

Supplementary Figure 1.

(a) Examples of purified HeLa nuclei. Nuclei were stained with the DNA dye Hoechst 33342 and the lipophilic dye DID. The staining showed that the purified nuclei appeared intact 3-D organelles, surrounded by membrane and maintaining not only their DNA content, but also preserving structures like the nucleoli (See Supplementary Movie 3 and 4). Images were acquired at the mid-point of the nucleus. Scale bar 10 μ m.



Supplementary Figure 2.

(a) Staining for filamentous actin. To assess whether the filamentous structures seen by the immunofluorescence staining of MVI were nuclear actin filaments, fixed HeLa cells and isolated nuclei were stained with Rhodamine-Phalloidin and Hoechst 3342. However, the lack of phalloidin staining in the nucleus suggested that these structures are not actin-based. Images were acquired at the mid-point of the nucleus. Scale bar 10 μ m.

(b) Prediction of DNA binding residues using BindN (http://bioinformatics.kstate.edu/bindn/). Bars indicate residues with a predicted confidence greater than 70%. A full list of residues are presented in Supplementary Table 1. A majority of sites are clustered in the CBD.

(c) Fluorescence anisotropy titrations of the CBD domain against various FAM-DNA substrates (50 nM). Data fitting was performed as described in Methods. K_d : ds₁₅ 500 +/- 45 nM, ss₂₀ 110 +/- 35 nM, dT₂₀ 120 +/- 25 nM, ss₄₀ 120 +/- 35 nM and ds₄₀ 100 +/- 20 nM. Data were averaged from three independent experiments.

(d) Fluorescence anisotropy was measured in stopped flow pre-steady-state measurements. MVI domains were rapidly mixed against 50 nM 40 bp FAM-DNA. Traces were fitted as described in the Methods to yield the rate constants plotted in d. Association and dissociation rate constants were calculated from linear fits to the data (Supplementary Table 3). Data were averaged from three independent experiments.

(e) Titration of Folch vesicles against 1 μ M CBD or CBD_{Δ SiteA}. Data were fitted as described in Methods, following background subtraction of the folch auto-fluorescence. Fitting gives an indication of the affinity with K_d 39 +/- 4 mg/ml. Due to the unknown mixed composition, it was not possible to have molar concentrations. Poor binding by CBD_{Δ SiteA} means data could not be fitted to the model.



Supplementary Figure 3.

(a-b) Intracellular distribution of EGFP-LI-MVI transiently expressed in HeLa cells. The EGFP-LI-MVI localisation was restricted to the cell periphery, being excluded from the nucleus. This is highlighted by the plot of mean intensity of Hoechst (Hst) and EGFP across each image stack. The position is measured relative to the mid-point of the nucleus. The intensity was measured within a region of interest through the centre of the nucleus. Scale bar 10 μ m.

(c) Intracellular distribution of EGFP-LI-MVI(M1062Q). The mutant, in which the LI helix is destabilised, displayed a similar distribution to the NI isoform. Images were acquired at the mid-point of the nucleus. Scale bar 10 μ m.



Supplementary Figure 4.

(a) Raw fluorescence intensity data for titration of NMVITAIL against CBD in Fig. 3a.

(b) Raw intensity data as shown in (a) but on a smaller scale.

(c) Raw fluorescence intensity data for titration of NMVITAIL against CBD in the presence of DNA (Blue is FITC intensity and Red is AF555 intensity).

(d) Fluorescence anisotropy titration of $_{N}MVI_{TAIL}$ against FITC-CBD and CBD against FITC- $_{N}MVI_{TAIL}$. Data were fitted as described in Methods for the DNA substrates with K_{d} +/- SEM.

(e) Raw fluorescence intensity data for titration of NMVITAIL against CBD in the presence of NDP52. (Blue is FITC intensity and Red is AF555 intensity)

(f) Fluorescence anisotropy titrations of FL MVI against a 40 bp FAM-DNA (50 nM) in the presence of F-Actin (5 μ M), Ca²⁺ (1 mM), ATP (2 mM) and Actin-ATP. Data fitting

was performed as described in Methods. Data were averaged from three independent experiments.

(g) Pull-down of CBD (5 μ M) by His-tagged Motor₁₋₁₀₆₀ and Motor₁₋₈₁₄ both at 10 μ M +/- 1 mM Ca²⁺. P and S represent pellet and supernatant fractions, respectively. Interaction of the CBD with the motor constructs is shown in the cartoon scheme.



Supplementary Figure 5.

(a) Fluorescence intensity titration of NDP52 against a 40 bp FAM-DNA substrate (50 nM). Data fitting was performed as described in Methods. Data were averaged from three independent experiments. K_d : ds₄₀ 80 +/- 25 nM

(b) Immunofluorescence staining against NDP52 (green), MVI (magenta) and DNA (cyan) on isolated HeLa nuclei, as performed in Fig. 1b. Insert depicts co-localising foci. Scale bar 10 μ m.

(c) Representative images of transiently expressed NI-, LI- and LI (M1062Q) -EGFP-MVI in HeLa cells stained against DNA (Cyan) and immune-stained against endogenous NDP52 (magenta). Scale bar 10 μ m. These images were used to assess the significance of the Pearson's coefficient regarding MVI and NDP52 co-localisation. We have established how the NI and LI (M1062Q) can form interactions with NDP52. Therefore, to assess this *in vivo* through imaging, we performed the Pearson's coefficient test (d) with the three MVI constructs. Here a region of interest was drawn around the cells and images were analysed from 5 different stacks. LI MVI shows a significantly lower co-localisation that the NI or mutant **p <0.001.



Supplementary Figure 6.

(a) Actin co-sedimentation assay. 1 μ M MVI was incubated with the indicated concentrations of MVI_{TAIL} before sedimentation in the presence of 5 μ M F-actin, as described in the Methods. Except for the 4 μ M* condition where 2 μ M MVI was used. P and S represent pellet and supernatant fractions, respectively.

(b) Gel densitometry was used to determine the relative % of MVI_{TAIL} in the actin pellet (Green bars). The % bound was then converted into the concentration of bound species (Red bars) (Error bars represent SEM).

(c) Plots of MVI or Motor₁₋₁₀₆₀ ATPase rate constants in the presence of 5 μ M DNA or NDP52 under conditions described in the Methods. Error bars represent SEM.

(d) Velocity histogram from sliding filament assay with MVI immobilised through the stated concentrations of NDP52.



Supplementary Figure 7.

(a) Raw fluorescence intensity data for titration of AF555-MVI_{TAIL} against FITC-MVI_{TAIL} in the presence of 20 μ M NDP52. (a-c) Blue is FITC intensity and Red is AF555 intensity.

(b) Raw fluorescence intensity data for titration of AF555-MVI_{TAIL} against FITC-MVI_{TAIL} in the presence of 20 μ M DNA/NDP52.

(c) Raw fluorescence intensity data for titration of AF555-MVI_{TAIL} against FITC-MVI_{TAIL}.

(d) FRET titration of AF555-MVITAIL against FITC-MVITAIL over a larger concentration range.

(e) FRET titration of AF555-CBD against FITC-CBD.

(f) FRET titration of AF555-NMVITAIL against FITC-NMVITAIL.

(g) FRET titration of AF555-CBD against FITC-CBD in the presence of 5 μ M or 20 μ M NDP52. For F-G, fitting was performed as described in the Methods.

(h) FRET titration of AF555-MVI_{TAIL} against FITC-MVI_{TAIL} in the presence of 5 μ M or 20 μ M NDP52.



Supplementary Figure 8.

(a) *In vitro* transcription by HelaScribe extracts. A control reaction was performed under standard manufacturer's instructions. Antibody depletion was performed as described in Methods. Here, Anti-Halotag represents an antibody negative control to highlight the specific isolation of target proteins. Samples were normalized to a non-depleted control reaction (error bars represent SEM).

(b) Confirmation of antibody depletion. Western-blots are shown confirming the presence of MVI, NDP52 and RNAPII in the HelaScribe extracts (Pre). Depletion of the proteins by the corresponding antibodies is shown in the Post samples.

(c) *In vitro* transcription by HelaScribe extracts in the presence of competitor *Bacilus subtilis* Smc and ParB at 25 μ M. Both proteins were used as controls for DNA binding which may block transcription. As no decrease in transcription occurred, we conclude that the decrease in transcription observed with the CBD is related to its interaction with the RNAPII complex and not the interference by DNA binding. Samples were normalized to the control sample in Fig. 6e (error bars represent SEM).

(d) siRNA Knock-down of myosin VI. Western-blot on myosin VI in MCF7 cells under mock conditions and following transfection with MVI siRNA and control scrambled siRNA.



Supplementary Figure 9.

(a-b) Loading controls of recombinant proteins for assays performed in Fig. 4f and 7c.

(c) Loading controls of recombinant proteins for assay performed in Fig. 6c.



Supplementary Figure 10.

Uncropped Western-blots. (a) MVI From Fig. 1c. (b) NDP52 From Fig. 4c. (c) Tubulin from cell fractionation Fig. 1c and 4c. (d) Lamin B from cell fractionation Fig. 1c and 4c. (e) From Fig. 4f. (f) From Fig. 6c and 7c. (g) From Fig. 7c. (h) From Supplementary Fig. 8b.

Residue	Amino acid	Output	Prediction	Confidence (0-1)
80	R	1.6914	+	0.9539
83	К	1.4826	+	0.9152
85	R	2.0163	+	0.9862
118	К	1.5894	+	0.9373
205	R	1.6289	+	0.9447
276	R	1.7193	+	0.9594
280	R	1.9585	+	0.9825
365	K	1.6639	+	0.9493
368	S	1.4254	+	0.9005
393	R	1.854	+	0.9705
632	K	1,4439	+	0.9023
667	R	1 4925	+	0.9189
708	R	1.9088	+	0.9788
792	R	1 7813	+	0.965
825	R	1 5331		0.9309
831	R	1 4709	+	0.9124
832	R	1 3556	+	0.8830
83/	K	1 1633	+	0.8166
947	K K	1.1033		0.0100
840	D	1.1970	+	0.8541
996		1 10/2	+	0.8353
000	r.	1.1943	+	0.8515
937	R	1.2040	+	0.8627
940	R	1.1010	+	0.824
948	R	1.1761	+	0.823
1035	R	0.9922	+	0.8235
1036	R	1.038	+	0.8475
1044	K T	0.9667	+	0.806
1049		0.9875	+	0.8217
1050	ĸ	1.0849	+	0.8714
1055	S	0.9608	+	0.8041
1056	ĸ	1.11/3	+	0.8862
1063	R	1.1307	+	0.8917
1085	R	1.0471	+	0.8548
1094	S	1.4206	+	0.8995
1095	K	1.9281	+	0.9816
1096	N	1.3317	+	0.8774
1097	K	1.4436	+	0.9023
1098	K	1.7798	+	0.965
1099	R	1.6503	+	0.9484
1100	Ν	1.2231	+	0.8433
1106	R	1.0999	+	0.8797
1109	К	1.507	+	0.9235
1112	Т	1.0336	+	0.8447
1126	R	1.0418	+	0.8502
1137	R	1.1627	+	0.8147
1155	S	1.1766	+	0.823
1156	ĸ	1.539	+	0.9318
1157	K	1 1108	+	0.8797
1158	ĸ	1 5536	+	0.9336
1205	Т	0.0608	· · · · · · · · · · · · · · · · · · ·	0.8041
1205	R	1 8287		0.9687
1200	K	1 1256		0.8008
1207	D	1.1200	- -	0.0300
1200		1.4000	+	0.9113
1231		1.4/31	+	0.9134
1240	<u>к</u>	1.7045	+	0.9348
1242		1.1889	+	
1243	<u>ү</u>	1.2152	+	0.8424
1245		1.2904	+	0.8645

Supplementary Table 1. DNA Binding Prediction

Construct	DNA	Conditions	Figure	<i>Kd</i> (μM)
	Substrate			
MVI	ds40		2a	3.5 +/- 0.7
Motor1-814	ds40		2a	-
MVITAIL	ds40		2a	2.5 +/- 0.4
NMVITAIL	ds40		2b	1.2 +/- 0.35
CBD	ds40		2b	0.1 +/- 0.02
CBD ΔΣιτεΑ	ds40		2e	0.11 +/- 0.04
NCBD	ds40		2e	1.3 +/- 0.7
CCBD	ds40		2e	0.12 +/- 0.03
$_{CCBD}$ ΔΣιτε B	ds40		2f	0.7 +/- 0.18
$\cosh\Delta\Sigma\iota\tau\epsilon X$	ds40		2f	0.5 +/- 0.15
MVITAIL	ds40		3e	0.3 +/- 0.03
(W1221A)				
CBD	ds15		Sup Fig 2a	0.5 +/- 0.045
CBD	ss20		Sup Fig 2a	0.11 +/- 0.035
CBD	dT20		Sup Fig 2a	0.12 +/- 0.025
CBD	ss40		Sup Fig 2a	0.12 +/- 0.035
MVI	ds40	$5 \mu M$ F-actin	Sup Fig 3f	2.4 +/- 1.3
MVI	ds40	1 mM Ca2+	Sup Fig 3f	1.7 +/- 0.9
MVI	ds40	2 mM ATP	Sup Fig 3f	2.5 +/- 1.1
MVI	ds40	F-actin + ATP	Sup Fig 3f	2.7 +/- 1.3
NDP52	ds40		Sup Fig 4f	0.08 +/- 0.025

Supplementary Table 2. DNA Binding dissociation constants.

Supplementary Table 3. Myosin VI – DNA Pre-steady-state kinetics.

Construct	Association rate constant	Dissociation rate constant (s ⁻¹)
	(μM⁻¹ s⁻¹)	
MVI	9.1 +/- 1.5	21.3 +/- 3.3
MVI _{TAIL}	9.4 +/- 1	25.6 +/- 2.2
NMVITAIL	17.4 +/- 2.2	27.8 +/- 4.7
CBD	42.2 +/- 3.8	13.3 +/- 3.9

Construct A	Construct B	Technique	Conditions	Figure	<i>Kd</i> (µМ)
NMVITAIL	CBD	FRET		За	4.5 +/- 0.6
NMVITAIL	CBD	Anisotropy		Sup Fig 3d	4 +/- 0.4
CBD	NMVITAIL	Anisotropy		Sup Fig 3d	4.2 +/- 0.4
NDP52	MVITAIL	FRET		4d	3.2 +/- 1.2
NDP52	MVITAIL (LI)	FRET		4d	9.8 +/- 4.3
NDP52	MVITAIL (RRL/ARL)	FRET		4d	4.6 +/- 1.2
NDP52	MVITAIL (RRL/RAL)	FRET		4d	55 +/- 23
NDP52	MVITAIL (RRL/RRA)	FRET		4d	21 +/- 11
NDP52	MVITAIL (RRL/AAA)	FRET		4d	63 +/- 15
AF555-MVITAIL	FITC-MVITAIL	FRET		5a	27 +/- 4.3
AF555-MVITAIL	FITC-MVITAIL	FRET	20 μM NDP52	5a	2.3 +/- 0.8
AF555-MVITAIL	FITC-MVITAIL	FRET	20 µM DNA	5a	29 +/- 5
AF555-MVITAIL	FITC-MVITAIL	FRET	20 μM NDP52/DNA	5a	2.5 +/- 1.2
AF555-CBD	FITC-CBD	FRET		Sup Fig 5g	58 +/- 9.5
AF555-CBD	FITC-CBD	FRET	20 μM NDP52	Sup Fig 5i	51 +/- 7
AF555- NMVITAIL	FITC-NMVITAIL	FRET		Sup Fig 5f	3.1 +/- 1.4

Supplementary Table 4. Protein-protein dissociation constants.

Supplementary Table 5. Recombinant DNA.

Construct (Residue numbers)	Source	
Human pEGFP-C3 myosin VI (large insert) (1-1284)	F. Buss (CIMR)	
Human pEGFP-C3 myosin VI M1062Q (large insert) (1-1284)	S. Polo (IFOM)	
Human pEGFP-C3 myosin VI (non insert) (1-1253)	F. Buss (CIMR)	
Human pEGFP-C3 myosin VI WKSKNKKR/WASANNNR (1-1253)	F. Buss (CIMR)	
Human pFastBacHTB GFP NI myosin VI	F. Buss (CIMR)	
Xenopus pFastBac1 Calmodulin (1-end)	J. Sellers (NIH)	
Bacillus subtilis pET28 Smc hinge	S.Gruber (MPIB)	
Bacillus subtilis pET28 ParB (1-end)	S.Gruber (MPIB)	
pSG1365	S.Gruber (MPIB)	
Human pET151 GST ERalpha (1-end)	Synthetic Gene – This Study	
Human pET151 NDP52 (1-end)	Synthetic Gene – This Study	
Human pET151 NDP52 (365-end)	Synthetic Gene – This Study	
Human pET151 EGFP-MVI _{TAIL} -RFP (814-1253)	Synthetic Gene – This Study	
Human pET151 MVI _{TAIL} (814-1253) W1221A	Synthetic Gene – This Study	
Human pET151 CBD (1060-end) W1221A	Synthetic Gene – This Study	
Human pET151 CoCoA (1-end)	Synthetic Gene – This Study	
Human pMX MVI (1184-1253)	Synthetic Gene – This Study	
Human pET151 MVI _{TAIL} 814-1253	This Study	
Human pET151 _N MVI _{TAIL} 814-1060	This Study	
Human pET28 CBD 1060-1253	This Study	
Human pET28 NCBD1060-1120	This Study	
Human pET28 _C CBD ₁₁₂₁₋₁₂₅₃	This Study	
Human pET28 MVI _{TAIL} (large insert) (814-1284)	This Study	
Human pET28 CBD (large insert) (1037-1284)	This Study	
Human pET28 CBD M1062Q (large insert) (1037-1284)	This Study	
Human pFastbacHTB NI MVI (1-1253)	This Study	
Human pFastbacHTB Motor ₁₋₈₁₄	This Study	
Human pFastbacHTB Motor ₁₋₁₀₆₀	This Study	
Human pET28 CBD ∆Site A WKSKNKKR/WASANNNR (1060-	This Study	
Xenopus pET28 Calmodulin	This Study	
Human pET28 _c CBD ∆Site B SKKK/AAAA	This Study	
Human pET28 cCBD ∆Site C TRKR/AAAA	This Study	
Human pET28 CBD RRL/RAL	This Study	
Human pET28 CBD LQSLL/AQSAA	This Study	
Human pET28 MVI _{TAIL} RRL/RAL	This Study	
Human pET28 MVI _{TAIL} RRL/ARL	This Study	
Human pET28 MVI _{TAIL} RRL/RRA	This Study	
Human pET28 MVI _{TAIL} RRL/AAA	This Study	

Supplementary Table 6. Primers for qPCR.

Sequence	Use
CATGGAGAACAAGGTGATCTG	TFF1/PS2 qPCR For
CACTGTACACGTCTCTGTCTG	TFF1/PS2 qPCR Rev
ATGGGAAATTCTTACGCTGGAC	GREB1 qPCR For
CACTCGGCTACCACCTTCT	GREB1 qPCR Rev
AGAGCTACGAGCTGCCTGAC	Human B-Actin qPCR For
AGCACTGTGTTGGCGTACAG	Human B-Actin qPCR Rev
AAGCTTCGATGATGGGCTTA	ESR1 qPCR For
AGGTGGACCTGATCATGGAG	ESR1 qPCR Rev
CCGAGCTCATCAGTGATGAGGC	Myosin VI qPCR For
CCAAGCATGATACACTTTTAGTCTCC	Myosin VI qPCR Rev
AAGGGCATCGACTTCAAGGA	CMV GFP RT-qPCR For In vitro
	Transcription
GGCGGATCTTGAAGTTCACC	CMV GFP RT-qPCR Rev In vitro
	Transcription

Supplementary Table 7. DNA Substrates.

Sequence	Use
TTAGTTGTTCGTAGTGCTCGTCTGGCTCTGGATTACCCGC*	ds40 A 3'FAM
GCGGGTAATCCAGAGCCAGACGAGCACTACGAACAACTAA	ds40 B
TTAGTTGTTCGTAGTGCTCGTCTGGCTCTGGATTACCCGC*	ss40 A 3'FAM
TCTGGCTCTGGATTACCCGC*	ds20 A 3'FAM
GCGGGTAATCCAGAGCCAGA	ds20 B
TCTGGCTCTGGATTACCCGC*	ss20 A 3'FAM
TTAGTTGTTCTCTGG*	15 bp A 3'FAM
CCAGAGAACAACTAA	15 bp B
ТТТТТТТТТТТТТТТТТТТ	dT ₂₀

Supplementary Table 8. PCR Primers.

Sequence	Use
AGCTAGCTGCATGGCTGACCAACTG	pET28 Calmodulin For
TTTTGCGGCCGCTCACTTTGCTGTCATC	pET28 Calmodulin Rev
CTAGGCGGCCGCCCGAGGATGGAAAGCCCGTTTG	pFastbacHTB NI Myosin VI
TTTTCTCGAGTTATTTCAACAGGTTCTGCAGCATG	pFastbacHTB NI Myosin VI
CACC GAAGCCTGCATTAAAATGC	MVI _{TAIL} 814-1253 For
CTATTTCAACAGGTTCTGCAGCAT	MVI _{TAIL} 814-1253 Rev
CTAGGCGGCCGCCCGAGGATGGAAAGCCCGTTTG	pFastBacHTB NI MVI
TTTTCTCGAGCTAGCATTTTAATGCAGGCTTC	pFastBacHTB NI MVI
CTAGGCGGCCGCCCGAGGATGGAAAGCCCGTTTG	pFastBacHTB NI MVI
TTTTCTCGAGCTAGATGGTATCACGTAGTTCTGC	pFastBacHTB NI MVI
AGCTAGCGCAGAACTACGTGATACCATC	CBD For
TTTTGCGGCCGCTATTTCAACAGGTTCTGCAGCAT	CBD Rev
AGCTAGCGCAGAACTACGTGATACCATC	NCBD For
TTTTGCGGCCGCTATGCTGGGTTTTGCTGAGG	_N CBD Rev
AGCTAGCGCTCAGATTCCTGCCAGG	cCBD For
TTTTGCGGCCGCTATTTCAACAGGTTCTGCAGCAT	cCBD Rev
AGCTAGCGGGGCAGAACTCAGCACTG	CBD Plus LI For
TTTTGCGGCCGCTATTTCAACAGGTTCTGCAGCAT	CBD Plus LI Rev
CACC GAAGCCTGCATTAAAATGC	NMVITAIL For 814-1060
CTAGATGGTATCACGTAGTTCTGC	NMVI _{TAIL} Rev 814-1060
AGCTAGCGCCGAACTGCGTGATACCA	CBD W1221A For
TTTTGCGGCCGCTATTATTTCAGCAGGCTCTGCAGC	CBD W1221A Rev
CTTGCAGAGAAGAATTTCATGCGGCAGCAAAAGTGTATCATGC	RRL/AAA For
GATTTCCAAGCATGATACACTTTTGCTGCCGCATGAAATTCTTC	RRL/AAA Rev
CTTGCAGAGAAGAATTTCATAGGAGAGCAAAAGTGTATCATGC	RRL/RRA For (L1118A)
GATTTCCAAGCATGATACACTTTTGCTCTCCTATGAAATTCTTC	RRL/RRA Rev (L1118A)
CTTGCAGAGAAGAATTTCATGCGAGACTAAAAGTGTATCATGC	RRL/ARL For (R1116A)
GATTTCCAAGCATGATACACTTTTAGTCTCGCATGAAATTCTTC	RRL/ARL Rev (R1116A)
GCAGAGAAGAATTTCATAGGGCACTAAAAGTGTATCATGCTTG	RRL/RAL Rev (R1117A)
CCAAGCATGATACACTTTTAGTGCCCTATGAAATTCTTCTCTGC	RRL/RAL Rev (R1117A)
GACCCTCAGAGTGCGGCAGCAGGCTGGTGGTATGC	SKKK/SAAA For
GCATACCACCAGCCTGCTGCCGCACTCTGAGGGTC	SKKK/SAAA Rev
GACTGGCCTGACTCGGAAGCGTGGTGCTGAGATCTTG	TRKR/TAAA For
CAAGATCTCAGCACCACGCTTCCGAGTCAGGCCAGTC	TRKR/TAAA Rev
GCCATGGCGCAGAACGCGGCGAAATAAGCCGAAG	LQSLL/AQSAA For
CTTCGGCTTATTTCGCCGCGTTCTGCGCCATGGC	LQSLL/AQSAA Rev
GATCGATAGTACATAAGGATTTCCTTACGCG	500bp 5'Bio-Teg
CCAATTTTCGTTTGTTGAACTAATGGGTGC	500 bp Rev