Multiple S100 protein isoforms and C-terminal phosphorylation contribute to the paralog-selective regulation of nonmuscle myosin 2 filaments

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Nonmuscle myosin 2 (NM2) has three paralogs in mammals, NM2A, NM2B, and NM2C, which have both unique and overlapping functions in cell migration, formation of cell–cell adhesions, and cell polarity. Their assembly into homo- and heterotypic bipolar filaments in living cells is primarily regulated by phosphorylation of the N-terminally bound regulatory light chain. Here, we present evidence that the equilibrium between these filaments and single NM2A and NM2B molecules can be controlled via S100 calcium-binding protein interactions and phosphorylation at the C-terminal end of the heavy chains. Furthermore, we show that in addition to S100A4, other members of the S100 family can also mediate disassembly of homotypic NM2A filaments. Importantly, these proteins can selectively remove NM2A molecules from heterotypic filaments. We also found that tail phosphorylation (at Ser-1956 and Ser-1975) of NM2B by casein kinase 2, as well as phosphomimetic substitutions at sites targeted by protein kinase C (PKC) and transient receptor potential cation channel subfamily M member 7 (TRPM7), down-regulates filament assembly in an additive fashion. Tail phosphorylation of NM2A had a comparatively minor effect on filament stability. S100 binding and tail phosphorylation therefore preferentially disassemble NM2A and NM2B, respectively. These two distinct mechanisms are likely to contribute to the temporal and spatial sorting of the two NM2 paralogs within heterotypic filaments. The existence of multiple NM2A-depolymerizing S100 paralogs offers the potential for diverse regulatory inputs modulating NM2A filament disassembly in cells and provides functional redundancy under both physiological and pathological conditions.

In vertebrates, there are three nonmuscle myosin 2 (NM2) paralogs: NM2A, NM2B, and NM2C (1–3). Myosin 2 heavy chain (NMHC2) consists of an N-terminal motor domain, a neck region involved in force generation, which also binds regulatory (RLC)3 and essential light chains (ELC), and a long α-helical tail terminating in a short nonhelical tailpiece. The two α-helical tail sequences form a coiled-coil creating the functional hexameric structure containing two NMHCs and two pairs of light chains (Fig. 1) (4). NMHC2 paralogs display 64–80% sequence identity, but they differ in ATPase kinetics, motility rates (5–8), and intracellular localization (9–11), and they perform distinctive and overlapping cellular functions (12–14). NM2 paralogs can assemble to form functional filaments in cells. The primary regulatory step of this self-assembly is the phosphorylation of the regulatory light chain by myosin light chain kinase (MLCK) and Rho-associated protein kinase (15–18). In the absence of phosphorylation and in the presence of ATP at physiological ionic strength, NM2 molecules adopt a structure wherein the two heads make an asymmetric interaction, and the tail region makes hairpin turns in two places to fold into a compact conformation. This conformational state binds only weakly to actin and has no actin-activated ATPase activity. Upon phosphorylation of the RLC, myosin adopts an extended conformation (19).

To form filaments, the charged ends of the coiled-coil rod domains, termed the assembly competence domains (ACDs), associate with each other via ionic interactions in a parallel and antiparallel manner (8, 20–22). Phosphorylation by casein kinase 2 (CK2), protein kinase C (PKC), and transient receptor potential melastatin 7 (TRPM7) in or near the tailpiece (23–27) and binding of partner proteins like S100A4 (28–32), lethal giant larvae (Lgl1) protein (33, 34), and S100P (35) to the C terminus of NMHC were shown to further regulate filament assembly/disassembly. The sequences of the three NM2 paralogs differ significantly in this region, mostly in the nonhelical tailpiece (36). NM2A was found to have a single CK2 phosphorylation target site, whereas NM2B and NM2C have multiple

The abbreviations used are: RLC, regulatory light chain; ELC, essential light chain; NMHC, nonmuscle myosin heavy chain; MLCK, myosin light chain kinase; ACD, assembly competence domain; PKC, protein kinase C; SCS, secondary chemical shift; FFP, fluorescent fusion protein; FP, fusion protein; TCEP, tris(2-carboxyethyl)phosphine; TEV, tobacco etch virus; HSQC, heteronuclear single quantum coherence; TIRF, total internal reflection fluorescence.

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CK2, PKC, and TRPM7 phosho-sites in the tailpiece (36). Several investigations found that phosphorylation events in the nonhelical tailpiece show paralog-specific effects disrupting selectively NM2B and not NM2A filaments (25, 26, 37). S100A4, S100P, and Lgl1, however, selectively bind NM2A, inhibiting assembly and promoting disassembly, and have only a slight effect on NM2B (38, 39). These findings led to suggestions that binding of S100A4 and other interacting proteins and C-terminal tail phosphorylation could selectively regulate NM2A and NM2B filaments, respectively, imposing a secondary regulatory mechanism of their functions (29, 40). Different results were reported by Dulyaninova et al. (23, 41) showing that CK2 phosphorylation prevented NM2A assembly similarly to NM2B resulting in increased cell motility. As a further regulation, they also demonstrated that phosphorylation of NM2A by CK2 can inhibit the binding of S100A4, protecting filaments and promoting assembly in the presence of S100A4 (23). It is important to note that this additional regulation was not found later by the same group using a phosphomimetic mutant of CK2-phosphorylated NM2A (41) and that they showed that solely CK2 phosphorylation negatively affected NM2A filament formation, simultaneously protecting filaments from S100A4 mediated disassembly (23). These findings suggest a more complex regulatory mechanism, but the effect of cross-talk between phosphorylation and S100A4 binding is yet unclear.

It has been recently demonstrated that NM2A and NM2B can form heterotypic filaments (hereafter referred to as heterofilaments) in vitro (11, 42, 43). However, the effects of S100A4 and C-terminal phosphorylation on mixed filaments were not investigated. The question therefore remains whether the equilibrium between heterofilaments and homofilaments can be selectively shifted either by S100A4 binding or C-terminal phosphorylation, representing a novel secondary regulatory mechanism of nonmuscle myosins. These questions were addressed here using in vitro reconstituted filaments from full-length myosins and from novel artificial filament-forming myosin derivatives.

S100A4 and S100P are structurally similar members of the vertebrate-specific, small Ca<sup>2+</sup>-binding S100 protein family, forming homodimers and having two EF-hand sites in each chain. The conformational change caused by Ca<sup>2+</sup> binding results in the exposure of two identical hydrophobic binding surfaces (44). S100A10 is a known exception in the family because it is in the open conformation in the apo-form, making its activity Ca<sup>2+</sup>-independent. The wider S100 family of proteins has important pathological roles in cancer and metastasis formation, chronic inflammation, such as rheumatoid arthritis, and various forms of fibrosis (45–47). S100A4, also known as metastasin, is the best characterized member of the family and is an important biomarker for metastatic tumors with poor survival rates (48–51). It was demonstrated that metastasis-associated motility is connected with S100A4–NM2A interaction (28, 31, 32). Because of the structural similarities and the previously demonstrated binding of S100P to NM2A, we considered the possibility that other S100 proteins may also be able to regulate the assembly of NM2B filaments, potentially providing functional redundancy with S100A4 or allowing tissue-specific nonmuscle myosin regulation.

Results

Identification of the major CK2 phosphorylation sites in NM2A and NM2B peptides

Previous works have shown that phosphorylation of NM2A and NM2B at the C-terminal end of the heavy chain by CK2 affects their assembly capabilities; however, there is disagreement about the selectivity of this regulation. It was found that NM2A has only one distinct CK2 site at Ser<sup>1943</sup> (23, 37), and the location and number of NM2B sites inside the nonhelical tailpiece were not fully clarified. In addition to the NM2A equivalent site, residue Ser<sup>1956</sup> in this case, there are at least three more potential phosphorylatable Ser residues and one Thr residue in NM2B (Fig. 1). To precisely determine these phosphorylation sites and to study whether CK2 phosphorylation causes any conformational change that inhibits S100A4 binding, as suggested by Dulyaninova et al. (23), we expressed three recombinant peptides: M111A (NM2A(1850–1960)), M121B (NM2B(1856–1976)), and M121AB chimera consisting of the coiled-coil region from NM2A(1850–1924) and the nonhelical tailpiece of NM2B (1932–1976). The last construct was used for NMR studies because M121B was difficult to keep in solution under NMR conditions. These constructs were previously shown to form coiled-coil dimers but not assemble to higher order filaments (52).

We identified phosphopeptides using MS of trypsin-digested fragments after in vitro phosphorylation, and we observed the known CK2 site of NM2A and a maximum of three sites on NM2B. Because of technical difficulties, the precise identification of the three sites was not successful using this technique. To further investigate the phosphorylatable residues, chemical shifts of M111A, M111A-P, M121AB, and M121AB-P fragments were examined by NMR spectroscopy. The disordered C-terminal tailpiece and the unfolded N-terminal part of the truncated and incomplete coiled-coil could be assigned in the <sup>1</sup>H–<sup>15</sup>N HSQC spectra of the fragments (Fig. 2, A and B). Residues belonging to the predicted coiled-coil regions were unde-
Selective regulation of NM2 heterofilaments

Figure 2. NMR spectroscopic characterization of native and CK2-phosphorylated M111A, M111A-P, M121AB, and M121AB-P. Assigned 1H–15N HSQC spectra for M111A (red) and M121AB (green) and their CK2-phosphorylated forms (M111A-P (blue) and M121AB-P (purple)) are shown. For M111A, only the Ser1943 but for M121AB the Ser1956 and Ser1975 cross-peaks show significant changes upon phosphorylation. C, chemical shift variation along the M111A amino acid sequence reveals that the effect of phosphorylation at Ser1943 is the strongest on the neighboring residues, and it weakens constantly with the distance from the phosphorylation site, whereas for residues further than 5 amino acids both in the N- or C-terminal direction, no difference can be detected. D, chemical shift variation along M121AB reveals a different picture, as for this paralog phosphorylation effects are minor already on the neighboring residues. Changes in SCS (ppm) for M111A and F for M121AB upon phosphorylation indicate that in both paralogs the secondary structure is not perturbed, and the effect of phosphate moiety introduction is only local.

Figure 2, A, B, C, and D: Assigned 1H–15N HSQC spectra for M111A (red) and M121AB (green) and their CK2-phosphorylated forms (M111A-P (blue) and M121AB-P (purple)) are shown. For M111A, only the Ser1943 but for M121AB the Ser1956 and Ser1975 cross-peaks show significant changes upon phosphorylation. C, chemical shift variation along the M111A amino acid sequence reveals that the effect of phosphorylation at Ser1943 is the strongest on the neighboring residues, and it weakens constantly with the distance from the phosphorylation site, whereas for residues further than 5 amino acids both in the N- or C-terminal direction, no difference can be detected. D, chemical shift variation along M121AB reveals a different picture, as for this paralog phosphorylation effects are minor already on the neighboring residues. Changes in SCS (ppm) for M111A and F for M121AB upon phosphorylation indicate that in both paralogs the secondary structure is not perturbed, and the effect of phosphate moiety introduction is only local.

Filament formation properties of fluorescent protein-fused full-length NM2 tail constructs

The NM2 tail fragments used here for the phosphosite studies and similar but longer tail fragments used by other groups tend to form paracrystal-like structures or large and heterogeneous filaments of unknown polarity (21, 53). These structures do not resemble the small bipolar filaments formed by full-length NM2 paralogs (22, 54) and therefore are not adequate models of the native myosins. To study the effect of CK2 phosphorylation on NM2A assemblies, with constructs that more closely approach native filaments, we expressed GFP- and mCherry-fused NM2A (GFP–2A-tail, mCherry–2A-tail) and NM2B (GFP–2B-tail) coiled-coil tail fragments in *Escherichia coli* cells. In agreement with Melli *et al.* (8), in which similar proteins were expressed in *S. cerevisiae* cells, we found that capping the N terminus of these tail fragments with a fluorescent fusion protein (FPP, which is when the FP moiety is fused to a myosin tail) allowed the fragments to form small bipolar filaments that were similar in size to those formed by full-length NM2 molecules. Furthermore, the use of different fluorescent moieties in place of the myosin head made it possible to easily differentiate NM2A and NM2B in experiments looking at heterofilaments using fluorescence microscopy.

Negative staining electron microscopy (EM) of the FFP-capped tail fragments revealed the presence of bipolar filaments at near-physiological salt concentration (Fig. 3, A and C). Measurement of the total contour length and width of the bare zones at the center of 100 filaments from each paralog revealed no significant differences between paralogs (length and width of GFP–2A-tail: 287 ± 58 nm and 12.1 ± 2.9 nm, and GFP–2B-tail: 282 ± 48 nm and 12.1 ± 2.8 nm) (Fig. 3, E and F). Two general conformations were distinguished, one with the GFP moieties in close apposition to the filament shaft, and one where the moieties are spread outwards from the backbone. GFP–2A-tail filaments were less compact than the GFP–2B-tail at the filament ends under these conditions, suggesting somewhat looser packing of molecules. The average dimensions and distributions of the FFPs of these filaments are remarkably similar to those shown by Billington *et al.* (54) using full-length
myosins and of tail filaments capped with a HALO-tag (8), indicating that these artificial tail constructs are indeed appropriate tools to study this level of organization.

To characterize the assembly properties of these constructs, sedimentation assays were performed. We found that GFP–2A-tail filaments are depolymerized at lower NaCl concentrations, and their assembly is more cooperative than those of GFP–2B-tail (50% solubility = 204 ± 4 mM NaCl, Hill coefficient 5.4 ± 0.4, this refers to GFP–2A-tail, and 229 ± 3 mM NaCl, Hill coefficient 3.9 ± 0.2, this refers to GFP–2B-tail), being in good agreement with previous studies (Fig. 3, G and H) (23, 29). At 150 mM NaCl concentration around 80% of both paralogs formed filaments, but a slight increase in salt level had affected GFP–2A-tail filaments more than the GFP–2B-tail filaments.

**Effects of CK2 and phosphomimetic mutations on GFP-tail fragments**

To determine the outcome of CK2 phosphorylation on these filaments, GFP-tail constructs were phosphorylated similarly to the short peptides. In a filament disassembly assay, we found that CK2 phosphorylation did not prevent filament formation of either GFP–2A-tail or GFP–2B-tail filaments. However, it effectively destabilized GFP–2B-tail assemblies, increasing their solubility (50% solubility = 175 ± 3 mM NaCl, and at 150 mM NaCl less than 65% of the molecules remained in filaments) (Fig. 3H). Phosphorylation of the GFP–2B-tail also affected filament width and length (Fig. 3, D–F). The phosphorylated filaments were significantly shorter (215 ± 58 nm versus 282 ± 48 nm, p < 0.01 using the Mann-Whitney test), but we found less effect on the filament thickness (10.1 ± 2.5 nm versus 12.1 ± 2.8 nm, p = 0.12). In contrast, GFP–2A-tail showed no appreciable change (50% solubility = 200 ± 4 mM NaCl) (Fig. 3G), and only slight shortening was detected compared with the unphosphorylated form (278 ± 58 nm in length and 12.2 ± 2.5 nm in width) (Fig. 3, B, E, and F). Examination of the phosphorylated GFP–2B-tail filaments showed a tendency of filament ends to splay, and in many cases general disruption of the whole filament was visible. In contrast, GFP–2A-tail filaments observed by EM appeared to remain intact and unaffected (Fig. 3, B and D). Although the precise arrangement and interaction of coiled-coils within a bipolar filament is currently unknown, the filament is likely to be constructed from tails with a range of different sized overlaps between adjacent molecules. The effect of phosphorylation is to cause electrostatic repulsions (or weaken the overall electrostatic interactions) between the tails. Molecules with short overlaps are likely to be preferentially removed.

Figure 3. Selective regulation of GFP–2B-tail by C-terminal phosphorylation. GFP–2A-tail and GFP–2B-tail and their CK2-phosphorylated forms were diluted in buffer containing 150 mM NaCl to study the effects of CK2 on filaments. Filaments were visualized using negative staining EM. Filaments were composed of GFP–2A-tail (A), GFP–2A–P-tail (B), GFP–2B-tail (C), and GFP–2B–P-tail (D). The -P indicates that samples were phosphorylated with CK2. Small white dots around filaments are the N-terminal GFPs (black arrows). Note that there is some heterogeneity in the lengths, widths, and conformation (tightly packed, black asterisk; GFPs spread apart, white asterisk) of these filaments. White bar represents 100 nm. E, quantification of the length distribution of the filaments. F, quantification of the bare zone widths of filaments. In each case, the lengths and widths of 100 filaments were measured, and the means ± S.D. values were calculated. The length of GFP–2B–P-tail filaments significantly differs from the GFP–2B-tail filaments but not the width (p < 0.01 in Mann-Whitney U test for length and p = 0.12 for width), but there was no real phosphorylation-induced change for GFP–2A-tail filaments. G, salt dependence studies were carried out by titrating GFP–2A-tails (□) and GFP–2A–P tails (■) with NaCl. For comparison, in the small inset GFP–NM2A-tail (●) and GFP–NM2B-tail (○) filaments were titrated with salt. All samples were centrifuged, and the fluorescence of the supernatants was measured. The values represent the means ± S.D. of three independent measurements, and sigmoidal curves (dashed lines, unphosphorylated; straight lines, CK2-phosphorylated) were fitted in all cases to determine the 50% solubility values of filament. H, similar experiment conducted with GFP–2B-tail (□), GFP–2B–P-tail (■), and the phosphomimetic GFP–2B–SS/EE–P-tail (○) and GFP–2B–SS/EE–P-tail (●). For GFP–2B–SS/EE–tail, Ser1945 and Ser1937 were replaced by Glu residues. For GFP–2B–SS/EE–P-tail, this construct was additionally incubated with CK2.
by this mechanism as there are insufficient electrostatic interactions between the tails to compensate. A consequence of this is that phosphorylation leads to a slight decrease in the filament length.

NM2B was predicted to also be a target for PKC and TRPM7-driven phosphorylation. Note here that NM2A was also reported to be the target for PKC (Ser<sup>1917</sup>) (55) and TRPM7 (Thr<sup>1800</sup>, Ser<sup>1803</sup>, and Ser<sup>1808</sup>) phosphorylation, but the phosphosites are located in the coiled-coil region of NM2A rather than in the disordered tailpiece (36). Previous in vivo and in vitro (with a 600-residue-long tail fragment) studies found that introducing the phosphomimetic mutations in NM2B at Ser<sup>1935</sup> and Ser<sup>1937</sup> promotes filament disassembly (26, 56). To study the effect of phosphorylation of these residues, we mutated the above-mentioned two Ser residues to Glu residues to mimic phosphorylation. These changes increased the solubility of GFP–2B-tail (50% solubility = 202 ± 3 mM NaCl), which was further raised by CK2 phosphorylation (50% solubility = 144 ± 2 mM NaCl). After this “hyperphosphorylation,” less than half of the molecules remained in filaments at 150 mM NaCl (Fig. 3H). These findings show that phosphorylation by CK2 or mutations mimicking PKC or TRPM7 phosphorylation on the C-terminal tail of GFP–2B-tail do not inhibit filament formation completely, but each modification gradually destabilizes the filaments and shifts the equilibrium toward monomeric myosins.

**NM2A filaments are regulated by S100 proteins**

As shown previously with a number of different NM2A tail constructs, addition of S100A4 and S100P effectively depolymerized NM2A filaments in a concentration- and calcium-dependent manner (29, 35, 38). To determine whether other S100 family members were able to affect NM2 filament stability, filament disassembly assays were carried out using GFP–NM2-tail constructs. Because S100A4 forms an asymmetric complex, one dimer per one NMHC2 (30), we assumed the same stoichiometry with other S100s. Therefore, the depolymerization constant (K<sub>dep</sub>) values (which we define as the concentration of S100 needed to depolymerize 50% of filaments) are derived assuming that S100 protein binds as a dimer. Titration of 500 nM GFP–2A-tail with S100 proteins showed that S100A2 is nearly as effective as dissociating filaments (K<sub>dep</sub> ~ 300 nM) as S100A4 (K<sub>dep</sub> ~ 160 nM). The K<sub>dep</sub> of S100A1 binding was somewhat lower but was still in the nanomolar range (K<sub>dep</sub> ~ 460 nM), whereas S100A6 and S100P were in the micromolar range (K<sub>dep</sub> ~ 2 and 5 μM, respectively) (Fig. 4A). Other S100 proteins disassembled filaments with a K<sub>dep</sub> over 10 μM except S100A5 (K<sub>dep</sub> ~ 6 μM) and S100B (K<sub>dep</sub> ~ 7 μM) (Table 1). Selectivity for NM2A over NM2B was observed with only S100s. Of the proteins tested, S100A2 and S100A4 were the most effective at depolymerizing GFP–2B-tail filaments, although the effect was still very weak (K<sub>dep</sub> ~ 35 and 36 μM, respectively) (Fig. 4B). On average, there were more than 2 orders of magnitude difference between the K<sub>dep</sub> values of GFP–2A-tail and GFP–2B-tail filament disassembly by S100 proteins making them essentially NM2A-specific.

![Figure 4. Filament disassembly caused by S100 proteins at 150 mM NaCl.](Image)

Here, we investigated the effect of S100A4, being the most effective S100 protein, by adding it directly to preformed full-length NM2A filaments. In the presence of Ca<sup>2+</sup>, S100A4 depolymerizes filaments, which were formed either by MLCK-induced RLC phosphorylation of NM2A in the presence of ATP or by unphosphorylated myosin in the absence of ATP. Disassembly appeared to be more effective in the case of filaments formed upon RLC phosphorylation, indicating possible differences in the overall stability of the two types of filaments, similarly to the results of Liu et al. (17). Fitting of co-sedimentation data using a quadratic binding equation yielded a K<sub>dep</sub> of 26 nM for filaments formed by RLC phosphorylation and a K<sub>dep</sub> of 139 nM for unphosphorylated RLC filaments. In the absence of Ca<sup>2+</sup>, S100A4 was unable to induce filament disassembly (K<sub>dep</sub> > 200 μM) (Table 1).

In addition to the effect on filament assembly, we also found that S100A4 is capable of disrupting an antiparallel dimeric interaction that exists between the folded full-length molecules
repeated the filament disassembly assay with myosins that were phosphorylated in their tail regions. CK2 phosphorylation of the GFP–2A-tail caused no detectable inhibition of the filament-disrupting effect of S100A4 or S100A2. In fact, both S100 proteins disassembled filaments with slightly higher affinities ($K_{\text{dep}}$ was around 138 and 103 nM for S100A2 and S100A4, respectively; see Table 1). Similarly to CK2 phosphorylation, elevated salt concentrations increased the effect of S100A4 on GFP–2B-tail filaments (at 250 mM NaCl, the $K_{\text{deg}}$ was 11.34 ± 1.42 μM) (Fig. 6).

**S100A2 and S100A4 can disassemble NM2A heterofilaments**

It has recently been found that NM2A and NM2B can form heterofilaments in vivo and in vitro (8, 11, 42, 43). To test whether the two high-affinity S100 paralogs can selectively remove NM2A from preformed heterofilaments, two assays were used. In one, we made co-complexes of full-length NM2A and a fluorescent GFP–2B-tail construct and studied them by EM. In clear images, the smaller GFP moieties and the larger lobe-like myosin heads could be distinguished within filaments (Fig. 7A). Addition of S100A4 without Ca$^{2+}$ caused no change to these heterofilaments, but in the presence of Ca$^{2+}$, filaments appeared more similar to GFP–2B-tail homofilaments with only few full-length molecules being visible within the filament (Fig. 7B).

In a separate assay, total internal reflection fluorescence (TIRF) microscopy was used to visualize the composition of the heteropolymeric filaments formed by mCherry–2A-tail and GFP–2B-tail constructs. Initially, the average fluorescence intensity of a single GFP or mCherry was determined by examining the stepwise photobleaching of FFP-tail fragments at high salt (500 mM NaCl), where the myosin is unpolymerized. For this, the fluorescence intensity change of spots was followed over time to detect bleaching (Fig. 8). The initial intensities of spots, having well-separated bleaching steps, were determined using a custom spot-finding macro in Fiji. The number of bleaching steps indicates the number of FFPs (matured and not bleached before measurement), whereas the initial intensity indicates the total intensity of those FFPs together in the spot. Dividing the initial intensity values with the associated number of bleaching steps, the fluorescence intensity of a single FFP in each spot can be calculated. Averaging intensity values of these single FPUs from 10 different spots gave us the average fluorescence intensity of one fluorescent moiety. Knowing this value for both fused mCherry and GFP allowed us to determine the approximate number of mCherry–2A-tail and GFP–2B-tail in individual filaments by dividing the fluorescence intensity of an observed spot (representing a filament), measured at each wavelength using the same spot-finding macro as was used to measure the initial intensities of spots in photobleaching experiments, by the average intensity of each fused fluorophore. Note that only around 75% of the FPs in cells fold to become fluorescent proteins (57). Thus, dividing the total fluorescent intensity of a tagged filament by the unitary fluorescent increment determined by stepwise photobleach will result in an underestimation of the actual number of myosins in a filament.

**Effect of CK2 phosphorylation on S100A4 and S100A2 binding**

It has previously been suggested that CK2 phosphorylation can disrupt S100A4–NM2A interaction preventing filament disassembly (23, 41). To further investigate these findings, we presented the results demonstrate that S100A4 can also control the multimerization properties of the enzymatically inactive form of NM2A, in addition to controlling the assembly state of enzymatically active molecules.

**Selecting regulation of NM2 heterofilaments**

Table 1

| Filament disassembly caused by S100 proteins |
|---------------------------------------------|
| Depolymerization constants were determined by filament disassembly assays using 500 nM NM2 variants. Because of the known asymmetric interaction of S100A4 with NM2A peptide, the $K_{\text{dep}}$ values refer to the interactions of NM2 with S100 dimers. Values represent the mean ± S.D. of the fitted parameters. The five most effective S100s are shown in boldface type. Proteins marked with an asterisk had the opposite effect on filaments and caused association rather than dissociation. |

| S100A1 | 0.46 ± 0.11 | 43 ± 10 |
| S100A2 | 0.30 ± 0.12 | 35 ± 4 |
| S100A3 | > 30 | > 200 |
| S100A4 | 0.15 ± 0.02 | 36 ± 2 |
| S100A5 | 5.8 ± 1.4 | > 200 |
| S100A6 | 2.16 ± 0.15 | 163 ± 58 |
| S100A7 | > 30 | no effect |
| S100A8 | 11.4 ± 2.6 | > 200 |
| S100A9 | 19.5 ± 7.7 | no effect |
| S100A10 | 17.1 ± 1.3 | > 200 |
| S100A11 | > 30 | > 200 |
| S100A12 | 11.2 ± 5.3 | > 200 |
| S100A13 | > 30 | > 200 |
| S100A14 | > 30 | 178 ± 108 |
| S100A15 | > 30 | < 200 |
| S100A16 | 14.9 ± 2.8 | 23 ± 2 |
| S100B | 7.4 ± 1.1 | > 200 |
| S100G | no effect | > 200 |
| S100P | 5.2 ± 1.6 | 190 ± 32 |
| S100Z | 29.9 ± 8.9 | 112 ± 10 |

| GFP-2A-tail disassembly | GFP-2B-tail disassembly |
|--------------------------|--------------------------|
| $K_{\text{dep}}$ (μM)    | $K_{\text{dep}}$ (μM)    |
| S100A2                   | 0.13 ± 0.02              | 12.0 ± 2.3               |
| S100A4                   | 0.10 ± 0.01              | 15.4 ± 1.1               |

With unphosphorylated RLC in the presence of ATP (Fig. 5). The results demonstrate that S100A4 can also control the multimerization properties of the enzymatically inactive form of NM2A, in addition to controlling the assembly state of enzymatically active molecules.
Selective regulation of NM2 heterofilaments

Table 2

| [NaCl] | - S100A4 | + S100A4 |
|--------|----------|----------|
| 150 mM |          |          |
| 25 mM  |          |          |

Figure 5. Effects of S100A4 on full-length NM2A filaments. A and B, many unphosphorylated NM2A molecules form compact antiparallel dimers of two folded NM2A molecules in the presence of ATP when no S100A4 is present. C and D, in the presence of calcium S100A4 binds to the end of the tail of an NM2A molecule and disrupts the antiparallel folded dimer. In both cases, the averages of molecules are shown in smaller images beside the respective EM images. S100A4 is visible at the C-terminal end of the single coiled-coil molecules (white arrows). A and C show the ability of S100A4 to disrupt antiparallel interactions under conditions approximating physiological ionic strength. B and D, salt concentration is reduced to promote antiparallel interactions and highlight the effect. E, filament disassembly assay of RLC phosphorylated (with 20 μM ATP present) (●) and unphosphorylated (without ATP) full-length NM2A with (○) and without (□) CaCl₂ in samples titrated with S100A4. Each data point represents the means ± S.D. of three independent experiments. Solid lines indicate the data fitting to the quadratic binding equation.

suggesting that mostly single myosin coiled-coil tail constructs were present (Fig. 9A). A few spots containing more than two FFPs were also detected even at high salt, similarly to Billington et al. (58) using full-length myosin. We suggest that these brighter spots are a result of small scale aggregation, aspecific grouping, or random positioning of two or more myosin molecules within a diffraction-limited area. Mixed filaments can be formed in two ways in vitro from these typically single coiled-coil constructs. We term the filaments prepared by the first method as “copolymerized” filaments. Here, the two paralogs are first mixed in equal concentrations in high-salt buffers before the initiation of polymerization by reduction of salt concentration. The filaments that are formed by this method contain both myosin paralogs in minutes needed for visualizing them by TIRF microscopy (Fig. 9C). The second method involved the mixing of preformed homofilaments of the two myosins and allowing them to equilibrate through exchange of myosin paralogs. We termed these filaments “exchanged” filaments and found that the exchange rate is slow (Fig. 9, B and D).

The quantification of moieties in the equilibrated system (200 nM mCherry–2A-tail and 200 nM GFP–2B-tail was used in the first method) showed more GFP–2B-tail in heterofilaments. Our results from filament disassembly studies suggest that this deviation might come from the characteristics of GFP–2B-tail forming tighter and less dynamic filaments at physiological salt. Filaments that were larger than 10 tail constructs (arbitrarily chosen to study spots that are or nearly filament-sized and to make data sets more comparable) contained an average of 19.1 GFP–2B-tail constructs and only 11.5 mCherry–2A-tail constructs (Table 2). Note that the numbers here represent the number of FFPs. If all FFPs were fluorescent, dividing this number by two would give the exact number of myosin molecules in the filament. Interestingly, this fraction of mCherry–2A-tail in heterofilaments (~0.4) is similar to the fraction of full-length NM2A in heterofilaments found by Melli et al. (8). The population of filaments were very diverse in fluorescent intensity suggesting that the sizes of the filaments were distributed similarly to that observed by EM.

As visualized by TIRF microscopy within a few minutes, the addition of 2.5 μM S100A2 or S100A4, in the presence of 100 μM CaCl₂, to existing copolymerized heterofilaments drastically reduced the amount of mCherry–2A-tails in the filaments but not the amount of GFP–2B-tail molecules. There were 5.6 mCherry–2A-tail and 17.9 GFP–2B-tail constructs when S100A4 was added, and 7.8 and 18.5 when S100A2 was added, respectively. Incubating these S100-copolymerized filament mixtures for 30 min at room temperature before reimaging the samples resulted in an increase in the relative amount of GFP–2B-tail in the filaments (mCherry–2A-tail: 5.5; GFP–2B-tail: 23.9 for S100A4 and 6.9 and 21.9 for S100A2, respectively) indicating the reorganization of filaments (Fig. 10 and Table 2). Note here that the S100-binding site on the remaining mCherry–2A-tails in the filament are likely to be masked by GFP–2B-tails. Because the rearrangements of these deeply located molecules are very slow, we suggest that more time would have been needed for more complete disassembly of mCherry–2A-tails.

Using the CK2-phosphorylated GFP–2B-tail to form copolymerized heterofilaments with the unphosphorylated mCherry–2A-tail, we found that the ratio of 2A:2B tails shifted mostly due to a decrease of GFP–2B-tail molecules in filaments (number of phosphorylated GFP–2B-tail per filament was around 15.2,
whereas the number of mCherry–2A-tail per filament was 12.2). These data show that CK2 phosphorylation not only destabilizes GFP–2B-tail homofilaments but also affects the composition of mixed filaments.

Mixing the separately formed homofilaments showed no sign of paralog exchange when visualized by TIRF microscopy a few minutes after mixing. After a 2-h incubation at room temperature, the aged molecules rearranged into heterofilaments; however, even this amount of time was not enough to reach the same 2A:2B tail ratio found using copolymerized filaments (average number of GFP–2A tail: 17.4 and mCherry–2A-tail: 12.7) (Table 2). The addition of 2.5 and 1 µM dimer S100A2 and S100A4, respectively, to these 2-h aged exchanged filaments followed by the incubation of the mixture for 30 min resulted in drastically reduced numbers of mCherry–2A-tails in filaments (only 4.9 mCherry–2A-tail remained in case of S100A4 and for S100A2 there were 5.1). The remaining filaments were enriched with GFP–2B-tails (GFP–2B-tail in case of S100A2 and S100A4 were 23.2 and 25.4, respectively) (Table 2).

It is worth noting that the selective depolymerization of filaments by S100A2 and S100A4 requires the presence of Ca²⁺. Addition of EGTA to the system deactivated the effect of S100s and allowed the reassembly of mCherry–2A-tails in minutes. The smaller existing GFP–2B-tail–enriched homofilaments were complemented, and new filaments containing mostly mCherry–2A-tail also appeared (Fig. 10, C and F). These results show that S100A2 and S100A4 can be regulators of NM2 heterofilaments either by inhibiting the co-assembly of NM2A into these filaments or promoting paralog sorting by selectively dissociating NM2A from heteromeric filaments.

Discussion

In this paper we studied the regulation of NM2A and NM2B filament assembly by tail phosphorylation and binding of S100 protein family members. To improve on previous studies using isolated myosin tail fragments, we constructed chimeric myosin fragments replacing the head domain with a fluorescent protein (GFP or mCherry), and in some experiments we also used full-length NM2 molecules. Importantly, these tail fragments assemble into bipolar filaments closely resembling those formed by the native NM2. Furthermore, they co-assemble with full-length NM2 molecules into bipolar filaments. Uncapped myosin tail fragments typically form large (>1 µm) aggregates or paracrystals of unknown polarity (21, 53). The bipolar nature and relatively homogeneous size of the capped (FFP-tail) filaments coupled with the ability to produce the protein in large quantities in bacterial expression systems makes them ideal for in vitro studies of myosin assembly and disassembly.

Using NMR spectroscopy we found that Ser¹⁹⁴³ of NM2A and Ser¹⁹⁵⁶ and Ser¹⁹⁷⁵ of NM2B are primary targets of CK2 in vitro. Other predicted NM2B sites (Ser¹⁹⁵², Thr¹⁹⁶⁰, and Ser¹⁹⁶⁵) are phosphorylated only with very low probability (or cannot be phosphorylated at all) despite the fact that they have consensus sequence for CK2. A possible explanation is that one negative amino acid following the phosphorylatable Ser or Thr residue here is insufficient for high-affinity recognition by CK2 (59).

As for the functional consequence of phosphorylation, we found that CK2 phosphorylation and phosphomimetic mutation of Ser residues that are targets of PKC and TRPM7 (Ser¹⁹³⁵ and Ser¹⁹³⁷) alone cannot disassemble GFP–2B-tail filaments completely, but these events have synergistically negative effects on filament formation. We suggest that as more sites are phosphorylated at the tail, the C terminus becomes more neg-
Selective regulation of NM2 heterofilaments

Figure 7. Copolymerized heterofilaments formed by GFP–2B-tail with full-length NM2A and the effect of S100A4. Negatively stained EM images of copolymerized heterofilaments at 150 mM NaCl show that GFP–2B-tail can co-assemble with full-length myosin 2A (A). The GFPs (representing NM2B) are seen as small dots (an example marked with black asterisk), whereas NM2A head domains form lobe-like structures (an example marked with white asterisk). Zoomed images of each of these structures (black squares) are shown in the small images. B, S100A4 (5 μM) in the presence of Ca²⁺ seemingly decreases NM2A heads, and mainly GFPs can be found attached to the filaments, but the effect is not absolute. White bar represents 100 nm in length.

Figure 8. Variations of photobleaching steps. Photobleaching of GFP–2B-tail (A) and mCherry–2A-tail (B) was followed using TIRF microscope. Images were acquired continuously over time, and the intensity of weak spots at 500 mM NaCl was followed in all frames. Counting the number of photobleaching steps (black lines), the number of FFPs (not bleached before measurement) could be determined indicating the number of fluorophores per spot. Note here that at 500 mM NaCl concentration mostly single coiled-coil myosins are present meaning mostly one or two photobleaching steps were observed. Spots were extracted from every 50 frames and aligned to visualize the intensity change (below the graphs).

Atively charged, shifting filaments toward disassembly by intermolecular repulsions. This mechanism might be similar to previous findings showing that filament assembly can be inhibited by eliminating parts of the relatively positive C terminus of NM2B. As longer sections were eliminated, less stable filaments were observed reaching a threshold where no filaments could form (53, 60). Following phosphorylation of one or more PKC or TRPM7 sites in the serine-rich region (1935–1939), other serine residues located there may become potential targets of hierarchical CK2 phosphorylation (61), further promoting disassembly of filaments. The GFP–2A-tail has only one CK2 site on the C-terminal tail and is weakly affected by this modification in vitro. This is in agreement with the results of Murakami et al. (25, 29) but is in disagreement with the findings of Dulyanina et al. (23, 41), who showed by using shorter uncapped myosin rod constructs that C-terminal phosphorylation and phosphomimetic mutation promote NM2A filament disassembly. Other groups also found in vivo that C-terminal phosphorylation has a role in the filament dynamics of NM2A in vivo (41, 62). This contradiction can be explained in two ways. First, in vivo other C-terminal sites of NM2A, as mentioned under “Results,” might also be phosphorylated, and these phosphosites destabilize bipolar filaments synergistically, whereas CK2 phosphorylation alone causes only minor effects on these longer constructs. Second, NM2A filaments might be regulated with a different and more complex mechanism (63, 64), requiring full-length proteins capable of forming a 10S-like folded state and not simply by destabilization of filaments via intermoo.
Figure 9. Heterofilament formation and selective disassembly of mCherry–2A-tail by S100A4. Filaments were observed by TIRF microscope detecting the fluorescence of mCherry–2A-tail (red) and GFP–2B-tail (green), and the approximate number of FFPs they contain was calculated. Scatter plots below the TIRF images represent the 30 most intense spots of 10 images representing the population of filaments in each scenario. A, GFP/mCherry–NM2-tail paralogs (200–200 nM) at 500 mM NaCl are not in filaments, and only weak intensity spots were detected (mostly with 2 or less FFPs). Note here that spots containing more than two FFPs are overrepresented because the most intense spots were picked from each image. Filaments were formed by decreasing NaCl concentration in two different methods. B, copolymerized filaments of mCherry–2A-tail and GFP–2B-tail were mixed at equimolar concentrations in high salt as monomers and then diluted to 150 mM NaCl to form filaments. The images were taken immediately after mixing. Note that under these conditions the observed filaments are heteropolymers. C, exchanged filaments, homopolymeric mCherry–2A-tail and GFP–2B-tail filaments were preformed by diluting them separately to 20 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, and 500 µM CaCl₂-containing buffer before mixing and were observed as soon as possible. Under these conditions, the observed filaments are mostly homopolymers. D, homopolymeric filaments formed in C were aged for 2 h and reimaged. Note that now most of the filaments are heteropolymeric but that the full exchange of paralogs has not occurred yet. E, F100A4 selectively decreases the number of mCherry–2A-tail molecules in copolymerized heterofilaments formed by co-dilution from mixed paralogs from high salt (B) or in aged exchanged filaments formed by slow exchange of subunits over time (E). Gray lines represent the linear fitting to the data. Dashed lines in E and F represent the lines from B and E to emphasize the change in filament composition that has occurred.
Selective regulation of NM2 heterofilaments

Table 2
Composition of heterofilaments and the effect of S100A2 and S100A4
The 30 most intense spots in 10 images were listed in all cases, and intensities were divided using the average intensity of a single mCherry or GFP to get the number of molecules the spots contain. From those 300 values, only filaments larger than 10 FFP "heads" together were averaged to get the values presented in this table. The numbers here are the number of FFPs indicating the number of single tail constructs and not the coiled-coil molecule. Errors represent S.E. values.

| Composition of heterofilaments and the effect of S100A2 and S100A4 | Average no. of mCherry–2A-tail | GFP–2B-tail |
|---------------------------------------------------------------|-------------------|-----------|
| Copolymerized filaments (150 mM NaCl)                        | 11.5 ± 0.4        | 19.1 ± 0.6|
| Addition of 2.5 μM dimer S100A4 to copolymerized filaments + 100 μM CaCl₂ | 5.6 ± 0.2        | 17.9 ± 0.8|
| 30-Min incubation of the mixture before reimaging             | 5.5 ± 0.2        | 23.9 ± 0.9|
| Addition of 500 μM EGTA to the incubated mixture             | 11.4 ± 0.4        | 21.2 ± 0.7|
| Addition of 2.5 μM dimer S100A2 to copolymerized filaments + 100 μM CaCl₂ | 7.8 ± 0.3        | 18.5 ± 0.7|
| 30-Min incubation of the mixture before reimaging             | 6.9 ± 0.2        | 21.9 ± 0.9|
| Addition of 500 μM EGTA to incubated mixture                 | 11.4 ± 0.7        | 19.6 ± 0.9|
| Aged "exchanged" filaments (150 mM NaCl + 2-h incubation)    | 12.7 ± 0.6        | 17.5 ± 0.8|
| Addition of 1 μM dimer S100A2 to aged "exchanged" filaments + 100 μM CaCl₂ + 30 mins | 4.9 ± 0.2        | 23.2 ± 0.9|
| Addition of 1 μM dimer S100A2 to aged "exchanged" filaments + 100 μM CaCl₂ + 30 mins | 5.1 ± 0.3        | 25.4 ± 1.4|
| Copolymerized filaments (NM2A and NM2B-P) (150 mM NaCl)      | 12.2 ± 0.4        | 15.2 ± 0.7|

Figure 10. Disassembly of copolymerized heterofilaments by S100A2 and S100A4. TIRF microscope images were evaluated by presenting the 30 most intense spots of 10 images representing the population of filaments in each condition. 200–200 nm mCherry–2A-tail and GFP–2B-tail were mixed at high salt and were diluted to 20 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl₂, and 100 μM CaCl₂-containing buffer to form copolymerized filaments. A, 1 μM S100A4 dimer was added to copolymerized filaments, and the sample was imaged immediately or after 30 min of incubation (same as in Fig. 9C) (B). C, EGTA (500 μM) was added to this incubated sample and was reimaged without any incubation time. D, 2.5 μM S100A2 dimer was added to copolymerized filaments, and the sample was imaged immediately or after 30 min of incubation (E). F, EGTA (500 μM) was added to this incubated sample and was reimaged without any incubation time. Gray lines represent the linear fitting to spots. Dashed lines represent the lines from A and E.

lecular repulsions as suggested here in the case of NM2B. This is supported by our finding that the phosphorylation of the M111A peptide caused local changes around Ser1943 in contrast to the regions around NM2B phosphorylation sites. These changes might be also important in other regulatory mechanisms of NM2A, recently been proposed (63, 65). Third, in vivo the C-terminal phosphorylation of NM2A may act to recruit another protein whose binding to the tail of NM2A destabilizes the filament. In conclusion, we found that the stability of GFP–2B-tail bipolar filaments are more sensitive to C-terminal tail phosphorylation in vitro than is GFP–2A-tail bipolar filaments. This could be the basis for a fine-tuning selective regulation mechanism of co-filaments of NM2A and NM2B. Future in vivo phosphorylation studies are required to prove this suggestion.

We found that S100 proteins, unlike C-terminal phosphorylation, selectively disassemble NM2A filaments and only weakly affect NM2B. Here, we expressed all members of the S100 family and studied their dissociation capabilities in vitro. Our data show that S100A4 is the most effective in disassembling NM2A filaments, whereas S100A2 and S100A1 also disrupt NM2A filaments with moderate affinities (K_{dep} < 0.5 μM), whereas they affect NM2B filaments only at physiologically less relevant concentrations (K_{dep} > 30 μM). We found no inhibition of S100-mediated NM2A filament disassembly via CK2 phosphorylation in contrast to the previous results of Dulyaninova et al.
Selective regulation of NM2 heterofilaments

(23). This can be explained by the fact that the CK2 phosphorylation site is far from the binding surface of S100s, and no conformational change takes place after phosphorylation to block the interaction. Note here that the effective S100A4 concentration for filament disassembly is around 3 orders of magnitude higher than the dissociation constant of a short NM2A peptide—S100A4 complex (30). These differences can be explained by assuming that the free energy of the very tight interaction of the NM2A-binding region with S100A4 is utilized partially to weaken the coiled-coil interaction between the heavy chains within the ACD and overtake the cohesive forces between myosin molecules thus promoting filament disassembly (30, 66). Elevating the ionic strength and the presence of the C-terminal phosphorylation weakens the intermolecular interactions and thus increases the effectiveness of S100 protein binding.

Recent studies have shown that NM2A and NM2B form heterofilaments in cells (11, 43). The physiological consequences of this is not known, but the two myosins do have unique kinetic and motile properties (8). This would allow cells to fine-tune the mechanical properties of a given NM2 filament within. How this assembly is regulated is not known, but our studies show that S100A4 can selectively remove NM2A from heterofilaments, giving one possible mechanism. This selectivity is due to the more than 2 orders of magnitude difference between the affinities of NM2A and NM2B for S100A4, which comes from the sequence differences in their C terminus as discussed previously (38). Another mechanism for regulated heterofilament composition would be the hyperphosphorylation of the NM2B tail, which decreases the tendency of this myosin to polymerize into filaments as suggested by our results. The selective removal of NM2 paralogs from heterotypic filaments could serve two purposes. On the one hand, it increases the monomeric pool of NM2 molecules in the cytoplasm, ready to form force-generating filaments elsewhere and/or the monomeric molecules serve some specific functions as suggested previously (67). On the other hand, the resulting, mostly homotypic filaments can function in a more paralog-specific manner. Our results are in good agreement with previous suggestions (40) that cells can and need to regulate myosin functions in paralog-specific ways.

It is inevitable that if activated myosins, in which the RLC is phosphorylated, are prevented from forming filaments, they are able to persist as enzymatically activated monomers. This offers a possible explanation for the existence of such activated monomers in cells, as has previously been demonstrated (43). In addition to this, we have demonstrated here that the interaction that holds together inactive, nonfilamentous myosin molecules in an antiparallel dimer arrangement is disrupted by S100A4. A possible role for this antiparallel dimer has been suggested as a dormant seed for rapid filament assembly (54) because light-chain phosphorylation will result in opening of the myosin into a species that is already arranged in the correct manner for filament growth to proceed. The role of S100A4 in regulating filament assembly dynamics may therefore consist of both an ability to suppress nucleation as well an ability to prevent subsequent growth through the effective sequestration of monomers.

Importantly, S100 interactions are Ca^{2+}-dependent, and S100s typically have micromolar Ca^{2+}-binding affinities. In the extracellular space, Ca^{2+} is not limiting, but intracellular resting Ca^{2+} concentration is around 0.1 μM, and upon activation it is unlikely to globally reach a saturating concentration for S100 proteins. This contradiction was previously studied, and S100A4 was found to bind and partially disassemble NM2A even at resting Ca^{2+} level if the concentration of either S100A4 or NM2A exceeded a few micromolars (52, 68, 69). It should also be noted that Ca^{2+} affinities of S100 proteins are inherently increased when their binding partners are present, due to thermodynamic reasons (52, 70). Ca^{2+} activation and influx do not necessarily cause more filaments to disassemble; however, pulses of increasing Ca^{2+} concentration over a few seconds allow the dissociation of NM2A in a larger scale, e.g. from heterotypic filaments, followed by quick reorganization and restoration of resting Ca^{2+} levels. In this way, pulsing of calcium may aid in the rearrangement of paralogs within heterotypic and also homotypic filaments. Additionally it was shown that TRPM7 caused Ca^{2+} influxes at the leading edge of fibroblast cells controlling the direction of cell migration (71–73). According to our findings, this Ca^{2+} influx might activate S100 proteins to disassemble NM2A filaments close to the leading edge, thus promoting protrusion formation locally by lifting the NM2A filament-dependent retrograde flow. Besides the increase in Ca^{2+} concentration, filament-disrupting activity can be further increased in cells highly overexpressing S100A4 under pathological conditions.

We expect that other S100 proteins, having the ability to effectively disrupt NM2A filaments, can function similarly to S100A4. In clinical studies, S100A1 (74), S100A2 (75–77), S100A6 (78), S100P (79), (80), and S100B (81) were found to be up-regulated in many tumors, and they are also believed to promote metastasis (82). A similar effect is seen when NM2A is targeted directly. Disruption of NM2A has been shown to increase the rates of cell migration in cultured cells and to promote the formation of more invasive tumors in mouse strains already predisposed to tumor development (13, 83). Our results show that other S100s are able to disassemble NM2A filaments, and previous studies (74, 78, 79) indicate that this allows them to promote metastasis formation in pathological cases when their expression level is up-regulated. Interestingly, S100A2, which we found to have the second highest potency for disassembling NM2A filaments after S100A4, was found primarily in gastric tumors (77, 84, 85) where S100A4 is missing. These data suggest that S100 proteins can substitute in the pathological role of other family members and that the particular expression levels of each S100 will be key to determining which one is the main driver of pathology.

In conclusion, in this paper we provide in vitro experimental evidence that binding of S100 proteins to the C-terminal end of the NM2A tail (and not only S100A4 but other family members, especially S100A2) and phosphorylation of Ser residues in the nonhelical tail of GFP–2B-tail are able to regulate NM2 filaments in a mutually exclusive way. Moreover, we found that this regulation can result in sorting of paralogs from nascent heterotypic NM2 filaments. If these regulatory events also occur inside the cell, they represent an unexplored level of nonmuscle myosin regulation.
Experimental procedures

Production of recombinant proteins

N-terminal GFP and mCherry-fused human NM2A(837–1960) and NM2B(844–1976) tail sequences (UniProt accession codes P35579 and P35580, respectively) and NM2 peptides M111A(1850–1960), M121B(1856–1976), and M121AB (NM2A (1850–1924) coiled-coil fragment fused with NM2B (1932–1976)) C-terminal tail) were cloned into a modified pET15b vector (pEV) consisting of an N-terminal tobacco etch virus (TEV)-cleavable His6 tag with Ndel and BamHI. Human S100 proteins (UniProt accession codes: S100A1, P23297; S100A2, P29034; S100A3, P33764; S100A4, P26447; S100A5, P33763; S100A6, P06703; S100A7, P31151; S100A8, P05109; S100A9, P06702; S100A10, P60903; S100A11, P31949; S100A12, P80511; S100A13, Q95854; S100A14, Q9HCY8; S100A15, Q86SG5; S100A16, Q96FQ6; S100B, P04271; S100G, P29377; S100P, P25815; and S100Z, Q8WXR8) were cloned as described previously (Kiss et al. (30)). QuikChange method was used to produce the GFP-NM2B(S1935/1937E) tail phosphomimetic mutant. The constitutively active casein kinase 2α (CK2) subunit was purchased from Addgene (plasmid 27083) and was cloned into pGEX-3X vector.

NM2 tails, fragments, CK2, and S100 proteins were expressed in E. coli BL21(DE3) cells. Transformed cultures were grown in Luria-Bertani (LB) broth supplemented with 100 μg/ml ampicillin at 37 °C until the optical density at 600 nm reached 0.8. After centrifugation and washing with PBS, 11N-labeled M111A- and M121AB-expressing cells were transferred to minimal broth (50 mM Na2HPO4, 20 mM KH2PO4, pH 7.5, 1000 mM NaCl buffer disassembling the myosin filaments formed at low ionic strength, they were separated by anion-exchange chromatography using HiTrap Q HP column at pH 8.5. S100A7, S100A15, and S100Z were purified using Superdex 75 column. The purified proteins were concentrated with Amicon ultracentrifugation filter units (Merck), and equimolar amount of TCEP was added.

M111A, M121B, and M121AB were dialyzed against 20 mM Tris, pH 8.5, 150 mM NaCl, and 0.1 mM TCEP-containing buffer. His tag and TEV were removed by subtractive Ni2+-affinity chromatography. The peptides were purified by reverse HPLC using Jupiter 300 C5 column (Phenomenex) and were lyophilized.

The pelleted cells containing CK2 were resuspended in 20 mM Tris, pH 7.5, 500 mM NaCl, 0.1 mM TCEP buffer, and DNase was added. After centrifugation CK2 was applied to GST-affinity column (Protno GSH-agarose 4B (Machery-Nagel)) and was eluted with 10 mM reduced GSH. CK2 was dialyzed in 50 mM Tris, pH 8, 100 μm NaCl, and 0.1 mM TCEP and was stored in this buffer complemented with 5 mM TCEP and 20% glycerol. All constructs were stored at −80 °C.

Phosphorylation of M111A, M121B, and M121AB was performed using 20 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.5 mM TCEP-containing buffer and were supplemented with ATP at 5 × the concentration of the peptide. After CK2 was added at 20× dilution compared with the myosin concentration, the samples were incubated for 4 h at room temperature. The peptides were purified using HPLC as described above. GFP–NM2 constructs were phosphorylated identically except they were incubated in 500 mM NaCl-containing buffer for 2 h to disassemble filaments to expose phosphorylation sites, followed by >3 h incubation in 150 mM NaCl-containing buffer (CK2 was newly added in 20× dilution) to increase the efficiency of CK2. Phosphorylation was monitored using MS, NMR, and filament disassembly assays.

The cDNAs of full-length human NM2A (MYH9) and NM2B heavy chains (MYH10) were amplified and cloned into a modified pFastBac1 vector (FLAG-pFastBac1) encoding an N-terminal FLAG tag (DYKDDDK) to aid purification. Full-length proteins were expressed using the baculovirus/Sf9 cells system (Invitrogen). Plasmid DNA was transformed into DH10-Bac E. coli cells and recombinant bacmid isolated following the manufacturer’s protocols. First-generation baculovirus was generated by transfecting Sf9 cells with a mixture containing bacmid DNA and polyethyleneimine (PEI; Max, M40000; Polysciences) at a ratio of 1:9 in PBS buffer. Baculovirus multiplicity of infection of 3–5 was used to infect the Sf9 cells for protein expression. For full-length proteins, Sf9 cells were infected with heavy chain virus along with viruses for each light chain (RLC = MYL12B; ELC = MYL6). Baculovirus-infected Sf9 cells were grown for 48–72 h and harvested by sedimentation. Cell pellets were stored at −80 °C.

Proteins expressed in Sf9 cells were purified according to previously published protocols (54, 86, 87). Briefly, frozen pellets were thawed and homogenized using a ground glass homogenizer in buffer A (10 mM MOPS (pH 7.4), 5 mM MgCl2, 0.1 mM EGTA) containing 0.5 mM NaCl, 2 mM ATP, 0.1 mM TCEP, and eluted with the wash buffer supplemented with 5 mM EGTA. S100A3, S100A8, S100A9, S100A10, S100A13, S100A14, S100A16, and S100G were dialyzed and separated by anion-exchange chromatography using HiTrap Q HP column at pH 8.5. S100A7 and S100Z were purified using Superdex 75 column. The purified proteins were concentrated with Amicon ultracentrifugation filter units (Merck), and equimolar amount of TCEP was added.

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Phosphorylation of M111A, M121B, and M121AB was performed using 20 mM HEPES, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.5 mM TCEP-containing buffer and were supplemented with ATP at 5 × the concentration of the peptide. After CK2 was added at 20× dilution compared with the myosin concentration, the samples were incubated for 4 h at room temperature. The peptides were purified using HPLC as described above. GFP–NM2 constructs were phosphorylated identically except they were incubated in 500 mM NaCl-containing buffer for 2 h to disassemble filaments to expose phosphorylation sites, followed by >3 h incubation in 150 mM NaCl-containing buffer (CK2 was newly added in 20× dilution) to increase the efficiency of CK2. Phosphorylation was monitored using MS, NMR, and filament disassembly assays.

The cDNAs of full-length human NM2A (MYH9) and NM2B heavy chains (MYH10) were amplified and cloned into a modified pFastBac1 vector (FLAG-pFastBac1) encoding an N-terminal FLAG tag (DYKDDDK) to aid purification. Full-length proteins were expressed using the baculovirus/Sf9 cells system (Invitrogen). Plasmid DNA was transformed into DH10-Bac E. coli cells and recombinant bacmid isolated following the manufacturer’s protocols. First-generation baculovirus was generated by transfecting Sf9 cells with a mixture containing bacmid DNA and polyethyleneimine (PEI; Max, M40000; Polysciences) at a ratio of 1:9 in PBS buffer. Baculovirus multiplicity of infection of 3–5 was used to infect the Sf9 cells for protein expression. For full-length proteins, Sf9 cells were infected with heavy chain virus along with viruses for each light chain (RLC = MYL12B; ELC = MYL6). Baculovirus-infected Sf9 cells were grown for 48–72 h and harvested by sedimentation. Cell pellets were stored at −80 °C.
phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science). The proteins were purified by FLAG-affinity chromatography using M2 FLAG–affinity gel (Sigma) and eluted in buffer A containing 0.5 mM NaCl and 0.5 mg/ml FLAG peptide. The eluted proteins were dialyzed overnight in buffer A containing 25 mM NaCl and 1 mM DTT to induce myosin polymerization into filaments. The protein pellet was then collected by centrifugation at 60,000 $\times$ g for 30 min, and the pellet was dissolved in an appropriate amount of buffer containing 500 mM NaCl, 10 mM MOPS, pH 7.4, 2 mM MgCl$_2$, 0.1 mM EGTA, 1 mM TCEP. Purity of proteins was verified using SDS-PAGE (Invitrogen). Proteins were flash-frozen in 20-$\mu$l aliquots and stored in liquid nitrogen until required.

For experiments using RLC-phosphorylated full-length myosins, proteins were phosphorylated overnight on ice using final concentrations of MLCK (10 nM), 0.1 mM ATP, 0.1 $\mu$m calmodulin, 0.2 mM CaCl$_2$ in buffer containing 500 mM NaCl, 10 mM MOPS (pH 7.4), 2 mM MgCl$_2$, 0.1 mM EGTA, and 1 mM TCEP.

**NMR spectroscopy measurements**

NMR measurements were performed on a Bruker Avance III 700 MHz spectrometer operating at 700.13 MHz for $^1$H, 176.06 MHz for $^{13}$C, and 70.94 MHz for $^{15}$N nuclei, equipped with a 5-mm z-gradient triple-resonance TXI probe-head at 298 K. Temperature was calibrated by standard methanol sample. Typical sample composition for backbone and side-chain assignment was as follows: ~0.5–1 mM $^{15}$N-labeled protein, 20 mM MES buffer, 150 mM NaCl, 3 mM NaN$_3$, pH 6.0–6.7, 10% D$_2$O. All chemical shifts were referenced to the internal 4,4-dimethyl-4-silapentane-1-sulfonic acid resonance, and $^{15}$N chemical shift values were referenced indirectly using IUPAC-IUB recommended protocols (http://www.bmr.bsc.edu/ref_info/cshift.html). Sequence-specific assignments for H$^N$, N, and H$^N$ chemical shift values were obtained from 2D $^1$H–$^{15}$N-HSQC, 3D HSQC–TOCSY (mixing time = 70 ms) and 3D HSQC–NOESY (mixing time = 150 ms) measurements. For the assignment of M111A fragment data available from Badyal et al. (52) were also used. Spectra were processed with TOPSPIN and analyzed using CARA software. Phosphorylation caused chemical shift changes were interpreted by the $\Delta \delta$ parameter shown in Equation 1.

$$ \Delta \delta = \sqrt{\left(\delta H_{\text{phos}} - \delta H_{\text{nat}}\right)^2 + \left(0.1(\delta N_{\text{phos}} - \delta N_{\text{nat}})\right)} \tag{Eq. 1} $$

Variations in secondary structure were evaluated from SSC data for H$^N$ environments, defined as follows: SSC(H$^N$) = $\delta_{\text{measured}} - \delta_{\text{random coil}}$. Random coil values were sequence-corrected according to Wishart et al. (88).

**Salt-dependent filament disassembly assays**

GFP–2A-tail, GFP–2B-tail, GFP–2B$^{51935/1937}$-tail, and their CK2-phosphorylated forms were used in 500 nM final concentration and were diluted in 20 mM HEPES, pH 7.5, 2 mM MgCl$_2$, 2 mM CaCl$_2$, and 0.1 mM TCEP-containing buffer with appropriate NaCl concentrations. In each dilution 500 nM protein was used. After a 10-min incubation, filaments were centrifuged at 19,000 $\times$ g for 10 min. The fluorescence of the supernatants was measured in 384-well plates (Corning catalog no. 3676) using a Synergy H4 multimode microplate reader (BioTek). The excitation wavelength was 488 nm, and the emission was detected at 509 nm. The experiment was carried out in triplicate. The data were fitted in Origin 8 using the Hill equation.

**EM**

In each experiment, 200 nM of each GFP-NM2 tail and full-length NM2 were diluted in 10 mM MOPS, pH 7.5, 1 mM MgCl$_2$, 0.1 mM EGTA, and 0.1 mM TCEP-containing buffer with appropriate NaCl to give a final NaCl concentration of 150 mM after dilution. The heterofilaments using the full-length NM2A and the GFP-NM2B rod fragment were mixed at 500 mM NaCl and diluted together (copolymerized filaments). After 30 min of incubation, S100A4 was added in the stated concentrations, initially in the absence of CaCl$_2$, and samples were measured. Activation of S100 protein was then (following ~30 min of incubation) initiated by adding CaCl$_2$ to a final concentration of 200 $\mu$m, and samples were measured after 5 min of activation. For all EM experiments, a 3-$\mu$l drop of sample was applied to a carbon-coated copper grid (pretreated with UV light to produce a hydrophilic surface) and stained with 1% uranyl acetate. Micrographs were recorded on a JEOI 1200EX II microscope operating at room temperature. Data were recorded on an AMT XR-60 CCD camera. Catalase crystals were used as a size calibration standard. Images were analyzed using Fiji. Length and width of filaments were determined using the main body of the filaments and excluding individual molecules that occasionally extended beyond this. Statistical analysis was carried out using Origin 8 program.

**S100-dependent filament disassembly assays**

For S100A4-dependent disassembly assays of unlabeled full-length myosin, NM2 and S100A4 proteins were diluted in buffer to give final concentrations of 400 nM myosin, 150 mM NaCl, 10 mM MOPS (pH 7.4), 2 mM MgCl$_2$, 0.1 mM EGTA, and 1 mM TCEP with 1 mM CaCl$_2$ where required, along with the stated S100A4 concentration. In experiments comparing RLC-phosphorylated myosins, the final mixtures also contained 20 $\mu$m ATP. Mixtures were incubated for 30 min on
Selective regulation of NM2 heterofilaments

ice, and samples were centrifuged in a Beckman Optima TLX ultracentrifuge using a TLA-100 rotor for 30 min. Supernatants and pellets were quantified by SDS-PAGE and gel densitometry.

Disassembly experiments were carried out in triplicates. The measured data were fitted to a modified quadratic binding equation in Origin 8 program to determine the \( K_{\text{dep}} \) values of filament disassembly.

**TIRF microscopy**

Measurements were carried out at 10 mM MOPS, pH 7.5, 1 mM MgCl\(_2\), 0.1 mM EGTA, and 0.1 mM TCEP buffer with appropriate NaCl to give a final NaCl concentration of 150 mM after dilution except for the high-salt measurements (500 mM NaCl). For the activation of S100 proteins 200 \( \mu \)M CaCl\(_2\) was used, and for the inactivation, 500 \( \mu \)M EGTA was used. Heterofilaments were formed using equimolar concentration of mCherry-NM2A and GFP-NM2B tail fragments (200 – 200 nm). For TIRF measurements, a final dilution step was performed, and samples were applied immediately (few minutes) to the coverslip at a total concentration of 1 nM to decrease the number of visible spots and the background. Cover slides were cleaned using a plasma cleaner (Diener ZEPTO) for 10 min, and samples were imaged using a Nikon Eclipse Ti-E microscope with an H-TIRF module attachment and a Nikon LU-N4 Laser Unit (equipped with four lasers: 405, 488, 561, and 640 nm). Images were taken using an EMCCD camera (Andor iXon Ultra 888 EMCCD, 1024 × 1024 array, 13 \( \mu \)m pixel).

For determination of photobleaching, steps images were taken at high salt (500 mM NaCl) for 10 min using an exposure time of 30 ms for GFP-NM2B–tail and 400 ms for mCherry-NM2A–tail. Intensity of spots was analyzed in Fiji. The initial intensities of spots, where bleaching steps were clearly visible, were determined using a custom Find Maxima process written in Fiji. The initial intensity of a spot equals the total intensity of all still fluorescent (matured and not photobleached) FFPs, and the number of bleaching steps indicate the number of those FFPs. The initial intensity of the spot divided with the respective number of bleaching steps (number of FFPs) equals the intensity of a single FFP in that spot. Averaging the intensities of single FFPs from 10 different spots gave us the average intensity of a single FFP in the sample. Using this average single fluorophore intensity and the same macro (custom Find Maxima) to determine the intensities of spots during heterofilament experiments, the total number of a given FFP in heterofilaments could be calculated by simply dividing the intensity values of the spots with the average intensity value of a single FFP. It is important to note here that the number of FFPs indicates the amount of myosin rod constructs in the filaments (not the coiled-coil hexamers), but this number is underestimated because FFPs might be photobleached before the measurements or not matured during expression. Individual spots were identified automatically using the custom Find Maxima process in Fiji with a value three times the average background used to select spots. A 2D Gaussian was fitted to each spot, and intensities were calculated by measuring the space under the Gaussian curve and correcting with the background at the base of the curves. In all experiments, the 30 most intense spots were measured with both wavelengths, and these spots from 10 different fields were presented. Statistics were carried out using Origin 8 program. To exclude small oligomers and single molecules, we restricted the statistic determination of the average number of FFP “heads” in each filament to spots having more than 10 calculated fluorophores.

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