Figure S1

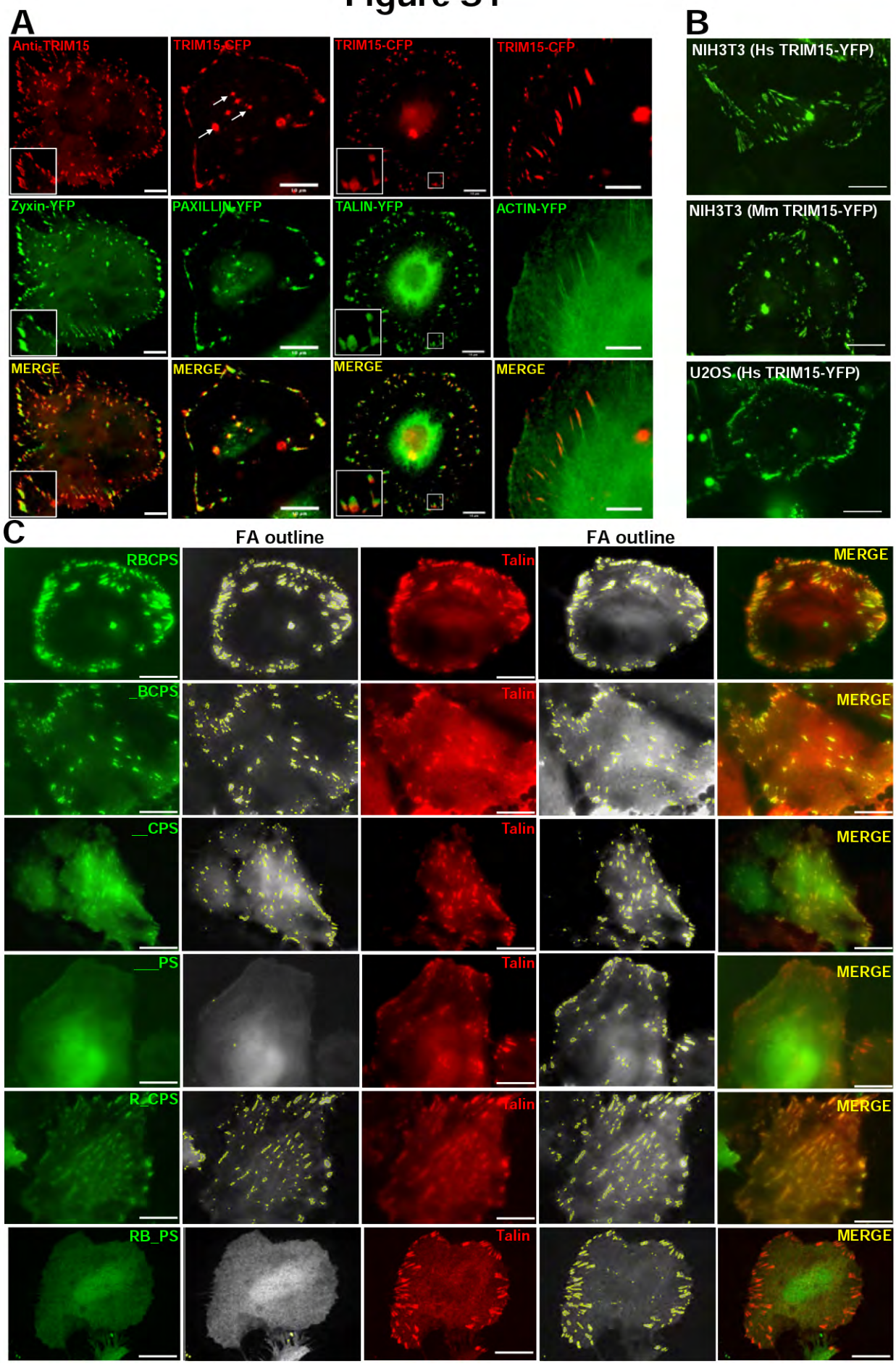

A
Anti-TRIM15
TRIM15-CFP
TRIM15-CFP
TRIM15-CFP
Zyxin-YFP
PAXILLIN-YFP
TALIN-YFP
ACTIN-YFP
MERGE
MERGE
MERGE
MERGE
B
NIH3T3 (Hs TRIM15-YFP)
NIH3T3 (Mm TRIM15-YFP)
U2OS (Hs TRIM15-YFP)
C
FA outline
FA outline
RBCPS
_BCPS
__CPS
___PS
R_CPS
RB_PS
Talin
MERGE

Figure S2

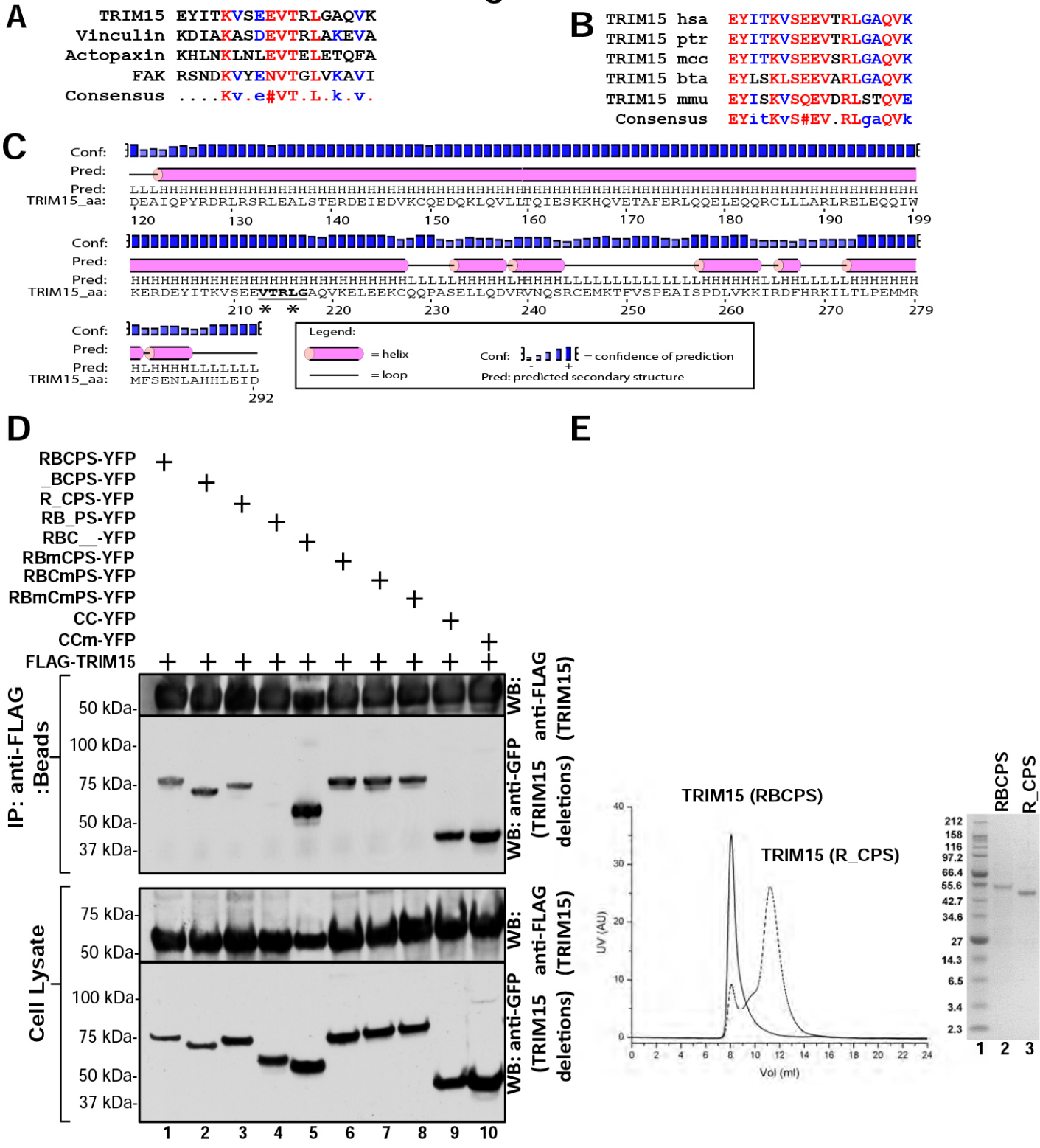
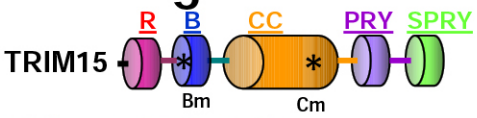
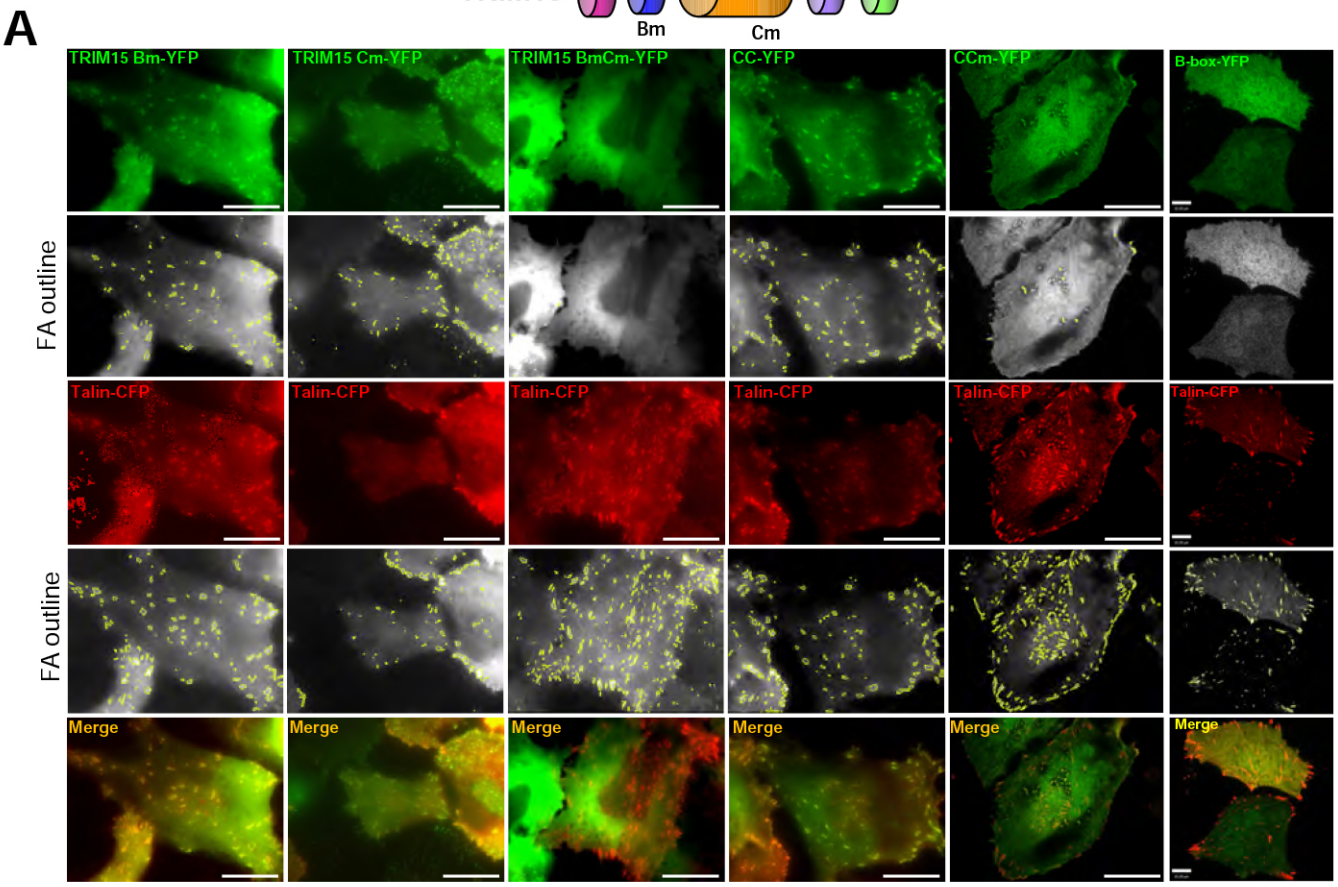
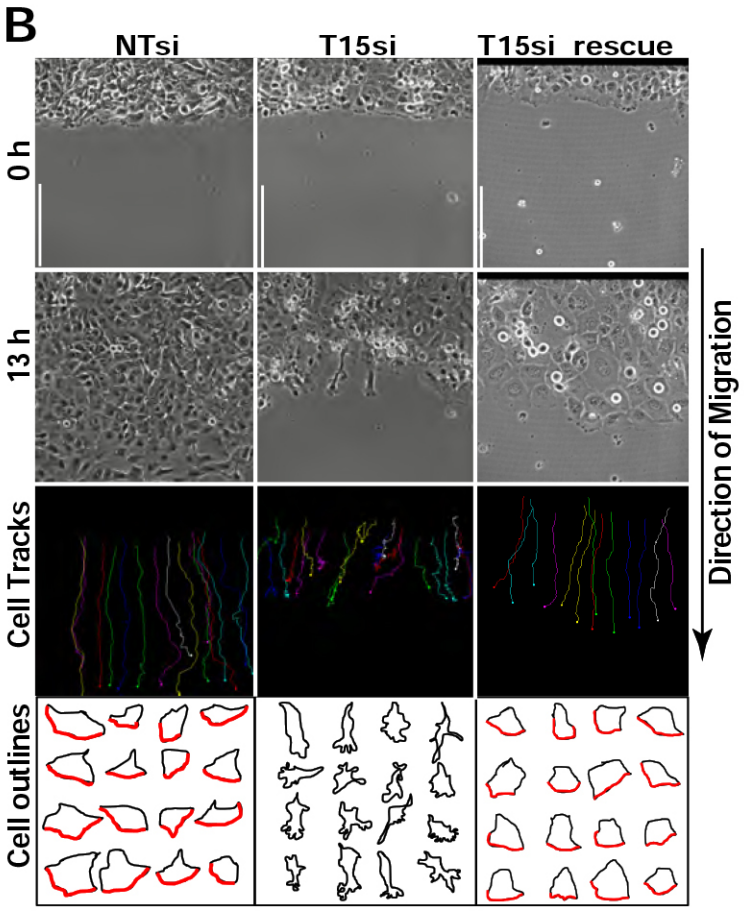
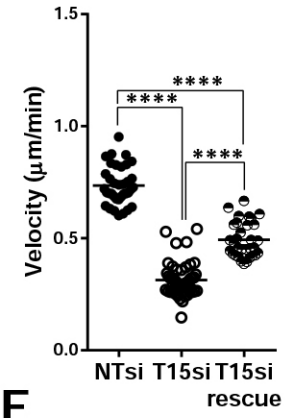

A
TRIM15 EYITKVSEEVTRLGAQVK
Vinculin KDIAKASDEVTRLAKEVA
Actopaxin KHLNKLNLEVTELETQFA
FAK RSNDKVYENVTGLVKAVI
Consensus ....Kv.e#VT.L.k.v.
B
TRIM15 hsa EYITKVSEEVTRLGAQVK
TRIM15 ptr EYITKVSEEVTRLGAQVK
TRIM15 mcc EYITKVSEEVSRLGAQVK
TRIM15 bta EYLSKLSEEVARLGAQVK
TRIM15 mmu EYISKVSQEVDRLSTQVE
Consensus EYitKvS#EV.RLgaQVk
C
Conf:
Pred:
TRIM15_aa:
Legend:
= helix
= loop
Conf: = confidence of prediction
Pred: predicted secondary structure
D
RBCPS-YFP
_BCPS-YFP
R_CPS-YFP
RB_PS-YFP
RBC__-YFP
RBmCPS-YFP
RBCmPS-YFP
RBmCmPS-YFP
CC-YFP
CCm-YFP
FLAG-TRIM15
IP: anti-FLAG :Beads
Cell Lysate
50 kDa
100 kDa
75 kDa
37 kDa
WB: anti-FLAG (TRIM15)
WB: anti-GFP (TRIM15 deletions)
1 2 3 4 5 6 7 8 9 10
E
TRIM15 (RBCPS)
TRIM15 (R_CPS)
UV (AU)
Vol (ml)
RBCPS
R_CPS
212
158
116
97.2
66.4
55.6
42.7
34.6
27
14.3
6.5
3.4
2.3
1 2 3

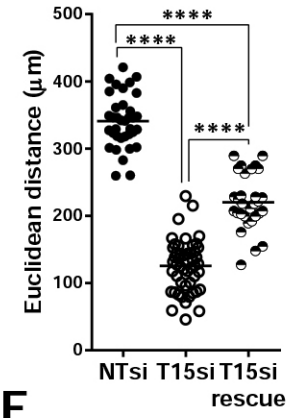
Figure S3

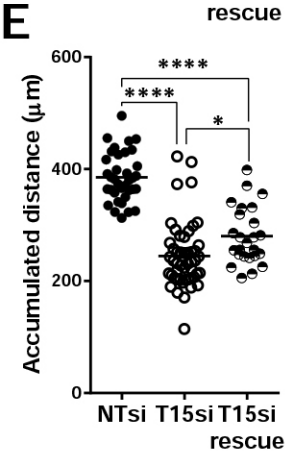

TRIM15
R
B
CC
PRY
SPRY
Bm
Cm


A
TRIM15 Bm-YFP
TRIM15 Cm-YFP
TRIM15 BmCm-YFP
CC-YFP
CCm-YFP
B-box-YFP
FA outline
Talin-CFP
FA outline
Merge


B
NTsi
T15si
T15si rescue
0 h
13 h
Cell Tracks
Cell outlines
Direction of Migration


C
Velocity (µm/min)
1.5
1.0
0.5
0.0
****
****
****
NTsi
T15si
T15si rescue


D
Euclidean distance (µm)
500
400
300
200
100
0
****
****
****
NTsi
T15si
T15si rescue


E
Accumulated distance (µm)
600
400
200
0
****
****
*
NTsi
T15si
T15si rescue

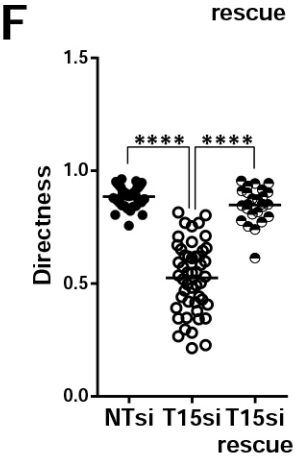
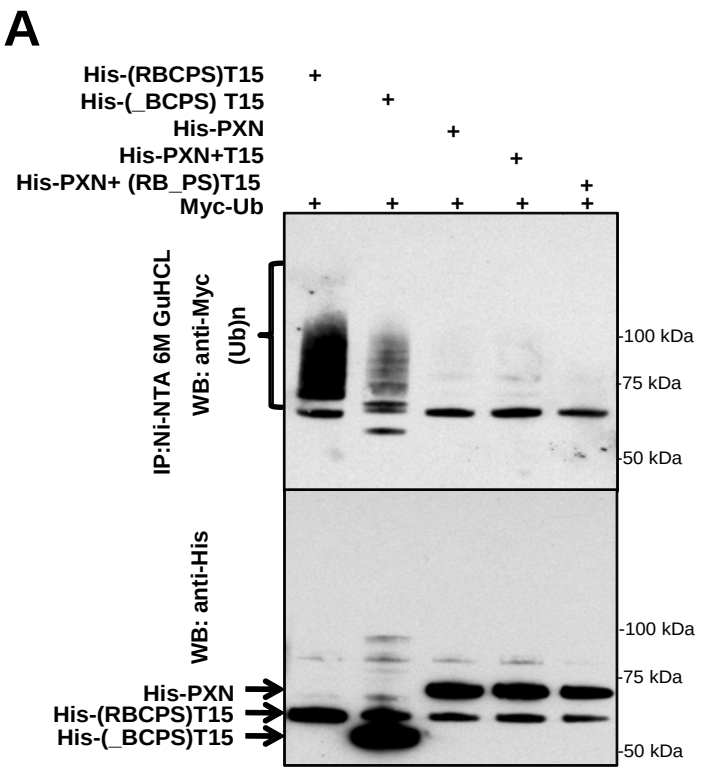
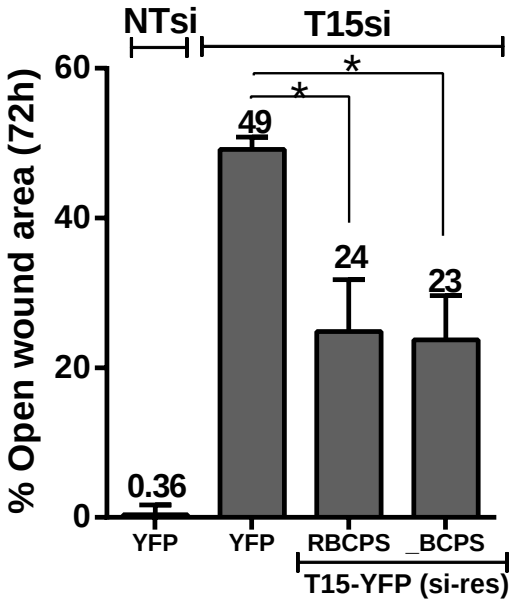

F
Directness
1.5
1.0
0.5
0.0
****
****
NTsi
T15si
T15si rescue

Figure S4


A
His-(RBCPS)T15
His-(_BCPS) T15
His-PXN
His-PXN+T15
His-PXN+ (RB_PS)T15
Myc-Ub
IP:Ni-NTA 6M GuHCL
WB: anti-Myc
(Ub)n
100 kDa
75 kDa
50 kDa
WB: anti-His
100 kDa
75 kDa
50 kDa
His-PXN
His-(RBCPS)T15
His-(_BCPS)T15


B
NTsi
T15si
% Open wound area (72h)
60
40
20
0
0.36
49
24
23
YFP
YFP
RBCPS
_BCPS
T15-YFP (si-res)

Online supplemental material

Supplementary figure and Movie legends

Fig. S1. The coiled coil domain of TRIM15 is essential for targeting to focal adhesions. (A) Columns 1-4 show split and merged images of HeLa cells expressing YFP-tagged (green) zyxin, paxillin, talin or actin and CFP-tagged or immunolocalized untagged TRIM15 (anti-TRIM15; red). The arrows in the third column indicate cytoplasmic bodies. Scale bar: 10 μm. (B) Localization of Human (Hs) or mouse (Mm) YFP-tagged TRIM15 in indicated cell types. Scale bar: 10 μm.

Fig. S2. The oligomerization activity of B-box contributes to interaction with paxillin in addition to coiled coil domain of TRIM15 that harbors the paxillin binding site. (A & B) Sequence alignment of a stretch in TRIM15 coiled coil region (aa 204-221) with the putative paxillin binding subdomain (PBS) of vinculin, actopaxin and FAK and with TRIM15 protein from hsa (*Homo sapiens;* human), ptr (*Pan troglodytes*; chimpanzee), mcc (*Macaca mulatta*; rhesus macaques), bta (*Bos taurus*; cow) and mmu (*Mus musculus*; mice) showing the conserved putative paxillin binding subdomain. (C) A secondary structure analyses predicted using the PSPIRED v3.3 software (Jones, 1999) for TRIM15 amino acid sequence (120-292) encompassing the coiled coil domain is shown along with confidence of prediction (blue). The cylindrical regions shown in pink depict the predicted helical regions [H] and the lines represent the loops [L]. The amino acids corresponding to putative PBS (VTRLG) near the end of the coiled coil helical region is underlined and asterisks are shown below the mutated amino acids. (D) The ability of mutant YFP-tagged TRIM15 to dimerize was tested by co-expression with FLAG-tagged wild-type TRIM15 in HEK293 cells. Cell lysates were immunoprecipitated using FLAG antibodies followed by western blotting with antibodies to FLAG and GFP. (E) Gel filtration profile of wild-type and B-box deleted TRIM15 purified from *E. coli*. Fraction eluted at ~8 ml represents higher-order oligomers while fraction eluted at ~12 ml corresponds to a molecular weight of ~114 kDa. The *E. coli* purified TRIM15 (RBCPS) and B-box deleted TRIM15 (R_CPS) are shown as coomassie stained gel along with molecular weight markers.

Fig. S3. (A) **Point mutations in coiled coil and B-box domain abolish focal adhesion targeting of TRIM15**. TIRFM images of HeLa cells expressing indicated TRIM15-YFP mutants (green) from Fig.

7B and the YFP fusion of the B-box domain of TRIM15 is shown here with talin-CFP as a focal adhesion reference marker to ensure that samples were imaged at the correct focal plane. This reference focal plane was critical to ascertain cytoplasmic fluorescence of TRIM15 mutants or individual domains that fail to localize to FAs. Individual FAs are outlined for clarity.Scale bar: 10 μm. (B) **TRIM15-depletion impairs cell migration and directional persistence in HT1080 cells**. HT1080 cell monolayers treated with indicated siRNAs with or without ectopic expression of siRNA-resistant TRIM15 (T15si rescue) for 72 h were wounded and cell motility followed by live cell imaging for 12-14 h. Selected frames of Movie S1 at 0 and 13 h after wounding are shown. Migration trajectories of individual cells are shown as dots and lines The outlines of representative cell boundaries and direction of migration are shown in the right to emphasize the single leading edge (shown in red) seen for NTsi- and T15si-treated rescued cells compared to multiple lateral lamellopodia in T15si-treated cells. (C-F) Plots of mentioned cell migration parameters for three experiments as in E are shown. The parameters for more than 40-60 individual cells (open and closed circles) were computed. Statistical significance values ****, * correspond to $p<0.0001$ and $p<0.05$, respectively. ns: not significant.

Fig. S4. TRIM15 is a self-ubiquitinating E3 ubiquitin ligase but does not ubiquitinate paxillin and E3 activity is not required for its function. (A)TRIM15 self- and paxillin-ubiquitination were tested by expressing wild-type and RING-deleted His-tagged TRIM15 or paxillin in the presence of myc-tagged ubiquitin. His-tagged TRIM15 or paxillin were isolated using Ni-NTA under denaturing conditions using 6 M Guanidinium HCL and the ubiquitination tested by western blotting with anti-myc antibodies. (B) Percent open wound area at 72 h determined using images analyzed by Tscratch software during the course of wound healing assay carried out in HeLa cells treated with either non-targeting (NT) or TRIM15 (T15) siRNA and transfected with 100 ng of plasmids expressing indicated siRNA resistant YFP fusions of full length or RING deleted (_BCPS) TRIM15 or YFP alone Data represent the mean ± SD of three experiments carried out in triplicates. Statistical significance values * corresponds to $p< 0.05$.

Movie S1. Time-lapse differential interference contrast imaging of wound healing assay. Confluent monolayer of HT1080 cells were treated with control non-targeting siRNA (NTsi) (top row)

or TRIM15 siRNA without or with ectopic expression of siRNA resistant TRIM15 (middle and bottom rows respectively). The cells were imaged from 0 to 13 h after wounding the monolayer. Tracked individual cells (colored dots) and their migration trajectories (colored lines) are shown in the right panels. Note the smooth trajectories for control and TRIM15si rescue panels compared to random migratory pattern of TRIM15 siRNA treated cells. In addition, TRIM15 siRNA treated cells display multiple lateral filopodia compared to a dominant leading edge exhibited by control and TRIM15si-treated rescued cells. See also Fig. S3 B-F. Scale bar: 100 μm.

Movie S2. TRIM15-depletion impairs two dimensional migration in HeLa cells. HeLa cells were treated for 48 h with control NT siRNA (NTsi; top panel) or TRIM15 siRNA (T15si) panel) without or with ectopic expression of siRNA resistant TRIM15-YFP for rescue (middle and bottom panels respectively). The cells were replated on fibronectin-coated coverslips and rested for 12-13 h before time-lapse differential interference contrast imaging with a 10 X objective lens. The first frame in the rescue panel shows an image acquired in the YFP channel (red) to identify the cell expressing siRNA resistant TRIM15-YFP. At this magnification, FAs cannot be seen and TRIM15-YFP expressing cells are identified by the presence of fluorescent cytoplasmic bodies. Tracked individual cells (colored dots) and their migration trajectories (colored lines) are shown as overlays. Note the restricted migration trajectories of TRIM15-depleted cells compared to long tracks displayed by control and the rescued cell. The longer migration trajectories seen, if any, in TRIM-depleted cells is a consequence of cell rounding, detachment and reattachment due to cell division. Scale bar: 10 μm.

Movie S3. A comparison of FA dynamics in control and TRIM15-depleted cells under steady-state conditions. HeLa cells stably expressing zyxin-YFP and treated for 48-72 h with control NT (NTsi; left panel) or TRIM15 (T15si; right panel) siRNA were subjected to time-lapse microscopy for 2-3 h to monitor FA dynamics. Examples of FA assembly and disassembly events are highlighted using green and red boxes respectively. FA assembly and disassembly rates were calculated from 6-8 time lapse movies encompassing >200 FAs using Focal Adhesion Analysis Server. See also Fig. 6 C, D. Scale bar: 10 μm.

Movie S4. A comparison of microtubule-induced FA disassembly in control and TRIM15-depleted cells. HeLa cells stably expressing zyxin-YFP and treated with control NT (NTsi; left panel) or TRIM15 (T15si; right panel) siRNA were treated with 10 μM nocodazole for 2-4 h to inhibit microtubule polymerization and prevent FA disassembly. The cells were then imaged for 2 h immediately after nocodazole was washed out to monitor FA disassembly. Note the rapid disassembly of FAs in control cells compared to that seen for TRIM15 siRNA treated cells. An example of FA disassembly event is shown in the boxed region. FA disassembly rates were calculated from atleast 6 movies for each condition encompassing ~175 FAs. See also Fig. 6L. Scale bar: 10 μm.

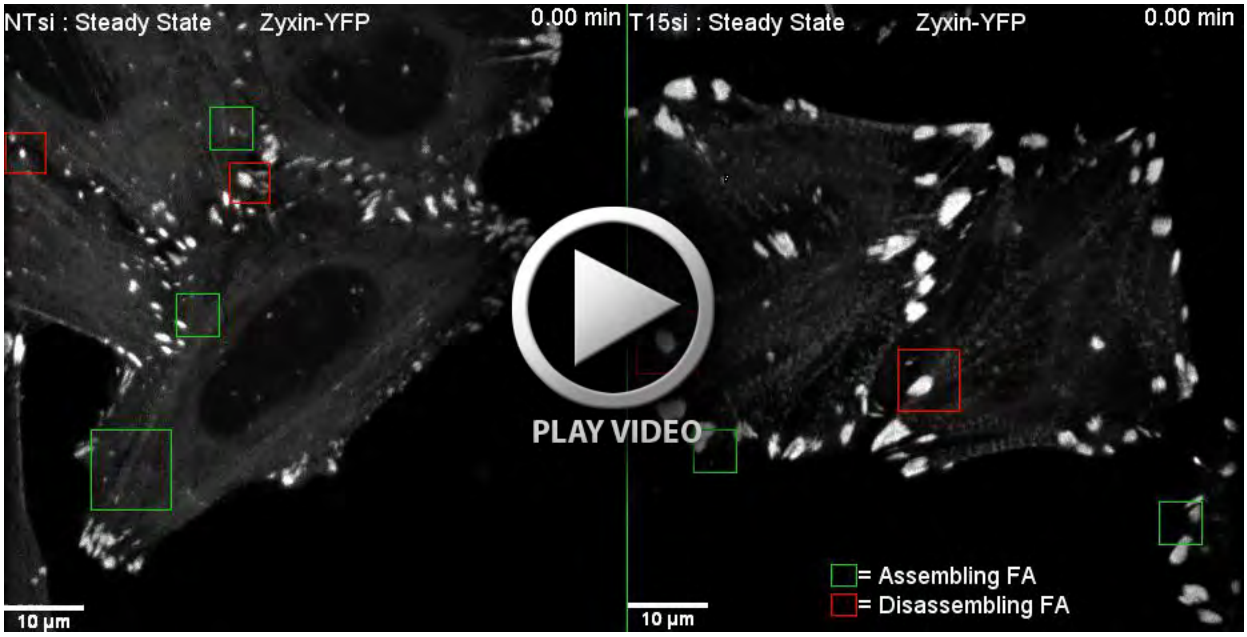
Movie S5. Disassembly modes of TRIM15 and Zyxin at focal adhesions. HeLa cells stably expressing TRIM15-YFP (left panel) or Zyxin-YFP (right panel) were subjected to time-lapse microscopy for 90 min at steady-state to monitor the way they disassemble from FAs. The red arrow heads point to disassembling FAs. Note that TRIM15 disassembles from FAs by collapsing into an oligomeric structure, to some extent instantaneously whereas zyxin dissipates slowly into the actin network and cytoplasm during disassembly. Scale bar: 10 μm

Supplementary References:

Jones, D. T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. *Journal of molecular biology* **292**, 195-202.

Movie 1.

Movie 2.



Movie 3.

Movie 4.

Movie 5.

Paxillin amino acid co-ordinates	Peptide Sequence
76-93	FIHQQPQSSSPVYGSSAK
111-123	VGEEEHVYSFPNK
265-277	ELDELMASLSDFK
363-375	KPIAGQVVTAMGK

Table S1. Details of peptides derived from paxillin that were identified by mass spectrometric analyses of TRIM15 immunoprecipitates.

Constructs	**Source**	**Reference**
CFP fusions of human zyxin and paxillin	Pekka Lappalainen (University of Helsinki, Finland)	(Hotulainen and Lappalainen, 2006)
CFP fusion of Human Talin1	Pietro De Camilli (Yale University, New Haven, CT)	(Lee et al., 2005).
Lentivirus expression construct for mTagRFPT-tubulin	Brett Lindenbach (Yale University, New Haven, CT)	(Counihan et al., 2011)

Table S2. Details of constructs used in the study.

siRNA/shRNA	**Sequence**	**Source**
TRIM15 siRNA #1	CCCUGAAGGUGGUCCAUGAUU	Dharmacon Inc.
TRIM15 siRNA #2	GCGAGAACGAUGCCGAGUUUU	Dharmacon Inc.
Paxillin siRNA	CCCUGACGAAAGAGAAGCCUAUU	Dharmacon Inc
control non-targeting (NT) luciferase siRNA	CUUACGCUGAGUACUUCGAAATT	Samchully Pharm CO., LTD (Seoul, Korea)
TRIM15 shRNA#1	CAGCGGTTGTTTTTACTTT	OpenBiosytems Inc
TRIM15 shRNA#2	AGCGGTTGTTTTTACTTTA	OpenBiosytems Inc
control non-targeting (NT) luciferase shRNA	TACAAACGCTCTCATCGACAAG	OpenBiosytems Inc.

Table S3. List of siRNA/shRNA used in the study and their sources