Supplemental Information
Molecular Cell, *Volume 39*

Spindle Position Is Coordinated with Cell-Cycle Progression through Establishment of Mitotic Exit-Activating and -Inhibitory Zones
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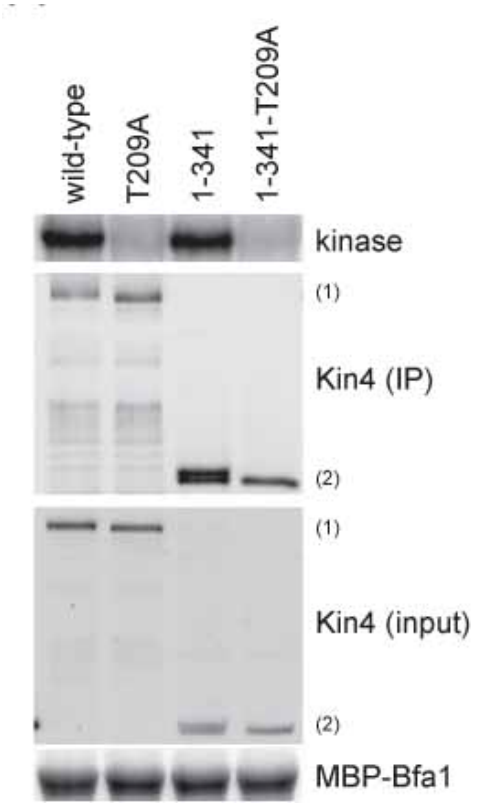

Figure S1. Kinase Activity Associated with Kin4(1-341) (Related to Figure 2)

Cells expressing Kin4-3HA (A11779), kin4-T209A-3HA (kinase dead) (A22119), kin4(1-341)-3HA (A14052) or kin4(1-341)-T209A-3HA (A22278) were grown to exponential phase and arrested with 15 μg/ml nocodazole for 2 hr. Kin4 associated kinase activity (top, Kin4 kinase), immunoprecipitated Kin4-3HA (second row, Kin4 (IP)), total amount of Kin4-3HA in extracts (third row, Kin4 (input)) and levels of Bfa1 substrate (as monitored by Coomassie stain) added to the kinase reaction (bottom, MBP-Bfa1) are shown. The band that is shown for Kin4 associated kinase activity and total Bfa1 substrate is the first major degradation product of MBP-Bfa1 as described in Maekawa et al., 2007 and was the dominant signal. (1) denotes full length and (2) denotes the kinase domain alone.

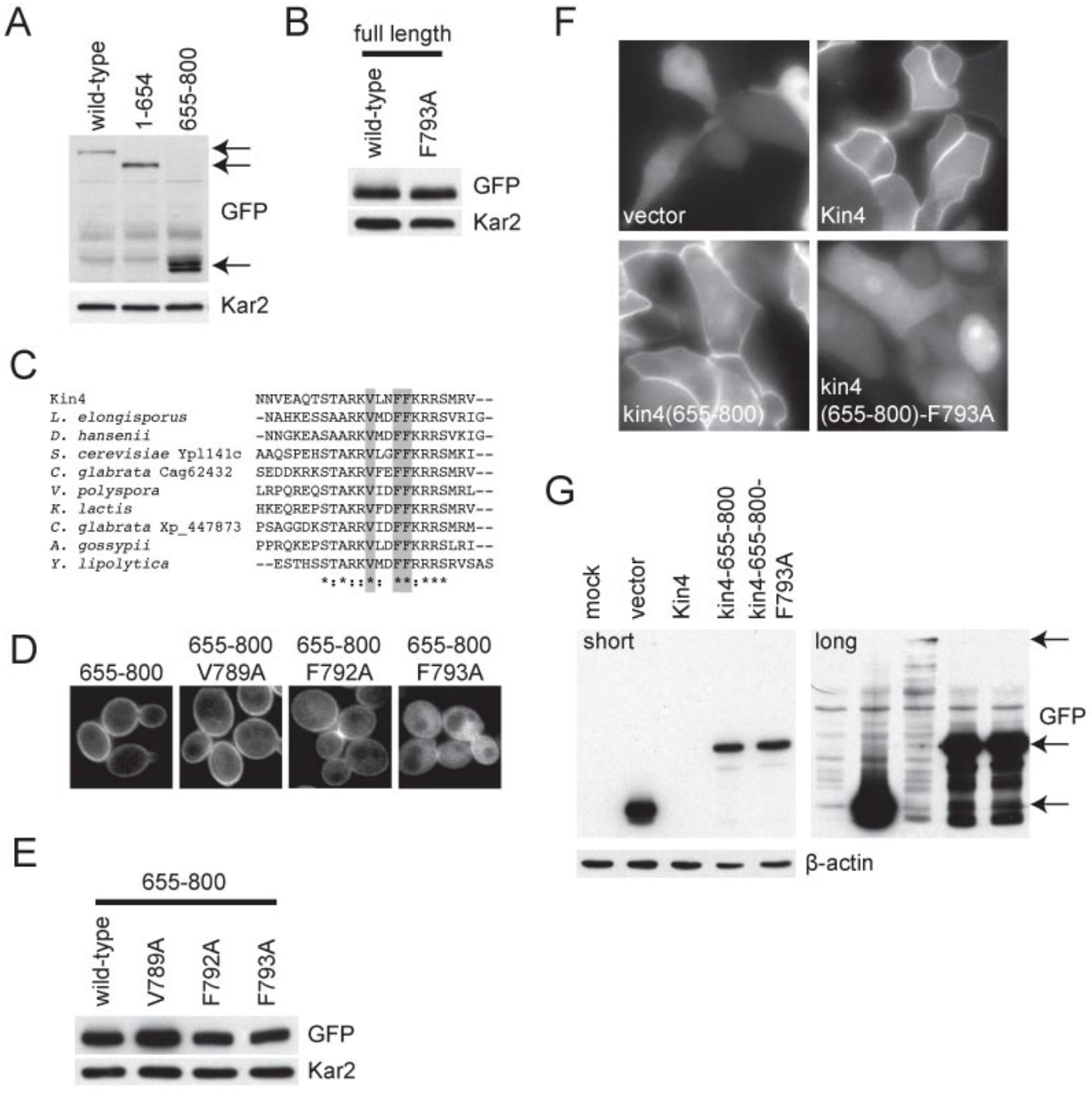
A
wild-type
1-654
655-800
GFP
Kar2
B
full length
wild-type
F793A
GFP
Kar2
C
Kin4 NNVEAQTSTARKVLNFFKRRSMRV--
L. elongisporus -NAHKESSAARKVMDFFKRRSVRIG-
D. hansenii -NNGKEASAARKVMDFFKRRSVKIG-
S. cerevisiae Ypl141c AAQSPEHSTAKRVLGFFKRRSMKI--
C. glabrata Cag62432 SEDDKRKSTAKRVFEFFKRRSMRV--
V. polyspora LRPQREQSTAKKVIDFFKRRSMRL--
K. lactis HKEQREPSTAKRVFDFFKRRSMRV--
C. glabrata Xp_447873 PSAGGDKSTARRVIDFFKRRSMRM--
A. gossypii PPRQKEPSTARKVLDFFKRRSLRI--
Y. lipolytica --ESTHSSTARKVMDFFRRRSRVSAS
:::*: **:***
D
655-800
655-800 V789A
655-800 F792A
655-800 F793A
E
655-800
wild-type
V789A
F792A
F793A
GFP
Kar2
F
vector
Kin4
kin4(655-800)
kin4 (655-800)-F793A
G
mock
vector
Kin4
kin4-655-800
kin4-655-800-F793A
short
long
GFP
β-actin

Figure S2. Analysis of Amino Acids 655-800 of Kin4 (Related to Figure 3)

(A) Cells expressing an mCherry-Tub1 fusion protein and Kin4-GFP (A19900), kin4(1-654)-GFP (A21575) or kin4(655-800)-GFP (A21576) were lysed and analyzed for expression of the GFP fusion protein by western blot. Arrows indicate the relevant bands. Kar2 was used as a loading control.

(B) Cells expressing mCherry-Tub1 and Kin4-GFP (A19900) or kin4-F793A-GFP (A21556) fusion proteins were analyzed as in (A).

(C) The sequence of the last 24 amino acids of Kin4 and its orthologs were aligned using T-Coffee. The residues V789, F792 and F793 are highlighted. An asterisk denotes a strictly conserved residue and a colon denotes a similarly conserved residue.

(D) Cells expressing GFP-kin4(655-800) (A18463), GFP-Kin4(655-800)-V789A (A18452), GFP-kin4(655-800)-F792A (A18451) or GFP-kin4(655-800)-F793A (A18450) from the galactose inducible promoter were grown to exponential phase in YePA + 2% raffinose and induced with the addition of 2% galactose for 90 min and imaged live.

(E) Cells in (D) were analyzed for expression of the Kin4 fusion protein as in (A).

(F) 293T cells were transiently transfected with plasmids expressing eGFP (pAM50), eGFP-Kin4 (pAM51), eGFP-kin4(655-800) (pAM52) or eGFP-kin4(655-800)-F793A (pAM53) and imaged live. The exposure times are approximately 5ms for eGFP, 100ms for eGFP-Kin4 and 20ms for eGFP-kin4(655-800) and eGFP-kin4(655-800)-F793A.

(G) Cells in (F) were lysed and analyzed for expression of the GFP fusion protein by western blot. Short and long exposures are shown due to the lower expression of eGFP-Kin4. Arrows indicate the relevant bands. Levels of β-actin were used as a loading control.

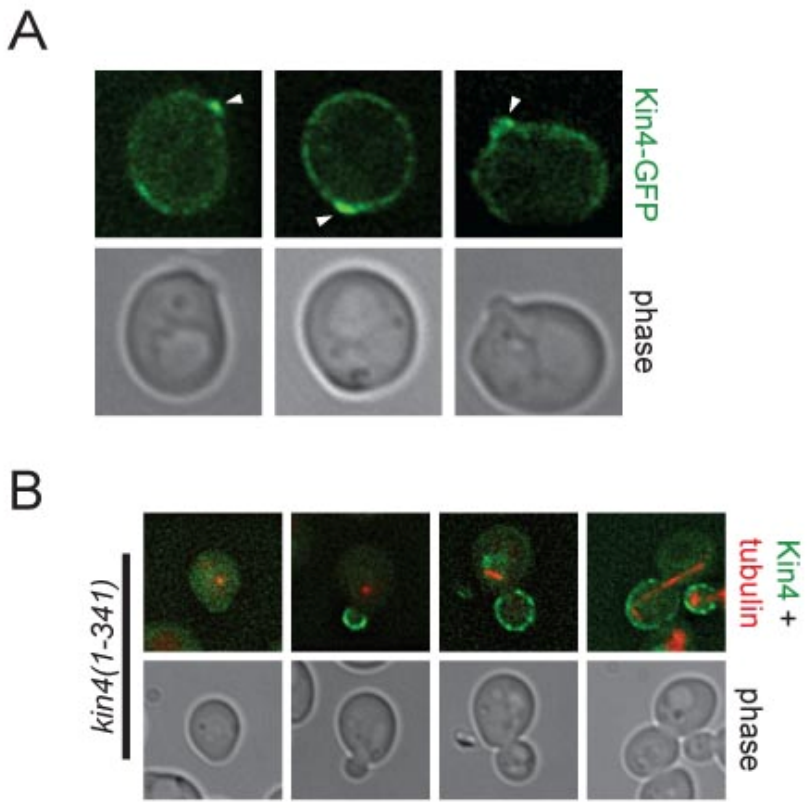

Figure S3. Localization of Kin4 in Nascent Budded Cells (Related to Figure 3)

(A) Cells expressing Kin4-GFP (A19900) were grown to exponential phase and imaged live. The deconvolved GFP signal shown is from 8 serial sections. Arrows indicate the position of the nascent bud.

(B) Cells expressing an mCherry-Tub1 fusion protein and kin4(1-341)-GFP (A23410) were grown to exponential phase and imaged live. The deconvolved GFP signal shown is from 8-10 serial sections. Kin4-GFP is in green and mCherry-Tub1 is in red.

Table S1. Table of Yeast Strains

A1828	*MATa TEM1-3MYC*
A1863	*MATa bub2Δ::HIS3*
A2587	*MATa ade2-1 leu2-3 ura3 trp1-1 his3-1115 can1-100 GAL [phi+] (W303)*
A4874	*MATa spo12Δ::HIS3*
A11779	*MATa KIN4-3HA:KanMx6*
A11997	*MATa His3Mx6:pGAL1-10-GFP-KIN4*
A14052	*MATa kin4(1-341)-3HA:His3Mx6*
A17349	*MATa dyn1Δ::URA3*
A17351	*MATa kin4Δ::KanMx6 dyn1Δ::URA3*
A17865	*MATa kin4Δ::KanMx6*
A18450	*MATa trp1:pGAL1-10-GFP-kin4(655-800)-F793A:TRP1*
A18451	*MATa trp1:pGAL1-10-GFP-kin4(655-800)-F792A:TRP1*
A18452	*MATa trp1:pGAL1-10-GFP-kin4(655-800)-V789A:TRP1*
A18463	*MATa trp1:pGAL1-10-GFP-kin4(655-800):TRP1*
A18792	*MATa His3Mx6:pGAL1-10-GFP-KIN4 bub2Δ::HIS3*
A19900	*MATa ura3:mCherry-TUB1:URA3 KIN4-GFP:His3Mx6*
A20608	*MATa KIN4-S508A-3HA:KanMx6*
A21298	*MATa kin4-F793A:KIN4-3'UTR:KanMx6 dyn1Δ::URA3*
A21299	*MATa KIN4-S508A:KIN4-3'UTR:KanMx6*
A21301	*MATa KIN4-S508A:KIN4-3'UTR:KanMx6 dyn1Δ::URA3*
A21555	*MATa kin4(Δ503-511)-GFP:His3Mx6 ura3:mCherry-TUB1:URA3*
A21556	*MATa kin4-F793A-GFP:His3Mx6 ura3:mCherry-TUB1:URA3*
A21557	*MATa KIN4-S508A-GFP:His3Mx6 ura3:mCherry-TUB1:URA3*
A21575	*MATa kin4(1-654)-GFP:His3Mx6 ura3:mCherry-TUB1:URA3*
A21576	*MATa trp1:kin4(655-800)-GFP:TRP1 ura3:mCherry-TUB1:URA3*
A22119	*MATa kin4-T209A-3HA:KanMx6*
A22262	*MATa kin4(1-341):KanMx6 dyn1Δ::URA3*
A22263	*MATa kin4(1-654):KanMx6 dyn1Δ::URA3*
A22278	*MATa kin4(1-341)-T209A-3HA:His3Mx6*
A22736	*MATa kin4-T209A:KIN4-3'UTR:KanMx6 dyn1Δ::URA3*
A23045	*MATa bub2Δ::KanMx6*
A23051	*MATa KIN4-S508A-GFP:His3Mx6 ura3:mCherry-TUB1:URA3 kar9Δ::His3Mx6*
A23052	*MATa KIN4-S508A-GFP:His3Mx6 ura3:mCherry-TUB1:URA3 dyn1Δ::His3Mx6*
A23055	*MATa KIN4-S508A-GFP:His3Mx6 ura3:mCherry-TUB1:URA3 clb4Δ::His3Mx6*
A23249	*MATa KIN4-GFP:His3Mx6 ura3:mCherry-TUB1:URA3 clb4Δ::HIS3*
A23250	*MATa His3Mx6:pGal1-10-GFP-kin4(1-341):KanMx6*
A23410	*MATa kin4(1-341)-GFP:HIS3Mx6 ura3:mCherry-TUB1:URA3*
A23686	*MATa KanMx6:pGAL1-10-URL-3HA-LTE1*
A24083	*MATa KIN4-S508A:KIN4-3'UTR:KanMx6 clb4Δ::HIS3*
A24084	*MATa KanMx6:pGAL1-10-URL-3HA-LTE1 KIN4-S508A:KIN4-*

	3'UTR:KanMx6
A24085	*MATa KanMx6:pGAL1-10-URL-3HA-LTE1 clb4Δ::HIS3*
A24086	*MATa KanMx6:pGAL1-10-URL-3HA-LTE1 KIN4-S508A:KIN4-3'UTR:KanMx6 clb4Δ::HIS3*
A24113	*MATa His3Mx6:pGAL1-10-GFP-kin4(1-341):KanMx6 bub2Δ::HIS3*
A24346	*MATa KanMx6:pGAL1-10-URL-3HA-LTE1 KIN4-S508A:KIN4-3'UTR:KanMx6 clb4Δ::NatMx4 bub2Δ::HIS3*
A24543	*MATa KanMx6:pGAL1-10-URL-3HA-LTE1 spo12Δ::HIS3*
A24586	*MATa kin4-SPC72(177-622):KanMX6*
A24587	*MATa kin4-SPC72(177-622):KanMX6 KanMx6:pGAL1-10-URL-3HA-LTE1*
A24588	*MATa kin4-SPC72(177-622):KanMX6 KanMx6:pGAL1-10-URL-3HA-LTE1 bub2Δ::HIS3*
A24761	*MATa KIN4-S508A:KIN4-3'UTR:CaURA3Mx4 clb4Δ::NatMx4 KanMx6:pGAL1-10-URL-3HA-LTE1 TEM1-3MYC*
A24805	*MATa clb4Δ::NatMx4 KanMx6:pGAL1-10-URL-3HA-LTE1 TEM1-3MYC*
A24806	*MATa kin4Δ::KanMx6 lte1Δ::NatMx4*
A24807	*MATa lte1Δ::NatMx4*
A24808	*MATa bub2Δ::KanMx6 lte1Δ::NatMx4*
A24816	*MATa KanMx6:pGAL1-10-URL-3HA-LTE1 KIN4-S508A:KIN4-3'UTR:KanMx6 kar9Δ::His3Mx6*
A24817	*MATa kin4-SPC72(177-622):KanMX6 KanMx6:pGAL1-10-URL-3HA-LTE1 kar9Δ::His3Mx6*
A24858	*MATa kin4-SPC72(177-622):KanMX6 KanMx6:pGAL1-10-URL-3HA-LTE1 clb4Δ::HIS3*
A25794	*MATa KIN4-GFP:His3Mx6 ura3:mCherry-TUB1:URA3 clb4Δ::NatMx6 kar9Δ::His3Mx6*

Table S2. Table of Plasmids

pA1419	YIplac204-*pGAL1-10-GFP-kin4(655-800)*
pA1425	YIplac204-*pGAL1-10-GFP-kin4(655-800)-F793A*
pA1426	YIplac204-*pGAL1-10-GFP-kin4(655-800)-F792A*
pA1427	YIplac204-*pGAL1-10-GFP-kin4(655-800)-V789A*
pA1607	YIplac211-*kin4(Δ503-511)-3HA*
pA1608	YIplac211-*kin4-F793A-3HA*
pA1609	YIplac211-*KIN4-S508A-3HA*
pA1624	YIplac204-*kin4(655-800)-GFP*
pA1760	pFA6a-*SPC72(177-622)-KanMx6*
pAM50	pEGFP-C1
pAM51	pEGFP-C1-*KIN4*
pAM52	pEGFP-C1-*kin4(655-800)*
pAM53	pEGFP-C1-*kin4(655-800)-F793A*

Supplemental Experimental Procedures

Yeast Strains

All strains are derivatives of W303 (A2587) and are listed in Table S1. *KIN4-3'UTR:KanMx6, lte1Δ::NatMx4*, *bub2Δ::KanMx6*, *kin4(1-341), kin4(1-341)-GFP, kin4(1-341)-3HA, GAL-GFP-kin4(1-341), kin4(1-341)-T209A-3HA, kin4(1-654), kin4(1-654)-GFP, kin4-F793A-GFP, kin4(Δ503-511)-GFP, KIN4-S508A-GFP, KIN4-S508A-3HA, GAL-URL-3HA-LTE1, kin4-SPC72(177-622)* and *clb4Δ::NatMx4* were constructed by standard PCR based methods (Goldstein and McCusker, 1999; Longtine et al., 1998). *kin4(Δ503-511), kin4-F793A* and *KIN4-S508A* were constructed by two-step gene replacement using the *URA3* gene from *Kluyveromyces lactis* and PCR products derived from plasmids pA1607, pA1608 and pA1609 respectively. pA1419, pA1425, pA1426 and pA1427 were digested with EcoRV and integrated at the *TRP1* locus to generate *pGAL1-10-GFP-kin4(655-800)-[V789A, F792A, F793A]* alleles. pA1624 was integrated as above to generate the *kin4(655-800)-GFP* allele.

Plasmid Construction

All plasmids used in this study are listed in Table S2. pA1419 was constructed by digesting YIplac204 and a PCR product containing *pGAL1-10-GFP-kin4(655-800)* with BamHI and SacI and ligating the two fragments together. pA1425 - pA1427 were constructed by site directed mutagenesis of pA1419. pA1607 was constructed by digesting pA1207 (D'Aquino et al., 2005) and a PCR fragment containing the Δ503-511 deletion (constructed by PCR mediated ligation of two overlapping fragments spanning the deletion) with BsrGI and MscI and ligating the two fragments together. pA1608 and pA1609 were generated by site directed mutagenesis of pA1207. pA1624 was generated by sequential cloning into YIplac204 of the promoter of *KIN4* (1kb upstream of the start codon) with HindIII and then a PCR fragment containing *kin4(655-800)-GFP* with SphI and SacI. pA1760 was generated by digesting pFA6a-3HA-KanMx6 and a PCR fragment containing *SPC72(177-622)* (Maekawa et al., 2007) with AscI and PacI and ligating the two fragments together. pAM51 and pAM52 were constructed by digesting pAM50 (pEGFP-C1 [Clontech]) with EcoRI and ligating in PCR fragments containing *KIN4* and *kin4(655-800)* digested with MfeI respectively. pAM53 was generated by site directed mutagenesis of pAM52.

Immunoblot Analysis

For immunoblot analysis of mammalian cell lysates, cells were harvested, washed once in PBS (100 mM sodium phosphate [pH 7.2], 0.9% NaCl), resuspended in lysis buffer (PBS + 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml Benzonase [Novagen], 100 mM DTT and complete protease inhibitor cocktail [Roche]), incubated on ice for 30 min and boiled after the addition of sample buffer. GFP fusion proteins were detected using a mouse anti-GFP antibody cocktail (Roche) at 1:2000 and β-actin was detected using a mouse anti-β-actin antibody (Sigma AC-74) at 1:15,000.

Fluorescence Microscopy

To image 293T cells, cells were seeded on a poly-lysine coated coverslip and transfected with the appropriate plasmid (see below for transfection details). Cells were imaged live 24 hr posttransfection on a Zeiss Observer.Z1 inverted scope with a 40X objective. Images were collected with a Hammamatsu ORCA-ER C4742-80 digital CCD camera and analyzed with Metamorph software (Molecular Devices).

Mammalian Cell Growth and Transfection

293T cells were cultured in DMEM supplemented with 10% NBCS, 4 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated at a density of 1×10^5 cells/cm^2 24 hr prior to transfection. Cells were transfected using TransIT-LT1 transfection reagent (Mirus) according to the manufacturer's recommendations. Cells were imaged and harvested for immunoblot analysis 24 hr posttransfection.

Supplemental References

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