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Supplementary Materials for

The Huntingtin-interacting protein SETD2/HYPB is an actin lysine methyltransferase

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Published 2 October 2020, *Sci. Adv.* **6**, eabb7854 (2020)
DOI: 10.1126/sciadv.abb7854

The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

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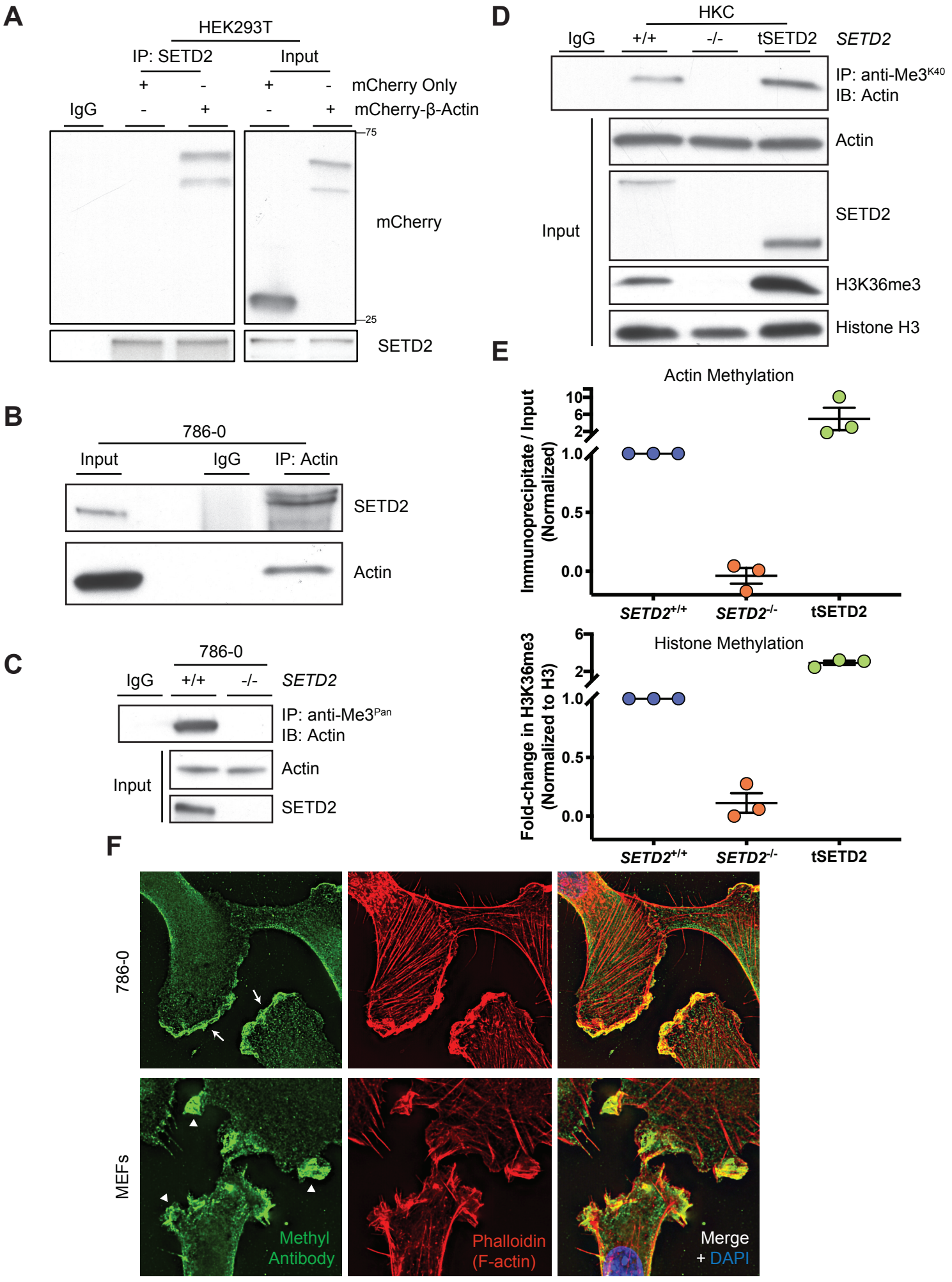
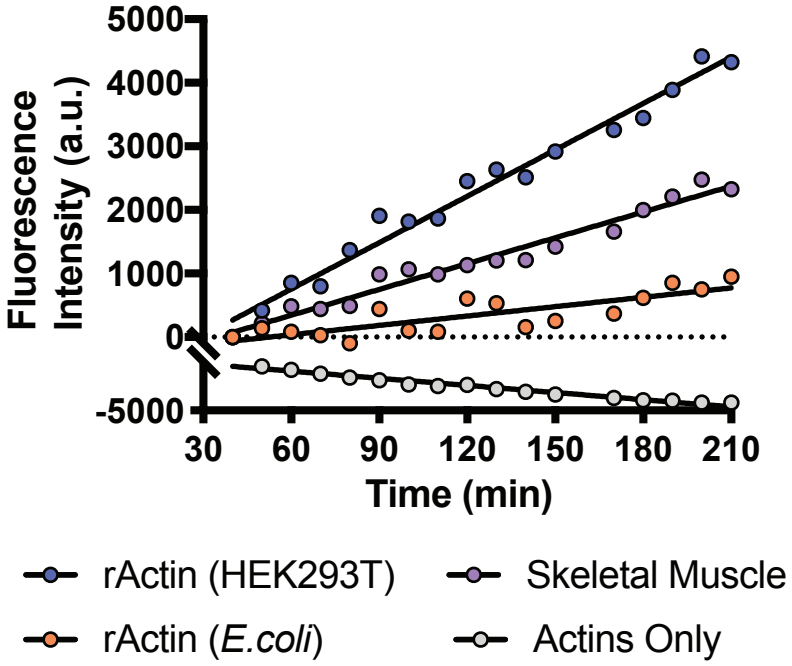
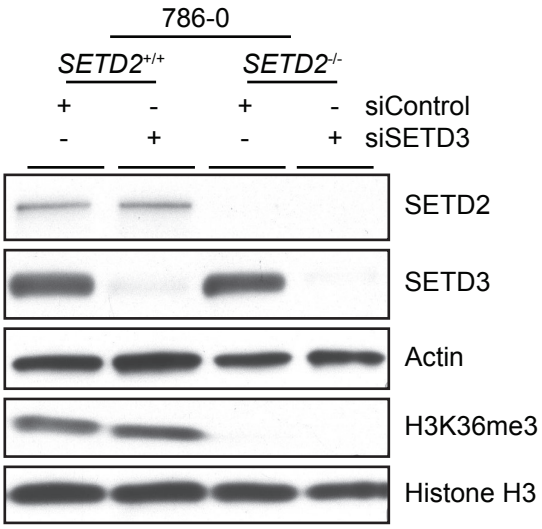

A
HEK293T
IP: SETD2
Input
mCherry Only
mCherry-β-Actin
IgG
75
25
mCherry
SETD2
B
786-0
Input
IgG
IP: Actin
SETD2
Actin
C
786-0
IgG
+/+
-/-
SETD2
IP: anti-Me3Pan
IB: Actin
Input
Actin
SETD2
D
HKC
IgG
+/+
-/-
tSETD2
SETD2
IP: anti-Me3K40
IB: Actin
Actin
Input
SETD2
H3K36me3
Histone H3
E
Actin Methylation
Immunoprecipitate / Input (Normalized)
10
6
2
1.0
0.5
0.0
SETD2+/+
SETD2-/-
tSETD2
Histone Methylation
Fold-change in H3K36me3 (Normalized to H3)
6
2
1.0
0.5
0.0
SETD2+/+
SETD2-/-
tSETD2
F
786-0
MEFs
Methyl Antibody
Phalloidin (F-actin)
Merge + DAPI

Figure S1. Interaction between SETD2 and actin. (A) Immunoblot analysis showing co-immunoprecipitation of endogenous SETD2 with mCherry-β-actin in HEK293T cells. **(B)** Co-immunoprecipitation of endogenous actin with endogenous SETD2 in 786-0 cells (reciprocal co-IP from data shown in in Fig. 1B). **(C)** Immunoprecipitation of actin from SETD2-proficient but not SETD2-deficient 786-0 cells using a pan-trimethyl lysine (anti-$Me3^{Pan}$) antibody. Data in (A) to (C) are representative of experiments repeated at least three times with similar results. **(D** and **E)** Immunoblot analysis (D) and quantitation (E) showing immunoprecipitation of actin using anti-$Me3^{K40}$ SETD2 methyl-epitope antibody in SETD2-proficient but not SETD2-deficient HKC cells, and rescued by re-expression of truncated SETD2 (tSETD2). Presence or absence of the H3K36me3 histone methyl mark used as a control for SETD2 methyltransferase activity. Data are mean ± S.E.M. for each biological replicate (n=3). **(F)** Deconvolution microscopy imaging of 786-0 and mouse embryonic fibroblast (MEF) cells showing localization of the SETD2 methyl epitope (green) to areas of high actin turnover, including ruffles (white arrowheads) and lamellipodia (white arrows) at the leading edge of cells. F-actin stained using phalloidin (shown in red).

A


5000
4000
3000
2000
1000
0
-5000
Fluorescence Intensity (a.u.)
30
60
90
120
150
180
210
Time (min)
rActin (HEK293T)
rActin (E.coli)
Skeletal Muscle
Actins Only

B


786-0
SETD2+/+
SETD2-/-
+ - + - siControl
- + - + siSETD3
SETD2
SETD3
Actin
H3K36me3
Histone H3

C


786-0 SETD2+/+
+ - siControl
IgG - + siSETD3
IP: Anti-Me3K36
IB: Actin
Input
SETD3
Actin

Figure S2. SETD2 methylation of actin. (A) Fluorescence-based quantitation of *in vitro* methylation using GST-tagged SETD2 catalytic SET domain (a.a. 1418-1714) with purified skeletal muscle actin (purple), and recombinant actin (rActin) from HEK293T cells (blue) or *E. coli* (orange). Actin proteins alone (grey) shown as negative control. Fluorescence signal calculated after subtracting automethylation from samples with SETD2 alone. **(B)** Immunoblot analysis showing expression of SETD3 in both parental and SETD2-deficient 786-0 cells, with *SETD3* knockdown used to demonstrate specificity for this antibody. **(C)** Immunoprecipitation of actin from control and *SETD3* knockdown 786-0 cells using SETD2 methyl-epitope antibody (anti-$Me3^{K36}$). The input blot showing SETD3 expression is a section of the blot shown in (C). Data are representative of experiments repeated at least three times with similar results.

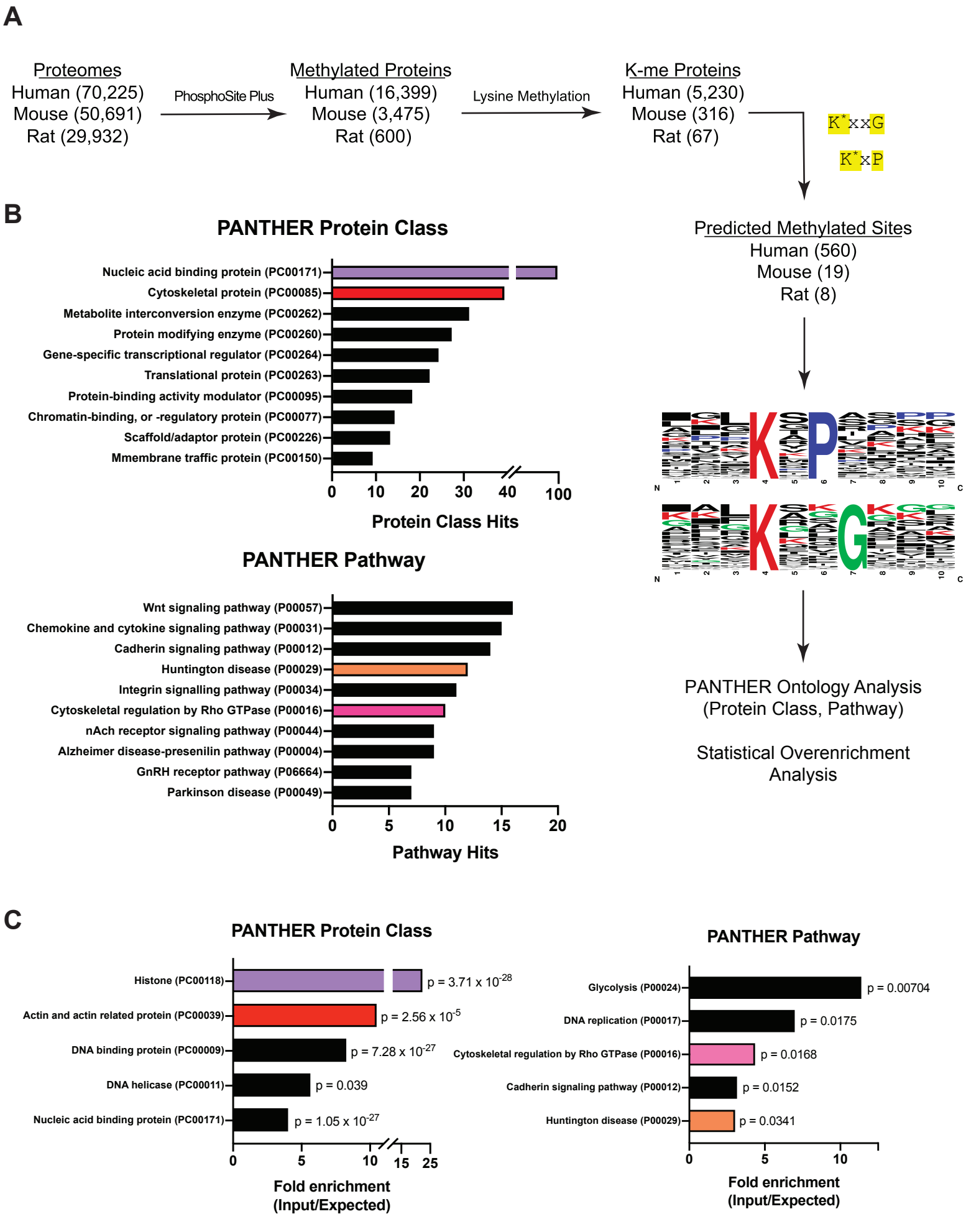

A
Proteomes
Human (70,225)
Mouse (50,691)
Rat (29,932)
PhosphoSite Plus
Methylated Proteins
Human (16,399)
Mouse (3,475)
Rat (600)
Lysine Methylation
K-me Proteins
Human (5,230)
Mouse (316)
Rat (67)
K*xxG
K*xP
B
PANTHER Protein Class
Nucleic acid binding protein (PC00171)
Cytoskeletal protein (PC00085)
Metabolite interconversion enzyme (PC00262)
Protein modifying enzyme (PC00260)
Gene-specific transcriptional regulator (PC00264)
Translational protein (PC00263)
Protein-binding activity modulator (PC00095)
Chromatin-binding, or -regulatory protein (PC00077)
Scaffold/adaptor protein (PC00226)
Mmembrane traffic protein (PC00150)
0 10 20 30 40 100
Protein Class Hits
PANTHER Pathway
Wnt signaling pathway (P00057)
Chemokine and cytokine signaling pathway (P00031)
Cadherin signaling pathway (P00012)
Huntington disease (P00029)
Integrin signalling pathway (P00034)
Cytoskeletal regulation by Rho GTPase (P00016)
nAch receptor signaling pathway (P00044)
Alzheimer disease-presenilin pathway (P00004)
GnRH receptor pathway (P06664)
Parkinson disease (P00049)
0 5 10 15 20
Pathway Hits
Predicted Methylated Sites
Human (560)
Mouse (19)
Rat (8)
PANTHER Ontology Analysis
(Protein Class, Pathway)
Statistical Overenrichment
Analysis
C
PANTHER Protein Class
Histone (PC00118)
p = 3.71 x 10-28
Actin and actin related protein (PC00039)
p = 2.56 x 10-5
DNA binding protein (PC00009)
p = 7.28 x 10-27
DNA helicase (PC00011)
p = 0.039
Nucleic acid binding protein (PC00171)
p = 1.05 x 10-27
0 5 10 15 25
Fold enrichment
(Input/Expected)
PANTHER Pathway
Glycolysis (P00024)
p = 0.00704
DNA replication (P00017)
p = 0.0175
Cytoskeletal regulation by Rho GTPase (P00016)
p = 0.0168
Cadherin signaling pathway (P00012)
p = 0.0152
Huntington disease (P00029)
p = 0.0341
0 5 10
Fold enrichment
(Input/Expected)

Figure S3. Identification of SETD2 methylation site on actin. (A) Schematic for pipeline identifying proteins with lysine methylation at known SETD2 recognition motif sites, using methylation site data downloaded from PhophoSitePlus®. Amino acid sequences for KxP- and KxxG- containing proteins generated using weblogo.berkeley.edu. **(B)** Ontology analysis showing the top 10 protein classes (top) and pathways (bottom) with proteins identified in (A) found to be methylated at a SETD2 recognition motif. **(C)** Overrepresentation analysis showing the top 5 protein classes (left) and pathways (right) from analysis shown in (A). Protein classes and pathways of interest in (B) and (C) are highlighted in color.

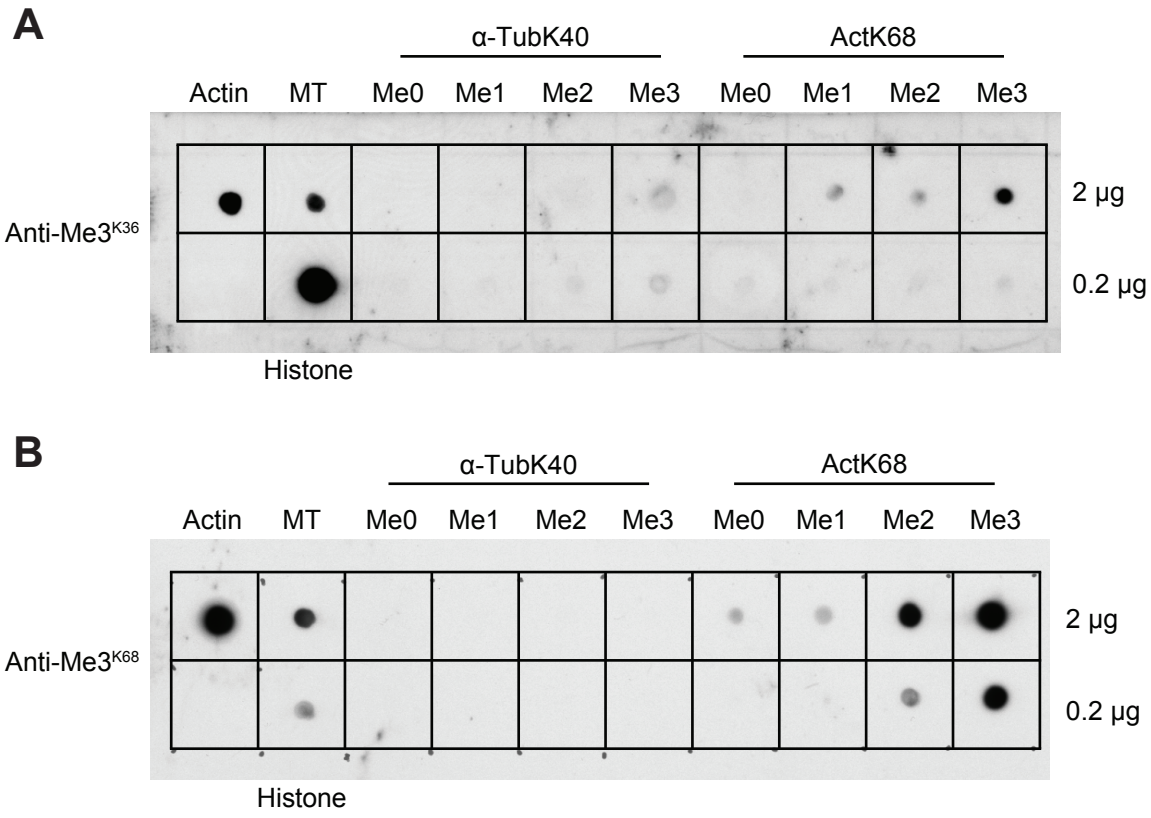

A
α-TubK40
ActK68
Actin
MT
Me0
Me1
Me2
Me3
Me0
Me1
Me2
Me3
Anti-Me3K36
2 µg
0.2 µg
Histone
B
α-TubK40
ActK68
Actin
MT
Me0
Me1
Me2
Me3
Me0
Me1
Me2
Me3
Anti-Me3K68
2 µg
0.2 µg
Histone
C
Cardiac Muscle Actin
Smooth Muscle Actin
- + + + + Actin
+ - + - + SETD2
50
37
In Vitro Methylation
Anti-Me3K68
Input
GST (SETD2)
Actin

Figure S4. Characterization of ActK68 methylation site. (**A** and **B**) Dot blot showing specificity of anti-Me3^{K36} (A) and anti-Me3^{K68} (B) antibodies. Methylated tubulin peptides, as well as purified muscle actin, microtubules (MT), and histone proteins also shown. The anti-Me3^{K36} antibody shows cross-reactivity with trimethylated ActK68 peptides. The anti-Me3^{K68} antibody shows little cross-reactivity with purified histones/nucleosomes. (**C**) Immunoblot analysis showing recognition of actin proteins by anti-Me3^{K68} antibody following *in vitro* methylation with GST-tagged SETD2 (a.a. 1418-1714). Data are representative of experiments repeated at least three times with similar results.

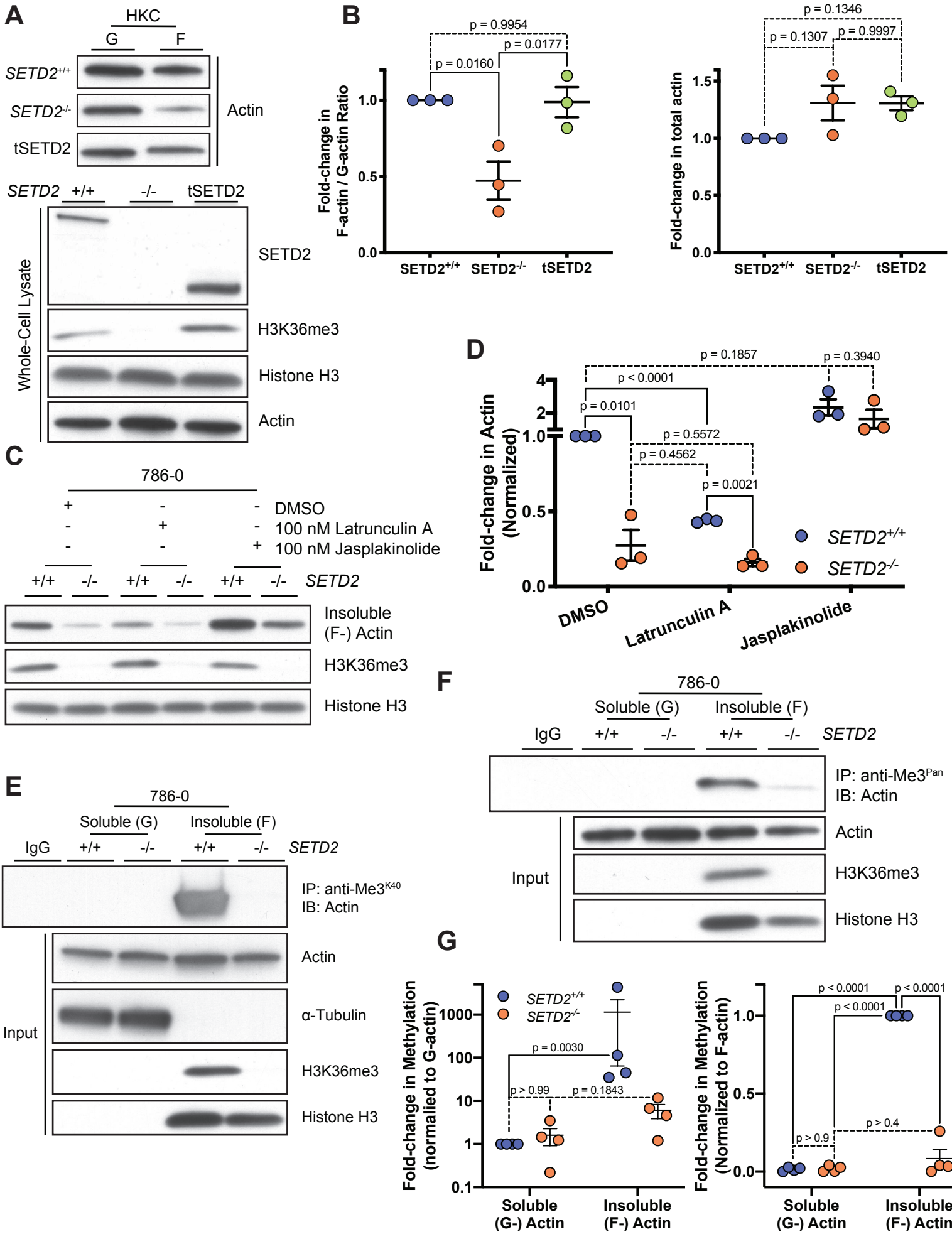

A
HKC
G
F
SETD2+/+
SETD2-/-
tSETD2
Actin
SETD2 +/+ -/- tSETD2
Whole-Cell Lysate
SETD2
H3K36me3
Histone H3
Actin
B
p = 0.9954
p = 0.0177
p = 0.0160
Fold-change in F-actin / G-actin Ratio
SETD2+/+ SETD2-/- tSETD2
p = 0.1346
p = 0.1307
p = 0.9997
Fold-change in total actin
SETD2+/+ SETD2-/- tSETD2
C
786-0
DMSO
100 nM Latrunculin A
100 nM Jasplakinolide
+/+ -/- +/+ -/- +/+ -/- SETD2
Insoluble (F-) Actin
H3K36me3
Histone H3
D
p = 0.1857
p = 0.3940
p < 0.0001
p = 0.0101
p = 0.5572
p = 0.4562
p = 0.0021
Fold-change in Actin (Normalized)
SETD2+/+
SETD2-/-
DMSO
Latrunculin A
Jasplakinolide
E
786-0
Soluble (G)
Insoluble (F)
IgG +/+ -/- +/+ -/- SETD2
IP: anti-Me3K40
IB: Actin
Actin
Input
α-Tubulin
H3K36me3
Histone H3
F
786-0
Soluble (G)
Insoluble (F)
IgG +/+ -/- +/+ -/- SETD2
IP: anti-Me3Pan
IB: Actin
Actin
Input
H3K36me3
Histone H3
G
SETD2+/+
SETD2-/-
p = 0.0030
p > 0.99
p = 0.1843
Fold-change in Methylation (normalized to G-actin)
Soluble (G-) Actin
Insoluble (F-) Actin
p < 0.0001
p < 0.0001
p < 0.0001
p > 0.9
p > 0.4
Fold-change in Methylation (Normalized to F-actin)
Soluble (G-) Actin
Insoluble (F-) Actin

Figure S5. Biochemical evidence for actin defect in SETD2-deficient cells. (A and **B)** Immunoblot analysis (A) and quantitation (B) showing decreased F-/G- actin ratio in SETD2-deficient HKC cells, rescued with re-expression of truncated SETD2 (tSETD2). Data are mean ± S.E.M. (n=3). **(C** and **D)** Immunoblot analysis (C) and quantitation (D) showing changes in insoluble F-actin in 786-0 cells treated with the depolymerizing agent Latrunculin A or the actin polymerizing agent Jasplakinolide. Data are mean ± S.E.M. (n=3). **(E** and **F)** Loss of methylation from the polymerized F-actin fraction in SETD2-deficient cells shown by immunoprecipitation of actin from the insoluble fraction of 786-0 cells using SETD2 methyl epitope anti-$Me3^{K40}$ (E) and anti-$Me3^{Pan}$ (F) antibodies. Data shown are representative of experiments repeated four times with similar results. H3K36me3 is used as a control to confirm loss of SETD2 in (C), (E). and (F). **(G)** Quantitation of data shown in (E), normalized to G-actin (left) and F-actin (left), showing a ~100- to 1000:1 ratio of methylated actin in the F-actin versus G-actin fractions. Data are mean ± S.E.M. (n=4). Quantitation representative of experiments performed with anti-$Me3^{K68}$ and anti-$Me3^{Pan}$ antibodies.

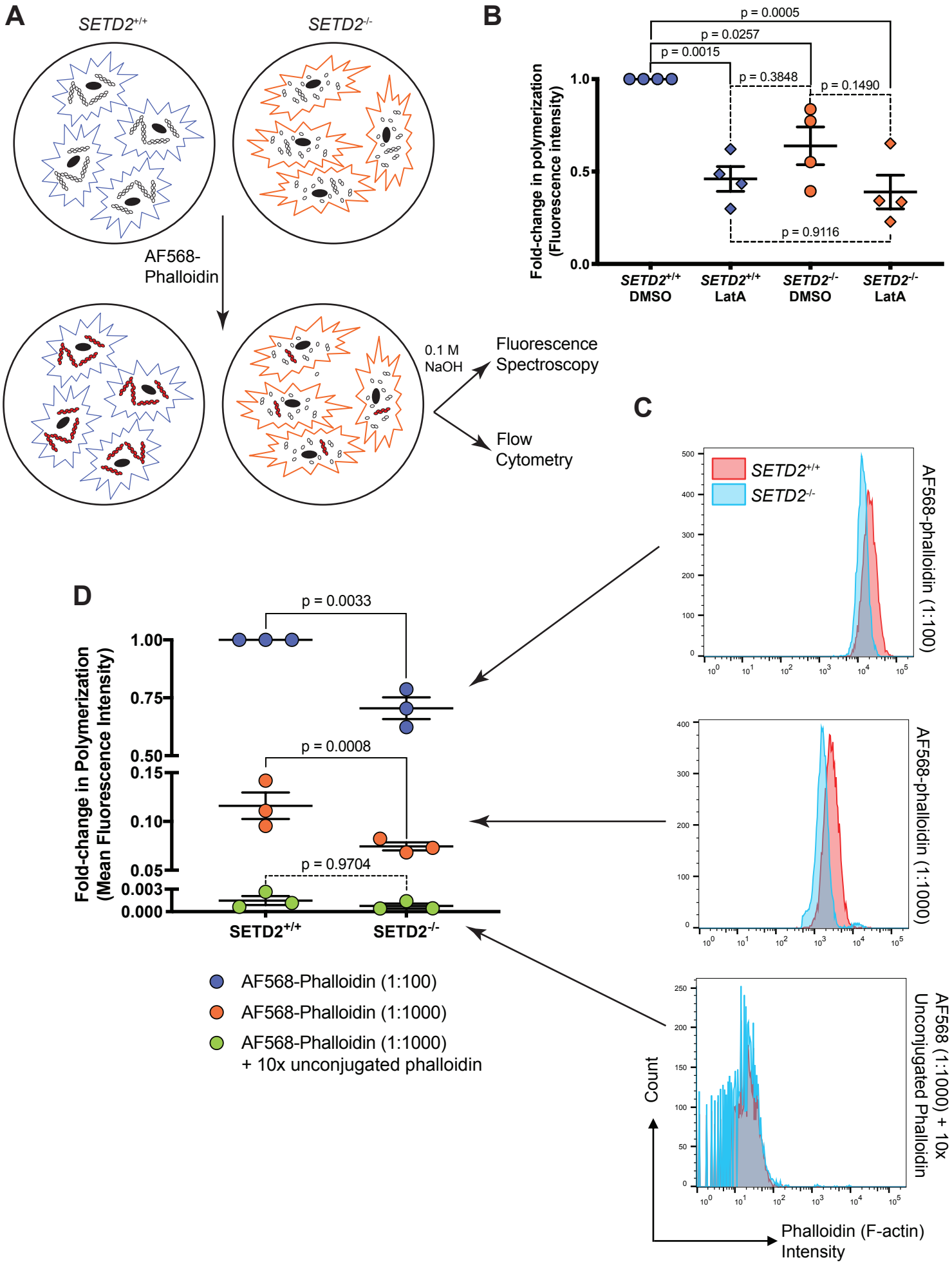

A
SETD2+/+
SETD2-/-
AF568-
Phalloidin
0.1 M
NaOH
Fluorescence
Spectroscopy
Flow
Cytometry
B
p = 0.0005
p = 0.0257
p = 0.0015
p = 0.3848
p = 0.1490
p = 0.9116
Fold-change in polymerization
(Fluorescence intensity)
1.0
0.5
0.0
SETD2+/+
DMSO
SETD2+/+
LatA
SETD2-/-
DMSO
SETD2-/-
LatA
C
SETD2+/+
SETD2-/-
AF568-phalloidin (1:100)
AF568-phalloidin (1:1000)
AF568 (1:1000) + 10x
Unconjugated Phalloidin
Count
Phalloidin (F-actin)
Intensity
D
p = 0.0033
p = 0.0008
p = 0.9704
Fold-change in Polymerization
(Mean Fluorescence Intensity)
1.00
0.75
0.50
0.15
0.10
0.05
0.003
0.000
SETD2+/+
SETD2-/-
AF568-Phalloidin (1:100)
AF568-Phalloidin (1:1000)
AF568-Phalloidin (1:1000)
+ 10x unconjugated phalloidin

Figure S6. Actin polymerization defect in SETD2-deficient cells. (A) Schematic drawing of experiments using fluorescent phalloidin to assess polymerized F-actin content in SETD2-proficient versus deficient cells. **(B)** Quantitation of spectrophotometric analysis of extracted fluorescent phalloidin from 786-0 cells treated with DMSO control or 100 nM Latrunculin A (LatA). Data are mean ± S.E.M. (n=4). **(C)** Flow cytometry plots showing intensity of fluorescent phalloidin in SETD2-proficient (red) and SETD2-deficient (blue) 786-0 cell populations. Data are shown for two concentrations of Alexa Fluor 568 (AF568) conjugated phalloidin (1:100, 1:1000), as well as for samples treated with AF568 phalloidin (1:1000) and 10x unconjugated phalloidin as a control. **(D)** Quantitation of mean fluorescence intensity from (C), with arrows indicating data from each phalloidin concentration. Y-axis split to reflect magnitude of change in phalloidin intensity between SETD2 -proficient and -deficient cells at the three different concentrations: 1:100 (blue) 1:1000 (orange), and 1:1000 + 10x unconjugated (green). Data are mean ± S.E.M. (n=3).

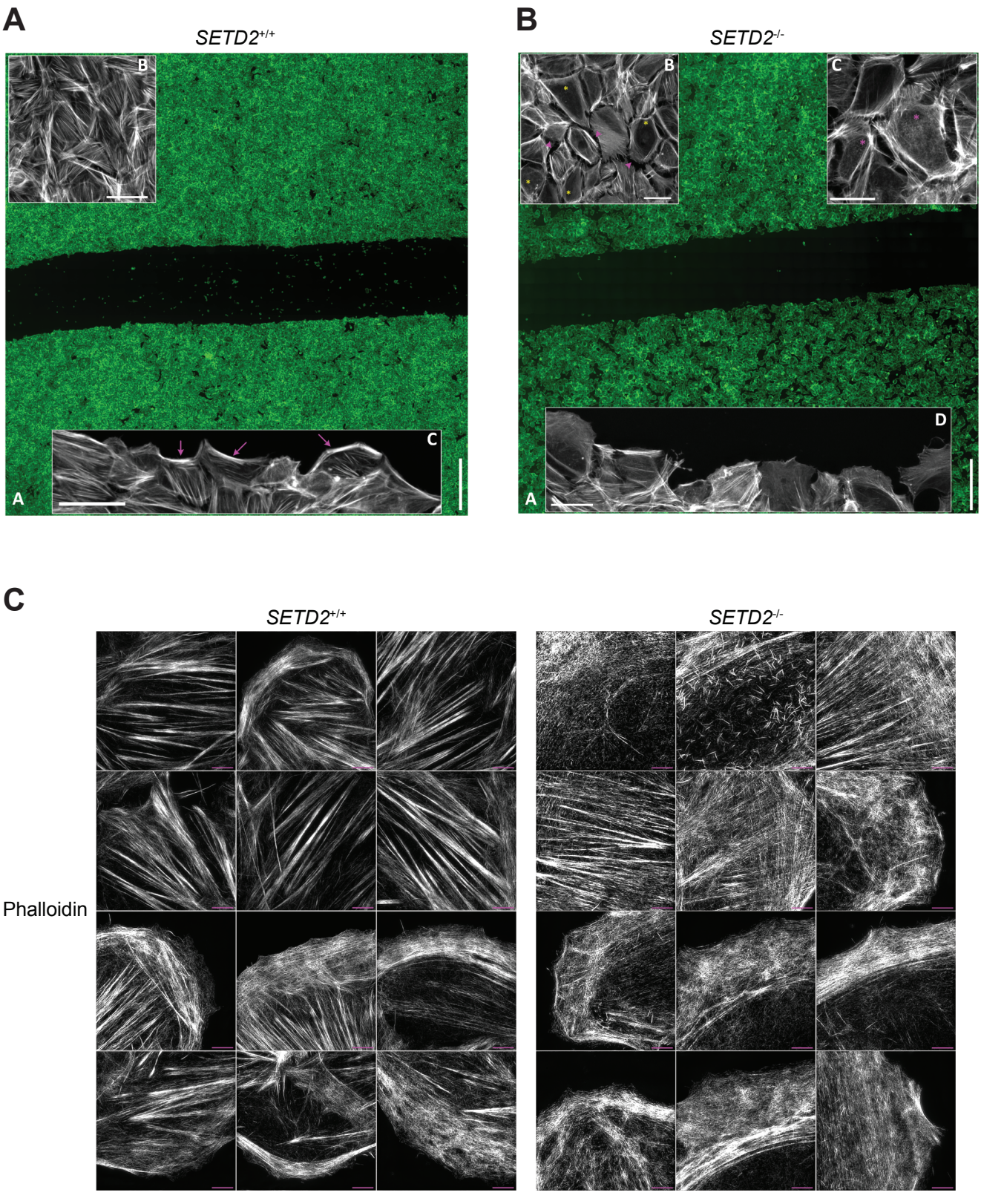

A
SETD2+/+
B
C
A
B
SETD2-/-
B
C
D
A
C
SETD2+/+
SETD2-/-
Phalloidin

Figure S7. High/Super-Resolution imaging of actin. (**A**) High-Resolution image stitching of SETD2-proficient HKC cells. Sub-panel A shows the ultra-large field-of-view afforded by this automated imaging approach (Scale bar, 1000 μm). Sub-panel B displays actual resolution of sub-panel A, to reveal highly confluent, tightly packed monolayer of cells, suggestive of robust cell-cell adhesion (Scale bar, 50 μm). Sub-panel C shows retrograde actin arcs (purple arrows) in leading-edge cells (Scale bar, 50 μm). (**B**) High-resolution imaging of SETD2-deficient HKC cells. Sub-panel A shows ultra-large field-of-view of all cells (Scale bar, 1000 μm). Sub-panel B shows dissolution of cell-cell contacts, as seen by increased pericellular space (purple arrowheads), and reduced stress fibers (yellow asterisks) in these cells (Scale bar, 50 μm). Sub-panel C shows apical actin-based protrusions suggestive of microvilli (purple asterisks) not observed in wild-type populations (Scale bar, 50 μm). Sub-panel D shows lack of robust actin arcs seen in wild-type cells, extended lamella, and cells lacking distinct actin signatures at the leading edge (Scale bar, 50 μm). (**C**) Super-Resolution imaging (3D-structured illumination microscopy) revealing robust stress fibers in SETD2-proficient cells (left), relative to the highly disorganized meshwork of actin representative of SETD2-deficient cells, along with appearance of microvilli in cells amongst the population (right). Scale bars, 5 μm.

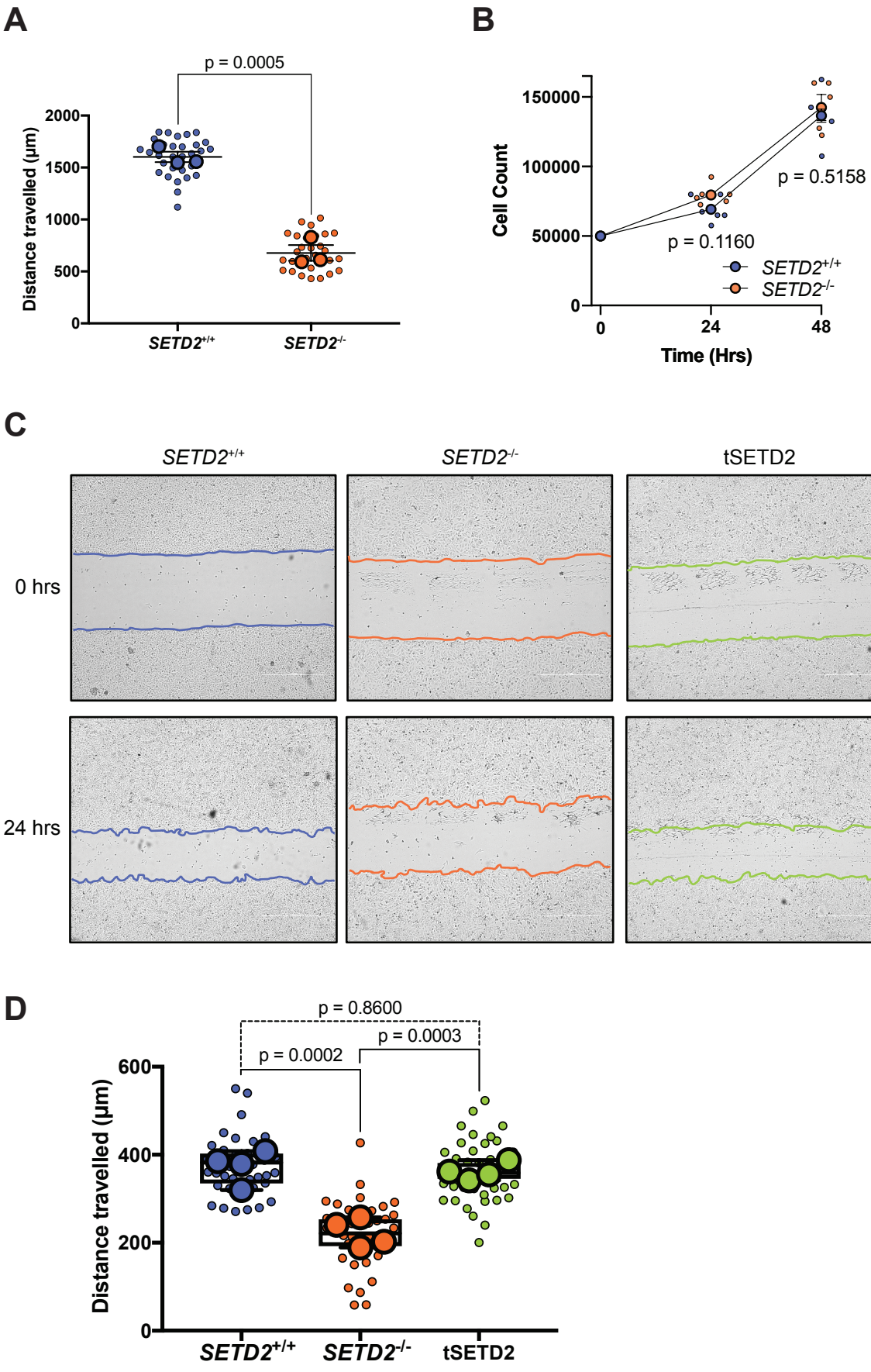

A
p = 0.0005
Distance travelled (µm)
2000
1500
1000
500
0
SETD2+/+
SETD2-/-
B
Cell Count
150000
100000
50000
0
p = 0.5158
p = 0.1160
SETD2+/+
SETD2-/-
0
24
48
Time (Hrs)
C
SETD2+/+
SETD2-/-
tSETD2
0 hrs
24 hrs
D
p = 0.8600
p = 0.0003
p = 0.0002
Distance travelled (µm)
600
400
200
0
SETD2+/+
SETD2-/-
tSETD2

Fig. S8. Proliferation-independent migration defect in SETD2-deficient cells. (A) Quantitation of scratch assay in 786-0 cells treated with cell proliferation inhibitor cytosine arabinoside (AraC). Small circles represent each independent measurement and large circles represent mean from 10 measurements from each independent biological replicate (n=3). **(B)** Cell counts for 786-0 cells over 48 hours (duration of the scratch assay from plating to final measurements). Small circles represent each independent measurement; large circles with error bars are mean ± S.E.M. for each independent biological replicate (n=2). **(C)** *In vitro* scratch assay at 0 and 24 hours after wound inflection illustrating decreased cell migration in SETD2-deficient versus SETD2-proficient HKC cells, and rescue of defect by re-expression of truncated SETD2 (tSETD2). Scale bars, 1000 μm. **(D)** Quantitation of data shown in (C). Small circles represent each independent measurement and large circles represent mean from a minimum of 9 measurements for each independent biological replicate (n=4).

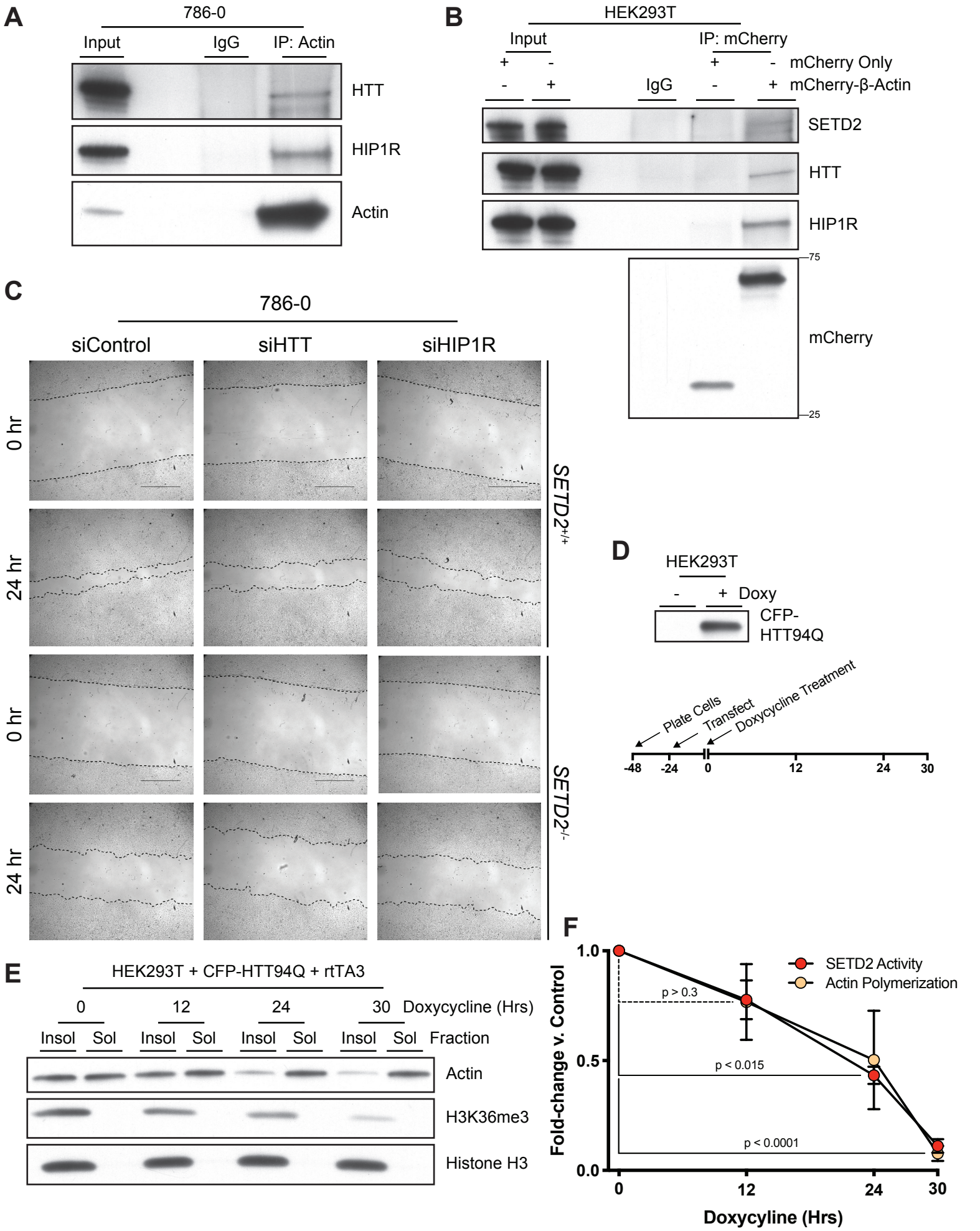
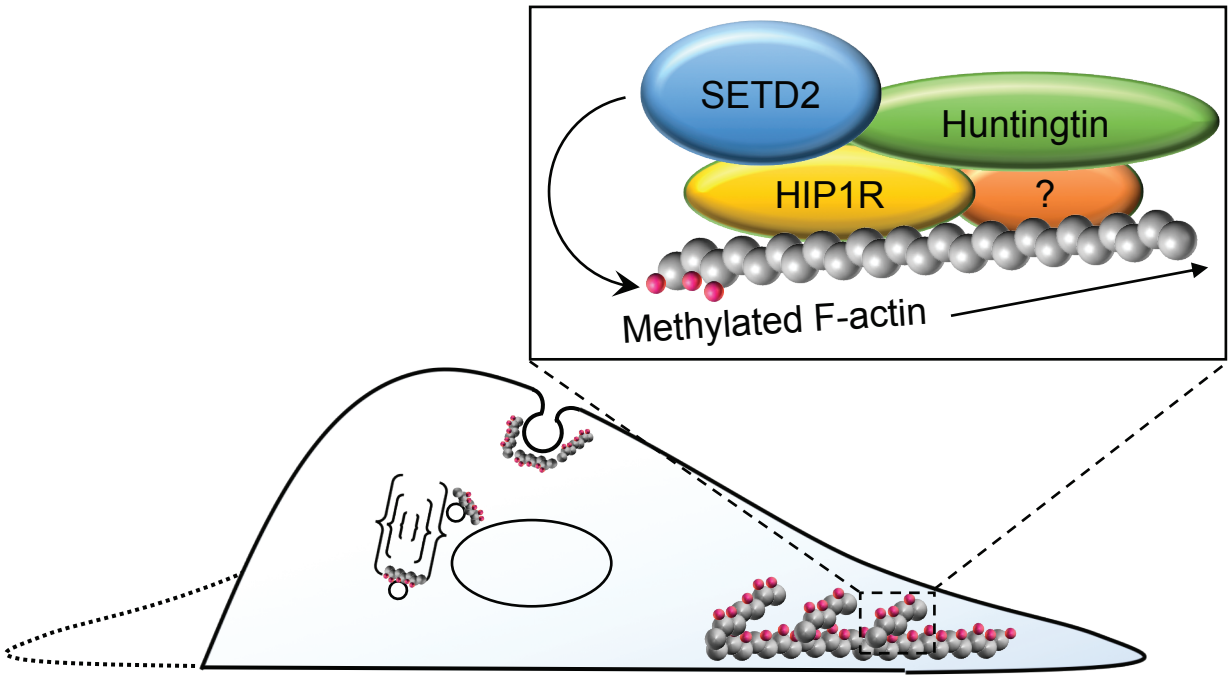
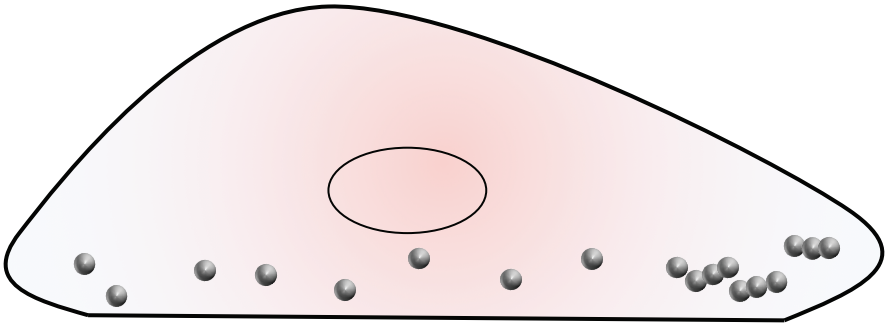

A
786-0
Input
IgG
IP: Actin
HTT
HIP1R
Actin
B
HEK293T
Input
IP: mCherry
+ -
- +
+ - mCherry Only
IgG
- + mCherry-β-Actin
SETD2
HTT
HIP1R
75
mCherry
25
C
786-0
siControl
siHTT
siHIP1R
0 hr
24 hr
SETD2+/+
0 hr
24 hr
SETD2-/-
D
HEK293T
- + Doxy
CFP-
HTT94Q
Plate Cells
Transfect
Doxycycline Treatment
-48
-24
0
12
24
30
E
HEK293T + CFP-HTT94Q + rtTA3
0
12
24
30
Doxycycline (Hrs)
Insol
Sol
Insol
Sol
Insol
Sol
Insol
Sol
Fraction
Actin
H3K36me3
Histone H3
F
SETD2 Activity
Actin Polymerization
p > 0.3
p < 0.015
p < 0.0001
Fold-change v. Control
1.0
0.5
0.0
0
12
24
30
Doxycyline (Hrs)

Fig. S9. SETD2-HTT-HIP1R actin methylation axis. (A and **B)** Immunoblot analysis showing co-immunoprecipitation of endogenous Huntingtin (HTT) and HIP1R with endogenous actin in 786-0 cells (A), and mCherry-β-actin expressed in HEK293T cells (B). Data shown are representative of experiments repeated three times with similar results. **(C)** *In vitro* scratch assay at 0 and 24 hours after wound inflection illustrating defects in cell migration in SETD2-proficient 786-0 cells after siRNA-mediated knockdown of *HTT* or *HIP1R*. Scale bars, 1000 μm. Quantitation shown in Fig. 7F. **(D)** Schematic drawing showing timeline for doxycycline treatment and expression of inducible CFP-tagged N-terminal mutant HTT construct (CFP-HTT94Q) in HEK293T cells. Inlay, immunoblot analysis showing expression of the mutant HTT construct after doxycycline (Doxy) treatment. **(E** and **F)** Immunoblot analysis (E) and quantitation (F) showing decrease in ratio of insoluble F-actin to soluble G-actin with expression of CFP-HTT94Q in SETD2-proficient HEK293T cells. H3K36me3 used as a control to assess SETD2 methylation activity in HEK293T cells expressing CFP-HTT94Q. Data are mean ± S.E.M. (n=3).



Wild-type migrating cell



Loss of SETD2

Fig. S10. Model of the SETD2-HTT-HIP1R axis for actin lysine methylation. Schematic diagram showing SETD2 methylates actin in a cellular complex containing the scaffolding protein huntingtin and the actin-binding adapter HIP1R. SETD2 methylation is required for normal cellular actin dynamics; loss of SETD2 negatively impacts actin dynamics and cell migration.

Supplemental Movies S1-S3. 3-dimensional rendering of cells shown in Figure S7. Movie S1 shows robust stress fibers in SETD2-proficient cells, which are less frequent and disorganized in SETD2-deficient cells (Movie S2). Additionally, while microvilli can be found in SETD2-proficient cells, they are found more frequently and appear more robust in SETD2-deficient populations (Movie S3).