Supporting Information

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SI Materials and Methods.

DNA Constructs. GFP-actin, GFP-actin-R62D, and 2GFP-actin constructs are based on pEGFP-C1 (Clontech), where human β-actin coding sequence, terminated by a stop codon has been cloned into HindIII-KpnI sites. In 2GFP-actin, the second GFP has been added into BspEI-BglII site of GFP-actin. To create Flag and HA-epitope tagged constructs for immunoprecipitation, GFP in either pEGFP-C1 or pEGFP-N3 (Clontech) was replaced by 2xFlag (DYKDDDDK) or with 2xHA (YPYDVPDYA) to create vectors 2Flag-C1, 2Flag-N3, and 2HA-C1. GFP was expressed from pEGFP-C1 and 2GFP from the same backbone to which another GFP was added with a BamHI-BamHI restriction. Two tags were included to increase the efficiency of the immunoprecipitation. To create 2Flag-actin, actin was cloned into HindIII-KpnI sites of the 2Flag-C1 vector. Mouse cofilin-1 was cloned into 2Flag-N3 into XhoI-EcoRI sites, and mouse importin-9 (Ipo9) into 2HA-C1 by using HindIII-KpnI sites. pQE-Ran-Q69L and GFP-RanBP1ΔNES were kind gifts from Maarten Fornerod and Caroline Hill, respectively. pBAC-GFP was a kind gift from Osamu Shimmi. RNAi-resistant Ipo9-mCherry construct was generated by creating silent point mutations (C ACA GAA GAA CAA ATC AAG) in the 19 bp Ipo9 siRNA target sequence (C ACC GAG GAG CAG ATT AAA) (Table S1) in mCherry-C1 vector, which is based on pEGFP-C1 (Clontech), where GFP has been exchanged for mCherry coding sequence. Flag nuclear localization signal (NLS) actin was created from Flag-actin plasmid by adding NLS sequence (PKKKRKVG).

Mammalian Cell Lines and Transfections. ***Cell lines.*** NIH 3T3 cells were cultured in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) (GIBCO) and penicillin-streptomycin (GIBCO) and maintained at 37 °C and 5% CO_2. NIH 3T3 cell line expressing MRTF-A-GFP upon tetracycline (Tet) induction has been described before (1). The stable, Tet-inducible cell lines expressing GFP-actin and GFP-actin-R62D were created similarly using the TetOn-system (Invitrogen) according to manufacturer's instructions. These stable cell lines were maintained in 5 μg/mL blasticidin and 250 μg/mL zeocin, and induced with tetracycline 24–48 h prior to the experiment. Flag-NLS-actin and Flag cell lines were generated from a single cell clone and maintained in 200 μg/mL G418 (Sigma) selection antibiotic.

DNA transfections. For plasmid transfections, 120,000 cells were seeded on 35 mm plates and transfected with 100 ng of plasmids by using Turbofect according to manufacturer's instructions. Where indicated, cells were treated with 20 nM leptomycin B (LMB) (Calbiochem) or 100 nM jasplakinolide (Calbiochem) prior to analysis.

Mammalian cell RNA interference. Cells were seeded at 20,000 per well in six well plates overnight and transfected with 10 nM gene-specific siRNA (Table S1) using interferin siRNA transfection reagent (Polyplus). The functionality of the siRNAs was determined by specific antibodies when available, and some siRNAs have been used before [e.g., Ipoβ (2) and cofilin-1 (3)]. For the remaining nuclear import receptors, a pool of two to three Flexiplate siRNAs was used to ensure efficient depletion. Cells were retransfected with 10 nM siRNA on day 2 and incubated for 4 d. Then cells were processed either for fluorescence microscopy or for cell fractionation (see main text). For Ipo9 rescue, 2,500 cells per 24 well of Tet-inducible cell line expressing GFP-actin were seeded on 13 mm coverslips and treated as described above. On day 4 of RNAi, cells were transfected with 100 ng RNAi-resistant Ipo9-mCherry, using lipofectamine transfection reagent according to the manufacturer·s protocol. Cells were induced with tetracycline and further incubated for 24 h before been processed for microscopy.

***Drosophila* Cell Culture and RNAi Experiments.** *Drosophila* S2R+ cells were maintained in a humidified chamber at 25 °C in Schneider's medium (Invitrogen) supplemented with 10% heat-inactivated FBS (GIBCO) and penicillin-streptomycin (GIBCO). For RNAi, complementary DNA from S2R+ cells was amplified with gene-specific primers (Table S1) containing the T7-binding site and double-stranded RNA (dsRNA) were generated and purified using the MEGAscript T7 Kit and NucAway Spin columns (Applied Biosystems), respectively.

Drosophila cell RNA interference. S2R+ cells in serum-free media were seeded at 50,000 on coverslips in a 24-well format. Gene-specific dsRNA (2 μg) was added and incubated for 30 min. In the double-knockdown experiments, cells were prediluted to 250,000 cells/mL in serum-free media and mixed with exportin 6 (Exp6) dsRNA to a final concentration of 10 ng/μL dsRNA. A volume of 200 μL of the cell suspension/dsRNA mix was seeded on cover slips in a 24-well plate before 2 μg dsRNA for specific genes were added to wells. Complete media was added after 30 min and cells were incubated for 5 d.

GFP localization. *Drosophila* S2R+ cells were seeded at 50,000 on 13 mm coverslips in 24-well plates. After 2 h, cells were cotransfected with dsRNA against either luciferase (luc) or Ran and pBAC-GFP plasmid using effectene transfection reagent (Qiagen). In brief, 1.2 μg dsRNA and 0.2 μg DNA were diluted in 60 μL EC buffer. A volume of 11.2 μL enhancer was added, mixed, and incubated at room temperature for 3 min before 5 μL effectene was added and the transfection mix was incubated for 15 min. Transfection mix was added to cells and incubated in a humidified chamber for 5 d.

1. Vartiainen MK, Guettler S, Larijani B, Treisman R (2007) Nuclear actin regulates dynamic subcellular localization and activity of the SRF cofactor MAL. *Science* 316:1749–1752.
2. Pawlowski R, Rajakyla EK, Vartiainen MK, Treisman R (2010) An actin-regulated importin alpha/beta-dependent extended bipartite NLS directs nuclear import of MRTF-A. *EMBO J* 29:3448–3458.
3. Hotulainen P, Paunola E, Vartiainen MK, Lappalainen P (2005) Actin-depolymerizing factor and cofilin-1 play overlapping roles in promoting rapid F-actin depolymerization in mammalian nonmuscle cells. *Mol Biol Cell* 16:649–664.

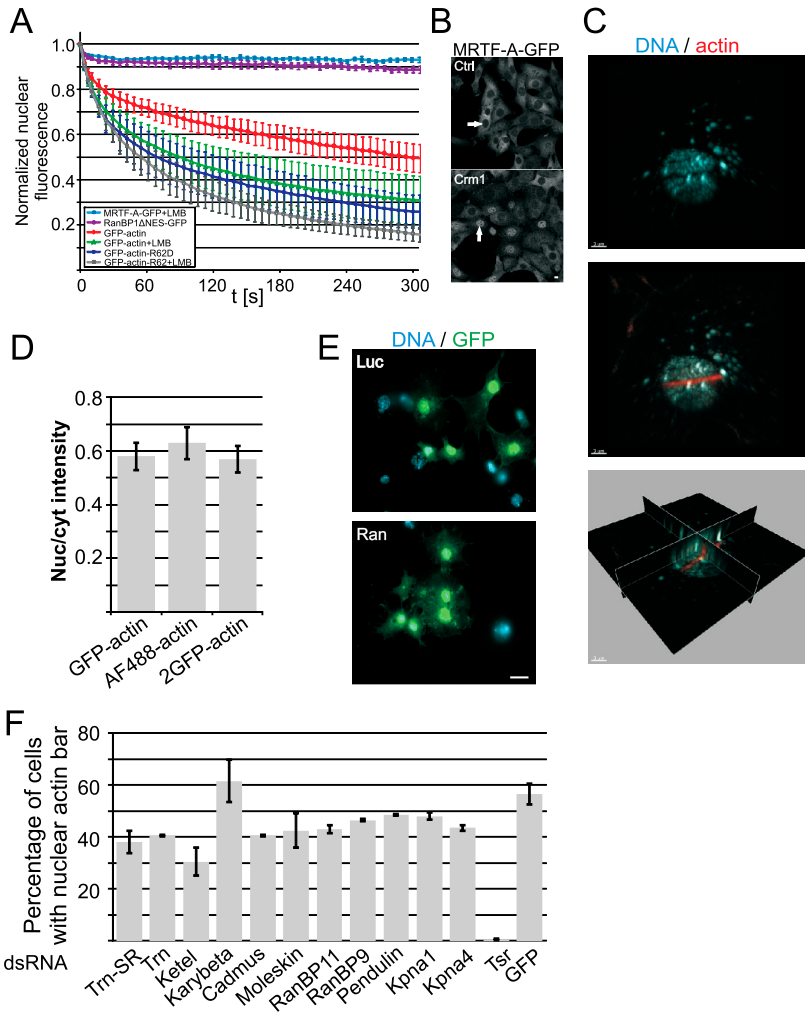

Fig. S1. (*A*) FLIP-curves of RanBP1ΔNES-GFP, GFP-actin, GFP-actin-R62D and GFP-actin, GFP-actin-R62D, and MRTF-A-GFP treated with LMB. Data represent mean nuclear fluorescence intensity +/ – std ($n = 6$–10). (*B*) NIH 3T3 cells expressing MRTF-A-GFP were transfected with control siRNA or siRNA against Crm1. MRTF-A-GFP accumulated in the nuclei of Crm1-depleted cells as indicated. Scale bar 10 μm. Ctrl, control. (*C*) Confocal microscope image of a typical Exp6-depleted S2R+ cell and projection of the nucleus showing actin bar in the nucleus. Scale bars, 3 μm. Ctrl, control; Cof1, cofilin 1. (*D*) Nuclear localization of fluorescent actin constructs. Fluorescence signal ratio of nucleus/cytoplasm (nuc/cyt) in NIH 3T3 cells microinjected with Alexa Fluor 488 labeled actin or expressing GFP-actin or 2GFP-actin from a transfected plasmid. Data represent mean signal ratios +/ – std from three independent experiments with $n = 6$–11. (*E*) Representative fluorescence microscope images of *Drosophila* cells transfected with empty GFP vector (green) and Ran or control (Luc) dsRNAs. DAPI; cyan. Scale bars, 10 μm. (*F*) RNA interference screen of known *Drosophila* cytoplasmic-nuclear transport factors. Quantifications of the percentage of cells with nuclear actin bars, which have been treated with indicated gene-specific dsRNA in addition to dsRNA against Exp6. Data represent mean and +/ – std from two independent experiments ($n = 200$).

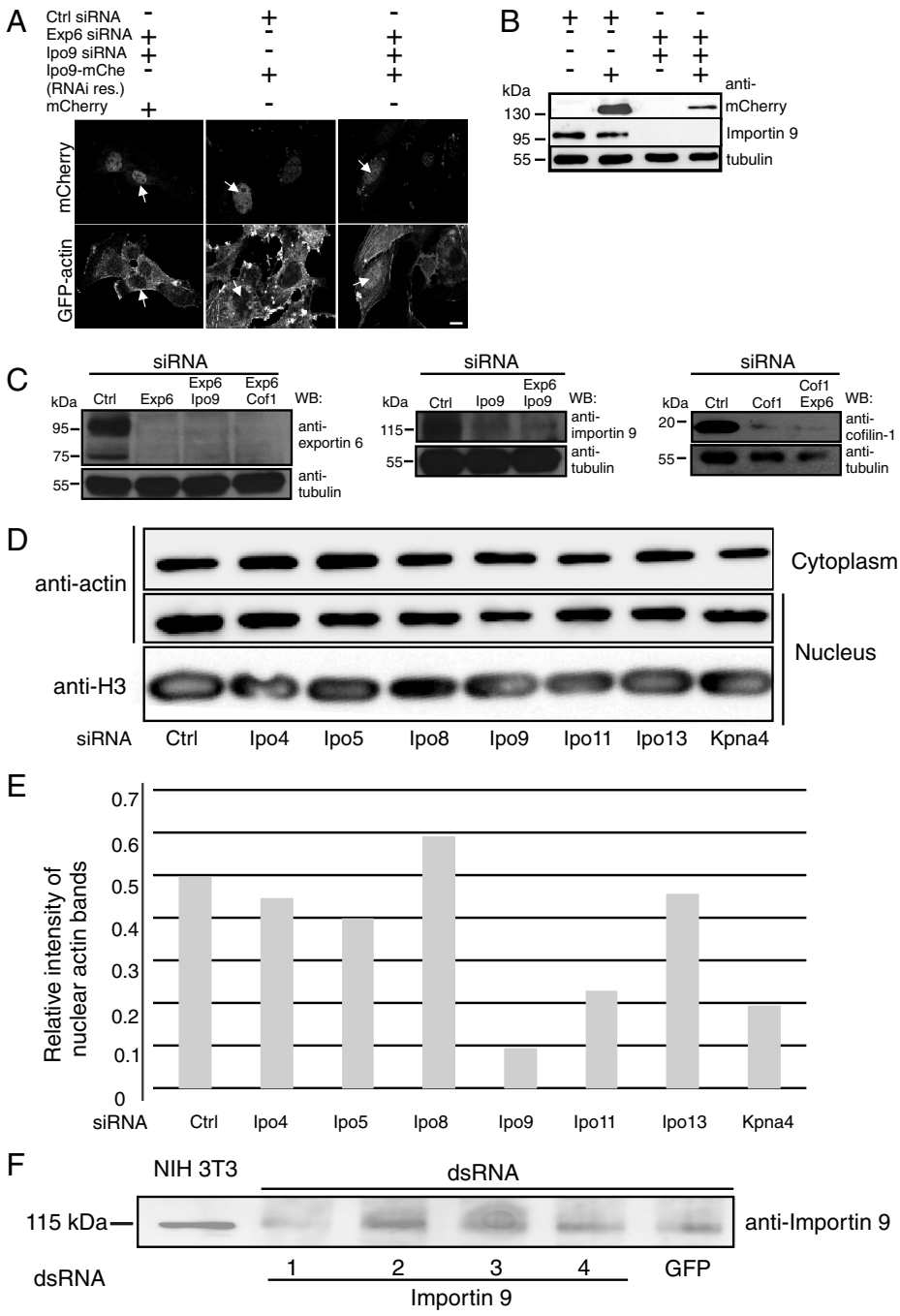

Fig. S2. (*A*) Rescue of nuclear import of actin after RNAi in cells expressing GFP-actin. Cells were transfected with either mCherry alone or RNAi resistant Ipo9-mCherry construct (Ipo9-mChe RNAi res.) after 4 d of RNAi as indicated. *Upper* arrows indicate mCherry and *Lower* arrows indicate corresponding actin levels in the nucleus. (*B*) Western blotting (WB) analysis of the levels of endogenous Ipo9 and Ipo9-mChe RNAi res. Of note, our Ipo9 antibody fails to recognize any transfected Ipo9 construct and thus does not also recognize the Ipo9res-mChe RNAi res. (*C*) WB analysis of RNAi efficiency in mammalian cells. Double silencing of two proteins using RNAi effectively decreased the proteins levels in all cases as shown. (*D*) RNAi screen of selected mammalian transport factors in cells. NIH 3T3 cells were fractionated into nuclear and cytoplasmic fractions after RNAi as indicated. Protein levels of nuclear and cytoplasmic fractions were determined by WB using antiactin. Histone H3 antibody was used as a loading control for nuclear fraction. (*E*) Relative intensities of the bands in *D*. Nuclear actin signal intensities were quantified using Quantity One and normalized to the corresponding histone H3 intensities. (*F*) RNAi mediated silencing of Ipo9 in *Drosophila* cells. S2R+ cells were treated with four different dsRNAs targeting Ipo9 as indicated. Cells treated with dsRNA against GFP were used as control. To test the functionality of the antibody, NIH 3T3 cell lysate was included in the blot and the blot was detected with mammalian anti-Ipo9.

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Table S1. A list of all dsRNA used in *Drosophila* RNAi experiments and a list of all siRNA used in mammalian RNAi experiments

	dsRNA		siRNA		
Gene	Amplicon ID/primer sequence	Gene description	Target sequence 1	Target sequence 2	Target sequence 3
Cas	DRSC03287	importin 4	CACCTTCGATGAAGTATACAA	CACTTTCTTGGTCAAAGTCAA	CAGGACCACTTCCCTAAGCTA
RanBP21	DRSC19447	importin 5	CCCGACAATGTGGTCCGGAAA	AACCTAGTGTTTATTCTGTAA	CAGAATTGGCCAGGAATTTAA
RanBP16	DRSC20160	importin 8	AAGATCCGTATGAATACATAA	TAGGTGATTCCTGAATTACAA	ATCATGTATTTCCATTATTAA
Tnpo3	DRSC06020	transportin 2 (†)	CAGGCCATGATGTATACACAA	CTCCATGGCTTCAAAGACCAA	ATGGGATGAAGTACTCTGAAA
Ntf2r	DRSC02019	Ipo9	CACCGAGGAGCAGATTAAA	CTCCTACACTGTTAGGATT	
Crm1	DRSC03528	importin 13	ACCGACCAGTATGAAAGCTTA	CTGATATTGTTGATTCATTTA	ACGGGTGAAGGAGATGGTGAA
Ran	DRSC20364	exportin 7	CCGGAGATCCCTATTTAACAA	CCGGAACTATGTGCTCAACTA	CAGACACAACTCATCCTTTAA
Ssh	DRSC16730	exportin, tRNA (*)	CCGGTTTAAATTGTTTATGTA	CAGGGATATATATTTCCTAAA	TAGCTGGACTAAATTAATTAA
Chic	DRSC03507	exportin 4	AACACTATTAATGGAATTGAA	AAGGGCAAACTTAGTAATTCA	TACAAGTGTATTCTAAGAATA
Trn-SR	DRSC00546	exportin 1	TACGGAAATAATAATGACTAA		
Trn	DRSC11309	transportin 3	CAGGTTGTCATCCCAATCTTA	CAGGTTGTTTCGATAGCTCAA	CAGCACTTTAATGGACTCCTA
Ketel	DRSC03328	RAN binding protein 9	TAGCAATGGTGTAATATCAAA	CCCAAGTTTACTTGAAAGAAA	CAGACTGTTCTAGAAGAATTA
Karybeta3	DRSC12355	RAN binding protein 10	CAGGTTGATGATAGCTTATTA	CCGGATTTCCCTACAGCTTAA	CAGGATGCCTTTAGCCTACTA
Cadmus	DRSC16213	RAN binding protein 17	AACAGTGTTACAAATATTCAA	CACAATTAGATGACTAACAAA	ACCGATCTCACTGAAAGAATA
Moleskin	DRSC11340	karyopherin alpha 1	CTGAGAGAACATGTTAGTTTA	CTGATGCATAATGACTATAAA	
RanBP11	DRSC07525	karyopherin alpha 2	CGGCGTCACCGGGAAATTTAA	TCGCCGCCGAATAGAAGTTAA	
Kpna1	DRSC11356	karyopherin alpha 3	CTTGGGCAATTAGCAACTTAA	AAGAGACGAACACTTACTGAA	
Kpna4	DRSC16976	karyopherin alpha 4	CAGGTTCATCTTTCAAATCTA	ACCGAGAAACATATATATATA	
Tsr	DRSC04718	karyopherin alpha 6	CAGGTCATTCTTAATTGTTCA	CAGGAGGAACTCCTGAGCAAA	
Exp6	DRSC18787	karyopherin alpha 7	CAGGGAAATGATCTCGCAGGA	ACCAATCACATTTCTTCGGAA	
Ipo9 (1st)†	DRSC16813	RAN binding protein 6	AACAGTGTAATAAATACTAAA	AAGAATTTATCTAGAACTGAA	
Ipo9 (2nd)†	'S' ATCATTGTCAAATGCCTGGG	importin 11	CCCAAATATTAAAGTCAGAAA	AAGAATAAGTATAAATCCCAA	
	'R' GTCTGCTCGTCATCCTCCTC	exportin 5	CTGGATATAGTTGAATCTGAA	CTGGTTCTCGTTAGCAACCAA	
Ipo9 (3rd)†	'S' TATGTGCTTGAAACAGTCCAGG	nuclear transport factor 2	TGGGTTGAAGTCCTAAATGTA	CCCAGGAAATCTTAACTAGTA	CTGGATAGTATAAAGCTACTA
	'R' TGTGAGTACAAACTGCATCGC	Exp6	CGTTGATATTGGACGCCAA		
Ipo9 (4th)†	'S' CTCTGTGCAAGCTGTTCGAG	importin beta	CAACTGAAACCATTAGTCA		
	'R' AGATCGGATATGTAACGCGG	cofilin 1	GGAGGACCTGGTGTTCATC		
Pendulin	'S' AAACGCGCTGCGTTATC				
	'R' TGATCTGTGCCAGTCACAAT				
GFP	'S' GAGGAGCTGTTCACC				
	'R' GGCGAGCTGCACGCTGCC				

For Ipo9, target sequence 1 was used in most experiments.