in the SH1-SH2 helix. NM2C subdomains are color coded according to Figure 1C and the nucleotide is shown in spheres representation. DOI: https://doi.org/10.7554/eLife.32742.011
Figure 5. Structural importance of R788 at the converter/Nter/lever junction. (A) Relay helix angle as a function of MD simulation time, as monitored by the angle between Cα atoms of residues S489, M510, and E521 along the trajectories. The relay helix straightens in NM2C with a steady increase in the HB population. (B) Comparison of the conformational states of R788 in WT and R788E in NM2C. (C) Structural comparison of WT and R788E in NM2C. (D) Fluorescence spectra of NM2C, NM2C + 0.5 mM ATP, and NM2C + 0.5 mM ADP. (E) Structural comparison of R788E in NM2C, R788E + 0.5 mM ATP, and R788E + 0.5 mM ADP. (F) Fluorescence spectra of R788E, R788E + 0.5 mM ATP, and R788E + 0.5 mM ADP.
angle of the relay helix from approximately 145° to 150°, while the angle does not change significantly in R788E and fluctuates around 145° throughout the 100 ns time course of the simulation. Values for the relay helix angle observed in crystal structures of pre-power stroke (PPS) and post-rigor (PR) are indicated by dotted lines. (B) Population of hydrogen bonds (HB) between R788 (NM2C) or E788 (R788E) and surrounding structural elements over the simulation time of 100 ns. The abbreviations s and m indicate side chain and main chain. (C) Dynamics and conformational changes in NM2C during MD simulations. Snapshots from the start (0 ns simulation time) and end conformations (100 ns simulation time) are shown in light cyan and colored cartoon representation, respectively. The relay helix is shown in red, the SH1-SH2 helix in purple and the converter in grey. Relay loop W525 is shown in red in stick representation. The insets show a close-up view of the conformational changes of W525 along the simulation trajectory. (D) Tryptophan fluorescence emission spectrum of 4 μM NM2C in the absence of nucleotide or the presence of 0.5 mM ATP or 0.5 mM ADP. (E) Dynamics and conformational changes in R788E during MD simulations. Snapshots from the start (0 ns simulation time) and end conformations (100 ns simulation time) are shown in light cyan and colored cartoon representation, respectively. The relay helix is shown in red, the SH1-SH2 helix in purple and the converter in grey. Relay loop W525 is shown in red in stick representation. The insets show a close-up view of the conformational changes of W525 along the simulation trajectory. (F) Tryptophan fluorescence emission spectrum of 4 μM R788E in the absence of nucleotide or presence of 0.5 mM ATP or 0.5 mM ADP.

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Figure 5—figure supplement 1. Stability of the salt bridge between switch-1 and switch-2 in MD simulations of NM2C, R788K, and R788E. (A) Monitored number of hydrogen bond (#HB) interactions between R261 (switch-1) and E483 (switch-2) along the simulation time of NM2C. (B) Monitored

Figure 5—figure supplement 1 continued on next page
number of hydrogen bond (#HB) interactions between R261 (switch-1) and E483 (switch-2) along the simulation time of R788E (B). Hydrogen bonds were detected with cutoff values for the donor-acceptor distance and angle of 3.5 Å and 30°. Note the intermediate breaking of the salt-bridge in R788E (B).

(C) Distance between main chain of G481 of switch-2 with the γ-phosphate of ATP during the 100 ns simulation time for NM2C. (D) Population of hydrogen bonds (HB) between structural elements of the active site of NM2C and ATP along the 100 ns simulation. The abbreviations s and m indicate side chain and main chain, respectively. (E) Population of hydrogen bonds (HB) between R788 (NM2C) or K788 (R788K) and surrounding structural elements along the simulation time. The abbreviations s and m indicate side chain and main chain, respectively. (F) Monitored number of hydrogen bond (#HB) interactions between R261 (switch-1) and E483 (switch-2) along the simulation trajectory of R788K. Simulations for NM2C, R788K, and R788E were performed in the presence of ATP in the active site.

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Figure 6. Proposed allosteric communication pathway from the converter to the NM2C active site. (A) Residue R788 connects the converter to the SH1 interface through main chain and side chain interactions in NM2C. This interface further interacts with Y518 of the relay helix and W525. The interactions are propagated to the active site and vice versa through the relay helix and through an interaction of relay helix residue N499 with the main chain of G481 from switch-2. The latter directly interacts with the nucleotide. Y599 of the wedge loop, that itself contacts the relay helix, establishes an interaction with switch-2 F482. This residue is in contact with S200 of the P-loop by main chain interactions and directly interacts with the nucleotide in the active site. Schematic not drawn to scale. (B) The interface between R788E and both, the SH1 and the relay helix, is perturbed and the allosteric communication from the active site to the converter compromised. The experimental observation that R788E does not change its intrinsic fluorescence upon nucleotide binding which is caused by a lacking conformational change of W525 indicates that the communication pathway is interrupted in the relay helix. The position of W525 in the relay loop at the distal end of the relay helix indicates that the pathway is interrupted before or at Y518, which is supported by the observation that the relay loop does not change its position during the time course of the MD simulation. As a consequence, Y518 cannot establish an interface with E788 and F732 of the converter and SH1 helix. Further, E788 cannot establish interactions with N776 and V791 and completely disrupts the structural integrity of the interface of converter, SH1-SH2 helix, relay helix and the lever arm and uncouples nucleotide-induced changes in the active site from the converter rotation. Only key residues involved are shown in the proposed mechanism. Schematic not drawn to scale.

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