Supplemental Materials

Molecular Biology of the Cell

Joshi and Inamdar

Rudhira/BCAS3 couples microtubules and intermediate filaments to promote cell migration for angiogenic remodeling.

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Inventory of Supplementary Information

Supplementary figure legends

Figure S1. Rudhira is required for cytoskeleton organization (related to Figure 1). (A) NS and KD cells expressing Vimentin-GFP were co-stained with Phalloidin to detect the IF and actin cytoskeleton organization, shown as the bottom, middle and top regions (projections of 12-16 optical slices each) and the entire cell volume. (B) Three-dimensional (3-D) reconstruction of the images in (A) showing the distribution of Vimentin IFs and actin stress fibres in the cell volume in shadow and surface modes. (C, D) NS and KD cells expressing Vimentin-GFP were kept untreated (C) or treated with Cytochalasin D to depolymerize actin stress fibres (D). Cells were counterstained with Phalloidin to validate actin depolymerization. Scale Bar: (A-D) 20 µm.

Figure S2. Rudhira regulates Plectin localization and MT-IF crosstalk (related to Figure 1). (A) NS and KD cells were analysed for Plectin organization by immunostaining. Boxed regions are magnified in the insets. (B) Validation of *plectin* knockdown by immunoblotting in SVEC. (C) SVEC cells were transfected with *plectin* shRNA and analysed for Rudhira localization by immunostaining. Cells were co-stained for Plectin to validate the knockdown. Arrows mark *plectin* knockdown cells and arrowheads mark the likely untransfected internal controls. Cells were counterstained with DAPI to mark nuclei (blue). Results shown are a representative of two independent experiments. (D) SVEC cells were transiently transfected with Rudhira-GFP and analysed for coalignment of Vimentin and MTs by co-immunofluorescence. Green arrows mark transfected cells and white arrowheads mark the likely untransfected internal controls. Graph shows the percent overlap between Vimentin and Tubulin pattern in Rudhira overexpressing cells (green bar) compared to untransfected internal controls (black bar) (30 cells). (E) Control and *plectin* knockdown cells were transiently transfected with Rudhira-GFP and analysed for coalignment of Vimentin and MTs by co-immunofluorescence. Green arrows mark transfected cells and white arrowheads mark the likely untransfected internal controls in each condition. Graph shows the percent

overlap between Vimentin and Tubulin pattern in Rudhira overexpressing cells (green bars) compared to untransfected internal controls (black bars), as indicated (22 cells). Results shown are a representative of two independent experiments. Statistical analysis was carried out using one-way ANOVA. Scale Bar: (A-E) 20 µm. *p<0.05, **p<0.01, ***p<0.001.

Figure S3. Rudhira is required for MT dynamics and binds to stable microtubules (related to Figures 2 and 3). (A) Time-lapse images of NS and KD cells incubated with SiR-Tubulin and imaged at 4-second intervals. Arrow indicates persistence of radial MT at cell periphery, while red asterisk indicates a MT bending away from the cell periphery. Green asterisk shows a MT travelling parallel to the cell periphery. 10 live cells each of NS and KD were imaged. (B) Time-projection of EB1-GFP tracks in Hela cells co-transfected with EB1-GFP and SNAP or Rudhira-SNAP vectors. Time-lapse images of EB1-GFP comets were analysed manually for calculating relative distance and average velocity (7 EB1-GFP comets from 3 cells) shown in the graphs. (C) Wild type SVECs were transiently transfected with GFP or Rudh-GFP, treated with Nocodazole and overlap between Rudhira-GFP and MTs was analyzed by immunostaining for Tubulin. Boxed regions are magnified in the insets. Validation of Rudhira-GFP overexpression by immunoblot in HEK293T cells. Results shown are a representative of at least three independent experiments. Scale Bar: (A) 2 µm, (B) 10 µm, (C) 5 µm.

Figure S4. Rudhira controls focal adhesion size indirectly (related to Figure 4). (A, B) Non-silencing control (NS) or *rudhir*a knockdown (KD) endothelial cells (SVEC) were analyzed by immunostaining for Paxillin (A) and p-Tyrosine (pY) (B) to detect FAs. Adjacent panels show threshold-set and binary-converted images. (C) Wild type cells were transiently transfected with Rudhira-2A-GFP (Rudh2AGFP) or EGFP vectors and analyzed for FA size by immunostaining for Paxillin. Green box and inset show FAs in transfected cell (marked by green arrows) and white box and inset show FAs in untransfected cell

(marked by white arrowheads). Graph shows the quantitation of FA size in cells overexpressing Rudhira as compared to the untransfected cells. Boxed regions in (A, B, C) are magnified in the insets. (D, D') Wild type SVEC cells were co-stained to detect the extent of overlap between Rudhira and FA markers Paxillin (D) and pY (D'). (E) Control or *plectin* knockdown SVEC cells were analysed by immunostaining for Paxillin to detect FAs. Cells were counterstained with Phalloidin to detect actin and DAPI to mark nucleus. Graph shows the quantitation of mean FA size (692 FAs from 20 cells). (F) *Plectin* knockdown cells were transiently transfected with Rudhira-GFP and analysed for FA size by immunostaining for Paxillin. Boxed regions in (F) are magnified in the insets. Green box and inset on top show FAs in transfected cell (marked by green arrows) and white box and inset on bottom show FAs in untransfected cell (marked by white arrowheads). Graph shows the quantitation of mean FA size (100 FAs from 10 transfected cells). Results shown are a representative of at least three independent experiments. Statistical analysis was carried out using one-way ANOVA. Scale Bar: (A, B, D) 10 µm, (C, E, F) 20 µm (D') 5 µm. *p<0.05, **p<0.01, ***p<0.001.

Figure S5. Rudhira regulates microtubule-mediated FA turnover downstream of FAK activation (related to Figure 4). Non-silencing control (NS) or *rudhira* knockdown (KD) SVECs were analysed for MT-mediated FA dynamics. (A) Recovery after Nocodazole treatment validated by α-Tubulin immunostaining of fixed cells to detect MTs. Cells were co-stained with Phalloidin to detect F-actin and DAPI to mark nuclei (Blue). (B, C) Immunostaining for FA signaling proteins pFAK, β1 Integrin and FAK. Results shown are a representative of at least three independent experiments. Scale Bar: (A) 20 µm, (B, C) 10 µm.

Figure S6. Rudhira organizes cytoskeleton and mediates cytoskeletal cross-regulation (related to Figure 5). (A, B) NS or KD cells expressing Vimentin-GFP were kept untreated (A) or treated with 10 µM

ROCKi (B) and analysed for Vimentin IF and actin organization by staining for Phalloidin. Scale Bar: (A, B) 20 μm.

Figure S7. Rudhira regulates MTs, Vimentin IFs and FA size and compensates for MT depolymerization or Vimentin IF depletion (related to Figures 4, 5). (A-D) NS and KD cells expressing Vimentin-GFP were kept untreated (A) or treated with 1 μM Nocodazole (to depolymerize MTs) (B) or 10 mg/ml Cycloheximide (CHX) (to aggregate IFs) (C) or 5 mM Acrylamide (also to aggregate IFs) (D) and analysed for FAs, MTs and Vimentin IFs, as indicated. Graph shows the quantitation of relative FA size in NS and KD cells upon various treatments (60 FAs for each condition). (E-G) SVEC cells transiently overexpressing Rudhira (Rudhira-IRES-GFP vector transfected) were kept untreated (E) or treated with 1 μM Nocodazole (F) or 10 mg/ml CHX (G) and analysed for FAs, Vimentin IFs and MTs, as indicated. Boxed regions in (E-G) are magnified in the insets. Green box and inset show FAs in transfected cell (marked by green arrows) and white box and inset show FAs in untransfected cell (marked by white arrowheads). Graph shows the quantitation of relative FA size upon various treatments (100 FAs from 5 transfected cells for each condition). Results shown are a representative of two independent experiments. Scale Bar: (A-G) 20 μm.

Figure S8. Structure prediction and functional analysis of Rudhira fragments (related to Figure 6). (A) Table summarising the observations from the bioinformatics analysis for Rudhira domain prediction and the deletion mutants generated. (B, B') Prediction of Rudhira full length protein (Full) structure using Phyre2 (B) and RaptorX (B'). (C) Distribution and frequency of the post-translational modifications (PTMs) in Rudhira protein (by Phosphosite plus). Boxed region indicates PTMs in the BCAS3+ fragment (461-928 aa). (D) Predicted MT-binding motifs in Rudhira protein (by MAPanalyzer). (E) Structure prediction of Rudhira mutants, as indicated, using RaptorX. (F, G) KD cells were transiently transfected

with pIRES2-EGFP vector or constructs of full-length Rudhira, BCAS3 or ΔBCAS3 mutants and analyzed for MT and actin organization (F) or FA size and MTs (G) by immunostaining. Boxed regions in (F) are magnified in the insets. Results shown are a representative of at least three independent experiments. Scale Bar: (F, G) 20 μm.

Supplementary video legends

Video S1. Loss of Rudhira results in unaligned MTs and MT bending before reaching cell periphery (related to Figure 2). Time-lapse images of SVEC NS and KD incubated with SiR-Tubulin monitored for 2 minutes, imaged at 4 second intervals (6 fps). Arrow indicates persistence of radial MT at cell periphery, while red asterisk indicates a MT bending away from the cell periphery. Green asterisk shows a MT travelling parallel to the cell periphery.

Video S2. Rudhira bridges microtubules and intermediate filaments for cell migration (related to Figure 2). Time-lapse images of SVEC NS and KD transiently expressing Vimentin-GFP incubated with SiR-Tubulin monitored for 4 minutes, imaged at 10 second intervals (6 fps). Arrow indicates persistent MT-IF coalignment and their similar dynamics, while arrowhead indicates absence of peripheral IFs and perturbed MT-IF association and dynamics in live migrating cells.

Video S3. Rudhira regulates microtubule growth and alignment towards cell periphery (related to Figure 2). Time-lapse images of the cell peripheral region of SVEC NS and KD transiently transfected with EB1-GFP monitored for 2-3 minutes imaged at 3-4 second intervals (6 fps). Fps: frames per second. Arrows indicate persistence of aligned EB1 positive MT growing end in NS and not in KD while asterisks

indicate a MT end not stabilized at the cell periphery. Also note the shorter EB1-GFP comets in KD as compared to NS.

Video S4. Rudhira regulates microtubule growth towards cell periphery (related to Figure 2). Time-lapse images of SVEC NS and KD transiently transfected with EB1-GFP monitored for 1-2 minutes imaged at 3 second intervals and manually tracked EB1-GFP positive comets (6 fps).

Video S5. Rudhira regulates focal adhesion disassembly (related to Figure 4). Time-lapse images of SVEC NS and KD transiently transfected with Paxillin-GFP monitored for 2-3 hours imaged at 5-minute intervals (4 fps). Arrow indicates a FA getting disassembled, while arrowhead indicates a FA persisting over time and not getting disassembled.

Video S6. BCAS3 domain of Rudhira is essential for MT dynamic organization (related to Figure 6). Time-lapse images of SVEC KD cell line transiently overexpressing Rudhira or RudhiraΔBCAS3, incubated with SiR-Tubulin monitored for 2 minutes, imaged at 4 second intervals (6 fps). Arrow indicates persistence of radial MT at cell periphery, while asterisk indicates a MT bending before reaching cell periphery. Note the MTs criss-crossing and not aligned towards cell periphery (red box) in vector/ΔBCAS3 transfected KD cells, as compared to aligned MTs (green box) in Rudhira transfected KD cells.

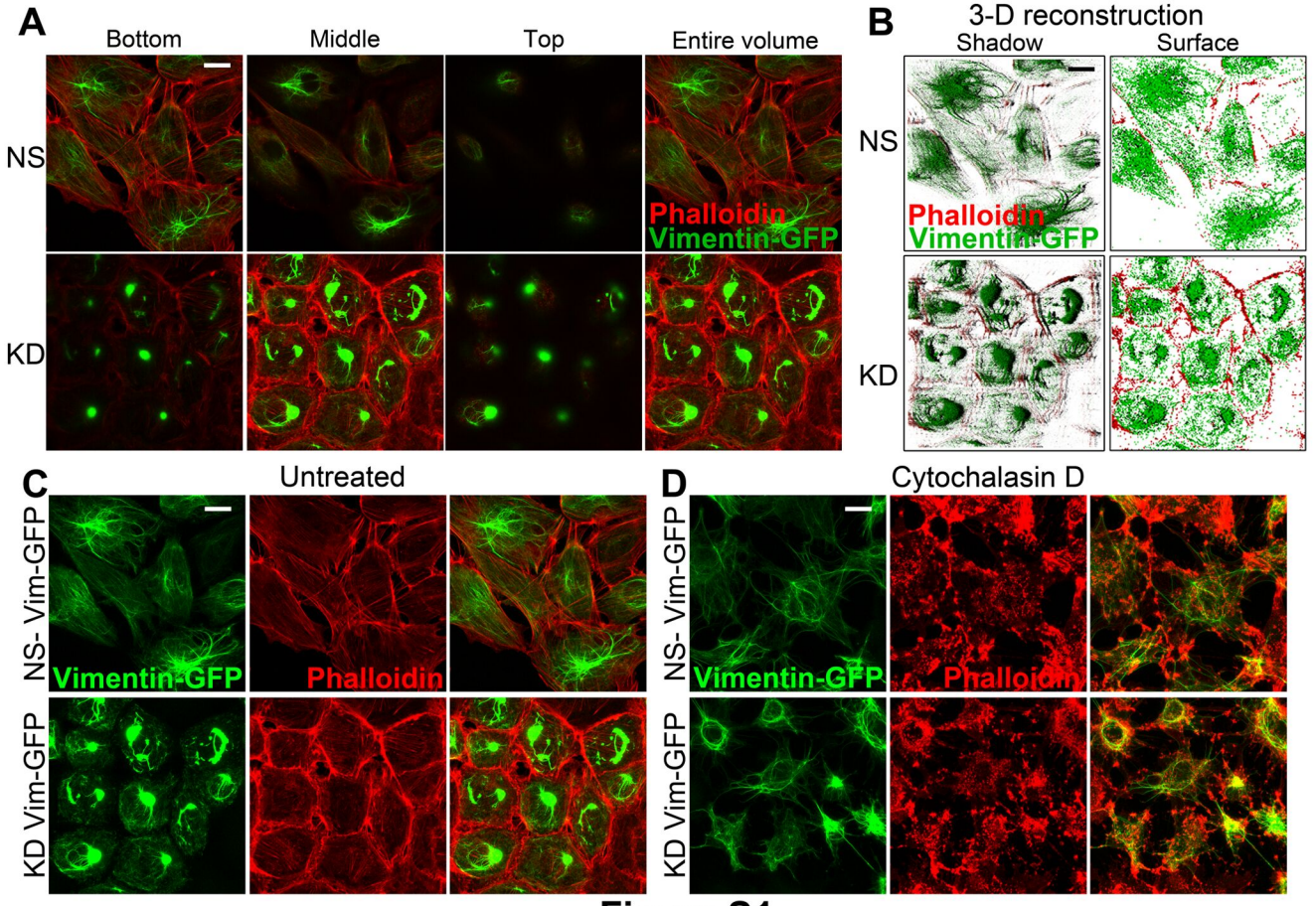
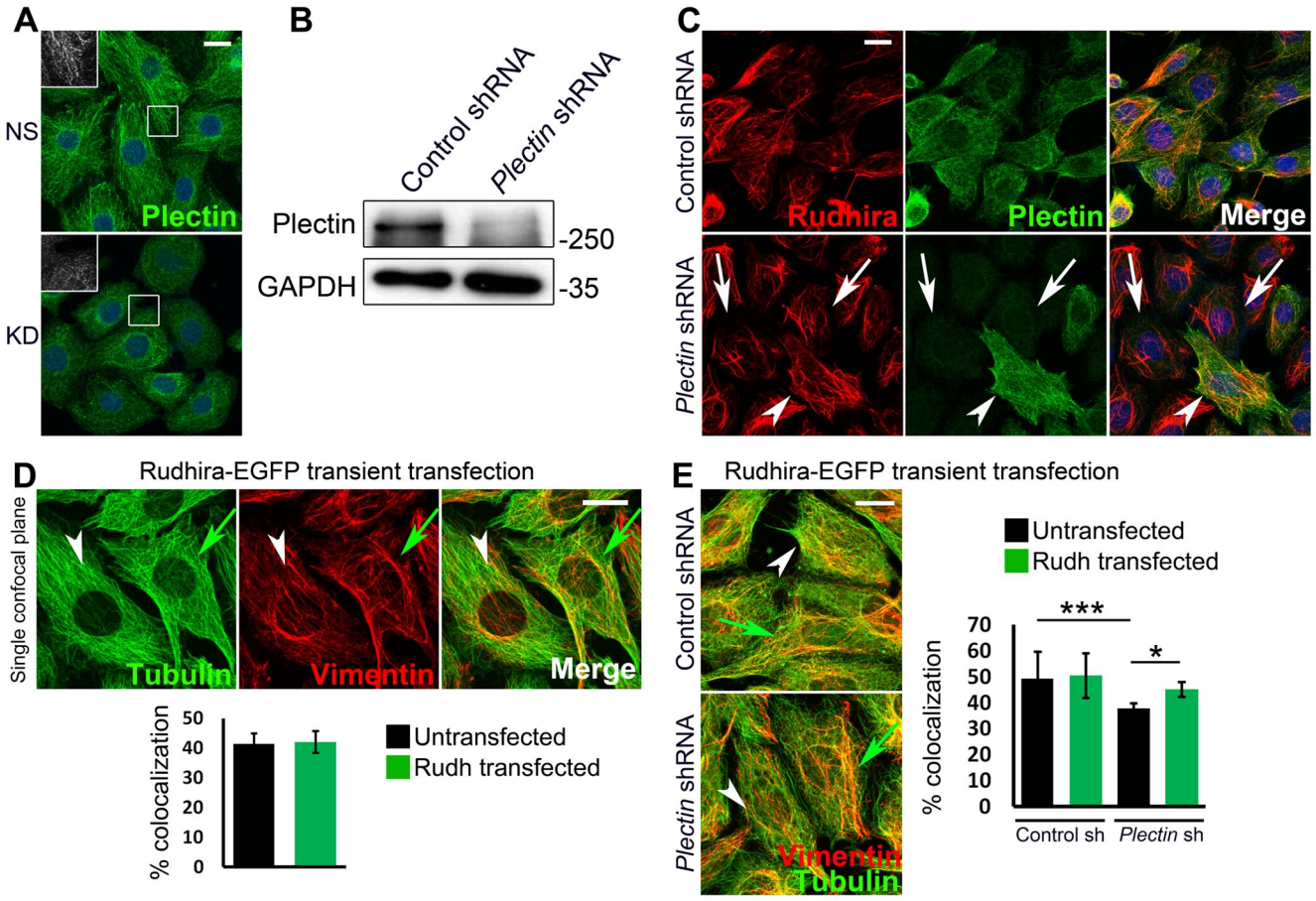

A
Bottom
Middle
Top
Entire volume
NS
KD
Phalloidin
Vimentin-GFP
B
3-D reconstruction
Shadow
Surface
NS
KD
Phalloidin
Vimentin-GFP
C
Untreated
NS- Vim-GFP
KD Vim-GFP
Vimentin-GFP
Phalloidin
D
Cytochalasin D
NS- Vim-GFP
KD Vim-GFP
Vimentin-GFP
Phalloidin

Figure S1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NS
Plectin
KD
B
Control shRNA
Plectin shRNA
Plectin
-250
GAPDH
-35
C
Control shRNA
Plectin shRNA
Rudhira
Plectin
Merge
D
Rudhira-EGFP transient transfection
Single confocal plane
Tubulin
Vimentin
Merge
% colocalization
50
40
30
20
10
0
Untransfected
Rudh transfected
E
Rudhira-EGFP transient transfection
Control shRNA
Plectin shRNA
Vimentin
Tubulin
Untransfected
Rudh transfected
% colocalization
70
60
50
40
30
20
10
0
***
*
Control sh
Plectin sh

Figure S2

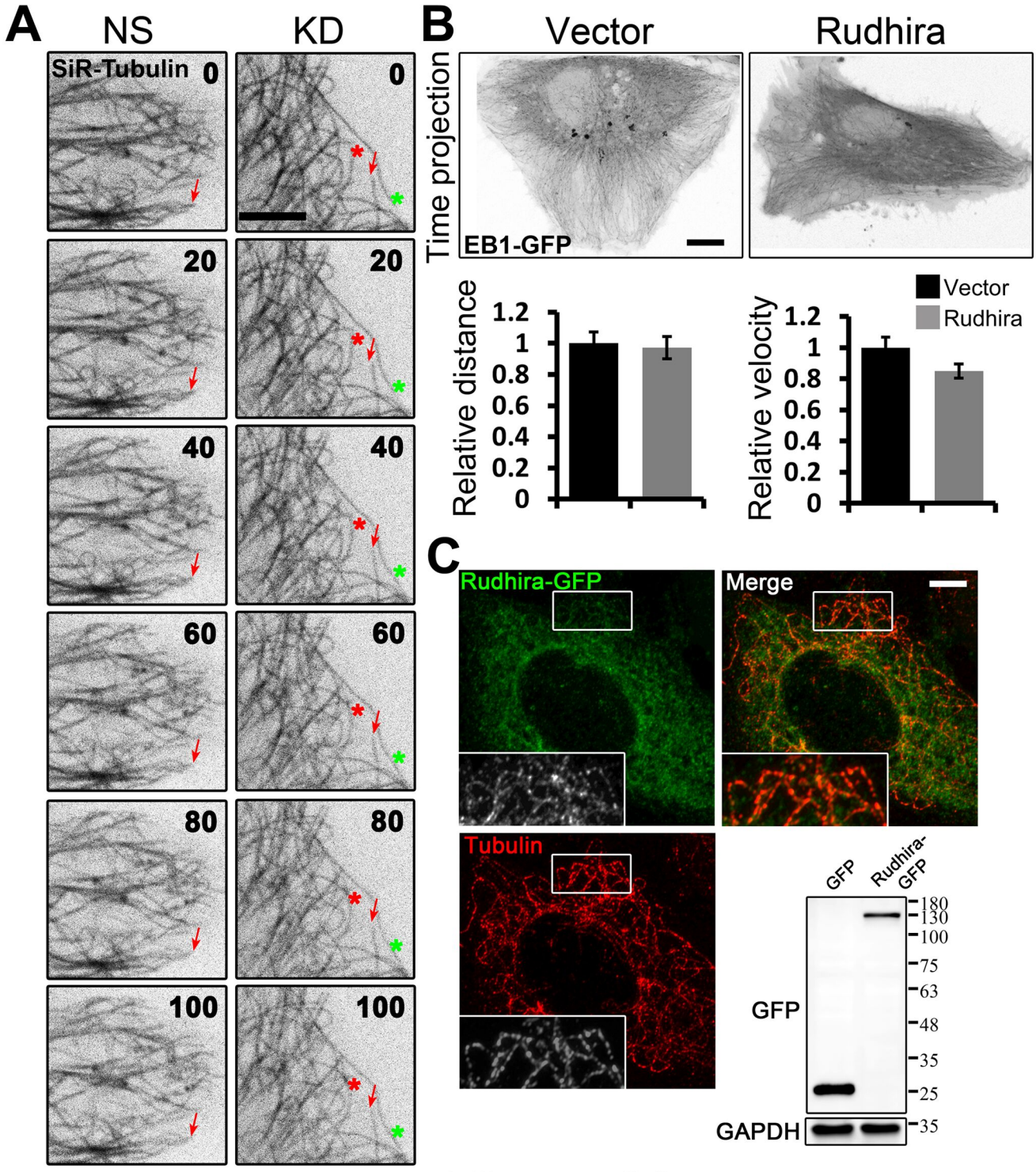

A
NS
KD
SiR-Tubulin
0
20
40
60
80
100
B
Vector
Rudhira
Time projection
EB1-GFP
Relative distance
Relative velocity
1.2
1
0.8
0.6
0.4
0.2
0
Vector
Rudhira
C
Rudhira-GFP
Merge
Tubulin
GFP
Rudhira-GFP
180
130
100
75
63
48
35
25
GAPDH
35

Figure S3

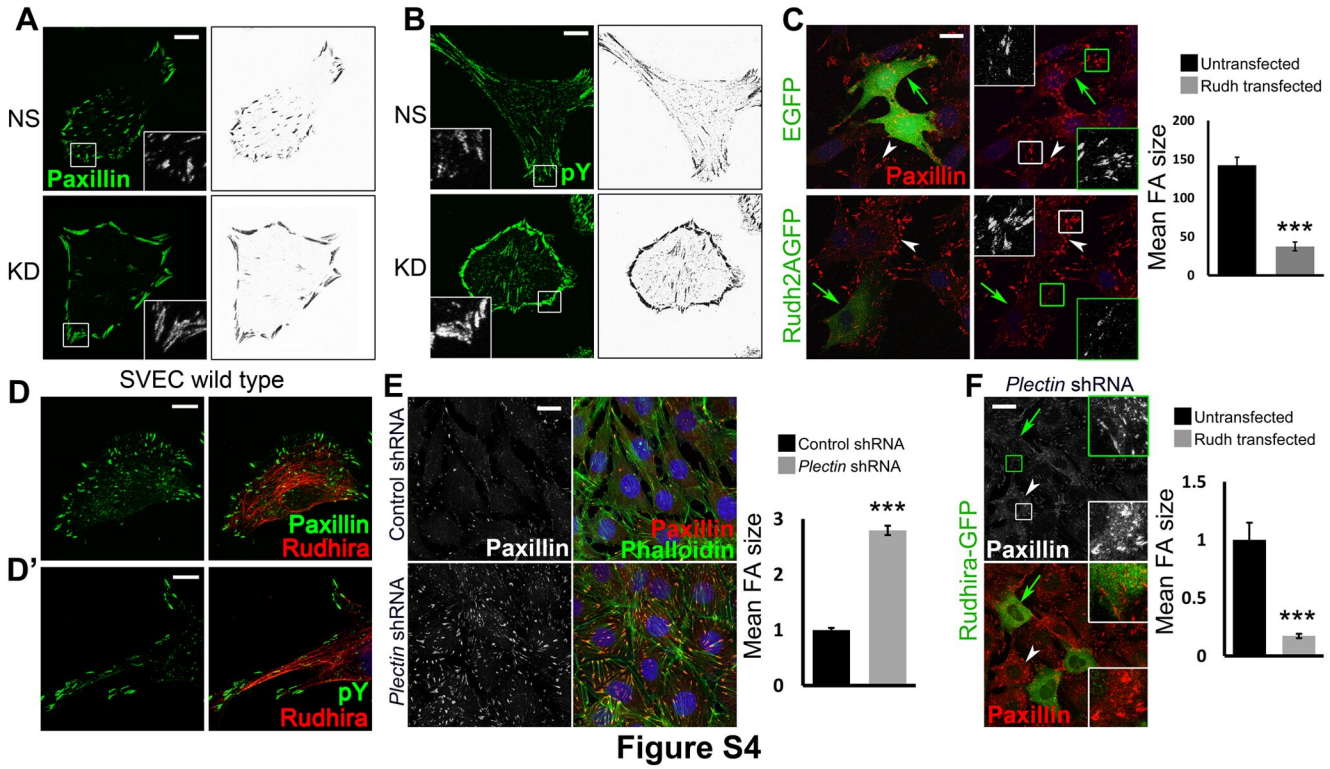
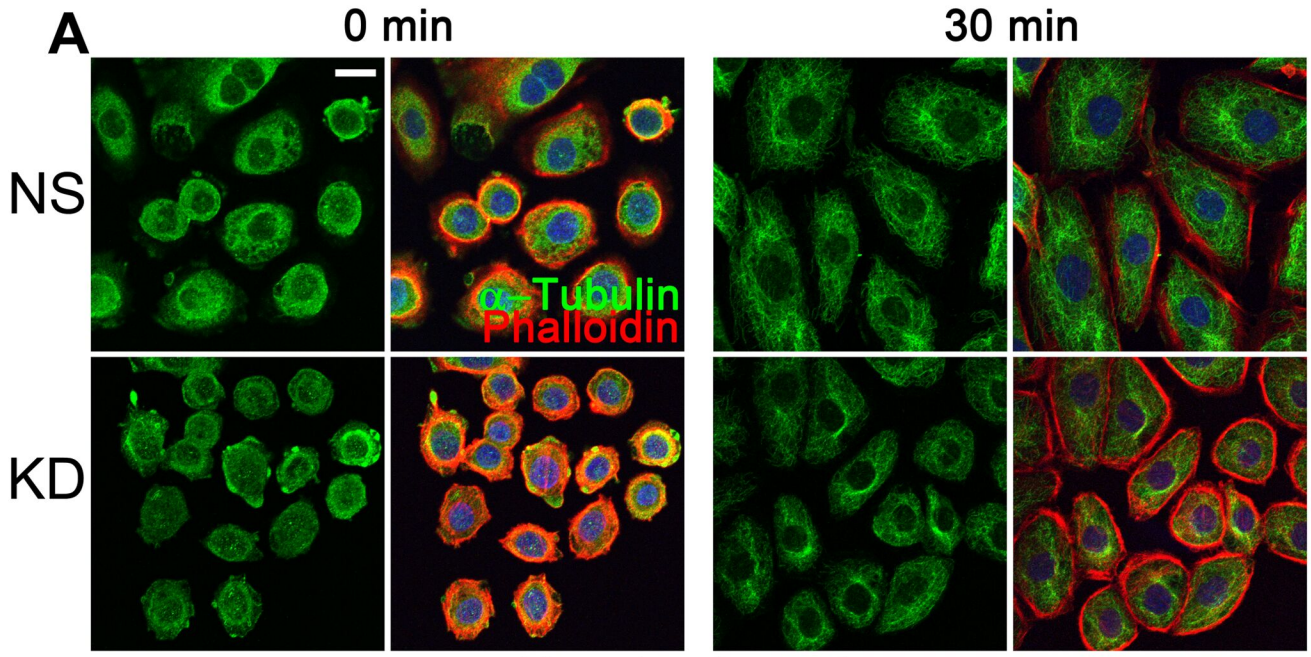

A
NS
KD
Paxillin
B
NS
KD
pY
C
EGFP
Rudh2AGFP
Paxillin
Untransfected
Rudh transfected
Mean FA size
200
150
100
50
0
***
D
SVEC wild type
D'
Paxillin
Rudhira
pY
Rudhira
E
Control shRNA
Plectin shRNA
Paxillin
Paxillin
Rhalloidin
Control shRNA
Plectin shRNA
Mean FA size
3
2
1
0
***
F
Plectin shRNA
Rudhira-GFP
Paxillin
Paxillin
Untransfected
Rudh transfected
Mean FA size
1.5
1
0.5
0
***

Figure S4


A
0 min
30 min
NS
KD
α–Tubulin
Phalloidin
Nocodazole washout

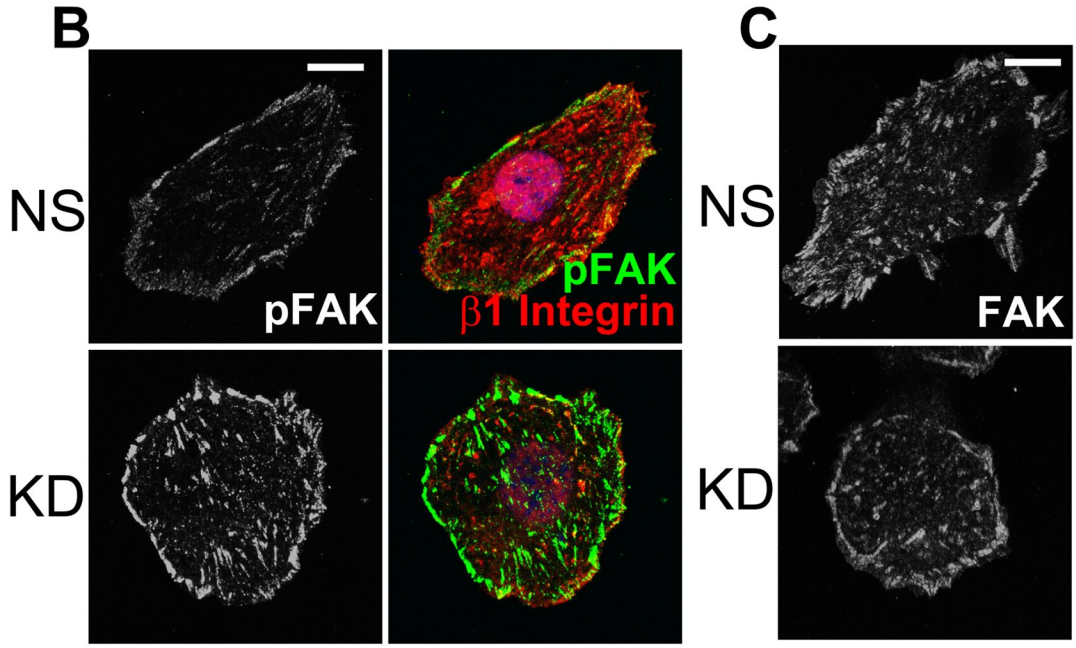

B
C
NS
KD
pFAK
pFAK
β1 Integrin
NS
FAK
KD

Figure S5

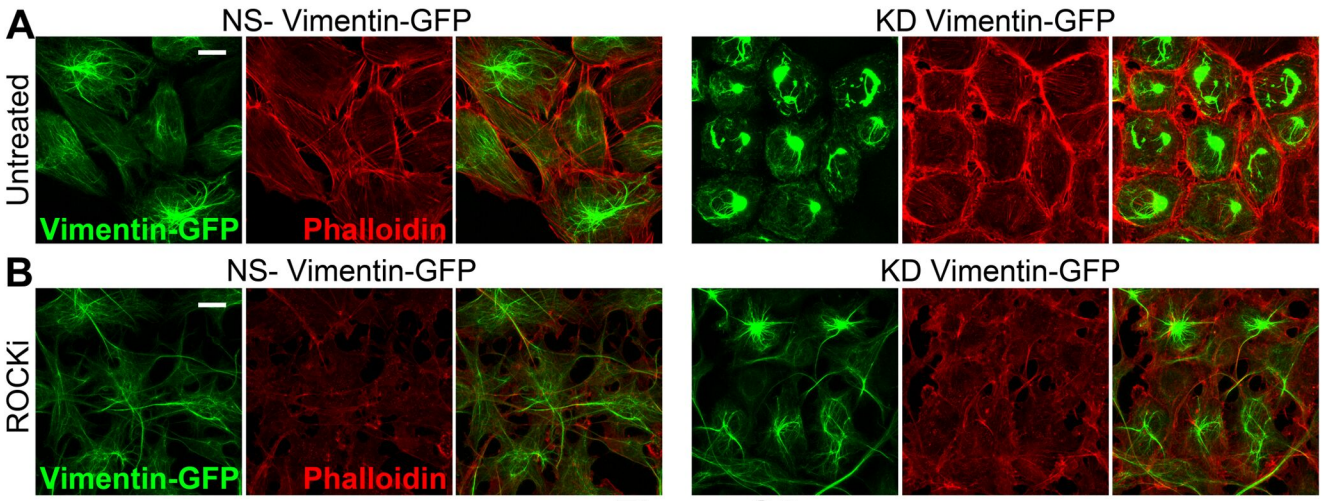

A
NS- Vimentin-GFP
KD Vimentin-GFP
Untreated
Vimentin-GFP
Phalloidin
B
NS- Vimentin-GFP
KD Vimentin-GFP
ROCKi
Vimentin-GFP
Phalloidin

Figure S6

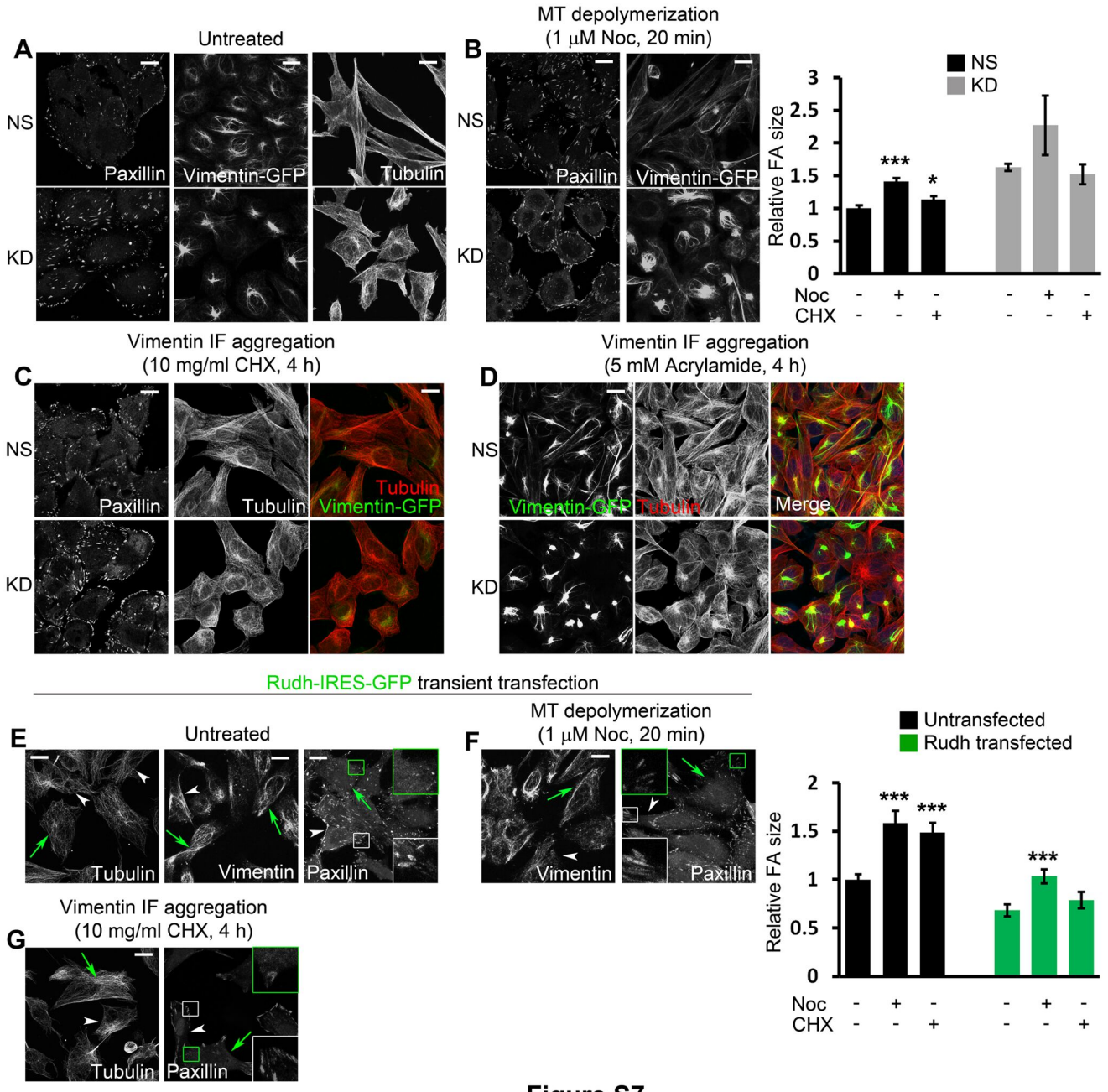

A
Untreated
NS
KD
Paxillin
Vimentin-GFP
Tubulin
B
MT depolymerization
(1 μM Noc, 20 min)
NS
KD
Paxillin
Vimentin-GFP
NS
KD
Relative FA size
3
2.5
2
1.5
1
0.5
0
***
*
Noc - + - - + -
CHX - - + - - +
C
Vimentin IF aggregation
(10 mg/ml CHX, 4 h)
NS
KD
Paxillin
Tubulin
Tubulin
Vimentin-GFP
D
Vimentin IF aggregation
(5 mM Acrylamide, 4 h)
NS
KD
Vimentin-GFP
Tubulin
Merge
Rudh-IRES-GFP transient transfection
E
Untreated
Tubulin
Vimentin
Paxillin
F
MT depolymerization
(1 μM Noc, 20 min)
Vimentin
Paxillin
Untransfected
Rudh transfected
Relative FA size
2
1.5
1
0.5
0
***
***
***
Noc - + - - + -
CHX - - + - - +
G
Vimentin IF aggregation
(10 mg/ml CHX, 4 h)
Tubulin
Paxillin

Figure S7

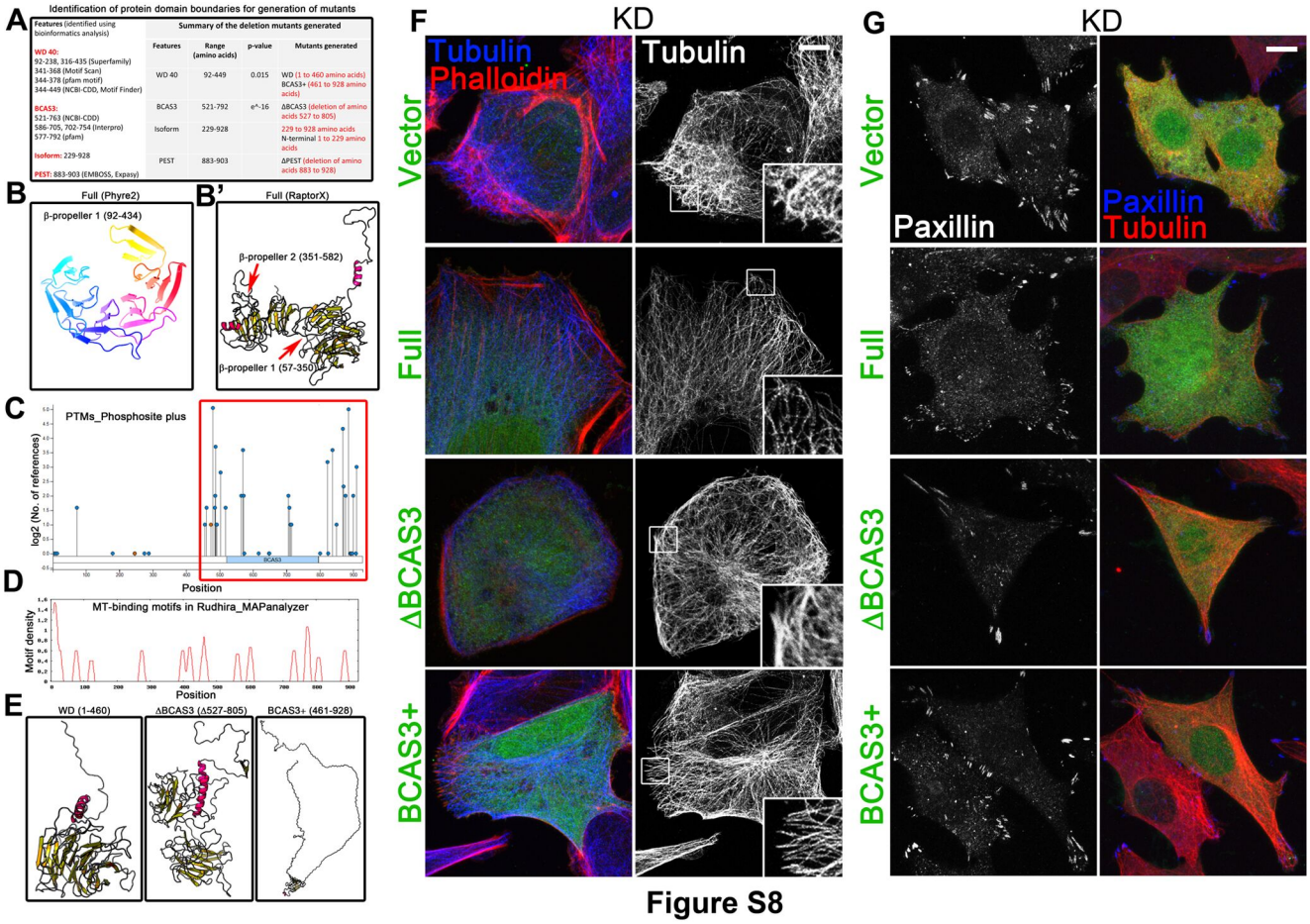

A
Identification of protein domain boundaries for generation of mutants
Features (identified using bioinformatics analysis)
WD 40:
92-238, 316-435 (Superfamily)
341-368 (Motif Scan)
344-378 (pfam motif)
344-449 (NCBI-CDD, Motif Finder)
BCAS3:
521-763 (NCBI-CDD)
586-705, 702-754 (Interpro)
577-792 (pfam)
Isoform: 229-928
PEST: 883-903 (EMBOSS, Expasy)
Summary of the deletion mutants generated
Features
Range (amino acids)
p-value
Mutants generated
WD 40
92-449
0.015
WD (1 to 460 amino acids)
BCAS3+ (461 to 928 amino acids)
BCAS3
521-792
e^-16
ΔBCAS3 (deletion of amino acids 527 to 805)
Isoform
229-928
229 to 928 amino acids
N-terminal 1 to 229 amino acids
PEST
883-903
ΔPEST (deletion of amino acids 883 to 928)
B
Full (Phyre2)
β-propeller 1 (92-434)
B'
Full (RaptorX)
β-propeller 2 (351-582)
β-propeller 1 (57-350)
C
PTMs_Phosphosite plus
log2 (No. of references)
BCAS3
Position
D
MT-binding motifs in Rudhira_MAPanalyzer
Motif density
Position
E
WD (1-460)
ΔBCAS3 (Δ527-805)
BCAS3+ (461-928)
F
KD
Tubulin
Phalloidin
Tubulin
Vector
Full
ΔBCAS3
BCAS3+
G
KD
Paxillin
Paxillin
Tubulin
Vector
Full
ΔBCAS3
BCAS3+

Figure S8