Supplementary Material

Controllable molecular motors engineered from myosin and RNA

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Supplementary Movie Legends

Supplementary Movies 1a-b. Gliding filament assays for M6-RB:ktL. Plus-ends of actin filaments are labeled using gelsolin-capped seeds containing Cy5-actin (red). All fluorescence videomicroscopy movies have a timestamp (in seconds) located in the top right corner, and a scale bar (3 µm) located in the bottom right corner. Filament bodies are labeled with TMR-phalloidin (green). ktL was incubated at (a) 200 nM or (b) 20 nM RNA.

Supplementary Movie 2. Cryo-EM reconstruction of actin filament decorated with M6-RB:ktLshort (apo). The actin filament (shaded blue) is oriented with the (+) end down.

Supplementary Movies 3a-c. Rigid and flexible fitting of myosin:RNA model into cryo-EM density. The segmented density map of M6-RB:ktLshort (apo) bound to F-actin is shown together with (a) a rigid-body fit of the initial naïve model for M6-RB:ktLshort; (b) the model generated by flexible fitting using Direx; and (c) a morph between the initial and flexible fitting models.

Supplementary Movies 4a-b. Comparison between ADP and apo conformations for M6-RB:ktLshort bound to F-actin. (a) Overlaid segmented density maps for M6-RB:ktLshort reconstructions obtained in the presence of ADP (mesh) and without nucleotide (solid). (b) Overlaid M6-RB maps with difference maps showing density attributable to RNA in the presence of ADP (pink) and without nucleotide (purple).

Supplementary Movies 5a-b. Gliding filament assays for M6-RB:ktRS1 (no switch strand). ktRS1 was incubated at (a) 200 nM or (b) 20 nM RNA.

Supplementary Movies 6a-b. Gliding filament assays for M6-RB:ktRS1 + switch. ktRS1 was incubated at (a) 200 nM or (b) 20 nM RNA.

Supplementary Movies 7a-b. Gliding filament assays for M6-RB:ktRS1 + switch + switchback. ktRS1 was incubated at (a) 200 nM or (b) 20 nM RNA.

Supplementary Movie 8. Gliding filament assay for M6-RB:ktRS1 with dynamic switching. Buffer exchanges were used to successively introduce switch and switchback strands (indicated top left).

Supplementary Movie 9. Single-molecule imaging of M6-RB:ktL:tet. Plus-ends of actin filaments are labeled using gelsolin-capped seeds containing Cy5-actin (red). Filament bodies are labeled with Alexa 488-phalloidin (blue). Motor tetramers are labeled with Cy3-DNA (green).

Supplementary Movie 10. Single-molecule imaging of M6-RB:ktRS1:tet (no switch strand).

Supplementary Movie 11. Single-molecule imaging of M6-RB:ktRS1:tet + switch.

Supplementary Movie 12. Single-molecule imaging of M6-RB:ktRS1:tet + switch + switchback.

Supplementary Movie 13. Gliding filament assay for M6-RB:ktRS2. ktRS2 was incubated at 20 nM RNA.

Supplementary Movies 14a-b. Sequence-specific control of directionality in (a) M6-RB:ktRS1 and (b) M6-RB:ktRS2. A set of four gliding filament assays is shown for each RNA construct, showing responses to complementary and orthogonal control strands. Text overlays (top left of each assay) indicate the combination of RNA, switch, and switchback strands used. ktRS1 and ktRS2 were incubated at 20 nM RNA.
Supplementary Figures

Supplementary Fig. 1. Protein construct diagram. Sequence detail shows the junction between myosin VI and L7Ae. ‘\( \sim \)', \((\text{GSG})_4\), flexible linker.

Supplementary Fig. 2. RNA construct diagrams. Sequences and predicted secondary structures are shown with modules colored: kink-turn (pink), reverse motif (blue), and switching loop (green). a, ktLinear. b, ktLshort. c, ktReverseSwitch1. d, ktReverseSwitch2.
**Supplementary Fig. 3.** Cryo-EM data collection and statistics. **a,** Representative micrographs for the four specimens used to obtain reconstructions in this study. **b,** Cryo-EM 3D reconstructions for the four specimens. **c,** Fourier Shell Correlation (FSC) curves of independently reconstructed half-reconstructions for the indicated specimens. Dashed line represents FSC=0.143. **d,** Myosin:RNA modeling based on cryo-EM. The initial naïve model (orange) for M6-RB:ktLshort (apo) is superimposed with the model (teal) obtained by flexible fitting to the cryo-EM density.
Supplementary Fig. 4. Full MOHCA-seq proximity maps and comparison of MOHCA-seq data to naïve 3D model. a, Proximity maps of ktRS1 RNA probed by MOHCA-seq in the absence (left) or presence (right) of bound switch strand. The ktRS1 RNA includes the 3’ overhang used for surface attachment or multimerization in functional assays (gray region on x- and y-axes), and the additional signal in lower-right-hand region (below the line marked “3’ overhang”; excluded in Fig. 3) may arise from folding back of the overhang to interact with the lever arm helix. b, Proximity map of non-switched ktRS1 RNA together with overlaid contours (purple) predicted from a naive approximate 3D model for this design (at right). Regions enclosed within contours represent pairs of residues for which the 2'-OH of the x-axis residue is 30 Å or less from the C4' of the y-axis residue in the model. Scored MOHCA-seq hits (Supplementary Table 4) are indicated on the proximity map by red circles, and on the 3D model by bars connecting the two residues: Strong hits = thick circles or bars; weak hits = thin circles or bars; satisfied constraints (2'-OH-to-C4' distance ≤ 30 Å) = solid circles or red bars; unsatisfied constraints (distance > 30 Å) = dashed circles or white bars.
Supplementary Fig. 5. Nucleic acid tetramer assembly gels. All gels are non-denaturing 1X TAE. For detailed information on the gel conditions and complex formation see Supplementary Methods - Gel Electrophoresis. a, SYBR-gold stained gel showing formation of DNA tetramer complex that included cyclized tet (lane 2) bound to four t-c3 (lane 1) strands. In lanes 9-13, t-c3 was titrated against tet to find saturating conditions where all four sites were occupied. A ratio of 1:6, of tet to t-c3 (lane 7), was used for the single-molecule microscopy experiments. The lanes marked with ‘x’ contain constructs not included in the present study. b, Cy3 fluorescent scan of the same gel as in a. c, SYBR-gold stained gel showing formation of the ktRS1 tetramer (ktRS1:tet:t-c3), consisting of four ktRS1 RNAs bound to tet:t-c3. In lanes 4-9, ktRS1 was titrated against tet:t-c3 to find saturating conditions where four ktRS1 RNAs were bound. d, Cy3 fluorescent gel scan of the same gel in c.
Supplementary Fig. 6. Myosin:RNA:DNA tetramer assembly gel. Combined fluorescent scan (Cy5 and YFP) of gel showing the formation of the tetramer including the protein M6-RB. In this false-color CMY image, Cy5 signal is displayed in magenta and YFP signal is displayed in cyan; bands containing both signals appear purple. In lanes 4-8, M6-RB was titrated against kRS1:tet:t-c5. At substoichiometric [M6-RB] (lane 6), a ladder of five kRS1:tet:t-c5 bands can be seen, corresponding to 0-4 bound M6-RB proteins. When an excess of M6-RB over RNA is used, the major kRS1:tet:t-c5 band (~70% on the basis of Cy5 fluorescence) corresponds to four bound M6-RB proteins. A secondary band (*) may be attributed to an incomplete assembly, lacking the full complement of RNA molecules and motor proteins. Incomplete assemblies (**) can also be seen as a minor contaminant in the starting nucleic acid material.
Supplementary Table 1

Gliding filament results. Data included in each numbered experiment (column 1) were obtained with the same material preparations on the same day. Conditions represented in experiments 1-8 were tested with at least two different preparations of RNA and two different preparations of protein. Experiments 9 and 10 were performed with the same set of RNA and protein preparations. Experiments were performed as described in Methods, using either 20 nM or 200 nM RNA as indicated. n (+), number of polarity-marked filaments scored moving with plus-ends trailing; n (-), number of polarity-marked filaments of scored moving with minus-ends trailing. A subset of directionality-scored filaments were used for velocity analysis. Mean filament velocities are reported ± s.d., with n reporting the number of filaments used for the velocity measurement; directionality statistics for this filament subset are shown in parentheses when non-uniform.

| Experiment # | Condition | [RNA] (nM) | n (-) | n (+) | Velocity (nm/s) | Movie # |
|--------------|-----------|------------|-------|-------|-----------------|---------|
| 1            | ktL       | 20         | 8     | 0     | - 22.3 ± 3.1    | n = 8   | -       |
| 2            | ktRS1     | 20         | 1     | 21    | + 5.5 ± 3.5     | n = 17 [1 (-), 16 (+)] | -       |
|              | ktRS1     | 20         | 0     | 11    | + 6.8 ± 2.1     | n = 10  | -       |
|              | ktRS1 + sw1 | 20      | 15    | 0     | - 22.6 ± 3.7    | n = 15  | -       |
|              | ktRS1 + sw1 + sb1 | 20 | 1     | 17    | + 7.4 ± 5.5     | n = 15 [1 (-), 14 (+)] | -       |
| 3            | ktRS1     | 200        | 0     | 47    | + 8.1 ± 1.8     | n = 21  | -       |
|              | ktRS1 + sw1 | 200     | 44    | 0     | - 11.4 ± 1.3    | n = 20  | -       |
|              | ktRS1 + sw1 + sb1 | 200 | 0     | 28    | + 4.9 ± 1.6     | n = 15  | -       |
|              | ktL       | 200        | 29    | 0     | - 25.6 ± 2.0    | n = 20  | 1a      |
| 4            | ktRS1     | 200        | 0     | 34    | + 6.7 ± 1.0     | n = 20  | 5a      |
|              | ktRS1 + sw1 | 200     | 14    | 0     | - 15.2 ± 0.9    | n = 12  | 6a      |
|              | ktRS1 + sw1 + sb1 | 200 | 0     | 30    | + 3.1 ± 0.6     | n = 20  | 7a      |
|              | ktL       | 200        | 10    | 0     | - 23.8 ± 3.1    | n = 8   | -       |
| 5            | ktRS1     | 20         | 0     | 83    | + 4.9 ± 1.1     | n = 28  | -       |
| 6            | ktRS1 + sw1 | 20       | 24    | 1     | - 7.2 ± 4.3     | n = 17 [16 (-), 1 (+)] | -       |
|              | ktRS1 + sw1 + sb1 | 20 | 0     | 19    | + 4.7 ± 1.4     | n = 12  | -       |
| 7            | ktRS1     | 20         | 0     | 73    | + 1.7 ± 0.5     | n = 26  | 5b      |
|              | ktRS1 + sw1 | 20       | 80    | 0     | - 8.9 ± 0.9     | n = 32  | 6b      |
|              | ktRS1 + sw1 + sb1 | 20 | 0     | 22    | + 2.3 ± 0.9     | n = 19  | 7b      |
|              | ktL       | 20         | 60    | 0     | - 21.5 ± 2.5    | n = 23  | 1b      |
| 8            | ktL       | 20         | 59    | 0     | - 23.1 ± 1.8    | n = 24  | -       |
| 9            | ktRS1 + sw2 | 20       | 0     | 59    | + 3.1 ± 1.2     | n = 42  | -       |
|              | ktRS1 + sw1 + sb2 | 20 | 77    | 0     | - 11.1 ± 1.7    | n = 65  | -       |
|              | ktRS2 + sw1 | 20       | 68    | 0     | + 3.5 ± 1.4     | n = 58  | -       |
|              | ktRS2 + sw2 + sb1 | 20 | 70    | 0     | - 4.9 ± 1.6     | n = 53  | -       |
|              | ktRS2     | 20         | 0     | 85    | + 4.6 ± 1.1     | n = 67  | -       |
|              | ktRS2 + sw2 | 20       | 78    | 0     | - 17.8 ± 1.6    | n = 60  | -       |
|              | ktRS2 + sw2 + sb2 | 20 | 2     | 57    | + 2.7 ± 1.4     | n = 50 [2 (-), 48 (+)] | 14b     |
|              | ktRS1 + sw2 | 20       | 0     | 44    | + 5.4 ± 1.6     | n = 38  | 14a     |
|              | ktRS1 + sw1 | 20       | 20    | 0     | - 13.5 ± 1.5    | n = 20  | 14a     |
|              | ktRS1 + sw1 + sb1 | 20 | 1     | 27    | + 2.6 ± 1.6     | n = 24 [1 (-), 23 (+)] | 14a     |
|              | ktRS1 + sw1 + sb2 | 20 | 74    | 0     | - 11.9 ± 2.1    | n = 62  | 14a     |
|              | ktRS2 + sw1 | 20       | 0     | 38    | + 10.7 ± 2.8    | n = 36  | 14b     |
|              | ktRS2 + sw2 | 20       | 55    | 0     | - 17.9 ± 2.0    | n = 46  | 14b     |
|              | ktRS2 + sw2 + sb2 | 20 | 55    | 0     | - 17.9 ± 2.0    | n = 46  | 14b     |
|              | ktRS2 + sw2 + sb1 | 20 | 0     | 23    | + 2.4 ± 1.5     | n = 21  | -       |
|              | ktRS1     | 20         | 0     | 37    | - 11.3 ± 3.0    | n = 31  | 14b     |
|              | ktRS2     | 20         | 0     | 42    | + 4.8 ± 1.4     | n = 40  | 13      |
### Supplementary Table 2

| Experiment # | Condition | [RNA] (nM) | n (-) | n (+) | Velocity (nm/s) |
|--------------|-----------|-----------|-------|-------|-----------------|
| 1, 7, 8      | ktL       | 20        | 127   | 0     | -22.3 ± 2.4     | n = 55 |
| 3 and 4      | ktL       | 200       | 39    | 0     | -25.1 ± 2.5     | n = 28 |
| all          |           | 20 and 200| 166   | 0     | -23.2 ± 2.8     | n = 83 |
| 2, 5, 7, 9   | ktRS1     | 20        | 1     | 225   | +4.4 ± 2.4      | n = 115 [1 (-), 114 (+)] |
| all          |           | 20 and 200| 1     | 306   | +5.2 ± 2.6      | n = 156 [1 (-), 155 (+)] |
| 2, 6, 7, 10  | ktRS1 + sw1 + sb1 | 200       | 0     | 81    | +7.4 ± 1.6      | n = 115 [1 (-), 114 (+)] |
| all          |           | 20 and 200| 1     | 143   | +3.9 ± 2.9      | n = 105 [2 (-), 103 (+)] |
| 9 and 10     | ktRS1 + sw2 | 20        | 0     | 103   | +4.2 ± 1.8      | n = 80  |
| 9 and 10     | ktRS2 + sw2 + sb2 | 20        | 0     | 151   | -11.5 ± 2.0     | n = 127 |
| 9 and 10     | ktRS2 + sb2 | 20        | 0     | 133   | -17.9 ± 1.8     | n = 106 |
| 9 and 10     | ktRS2 + sw1 + sb1 | 20        | 0     | 80    | +2.6 ± 1.4      | n = 71  [2 (-), 69 (+)] |
| 9 and 10     | ktRS2 + sb1 | 20        | 0     | 106   | +6.3 ± 4.0      | n = 94  |

**Supplementary Table 2.** Compiled gliding filament results. Results are totaled over all experiments for each condition from Supplementary Table 1.

### Supplementary Table 3

| Condition | RNA Prep | n (-) | n (+) | Velocity (nm/s) | Distance Traveled (µm) | Movie # |
|-----------|----------|-------|-------|-----------------|-------------------------|---------|
| ktL       | 1        | 61    | 0     | -37.8 ± 1.1     | 1.39 ± 0.10              | -       |
|           | 2        | 72    | 0     | -36.9 ± 1.1     | 1.54 ± 0.14              | 9       |
|           | 3        | 36    | 0     | -32.3 ± 0.6     | 2.05 ± 0.18              | -       |
| ktRS1     | 1        | 3     | 74    | 20.8 ± 1.5      | 1.21 ± 0.08              | 10      |
|           | 2        | 0     | 54    | 19.4 ± 1.0      | 1.59 ± 0.17              | -       |
| ktRS1 + sw1 | 1    | 48    | 2     | -12.4 ± 1.5     | 1.39 ± 0.14              | -       |
|           | 2        | 50    | 3     | -12.8 ± 1.2     | 1.16 ± 0.14              | 11      |
| ktRS1 + sw1 + sb1 | 1 | 15   | 115   | 19.1 ± 1.2      | 1.52 ± 0.08              | 12      |
|           | 2        | 1     | 10    | 18.4 ± 3.6      | 1.61 ± 0.31              | -       |
| ktL       | 1, 2, 3  | 169   | 0     | -36.2 ± 0.6     | 1.59 ± 0.08              | -       |
| ktRS1     | 1, 2     | 3     | 128   | 20.2 ± 1.0      | 1.36 ± 0.08              | -       |
| ktRS1 + sw1 | 1, 2   | 98    | 5     | -12.6 ± 0.9     | 1.28 ± 0.10              | -       |
| ktRS1 + sw1 + sb1 | 1, 2 | 16   | 125   | 19.1 ± 1.1      | 1.53 ± 0.08              | -       |

**Supplementary Table 3.** Single-molecule tracking results. Each RNA construct and condition was tested with at least two different preparations of RNA. The numbers of motor traces moving toward the (+) and (-) ends of the actin filaments are tabulated for each condition along with the velocity (± s.e.m.) and absolute distance traveled (± s.e.m) averaged over all traces for that condition. Distances traveled are reported as raw averages, and are not equivalent to processive run lengths. Results compiled over all experiments are shown in the bottom portion of the table, below the dashed line.
**Supplementary Table 4**

| ktRS1 (non-switched) | Modification position | Cleavage position | Strength of hit |
|----------------------|-----------------------|-------------------|-----------------|
| 69                   | 111                   | Strong            |
| 69                   | 143                   | "                 |
| 82                   | 149                   | "                 |
| 95                   | 160                   | "                 |
| 98                   | 145                   | "                 |
| 56                   | 68                    | Weak              |
| 56                   | 87                    | "                 |
| 56                   | 95                    | "                 |
| 57                   | 79                    | "                 |
| 63                   | 108                   | "                 |
| 64                   | 86                    | "                 |
| 64                   | 97                    | "                 |
| 64                   | 73                    | "                 |
| 73                   | 155                   | "                 |
| 74                   | 90                    | "                 |
| 74                   | 143                   | "                 |
| 79                   | 160                   | "                 |
| 87                   | 103                   | "                 |
| 87                   | 149                   | "                 |
| 88                   | 160                   | "                 |
| 119                  | 133                   | "                 |

**Supplementary Table 4.** Pairwise MOHCA-seq hits. Two categories of hits were identified, corresponding to "strong" and "weak" hits as described previously.
Supplementary Methods

Molecular Design

Protein Modeling. An initial structural model for M6-RB in the post-stroke state was constructed by fusing myosin VI (PDBID: 2BKH, residues 4-817) with L7Ae (PDBID: 1RLG, residues 9-118) after using PyMOL’s native builder to rebuild and extend 2BKH from 810 to 817, extending the N-terminal α-helix of 1RLG with three residues that overlap 2BKH (815-817), and aligning the terminal helices. A model for the pre-stroke state was constructed by α-helical alignments using a pre-stroke structure of myosin VI (4PFO) up to residue 787, myosin VI insert 2 (4ANJ) for residues 788-811, and the previously constructed post-stroke model of M6-RB from residue 812 onward.

ktLinear Design. The sequence for the kink-turn4 was fixed and NUPACK5 was used to design the sequence for the 40 bp duplex arm extension.

ktRS1 Design. Initial NUPACK runs were performed while keeping the sequences for the both the kink-turn and reverse-motif6 fixed. The duplex arm extension sequence was then modified by hand to replace standard Watson Crick base pairs with G-U wobble pairs, roughly every 5 base pairs, along the length of the duplex extension to minimize secondary structure in the DNA template and facilitate sequencing. The switch-loop sequence and the adjacent short duplex were then further modified to function as part of a stem-loop strand inversion system with the switch and switchback strands. Assemble2 (with Chimera) was used to construct a structural model of ktRS1 (Supplementary Fig. 4b). In Assemble2, a small library of the relevant RNA motifs (the two fragments for the reverse motif, and the kink-turn7) was compiled and used for threading. Inspired by Geary et al., we modeled the reverse junction using fragments from PDB entries 1JJ2 and 1U6B. The crossover junction fragment was taken from 1JJ2 AGG (1632-1634), UC (1569-1570), and GG (1627-1628). The GAAA/11 nt interaction fragment was taken from 1U6B GAAA (24-27), CUAG (147-152), CUAUG (159-163). After importing the sequence for ktRS1, the secondary structure was constructed. The three-dimensional model was then generated by threading the sequence onto the secondary structure, the structures of the two fragments for the reverse motif, and the structure of the kink-turn, and finally fitting the pieces together at helical junctions. An initial version of the ktRS1 model with similar overall geometry was also constructed by using Pymol to manually assemble fragments from the 1JJ2 junction, a fragment from the 11 nt receptor in 1U6B, and helical segments generated in Assemble1, then refining in Assemble1 and later modifying and rethreading in Assemble2.

ktRS2 Design. The sequence of ktRS2 was generated modifying the loop sequence of ktRS1, both by hand and in NUPACK, in order to reduce sequence overlap with ktRS1.

Docking and Modeling of Hybrid Motor Assemblies. RNA models were docked to protein models by using four bases in the kink-turn (GUGA) to align the RNA with a model of L7Ae bound to a kink-turn (1RLG) using PyMOL’s pair_fit command. Docked models were used for iterative feedback to manually optimize phasing of secondary structure elements in the RNA designs. ktL and ktRS were docked with pre- and post-stroke models to generate three-dimensional models of the RNA-myosin chimeras. Finally, as previously described17, actin-bound models were created to visualize predicted strokes, guided by a previous electron microscopy-based model of an actomyosin rigor complex (PDBID: 1M8Q).

Gel Electrophoresis

Nucleic acid tetramer complex ktRS1 + tet:t-c3. 160 nM of ktRS1 was annealed in TAB buffer (see motility assay protocol for TAB) containing 8 mM MgCl₂ and no DTT, for 2 min at 90 C, and then snap-cooled and stored on ice. To prepare the tet:t-c3 complex, 100 nM tet and 500 nM of t-c3 (see RNA and DNA sequences) were annealed together in TAB buffer (8 mM MgCl₂ and no DTT) for 5 min. at 90 C, 10 min. at 65 C, 15 min. at 45 C, 20 min. at 37 C, and then RT for at least 20 min. Then, in 10 µl volumes, ktRS was annealed to tet:t-c3 at RT for 30 minutes, with tet:t-c3 held at 15 nM, and ktRS1 at increasing concentrations of: 20 nM, 40 nM, 60 nM, 80 nM, 100 nM and 120 nM. The titrations, and the individual components, were then run on a 4 % non-denaturing PAGE gel containing 1X TAE (2 mM EDTA) and 8 mM MgCl₂.
Nucleic acid-protein tetramer complex ktRS1:tet:t-c5 + M6-RB. 180 nM of ktRS1 was annealed in TAB buffer (see motility assay protocol for TAB) containing 8 mM MgCl₂ and no DTT, for 2 min at 90 C, and then snap-cooled and stored on ice. To prepare the tet:t-c5 complex, 100 nM tet and 600 nM of t-c5 (see RNA and DNA sequences) were annealed together in TAB buffer (8 mM MgCl₂ and no DTT) for 5 min. at 90 C, 10 min. at 65 C, 15 min. at 45 C, 20 min. at 37 C, and then RT for at least 20 min. To form the nucleic acid complex, 135 nM ktRS1 was annealed to 15 nM tet:t-c5 at RT for 30 minutes. Finally, 10 nM of ktRS1:tet:t-c5 was combined with M6-RB at a series of protein concentrations as indicated in Supplementary Fig. 6. The titrations, and control lanes containing the individual components, were then run on a 4 % non-denaturing PAGE gel containing 1X TAE (2 mM EDTA) and 10 mM MgCl₂.

Imaging. Immediately after electrophoresis, all three gels were scanned on a laser gel scanner (Amersham Typhoon) using filter sets for cy3 or cy5, to image the fluorescently labeled tethers (t-c3 or t-c5). The gel containing YFP-labeled M6-RB protein (Supplementary Fig. 6) was scanned a second time using a filter set for YFP, to image the fluorescent protein. After initial scanning, the two nucleic acid gels (Supplementary Fig. 5) were stained with SYBR-gold and rescanned to image all nucleic acids (Supplementary Fig. 5a and 5c).

Cryo-EM

Cryo-EM sample preparation. For samples including RNA, myosin:RNA complexes were prepared beforehand: ktLshort was annealed in TAB by heating to 90C for 2 minutes and snap-cooling on ice, and then added at 2X molar excess to protein (M6-RB stored in 150 mM KCl, 5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 20 mM imidazole pH 7.5, 3 mM DTT, supplemented with 5 µM calmodulin), and incubating for 15 min at RT before storage of complexes for up to one day on ice. For all samples, F-actin and myosin (or myosin:RNA) were diluted to 0.03-0.6 µM and 2-4 µM, respectively, in KMEI (50 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 10 mM imidazole pH 7.0, 1 mM DTT). For nucleotide free conditions, myosin samples were supplemented with 5 mM Mg-ADP pH 7.0. F-actin (3 µL) was applied to a plasma-coated holey carbon grid (Protochips) in the humidified chamber of a Leica GP plunge freezer and incubated for 60 s at 25 °C. Myosin (3 µL) was then applied and incubated for 60 s. Solution (3 µL) was then removed and an additional 3 µL of myosin was applied. After an additional 60 s, 3 µL of solution was removed, and then the grid was blotted for 2-3 s from the back with filter paper (Whatman no. 5) and plunged-frozen in liquid ethane.

Cryo-EM data collection. Cryo-EM data were collected with the Leginon software on a Tecnai F20 operating at 200 kV using a Gatan K2 Summit direct electron detector in counting mode. Movies were collected with an exposure of 0.25 s/frame for a total of 6.0 s at a dosage of 6 e⁻/Å²/s ( < 10 e⁻/pix/s) yielding a total cumulative dose of 36 e⁻/Å². Data were collected at 1.5-3 µm underfocus at a nominal magnification of 29,000x, corresponding to a calibrated pixel size of 1.27 Å at the specimen level.

Image Processing. Image frames were aligned and summed with Unblur. Contrast transfer function (CTF) estimation and extraction of segments was performed in the Appion data-processing environment. Unless otherwise specified, 2D image processing operations were carried out using proc2d from the EMAN processing package. CTF parameters were estimated with CTFIND3. Segments were windowed in 512-pixel boxes with 81Å of non-overlap corresponding to a step-size of 3 actin protomers, normalized with xmipp_normalize, then binned by 2. Segments were extracted for each of four separate conditions: M6-RB (ADP) (27,655 segments from 506 images), M6-RB:ktLshort (ADP) (10,748 segments from 226 images), M6-RB (apo) (13,205 segments from 318 images), and M6-RB:ktLshort (apo) (27,856 segments from 374 images).

For 3D refinement and reconstruction, we adapted the IHRSR protocol recently described in Kim et al. which performs initial refinement and reconstruction using functions from the SPARX/EMAN2 libraries and helical search using the program hsearch_lorentz of Egelman, followed by final refinement and reconstruction using FREALIGN. Briefly, segments were extracted from phase-flipped images, then refined against an initial model generated by low-pass filtering an actin reconstruction (EMD-1990) to 35 Å. The reconstruction obtained from this refinement run was then low-pass filtered to 35 Å and used an initial model for a second round of refinement, where poorly aligning segments were excluded using a cross-correlation cutoff of 1.5 σ. Segments with correlation scores below the cutoff were then divided into two random half-datasets, and independent refinement of these half-datasets (to minimize noise bias) was re-initialized using the same low-pass filtered initial model. After each round of refinement, the asymmetric reconstructions of the half-datasets were summed, and the sum was used to calculate new helical parameters. These helical parameters were then applied to each half-reconstruction independently, which were then compared and low-pass filtered based on the Fourier Shell Correlation (FSC) to provide the references for the next round of refinement.
After refinement in EMAN2/SPARX, un-binned segments were re-extracted using alignparts_lmbfgs\textsuperscript{22} to correct for non-uniform beam-induced drift and apply an exposure-dependent filter to maximize signal at all spatial frequencies\textsuperscript{10}. Parameters from the half-data sets were recombined, then final refinement and reconstruction was performed with FREALIGN v 9.11 using fixed helical parameters and a strict low-pass filter of 12 Å. The final resolutions were determined based on the FSC 0.143 criterion\textsuperscript{23} as 7.4 Å (M6-RB(ADP)), 6.7 Å (M6-RB:ktLshort (ADP)), 6.8 Å (M6-RB(apo)), and 6.1 Å (M6-RB:ktLshort (apo)) (Supplementary Fig. 3). Although all four reconstructions reached sub-nanometer resolution globally, the L7Ae domain and bound RNA moiety were only visible when maps were low-pass filtered at 13 Å. The high-resolution features of these maps, which describe the myosin-VI-actin interface, will be described elsewhere (manuscript in preparation). Difference maps were calculated using the program DIFFMAP (http://grigoriefflab.janelia.org/diffmap).

**MOHCA-seq**

**RNA preparation for MOHCA-seq.** RNA was prepared for MOHCA-seq based on a previously published protocol\textsuperscript{3} with minor modifications. The double-stranded DNA (dsDNA) template for transcribing RNA for MOHCA-seq was generated by PCR from the ktRS1 plasmid with primers (see DNA and RNA sequences) purchased from IDT (Integrated DNA Technologies, San Diego, CA) using standard Phusion DNA polymerase conditions in HF buffer (New England Biolabs [NEB], Ipswich, MA), except that 4 µM of each primer was used. The PCR protocol was: 98°C for 30 sec; 35 cycles of 98°C for 10 sec, 65°C for 30 sec, and 72°C for 30 sec; and 72°C for 10 min. The ktRS1 RNA was transcribed at 37°C for 4 hr in an 840 µl reaction containing 0.2 µm dsDNA template, 100 mM Tris–HCl, pH 8.1, 200 mM MgCl\textsubscript{2}, 3.5 mM spermidine, 0.1% Triton X-100, 40 mM DTT, 4% PEG 8000, 4200 U T7 RNA Polymerase (NEB), 1 mM NTPs, and 0.5 mM 2'-NH\textsubscript{2}-2'-deoxy-ATP (TriLink BioTechnologies, San Diego, CA). The yield of transcription was ~3.5 nmol after purification using an RNA Clean & Concentrator 100 column (Zymo Research, Irvine, CA). The hydroxyl radical source, isothiocyanobenzyl-EDTA chelating Fe(III) (ITCB-Fe(III)-EDTA) (Dojindo Molecular Technologies, Inc., Santa Clara, CA), was covalently attached to the 2'-NH\textsubscript{2} groups on the RNA backbone using a two-step process. First, ITCB-EDTA was coupled to the RNA by mixing 60 µl 100 mM ITCB-EDTA dissolved in 0.4 M KPO\textsubscript{4}, pH 8.5 with 70.6 µL 20 mM ktRS1 RNA and 70.6 µL 0.8 M KPO\textsubscript{4}, pH 8.5 and incubating at 37°C for 20 hr. Then, 67.1 µL 200 mM FeCl\textsubscript{3} was added and the coupling reactions were mixed and incubated at room temperature for 15 min, after which 44.7 µl 500 mM Na-EDTA, pH 8.0 was added, reactions were mixed, and RNAs were purified with RNA Clean & Concentrator columns.

**Folding and native PAGE purification.** To generate purified native ktRS1 and ktRS1-switch strand complexes, switch strand invasion reactions were performed on folded ktRS1, and complexes were PAGE-purified. For the ktRS1 folding reactions, RNA was prepared in 50 mM Na-HEPES, pH 8.0, and 10 mM MgCl\textsubscript{2} as follows. First, the RNA was heated to 65°C in HEPES buffer for 3 min, then cooled to room temperature for 10 min; then MgCl\textsubscript{2} was added and the RNA was heated to 50°C for 5 min, then cooled to room temperature for 10 min. After folding, strand invasion was performed by adding an equimolar amount of switch strand and incubating at room temperature for 30 min. The folded complexes were then purified on a 6% 29:1 acrylamide:bis polyacrylamide native gel containing 1X TBE. A series of gel slices were excised based on prior experiments where the positions of the native complexes relative to loading dye were determined by UV-shadowing. Gel slices were then incubated in 300 µL folding buffer (50 mM Na-HEPES, pH 8.0, and 10 mM MgCl\textsubscript{2}) at 4°C overnight, and RNAs were purified by AMPure XP beads (Beckman Coulter, Indianapolis, IN) plus 12% PEG 8000 and eluted into folding buffer. The purified samples were run on a 10% native PAGE gel and stained with SYBR Gold (Thermo Fisher Scientific, Waltham, MA) to verify which gel slices corresponded to the folded and switch strand-bound complexes.

**MOHCA-seq experiment.** MOHCA-seq libraries were prepared for ktRS1 and ktRS1-switch strand complexes following the previously published protocol\textsuperscript{3}. Briefly, RNA was fragmented by exposure to 10 mM ascorbate for 10 min at room temp, followed by quenching with 10 mM thiourea and purification by ethanol precipitation with GlycoBlue (Life Technologies, Carlsbad, CA). Fragmented RNAs were end-repaired with T4 PNK in 3'-phosphatase conditions and purified by ethanol precipitation again. An ssDNA universal adapter sequence (Universal mrRNA cloning linker, NEB) was ligated to the RNAs using T4 RNA ligase 2 truncated, KQ mutant (NEB), and the RNAs were purified using RNA Clean & Concentrator columns (Zymo Research). The RNAs were then reverse transcribed using barcoded
primers containing the Illumina TruSeq Universal adapter sequence (RTU048-051 and 101-104 in Supplementary File 1 from Ref. 26), and the reactions were divided into halves for purification. One half was purified using DynaBeads magnetic beads (Life Technologies) conjugated to double-biotin-labeled ssDNA complementary to the TruSeq adapter, and the other was purified using AMPure XP beads (Beckman Coulter), to provide size-selection for larger fragments corresponding to tertiary proximities between more distant sequence positions. The cDNAs were then ligated with second sequencing adapters (from Supplementary File 1 in Ref. 26, ‘second ligation adapter 1’ for DynaBeads-purified samples and ‘second ligation adapter 2’ for AMPure XP-purified samples) using CircLigase (Epicentre, Madison, WI). Library concentrations were estimated as described previously3. The libraries were sequenced in two runs using 150-cycle MiSeq v3 kits on Illumina MiSeq instruments with 101 cycles for read 1, 6 cycles for indexing, and 50 cycles for read 2. Elution of libraries from beads and preparation for sequencing were performed as described previously4, except that the 1 mL sample after dilution into HT1 buffer was heated to 95°C for 2 min and cooled on ice for more than 5 min before loading into the MiSeq kit.

MOHCA-seq data analysis. The FASTQ files from the sequencing runs were combined and analyzed using the MAPseeker v1.2 software (available through https://rmdb.stanford.edu/tools/) as described previously3, following the instructions in the tutorial that is part of the README file for the package. Briefly, this analysis included (1) alignment and quantification of sequencing reads from the FASTQ files, (2) combination of the data for the size-selected AMPure XP-purified samples with the data for the non-size-selected DynaBeads-purified samples, and (3) determination of a two-point correlation signal using Closure-based OH Correlation Analysis (COHCOA). COHCOA calculates the two-point correlation signal by estimating a two-dimensional background arising from one-dimensional (uncorrelated) reverse transcription stop and cleavage profiles and subtracting this background from the quantified sequencing data. We used the default settings in COHCOA (the script cohcoa_classic.m), changing only the setting ‘percentile_cut’ from 0.1 to 0.4, which scales up the estimated uncorrelated background that is subtracted from the quantified sequencing data and reduced background signals that varied between replicates. The difference dataset was calculated by scaling the ktRS1 non-switched data by the ratio of the means of the positive values in the switched and non-switched state data, then subtracting the switched from the scaled non-switched state data. To visualize the COHCOA-analyzed data and difference data as 2D proximity maps (Fig. 3c-e and Supplementary Fig. 4a), we applied a filter to remove points with signal-to-noise < 2, followed by a 2D smoothing algorithm to aid visualization of the strongest features. To compare the MOHCA-seq proximity map to the naïve 3D model of ktRS1 generated in Assemble, we plotted contours on the proximity map delineating which pairs of residues are within 30 Å of each other (2’-OH of the 3’ residue is 30 Å or less from the C4’ of the 5’ residue) in the naïve model (Supplementary Fig. 4b). Additionally, we determined pairwise tertiary hits based on MOHCA-seq data for the non-switched state by identifying peaks in the proximity maps that were (1) distinguishable from local background by unbiased inspection and (2) not attributable to secondary structure, based on the expected secondary structure for ktRS126. These tertiary hits are overlaid on the proximity map and annotated on the naïve model in Supplementary Table 4. The MOHCA-seq data have been deposited in the RMDB with the following accession IDs: KTRSRNA_MCA_0000 (ktRS1 non-switched, COHCOA analyzed); KTRSRNA_MCA_0001 (ktRS1 switched, COHCOA analyzed); KTRSRNA_MCA_0002 (ktRS1 non-switched, raw data); KTRSRNA_MCA_0003 (ktRS1 switched, raw data).
RNA and DNA sequences

RNA lever arms

ktLinear (ktL):
GGGUUACGACCAGCGUUGACCAGAUGAGAAGGCUGAUGAGCAAGGCUCUGACCUCUACUGAC
AUCGGAUGGCUACCAGCUUGCGUACACCCACCACUACCACUACCACUCCC

ktLshort:
CAGUGAAGGCGUGAUGAGCAAAGGCUCUGACCUUCACUG

ktReverseSwitch1 (ktRS1):
GGGAUCAGAUCUGUUACAGAUAUCUGUAAAGUUCAGAUGGCUAGGGAAGUGGAAAC
GACGGCGUGAUGAGGUUCGCUACCCACUACUAACUCUACCACCGUAACUA
UGUCAUUCGUUCGUUCAUCUUACAUCAUCAGACUAAUAUGAAUAAACAGUUGAUUCACU
CCACCUUACCACUCCC

ktReverseSwitch2 (ktRS2):
GGGAUCAGAUCUGUUACAGAUAUCUGUAAAGUUCAGAUGGCUAGGGAAGUGGAAAC
GACGGCGUGAUGAGGUUCGCUACCCACUACUAACUCUACCACCGUAACUA
UGUCAUUCGUUCGUUCAUCUUACAUCAUCAGACUAAUAUGAAUAAACAGUUGAUUCACU
CCACCUUACCACUCCC

DNA strands

switch1 (sw1):
GTGTGAGTGAGTTGAAGATTGTGATAGCCTTAGC

switchback1 (sb1):
GCTAAGGCTATGACAATGCTCTCAACTCACTCACAC

switch2 (sw2):
GTGTGAGAGTAAAGTGGAGTTCGATAGCCTTAGC

switchback2 (sb2):
GCTAAGGCTATCGAACTCCACTTTACTCTCACAC

tetherBtn1 (tbtn1):
/5biotin/TTGAAATGAGAGGTAGGTAGGTTTTGGAGGTAGAAGGTGGAGT

tetherBtn2 (tbtn2):
/5biotin/TTGAAATGAGAGGTAGGTAGGTTTTGGAGGATGGACATATAGAC

tetherCy3 (t-c3):
/5Cy3/ACCTACCTACCTCTCTTCAATTGAGGATGAAGATGAGT

tetherCy5 (t-c5):
/5Cy5/ACCTACCTACCTCTCTTCAATTGAGGATGAAGATGAGT

tet-join:
ACCTACCTACCTCTCTTCA
tetramerize (4tet):
/5Phosphate/GTTAGGTAGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAATGAGAGGTTAGGTAGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAATGAGAGGT

Gibson cloning primers

ktL:
p1_ktL
CGGCTGGGCTGATACCTATAGTGTGATCTATGFATAGCTGGGAATCGAGTATGGGTCAGTAG
p2_ktL
TATAAGGAUUGCAGCGGCGGACCGGCAAGCTGCGAGGCTGCTGGGGAGC
p3_ktL
ACAGCCCGGCGGTATCTCGAGAAGGCTTGAGGCTGGGCTGCTGGGGAGC
p4_ktL
GACGGCCGCTGAGGCTTGTGGGGAGC

ktRS1:
t1_ktRS1/ktRS2
GCTTTTAAATCGACTCACCTATAGGGAATGACCTGGGAAATGGAGGATG
b1_ktRS1/ktRS2
TATAGTGAGCTCTATACAGATTGGGCTGGAATGAGGATG
b2_ktRS1/ktRS2
TCAGCCACAGAACTTACAGACGTCAGTGTTTTCGACGACTGGGGAGG
b3_ktRS1/ktRS2
ACATCTACGCTCTTACACACCTTGACGTCAGTGTTTTCGACGACTGGGGAGG
b4_ktRS
GGTTAGGTGAATGAGGAGCTCTACACACCTTGACGTCAGTGTTTTCGACGACTGGGGAGG
b5_ktRS
TGACTGAAATAAGTTAGGACGAGGACCTGTCACTGGGACGAGGACCTGTCACTGGG
b6_ktRS
GGAGGTGAGGAGGAGGAGGAGGACCTGTCACTGGGACGAGGACCTGTCACTGGG

ktRS2:
t1_ktRS1/ktRS2
GCTTTTAAATCGACTCACCTATAGGGAATGACCTGGGAAATGGAGGATG
b1_ktRS1/ktRS2
TATAGTGAGCTCTATACAGATTGGGCTGGAATGAGGATG
b2_ktRS1/ktRS2
TCAGCCACAGAACTTACAGACGTCAGTGTTTTCGACGACTGGGGAGG
b3_ktRS1/ktRS2
ACATCTACGCTCTTACACACCTTGACGTCAGTGTTTTCGACGACTGGGGAGG
b4_ktRS
GGTTAGGTGAATGAGGAGCTCTACACACCTTGACGTCAGTGTTTTCGACGACTGGGGAGG
b5_ktRS
TGACTGAAATAAGTTAGGACGAGGACCTGTCACTGGGACGAGGACCTGTCACTGGG
b6_ktRS
GGAGGTGAGGAGGAGGAGGAGGACCTGTCACTGGGACGAGGACCTGTCACTGGG
t6_ ktRS2
AACAAGTTCGTTCCGATATATGCATCTGCAGGACTCTAGAGGA
b1_ ktRS1/ktRS2
TATAGTGAGTCTATATTTAAACTACTAGCATGTCATAG
b2_ ktRS1/ktRS2
TCAGCCATCGAAGTTACAGATATCTCTAACAGTCTGATCC
b3_ ktRS1/ktRS2
ACCTCATACGCGTCGTTCTCATTCTGTGCCAACTTAC
b4_ ktRS2
GTTGATAATGGAGTCGATAGTATAGTAGCGACTGAGCGGCG
b5_ ktRS2
TGACTGAAATAAGTTAGAACAGAATGACTAGCTACCTGT
b6_ ktRS2
GAGGATGACATATAGACGTCAGAACTGTTAAATGATATTCAAGTCTCG

**Primers for PCR of MOHCA-seq DNA template from ktRS plasmid:**

ktRS-Fw
TTCTAATACGACTCTATAGGATCGACTGTTAGAG
ktRS-Rv
GGAGTTAGAGGATGGTGAATC
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