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How Myosin VI Traps its Off-State, is Activated and Dimerizes

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Abstract

Myosin VI (Myo6) is the only minus-end directed nanomotor on actin, allowing it to uniquely contribute to numerous cellular functions. As for other nanomotors, proper functioning of Myo6 relies on precise spatio-temporal control of motor activity via a poorly defined off-state and interactions with partners. Our structural, functional, and cellular studies reveal key features of myosin regulation and indicate that not all partners can activate Myo6. TOM1 and Dab2 cannot bind the off-state, while GIPC1 binds Myo6, releases its auto-inhibition and triggers proximal dimerization. Myo6 partners thus differentially recruit Myo6. We solved a crystal structure of the proximal dimerization domain, and show that its disruption compromises endocytosis in HeLa cells, emphasizing the importance of Myo6 dimerization. Finally, we show that the L926Q deafness mutation disrupts Myo6 auto-inhibition and indirectly impairs proximal dimerization. Our study thus demonstrates the importance of partners in the control of Myo6 auto-inhibition, localization, and activation.

48 Introduction

49
50 Myosin motor proteins generate force and/or movement from ATP hydrolysis when associated
51 with actin filaments. Conformational changes in the motor as it progresses from ATP hydrolysis to
52 release of inorganic phosphate and ADP on actin are amplified into large movements via a calmodulin
53 (CaM) or light chain binding region referred to as the “Lever arm” (Fig. 1A). To control the functions of
54 myosin motors in cells, the ATPase activity of the motor and its ability to interact with actin must be
55 regulated both spatially and temporally. Thirteen different classes of myosin motors serve diverse
56 cellular functions in mammalian cells¹. The regulation of their motor activity is however poorly
57 characterized. A general theme for the control of motor activity is the formation of intra-molecular
58 interactions involving the C-terminal Tail region and the Motor domain of these motors. In the active
59 form of the motor, the Tail region interacts with itself or cellular partners. The best understood is the
60 case of the dimeric myosin II (Myo2) class² and myosin V (Myo5) class³. In cardiac muscle, impairment
61 in the stabilization of the myosin off-state leads to severe cardiomyopathies⁴. Whether lack of
62 regulation of unconventional myosins can also lead to pathology has not been demonstrated.
63

64 Perhaps the most divergent form of regulation is emerging for Class VI (Myo6), VIIa (Myo7a),
65 and X (Myo10) myosins, which all contain regions of extended stable single alpha helices (SAH)⁵.
66 Indeed, while they are back-folded monomers in their inactive form⁶⁻⁹, these motors can self-associate
67 to form active dimers upon activation¹⁰⁻¹². How back-folding is stabilized is unknown and the nature of
68 the dimerization following unfolding has only been elucidated for Myo10¹², which forms an anti-
69 parallel coiled-coil immediately following the SAH. The SAH thus extends the Lever arm in the case of
70 Myo10¹². Whether this is also the case for the dimeric Myo6 is debated and requires elucidation of its
71 dimerization region^{11,13-18}. The manner in which the motor is dimerized and the composition of its
72 Lever arm greatly influence its function. A distinctive dimerization region in Myo10 allows the dimer
73 to easily reach out for neighboring actin tracks and participate in filopodia formation¹², unlike the
74 vesicle transporter, Myo5, that makes multiple steps on a single actin track.
75

76 As a minus-end directed actin motor, Myo6 performs unique cellular roles (reviewed in¹⁹),
77 including endocytic vesicle trafficking and maturation, stereocilia maintenance²⁰ and melanosome
78 maturation²¹, among many others. For these cellular functions, Myo6 must associate with different
79 binding partners, such as Dab2, GIPC1 and TOM1 in distinct endosomal compartments²²⁻²⁴. Initially
80 characterized as a deafness gene²⁰, Myo6 is also overexpressed in aggressive cancers^{25,26} and its
81 depletion reduces cell migration and proliferation^{25,26}.
82

83 Full-length Myo6 (FLMyo6) was characterized as a back-folded monomer *in vitro*^{8,9}, which was
84 confirmed to exist in cells by FLIM (Fluorescence lifetime imaging microscopy)²⁷. TOM1 and Dab2 bind
85 Myo6 through its WWY motif on the CBD^c, C-terminus part of its cargo-binding domain (CBD; Fig. 1A)
86 while GIPC1 binds the RRL motif on the CBDⁿ (Fig. 1A). FRET studies showing that these partners can
87 unfold constructs lacking the Myo6 Motor domain (MD) led to the proposition that all partners could
88 activate Myo6 upon binding²⁷⁻²⁹. However, whether the WWY and RRL motifs are both accessible in
89 the FLMyo6 back-folded state is unknown. Detailed studies of Myo6 recruitment are required to
90 investigate the role of partners in the spatio-temporal regulation of its cellular activity.
91

92 The configuration of the Myo6 active state, the nature of its Lever arm and its oligomeric state
93 can make critical differences in the way the force produced by the motor is used^{16,30,31}. In fact, Myo6
94 is well adapted to transport as well as to anchor, depending on the load it is working against, according

95 to single molecule assays³². Although the capacity of partners to favor either monomeric, dimeric or
96 oligomeric assemblies has been described^{13,27–29,31,33–35}, the active configuration required to perform
97 the different cellular roles of Myo6 is unknown. *In vitro* studies have identified a proximal dimerization
98 region^{11,17}, but its role for the cellular function of Myo6 is not established, nor is the structure of this
99 region. Furthermore, it is not known whether the dimerization occurs following partner binding, and
100 whether all partners lead to the same motor configuration, which ultimately will determine the nature
101 of the effective Lever arm and mechanical performance of the motor.

102
103 A detailed description of the Myo6 off-state, a structural characterization of the proximal
104 dimerization region, and the role of partners in Myo6 regulation are all essential to understand how
105 Myo6 function is regulated in cells. Here we used structural and functional assays to thoroughly
106 investigate these properties of Myo6. We demonstrate that not all partners can relieve Myo6 auto-
107 inhibition since not all binding sites are accessible, and importantly we solved the structure of the
108 proximal dimerization domain and demonstrate its validity.

110 Results

111 **ADP.P_i bound to the motor strongly stabilizes the off-state conformation of Myo6**

112
113 Previous biophysical characterizations of the Myo6 back-folded state identified contacts
114 between the Myo6 Lever arm and CBD (Fig. 1A)^{27,36}. However, a possible role of the Motor domain in
115 back-folding remains to be clarified. Size Exclusion Chromatography coupled with Multi-Angle Light
116 Scattering (SEC-MALS) and SEC coupled with Small-Angle X-ray Scattering (SEC-SAXS) experiments (Sup
117 Fig. 1A-C; Fig. 1B) indicate that FLMyo6 adopts a compact conformation in the presence of ADP.P_i
118 analogs (Radius of gyration (R_g) = 49.23 ± 0.92 (standard deviation; SD) Å) (Fig. 1B-C; Sup Fig. 1C, Sup
119 Data 1) even at high salt concentration (~425 mM NaCl) (Sup Fig. 1A-B). In contrast, when no nucleotide
120 is present (nucleotide-free (NF) condition), FLMyo6 shifts from a compact to an elongated
121 conformation in a salt concentration-dependent manner (at high salt, R_g = 84.18 ± 4.33 (SD) Å, elution
122 1 mL earlier from SEC-MALS) (Fig. 1B; Sup Fig. 1A-C). Overall, high salt dependency of FLMyo6 opening,
123 combined with the lack of salt dependency in presence of a nucleotide, suggests that the Lever arm
124 and the Tail are held together *via* electrostatic interactions, while the interactions that keep the Tail
125 back-folded on the Head require the Motor domain to be in a nucleotide-bound state of its cycle (Fig.
126 1D). At very low salt (10 mM KCl) and in the presence of actin, FLMyo6 consumes ATP ~10 fold slower
127 than the Tail-less construct MD^{Ins2} (Fig. 1A; Sup Fig. 1D), indicating that the back-folded state is auto-
128 inhibited.

130 131 **3D reconstruction of the Myo6 off-state**

132
133 To further characterize the Myo6 off-state, negative staining electron microscopy (EM) of
134 FLMyo6 in ADP.VO₄ (ADP.P_i analog) (Sup Fig. 2A) resulted in heterogeneous 2D classes, likely due to
135 the intrinsic flexibility of the protein particles. Previous FLIM demonstrated that fusion of the N- and
136 C-termini to fluorescent proteins is compatible with Myo6 back-folding²⁷. Thus, we fused the N- and C-
137 termini of Myo6 to two covalent bonding subdomains of *Streptococcus pneumoniae* pilus adhesin
138 RrgA: Jo and In³⁷ (the Jo-Myo6-In construct) to attempt to limit the inherent flexibility of the back-
139 folded monomer.

141 To show that the fusion does not disrupt the Myo6 back-folding, we confirmed that the Jo-
142 Myo6-In heavy chain could bind to two CaM using SDS-PAGE and that the Jo-Myo6-In behaved as a
143 compact folded protein, even in NF/high salt condition using SEC-MALS and SEC-SAXS (Sup Fig. 2B-D,
144 Sup Data 3A). Actin-activated ATPase measurements revealed a very slow steady state turnover rate
145 for Jo-Myo6-In compared to earlier measurements on wild-type Myo6³⁸, indicating that the
146 conformational changes required to cycle on actin were greatly slowed (Sup Fig. 2E). Finally, negative
147 staining EM images of Jo-Myo6-In in ADP.VO₄, low salt were collected (Fig. 1E). The 3D reconstruction
148 of the Myo6 off-state at ~17 Å resolution (Fig. 1F, Sup Movie 1) is consistent in shape and dimensions
149 with SAXS data of FLMyo6 (Fig. 1C, Sup Data 2C).

150 151 **Structural model of the Myo6 off-state**

152
153 The distinct EM density for the Jo-In fusion clearly defines the position of the N- and C-termini
154 of FLMyo6 and demonstrates how it can lock the off-state. We used available Myo6 crystallographic
155 structures to build a model inside the 3D reconstruction (Fig. 1F, see details in Methods). By defining
156 the orientation of the Lever arm, the model revealed that the flexible joint allowing back-folding must
157 be localized around aa 912-918 prior to the SAH. The ~10 nm long SAH ends up close to the Myo6 N-
158 terminus and Converter, where the rest of the Tail can also participate in stabilizing interactions.
159 Importantly, only the pre-powerstroke structure of the Motor domain (which traps ADP.P_i, Fig. 1F), not
160 the Rigor (NF) structure (Sup Fig. 2F) leads to good model-to-map agreement. We challenged this
161 model by measuring affinities between Myo6 CBD^{1035-end} and Myo6 Head fragments (Table 1, Sup Fig.
162 3). The CBD binds to the Motor domain with low affinity, and the strongest interaction (K_D ~150 nM)
163 was measured for MD^{Ins2/IQ/3HB}. Removal of the IQ-3HB region (MD^{Ins2}) reduces the affinity by 2-fold.
164 Last, the interaction between Myo6 CBD and MD^{Ins2/IQ/3HB} drops from K_D ~150 nM to ~750 nM upon
165 nucleotide removal (Table 1, Sup Fig. 3). These data indicate an interaction of the CBD with both the
166 Motor domain and the Lever arm and highlight the importance of the nucleotide state for optimal
167 interaction.

168
169 To define the CBD region that interacts with the MD^{Ins2/IQ/3HB}, we introduced four missense
170 mutations (D1157V.Y1159D.D1161R.Q1163V: CBD^c loop mutant) in a conserved and exposed loop of
171 the CBD (Fig. 1G). These mutations abolished the ability of the CBD to bind to MD^{Ins2/IQ/3HB}, suggesting
172 a key role of the CBD^c, and this specific loop, in the interaction (Table 1). When this information is used
173 to dock the CBD^c, the Myo6 C-terminus is oriented towards the surface, close to the N-terminus
174 consistent with our Jo-Myo6-In model (Fig. 1H, Methods). Finally, we performed a crosslinking mass
175 spectrometry analysis of the purified FLMyo6 to validate our structural model (Sup Text, Sup Fig. 4A-
176 C, Sup Table 1).

177 178 **Auto-inhibition of Myo6 and hearing loss**

179
180 The back-folded model predicts that a sharp kink occurs at the junction between the 3HB and
181 the SAH (Fig. 1F). The N-terminus region of the SAH (aa 922-935) is thus positioned alongside the 3HB
182 and could participate in the stabilization of the Myo6 off-state via apolar residues found in its atypical
183 sequence (Fig. 2A). The importance of the sequence following the 3HB for back-folding was
184 characterized using the previously published Myo6 (SAHmimic) mutant¹⁶, in which all apolar residues
185 in the SAH were replaced by charged residues to match the i, i+4 alternance of a “perfect SAH
186 sequence” (Fig. 2A). SEC-SAXS and SEC-MALS experiments indicated that FLMyo6 (SAHmimic) adopts

187 an elongated conformation, even upon addition of an ADP.P_i analog, confirming the importance of the
188 residues 922-935 for stabilization of the Myo6 off-state (Fig. 2B-C and Sup Fig. 5A).

189
190 Interestingly, a missense mutation present in this region of the SAH (L926Q) leads to deafness
191 in humans³⁹. Positioned away from the Motor domain or from Tail regions involved in recruitment (Fig.
192 2A), the effect of the mutation on Myo6 function had remained elusive. SEC-MALS (Sup Fig. 5A) and
193 SEC-SAXS experiments (Fig. 2B-C) indicate that the L926Q mutation destabilizes the back-folded state.
194 Both FLMyo6 (L926Q) and FLMyo6 (SAHmimic) mutants display higher ATPase rates (2.86 ± 0.12 (SD)
195 and 4.83 ± 0.11 (SD) s⁻¹.Head⁻¹ respectively) than the wild-type (0.65 ± 0.08 (SD) s⁻¹.Head⁻¹) (Fig. 2D),
196 which confirms the destabilization of the off-state.

197
198 We then investigated the impact of back-folding misregulation in the human pigmented
199 melanoma cell line (MNT-1). Myo6 localizes to dot-like subdomains on the surface of pigmented
200 melanosomes to promote membrane constriction and fission for the release of tubular carriers²¹.
201 MNT-1 cells were transiently co-transfected with plasmids encoding (1) fluorescent components
202 associated with pigmented melanosome^{iRFP}VAMP7²¹ and ^{mCherry}MST⁴⁰, and with (2) either FLMyo6 WT,
203 SAHmimic, L926Q or Jo-Myo6-In, all fused to GFP. All ^{GFP}Myo6 constructs localize as dots on
204 melanosomes (Sup Fig. 5B), although at distinct levels (Sup Fig. 5C). The co-distribution of ^{GFP}FLMyo6
205 (SAHmimic) or ^{GFP}FLMyo6 (L926Q) with melanosomal components was greater than that of the
206 ^{GFP}FLMyo6 (WT) (~1.2 fold increase, $p \leq 0.001$, Sup Fig. 5C). However, the co-distribution of ^{GFP}Jo-Myo6-
207 In with melanosomes was reduced ~3-fold compared to ^{GFP}FLMyo6 (Sup Fig. 5C) and the associated
208 cytosolic and diffuse fluorescent signal was more readily observed (Sup Fig. 5B).

209
210 Collectively, these data indicate that Myo6 auto-inhibition drastically reduces endogenous
211 recruitment to melanosomes while impairment of Myo6 back-folding can result in over-recruitment.
212 These results highlight the importance of the 3HB-SAH region for Myo6 auto-inhibition since the
213 deafness L926Q mutation is sufficient for over-recruitment of the motor. Thus, destabilization of the
214 off-state can lead to pathology.

215 216 **Differential binding and activation of FLMyo6 by distinct cellular partners**

217
218 We next aimed at distinguishing whether partners can bind to FLMyo6 in the back-folded state
219 and if binding depends on the specific binding site. Partners interacting either with the RRL motif
220 (GIPC1³⁴) or the WWY motif (TOM1³⁵ and Dab2¹³) (Fig. 1A) were examined as our model suggests that
221 in the FL off-state, the WWY motif of the CBD^c is buried and unavailable for binding (Fig. 3A-B). We first
222 looked at the ability of ^{His}GIPC1 and ^{His}TOM1 to bind FLMyo6 using an anti-His pull-down assay on
223 purified proteins, in conditions promoting either Myo6 back-folding (addition of ADP.VO₄) or opening
224 (NF, use of the SAHmimic mutant, or addition of Ca²⁺ as previously proposed^{33,36}) (Fig. 3D, Sup Fig. 6).
225 Both TOM1 and GIPC1 were able to retain Myo6 in conditions that favor Myo6 opening. In contrast,
226 upon ADP.VO₄ addition, the interaction of Myo6 with GIPC1 is maintained, but the interaction with
227 TOM1 is weakened, suggesting that binding of TOM1 requires Myo6 opening.

228
229 Next, we assessed the ability of GIPC1, Dab2 and TOM1 to stimulate the ATPase activity of
230 FLMyo6 (Fig. 3E), and found that GIPC1 increases the Myo6 ATPase rate in a concentration-dependent
231 manner, while addition of Dab2 or TOM1 has little impact. Note that partner affinities for Myo6^{YFP}CBD
232 are all in the submicromolar range (*i.e.*, sufficient to ensure binding in our ATPase assays) (Sup Fig. 7).
233 Lack of activation by TOM1 and Dab2 must thus be due to inaccessibility of the WWY motif in the back-

234 folded FLMyo6. Interestingly, TOM1 and Dab2 increase the ATPase rate of the FLMyo6 (SAHmimic)
235 mutant, as does GIPC1, which indicates that all partners can bind to and stabilize the unfolded state
236 (Fig. 3E).

237
238 In this context, we postulate that the RRL motif required for GIPC1 binding to CBDⁿ (Fig. 1A)
239 must be exposed on the surface of the back-folded Myo6, as opposed to the WWY motif. The CBDⁿ
240 fragment (PDB: 5V6E [<https://doi.org/10.2210/pdb5V6E/pdb>]) was thus positioned in the unexplained
241 density lying in continuity to CBD^c in our Jo-Myo6-In 3D reconstruction (Fig. 3A-C, Sup Movie 1).

242
243 Collectively, these results demonstrate that not all partners can induce activation of Myo6.
244 Partners Dab2 and TOM1 require another factor promoting Myo6 opening prior to their binding. In
245 contrast, GIPC1 can directly activate FLMyo6, consistent with a previous study³³.

246 247 **Assessing the specific recruitment of Myo6 to native organelles by distinct partners**

248
249 If some partners can relieve Myo6 from auto-inhibition, we reasoned that artificial targeting
250 of these partners to specific cellular membranes would lead to massive recruitment of Myo6. We thus
251 decided to artificially drive GIPC1, TOM1 and Dab2 to melanosome membranes by fusing them to the
252 melanosome-targeting tag (MST)⁴⁰ (Sup Fig. 8) and verifying their ability to recruit either open
253 (SAHmimic and L926Q) or locked (Jo-Myo6-In) Myo6. To do so with an optimized signal to noise
254 measurement, we introduced the point mutation I1072A in our Myo6 constructs since it drastically
255 reduces endogenous recruitment of Myo6 to the melanosomes (GFPFLMyo6 (I1072A), 3.7-fold
256 reduction ($p > 0.0001$) compared to GFPFLMyo6; Fig. 4A; Sup Fig. 9A-B, 10A), while it is not part of the
257 interface with either GIPC1 (Sup Fig. 7), TOM1 or Dab2. Hence, the I1072A mutation provides an easy
258 way to reduce endogenous recruitment to melanosomes and offers a powerful tool to test the ability
259 of distinct exogenous partners to recruit Myo6.

260
261 We transiently transfected MNT-1 cells with plasmids encoding for mCherryMST-GIPC1,
262 mCherryMST-TOM1, or mCherryMST-Dab2 for melanosome targeting. Co-transfection with plasmid
263 encoding for GFPMyo6 (I1072A), GFPJo-Myo6-In (I1072A), GFPFLMyo6 (SAHmimic.I1072A) or GFPFLMyo6
264 (L926Q.I1072A) provided a quantitative way to compare the ability of these partners to recruit Myo6
265 to specific organelles such as the melanosomes in cells (see Methods).

266
267 Expression of mCherryMST-GIPC1 resulted in ~90% of Myo6-positive melanosomes for all the
268 Myo6 constructs tested (Fig. 4B, E; Sup Fig. 10B), indicating that exogenous GIPC1 can recruit Myo6 to
269 melanosomes independently of Myo6 being open or closed. In contrast, expression of mCherryMST-
270 TOM1 or mCherryMST-Dab2 did not significantly increase the amount of GFPJo-Myo6-In (I1072A) positive
271 melanosomes ($p = 0.5005$ and $p = 0.344$ respectively). This confirms the ineffectiveness of WWY partners
272 in recruiting back-folded FLMyo6. Yet, their expression results in a 1.3/1.4-fold increase of
273 melanosomes containing active Myo6 mutants GFPFLMyo6 (SAHmimic.I1072A) and GFPFLMyo6
274 (L926Q.I1072A) ($p = 0.003$ or lower) (Fig. 4C-E; Sup Fig. 10C-D).

275
276 Interestingly, I1072A moderately affects the recruitment of Myo6 mutants impaired in auto-
277 inhibition. Compared to GFPFLMyo6 (SAHmimic) and GFPFLMyo6 (L926Q), we observe reductions of 1.4
278 and 1.7-fold in the co-distribution with melanosome components for GFPFLMyo6 (SAHmimic.I1072A)
279 and GFPFLMyo6 (L926Q.I1072A), respectively (Fig. 4A, 4E; Sup Fig. 5B-C), which are interestingly similar
280 to the 1.6-fold reduction in recruitment observed for the CBD alone carrying the mutation I1072A (Sup
281 Fig. 9C-D). We thus conclude that the I1072A mutation must reduce the affinity of the CBD for

282 partner(s) responsible for Myo6 endogenous recruitment to melanosomes. In addition, the drastic
283 reduction in ^{GFP}FLMyo6 (I1072A) recruitment evidences the role of endogenous partners to promote
284 Myo6 unfolding and indicates the major role of the I1072 residue in this process.
285

286 These results illustrate the importance of the recognition of the inactive state, and the distinct
287 ways signaling factors can trigger association or activation of the back-folded state in a compartment
288 for spatial and timely control of motor activity.
289

290 **A hinge that dimerizes**

291
292 While we have demonstrated the key role of the sharp kink (hinge) at the 3HB/SAH junction
293 for Myo6 auto-inhibition (Fig. 2; Sup Fig. 5), previous evidence by single molecule motility assays^{11,16}
294 already suggested that this region is key for Myo6 proximal dimerization. Since Myo6 proximal
295 dimerization might be critical for a number of functional properties, we wanted to elucidate the
296 structure of the dimerization region.
297

298 SEC-MALS with six fragments derived from the 3HB/SAH junction (Sup Fig. 11A, C-E) indicated
299 that a rather conserved region (aa 875-940) can self-dimerize with K_D^{APP} of $\sim 19 \mu\text{M}$ ($\Delta G^\circ \sim -6.4 \text{ kcal/mol}$)
300 obtained from titration (Sup Fig. 11A, C). This minimal region corresponds to the last half of the 3HB
301 (*i.e.*, the 2nd and 3rd helix) and the first part of the SAH (Sup Fig. 11A-C). In contrast, no dimerization
302 was observed when peptides included the whole 3HB domain, even when peak concentration of 30
303 μM was reached for the 834-955 peptide (Sup Fig. 11A, D). This data is consistent with previous findings
304 indicating that proximal dimerization requires unfolding of the 3HB (¹⁶, Sup Fig. 12A).
305

306 Crystals of the 875-940 peptide diffracted to 2.1 Å resolution (Fig. 5A; Sup Table 2). Clear
307 electron density for all residues from 876 to 937 indicates that they form an extended helix that
308 dimerizes in an anti-parallel manner (Fig. 5B; Sup Fig. 13). This anti-parallel dimerization is stabilized
309 by multiple apolar contacts involving 13 residues from each helix, and six polar interactions involving
310 R892 with D900, and T888 with S906 (*via* a water molecule) (Fig. 5A; Sup Fig. 11B).
311

312 At the center of the interface, the structure highlights how residues T888, R892 and V903
313 contribute to the dimerization (Fig. 5A; Sup Fig. 11B). Three mutations (T888D.R892E.V903D) were
314 introduced into the 875-940 peptide to assess the impact on proximal dimerization. Importantly, these
315 residues were chosen on the surface of the 3HB so that the mutations would not disrupt the 3HB
316 stability (Fig. 5C). (Note that residue 892 can be a Gln or an Arg depending on the species (Sup Fig. 11B)
317 but both are compatible with the formation of the dimer). SEC-MALS confirms that the
318 T888D.R892E.V903D mutant stays monomeric even up to 43 μM (peak concentration in the SEC-MALS
319 experiment) while the WT counterpart is dimeric in similar conditions (Fig. 5D).
320

321 Finally, a model of the active dimeric configuration of Myo6 bound to F-actin including this
322 crystal structure was built (Fig. 5E, see Methods). The inter-head distance is indeed compatible with
323 the large ($\sim 30 \text{ nm}$) stepping previously reported when Myo6 walks processively^{11,41}. Taken together,
324 our results strongly support that proximal dimerization requires the formation of an extended anti-
325 parallel coiled-coil, which can form following destabilization of the 3HB.
326

GIPC1 promotes unfolding of the Myo6 monomer and proximal dimerization

We further characterized this proximal dimerization region and investigated the ability of partners to promote proximal dimerization of Myo6 using an actin-based ATPase assay. Such dimerization indeed leads to “gating”, *i.e.* coordination between the two Heads of the dimer that translates into slowing of ATP binding to the lead Head while the rear Head is attached⁴². This results in a 50% drop of ATPase rate per Head when Myo6 is dimerized compared to a monomer. The ATPase rate of zippered dimer (Myo6 truncated at R991 followed by a leucine zipper to create a constitutive dimer) in which gating has been characterized⁴² is indeed ~50% that of the monomeric MD^{Ins2} ATPase rate (Fig. 5F).

Indeed, upon addition of GIPC1, we found a ~50% reduction in the maximal ATPase activity per Head for FLMyo6 (WT) compared to MD^{Ins2} (Fig. 5F), consistent with GIPC1 promoting proximal dimerization of FLMyo6. In contrast, addition of GIPC1 to the FLMyo6 (SAHmimic) mutant is similar to that measured with MD^{Ins2}, consistent with a role of GIPC1 in fully freeing the Motor domain from Tail inhibition upon stabilizing an extended, monomeric conformation. Importantly, the FLMyo6 (T888D.Q892E.V903D) exhibits ~2-fold higher maximal ATPase rate upon GIPC1 addition, consistent with loss of gating (Fig. 5F).

This additional evidence strongly validates the role of these residues in antiparallel proximal dimerization, and the role of this region in controlling motor mechanical properties. Furthermore, we demonstrate for the first time that proximal dimerization (involving 3HB unfolding) can be triggered upon GIPC1 binding.

The L926Q deafness mutation indirectly impairs proximal dimerization

Interestingly, when we used GIPC1 to activate the FLMyo6 (L926Q) construct (Fig. 5F), the maximal ATPase activity that we found was intermediate between monomeric and dimeric FLMyo6. Since our proximal dimerization structure indicates that the L926Q missense mutation does not impact the anti-parallel coiled-coil region itself (Sup Fig. 12B), and since we found that 3HB unfolding is essential for proximal dimerization, we hypothesized that L926Q impairs Myo6 dimerization by perturbing the unfolding of the 3HB. This was previously reported for the FLMyo6 (SAHmimic) mutant¹⁶.

To monitor 3HB unfolding, we introduced cysteines at two positions of the 3HB surface (T845 and A880), for tetramethylrhodamine (TMR) labelling (Fig. 5C, Sup Fig. 12A). As previously described¹⁷, a low TMR fluorescence ratio is found when 3HB is folded (fluorescence quenching due to stacking of the rhodamine rings; MD^{Ins2/IQ/3HB} T845C, A880C). This value increases upon 3HB opening (Sup Fig. 12A), and a high TMR fluorescence ratio indicative of 3HB unfolding has been reported for the Myo6 zippered dimer T845C, A880C¹⁷. Introducing the L926Q mutation in this zippered construct led to an intermediate fluorescence intensity, indicating limited unfolding of the 3HB for the deafness mutant compared to control (Table 2). This suggests a role for the L926Q mutation in limiting the conformational changes of the 3HB required for dimerization, in addition to its effect in destabilizing the off-state.

372 Importance of proximal dimerization in cells

373
374 To further demonstrate that proximal dimerization of Myo6 occurs via the anti-parallel coiled-
375 coil seen in our structure, we compared the ability of FLMyo6 (WT) and FLMyo6 (T888D.R892E.V903D)
376 to rescue Myo6-mediated transferrin uptake¹⁶ in HeLa cells whose Myo6 was knocked out for Myo6
377 by using CRISPR/Cas9 (Sup Fig. 14A). FLMyo6 (WT) and the FLMyo6 (T888D.R892E.V903D) were
378 transiently expressed, and the transferrin internalized during a 10 min pulse was quantified. As
379 summarized in Fig. 5G and Sup Fig. 14B, expression of the T888D.R892E.V903D mutant, unable to form
380 the proximal dimer, profoundly decreases the rate of uptake of endocytic vesicles, providing evidence
381 for the need of proximal dimerization to optimize Myo6 function during endocytosis. Furthermore,
382 this also strengthens the evidence that proximal dimerization occurs via an anti-parallel coiled-coil as
383 depicted in our crystal structure.

384 **Discussion**

385
386
387 Despite the significance of controlling where and when myosin motors generate forces and
388 move cargoes in cells, careful investigation of how the function of myosin motors is regulated has only
389 been performed for a few classes of myosin^{2,3,43,44}, and most extensively for Myo2. The results of this
390 study highlight the importance of regulated inhibition of the Myo6 motor until it reaches its target in
391 a cell and it is activated. Myo6 must cross actin-rich regions in order to diffuse and reach its binding
392 partners which selectively activate motor activity (Fig. 6A). If the motor was not blocked from
393 interacting with and cycling on actin, Myo6 would bind to actin filaments throughout the cell, retarding
394 diffusion to its target sites at the cell membrane. The fact that the L926Q mutant disrupts the folding
395 and regulation of Myo6 (Fig. 2) and causes deafness in humans³⁹ attests to the critical need for the
396 regulation of this class of myosin motors.

397
398 Our structural and functional studies provide a more precise model to account for the
399 interactions stabilizing Myo6 back-folding (Fig. 1H, Table 1). Among the major differences compared
400 to previous models^{8,36}, we show that (1) ADP.P_i bound to the Motor domain is essential to lock Myo6
401 in its back-folded state (Fig. 1B, Table 1); (2) back-folding involves a specific loop of the CBD^c (Table 1),
402 which was previously predicted to be external to the folded complex by AlphaFold⁴⁵ (Sup Fig. 15); and
403 (3) the 3HB/SAH junction acts as a critical hinge to control the equilibrium between on/off states of
404 the motor (Fig. 1G-H, Fig. 2). Earlier studies of the folded monomers^{27-29,36} focused only on interactions
405 within a full-length construct in the absence of nucleotide or within a Motor-less construct, and thus
406 did not fully represent what is happening with the full-length Myo6 monomer saturated with
407 nucleotide.

408
409 Intriguingly, a single amino acid change (L926Q) causes deafness³⁹ and is in fact sufficient to
410 destabilize the back-folded monomer (Fig. 2). This SAH mutation flanks the hinge region that we
411 identified as essential for the off-state of this motor. To further investigate the impact of Myo6 back-
412 folding in myosin recruitment, we used the FLMyo6 (L926Q) and FLMyo6 (SAHmimic) mutants to probe
413 their impact on Myo6 recruitment on melanosomes. What was observed (Sup Fig. 5B-C) was that both
414 constructs lead to greater recruitment than the FLMyo6 (WT). This is not a gain of function, but rather
415 a loss of regulation as, (1) the normal cellular control over the spatial and temporal recruitment of
416 Myo6 has been lost, and (2) fluorescence quenching assays show that both SAHmimic¹⁶ and L926Q
417 (Table 2) mutations impair proximal dimerization and thus Myo6 function. Deafness due to the L926Q

418 mutation in humans may therefore result from the inability of Myo6 monomers to reach their target
419 sites in hair cells due to loss of folded regulation.

420
421 We next examined the ability of some of the Myo6 binding partners that recognize different
422 regions of the CBD to induce unfolding and recruitment of Myo6. Folding not only prevents cycling of
423 the motor on actin until the cellular target has been reached, but as shown by our actin-activated
424 ATPase (Fig. 3D), pull-down (Fig. 3E) and recruitment assays on melanosomes (Fig. 4), the folding can
425 also prevent interaction with a subset of cellular partners until unfolding occurs, by either a different
426 class of partners, or potentially by a spike in cellular Ca^{2+} concentration^{33,36} or PIP_2 recognition⁴⁶. We
427 thus propose a model of the folded off-state of FLMyo6 in which the GIPC1 binding site is available for
428 binding, while the TOM1/Dab2 site is masked (Fig. 3A-C). Interestingly, this demonstrates that not all
429 partners are equivalent in their potential for binding the auto-inhibited form of the motor and to
430 activate Myo6.

431
432 While TOM1 and Dab2 cannot trigger Myo6 initial unfolding, once bound they prevent the
433 formation of the off-state due to their incompatibility with it, as previously proposed^{27,28}. Depending
434 on the nature of the partner and its distribution, binding will activate the Myo6 motor and could drive
435 either proximal dimerization (Fig. 5F), distal dimerization^{13,14} or maintain an activated, monomeric
436 form³⁵. Taken together, these results suggest unique roles for partners not only in Myo6 localization,
437 but also in the control of Myo6 activation and function (Fig. 6B).

438
439 Once unfolded, binding to its cargo brings two unfolded Myo6 monomers into close
440 apposition, favoring its dimerization^{11,13,14,16,17,27}. The experiments summarized in Fig. 5 provide the
441 previously unknown structure of the proximal dimerization region. We present both *in vitro* and
442 cellular evidence in support of the structure. This structure reveals the dimerization region to be an
443 anti-parallel coiled-coil, as for Myo10¹². Mutations of three of the amino acids that stabilize this coiled-
444 coil structure (T888D.Q892E.V903D) abolish dimer formation in *in vitro* assays, but with no impact on
445 back-folding of the monomer (Fig. 5F). Furthermore, introduction of this Myo6 triple mutant into cells
446 fails to rescue endocytosis (Fig. 5G), providing evidence for the need of proximal dimerization to
447 optimize this cellular function of Myo6.

448
449 Myo7A, Myo10 and Myo6 exist as folded monomers in cells until they are activated and
450 recruited by their partners. Formation of an antiparallel dimer may be the mode of dimerization for
451 the three classes that appear to undergo this folded monomer to dimer transition. The structure of
452 the active form of Myo6 has been a long-debated issue^{11,13-18,33-35}, which is resolved by our structure
453 for the proximal dimerization region. As shown in Fig. 5E, Myo6 is unique in that its antiparallel coiled-
454 coil and Lever arm in the dimer are derived from unfolding of a 3HB, with contribution of the SAH. The
455 resulting Lever arm formed by the CaM binding region and the unfolded 3HB (half of which contributes
456 to the coiled-coil) is sufficiently long to account for the ability of Myo6 to take steps that average ~30
457 nm on actin^{11,41}. These findings provide a structural framework that can be applied to understanding
458 how motors are recruited and how partners influence motor functions in cells.

459

460 Methods

461 Constructs cloning, expression and purification

462 A list of all the primers and cloning techniques used to clone our constructs can be found in [Sup](#)
463 [Table 3](#).

464 Cloning, expression and purification from Sf9/baculovirus system.

465 The full-length wild-type Myo6 (FLMyo6) was generated using human [MYO6](#) cDNA splice form without
466 the large insertion (Q9UM54-2 in UNIPROT). The small insert was removed through sub cloning to
467 obtain a FLMyo6 construct without any spliced insert (corresponding to isoform Q9UM54-5). The
468 FLMyo6 (no inserts) construct was then used in all *in vitro* experiments requiring a full-length construct
469 except for the Anti-His pull-down experiment, for which the small insert isoform was used.

470 The deafness mutant (L926Q) and triple mutant (T888D.Q892E.V903D - R892 in mouse corresponds to
471 Q892 in human, see [Sup Fig. 11B](#)) in the anti-parallel dimerization region were produced from FLMyo6
472 with no insert by [Quikchange](#) and reverse PCR [respectively](#). The mutant FLMyo6 (SAHmimic)¹⁶ was
473 made where the residues from Glu922 to Glu935 (EAERLRRIQEEMEK) were replaced with alternate
474 acidic and basic residues (EERKRREEERKKREEE) to match the (i, i+4) phasing observed in the predicted
475 Myo6 SAH domain.

476 For microscale thermophoresis and ATPase assays, previously described constructs were used: MD (1-
477 789)⁴⁷, MD^{Ins2} (2-816)⁴⁸, MD^{Ins2/IQ/3HB}(1-917)¹⁷. The Myo6 zippered dimer³⁸ was created by truncation at
478 R991 followed by a leucine zipper.

479 For the bundle unfolding experiments, a monomeric “cys-lite” construct was made by C-terminal
480 truncation at amino acid Q919 and introduction of C321S, C362S, and C611A. To this construct, either
481 a T845C mutation alone, or the combination of T845C and A880C mutations was introduced for
482 rhodamine labeling¹⁷. A dimeric “cys-lite” construct was made by introduction of the C321S, C362S,
483 C611A mutations in the Myo6 zippered dimer³⁸. Into this construct, either a T845C mutation alone, or
484 the combination of T845C and A880C mutations were introduced for rhodamine labeling¹⁷, with or
485 without the addition of a deafness-causing mutation (L926Q).

486 Each of these constructs had a Flag tag (GDYKDDDDK) at its N-terminal end to facilitate purification.
487 Expression in baculovirus system and purification were performed as [follows](#)¹⁶:

488 Sf9 cells were infected with recombinant baculovirus driving high level expression of our Myo6
489 construct, and co-infected with recombinant virus containing human CaM. Three days after infection,
490 Sf9 cells were either flash frozen for later purification, or directly used for the protein purification. Cells
491 were mechanically lysed by 7 shots in a dounce homogenizer in buffer: 200 mM NaCl; 20 mM HEPES
492 pH 7,5; 4 mM MgCl₂; 0.5 mM EDTA; 1 mM EGTA; 0,5% Igepal ; 7% Sucrose; 1 mM NaN₃; 10 µg/mL
493 Aprotinin; 10 µg/mL Leupeptin; 2 mM DTT; 2 mM ATP. The lysate was centrifuged at 20,000 rpm during
494 45 minutes in a 25.50 rotor. The supernatant was incubated with anti-Flag epitope antibody affinity
495 resin under stirring during 2h at 4°C. The anti-flag resin was then loaded on a column. The resin was
496 washed with 200 mL of buffer: 150 mM KCl; 20 mM imidazole pH 7,5; 5 mM MgCl₂; 1 mM PMSF; 3
497 mM DTT; 1 mM EDTA; 1 mM EGTA; 10 µg/mL aprotinin; 10 µg/mL leupeptin; 3 mM ATP. The myosin
498 was then eluted via Flag peptide competition. The sample was then ultracentrifuged at 78000 rpm at
499 4°C during 15 minutes in a TLA110 rotor to spin out any actin that can still be bound to the myosin.
500 The protein was then microdialysed (for ATPase assays) or injected in a Superdex 200 Increase
501 column (Cytiva) and concentrated into the appropriate buffer for following experiments (with or
502 without nucleotide). Purity of all myosin preparations was confirmed on SDS-PAGE gels.

503 Jo and In-Flag sequences³⁷ were synthesized (Eurofins genomics) and fused to Myo6 N-terminus (linker
504 Gly-Ser) and C-terminus (linker Gly), in pVL1392 for expression in Sf9 cells. Purification was achieved
505

508 using the same protocol as for FLMyo6 except that, for EM studies, purification was performed by
509 replacing ATP with ADP.VO₄ in the lysis buffer. For increased purity, a SEC step was performed using a
510 Superdex 200 Increase column (Cytiva) developed in 10 mM Hepes; 80 mM NaCl; 5mM NaN₃; 1 mM
511 MgCl₂; 0.1 mM TCEP; 0.1 mM ADP; 0.2 mM VO₄; 0.1 mM EGTA; pH 7.5.

512

513 **Constructs cloning, expression and purification from *Escherichia coli***

514 **Cloning Myo6 constructs**

515 Ins2/IQ/3HB was generated using our human FLMyo6 NI construct (see previous section), DNA
516 sequence encoding for aa783-917 was transferred into pPROX-HTB plasmid containing in N-terminus
517 6XHis tag and a TEV cleavage sequence (coding for ENLYFQG).

518 Myo6^{YFP}CBD was generated through several rounds of subcloning from cDNA mouse **MYO6** (E9Q3L1
519 in UNIPROT). **MYO6** was incorporated in pET14 plasmid containing an N-terminus 6XHis-tag, a yellow
520 fluorescent protein (YFP) and a TEV cleavage sequence. Finally, **MYO6** was truncated in N-terminus at
521 position corresponding to aa M1032 through reverse PCR. The ^{YFP}CBD (D1157V.Y1159D.D1161R.
522 Q1163V) mutant was generated through point mutations addition using reverse PCR on the ^{YFP}CBD
523 (WT) construct.

524

525 **Cloning partner constructs**

526 In order to avoid partner (GIPC1, TOM1 and Dab2) degradation and auto-inhibition as previously
527 reported^{28,34}, we used truncations containing the published Myo6-binding domains^{13,22,34,35} instead of
528 FL constructs.

529 For microscale thermophoresis assays and his-pull-down assays, His⁶GIPC1 construct was generated
530 using cDNA full length mouse **GIPC1** (UNIPROT Q9Z0G0). **GIPC1** was incorporated in pProEX-HTB
531 plasmid containing in N-terminus 6XHis-tag and a TEV cleavage sequence. Finally, **GIPC1** was truncated
532 in N-terminus at the position corresponding to aa D255 through reverse PCR in order to keep only the
533 GH2 domain, which is sufficient for the interaction with Myo6³⁴.

534 For ATPase assays, we used GIPC1 in fusion with the mNeonGreen tag: GIPC1 DNA sequence encoding
535 for residues 238-end was incorporated in the pET28 plasmid containing in the N-terminus 6XHis-tag
536 and a mNeonGreen tag using homemade Gibson Assembly mix.

537 For microscale thermophoresis and his-pull-down assays, a non-fluorescent His⁶TOM1 construct was
538 generated using full length human **TOM1** (UNIPROT O60784-1) cDNA. TOM1 207-end was incorporated
539 into pET14 in-frame with an N-terminus 6XHis-tag and a TEV cleavage sequence.

540 For ATPase assays, we used the TOM1 436-461 peptide described as the minimal sequence required
541 for TOM1 binding to Myo6³⁵. DNA sequence coding for aa436-461 was incorporated in pET14 plasmid
542 containing in N-terminus 6XHis-tag, yellow fluorescent protein (YFP) and a TEV cleavage sequence.

543 Dab2 His-650-end (tDab2²⁸) was a kind gift of Christopher Toseland.

544 To identify the minimal sequence involved in proximal dimerization, several Myo6 truncations were
545 generated from mouse **MYO6** cDNA (UNIPROT: E9Q3L1). Constructs encoding for aa 875-940 and 875-
546 955 were cloned with a N-terminal 6xHis-tag into pET14 plasmid. The construct encoding for aa 834-
547 955 was cloned into pET14 with a N-terminal 6xHis-tag followed with a thrombin cleavage site (coding
548 for LVPRGSH). Constructs encoding for aa 880-940 and 888-940 were cloned with a C-terminal 6xHis-
549 tag into pET14 by PCR and blunt-end ligation. The 912-end construct was cloned into pProEX-HTb with
550 a N-terminal 6xHis-tag through several rounds of sub cloning. For crystallization assays, the construct
551 encoding for aa 875-940 was generated with a N-terminal 6xHis-tag and a TEV cleavage sequence into

552 pProEX-HTb using homemade Gibson Assembly mix. The point mutations T888D, R892E, V903D were
553 added in the backbone encoding for his-rTEV-875-940.

554

555 **Protein expression and purification**

556 Constructs were expressed in E.coli BL21 (DE3) cells (NEB). Cells were grown in 2xYT media until
557 $OD_{560} \sim 0.8$, expression was then induced by addition of 200 μ M isopropyl β -D-1-thiogalactopyranoside
558 (except for Dab2 expression, where 1 mM isopropyl β -D-1-thiogalactopyranoside was used). Cells were
559 lysed by sonication. For purification, the lysate soluble fraction was loaded on an IMAC column
560 (cComplete 5mL, Roche for all constructs except ^{YFP}CBD (WT and mutant) for which HisTrap-FFcrude
561 5mL, Cytiva was used instead), and proteins were eluted with 200 mM or 300 mM Imidazole. Purest
562 fractions were identified by SDS-PAGE. If needed, pooled fractions were concentrated using Vivaspin
563 concentrators (Sartorius) up to ~ 5 mL. Concentrated samples were injected in Superdex 200 or 75
564 16/600 columns (Cytiva) depending on the molecular weight of the target protein. Purest fractions and
565 the final sample were concentrated by ultrafiltration, and protein concentration was determined using
566 Nanodrop 2000 (ThermoScientific). The final sample containing concentrated protein was flash-frozen
567 in liquid nitrogen and stored at -80°C .

568 For proteins containing a TEV cleavable His-tag, prior to the gel filtration, His-tag was removed by
569 incubation with homemade rTEV protease overnight in a 1/50 mass ratio. The incubate was passed
570 through the cComplete His-Tag Purification Column again to remove rTEV and the uncleaved fraction,
571 then concentrated and loaded in a Superdex 75-16/60 gel filtration column.

572 Purification buffers are detailed in [Sup Table 4](#).

573

574 **Constructs cloning for expression in cells**

575 For expression in MNT-1, FLMyo6 was generated from cDNA of full length human **MYO6**, no inserts
576 isoform (UNIPROT: Q9UM54-5) with shRNA resistance. DNA was transferred to the pEGFP-C1 vector
577 via a XbaI restriction enzyme site. SAHmimic mutations (Glu922 to Glu935 (EAERLRRIQEEMEK)
578 replaced with alternate acidic and basic residues (EERKRREEEERKKREEE) were introduced by reverse
579 PCR. The L926Q mutation was introduced using Quikchange. Transfer of Jo-Myo6-In from baculovirus
580 vector to P-EGFP-C1 was ordered from GenScript. Myo6 CBD was generated by transferring DNA
581 encoding G1037-end from human **MYO6**, no inserts isoform (UNIPROT: Q9UM54-5) into pEGFP-C1
582 plasmid using the XbaI restriction enzyme site.

583 I1072A was introduced in previously cloned constructs (see above) using reverse PCR.

584 Mouse GIPC1 (239-end), human TOM1 (299-end as described in²²) and human Dab2 (650-end) were
585 transferred in a modified pmCherry-C1 plasmid containing in N-terminus a melanosome-targeting tag
586 (MST tag, aa 1-139 from Mouse **MREG** – UNIPROT: Q6NVG5) as described in⁴⁰. The MST tag and
587 mCherry are separated by a GGSGGTGG linker. In the ^{mCherry}MST-partners constructs, mCherry and
588 GIPC1, TOM1 or Dab2 sequences are separated by the polylinker multiple cloning site
589 SGLRSRAQASNSLTSK.

590 **For expression in HeLa cells, our previously existing ^{Flag}FLMyo6(WT) (pig/mouse) with small insert¹¹ was**
591 **introduced in TREX Pcdna4/TO plasmid together with a C-terminal mApple for detection. Mutations**
592 **(T888D, R892E and V903E) were successively introduced in this construct.**

593

594

595

596 **SEC-SAXS**

597 SAXS data were collected on the SWING beamline at synchrotron SOLEIL (France)⁴⁹ in HPLC mode at
598 $\lambda = 1.0332150494700432 \text{ \AA}$ using a Dectris EIGER-4M detector at a 2 m distance. Protein samples were
599 injected at 0.1 mL/min on Superdex 3.2/300 column pre-equilibrated in 20 mM HEPES; 200 mM NaCl;
600 2 mM MgCl₂; 1 mM NaADP; 1 mM AlF₄, 0.1 mM EGTA, 1 mM DTT; or 20 mM HEPES; 400 mM NaCl; 2
601 mM MgCl₂, 0.1 mM EGTA, 1 mM DTT; pH 7.5 prior to data acquisition in the SAXS capillary cell. 150
602 frames of buffer scattering (before the void volume), then 719 frames of elution sample scattering
603 were collected. Exposure time was 1990 ms/frame. Images were processed using the Foxtrot 3.5.10-
604 3979⁴⁹ developed at the SOLEIL synchrotron: buffer averaging, buffer subtraction from the
605 corresponding frames at the elution peak, and sample averaging were performed automatically.
606 Further data analysis to obtain R_g, I(0), D_{max} and molecular weight estimation was done with PRIMUS
607 from ATSAS suite⁵⁰. Dimensionless Kratky plot ($(q \cdot R_g)^2 \cdot I(q) / I(0)$ versus $q \cdot R_g$) was generated using
608 Microsoft Excel based on I(0) and R_g values found with PRIMUS. 20 envelopes were generated
609 independently with GASBOR⁵¹ and averaged with DAMAVER⁵².

610

611 **SEC-MALS**

612 For SEC-MALS analysis, samples were injected in a Superdex 200 10/300 Increase (Cytiva) previously
613 equilibrated in the corresponding buffer, and developed at 0.5 ml/min. Data collection was performed
614 every 0.5 sec with a Treos static light scattering detector, and a t-Rex refractometer (both from Wyatt
615 Technologies). Concentration and molecular mass of each data point were calculated with the
616 software Astra 6.1.7 (Wyatt Technologies).

617

618 **Microscale Thermophoresis measurements between Myo6 Tail and Head**

619 Microscale thermophoresis experiments were performed on a Monolith NT.115 system (NanoTemper
620 Technologies) using YFP-fusion proteins.

621 The non-fluorescent protein was first treated with 0.5 mM EGTA (+/- 2 mM MgADP; 2 mM Na₃VO₄ for
622 some experiments); then dialysed against 20 mM Hepes pH 7.5, 50 mM NaCl, 2.5 mM MgCl₂, 1 mM
623 TCEP and 0.05% (v/v) Tween-20 (+/- 2 mM MgADP; 2 mM Na₃VO₄ for some experiments). Two-fold
624 dilution series (16 in total) of the non-fluorescent protein (Head- sample) were performed at 25°C in
625 the same buffer. The YFP-fused partner was kept at a constant concentration of 100 nM. The samples
626 were loaded into premium capillaries (Nanotemper Technologies) and heated for 30 sec at 60% laser
627 power. All experimental points were measured twice. The affinity was quantified by analyzing the
628 change in thermophoresis as a function of the concentration of the titrated protein using the
629 NTAnalysis software provided by the manufacturer.

630

631 **Microscale Thermophoresis measurements with Myo6 partners**

632 Two-fold dilution series (16 in total) of the non-fluorescent protein (Myo6 partner) were performed at
633 25°C in the MST buffer: 20 mM Bis-Tris pH 6.5, 100 mM KCl, 1 mM DTT and 0.05% (v/v) Tween 20. The
634 YFP-fused partner was kept at a constant concentration of 100 nM. Microscale thermophoresis
635 experiments were then performed in similar conditions as above in 20 mM Bis-Tris pH 6.5, 100 mM
636 KCl, 1 mM DTT and 0.05% (v/v) Tween 20. Capillaries were heated for 30 sec at 50% laser power.

637

638

639

640

641 **Protein cross-linking and mass spectrometry detection**

642 Before the cross-linking reactions, and to prevent cross-linking reactions with MgATP/ADP,
643 concentrated full-length myosin 6 was buffer exchanged by 20-fold dilution and concentration (twice)
644 using EDTA buffer (10 mM HEPES, 50 mM NaCl, 2 mM EDTA, 0.1 mM DTT, pH 7.4) to strip the
645 nucleotide from the motor domain. Then, the protein was diluted and concentrated again using Mg
646 buffer (10 mM HEPES, 50 mM NaCl, 2 mM MgCl₂, 0.1 mM DTT, pH 7.4) until a concentration of ~4-5
647 mg/ml. Finally, 0.5 mM 2-chloroadenosine-5'-triphosphate (Cl-ATP) were added to the protein
648 solution.

649 Protein cross-linking reactions were carried out at room temperature for 60 minutes at a 1:300 protein
650 to DSSO ratio and quenched with the addition of Tris buffer to a final concentration of 50 mM. The
651 quenched solution was reduced with 5 mM DTT and alkylated with 20 mM iodoacetamide. The SP3
652 protocol as described in^{53,54} was used to clean-up and buffer exchange the reduced and alkylated
653 protein. Shortly, proteins are washed with ethanol using magnetic beads for protein capture and
654 binding. The proteins were resuspended in 100 mM NH₄HCO₃ and were digested with trypsin
655 (Promega, UK) at an enzyme-to-substrate ratio of 1:20, and protease max 0.1% (Promega, UK).
656 Digestion was carried out overnight at 37 °C. Clean-up of peptide digests was carried out with HyperSep
657 SpinTip P-20 (ThermoScientific, USA) C18 columns, using 80 % Acetonitrile as the elution solvent.
658 Peptides were then evaporated to dryness *via* Speed Vac Plus. Dried peptides were suspended in 3 %
659 Acetonitrile and 0.1 % formic acid and analysed by nano-scale capillary LC-MS/MS using a Ultimate
660 U3000 HPLC (ThermoScientific, USA) to deliver a flow of approximately 250 nl/min. Peptides were
661 trapped on a C18 Acclaim PepMap100 5 µm, 100 µm x 20 mm nanoViper (ThermoScientific, USA)
662 before separation on PepMap RSLC C18, 2 µm, 100 A, 75 µm x 50 cm EasySpray column
663 (ThermoScientific, USA). Peptides were eluted on a 90 minute gradient with acetonitrile an interfaced
664 *via* an EasySpray ionisation source to a quadrupole Orbitrap mass spectrometer (Q-Exactive HFX,
665 ThermoScientific, USA). MS data were acquired in data dependent mode with a Top-25 method, high
666 resolution scans full mass scans were carried out (R = 120,000, m/z 350 – 1750) followed by higher
667 energy collision dissociation (HCD) with stepped collision energy range 21, 27, 33 % normalised
668 collision energy. The tandem mass spectra were recorded (R=30,000, isolation window m/z 1.6,
669 dynamic exclusion 50 s). Cross linking data analysis: Xcalibur raw files were converted to MGF files
670 using ProteoWizard⁵⁵ and cross links were analysed by MeroX⁵⁶. Searches were performed against a
671 database containing known proteins within the complex to minimise analysis time with a decoy data
672 base based on peptide sequence shuffling/reversing. Search conditions used 3 maximum missed
673 cleavages with a minimum peptide length of 5, cross linking targeted residues were K, S, T, and Y, cross
674 linking modification masses were 54.01056 Da. Variable modifications were carbamidomethylation of
675 cysteine (57.02146 Da) and Methionine oxidation (15.99491 Da). False discovery rate was set to 1 %,
676 and assigned cross linked spectra were manually inspected.

677

678 **ATPase assays**

679 **Steady-state ATPase activities were measured at 25°C using an NADH-coupled assay**³⁸. ATPase rate
680 determined from 2-3 preps with 2-3 independent assays per prep. Myo6 was used at 150 nM, F-Actin
681 was used at 40 µM (unless otherwise noted) and 2.5 µM additional CaM were added in all our
682 experiments. The experiments were all carried out in 10 mM MOPS pH 7.0; 10 mM KCl; 1 mM DTT; 1
683 mM MgCl₂; 1 mM EGTA.

684

685

686 **Anti-His Pull-down assay**

687 FLMyo6 SI (WT) or (SAHmimic) were used alone or mixed with partner GIPC1 (His-rTEV-GIPC1 255-end)
688 or TOM1 (His-TOM1 207-492) in a ratio (1/1) (10 μ M) and 1 μ M of extra Calmodulin was added in a
689 total volume of 20 μ L. The input was incubated with 40 μ L of Ni²⁺ beads from cComplete column
690 (Roche), which were previously equilibrated either in ADP.VO₄ Buffer (10 mM HEPES pH 7.5; 100 mM
691 NaCl; 5 mM NaN₃; 1 mM MgCl₂; 0.1 mM TCEP; 1 mM NaADP; 1 mM Na₃VO₄; 0.1 mM EGTA; 4 mM
692 imidazole) or ADP.VO₄-CaCl₂ Buffer (10 mM HEPES pH 7.5; 100 mM NaCl; 5 mM NaN₃, 1 mM MgCl₂,
693 0.1 mM TCEP, 1 mM NaADP; 1 mM Na₃VO₄; 4 mM imidazole; 1 mM CaCl₂) or NF Buffer [10mM HEPES
694 pH 7.5; 100 mM NaCl; 5 mM NaN₃; 1 mM MgCl₂; 0.1 mM TCEP; 0.1 mM EGTA; 4 mM imidazole]. All
695 steps were performed at 4°C. Beads were washed by centrifugation after 1 hour of gentle agitation.
696 Bound proteins were eluted in 600 mM imidazole in the corresponding buffer.

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698 **Electron Microscopy**

699 Purified Jo-Myo6-In at 50 μ g/ml in 10 mM HEPES; 80 mM NaCl; 1 mM MgCl₂; 0.1 mM TCEP; 0.1 mM
700 ADP; 0.2 mM Na₃VO₄; 0.1 mM EGTA, pH 7.5 was transferred to Carbon Film 300 mesh copper grids
701 (Electron Microscopy Sciences), then stained with 2% uranyl acetate. A total of 284 images were
702 collected with a 200 kV Tecnai G2 microscope under low dose condition with a 4Kx4K F416 TVIPS
703 camera at 0.213 nm/px and treated with the software CryoSPARC⁵⁷. Following CTF determination,
704 template picking was carried out using an initial set of 100 manually picked particles. The resulting
705 711,671 particles were submitted to a few rounds of 2D classification from which 93,293 particles were
706 selected. These were used in the ab-initio reconstruction that produced the map at 17 Å resolution
707 (FSC).

708

709 **Model of the Myo6 off-state**

710 We first positioned the Motor domain-Lever arm (residues 1-917) in the Jo-Myo6-In map from negative
711 staining EM. Best model-to-map agreement was obtained with PDB 4ANJ
712 [<https://doi.org/10.2210/pdb4ANJ/pdb>] (Motor domain and insert-2/Ca²⁺-CaM with ADP.P_i analog
713 bound, in pre-powerstroke state (PPS)). The Lever arm from PDB 3GN4
714 [<https://doi.org/10.2210/pdb3GN4/pdb>] was then superimposed to PDB 4ANJ
715 [<https://doi.org/10.2210/pdb4ANJ/pdb>] by using the insert-2/Ca²⁺-CaM region, present in both
716 structures, as reference. In the negative staining reconstruction, Jo-In PDB 5MKC
717 [<https://doi.org/10.2210/pdb5MKC/pdb>] was placed in the distinct density that corresponds to it, as
718 expected, with the N- and C-termini pointing towards the center of the main density body occupied by
719 Myo6. The structure of the C-terminal half of the CBD from PDB 3H8D
720 [<https://doi.org/10.2210/pdb3H8D/pdb>] was placed according to structural and biochemical
721 restrictions as follows: (1) the CBD^c C-terminus must be near the N-terminus of the fusion protein In;
722 (2) residues D1157, Y1159, D1161 and Q1163 are in contact with the MD^{ins2}; (3) there is still density to
723 be filled close to the N-terminus of the CBD^c, that can be filled by CBDⁿ. (Note that this proposed
724 position is opposite to that currently predicted by AlphaFold⁴⁵ for uniprot entry : Q9UM54, due to lack
725 of data for the intermolecular interactions when that model was built). At last, the NMR structure of
726 the SAH domain (residues 919-998; PDB: 6OBI [<https://doi.org/10.2210/pdb6OBI/pdb>]) was
727 accommodated in the density. This density is narrow up to residue ~955 and then becomes much larger
728 to account for the rest of the model, in which no distinct subdomain can be identified. Thus, our current
729 model lacks the distal Tail (a compact domain of 3 nm in diameter⁸) and CBDⁿ, for which there seems
730 to remain enough density to be fitted. Model and figure were prepared with Pymol⁵⁸. The complete
731 model of the Myo6 off-state is presented in Fig. 3A. Placement of the different domains is further

732 supported by the crosslink experiment presented in [Sup Fig. 4](#) and [Sup Table 1](#). Its ability to fit our off-
733 state Myo6 SAXS data was tested ([Sup Data 2D-F](#) and [Sup Data 3B-C](#)).

734

735 **MNT-1 cell transfection**

736 MNT-1 cells (human pigmented melanoma cell line kindly provided by Pr. Michael S. Marks
737 (Department of Pathology and Laboratory Medicine, Children's Hospital of Philadelphia Research
738 Institute, Philadelphia, PA; Department of Pathology and Laboratory Medicine and Department of
739 Physiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA)) were cultured
740 in DMEM supplemented with 20 % FBS, 10 % AIM-V medium, 1 % sodium pyruvate, 1 % nonessential
741 amino acids, and 1 % penicillin-streptomycin. For plasmid transfection, 400 000 MNT-1 cells were
742 transfected using nucleofection (NHEM kit, Lonza) on Amaxa device 2 (program T20) with 1.5 µg of
743 ^{iRFP}VAMP7 plasmid; 1 µg of ^{mCherry}MST plasmid and 3 µg of ^{GFP}FLMyo6 plasmid. After transfection cells
744 were seeded in fluorodish containing 1 mL RPMI medium, then 1 mL of complete MNT-1 medium
745 supplemented by 10 % FBS was added 6 h post-transfection. Medium was changed 1-day post-
746 transfection by complete medium then cells were fixed with 4 % PFA at 48 h post-transfection. Cells
747 were stored in the dark at 4 °C in PBS medium until imaging. Fluorescence intensity of each ^{mCherry}MST
748 construct was analyzed to ensure equivalent expression levels between the different partners ([Sup Fig.
749 16](#)).

750

751 **Super resolution imaging and analysis**

752 Samples were imaged in fluorodish using a 100x/1.4 NA oil immersion objective on an inverted
753 Spinning disk confocal microscope (Inverted Eclipse Ti-E Nikon, Spinning disk CSU-X1, Yokogawa)
754 equipped with a Photometrics sCMOS Prime 95B Camera (1200 x 1200 pixels). Z images series were
755 acquired every 0.2 µm. Images were processed with a Live super Resolution module (Live-SR; Gataca
756 systems) based on structured illumination with optical reassignment technique and online processing
757 leading to a two-time resolution improvement⁵⁹. For the figures, Z maximum projection and a substract
758 background (50 pixels) were applied on SR images using the FIJI software. Analysis was done on raw
759 SR images. Melanin pigments (black spots) were automatically detected in a defined region of interest
760 (ROI) (here, cell outlines that were manually drawn) in BrightField images by creating a MIN-intensity
761 z-projection and considering the lowest values, defined using the 'Find Maxima' function of Image J/Fiji
762 and whose spatial coordinates were recorded. To quantify the percentage of melanosomes containing
763 ^{iRFP}VAMP7 / ^{mCherry}MST / ^{GFP}Myo6 proteins at the membrane, additional ROIs centered around each
764 individual detected pigment were generated whose size was defined (0.350 µm diameter). Then, for
765 each detected brightfield spot, an additional automatic detection in the fluorescent channel(s) of
766 interest was performed by creating a MAX intensity z-projection in the ROI around the pigments and
767 considering the highest values. Detected pigments were considered positive for the marker of interest
768 above a threshold (defined by Triangle's automatic thresholding method, calculated on the MAX
769 intensity projection, or manual thresholding in the case of cells expressing the lowest GFP-Myo6), and
770 the percentage of which was calculated. Pigments that were automatically detected very close to each
771 other (within 4 pixels in XY and 2 pixels in Z) and that had overlapping ROIs were automatically
772 removed and eliminated from the analysis to avoid data duplication. Moreover, automatically
773 detected pigmented that were negative for ^{iRFP}VAMP7 and/or ^{mCherry}MST fluorescent signal were
774 excluded from the analysis because not considered as pigmented melanosomes (positive for
775 membrane-associated components). For each cell, a percentage of Myo6 positive melanosome was

776 calculated and normalized to the total number pigmented melanosomes (co-positive for pigment and
777 ^{iRFP}VAMP7 and/or ^{mCherry}MST).

778

779 **Proximal dimer Crystallization, Data Collection, and Structure Determination**

780 The Myo6 875-940 construct was crystallized by hanging drop vapor diffusion at 290 K by mixing 1 μ L
781 of 9.8 mg/mL protein solution with 1 μ L of reservoir solution (27% PEG 4000, 10 mM MgCl₂ and 0.2 M
782 imidazole / malate, pH 6.0). Crystals grew spontaneously as rods 1 to 7 days after. After additional 3
783 weeks, they were cryo-cooled in liquid nitrogen in a solution containing 28% PEG 4000, 10 mM MgCl₂,
784 0.2 M imidazole / malate, pH 6.0, and 27% ethylene glycol. One exploitable X-ray dataset was collected
785 at the Proxima 1 beamline (Synchrotron Soleil, Gif-Sur-Yvette) and processed with Autoproc⁶⁰.
786 Diffraction limits after treatment with Staraniso⁶¹ with cut-off of 1.2 I/sI were 2.566 Å in two directions,
787 and 2.077 Å⁶¹ in one direction. Initial structure factors were obtained by molecular replacement with
788 Phaser⁶² using a helix comprised of 30 serine residues as search model. Initial sequence attribution was
789 obtained with Phenix AutoBuild⁶³, followed by several cycles of iterative edition with Coot⁶⁴ and
790 refinement with Buster⁶⁵. Resolution was automatically cut by Buster to 2.2 Å based on model-map
791 cross-correlation. The dimer is defined by one of the 2-fold symmetry axis, with crystal contacts
792 between the N-terminus of one dimer and C-terminus of neighboring dimers (Sup Fig. 13B-C, Sup
793 Movie 2). When the carbons are colored according to B-factors (Fig. 5A), the lowest values are found
794 between residues 885 and 913, suggesting that the dimerization interface is comprised within those
795 boundaries.

796

797 **Model of the Myo6 proximal dimer**

798 The cryo-EM structure of Myo6 bound to actin (PDB: 6BNP [<https://doi.org/10.2210/pdb6BNP/pdb>])
799 was used as basis for placing two Myo6 Motor domains in rigor and PPS states (grey) at a distance
800 compatible with the average step size of ~30 nm previously measured for the truncated constructs (at
801 991 or 1050), the zippered dimer and the full-length protein¹¹ (Fig. 5E). On each side, the N-terminus
802 of the Lever arm bound to two light chains (pink, PDB: 3GN4 [<https://doi.org/10.2210/pdb3GN4/pdb>])
803 was aligned to the corresponding residues in the Converter. The crystallized dimerization domain
804 (blue) was then placed with minimal distance from the two Lever arms, leaving a gap of 4.6 nm from
805 each side. This gap needs to be filled by a stretch of 26 amino acid residues that would make 3.9 nm if
806 in a theoretical helix, or up to 9.9 nm if fully extended. SAH (green) (PDB: 6OBI
807 [<https://doi.org/10.2210/pdb6OBI/pdb>]) was connected to the C-terminus of the dimerization region
808 *via* a putative kink. Model and figure were prepared with Pymol⁵⁸.

809

810 **Bundle Unfolding Assay**

811 As previously described¹⁷, cysteine residues were introduced to replace T845 and A880 in Myo6-917
812 (MD^{Ins2/IQ/3HB}) and Myo6-991-leucine zipper (zippered dimer) constructs with no reactive cysteines.
813 Control constructs contained one reactive cysteine, T845C. One mg of each protein was labeled with a
814 10-fold molar excess of TMR 5-iodoacetamide (5-TMRIA; Anaspec, San Jose, CA) per cysteine (from a
815 stock concentration of 20 mM in dimethylformamide) at 4°C for 1–3 hr. Unbound rhodamine was
816 removed by gel filtration and overnight dialysis. Absorption spectra were measured in a HP Diode Array
817 Spectrophotometer, and fluorescence spectra were obtained in a PTI QM3 luminescence
818 spectrofluorometer. The excitation and emission spectra were measured at 552 and 575 nm,
819 respectively.

820

821 **Generation of Myo6 null HeLa cells**

822 The **MYO6** gene of HeLa cells (ATCC CCL-2) was inactivated by CRISPR/Cas9 gene editing approach.
823 Briefly, HeLa cells were transfected using a combination of three human Myo6 CRISPR plasmid variants
824 (Santa Cruz Biotech SC-401815) – each driving expression of Cas9, GFP and one of the following human
825 Myo6-specific 20-nucleotide gRNAs (5'-3': taatatacaagttcgatata, acattctgattgcagtgaatc,
826 ccaagtgttctgcagaag). Clones of transfected HeLa cells were selected on the basis of GFP fluorescence,
827 and PCR of isolated DNA using primers flanking the targeted genomic sequences. Loss of Myo6
828 expression was confirmed by western blot using an anti-Myo6 antibody (rb pc)(EMD-Millipore, Cat#
829 **ABT42, Lot# 3011368, used at 1:1000 dilution for the western blot**).

831 **Transferrin Endocytosis Assay**

832 Normal and Myo6 null HeLa cells were grown in multi-well tissue culture plates on coverslips coated
833 with rat collagen I (Corning). FLMyo6 (WT) or FLMyo6 (T888D.Q892E.V903D) were tagged with a C-
834 terminal mApple for identification of expressing cells. Transfections were performed using the X-
835 tremeGENE 9 DNA transfection reagent (Sigma-Aldrich) following the manufacturer's instructions.
836 Cells were serum starved, but otherwise maintained in normal growth conditions – at 37°C with 5%
837 CO₂, by incubation in serum-free Dulbecco's modified Eagle's medium for 2.5 h. Serum-free medium
838 was supplemented with genistein (600 μM, Cayman Chemical Company) to inhibit caveolae-mediated
839 uptake of transferrin following Myo6 depletion as previously reported¹⁶. During the final 10 min of
840 serum starvation, Alexa fluor 488-conjugated transferrin (ThermoFisher) was added to the culture
841 medium at 25 μg/ml. Following serum starvation, plates were placed on ice and washed twice with 10
842 mM HCl and 150 mM NaCl to remove cell surface-bound transferrin. The cells were fixed with ice-cold
843 4 % paraformaldehyde for 20 min and stained with rabbit anti-dsRed antibody (rb pc) (TakaraBio, Cat#
844 **632496, Lot# 2103116, used at 1:4000 dilution for IF**), and Alexa fluor 568-conjugated anti-rabbit
845 secondary antibody to identify cells expressing mApple-tagged Myo6. Image acquisition was
846 performed with a Leica Application Suite X software on Leica TSC-8 confocal system using a 40X oil
847 immersion objective lens (n.a. = 1.3). Transferrin uptake was determined using ImageJ software: the
848 total transferrin-conjugated fluorescence intensity from sum slice projections of individual cells was
849 subsequently normalized by cell size. Comparative samples were stained, imaged, and processed
850 simultaneously under identical conditions. Data were subjected to one-way analysis of variance with
851 Tukey post-hoc comparison of individual groups to determine statistical significance.

853 **Statistics & Reproducibility**

854 For the transferrin endocytosis assay, cells examined over 2 independent experiments (number of cells
855 examined by conditions: WT=62, KO=58, KO+ FLMyo6 (WT)=79, KO+ FLMyo6
856 (T888D.R892E.V903D)=66). All fluorescence images were acquired from random fields of view. A one-
857 sided one-way analysis of variance (ANOVA) with Tukey post-hoc comparison was performed. For
858 studying Myo6 recruitment to melanosomes, ~20-30 cells were examined over 2-3 independent
859 experiments for each condition. For statistical analysis, a two-sided unpaired t test with Welch's
860 correction was performed. Only cells for which both Myo6 construct, and MST construct transfection
861 were successful were taken into account. Non or less-pigmented cells, cells with low fluorescence
862 intensity and/or low expression precluding signal thresholding and further quantification were
863 excluded (~11% of cells excluded). Statistical analyses were performed with GraphPad Prism. Sample
864 sizes were chosen to reach statistical significance, and data were reproducible. The investigators were
865 not blinded.

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Data availability

The atomic model of the Myo6 proximal dimer is available on the PDB⁶⁶ under the accession code 8ARD [<https://doi.org/10.2210/pdb8ard/pdb>]. The mass spectroscopy data supporting Sup Fig. 4 data have been deposited to the ProteomeXchange Consortium via the PRIDE⁶⁷ partner repository with the dataset identifier PXD044767. Source data are provided with this paper.

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1020

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1037

1038 Author contributions

1039

1040 A.H. and H.L.S. designed and directed the research. L.C., V.J.P.-H., K.J.P. and C.D. were involved in
1041 project management. H.S., X.L., L.H., L.C., T.L., C.K., L.V., A.D., E.F., P.H., V.J.P.-H., D.M. cloned,
1042 produced and purified constructs. L.C., T.L., C.K., H.S., L.H., A.D., L.V., P.H., V.J.P.-H., D.M. performed
1043 biophysical and biochemical assays. C.K. performed grid optimization for negative staining EM that
1044 were collected by ADC. D.L. provided access to the 200kV microscope. E.D. provided access to mass
1045 spectrometry equipment. C.K. analyzed the negative staining data and performed the 3D
1046 reconstruction. H.S. characterized the limits of the proximal dimerization region, crystallized it and C.K.
1047 solved the structure. F.M., Y.I.L. and K.J.P. performed the cell-based assays. T.E.M. performed
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1052

1053 Competing interests

1054

1055 The authors declare no competing financial interests.

1056 Tables

1057

Head	Tail	Motor state	K_D (nM)	ΔG° (kcal/mol)	n
MD ^{Ins2/IQ/3HB}	YFP ^{CBD}	ADP.VO ₄	144 ± 61	-9.3	3
MD ^{Ins2}	YFP ^{CBD}	ADP.VO ₄	343 ± 197	-8.8	3
MD	YFP ^{CBD}	ADP.VO ₄	3920 ± 1453	-7.4	4
Ins2/IQ/3HB	YFP ^{CBD}	-	250 ± 86	-9.0	4
MD ^{Ins2/IQ/3HB}	YFP ^{CBD}	NF	726 ± 480	-8.4	2
MD ^{Ins2/IQ/3HB}	YFP ^{CBD} _{D1157V.Y1159D.D1161R.Q1163V}	ADP.VO ₄	n.b.	-	3

1058

1059 **Table 1** – Main contacts that stabilize the back-folded conformation

1060 Dissociation constant (K_D) ± K_D confidence (with a 68% confidence using the NTAanalysis software)
1061 determined by microscale thermophoresis of Myo6 Head constructs against Myo6 Tail constructs
1062 (constructs schematized in Fig. 1A). Standard Gibbs free energy were obtained from the K_D values, using
1063 the quantitative relationship $\Delta G = RT \ln(K_D)$. Microscale thermophoresis profiles are presented in Sup Fig.
1064 3. Source data are provided as a Source Data file.

1065

1066

Construct	Fluorescence Ratio without Actin + ATP	Fluorescence Ratio with Actin + ATP	Molar Ratio of Labeling per Myosin Head
MD ^{Ins2/IQ/3HB} T845C	256.1 ± 24.4	243.8 ± 14.5	1.03

MD ^{Ins2/IQ/3HB} T845C, A880C	22.5 ± 5.8	22.6 ± 7.2	2.10
zippered dimers A880C	238.2 ± 23.7	232.3 ± 30.4	1.11
zippered dimers T845C, A880C	206.5 ± 19.6	214.3 ± 12.6	2.22
zippered dimers T845C, A880C, L926Q	147.53 ± 30.4	164.6 ± 18.7	2.14

1067

1068 **Table 2 – L926Q stabilizes the 3HB.**

1069 Fluorescence observed by TMR labeling of one or two cysteine residues inserted into the three-helix
 1070 bundle of monomers (MD^{Ins2/IQ/3HB}) and zippered dimers. Fluorescence was analyzed by a ratio of the
 1071 emission values to that of the absorption values for each construct from four independent
 1072 measurements (n=4). Mean values (±SD) are reported. The molar ratio was calculated by comparing
 1073 the myosin concentration to the concentration of the incorporated TMR.

1074

1075 Figure Legends/Captions

1076

1077 **Figure 1 – Importance of ADP.P_i for the compact, back-folded Myo6 conformation.**

1078 (a) Schematic representation of FLMyo6 with the Motor domain (MD, grey), CaM binding sites
 1079 (Ins2/IQ, purple/red), CaM (lilac/pink), 3-helix bundle (3HB, blue), single alpha helix (SAH, green), distal
 1080 Tail (DT, orange) and CBD (brown). Residue numbers correspond to human Myo6, Uniprot entry
 1081 Q9UM54-2. (b) Dimensionless Kratky plot representation from SEC-SAXS. FLMyo6 in the presence of
 1082 ADP.AIF₄ (a widely used ADP.P_i analog that stabilizes the pre-powerstroke of Myo6) (green) results in a
 1083 bell-shaped spectrum with a maximum close to the intersection of the dashed lines (v3:1.104), typical
 1084 of a globular protein. The spectrum for FLMyo6 in NF/high salt (black) suggests a much more elongated
 1085 shape. Source data are provided as a Source Data file. (c) Representation of the *ab initio* SAXS envelope
 1086 of Myo6 in ADP.AIF₄ condition (green) with MD^{Ins2-IQ-3HB} docked. Myo6 adopts a compact conformation
 1087 that requires Myo6 to fold back after the 3HB domain (see Methods and Sup Data 2A-B). (d) Scheme
 1088 representing the interactions stabilizing the Myo6 back-folded state. (e) Example of a negative staining
 1089 micrograph of Jo-Myo6-In in ADP.VO₄ (representative of 25 grids prepared with 2 different protein
 1090 batches) with selected 2D classes overlaid (from left to right: 8630; 9284; 9261; 7822 and 7179
 1091 particles averaged, respectively). (f) EM density for Jo-Myo6-In (grey mesh) obtained by negative
 1092 staining. Myo6 fragments and Jo-In were manually docked inside the negative staining 3D
 1093 reconstruction (see Methods). Negative staining 3D-reconstruction and the *ab initio* SAXS envelope
 1094 exhibit similar overall size and shape (Sup Data 2C). (g) (Top) Crystal structure of the Myo6 C-terminus
 1095 (CBD^c) (PDB: 3H8D <https://doi.org/10.2210/pdb3h8d/pdb>). Star: highly conserved and exposed loop
 1096 between the β_A and β_B strands. (Bottom) Alignment of Myo6 CBD^c domain (aa 1143 to 1262 in
 1097 Q9UM54-2) from different species. Strictly conserved and similar residues are shown in blue and red,
 1098 respectively. Stars: residues implicated in binding to the Myo6 Head (Table 1). (h) CBD^c (brown) added
 1099 to the negative staining-based model pictured in (F), (see Methods). The distances between Jo C-
 1100 terminus and Myo6 N-terminus; and between Myo6 C-terminus and In N-terminus are indicated.

1101

1102

1103

1104 **Figure 2 – Role of the proximal Myo6 sequence in the stabilization of the off-state.**

1105 (a) Model of Myo6 opening/back-folding. Back-folding requires the SAH to fold back on the 3HB. The
 1106 L926 residue (red cross) leads to deafness when mutated into Gln³⁹. (Insert) Mutations of the apolar
 1107 residues at the N-terminus of the SAH to turn Myo6 into a constitutive monomer¹⁶ (SAHmimic). (b)

1108 Dimensionless Kratky plot representation from SEC-SAXS. FLMyo6 in NF/high salt is pictured in black.
1109 In the presence of ADP.AIF₄ (ADP.P_i analog), FLMyo6 (L926Q) (yellow) and FLMyo6 (SAHmimic) (light
1110 blue) spectrums correspond to an elongated shape, as opposed to FLMyo6 WT (green). (c) R_g of
1111 FLMyo6 WT, L926Q and SAHmimic determined by SEC-SAXS experiments (n=1) in the presence of
1112 ADP.AIF₄ (ADP.P_i analog) and FLMyo6 in NF/high salt. R_g values were extracted from linear fits of the
1113 Guinier plots shown in Sup Fig. 1C using primusqt (ATSAS suite⁵⁰). Mean ±SD. (d) Actin-activated
1114 ATPase rate of FLMyo6 WT, L926Q, SAHmimic and MD^{Ins2} (n=6). Mean ±SD. (b-d) Source data are
1115 provided as a Source Data file.

1116
1117 **Figure 3 – GIPC1 can bind to and activate the back-folded form of Myo6, while Dab2 and Tom1 can**
1118 **only bind Myo6 once the motor has been primed open.**

1119 (a) EM density for the Jo-Myo6-In (grey mesh) obtained by negative staining, as in Fig. 1H and Sup
1120 Movie 1. The WWY motif (red spheres) of CBD^c is buried. The CBDⁿ fragment (beige) (PDB: 5V6E
1121 [<https://doi.org/10.2210/pdb5V6E/pdb>]) is positioned in the remaining, uninterpreted part of the
1122 density so that the RRL motif (red spheres) on CBDⁿ and the I1072 (blue sphere) proposed to mediate
1123 interaction between ubiquitin and Myo6⁶⁸ are both exposed. Note that no experimental model exists
1124 for 36 missing residues between the CBDⁿ and CBD^c (dashed lines), and that the position of the CBDⁿ is
1125 consistent with the crosslinks found between CBDⁿ and the rest of the Myo6 molecule through
1126 crosslinking mass spectrometry of the purified FLMyo6 with disuccinimidyl sulfoxide (DSSO) (Sup Text,
1127 Sup Fig. 4A-C, Sup Table 1). Placement of elements of the Myo6 Tail within the model improved the
1128 fitting between our atomic model and the SAXS data (Sup Data 2D-F and Sup Data 3B-C). (b) Fitting of
1129 CBD^c-TOM1 structure (PDB: 6J56 [<https://doi.org/10.2210/pdb6J56/pdb>]) with CBD^c (brown) in the
1130 model presented in Fig. 1H. TOM1 (yellow) binding would result in clashes with SAH (green) and CaM
1131 (lilac). (c) Fitting of CBDⁿ (beige)-GIPC1 (light blue) structure (PDB: 5V6E
1132 [<https://doi.org/10.2210/pdb5V6E/pdb>]) as for CBDⁿ alone. GIPC1 binding seems compatible with
1133 Myo6 back-folded conformation. (d) Elutions of anti-His pull-down assays (FLMyo6 against His⁶TOM1
1134 and His⁶GIPC1) revealed using SYPRO⁶⁹ (Input and last wash pictured in Sup Fig. 6). Crosses:
1135 quantification of retained Myo6 (Image-Lab software, Bio-Rad) followed by stoichiometric
1136 normalization based on partner concentration (n=4 for WT and n=2 for SAHmimic). + means less than
1137 10% Myo6 retained; +++ means more than 20% Myo6 retained. (e) ATPase rates of FLMyo6 (WT) and
1138 FLMyo6 (SAHmimic) with 40 μM F-actin and increasing concentrations of GIPC1, TOM1 or Dab2 (n=6).
1139 Purple line: ATPase rate of MD^{Ins2} at 40 μM actin (n=6) for reference. Mean ±SD. (d-e) Source data are
1140 provided as a Source Data file.

1141
1142 **Figure 4 – GIPC1 recruits Myo6 to melanosomes independently of Myo6 closure; Dab2 and TOM1 can**
1143 **only recruit Myo6 after the motor has been primed open.**

1144 (a) Representative fixed MNT-1 cells co-expressing different GFPMyo6 (I1072A), mCherryMST and
1145 irFPVAMP7 constructs. (b) Representative fixed MNT-1 cells co-expressing different GFPMyo6 (I1072A)
1146 constructs with mCherryMST-GIPC1 and irFPVAMP7. (c) Representative fixed MNT-1 cells co-expressing
1147 different GFPMyo6 (I1072A) constructs with mCherryMST-TOM1 and irFPVAMP7. (d) Representative fixed
1148 MNT-1 cells co-expressing different GFPMyo6 (I1072A) constructs with mCherryMST-Dab2 and irFPVAMP7.
1149 (a-d) Green: Myo6 GFP; Cyan: irFPVAMP7; Magenta: mCherryMST partner. From left to right: entire cell,
1150 3 channels merged; 8x zoom on boxed region: GFPMyo6 / mCherryMST-partners merged, then individual
1151 channels. Scale bars: 10μm. Arrowheads: recruitment of Myo6 on melanosomes. (e) Myo6-positive
1152 melanosomes quantification of different GFPMyo6 mutants when different mCherryMST tagged partners
1153 are expressed (n=3, total cell number~30). Myo6-positive melanosomes are expressed in percentage
1154 and normalized to the total number of VAMP7-positive melanosomes. Cells were fixed 48h post-

1155 transfection then imaged and processed for quantification. Data are presented as the mean \pm SEM.
1156 Significant stars: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; n.s., not significant (two-sided unpaired t test
1157 with Welch's correction), for each ^{GFP}Myo6 construct, significance of experiments with partners
1158 compared to the control without partner (in black on the graph). P values are the following: FLMyo6
1159 (I1072A)/GIPC1: $p < 0.0001$, FLMyo6 (I1072A)/Dab2: $p = 0.698$, FLMyo6 (I1072A)/TOM1: $p = 0.0071$, Jo-
1160 Myo6-In (I1072A)/GIPC1: $p < 0.0001$, Jo-Myo6-In (I1072A)/Dab2: $p = 0.344$, Jo-Myo6-In (I1072A) /TOM1:
1161 $p = 0.5005$, FLMyo6 (SAHmimic.I1072A)/GIPC1, TOM1 or Dab2: $p < 0.0001$, FLMyo6
1162 (L926Q.I1072A)/GIPC1 or Dab2: $p < 0.0001$, FLMyo6 (L926Q.I1072A)/TOM1: $p = 0.003$. Source data are
1163 provided as a Source Data file.

1164

1165 **Figure 5** – Myo6 can form an anti-parallel dimer through residues 875-940 which allow large steps.

1166 (a) (Left) X-ray structure of mouse Myo6 875-940 antiparallel dimer colored according to B-factor from
1167 18.6 \AA^2 (dark blue) to 150.8 \AA^2 (red). (Right) Key residues for dimer stabilization. Apolar contacts are
1168 mediated by residues pictured in green. Dotted blue line: polar contacts. Residues mutated in our triple
1169 mutant (T888D.R892E.V903D) are underlined. (b) Close-up of the dimerization interface of Myo6 875-
1170 940 in the electronic density. (c) Triple helix bundle (PDB: 2LD3,
1171 <https://doi.org/10.2210/pdb2ld3/pdb>) domain. T888, R892, V903, T845C and A880C pictured as
1172 sticks are surface residues. (d) SEC-MALS profiles of Myo6 875-940 WT (red) and T888D.R892E.V903D
1173 mutant (blue), following injection of 50 μl at 10 mg/mL in 10 mM Tris-HCl pH 7.5; 50 mM NaCl; 5 mM
1174 NaN_3 ; 0.5 mM TCEP. Thin lines: static light scattering; thick lines: measured molecular mass. WT elutes
1175 as dimers (32 μM concentration at the peak, measured by the in-line refractometer) and
1176 T888D.R892E.V903D mutant elutes as monomers (43 μM at the peak). (e) Model of active FLMyo6
1177 dimer (see Methods). (f) ATPase rates (mean \pm SD) of FLMyo6 WT (green), T888D.Q892E.V903D (grey),
1178 SAHmimic (blue) and L926Q (yellow) at 40 μM F-actin and increasing concentrations of GIPC1 ($n=6$).
1179 ATPase rates of MD^{Ins2} and zippered dimer¹¹ without partner ($n=6$) plotted as purple and red thick
1180 lines (respectively) as references for monomeric and dimeric Myo6. (g) Fluorescence intensity of
1181 internalized transferrin was measured for each condition after treatment with genistein (cells
1182 examined over 2 independent experiments: WT=62, KO=58, KO+ FLMyo6 (WT)=79, KO+ FLMyo6
1183 (T888D.R892E.V903D)=66) ($p < 0.001$, one-way ANOVA; Tukey post-hoc comparisons; one-sided). P
1184 values are the following: WT vs KO: $p < 0.0001$, WT vs KO+ FLMyo6 (WT): $p = 0.3363$, WT vs KO+ FLMyo6
1185 (T888D.R892E.V903D): $p < 0.0001$ (***), KO vs KO + FLMyo6 (WT): $p = 0.0001$, KO vs KO + FLMyo6
1186 (T888D.R892E.V903D): $p = 0.5228$, KO + FLMyo6 (WT) vs KO+ FLMyo6 (T888D.R892E.V903D): $p = 0.0146$
1187 ($\$$). Whisker boxes (10-90 percentile with 2nd and 3rd quartiles within the box; white dot indicates the
1188 median) encased within a violin plot (generated with BoxPlotR⁷⁰). (d,f,g) Source data are provided as a
1189 Source Data file.

1190

1191 **Figure 6** – Importance of a folded monomer for regulation.

1192 (a) When auto-inhibited, Myo6 can diffuse across actin-rich regions and interacts weakly with F-actin.
1193 These weak actin interactions ($\sim 7 \mu\text{M}$ apparent affinity, estimated in Sup Fig. 2D) result in facilitated
1194 diffusion and in increasing the Myo6 concentration in actin-rich regions of the cell. Once recruited by
1195 a partner, Myo6 is activated and starts performing its cellular function. (b) Scheme representing
1196 possible activation mechanisms for Myo6. Myo6 domains are color coded: Myo6 MD (grey), Ins2/CaM
1197 (purple), IQ/CaM (red/pink), 3HB in blue, SAH (green), DT (orange), CBD (brown), and the partner
1198 binding sites (garnet). The binding site (WWY) for Dab2 and TOM1 is blocked, preventing recruitment
1199 of Myo6 without a prior unfolding signal prior to unblock their binding. GIPC1 can bind the accessible
1200 RRL motif resulting in Myo6 recruitment and opening. Other signals can act as unfolding factors such
1201 as Ca^{2+} , which can allow TOM1 to bind to Myo6. Such an activation cascade was previously proposed³⁶.
1202 Once unfolded, Myo6 potentially acts as a monomer, as previously proposed³⁵ upon TOM1 binding; or
1203 it can dimerize²⁹ through proximal dimerization, as demonstrated in this study with GIPC1 binding; or
1204 it dimerizes through distal dimerization upon Dab2 binding¹³, which may lead to proximal dimerization.









