

1 **Mechanistic Insights into the Active Site and Allosteric**
2 **Communication Pathways in Human Nonmuscle Myosin-2C**

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1 Abstract

2 The cyclical interaction of myosin with F-actin and nucleotides is the basis for contractility of the
3 actin cytoskeleton. Despite a generic, highly conserved motor domain, ATP turnover kinetics
4 and their activation by F-actin vary greatly between myosins-2 isoforms. Here, we present a
5 2.25 Å crystal structure of the human nonmuscle myosin-2C motor domain, one of the slowest
6 myosins characterized. In combination with integrated mutagenesis, ensemble-solution kinetics,
7 and molecular dynamics simulations approaches, this study reveals an allosteric communication
8 pathway that connects the distal end of the motor domain with the active site. Genetic disruption
9 of this pathways reduces nucleotide binding and release kinetics up to 85-fold and abolishes
10 nonmuscle myosin-2 specific kinetic signatures. These results provide insights into structural
11 changes in the myosin motor domain that are triggered upon F-actin binding and contribute
12 critically to the mechanochemical behavior of stress fibers, actin arcs, and cortical actin-based
13 structures.

14 Introduction

15 The extent to which filamentous actin (F-actin) can activate the enzymatic output of
16 conventional myosins-2 varies by more than two orders of magnitude (1, 2). Despite a
17 substantial sequence identity and a highly conserved actomyosin ATPase cycle, structural and
18 allosteric adaptations causative for the tremendous enzymatic and hence physiological
19 differences are largely unknown (**Figure 1A**) (1, 3).

20 Nonmuscle myosin-2C is one of the slowest myosins-2 as its steady-state ATPase
21 activity lacks potent F-actin activation (4). Transient kinetic signatures of the nonmuscle myosin-
22 2C enzymatic cycle include a high affinity for ADP, small kinetic (k_{AD}/k_D) and thermodynamic
23 (K_{AD}/K_D) coupling ratios, and a higher duty ratio than it is commonly found in myosins-2 (**Figure**
24 **1A – Figure supplement 1A**). Together, these signatures qualify nonmuscle myosin-2C as a
25 dynamic strain-sensing actin tether (3-5). The participation in the active regulation of
26 cytoplasmic contractility in cellular processes including cytokinesis, neuronal dynamics,
27 adhesion, and tension maintenance are in line with this interpretation (6-8). Nonmuscle myosin-
28 2C has recently received great attention as the near atomic resolution cryo electron microscopic
29 structure of its motor domain bound to F-actin has been determined (9). The structure of the
30 actomyosin complex, together with a detailed kinetic characterization of the motor properties,
31 qualifies nonmuscle myosin-2C as the ideal human myosin to decipher the structure-function
32 relationships in the myosin motor domain that underlie its kinetic signatures (4, 9).

33 To understand how structural adaptations lead to characteristic kinetic features, we
34 solved the 2.25 Å crystal structure of the human nonmuscle myosin-2C motor domain (NM2C)
35 (**Figure 1B, Table 1**). The NM2C pre-powerstroke state structure suggests that structural fine-
36 tuning at the active site and a reduced interdomain connectivity in the motor domain define its
37 kinetic signatures. Comparative structural analysis, ensemble solution kinetic studies, and *in*
38 *silico* molecular dynamics (MD) simulations collectively demonstrate a communication pathway
39 that connects the active site and the distal end of the motor domain. Disruption of the pathway
40 uncouples the myosin ATPase activity from actin-activation, alters the ATP/ADP sensitivity and
41 results in the loss of nonmuscle myosin-2 kinetic signatures. The allosteric coupling pathway
42 may be of significance in intermolecular gating and load-sensitivity of nonmuscle myosins-2 that
43

1 is important for their physiological function as cytoskeletal strain-sensor in the context of stress
2 fibers, actin arcs, and cortical actin-based structures.

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5 **Results**

6 **Overall topology of pre-powerstroke NM2C.** We determined a 2.25 Å pre-powerstroke state
7 structure of human NM2C, one of the slowest myosins-2 characterized. Crystallization was
8 achieved after truncation of the flexible N-terminal 45 residues and the fusion of the NM2C C-
9 terminus to *Dictyostelium* α -actinin tandem repeats 1 and 2 (**Figure 1B**, **Figure 1 – Figure**
10 **supplement 1B**, **Table 1**). This approach has been used successfully in prior structural and
11 kinetic studies on NM2C and allows the detailed analysis of structure-function relationships in
12 the myosin motor domain (4, 9).

13 The overall topology of NM2C shares the structural four-domain architecture of myosin-2
14 motor domains comprising the N-terminal subdomain (Nter), the upper 50 kDa subdomain (U50
15 kDa), the lower 50 kDa subdomain (L50 kDa), and the converter that terminates in the lever
16 (**Figure 1C**) (10). The nucleotide-binding active site is formed by structural elements of the U50
17 kDa and allosterically communicates with the actin binding interface that is formed by distant
18 structural elements of the U50 kDa and the L50 kDa. The NM2C C α atoms superimpose with a
19 root mean square deviation (*r.m.s.d.*) of 0.57 Å, 0.78 Å, and 0.73 Å to the closely related motor
20 domain structures from chicken smooth muscle myosin-2 (PDB entry 1BR2), scallop striated
21 muscle myosin-2 (PDB entry 1QVI), and *Dictyostelium* nonmuscle myosin-2 (PDB entry 2XEL)
22 (**Figure 1 – Figure supplement 1C**) in the pre-powerstroke state.

23 NM2C pre-powerstroke state characteristics include a closed conformation of the
24 nucleotide binding motifs switch-1 and -2 in the active site (**Figure 1B**). In this conformation, the
25 characteristic salt bridge between switch-1 R261 and switch-2 E483 that is pivotal for the
26 hydrolysis of ATP is formed (10-12). The relay helix is in a kinked conformation, the central
27 seven-stranded β -sheet is untwisted, and the converter domain and the adjacent lever arm in
28 the up-position (**Figure 1B**). NM2C switch-1 and lever dihedral ϕ , ψ angles are substantially
29 changed when compared to the pre-powerstroke state structures of chicken smooth muscle
30 myosin-2 (PDB entry 1BR2), indicating that small arrangements in the active site are coupled to
31 conformational changes at the distal end of the motor domain (**Figure 1 – Figure supplement**
32 **1C-D**, **Supplementary table 1**). Concomitantly, the relative orientation of converter and lever
33 arm deviates by ~ 8 - 10° when compared to the high resolution pre-powerstroke state crystal
34 structure of chicken smooth muscle myosin-2 (PDB entry 1BR2) and resembled the orientation
35 of the respective regions of scallop striated muscle myosin-2 (PDB entries 1QVI, 1DFL) motor
36 domain structures (**Figure 1 – Figure supplement 1D**).

37

38 **Unique structural rearrangements in the NM2C active site in the pre-powerstroke state.**
39 Switch-1, switch-2, P-loop, and the purine-binding A-loop are the prototypic nucleotide binding
40 motifs in the myosin active site that undergo conformational changes in response to nucleotide
41 binding and release. The active site is flanked by loop-3 and a loop that connects helices J and
42 K in the U50 kDa, hereafter referred to as JK-loop (**Figure 2A**). The JK-loop constitutes a major
43 connection between switch-1 and the nucleotide is of great functional significance as a hotspot

1 for human cardiomyopathy-causing mutations in cardiac myosin-2 and the site of exon 7 in
2 *Drosophila* muscle myosin-2 (**Figure 2 – Figure supplement 1A**) (13-15).

3 A slight reduction in the JK-loop length in NM2C causes an 8.8 Å shift between switch-1
4 and the U50 kDa and abolishes the formation of a tight interaction network with residues of the
5 active site found in other class-2 myosins (**Figure 2B,C, Figure 2 - Figure supplement**
6 **1A,B,C**). Comparative analysis of interactions between the JK-loop and the switch-1 region in
7 NM2C and scallop striated muscle myosin-2 (PDB entry 1QVI) shows that in the latter, JK-loop
8 N321 is in hydrogen bond interaction with switch-1 N238 and located at a distance of 4.6 Å to
9 the hydroxyl group of the C2' of the ADP ribose. The connectivity between switch-1 and the
10 nucleotide is further strengthened by a hydrogen bond between N237 and the ADP ribose.
11 Moreover, A-loop residue R128 forms hydrogen bonds with the ADP adenosine (3.2 Å) and
12 E184 (2.8 Å) of the P-loop in the active site of striated muscle myosin-2. Strikingly, NM2C lacks
13 all interactions described for scallop striated muscle myosin-2 due to the replacement of R128
14 with Q150 and the above mentioned JK-loop shortening. Both structural alterations increase the
15 distance to the adenosine in the active site to 5.8 Å and disrupt constraints between switch-1 and
16 the JK-loop (**Figure 2B,C,E**). The shift also increases the volume and hence the accessibility of
17 the NM2C active site when compared to the transition state structures of chicken smooth
18 muscle myosin-2 (PDB entry 1BR2), *Dictyostelium* nonmuscle myosin-2 (PDB entry 2XEL), and
19 scallop striated muscle myosin-2 (PDB entry 1DFL,1QVI) (**Figure 2A, Figure 2 – Figure**
20 **supplement 1B,D,E**). A 4.3-fold volume increase from 697 Å³ to 3054 Å³ in the active site is
21 observed for example between scallop striated muscle myosin-2 (PDB entry 1QVI) and NM2C
22 (**Figure 2 – Figure supplement 1D,E**). Strikingly, the enlarged accessibility of the active site
23 region observed in the NM2C pre-powerstroke state structure resembles the conformation of the
24 active site found in the actin-bound NM2C rigor state (PDB entry 5J1H), indicating that F-actin
25 binding does not induce major structural rearrangements in the vicinity of the active site (**Figure**
26 **2C**).

27 The temperature factors obtained from the refined crystal structure show that the NM2C
28 JK-loop is highly flexible despite its reduced length. We attribute the flexibility to the lack of
29 constraints with switch-1 residues and the bound nucleotide (**Figure 2A,B,C,E**). Specifically, the
30 connectivity of the JK-loop to switch-1 is reduced by the replacement of a conserved arginine
31 (R280 in PDB entry 1QVI) with cysteine (C299) in the loop preceding helix I. This arginine
32 further establishes an interaction with the highly conserved switch-1 K233 in scallop striated
33 muscle myosin-2. The arginine to cysteine substitution in NM2C also abolishes the formation of
34 a salt bridge with JK-loop residue E341 and hence disrupts the connection between the JK-loop
35 and switch-1 (**Figure 2C,E, Figure 2 – Figure supplement 1C**). Consequently, the JK-loop
36 loses its ability to sense conformational changes in the nucleotide binding motif switch-1 in
37 response to ATP binding, hydrolysis, and product release.

38 The interaction between the JK-loop and the nucleotide is weakened by the replacement
39 of an asparagine with G339, which abolishes hydrogen bond formation with switch-1 D257.
40 Comparison with data from previous crystallographic studies shows that D257 replaces an
41 asparagine (N238 in PDB entry 1QVI) in the switch-1 in striated muscle myosins-2. The
42 asparagine interacts weakly with the ADP moiety in the pre-powerstroke state (**Figure 2B,D,E,**
43 **Figure 2 figure supplement 1B,C**) and strongly in the near-rigor state (16, 17). The nucleotide
44 coordination in NM2C is further weakened by residue Q150 that replaces an arginine (R128 in

1 PDB entry 1QVI) in the A-loop of scallop striated muscle myosin-2. The substitution disrupts
2 coordinating interactions between the A-loop and the ADP adenosine (**Figure 2D, Figure 2**
3 **figure supplement 1B,C**). The replacement additionally impairs the formation of a salt bridge
4 with the invariant E206 (E184 in PDB entry 1QVI) at the distal end of the P-loop that is predicted
5 to be involved in nucleotide recruitment to the active site (**Figure 2D, Supplementary table 2**)
6 (16).

7 Previous studies established that the number of hydrogen bond interactions between P-
8 loop, switch-1, and the Nter positively correlate with the thermodynamic coupling (K_{AD}/K_D), the
9 efficiency of F-actin to displace ADP during the catalytic cycle (16). Substitution of an arginine
10 with K255 and a glutamate with H700 in NM2C is expected to weaken the tight and highly
11 conserved interaction network and is expected to contribute to the low thermodynamic (K_{AD}/K_D)
12 and kinetic (k_{AD}/k_D) coupling efficiency, and the slow actin-activated ADP release in nonmuscle
13 and smooth muscle myosins-2 compared to cardiac and striated muscle myosins-2
14 (**Supplementary table 2**) (16).

15 Taken together, comparative structural analysis of the active site of myosins-2 suggests
16 that the interconnectivity of the highly conserved nucleotide switches and myosin subdomains is
17 weaker in NM2C and other nonmuscle myosins-2 compared to fast sarcomeric myosins-2.
18

19 **Interdomain connectivity between converter, Nter, and lever.** The interdomain connectivity
20 between the converter and the Nter in the myosin-2 motor domain is established by the relay
21 and the SH1-SH2 helix. The Nter controls the movement of the converter, which undergoes a
22 large-scale rotation that drives the powerstroke during force generation (18-22).

23 Residue R788 at the interface of converter and lever is highly conserved in nonmuscle
24 and smooth muscle myosins-2 and the only connecting hub between structural elements of the
25 L50 kDa and the Nter (**Figure 3A,B**). Notably, replacement of R788 with a lysine in muscle
26 myosins-2 weakens the interaction between the aforementioned structural elements in all states
27 of the myosin and actomyosin kinetic cycle (**Figure 3 – Figure supplement 1A,B**). In NM2C,
28 R788 forms 3 main chain and 5 side chain interactions with residues of the SH1-SH2 helix, the
29 converter, and the lever in the pre-powerstroke state. Specifically, the guanidinium group of the
30 R788 side chain of NM2C forms hydrogen bonds with the main chain carbonyls of residues
31 Q730 and G731 of the SH1 helix. The hydrophobic methylene groups of R788 are stabilized by
32 SH1 helix F732 and the main chain oxygen and hydroxyl groups of N776 of the converter. Y518
33 from the relay helix interacts with residues G731 and F732 of the converter, thereby interlinking
34 R788 with both structural elements. The side chain of R788 is in van der Waals distance (4.8 Å)
35 from W525 of the relay loop. The main chain carbonyl of R788 further interacts with V791 at the
36 converter/lever junction (**Figure 3A,B**). This interaction is important for the stabilization of the
37 converter fold and the interface with the lever in the absence of F-actin. Notably, the R788 side
38 chain-main chain interaction is also evident in crystal structures of nonmuscle myosins-2 in the
39 nucleotide-free (PDB entry 4PD3) and the phosphate-release state (PDB entry 4PJK) (not
40 shown). We therefore hypothesize that (i) the tight interaction network formed by R788 is
41 required for the precise positioning and coupling of the converter, the SH1-SH2 helix, the relay
42 helix, and the lever arm throughout all steps of the myosin ATPase cycle (**Figure 1 - Figure**
43 **supplement 1A**), and (ii) different communication pathways between the active site and the
44 distal end of the motor domain are employed by NM2C in the presence and absence of F-actin.

1

2 **Kinetic consequences after the disruption of the converter/Nter/lever interface.** To
3 experimentally probe for a possible effect of R788 on myosin motor function, we performed
4 comparative ensemble solution kinetic studies with NM2C and R788E, a mutant in which a
5 glutamate replaces the converter R788. Rationale for the design of the R788E charge reversal
6 mutant was to disrupt side chain:side chain and side chain:main chain interactions, an approach
7 that has been successfully used in *in vitro* and *in vivo* studies to probe for the influence of the
8 converter/U50 kDa interface on myosin-2 performance (19, 23-25).

9 R788E shows prominent changes in virtually all parameters of the myosin and
10 actomyosin ATPase cycle (**Supplementary table 3**). Confirming our hypothesis, transient
11 kinetic changes mainly affect nucleotide binding and release kinetics and are more pronounced
12 in the absence of F-actin, as seen in single-turnover measurements (**Figure 4A,B**). Most
13 importantly, nonmuscle myosin-2 specific transient kinetic signatures such as a high k_{+AD}/K_1k_{+2}
14 ratio ($k_{+AD}/K_1k_{+2} \sim 2$ -20) are absent in R788E ($k_{+AD}/K_1k_{+2} \sim 0.26$) due to a 20-fold acceleration of
15 the second-order rate constant for ATP binding (K_1k_{+2}) by F-actin (**Table 2, Figure 4 – Figure**
16 **supplement 1A-D**) (4, 26, 27). This feature is also described for conventional myosins-2 from
17 cardiac and striated muscle that bind ATP and ADP with similar rates (**Supplementary table 2**)
18 (28). Actin-activation of the ADP release ($k_{-AD} = 0.68 \pm 0.01 \text{ s}^{-1}$ for NM2C and $k_{-AD} = 0.19 \pm 0.01 \text{ s}^{-1}$
19 for R788E) results in a kinetic coupling constant k_{-AD}/k_{-D} of ~ 2.5 for R788E, whereas neutral or
20 negative kinetic coupling is a feature of NM2C ($k_{-AD}/k_{-D} = 0.7$) and other human nonmuscle
21 myosins-2 (**Table 2**) (4, 26, 27). The changes in ADP binding and release kinetics of R788E
22 result in a 10-fold increase in the ADP dissociation equilibrium constant K_{AD} ($K_{AD} \sim 0.29 \mu\text{M}$ for
23 NM2C and $\sim 2.68 \mu\text{M}$ for R788E). The thermodynamic coupling ($K_{AD}/K_D = 5$) for R788E is 42-
24 times higher than for NM2C (**Supplementary table 3**). R788E displays an extraordinary slow
25 ADP binding rate constant ($k_{+AD} = 0.03 \pm 0.001 \mu\text{M}^{-1}\text{s}^{-1}$) (**Table 2, Figure 4 – Figure supplement**
26 **1D**) in the presence of F-actin whereas the kinetic constants for the interaction between
27 actomyosin and ATP, K_1k_{+2} , k_{+2} , and $1/K_1$ are marginally affected when compared to NM2C
28 (**Supplementary table 3**). The F-actin affinity in the absence and presence of ADP (K_A , K_{DA}) of
29 NM2C and R788E is similar (**Supplementary table 3**) and F-actin can activate the steady-state
30 ATPase activity of R788E to approximately half the k_{cat} of NM2C ($k_{cat} = 0.2 \pm 0.01 \text{ s}^{-1}$ for R788E
31 and $k_{cat} = 0.37 \pm 0.02 \text{ s}^{-1}$ of NM2C) (**Supplementary table 3**) under steady-state conditions. The
32 duty ratio at an F-actin concentration of $190 \mu\text{M}$ increases from ~ 0.3 for NM2C to ~ 1 for R788E
33 due to the decreased k_{cat} and the decreased actin-activated ADP release rate k_{-AD} . This feature
34 makes k_{-AD} likely to rate-limit the kinetic cycle of R788E, whereas the actin-activated P_i release
35 is expected to rate-limit the kinetic cycle of NM2C and other myosins-2 (1, 4, 26, 27, 29, 30).

36 The slow nucleotide binding and release kinetics of R788E suggest that the R788-
37 mediated interaction at the converter/Nter/lever interface is allosterically communicated to the
38 active site. This is in line with the observation that R788E does not show a nucleotide-induced
39 change in the intrinsic tryptophan fluorescence signal during steady-state and transient-state
40 kinetic assays (**Figure 5D,F, Figure 4 - Figure supplement 1E, Table 3**). The fluorescence
41 change observed with NM2C is attributed to a conformation-induced change in the
42 microenvironment of the conserved relay loop W525, which is in van der Waals distance to
43 R788 and a direct indicator for the switch-2 induced converter rotation (31).

44

1 **Allosteric communication pathway between the active site and the distal end of the**
2 **motor domain.** R788 is a hub amino acid and forms the center of a cluster of interactions that
3 connect the converter, the SH1-SH2 helix, the relay helix, and the lever (**Figure 3A,B**). To
4 understand its dynamic interactions that are allosterically communicated to the active site, we
5 performed comparative molecular dynamics simulations of NM2C and R788E in explicit water
6 with Mg^{2+} -ATP bound to the active site over a time of 100 ns. As expected, the characteristic
7 salt bridge between switch-1 R261 and switch-2 E483 of the active site that is critical for the
8 hydrolysis of ATP is stable throughout the time course of the simulation for NM2C (**Figure 5 -**
9 **Figure supplement 1A**). The relay helix that connects the active site and the converter
10 straightens and likewise the converter undergoes a 27° rotation that directs lever arm motion
11 (**Figure 5A,C**). This straightening is caused by the exchange of the co-crystallized $ADP \cdot VO_4$ to
12 ATP, since the replacement with ADP did not lead to either relay straightening nor converter
13 rotation in a control simulation. Hence, ATP appears to trigger the structural transition from the
14 pre-powerstroke state (up position) towards the post-rigor state (down position). After 100 ns,
15 the position of the converter and the adjacent lever show an orientation between the post-rigor
16 and pre-powerstroke state, as compared to the crystal structures of *Dictyostelium* nonmuscle
17 myosin-2 motor domain (PDB entries 1FMW, 2XEL) (**Figure 5C**). With the movement of the
18 relay helix, the indole ring of relay loop W525 changes its conformation by $70-80^\circ$, which agrees
19 with the experimentally observed nucleotide-induced change in the intrinsic tryptophan
20 fluorescence signal in NM2C (**Figure 5B,C, Figure 4 - Figure supplement 1E**). The hub amino
21 acid R788 is in transient interactions with main chain and side chain atoms of 10 amino acids of
22 the SH1 helix and the converter during the 100 ns time course of the simulation (**Figure 5B**).

23 MD simulations for R788E indicate that all interdomain interactions between R788E of
24 the converter and the SH1 helix are lost (**Figure 5B**). The straightening angle of the relay helix
25 remains constant and abolishes a detectable converter rotation (**Figure 5A,E**). This allosteric
26 decoupling at the distal end of the myosin motor domain is translated further upstream via the
27 relay helix and results in a pronounced 6 Å conformational change of a loop that connects the γ -
28 phosphate sensor switch-2 of the active site and the relay helix. As a consequence, the salt
29 bridge between switch-2 E483 and switch-1 R261 appears less stable in the MD simulations of
30 R788E as compared to NM2C (**Figure 5 - Figure supplement 1A,B**). The importance of the
31 salt bridge between both switches for the ATP hydrolysis is established and expected to directly
32 contribute to the experimentally observed impaired nucleotide binding, hydrolysis, and release
33 kinetics of R788E in transient-state kinetic assays (**Supplementary table 3, Figure 4A, Figure**
34 **4 - Figure supplement 1A,D**) (11, 32, 33). Moreover, the side chain of switch-1 N256 changes
35 its orientation, thereby impairing hydrogen bond interactions with the α - and β -phosphate
36 moieties of the nucleotide and constrains of switch-1. The lack of coordinating interactions is
37 reflected in the very slow nucleotide binding and release rate constants (**Figure 4A, Figure 4 -**
38 **Figure supplement 1A, Supplementary table 3**).

39 The side chain of relay loop W525 does not undergo a conformational change
40 throughout the time course of the simulation for R788E, supporting our experimental
41 observation that R788E does not exhibit a nucleotide-induced change in its intrinsic tryptophan
42 fluorescence signal of R788E (**Figure 5D,F, Figure 4 - Figure supplement 1E**). The direct
43 comparison of the dynamic interaction and conformational signatures of W525 in NM2C and
44 R788E reveals that R788 is in van der Waals distance (5.4 Å) from relay loop W525 at the start

1 of the simulations. W525 transiently interacts with I523 (18%) and Q519 (14%), changes its
2 conformation by 70-80° and increases the distance to NM2C R788 to 7.3 Å after 100 ns
3 simulation time. In comparison, the distance between W525 and E788 increases to 9.9 Å during
4 the simulation for R788E. The lack of a conformational change in W525 along the simulation
5 trajectory results in transient interactions with Q515 (55%) and Q730 (14%) instead of the
6 interactions with I532 and Q519 as seen in NM2C.

7 Taken together, our *in silico* and experimental *in vitro* data support a model in which
8 R788 is a distant allosteric modulator of switch-2 dynamics at the active site that impacts
9 nucleotide binding and release kinetics in the actin-detached states. Moreover, our data support
10 a model of R788 as a quencher of W525 fluorescence.

11 12 13 **Discussion**

14 The experiments presented here collectively demonstrate an allosteric communication pathway
15 from the distal end of the myosin motor domain that, together with substitutions of several key
16 residues in or in vicinity to the active site, account for NM2C-specific kinetic properties.
17 Disruption of the pathway by mutation of R788 to a glutamate causes the loss of its enzymatic
18 signatures and results in a high duty ratio motor.

19 It is of note that several key residues involved in the communication pathway are near
20 residues that are mutated in patients with autosomal dominant hearing loss (34). Missense
21 mutation G376C is in proximity to residues C324 of helix J and R328 of the JK-loop (**Figure 4 –**
22 **Figure supplement 1F**). Based on its location, we suggest that this substitution may interfere
23 with the nucleotide binding and release kinetics from the NM2C active site. Missense mutation
24 R726S is in the SH1-SH2 helix and the guanidinium group of the wild type arginine interacts
25 (3.3 Å) with the NM2C Nter. The serine residue is expected to disrupt this interaction because of
26 the shorter side chain and different charge when compared to arginine. It is therefore likely that
27 these mutations impact the interaction of the motor domain with nucleotides, thereby
28 contributing to impaired tension-sensing and maintenance of nonmuscle myosin-2C in the
29 human cochlea (34-36).

30 31 **R788 is part of a conserved pathway that connects the active site and the converter.**

32 R788 is part of an allosteric communication pathway that connects the converter at the distal
33 end of the myosin motor domain via the relay helix with switch-2 of the active site. Uncoupling of
34 the converter from the motor domain in R788E slows down all kinetic steps of the myosin
35 ATPase cycle and decreases its actin-activation, due to altered switch-2 dynamics. The
36 observed kinetic phenotype of the R788E myosin ATPase cycle is similar to the *Dictyostelium*
37 nonmuscle myosin-2 non-hydrolyzer mutants in which the salt bridge between switch-1 and
38 switch-2 is destroyed by mutagenesis (11, 32). Non-hydrolyzer mutants are characterized by
39 long-lived ATP states and reduced nucleotide release and binding kinetics, including a drastic
40 decrease in k_{+AD} (11, 32). Like R788E, *Dictyostelium* myosin-2 non-hydrolyzer mutants do not
41 exhibit a nucleotide-induced change in the intrinsic fluorescence signal (11). It is of note that the
42 lack of an intrinsic fluorescence signal in R788E is caused by the blockage of the
43 communication pathway on the relay helix before or at Y518 (**Figure 6A,B**) whereas the
44 impairment of the nucleotide switches to form a salt bridge and the resulting lack of the switch-2

1 induced conformational change of the relay helix is the expected cause in the non-hydrolyzer
2 mutants.

3 F-Actin affinities are largely unaffected by the R788E mutation, which is in line with the
4 observation that switch-1 dynamics are only marginally affected in comparative MD simulations.
5 The presence of F-actin however establishes ATP/ADP selectivity in the actomyosin ATPase
6 cycle: ActoNM2C preferentially binds ADP over ATP, whereas actoR788E preferentially binds
7 ATP over ADP (**Supplementary table 3**). The ATP selectivity is caused by a 85-fold decreased
8 second-order ADP binding rate constant k_{+AD} , a key signature of R788E actomyosin ATPase
9 cycle. ATP/ADP selectivity is established by actin-induced conformational changes in the
10 myosin motor domain and underlines that the coupling mechanism from the active site to the
11 converter and *vice versa* is different in the presence and absence of F-actin. This observation is
12 in line with recent reports on distinct pathways for the myosin and actin-activated ATPase cycle
13 (21).

14 **Implications for NM2C function *in vivo*.** Nonmuscle myosin-2C assembles into small
15 bipolar filaments that dynamically tether actin filaments in cells (8, 37). The actomyosin
16 interaction and hence the tension exerted by a sarcomeric array of nonmuscle myosin-2C is of
17 importance for the function and organization of the apical junctional complex in the organ of
18 Corti and actin-rich structures including stress fibers and actin arcs (8).

19 Based on our kinetic data, the calculated duty ratio of NM2C suggests that ~ 14 motor
20 domains would be needed to be geometrically capable of interacting with F-actin at any given
21 time. This number is identical to the number of motor domains per nonmuscle myosin-2C half
22 filament, suggesting that the filament may be at the threshold of being processive in the
23 absence of external loads (37). It is likely that this threshold is crossed in the presence of other
24 actin binding proteins, intermolecular loads, and gating between the F-actin bound motor
25 domains (38).

26 The structural prerequisites underlying gating and load-sensitivity in nonmuscle myosins-
27 2 have not been investigated but include a distortion at the converter/lever or the
28 converter/motor domain interface in the nanometer range that is allosterically communicated
29 through the lever arm via the converter to the active site (39). Resisting load applied to the lever
30 slows down the actin-activated ADP release from the lead motor of nonmuscle myosin-2A
31 around 5-fold thereby increasing the duty ratio, but does not alter the rate of ATP binding (38,
32 39). The kinetic signatures of the strained nonmuscle myosin-2A lead motor are very similar to
33 the observed kinetic features of R788E. The reduction of the steady-state ATPase of R788E
34 and the concomitant increase in duty ratio and a rate-limiting ADP release rate goes in line with
35 this finding. We propose that internal strain in the nonmuscle myosin-2 dimer distorts the lead
36 motor at the converter/lever interface and leads to its axial translation as seen in electron
37 microscopic studies (40). This translation abolishes the interaction of the converter R788 with
38 residues of the SH1-SH2 helix and the relay helix, thereby uncoupling the myosin subdomains
39 and disrupting the communication pathway from the converter to the active site, which is
40 expected to result in a similar kinetic effect as in R788E. Consequently, a motor in a nonmuscle
41 myosin-2 filament would decrease its enzymatic activity and stay strongly bound to F-actin. This
42 feature is required to generate processivity and cytoskeletal tension and of physiological
43 significance in the maintenance of cell shape and tensional homeostasis in the actin
44 cytoskeleton.

1 **Material and Methods**

3 **Protein production**

4 For structural studies, a His₈-tagged human NM2C construct comprising amino acids 45-799
5 directly fused to spectrin repeats 1 and 2 from α -actinin was generated based on the vector
6 pFastBac1-NMHC-2C0-2R-His₈ (4). Mutagenesis was accomplished by sequence-specific
7 deletion using a whole-plasmid amplification approach. For kinetic studies, an equivalent motor
8 domain construct comprising amino acids 1-799 of NM2C was cloned into a modified pFastBac1
9 vector containing a cDNA sequence encoding a C-terminal Flag-tag (**Figure 1 – Figure**
10 **supplement 1B**). This construct was used as a template to introduce the R788E mutation by In-
11 Fusion cloning (Clontech, Mountain View, CA 94943, USA). All proteins were recombinantly
12 overproduced in the *Sf9*/baculovirus system, purified to electrophoretic homogeneity, and
13 concentrated to ~10 mg/ml using Vivaspin ultrafiltration units (Sartorius, Göttingen, Germany)
14 as previously described (4, 41). Throughout this manuscript, numbering refers to the amino acid
15 sequence of full-length nonmuscle myosin-2C (GenBank accession number NP_079005).

17 **Crystallization of NM2C**

18 NM2C at a concentration of ~10 mg/ml was complexed with the ATP analogue ADP·VO₄ and
19 crystallized using the hanging drop vapor diffusion method by mixing 2 μ l of protein solution with
20 2 μ l of reservoir solution containing 50 mM Tris pH 8.2, 10 % (w/v) PEG-5K MME, 1 % (v/v)
21 MPD, and 0.2 M NaCl at 8°C. Rectangular plate shaped crystals grew up to 400 x 300 x 400
22 μ m³ within 4 weeks. Crystals were soaked in the corresponding mother liquor supplemented
23 with 100 mM NaCl and 20 % (w/v) ethylene glycol. Protein crystals were transferred in serial
24 steps of increasing concentrations of the cryo-solution. Crystals were transferred into liquid
25 nitrogen using MiTeGen loops and stored at 100 K until data collection.

27 **Data collection, processing and refinement**

28 X-ray diffraction data of NM2C crystals were collected at the beam line BL14.1 at Bessy II
29 (Helmholtz-Zentrum, Berlin, Germany) to 2.25 Å resolution (**Table 1**). Data processing was
30 performed using XDS and SADABS (42). The structure of NM2C in the pre-powerstroke state
31 was solved by molecular replacement using Phaser (43). The crystal structure of the chicken
32 smooth muscle myosin-2 motor domain (PDB entry 1BR4) was used as a search model and the
33 two α -actinin repeats were manually traced and rebuilt. The electron density map was
34 sharpened using Coot (44) to ensure the directionality and identity of the α -helices for the two α -
35 actinin repeats. Maximum likelihood crystallographic refinement was performed using iterative
36 refinement cycles in autoBUSTER (45). Iterative cycles of model building were performed using
37 Coot, and model bias was minimized by building into composite omit maps. The model was
38 initially validated in Coot and final validation was performed using MolProbity (46).

40 **Kinetic experiments**

41 The actin-activated ATPase assays under steady-state conditions was performed as described
42 earlier in buffer containing 10 mM MOPS pH 7.0, 50 mM NaCl, 2 mM MgCl₂, 2 mM ATP, 0.15
43 mM EGTA, 40 U/ml l-lactic dehydrogenase, 200 U/ml pyruvate kinase, 200 μ M NADH, and 1
44 mM phosphoenolpyruvate at 25°C with a Cary 60 Bio spectrophotometer (Agilent Technologies,

1 Wilmington, DE 19808, USA) (41). Transient state kinetic assays were carried out as described
2 previously with a TgK Hi-tech Scientific SF-61 DX stopped-flow system (TgK Hi-tech Scientific
3 Ltd., Bradford-on-Avon, UK) in SF-buffer (25 mM MOPS pH 7.0, 100 mM KCl, 5 mM MgCl₂ and
4 0.1 mM EGTA) unless stated otherwise (4). Initial data fitting was performed with Kinetic Studio
5 Version 2.28 (TgK Hi-tech Scientific Ltd., Bradford-on-Avon, UK). Plots were generated with
6 OriginPro 8.5 (OriginLab Corp., Northampton, MA 01060, USA). Data interpretation is according
7 to the kinetic scheme of the myosin and actomyosin ATPase cycle as presented in **Figure 1 –**
8 **Figure supplement 1A.**

9 10 **Fluorescence measurements**

11 Tryptophan fluorescence emission spectra of 4 μM NM2C or R788E in the presence and
12 absence of 0.5 mM ADP and 0.5 mM ATP, respectively were measured after excitation at 297
13 nm at a temperature of 20 °C in a QuantaMaster fluorescence spectrophotometer (Photon
14 Technology International, Birmingham, NJ 08011, USA) as described previously (47). Prior to
15 the assay, proteins were transferred to SF-buffer with zebra spin desalting columns (Thermo
16 Fisher Scientific GmbH, Dreieich, Germany). The fluorescence was normalized to the maximum
17 fluorescence of NM2C or R788E in the absence of nucleotide.

18 19 **Molecular dynamics simulations**

20 Molecular dynamics based *in silico* site directed mutagenesis and simulations were performed
21 using NAMD 2.9 and the CHARMM27 force field (48, 49). The X-ray crystal structure of the
22 NM2C motor domain in the pre-powerstroke state, encompassing residues 49-807, served as
23 the starting structure for NM2C and R788E simulations. Mutations were introduced *in silico* and
24 the proteins were prepared and optimized prior to MD simulations using the Protein Preparation
25 Wizard of the Schrödinger software suite (Schrödinger Suite 2012 Protein Preparation Wizard;
26 Epik version 2.3; Impact version 5.8; Prime version 3.1; Maestro version 9.3. Schrödinger LLC,
27 New York, NY, USA). The proteins were fully hydrated with explicit solvent using the TIP3P
28 water model and charge neutralization was accomplished by adding Na⁺ counter ions (50).
29 Short-range cutoffs of 12 Å were used for the treatment of non-bonded interactions; while long-
30 range electrostatics was treated with the particle-mesh Ewald method (51). All simulations were
31 carried out in an NpT ensemble at 310 K and 1 atm using Langevin dynamics and the Langevin
32 piston method. A 1 fs time step was applied. Prior to production runs the solvated systems were
33 subjected to an initial energy minimization and subsequent equilibration of the entire system for
34 5 to 10 ns. All MD simulations were carried out at the Computer Cluster of the Norddeutscher
35 Verbund für Hoch- und Höchstleistungsrechnen.

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40 computational resources and the Biophysics Core of the National Heart, Lung, and Blood
41 Institute (NHLBI) for advice, support and the use of the facility. Data deposition: The atomic
42 coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org
43 (PDB entry 5I4E).

1 **Author Contributions**

2 D.J.M. conceived the study. K.C. performed the crystallization, collected the diffraction data, and
3 solved the structure. S.M.H. cloned, expressed and purified the proteins, and performed the
4 biochemical and kinetic assays. M.P. performed and analyzed the molecular dynamics
5 simulations. K.C., S.M.H., M.P., J.R.S., and D.J.M. designed experimental approaches and
6 wrote the manuscript. All authors read and approved the final manuscript.

9 **Competing Interests**

10 The authors declare no conflicts of interest with the contents of this research article.

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35
36
37

1 Figure Legends

2 **Figure 1: Myosin-2 phylogeny, overall topology, and active site characteristics of human**

3 **NM2C.** (A) Phylogenetic analysis divides human myosins-2 in the three subfamilies (i)
4 nonmuscle and smooth muscle myosins-2, (ii) cardiac, (iii) and skeletal muscle myosins-2 (52).
5 Nonmuscle myosin-2s are essential for the structural integrity of the cytoplasmic architecture
6 during cell shape remodeling and motile events of eukaryotic cells whereas all other myosins-2
7 play eminent roles in the contraction of smooth, cardiac and striated muscle cells (53).
8 Abbreviations used: NM2A: nonmuscle myosin-2A, NM2B: nonmuscle myosin-2B; NM2C:
9 nonmuscle myosin-2C; SM: smooth muscle myosin-2; CardA: α -cardiac myosin-2; CardB: β -
10 cardiac myosin-2; EO2: extraocular myosin-2; EMB: embryonic myosin-2; PERI: perinatal
11 myosin-2; IIb: fast skeletal muscle myosin-2; IIx/d: skeletal muscle myosin-2; IIa: slow skeletal
12 muscle myosin-2. (B) Architecture of the crystallized NM2C construct in the pre-powerstroke
13 state. The myosin motor domain and the α -actinin repeats are shown in cartoon representation
14 in green and grey color. The nucleotide is shown in spheres representation. *Inset*, Conserved
15 key residues that interact with the nucleotide in the NM2C active site. The $F_o - F_c$ omit map of
16 $Mg^{2+} \cdot ADP \cdot VO_4$ is contoured at 4σ . The salt bridge between switch-1 R261 and switch-2 E483 is
17 highlighted. (C) Subdomain architecture of NM2C. The U50 kDa is shown in green, the L50 kDa
18 in purple, the converter in grey, and the Nter in blue. The region shown in orange corresponds
19 to the active site and the junction of U50 kDa and L50 kDa. The bound nucleotide is shown in
20 spheres representation. The location of the SH1-SH2 helix and the relay helix in the L50 kDa is
21 highlighted.
22

23 **Figure 2: Conformational changes of the JK-loop in the myosin active site.** (A) Top view

24 on the NM2C active site in the pre-powerstroke state (green) superimposed on pre-powerstroke
25 state structures from chicken smooth muscle myosin-2 (grey, PDB entry 1BR4), *Dictyostelium*
26 nonmuscle myosin-2 (blue, PDB entry 2XEL), and scallop striated muscle myosin-2 (orange,
27 PDB entry 1QVI). The nucleotide is shown in spheres representation. (B) Conformation of the
28 JK-loop in vicinity to the NM2C active site. The JK-loop flanks the active site and connects
29 helices J and K. The distance between the residue Q340 of the JK-loop in the U50 kDa and the
30 D257 of switch-1 of the active site is ~ 8.8 Å. The distance between residue S336 of the JK-loop
31 and switch-1 D257 is ~ 7.4 Å. Switch-1 residue N256 interacts with α -phosphate (3.1 Å) and β -
32 phosphate (3.5 Å) group of $ADP \cdot VO_4$ in the active site. NM2C is colored in green/orange, the
33 JK-loop is colored in brick red and $ADP \cdot VO_4$ is shown in spheres. (C) Interactions between the
34 JK-loop and the switch-1 region are compared between the NM2C (green) and scallop striated
35 muscle myosin-2 (orange, PDB entry 1QVI). A-loop residue R128 is coordinating the interaction
36 to the ADP adenosine in the active site of striated muscle myosin-2. The distance between the
37 residues is 3.2 Å. R128 further forms a hydrogen bond (2.8 Å) with E184 of the P-loop. JK-loop
38 N321 is in hydrogen bond interaction with switch-1 N238, located at a distance of 4.6 Å to the
39 hydroxyl group of the C2' of the ADP ribose. The connectivity between switch-1 and the
40 nucleotide is further strengthened by a hydrogen bond between N237 and the ADP ribose.
41 NM2C lacks all interactions described for scallop striated muscle myosin-2 due to the
42 replacement of R128 with Q150 and JK-loop shortening which increases the distance to the
43 adenosine in the active site to 5.8 Å and disrupts constraints between switch-1 and the JK-loop.
44

1 All residues in the JK-loop region are labeled for scallop striated muscle myosin-2 (PDB entry
2 1QVI). For NM2C only amino acid substitutions are labeled for legibility (**D**) Superimposition of
3 the NM2C pre-powerstroke state structure (green) and the actin-bound near-rigor actoNM2C
4 complex (red) shows that the nucleotide binding site does not undergo major structural
5 changes. Actin subunits are colored in shades of grey and the nucleotide in the in spheres
6 representation. (**E**) Sequence alignment of select structural elements in the myosin motor
7 domain that interact with the JK-loop. Interactions are of A-loop R128 are highlighted with
8 brackets for scallop striated muscle myosin-2 (PDB entry 1QVI). All highlights interactions are
9 absent in NM2C due to the presence of Q150 in the A-loop. Abbreviations used: *Hs* NM2C:
10 human NM2C (NP_079005.3); *Hs* NM2A: human nonmuscle myosin-2A (NP_002464.1); NM2B:
11 human nonmuscle myosin-2B (NP_005955.3); *Gg* SM: chicken smooth muscle myosin-2
12 (NP_990605.2); *Hs* CARD: human beta β -cardiac muscle myosin-2 (NP_000248.2); *Ai* ST:
13 scallop striated muscle myosin-2 (P24733.1). PDB entries are indicated when available.
14

15 **Figure 3: Interdomain connectivity at the converter/Nter/lever junction.** (**A**) Interaction
16 profile of R788 in the pre-powerstroke state. R788 is shown in orange colored sticks and the
17 converter is colored in white, the relay helix in red, the SH1-SH2 helix in purple, and the lever
18 arm in green colored cartoon representation. The inset shows a close-up view of the complete
19 R788 interaction profile and is rotated 137° respective to the main panel. The guanidinium group
20 of R788 forms hydrogen bonds (2.8 Å) with backbone oxygen atom of Q730 from the SH1 helix
21 and the backbone oxygen atom of G731 (3.0 Å) of the converter. The δ -nitrogen atom of R788
22 interacts (3.0 Å) with N776 backbone oxygen atom of N776. The R788 guanidinium group
23 interacts (3.1 Å) with the hydroxyl group of N776 of the converter. The backbone nitrogen atom
24 of R788 interacts (3.1 Å) with the carbonyl group of L777 of the converter. The backbone
25 carbonyl group of R788 interacts (3.4 Å) with the backbone nitrogen of V791 of the lever as well
26 as a water molecule (3.0 Å). The hydroxyl group from relay helix Y518 interacts with the
27 backbone nitrogen atom of G731 (2.8 Å) and backbone oxygen atom of F732 (3.4 Å). F732
28 forms hydrophobic interactions with the methylene groups of R788 with the latter positioned in
29 van der Waals distance to relay loop W525. All the amino acids involved in interactions with
30 R788 are shown as sticks and water molecules as spheres. (**B**) Sequence alignment of selected
31 regions from relay helix, SH1 helix, converter and lever arm shows the high sequence
32 conservation within the myosin-2 motor domain. The asterisk indicates the invariant, positively
33 charged residue corresponding to NM2C R788. The interactions of NM2C R788 with structural
34 elements of the L50 kDa, the converter, and the lever are highlighted. Abbreviations used: *Hs*
35 NM2C: human NM2C (NP_079005.3); *Hs* NM2A: human nonmuscle myosin-2A (NP_002464.1);
36 NM2B: human nonmuscle myosin-2B (NP_005955.3); *Gg* SM: chicken smooth muscle myosin-2
37 (NP_990605.2); *Hs* CARD: human beta β -cardiac muscle myosin-2 (NP_000248.2); *Ai* ST:
38 scallop striated muscle myosin-2 (P24733.1). PDB entries are indicated when available. Lysine
39 residues that replace R788 in cardiac and striated muscle myosins-2 highlighted in the boxed
40 area.
41

42 **Figure 4: Transient kinetic features of NM2C and R788E.** (**A,B**) Interaction between
43 NM2C/R788E with ATP under single-turnover conditions in the absence (**A**) and presence (**B**) of
44 F-actin. Binding 0.375 μ M mantATP to 0.5 μ M myosin/actomyosin results in a transient

1 fluorescence increase that is followed by a short plateau (hydrolysis) and a slow decrease in
2 mantADP fluorescence that is associated with its release. All three phases are reduced in
3 R788E (grey) compared to NM2C (green) and are more pronounced in the absence of F-actin
4 (A). The very slow decrease of the fluorescence signal in R788E in (A) indicates that either the
5 ATP hydrolysis rate or a subsequent release rate of the hydrolysis products are severely
6 decreased when compared to NM2C.

7
8 **Figure 5: Structural importance of R788 at the converter/Nter/lever junction.** (A) Relay
9 helix angle as a function of MD simulation time, as monitored by the angle between C_α atoms of
10 residues S489, M510, and E521 along the trajectories. The relay helix straightens in NM2C with
11 a steady increase in the angle of the relay helix from approximately 145° to 150°, while the
12 angle does not change significantly in R788E and fluctuates around 145° throughout the 100 ns
13 time course of the simulation. Values for the relay helix angle observed in crystal structures of
14 pre-power stroke (PPS) and post-rigor (PR) are indicated by dotted lines. (B) Population of
15 hydrogen bonds (HB) between R788 (NM2C) or E788 (R788E) and surrounding structural
16 elements over the simulation time of 100 ns. The abbreviations s and m indicate side chain and
17 main chain. (C, E) Direct comparison of the dynamics and conformational changes in NM2C (C)
18 and R788E (E) during MD simulations. Snapshots from the start (0 ns simulation time) and end
19 conformations (100 ns simulation time) are shown in light cyan and colored cartoon
20 representation, respectively. The relay helix is shown in red, the SH1-SH2 helix in purple and
21 the converter in grey. Relay loop W525 is shown in red in stick representation. The insets show
22 a close-up view of the conformational changes of W525 along the simulation trajectory. (D, F)
23 Tryptophan fluorescence emission spectrum of 4 μM NM2C (D) and 4 μM R788E (F) in the
24 absence of nucleotide or the presence of 0.5 mM ATP or 0.5 mM ADP.

25
26 **Figure 6: Proposed allosteric communication pathway from the converter to the NM2C**
27 **active site.** (A) Residue R788 connects the converter to the SH1 interface through main chain
28 and side chain interactions in NM2C. This interface further interacts with Y518 of the relay helix
29 and W525. The interactions are propagated to the active site and *vice versa* through the relay
30 helix and through an interaction of relay helix residue N499 with the main chain of G481 from
31 switch-2. The latter directly interacts with the nucleotide. Y599 of the wedge loop, that itself
32 contacts the relay helix, establishes an interaction with switch-2 F482. This residue is in contact
33 with S200 of the P-loop by main chain interactions and directly interacts with the nucleotide in
34 the active site. (B) The interface between R788E and both, the SH1 and the relay helix, is
35 disrupted. The allosteric communication from the active site to the converter is compromised.
36 The experimental observation that R788E does not change its intrinsic fluorescence upon
37 nucleotide binding which is caused by a lacking conformational change of W525 indicates that
38 the communication pathway is interrupted in the relay helix. The position of W525 in the relay
39 loop at the distal end of the relay helix indicates that the pathway is interrupted before or at
40 Y518, which is supported by the observation that the relay loop does not change its position
41 during the time course of the MD simulation. As a consequence, Y518 cannot establish an
42 interface with E788 and F732 of the converter and SH1 helix. Further, E788 cannot establish
43 interactions with N776 and V791 and completely disrupts the structural integrity of the interface
44 of converter, SH1-SH2 helix, relay helix and the lever arm and uncouples nucleotide-induced

1 changes in the active site from the converter rotation. Only key residues involved are showed in
2 the proposed mechanism. Small elliptical shapes show main chain interactions.

3

4 **Tables**

5

6 **Table 1: Crystallographic data collection and refinement statistics for human NM2C.**

7

Parameter	Hs NM2C-ADP-VO₄
<i>X-ray data reduction statistics</i>	
Space group	<i>P</i> 2 ₂ 1 ₂ 1
<i>Unit cell dimensions</i>	
<i>a, b, c</i>	81.12 Å, 125.61 Å, 153.96 Å
α, β, γ	90°, 90°, 90°
Resolution	97.33 Å – 2.25 Å
Last shell	2.35 Å – 2.25 Å
Total measurements	1116790
No. of unique reflections	75357
Last shell	9069
Wavelength	0.91841 Å
<i>R</i> _{merge}	0.12
Last shell	0.23
<i>I</i> / σ (<i>I</i>)	13.6
Last shell	2.63
Completeness	0.998
Last shell	1.00
Multiplicity	14.82
Last shell	14.97
<i>Refinement statistics</i>	
Resolution	34.77 Å–2.25 Å
Last shell	2.31 Å–2.25 Å
No. of reflections (working set)	71459
No. of reflections (test set)	3786
<i>R</i> -factor	0.2210
Last shell	0.2910
<i>R</i> -free	0.2410
Last shell	0.2920
<i>No. of non-hydrogen atoms</i>	
Macromolecules	7697
Ligands	33
solvent	666
<i>Average B-factor</i>	
Protein	76.06 Å ²
Ligands	35.59 Å ²
Solvent	63.37 Å ²
<i>RMS deviations from ideal values</i>	
Bond lengths	0.01 Å
Bond angles	1.16 ⁰
Ramachandran favored	96.22 %
Ramachandran allowed	3.78 %
Ramachandran outliers	0

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Table 2: Comparative analysis of kinetic signatures of monomeric myosin-2 motor domain constructs. Abbreviations used: NM2A: human nonmuscle myosin-2A, NM2B: human nonmuscle myosin-2B (PDB entry 4PD3); NM2C: human nonmuscle myosin-2C; SM: chicken smooth muscle myosin-2 (PDB entry 1BR2); CARD: human/chicken β -cardiac myosin-2 (PDB entry 4DB1); ST: rabbit/scallop striated muscle myosin-2 (PDB entry 1DFL).

Myosin	k_{cat}/K_{app}	k_{+AD}/K_1k_{+2}	K_{AD}/K_D	k_{-AD}/k_{-D}	K_{DA}/K_A	duty ratio
<i>Hs</i> NM2C	0.003	2.1	0.12	1	0.13	$\sim 0.3^{\$}$
<i>Hs</i> R788E	0.004	0.26	5	3	0.19	$\sim 1^{\$}$
<i>Hs</i> NM2A (26)	0.002	19.4	0.7	2.8	2	0.1
<i>Hs</i> NM2B (27)	0.002	10	0.2	0.7	0.4	0.37
<i>Gg</i> SM (28, 54)	0.07	4.4	4.2	12	6.9	<0.05
<i>Hs/Gg</i> CARD (28, 55)	0.07 [#]	1.6	42	103.3	23.9	<0.02
<i>Oc/Ai</i> ST (30, 56-58)	1.6	1.6	49	250	30	<0.04

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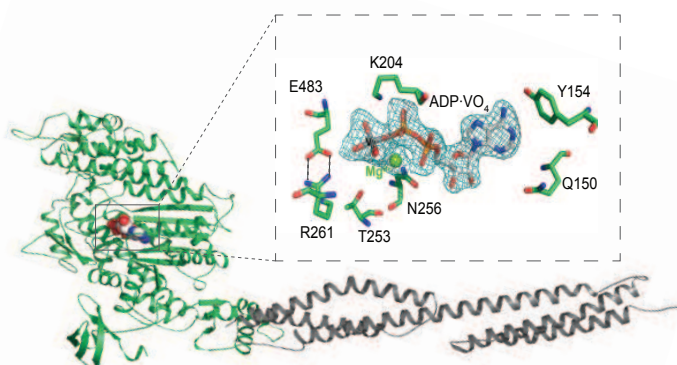
* Rabbit striated muscle myosin-2
Chicken cardiac muscle myosin-2
\$ Calculated at 190 μ M F-actin

Figure 1

A

	Gene	Protein	Localization	Function	ATPase	Duty Ratio
Nonmuscle and smooth muscle myosins-2	MYH14	NM2C	Cytosol	Strain sensor	Low	Elevated
	MYH11	SM	Smooth muscle	Force holder	Medium	Low
	MYH9	NM2A	Cytosol	Strain sensor	Low	Low
	MYH10	NM2B	Cytosol	Strain sensor	Low	Elevated
Cardiac myosins-2	MYH6	CardA	Cardiac muscle	Force holder	Medium	Low
	MYH7	CardB	Cardiac muscle	Force holder	Medium	Low
Skeletal myosins-2	MYH13	EO2	Skeletal muscle	Fast mover	High	Low
	MYH3	EMB	Skeletal muscle	Fast mover	High	Low
	MYH8	PERI	Skeletal muscle	Fast mover	High	Low
	MYH4	Ilb	Skeletal muscle	Fast mover	High	Low
	MYH1	Ix/d	Skeletal muscle	Fast mover	High	Low
	MYH2	Ila	Skeletal muscle	Fast mover	High	Low

B



C

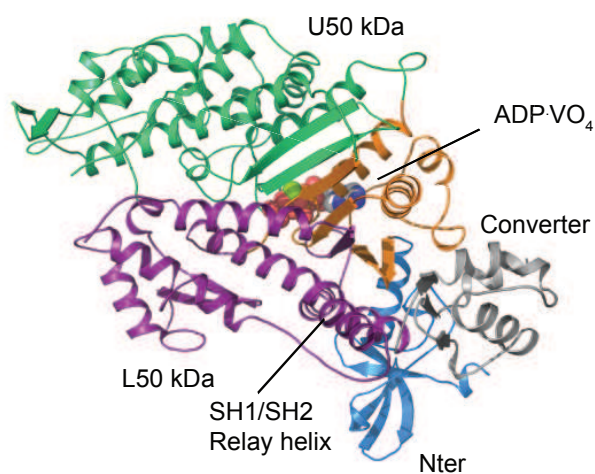


Figure 2

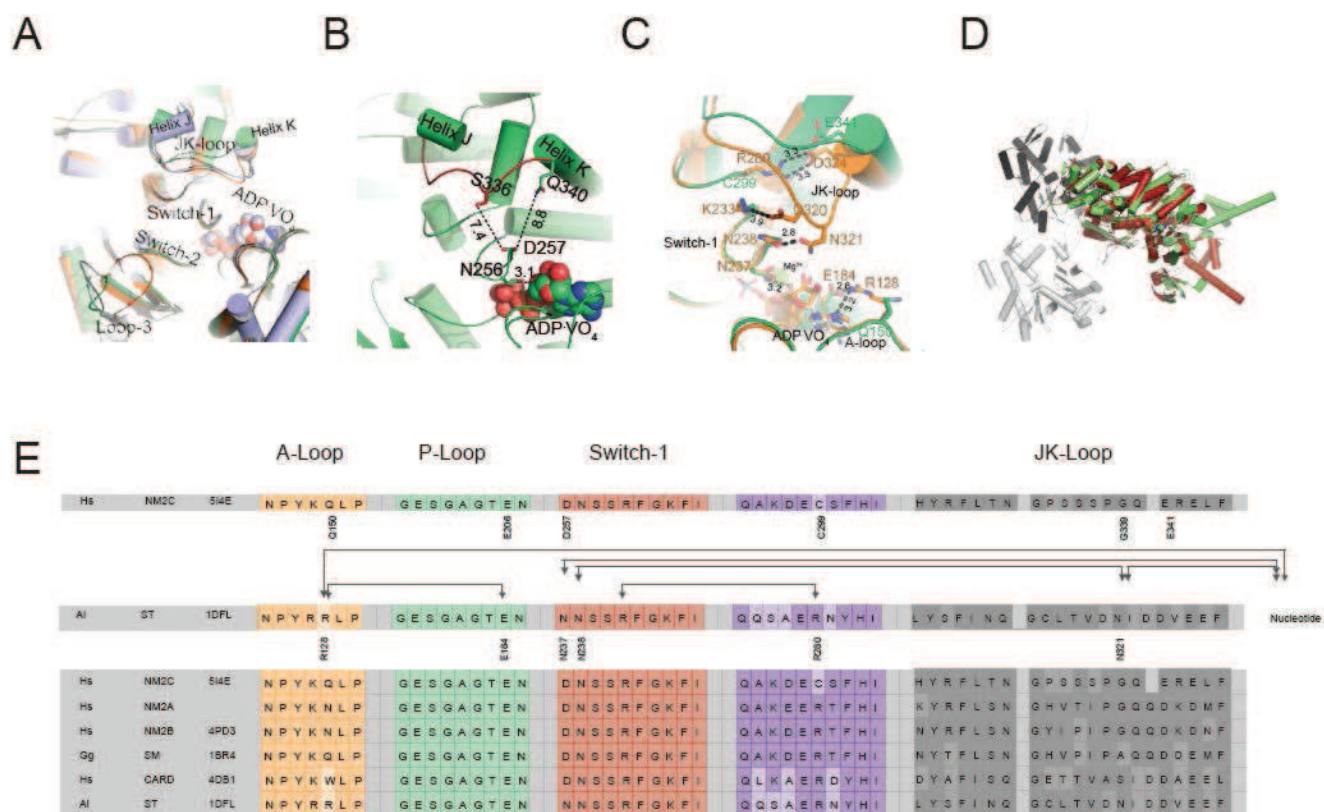
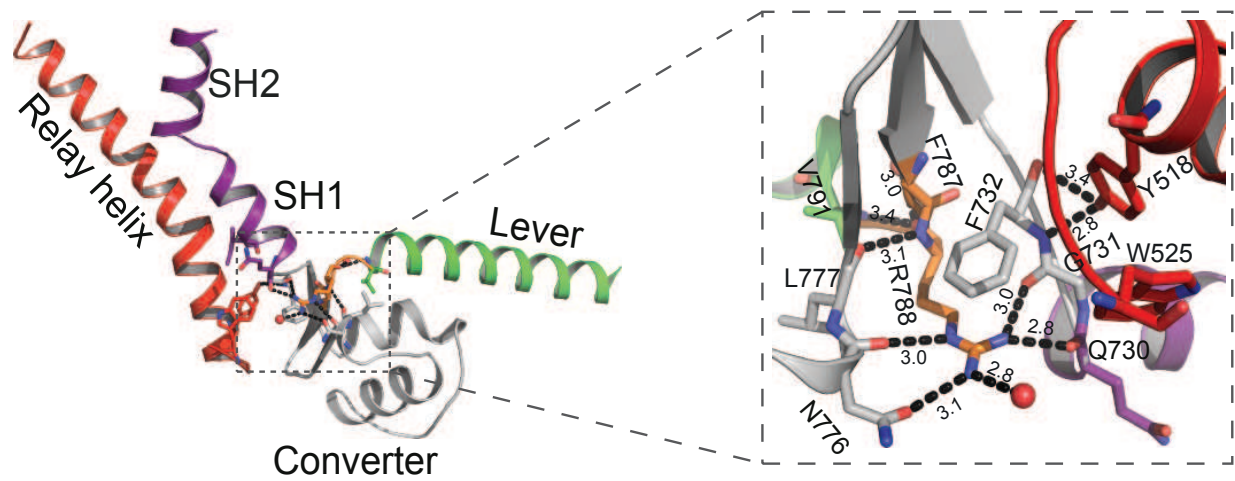


Figure 3

A



B

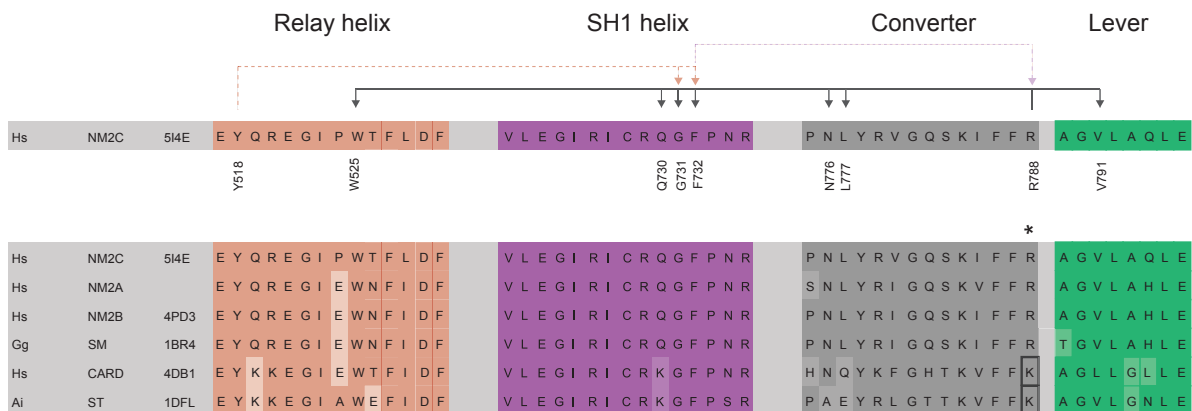
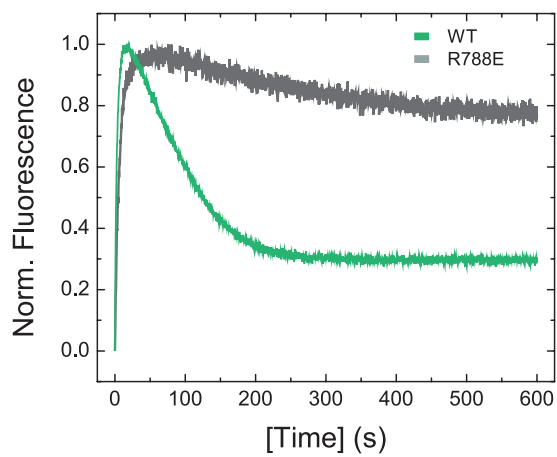


Figure 4

A



B

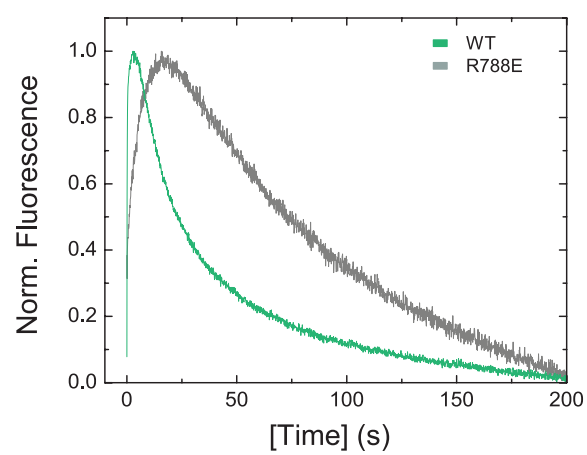


Figure 5

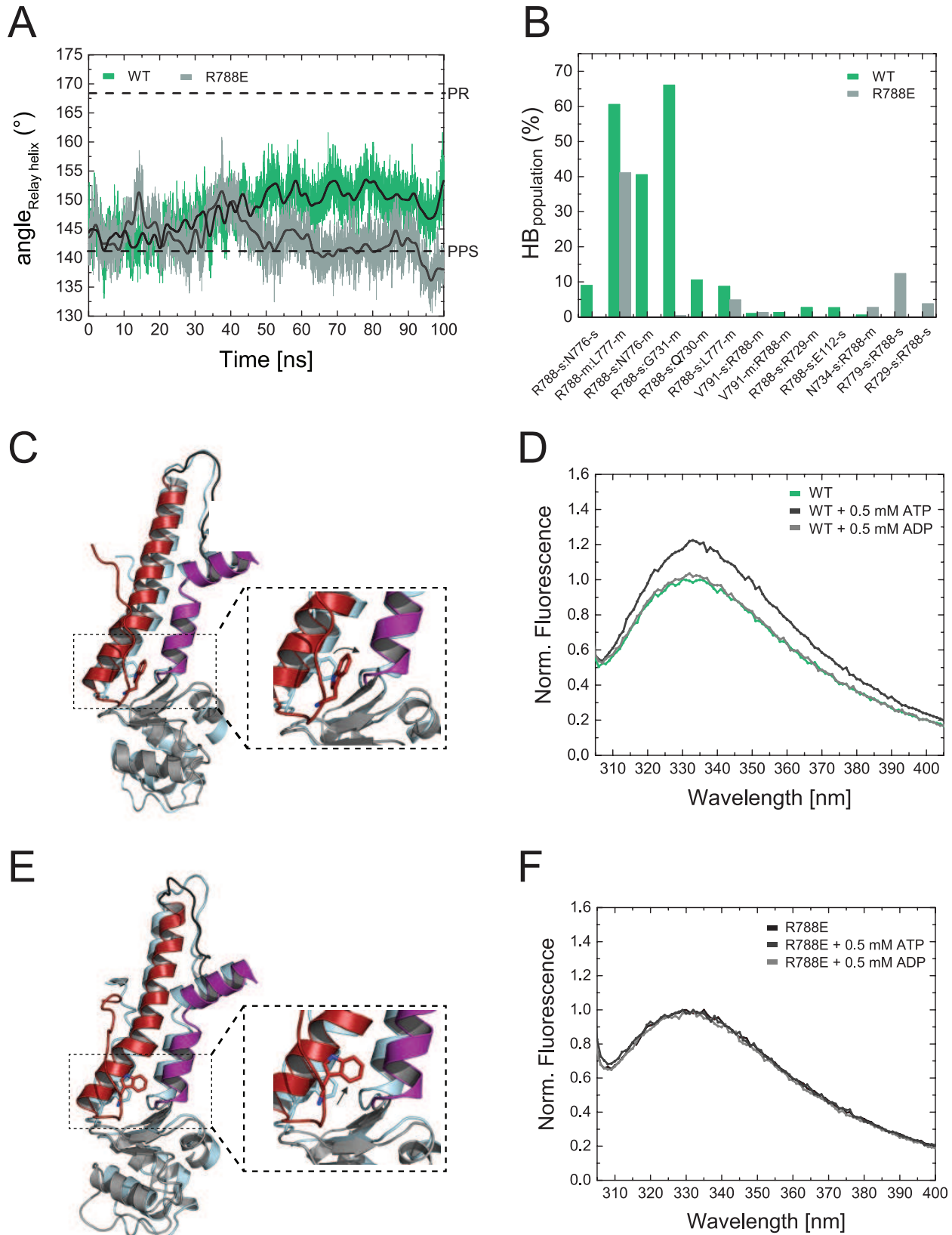
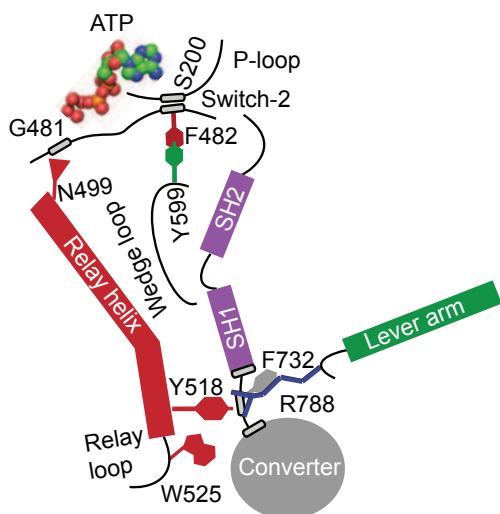
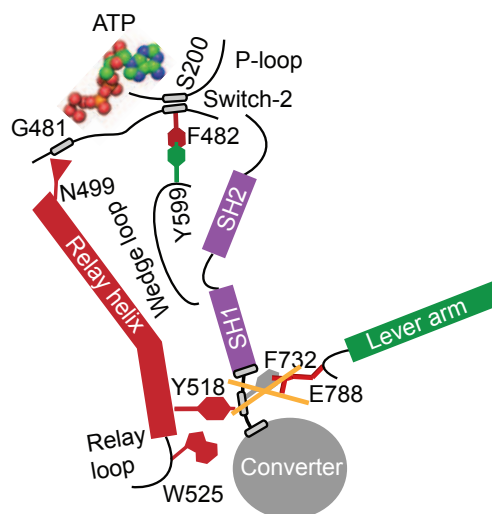


Figure 6

A



B



1 Supplementary Information

2
3 **Figure 1 - Figure supplement 1: Myosin-2 ATPase cycle, expression constructs, and**
4 **structural alignment of the NM2C C_α coordinates. (A)** Consensus scheme of the myosin and
5 actomyosin ATPase cycle. The upper part represents the myosin (M) ATPase cycle. The lower
6 part represents the ATPase cycle in the presence of F-actin (A). The asterisk denotes enhanced
7 states of the intrinsic myosin fluorescence, which are attributed to the nucleotide-induced
8 changes in the microenvironment of the conserved relay loop W525. Lowercase *k* denotes a
9 rate constant. $k_{+D}=k_{-6}$, $k_{-D}=k_{-6}$, $k_{+AD}=k_{-6}$, $k_{-AD}=k_{-6}$. An uppercase *K* denotes a dissociation
10 equilibrium constant ($K=k_{-x}/k_{+x}$) throughout this work. Normal and bold face notation denote the
11 respective kinetic constants in the absence and presence of F-actin. The main pathway of the
12 actomyosin ATPase cycle is highlighted in grey. Strong and weak actin binding states are
13 indicated. **(B)** Schematic representation of the expression constructs used in this study. *Top*,
14 Nonmuscle myosin-2C contains a N-terminal motor domain (green) followed by a neck and a tail
15 domain (dark grey). *Middle*, For kinetic studies of NM2C and R788E, the motor domain was
16 directly fused to spectrin repeats 1 and 2 from *Dictyostelium* α-actinin (light grey) which serves
17 as an artificial lever arm. The concept of the artificial lever arm has been successfully used in
18 structural and kinetic studies of myosins-2 (4, 11, 59, 60). *Bottom*, For structural studies, the N-
19 terminal 45 amino acids were deleted. The numbering refers to the amino acid sequence of the
20 full-length protein. **(C)** The NM2C C_α atoms (green) in ribbon representation superimpose with a
21 root mean square deviation (*r.m.s.d.*) of 0.57 Å to chicken smooth muscle myosin-2 (grey, PDB
22 entry 1BR2), with 0.78 Å to scallop striated muscle myosin-2 (orange, PDB entry 1QVI), and
23 0.73 Å to *Dictyostelium* nonmuscle myosin-2 (violet, PDB entry 2XEL), underlining a strong
24 correlation between C_α geometry and overall motor domain fold. The JK-loop and the nucleotide
25 are highlighted in color and spheres representation. **(D)** Relative orientation of converter and
26 lever in NM2C (green), chicken smooth muscle myosin-2 (PDB entry 1BR2, blue), and scallop
27 striated muscle myosin-2 (PDB entry 1QVI (orange), 1DFL (violet)) motor domain structures in
28 cartoon representation. α-helices are depicted as cylinders.

29
30 **Figure 2 - Figure supplement 1: Active site characteristics in myosin-2 motor domains.**

31 **(A)** Sequence alignment of myosin-2 JK-loops. The asterisk indicates the invariant, negatively
32 charged residues corresponding to NM2C E341. The abbreviations used are as follows: *Hs*
33 NM2C: human NM2C (NP_079005.3); *Hs* NM2A: human nonmuscle myosin-2A (NP_002464.1);
34 NM2B: human nonmuscle myosin-2B (NP_005955.3); *Gg* SM: chicken smooth muscle myosin-2
35 (NP_990605.2); *Hs* CARD: human beta β-cardiac muscle myosin-2 (NP_000248.2); *Ai* ST:
36 scallop striated muscle myosin-2 (P24733.1); *Dd* NM2: *Dictyostelium* nonmuscle myosin-2:
37 (XP_637740.1); *Dm* EMB: *Drosophila* embryonic body wall muscle myosin-2 (P05661 with
38 spliced exon 7a); *Dm* IFI: *Drosophila* indirect flight muscle myosin-2 (P05661 with spliced exon
39 7d). PDB entries are indicated when available. Cardiomyopathy-associated mutations in β-
40 cardiac myosin-2 on positions F312 (F312C), V320 (V320M), A326 (A326P), and E328 (E328G)
41 are highlighted in the boxed areas. **(B)** Superimposition of JK-loops of pre-powerstroke state
42 structures of NM2C (brick red), chicken smooth muscle myosin-2 (yellow, PDB entry 1BR2), and
43 *Dictyostelium* nonmuscle myosin-2 (purple, PDB entry 2XEL) motor domains are shown in
44 close-up view. The distance between the JK-loops of smooth muscle myosin-2 and

1 *Dictyostelium* nonmuscle myosin-2 and switch-1 is ~ 2.8 Å and 8.8 Å for NM2C. (C) The
2 distance between the residue P324 of the JK-loop (yellow) in the U50 kDa of chicken smooth
3 muscle myosin 2 (grey, PDB entry 1BR2) and the D243 of switch-1 is ~2.8 Å. Switch-1 residue
4 N242 interacts with α -phosphate (2.8 Å) and β -phosphate (3.1 Å) group of the ADP-ALF₄
5 complex in the active site is shown in spheres. (D,E) The volume of the active site, indicated by
6 the inclusion spheres, was determined to 3065 Å³ in NM2C (D) and 697 Å³ in scallop striated
7 muscle myosin-2 (PDB entry 1QVI) (E). Both structures are in the pre-powerstroke state.

8
9 **Figure 3 - Figure supplement 1: Interdomain connectivity at the converter/Nter/lever**
10 **junction in muscle myosins-2.** In contrast to NM2C (Figure 3), the substitution of R788 with a
11 lysine K762 in scallop striated muscle myosin-2 (PDB entry IQVI) (A) and K766 in human β -
12 cardiac muscle myosin-2 (PDB entry 4DB1) (B) reduces the number of side chain and main
13 chain interactions at the converter/Nter/lever junction. Coloring is according to Figure 3.

14
15 **Figure 4 - Figure supplement 1: Nucleotide binding characteristics of NM2C and R788E**
16 **and disease causing NM2C mutations.** (A) Dependence of the observed rate constants (k_{obs})
17 upon d-mantATP binding to 0.25 μ M NM2C/R788E on the nucleotide concentration. Linear fits
18 to the data result in second-order rate binding constants of $K_1k_{+2} = 0.48 \pm 0.01 \mu\text{M}^{-1}\text{s}^{-1}$ for NM2C
19 (green) and a 5-fold reduced binding rate constant of $K_1k_{+2} = 0.09 \pm 0.005 \mu\text{M}^{-1}\text{s}^{-1}$ for R788E
20 (grey). (B) The second-order ATP binding rate constant K_1k_{+2} , determined by the ATP-induced
21 dissociation of the pyrene-labeled actoNM2C (green) or actoR788E (grey) complexes are with
22 $K_1k_{+2} = 1.23 \pm 0.02 \mu\text{M}^{-1}\text{s}^{-1}$ and $K_1k_{+2} = 2.06 \pm 0.07 \mu\text{M}^{-1}\text{s}^{-1}$ similar. (C) The R788E (grey) mutation
23 decreases the second-order ADP binding rate constant 3-fold to $k_{+D} = 0.12 \pm 0.01 \mu\text{M}^{-1}\text{s}^{-1}$ when
24 compared to $k_{+D} = 0.39 \pm 0.01 \mu\text{M}^{-1}\text{s}^{-1}$ for NM2C (green). (D) The ADP binding rate constant k_{+AD}
25 = $0.03 \pm 0.001 \mu\text{M}^{-1}\text{s}^{-1}$, as calculated from the slope of the k_{obs} versus [d-mantADP] plot, is 85-fold
26 decreased for R788E (grey) when compared to $k_{+AD} = 2.54 \pm 0.18 \mu\text{M}^{-1}\text{s}^{-1}$ for NM2C (green). (E)
27 Time-dependent change in the intrinsic tryptophan signal upon mixing 0.5 mM ATP with 0.25
28 μ M NM2C (grey) or R778E (grey) in the presence of 5 μ M ADP. ATP binding increases the
29 fluorescence signal in NM2C but not R788E under identical conditions in a stopped-flow
30 spectrophotometer. (F) Missense mutations G376C and R726S are associated with autosomal
31 dominant hearing impairment (DFNA4) and their location in NM2C is shown in spheres
32 representation. G376C is in proximity to the JK-loop, R726S is in the SH1-SH2 helix. NM2C
33 subdomains are color coded according to Figure 1C and the nucleotide is shown in spheres
34 representation.

35
36 **Figure 5 - Figure supplement 1: Stability of the salt bridge between switch-1 and switch-2**
37 **in MD simulations of NM2C and R788E.** (A,B) Monitored number of hydrogen bond (#HB)
38 interactions between R261 (switch-1) and E483 (switch-2) along the simulation time of NM2C
39 (A) and R788E (B). Hydrogen bonds were detected with cutoff values for the donor-acceptor
40 distance and angle of 3.5 Å and 30°. Note the intermediate breaking of the salt-bridge in R788E
41 (B).

1 **Supplementary table 1: Dihedral angles of switch-1 and lever arm residues in crystal**
 2 **structures of NM2C and smooth muscle myosin-2 (PDB entry 1BR2).**
 3

<i>Hs</i> NM2C			<i>Gg</i> smooth muscle myosin-2		
Residue	φ	ψ	Residue	φ	ψ
<i>Switch-1</i>					
N256	-152.2	125.4	N242	-148.5	139.7
D257	-66.7	-15.6	D243	-78.1	-32.9
N258	-135.6	51.3	N244	-116.9	55.9
S259	-71.5	128.2	S245	-81.2	127.9
S260	-78.1	122.8	S246	-71.1	119.6
R261	-104.7	29.2	R247	-102.5	35.4
F262	-161.6	156.6	F248	-168.3	141.5
G263	-98.8	156.5	G249	-78.8	165.2
K264	-148.4	141.1	K250	-159.4	129.9
<i>Lever arm</i>					
F786	-95.4	133.3	F775	-96.73	131.6
F787	-124.1	154.2	F776	-113.62	151.8
R788	-72.2	159.4	R777	-73.08	164.5
A789	-55.2	139.0	T778	-54.84	137.1
G790	82.4	-3.7	G779	64.23	15.6
V791	-72.2	-41.3	V780	-81.43	-54.4
L792	-72.2	-41.2	L781	-58.17	-35.6
A793	-54.7	-36.7	A782	-48.66	-63.9
Q794	-78.2	-30.3	H783	-53.48	-37.6
L795	-75.2	-32.8	L784	-61.41	-42.1
E796	-67.5	-31.7	E785	-63.52	-52.9
E797	-79.4	-32.2	E786	-52.85	-42.9
E798	-74.2	-36.6	E787	-66.4	-36.1

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1 **Supplementary table 2: Structure function relationships in the myosin-2 motor domain.**
2 Interactions between residues in the active site involved in nucleotide binding and release
3 kinetics based on this work and previous biochemical and structural studies on myosin motor
4 domains (13, 16, 17, 61, 62). It is of note that myosin is a highly allosteric enzyme and
5 nucleotide binding and release kinetic involve numerous interactions and subtle structural
6 rearrangements of residues from different motor subdomains. Kinetic parameters from
7 monomeric myosin motor domain constructs that are associated with a structural interaction are
8 listed for direct comparison. An emerging trend from this analysis is that the myosin-2 kinetic
9 cycle does not have a selectivity of ATP *versus* ADP. The presence of F-actin results in different
10 allosteric communication pathways in myosins-2 and establishes ATP/ADP binding selectivity.
11 Overall, nucleotide binding rates are decreased for the group of nonmuscle and smooth muscle
12 myosins-2 compared to myosins-2 from cardiac and skeletal muscle. The lacking salt bridge
13 interactions between JK-loop, U50 kDa and switch-1 in nonmuscle myosins-2 results in
14 decreased second-order binding rate constants for ATP (K_1k_{+2}) and ADP (k_{+D}) (**Figure 1 -**
15 **Figure supplement 1A**). Either a salt bridge interaction or hydrophobic interactions between
16 the A-loop and the P-loop of muscle and cardiac myosins-2 at the active site favor fast
17 nucleotide binding kinetics and does not or only marginally discriminate between ADP and ATP.
18 The lack of a salt bridge interactions can have different effects dependent on the coordinating
19 residue in the A-loop: An asparagine in the A-loop of nonmuscle myosins-2A and -2B favors
20 ADP over ATP binding to actomyosin. A glutamine in the NM2C A-loop, which has a longer side
21 chain than asparagine, abolishes ATP/ADP sensitivity in NM2C and the closely related smooth
22 muscle myosin-2. The number of salt bridge interactions between P-loop, switch-1, and the Nter
23 correlates with the thermodynamic and kinetic coupling, and the actin-activated ADP release
24 rates in all myosins-2. Abbreviations used: NM2A: human nonmuscle myosin-2A; human NM2B:
25 nonmuscle myosin-2B (PDB entry 4PD3); NM2C: human nonmuscle myosin-2C; SM: chicken
26 smooth muscle myosin-2 (PDB entry 1BR2); CARD: human β -cardiac myosin-2 (PDB entry
27 4DB1); Oc ST: rabbit striated muscle myosin-2 (PDB entry 1DFL).
28

Myosin	Residue	Residue	Residue	Residue	Parameter	
	<i>JK-loop</i>	<i>U50 kDa</i>	<i>JK-loop</i>	<i>Switch-1</i>	K_1k_{+2}	k_{+D}
<i>Hs</i> NM2A (26)	T273	D315	G312	D230	0.56	0.55
<i>Hs</i> NM2B (27)	T280	D322	G319	D237	0.65	0.81
<i>Hs</i> NM2C	E341	C299	G339	D257	0.48	0.39
<i>Gg</i> SM (54)	D328	R285	A325	D243	2.1	1.8
<i>Hs</i> CARD (55)	D325	R281	S322	D239	1.5	1.5
<i>Oc</i> ST (30, 56)	R280	E324	N321	N238	3.9	1.7

	Residue	Residue	Parameter				
	<i>A-loop</i>	<i>P-loop</i>	k_{+AD}	K_1k_{+2}	k_{+D}	K_1k_{+2}	k_{+AD}/K_1k_{+2}
<i>Hs</i> NM2A (26)	N125	E182	2.72	0.14	0.55	0.56	19.4
<i>Hs</i> NM2B (27)	N130	E186	2.41	0.24	0.81	0.65	10
<i>Hs</i> NM2C	Q150	E206	2.54	1.23	0.39	0.48	2.1
<i>Gg</i> SM (54)	Q129	E185	3.6	2	1.8	2.1	4.4
<i>Hs</i> CARD (55)	W130	V186	4.4	1.1	1.5	1.5	1.6
<i>Oc</i> ST (30, 56)	R128	E184	1.6	2.5	1.7	3.9	1.6

	Residue	Residue	Residue	Parameter		
	<i>P-loop</i>	<i>Switch-1</i>	<i>Nter</i>	K_{AD}/K_D	k_{-AD}/k_{-D}	k_{-AD}
<i>Hs</i> NM2A (26)	E175	K228	H676	0.7	2.8	1.72
<i>Hs</i> NM2B (27)	E179	K235	H683	0.2	0.7	0.35
<i>Hs</i> NM2C	E199	K255	H700	0.12	1	0.65
<i>Gg</i> SM (54)	E177	K240	H688	4.2	12	15
<i>Hs</i> CARD (55)	E179	R237	E677	42	103.3	150
<i>Oc</i> ST (30, 56)	E177	R236	E675	49	250	500

1 **Supplementary table 3: Steady-state and transient state kinetic parameters of NM2C and**
 2 **R788E.** Numbering of the kinetic constants refers to **Figure 1 - Figure Supplement 1A.**
 3 Measurements of transient kinetic parameters that rely on a change in intrinsic tryptophan
 4 fluorescence are not experimentally accessible for R788E.
 5
 6

Parameter	Signal or calculation	NM2C	R788E
<i>Steady-state ATPase</i>			
k_{cat} (s^{-1})	NADH assay ^a	0.37±0.02	0.2±0.01
K_{app} (μM)	NADH assay ^a	129.4±17.2	45.9±13
k_{cat}/K_{app} ($\mu M^{-1}s^{-1}$)	NADH assay	~0.003	~0.004
<i>ATP interaction</i>			
K_1k_{+2} ($\mu M^{-1}s^{-1}$)	Tryptophan	0.39±0.01	not accessible [†]
K_1k_{+2} ($\mu M^{-1}s^{-1}$)	d-mantATP	0.48±0.01	0.09±0.005
$1/K_1$ (μM)	Tryptophan	44.2±6.23	not accessible [†]
k_3+k_{-3} (s^{-1})	Tryptophan	24.32±0.89	not accessible [†]
K_1k_{+2} ($\mu M^{-1}s^{-1}$)	Pyrene-actin	1.23±0.02	2.06±0.07
K_1k_{+2} ($\mu M^{-1}s^{-1}$)	d-mantATP	1.64±0.04	1.37±0.04
$1/K_1$ (μM)	Pyrene-actin	~318	~390
k_{+2} (s^{-1})	Pyrene-actin	643.24±23.45	578.53±13.65
<i>ADP interaction</i>			
k_{+D} ($\mu M^{-1}s^{-1}$)	d-mantADP	0.39±0.01	0.12±0.01
k_{-D} (s^{-1})	d-mantADP ^b	0.94±0.07	0.06±0.01
k_{-D} (s^{-1})	d-mantADP ^c	0.39±0.01	0.05±0.001
k_{-D} (s^{-1})	Tryptophan ^c	0.47±0.02	not accessible [†]
K_D (μM)	k_{-D}/k_{+D}	~1-2.4	~0.5
k_{+AD} ($\mu M^{-1}s^{-1}$)	d-mantADP	2.54±0.18	0.03±0.001
k_{-AD} (s^{-1})	d-mantADP ^b	0.65±0.06	0.09±0.03
k_{-AD} (s^{-1})	Light scattering ^c	0.39±0.01	0.15±0.01
k_{-AD} (s^{-1})	d-mantADP ^c	0.68±0.01	0.19±0.01
k_{-AD} (s^{-1})	Pyrene-actin ^c	0.48±0.01	0.14±0.01
K_{AD} (μM)	k_{-AD}/k_{+AD}	~0.29	~2.68
<i>Actin interaction</i>			
k_{+A} ($\mu M^{-1}s^{-1}$)	Light scattering ^{d,e}	2.49±0.07	0.66±0.03
k_{-A} (s^{-1})	Pyrene-actin ^{d,e}	~0.15	~0.031
K_A (nM)	k_{-A}/k_{+A}	~60	~47
k_{+DA} ($\mu M^{-1}s^{-1}$)	Light scattering ^d	0.53±0.01	0.21±0.01
k_{-DA} (s^{-1})	Pyrene-actin ^d	~0.004	~0.002
K_{DA} (nM)	k_{-DA}/k_{+DA}	~8	~9

7

8

9 ^a In 50 mM NaCl, 2mM MgATP, T=25 °C

10 ^b From y-intercept

11 ^c From chasing experiment

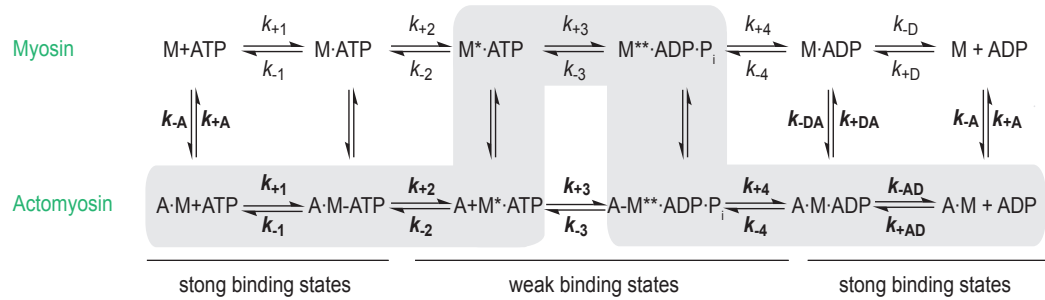
12 ^d Measured in salt free stopped-flow buffer

13 ^e Samples were treated with apyrase prior to the assay to ensure rigor conditions

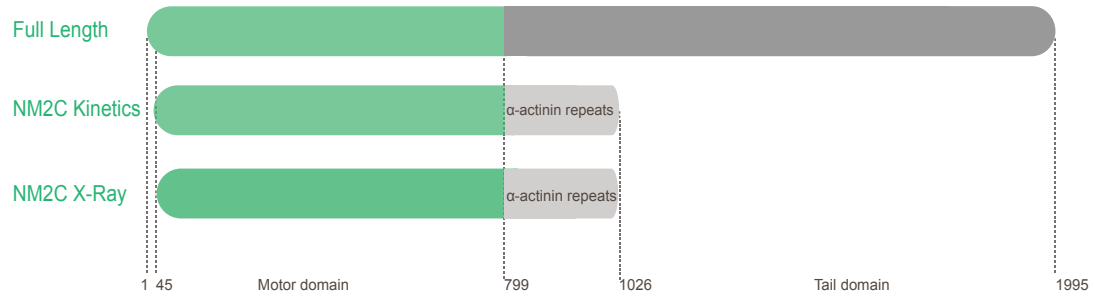
14 ^f The lack of a change in intrinsic fluorescence upon ATP binding to R788E precludes the direct
 15 measurement of this and associated kinetic parameter

Figure 1 - Figure supplement 1

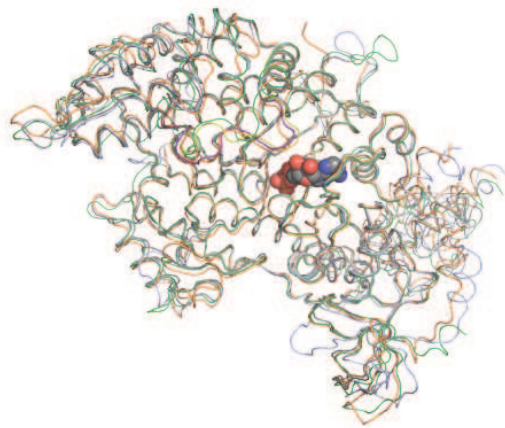
A



B



C



D

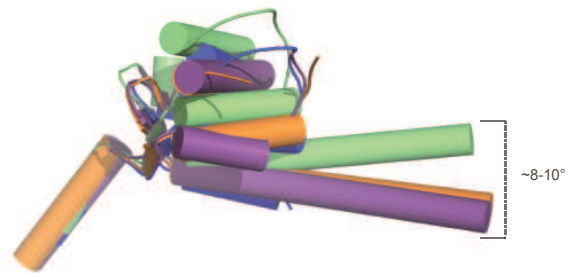
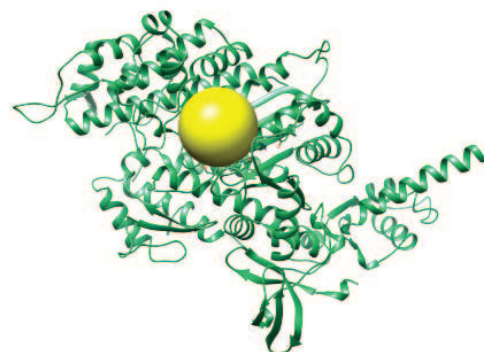


Figure 2 - Figure supplement 1

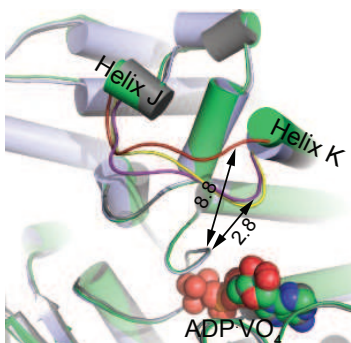
A

Hs	NM2C	514E	HYRFLTN - GPSSSPGQ	E	RELF
Hs	NM2A		KYRFLSN - GHVTIPGQQ	D	KDMF
Hs	NM2B	4PD3	NYRFLSN - GYIPIPGQQ	D	KDNF
Gg	SM	1BR4	NYTFLSN - GHVPIPAQQ	D	DEM F
Hs	CARD	4DB1	DYAFISQ - GETTVASID	D	AEEEL
Ai	St	1DFL	LYSFINQ - GCLTVDNID	D	VEEF
Dd	NM2	2XEL	SFNYLNQS GCVDIKGVSD	D	SEEF
*					
Dm	EMB/exon 7a		DYHIVSQ - GKVTVASID	D	AEEF
Dm	IF1/exon 7d		DYYNVSQ - GKVTVPNMD	D	GEEF

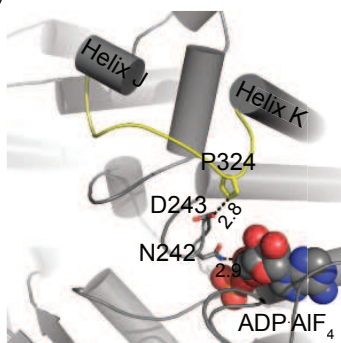
D



B



C



E

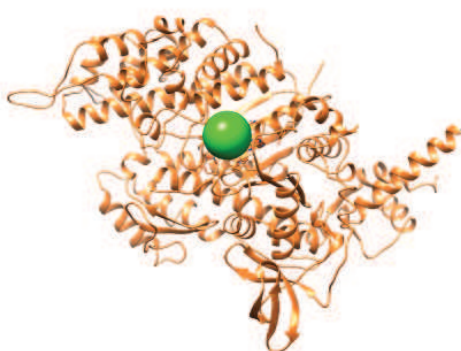
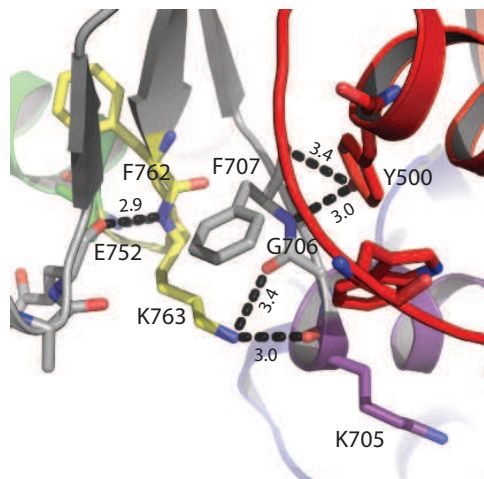


Figure 3 - Figure supplement 1

A



B

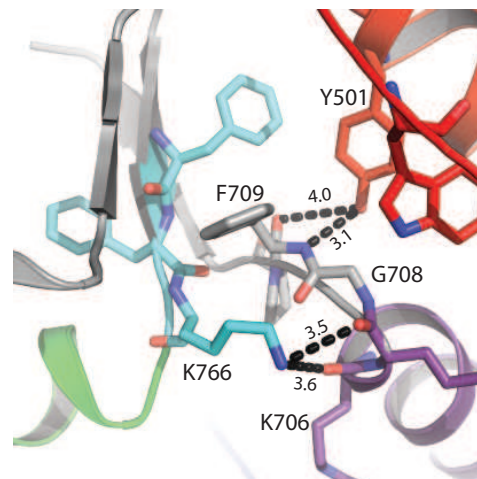


Figure 4 - Figure supplement 1

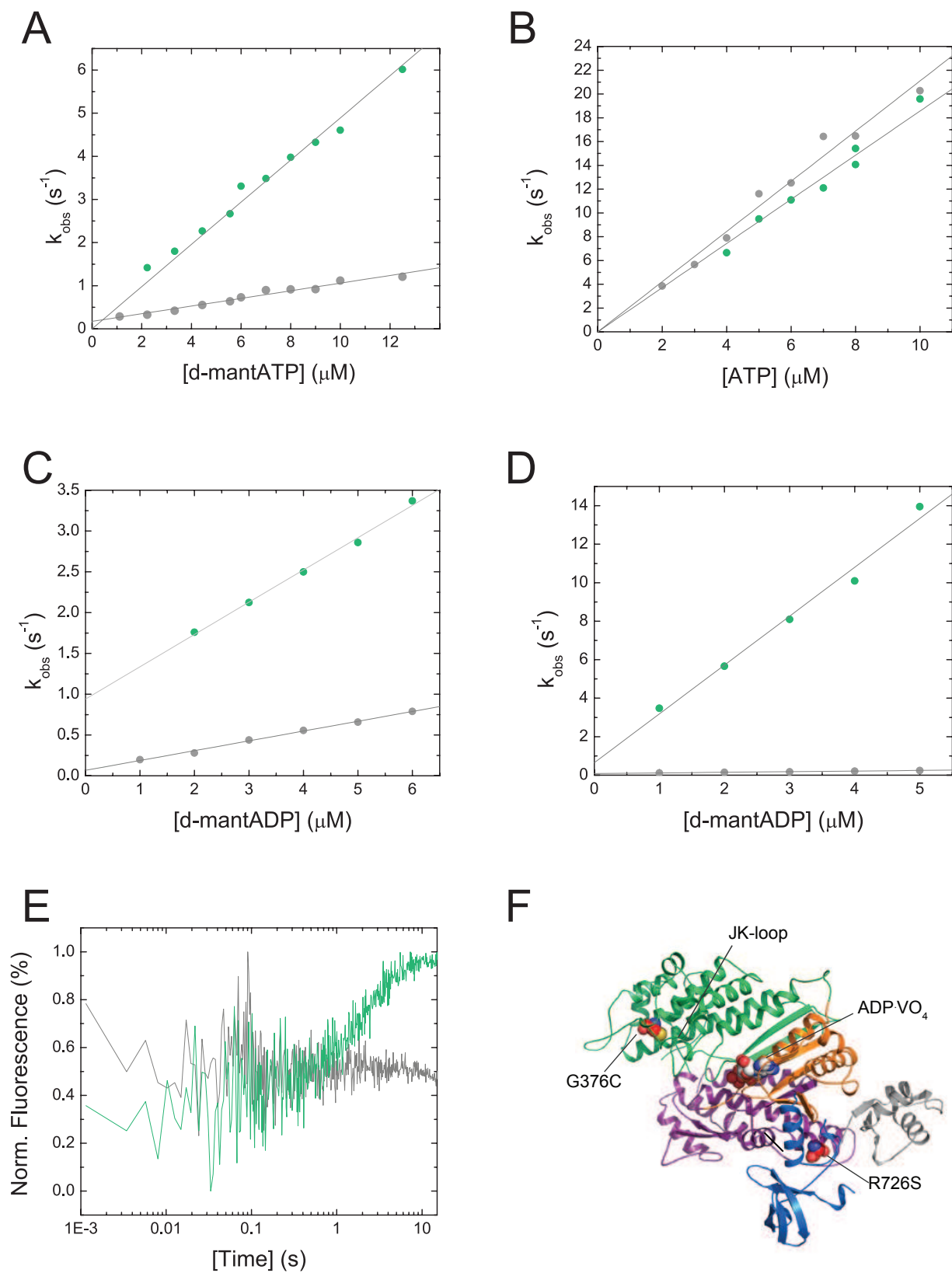


Figure 5 - Figure supplement 1

