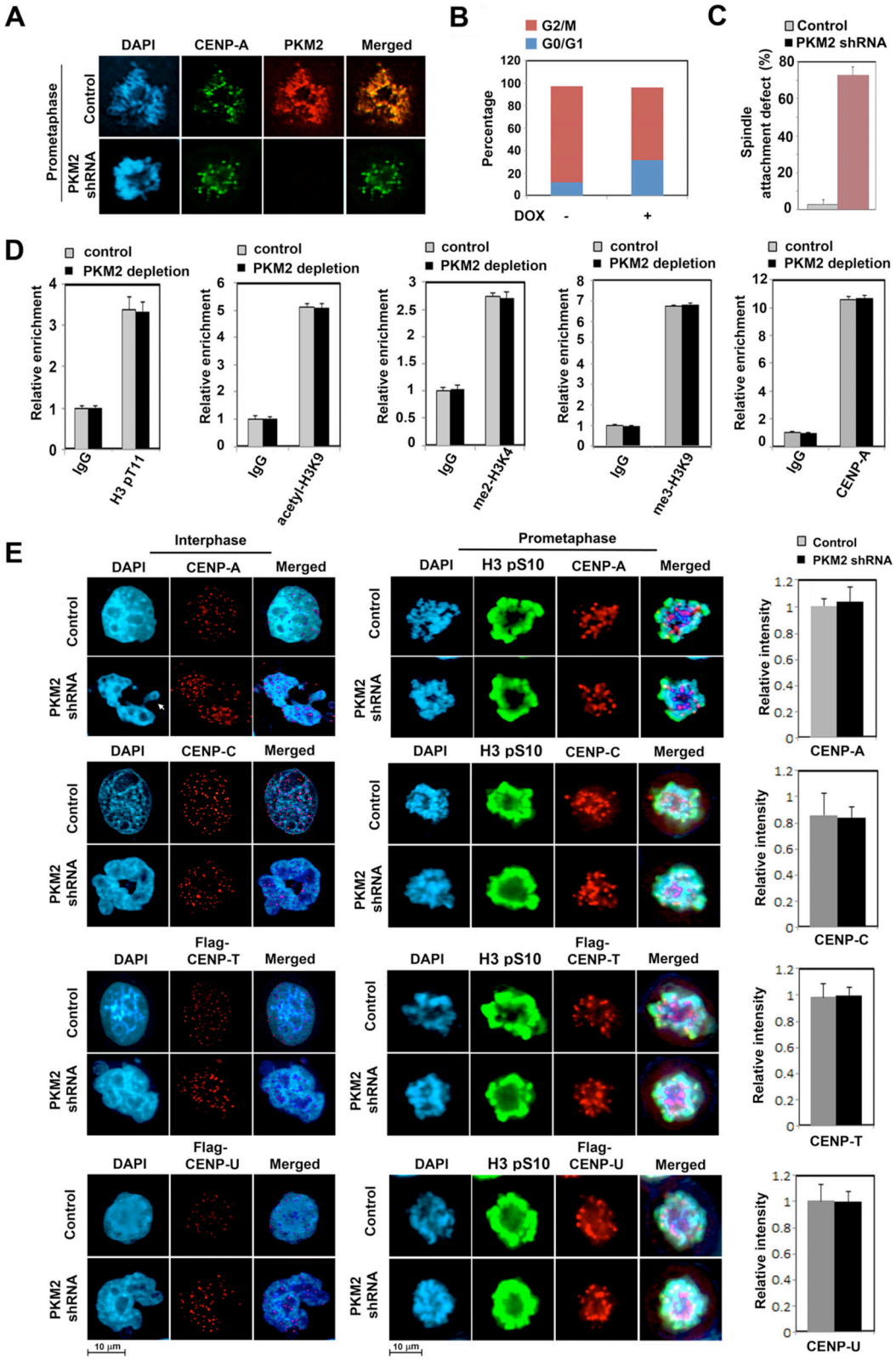
SUPPLEMENTAL INFORMATION



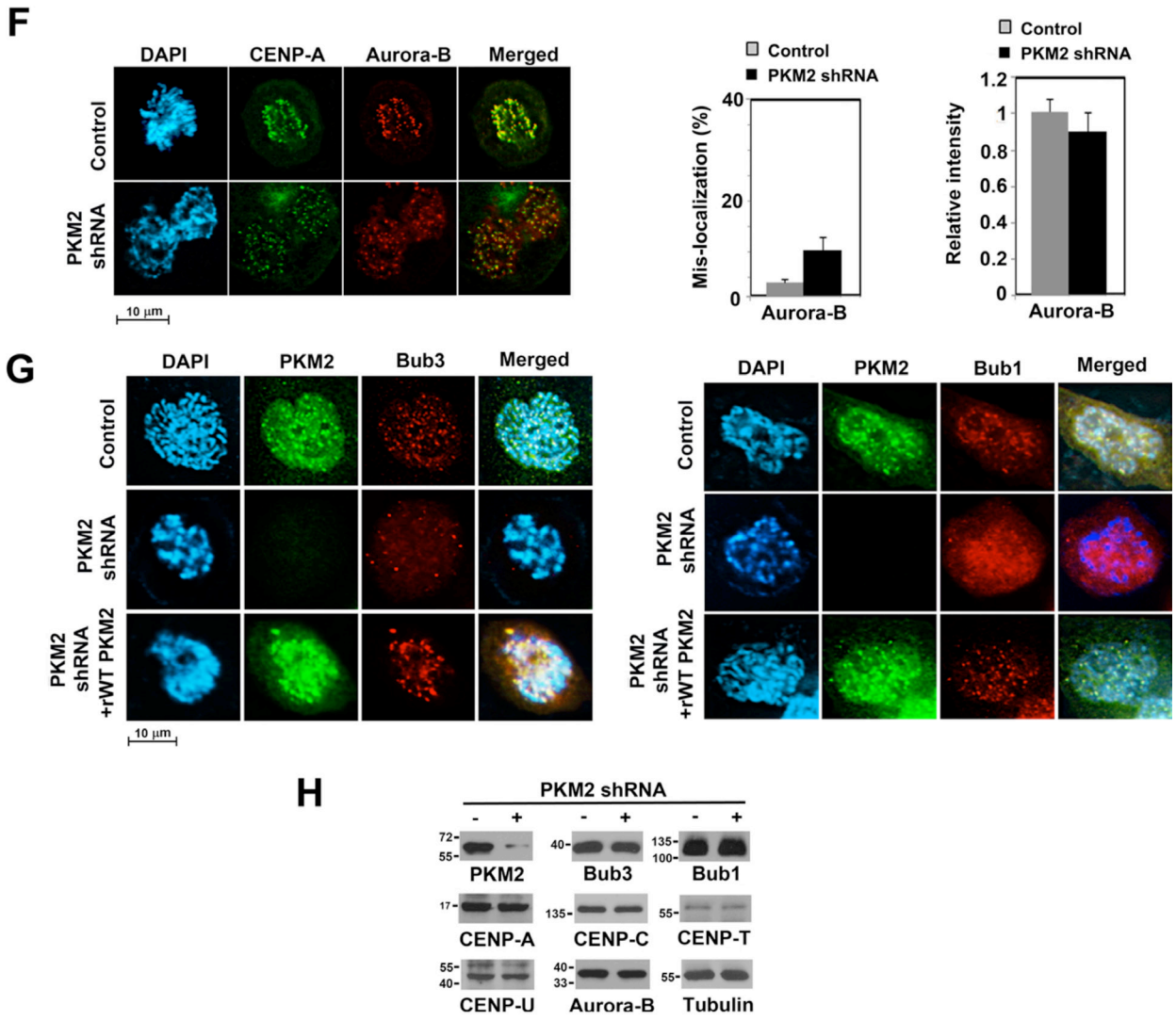


Figure S1, Related to Figure 1. PKM2 is required for the fidelity of chromosome segregation and kinetochore localization of Bub3 and Bub1

(A) HeLa cells with expression of PKM2 shRNA or scrambled shRNA as a control were immunostained with the indicated antibodies. Nuclei were stained with DAPI (blue).

(B) U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for 9 h. Doxycycline (500 ng/ml) was added or not added at the time point indicated in Figure 1C to induce PKM2 shRNA expression. Flow cytometric analyses were performed. Data represent the mean ± SD of three independent experiments.

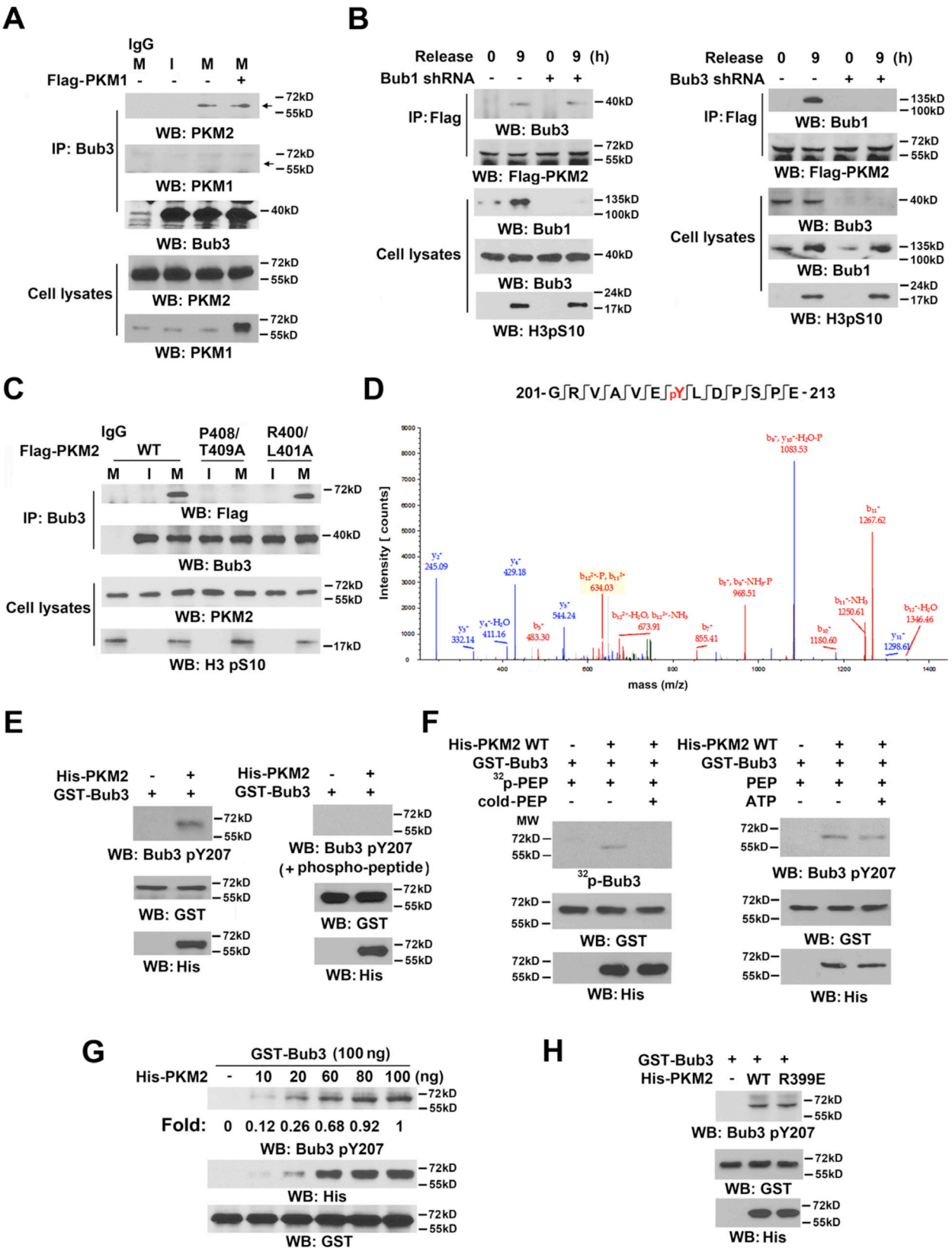
(C) U87/EGFRvIII cells expressing GFP-tubulin and mCherry-Histone H2B (for chromosome staining) synchronized by thymidine double block (2 mM) were released for 9 h. Doxycycline (500 ng/ml) was added with thymidine to induce PKM2 shRNA expression. Live-cell confocal time-lapse images were taken at the indicated time points. Thirty cells of each cell line were compared and quantified. Data represent the mean ± SD of three independent experiments.

(D) The lysates of HeLa cells with or without induced PKM2 depletion were immunoprecipitated with the indicated antibodies for ChIP assays. The specific primers for human centromeric satellite α (Satα) repeats were used for PCR reactions. Data represent the mean ± SD of three independent experiments.

(E, F) HeLa cells synchronized by thymidine double block (2 mM) were released for 9 h (prometaphase) or 12.5 h (interphase). Doxycycline (500 ng/ml) was added as indicated in Figure 1C to induce PKM2 shRNA expression. Immunofluorescence analyses were performed with the indicated antibodies (An anti-Flag antibody was used for detection of Flag-CENP-T and Flag-CENP-U). Nuclei were stained with DAPI (blue). The relative fluorescence intensity of the indicated proteins in one hundred cells of each cell line was compared and quantified (E, F). Localization of Aurora-B at kinetochores in one hundred cells in each cell line was analyzed (F). Data represent the mean ± SD of three independent experiments.

(G) U87/EGFRvIII and WT rPKM2-expressing U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for 9 h. Doxycycline (500 ng/ml) was added at the indicated time point (as shown in Figure 1C) to induce PKM2 shRNA expression. The cells were stained with the indicated antibodies.

(H) Immunoblotting analyses of U87/EGFRvIII cells with or without expression of PKM2 shRNA were performed with the indicated antibodies.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IgG
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72kD
55kD
WB: PKM2
IP: Bub3
WB: PKM1
40kD
WB: Bub3
Cell lysates
WB: PKM2
WB: PKM1
B
Release 0 9 0 9 (h)
Bub1 shRNA - - + +
IP: Flag
40kD
WB: Bub3
WB: Flag-PKM2
135kD
100kD
WB: Bub1
Cell lysates
WB: H3pS10
24kD
17kD
Release 0 9 0 9 (h)
Bub3 shRNA - - + +
WB: Bub1
WB: Flag-PKM2
WB: Bub3
WB: H3pS10
C
IgG
Flag-PKM2 WT
P408/ T409A
R400/ L401A
M I M I M I M
IP: Bub3
WB: Flag
WB: Bub3
Cell lysates
WB: PKM2
17kD
WB: H3 pS10
D
201-G⎡R⎡V⎡A⎡V⎡E⎡pY⎡L⎡D⎡P⎡S⎡P⎡E-213
Intensity [counts]
mass (m/z)
E
His-PKM2 - +
GST-Bub3 + +
WB: Bub3 pY207
WB: Bub3 pY207 (+ phospho-peptide)
WB: GST
WB: His
F
His-PKM2 WT - + +
GST-Bub3 + + +
32p-PEP + + +
cold-PEP - - +
MW
32p-Bub3
WB: GST
WB: His
PEP + + +
ATP - - +
WB: Bub3 pY207
G
GST-Bub3 (100 ng)
His-PKM2 - 10 20 60 80 100 (ng)
Fold: 0 0.12 0.26 0.68 0.92 1
WB: Bub3 pY207
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WB: GST
H
GST-Bub3 + + +
His-PKM2 - WT R399E
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WB: His

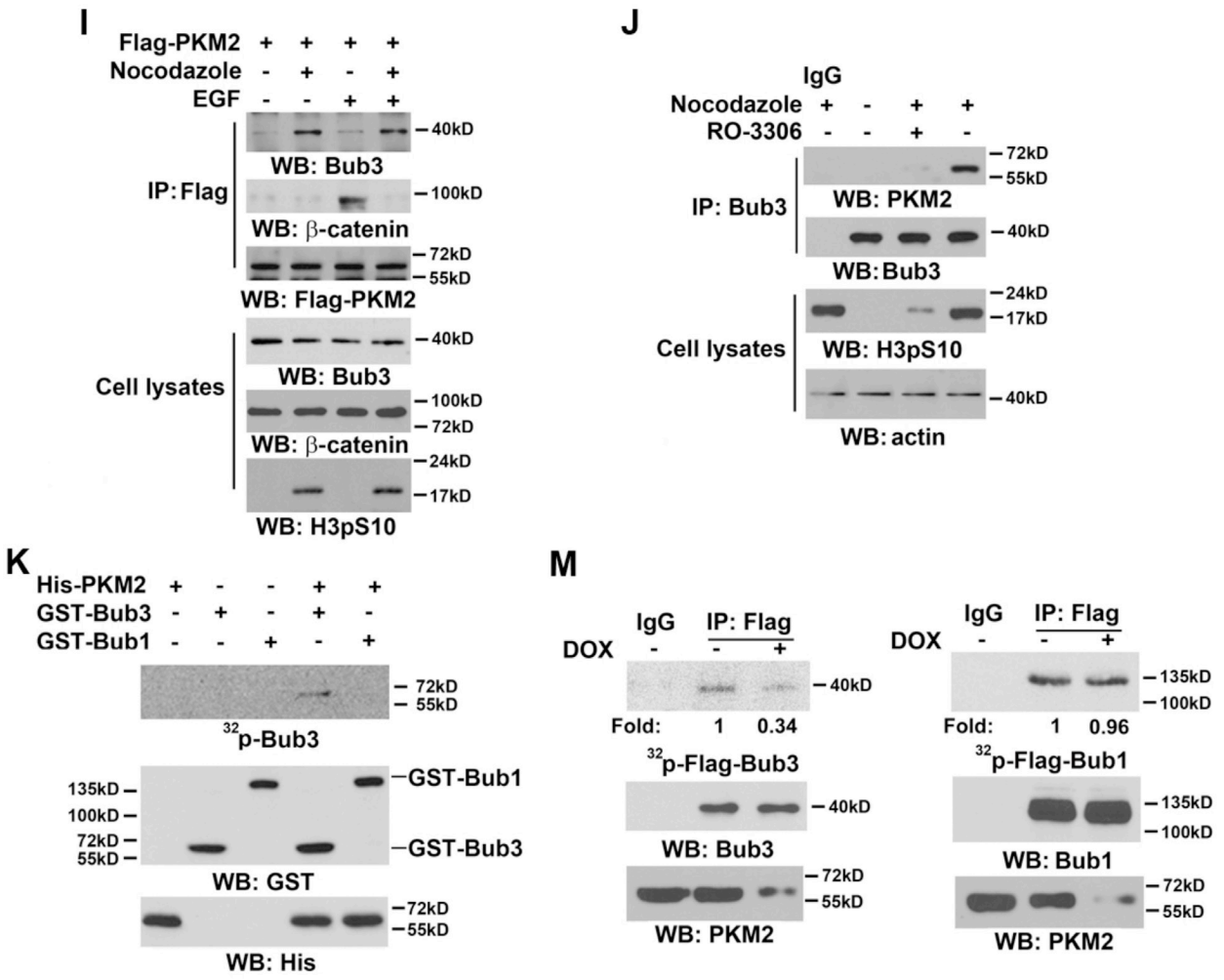


Figure S2, Related to Figure 2. PKM2, but not PKM1, phosphorylates Bub3 at Y207 in vitro and in vivo

(**A**) U87/EGFRvIII cells with or without overexpressed FLAG-PKM1 were synchronized in mitosis by thymidine double block (2 mM) and released for 9 h. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**B**) U87/EGFRvIII cells with overexpressed FLAG-PKM2 and with or without expression of Bub1 shRNA (left panel) or Bub3 shRNA (right panel) were synchronized in mitosis by thymidine double block (2 mM) and released for 9 h. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**C**) U87/EGFRvIII cells with PKM2 depletion and reconstituted expression of Flag-tagged PKM2 proteins were synchronized in mitosis by thymidine double block (2 mM) with release for 9 h. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**D**) In vitro phosphorylation analyses were performed by mixing purified recombinant GST-Bub3 proteins with purified His-PKM2. The mass spectrometry results of a fragment spectrum of a peptide at m/z 756.346 (mass error, -+4.2 ppm) matched to the doubly-charged peptide 201-GRVAVEYLDPSPE-213, suggesting that Y207 was phosphorylated. The Sequest score for this match was Xcorr = 3.3.

(**E**) In vitro phosphorylation analyses were performed by mixing purified recombinant GST-Bub3 proteins with purified His-PKM2 in the presence or absence of competing phospho-peptides for pY207 of Bub3. Immunoblotting analyses were performed with the indicated antibodies.

(**F**) In vitro phosphorylation assays were performed by mixing purified WT His-PKM2 with the indicated recombinant GST-Bub3 proteins in the presence of ^{32}P-labeled PEP and non-labeled PEP (left panel) or in the presence of PEP and ATP (right panel). Immunoblotting analyses were performed with the indicated antibodies.

(**G**) In vitro phosphorylation analyses were performed by mixing the indicated amount of purified His-PKM2 with the indicated recombinant GST-Bub3 protein in the presence of PEP.

(**H**) In vitro phosphorylation analyses were performed by mixing GST-Bub3 protein with purified WT His-PKM2 or His-PKM2 R399E in the presence of PEP. Immunoblotting analyses were performed with the indicated antibodies.

(**I**) U87/EGFR cells were treated with or without nocodazole (20 ng/ml) for 16 h, EGF (100 ng/ml) for 6 h, or treated with nocodazole (20 ng/ml) for 16 h in the presence of EGF (100 ng/ml) for last 6 h. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**J**) U87/EGFRvIII cells were treated with or without nocodazole (20 ng/ml) for 16 h in the absence or presence of RO-3306 (10 μM). Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**K**) In vitro phosphorylation analyses were performed by mixing purified His-PKM2 with the indicated recombinant GST-Bub3 or GST-Bub1 proteins in the presence of ^{32}P-labeled PEP. Immunoblotting analyses were performed with the indicated antibodies.

(**M**) U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for 9 h. Doxycycline (500 ng/ml) was added at 48 h before harvesting the cells to induce PKM2 shRNA expression. The cells were metabolically labeled with ^{32}P-phosphate (1 mCi/ml) for 12 h before harvesting. Immunoprecipitation with an anti-FLAG antibody and immunoblotting analyses with the indicated antibodies were performed.

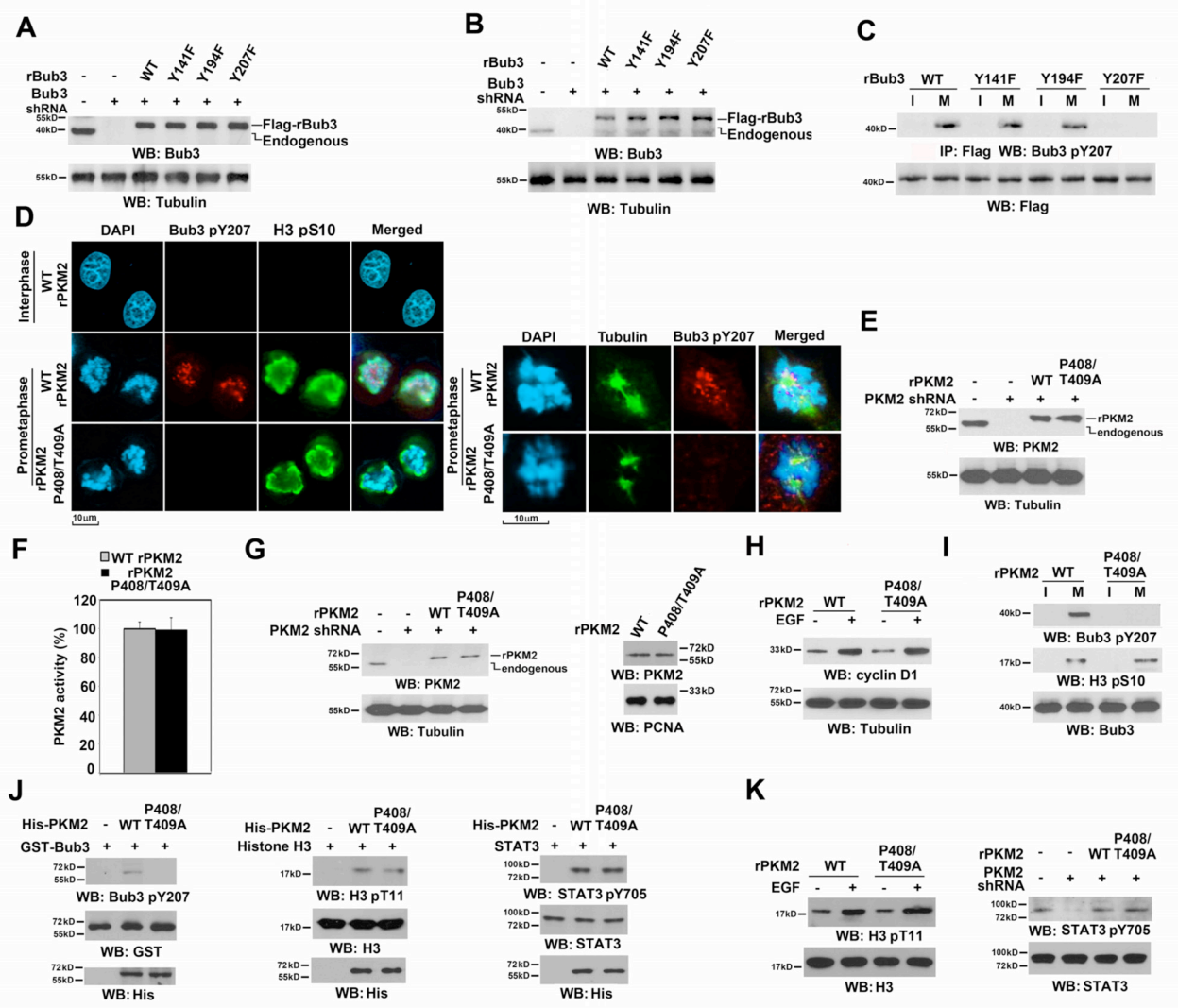


Figure S3, Related to Figure 2. P408/T409 of PKM2 is required for interaction of PKM2 with Bub3 and phosphorylation of Bub3 Y207

(**A**) Bub3 was depleted from HeLa cells with expression of Bub3 shRNA, and expression of the indicated Bub3 proteins was reconstituted. Immunoblotting analyses were performed with the indicated antibodies.

(**B**) Bub3 was depleted from U87/EGFRvIII cells with expression of Bub3 shRNA, and

expression of the indicated Bub3 proteins was reconstituted. Immunoblotting analyses were performed with the indicated antibodies.

(C) U87/EGFRvIII cells with depleted Bub3 and reconstituted expression of the indicated Bub3 proteins were synchronized by thymidine double block (2 mM) and released for 9 h. Immunoblotting analyses were performed with the indicated antibodies. I, interphase; M, mitosis.

(**D**) HeLa cells with PKM2 depletion and reconstituted expression of the indicated PKM2 proteins were immunostained with the indicated antibodies. The cells in interphase and prometaphase were examined.

(**E**) PKM2 was depleted from HeLa cells with expression of PKM2 shRNA, and expression of the indicated PKM2 proteins was reconstituted. Immunoblotting analyses were performed with the indicated antibodies.

(**F**) The pyruvate kinase activity of recombinant WT PKM2 and PKM2 P408/T409A proteins was examined. Data represent the mean ± SD of three independent experiments.

(**G**) U87/EGFRvIII cells with depleted PKM2 were reconstituted the expression of WT rPKM2 or rPKM2 P408/T409A (left panel). The nuclear lysates were prepared (Right panel). Immunoblotting analyses were performed with the indicated antibodies.

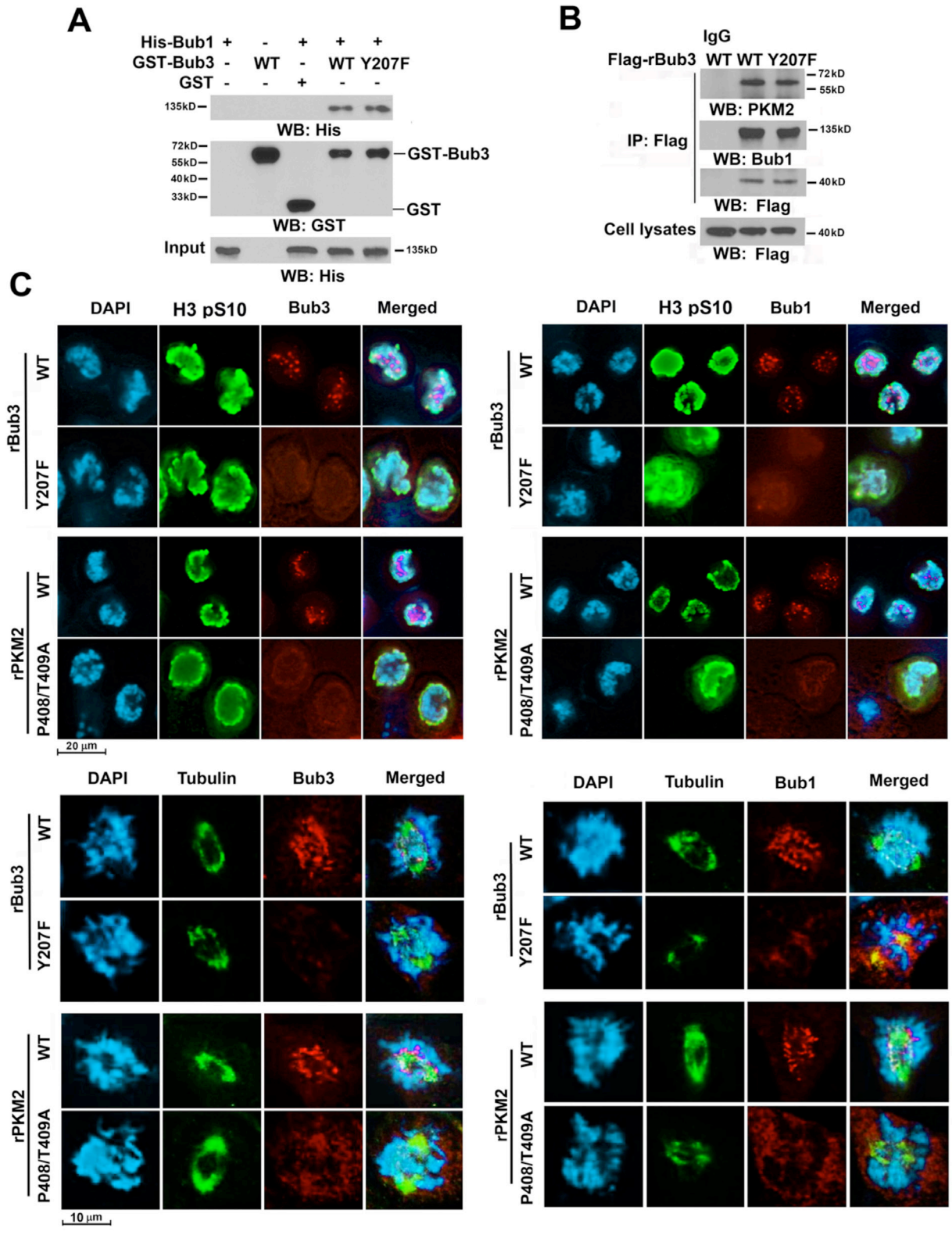
(**H**) U87/EGFR cells with PKM2 depletion and reconstituted expression of Flag-tagged PKM2 proteins were treated with EGF (100 ng/ml) for 24 h. Immunoblotting analyses were performed with the indicated antibodies.

(**I**) U87/EGFRvIII cells with depleted PKM2 and reconstituted expression of WT PKM2 or PKM2 P408/T409A were synchronized by thymidine double block (2 mM) with or without

release for 9 h. Immunoblotting analyses were performed with the indicated antibodies. I, interphase; M, mitosis.

(**J**) In vitro phosphorylation analyses were performed by mixing purified His-PKM2 with the indicated recombinant Bub3 protein (left panel), histone H3 protein (middle panel), and calf intestinal phosphatase-treated immunoprecipitated STAT3 protein (from HeLa cells; right panel) in the presence of PEP. Immunoblotting analyses were performed with the indicated antibodies.

(**K**) HeLa cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A were treated with or without EGF (100ng/ml) for 6 h (left panel). Nuclear extracts of HeLa cells with or without PKM2 depletion and reconstituted expression of WT rPKM2 and rPKM2 P408/T409A were prepared (right panel). Immunoblotting analyses were performed with the indicated antibodies.


A
His-Bub1 + - + + +
GST-Bub3 - WT - WT Y207F
GST - - + - -
135kD
WB: His
72kD
55kD
40kD
33kD
GST-Bub3
GST
WB: GST
Input
135kD
WB: His
B
IgG
Flag-rBub3 WT WT Y207F
72kD
55kD
WB: PKM2
IP: Flag
135kD
WB: Bub1
40kD
WB: Flag
Cell lysates
40kD
WB: Flag
C
DAPI
H3 pS10
Bub3
Merged
rBub3
WT
Y207F
rPKM2
WT
P408/T409A
20 μm
DAPI
H3 pS10
Bub1
Merged
rBub3
WT
Y207F
rPKM2
WT
P408/T409A
DAPI
Tubulin
Bub3
Merged
rBub3
WT
Y207F
rPKM2
WT
P408/T409A
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P408/T409A

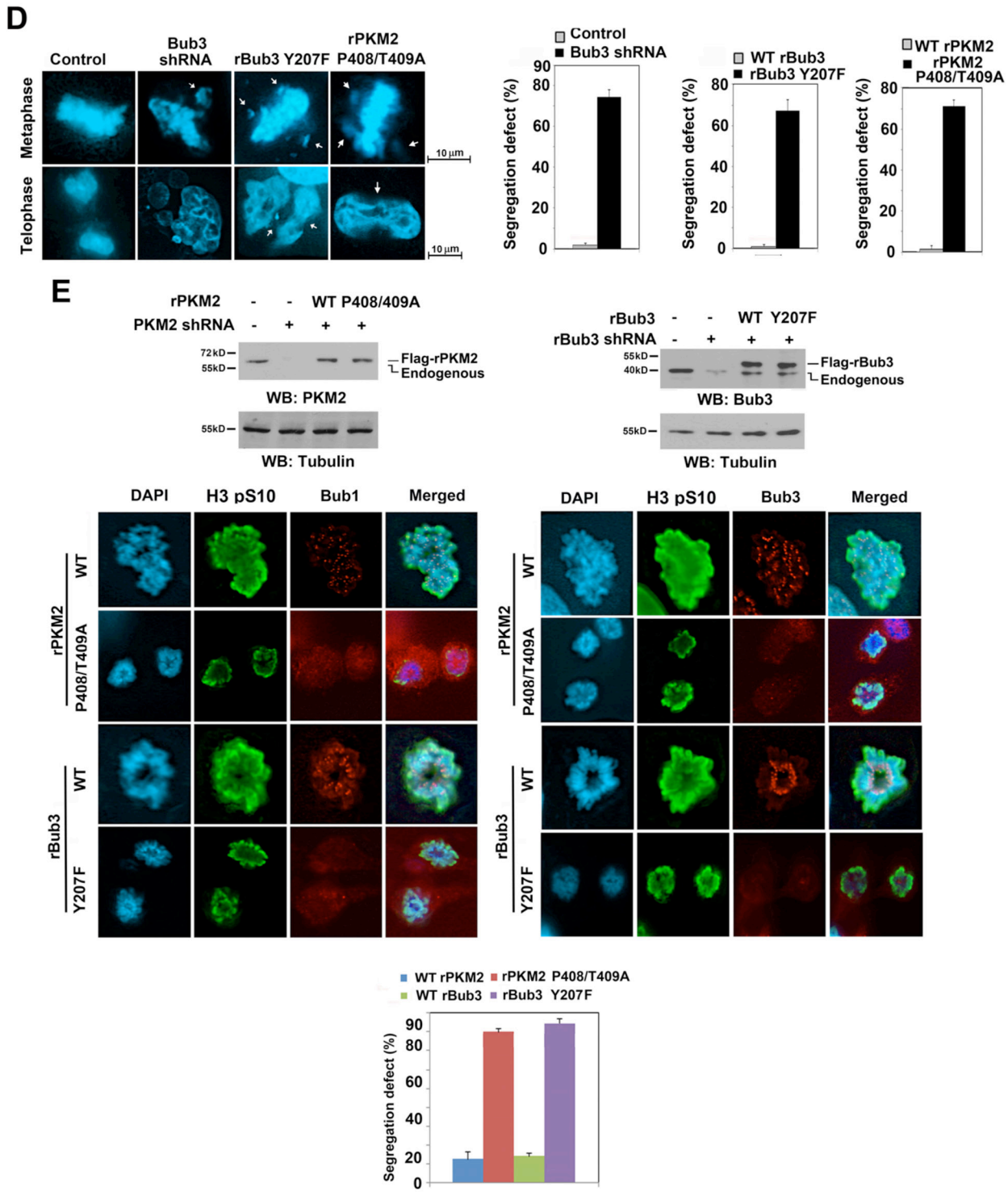


Figure S4, Related to Figure 3. PKM2-dependent Bub3 Y207 phosphorylation is required for accurate chromosome segregation

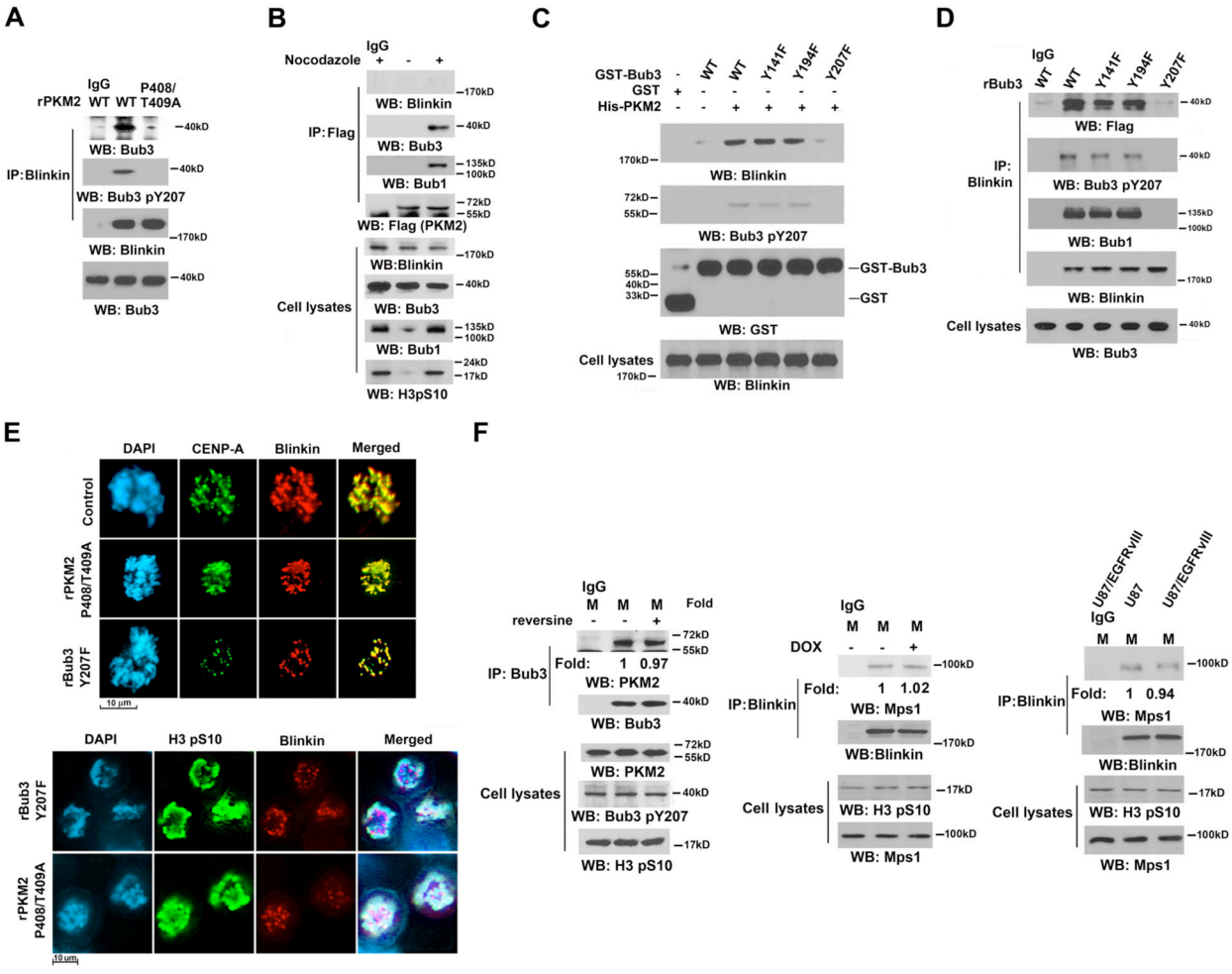
(**A**) Purified recombinant GST or GST-Bub3 proteins were mixed with purified recombinant His-Bub1. A GST pull-down assay was performed. Immunoblotting analyses were performed with the indicated antibodies.

(**B**) HeLa cells with Bub3 depletion and reconstituted expression of Flag-tagged WT rBub3 or rBub3 Y207F were synchronized by thymidine double block (2 mM) and released for 9 h. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**C**) HeLa cells with depleted Bub3 and reconstituted expression of WT rBub3 or rBub3 Y207F (top two panels) or depleted PKM2 and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A (bottom two panels) were immunostained with the indicated antibodies. The cells in prometaphase were examined.

(D) U87/EGFRvIII cells with depleted Bub3 and reconstituted expression of WT rBub3 and rBub3 Y207F or depleted PKM2 and reconstituted expression of WT rPKM2 and rPKM2 P408/T409A were immunostained with the indicated antibodies. The cells in metaphase and telophase were examined (left panel). One hundred cells in mitosis were analyzed. Data represent the mean ± SD of three independent experiments (middle and right panels).

(E) U251 cells with depleted Bub3 and reconstituted expression of WT rBub3 or rBub3 Y207F or depleted PKM2 and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A were immunoblotted and immunostained with the indicated antibodies. The cells (synchronized by thymidine double block and release) in metaphase were examined. One hundred cells in mitosis were analyzed. Data represent the mean ± SD of three independent experiments.


A
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WB: Bub3
WB: Bub3 pY207
WB: Blinkin
WB: Bub3
40kD
170kD
B
IgG
Nocodazole + - +
IP: Flag
WB: Blinkin
WB: Bub3
WB: Bub1
WB: Flag (PKM2)
Cell lysates
WB:Blinkin
WB: Bub3
WB: Bub1
WB: H3pS10
170kD
40kD
135kD
100kD
72kD
55kD
24kD
17kD
C
GST-Bub3 - WT WT Y141F Y194F Y207F
GST + - - - - -
His-PKM2 - - + + + +
WB: Blinkin
WB: Bub3 pY207
WB: GST
GST-Bub3
GST
Cell lysates
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72kD
55kD
40kD
33kD
D
IgG
rBub3 WT WT Y141F Y194F Y207F
IP: Blinkin
WB: Flag
WB: Bub3 pY207
WB: Bub1
WB: Blinkin
Cell lysates
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135kD
100kD
170kD
E
DAPI CENP-A Blinkin Merged
Control
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rBub3 Y207F
10 μm
DAPI H3 pS10 Blinkin Merged
rBub3 Y207F
rPKM2 P408/T409A
10 um
F
IgG
M M M Fold
reversine - - +
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Fold: 1 0.97
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WB: Bub3
Cell lysates
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WB: Bub3 pY207
WB: H3 pS10
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55kD
40kD
17kD
IgG
M M M
DOX - - +
IP:Blinkin
Fold: 1 1.02
WB: Mps1
WB:Blinkin
Cell lysates
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WB: Mps1
100kD
170kD
17kD
U87/EGFRvIII U87 U87/EGFRvIII
IgG
M M M
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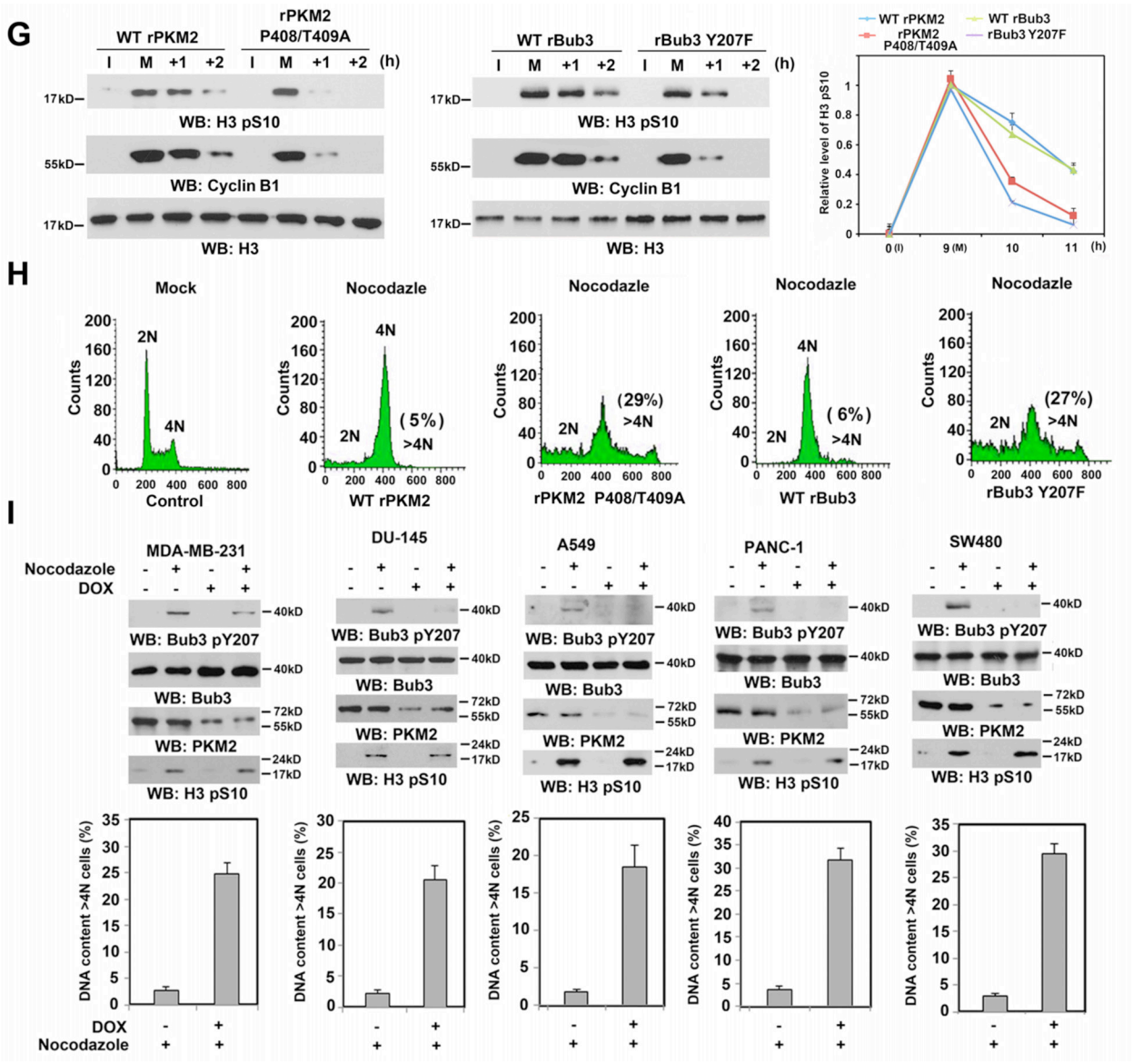


Figure S5, Related to Figure 4. PKM2-dependent Bub3 Y207 phosphorylation is required for the association of Bub3 and Bub1 with Blinkin, spindle assembly checkpoint, and accurate chromosome segregation in multiple human cancer cell lines

(**A**) U87/EGFRvIII cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408T409A were synchronized by thymidine double block (2 mM) and released for 9 h. Immunoprecipitation of endogenous Blinkin was performed.

(**B**) U87/EGFRvIII cells expressing Flag-PKM2 were synchronized by thymidine double block (2 mM) with or without release for 6 h were treated with nocodazole (20 ng/ml) for 12 h. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(**C**) Purified recombinant GST or GST-Bub3 proteins were mixed with or without purified recombinant His-PKM2 for an in vitro phosphorylation reaction in the presence of PEP. The reaction mixtures were then incubated with the lysates of endogenous PKM2-depleted U87/EGFRvIII cells synchronized by thymidine double block (2 mM) and arrested at mitosis by nocodazole (20 ng/ml) treatment for 9 h. GST pull-down assays were performed.

(**D**) U87/EGFRvIII cells with Bub3 depletion and reconstituted expression of Flag-tagged rBub3 proteins were synchronized by thymidine double block (2 mM) and released for 9 h. Immunoprecipitation analyses were performed with an anti-Blinkin antibody.

(**E**) HeLa cells (synchronized by thymidine double block and release for 9 h) with depleted Bub3 and reconstituted expression of WT rBub3 or rBub3 Y207F or depleted PKM2 and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A were immunostained with the indicated antibodies. The cells in prometaphase were examined.

(**F**) Immunoblotting and immunoprecipitation analyses were performed with the indicated antibodies.

Left panel: U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for 9 h, followed by MG132 (20 μM) and reversine (2 μM) for 5 h. Cell lysates were prepared.

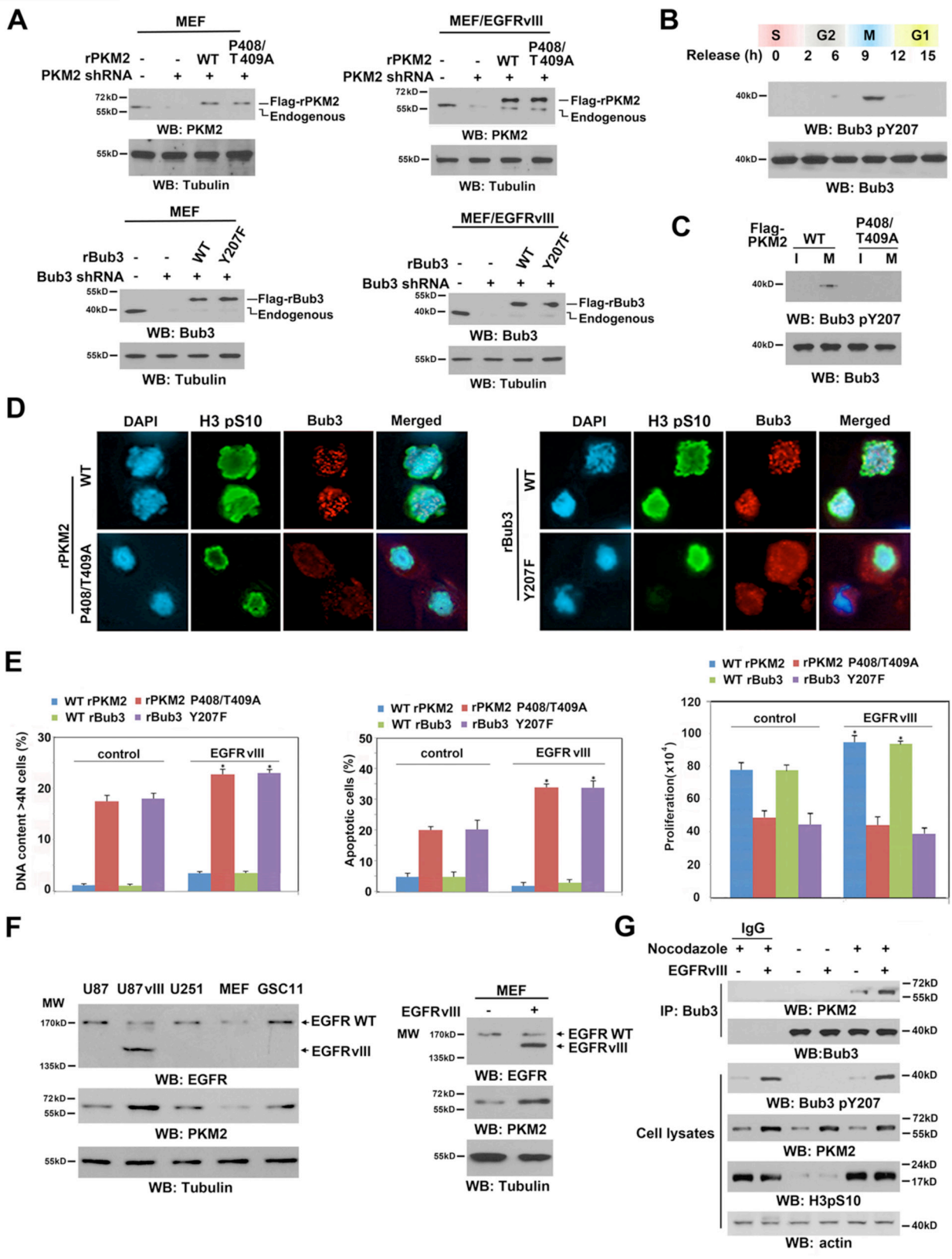
Middle panel: U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for 9 h. Doxycycline (500 ng/ml) was added or not added at the time point indicated in Figure 1C to induce PKM2 shRNA expression. Cell lysates were prepared.

Right panel: U87 or U87/EGFRvIII cells synchronized by thymidine double block (2 mM) were released for 9 h. Cell lysates were prepared.

(**G**) HeLa cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A (left panel) or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F (middle panel) were synchronized by thymidine double block (2 mM) and released for the indicated periods of time. Immunoblotting analyses were performed with the indicated antibodies. The intensity of H3 pS10 was quantified. Data represent the mean ± SD of three independent experiments (right panel).

(**H**) HeLa cells with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A (2nd and 3rd panel) or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F (4th and 5th panel) were synchronized by thymidine double block (2 mM) and released for 6 h, followed by nocodazole (20 ng/ml) treatment for 36 h. Flow cytometric analyses were performed. Five independent experiments were performed. Representative cell cycle profiles are shown.

(**I**) The indicated cells were synchronized by thymidine double block (2 mM) were released for 6 h followed by with or without nocodazole (20 ng/ml) treatment for 27 h for immunoblotting analyses or 36 h for flow cytometric analyses. Doxycycline (500 ng/ml) was added at the indicated time point in Figure 1C to induce PKM2 shRNA expression. Nocodazole was removed after doxycycline treatment for 48 h. Immunoblotting analyses with the indicated antibodies were performed.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rPKM2
PKM2 shRNA
WT
P408/ T409A
Flag-rPKM2
Endogenous
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rBub3
Bub3 shRNA
Flag-rBub3
WB: Bub3
B
S
G2
M
G1
Release (h) 0 2 6 9 12 15
WB: Bub3 pY207
C
Flag-PKM2
I M
D
DAPI
H3 pS10
Bub3
Merged
E
WT rPKM2
rPKM2 P408/T409A
WT rBub3
rBub3 Y207F
DNA content >4N cells (%)
Apoptotic cells (%)
Proliferation(x10⁴)
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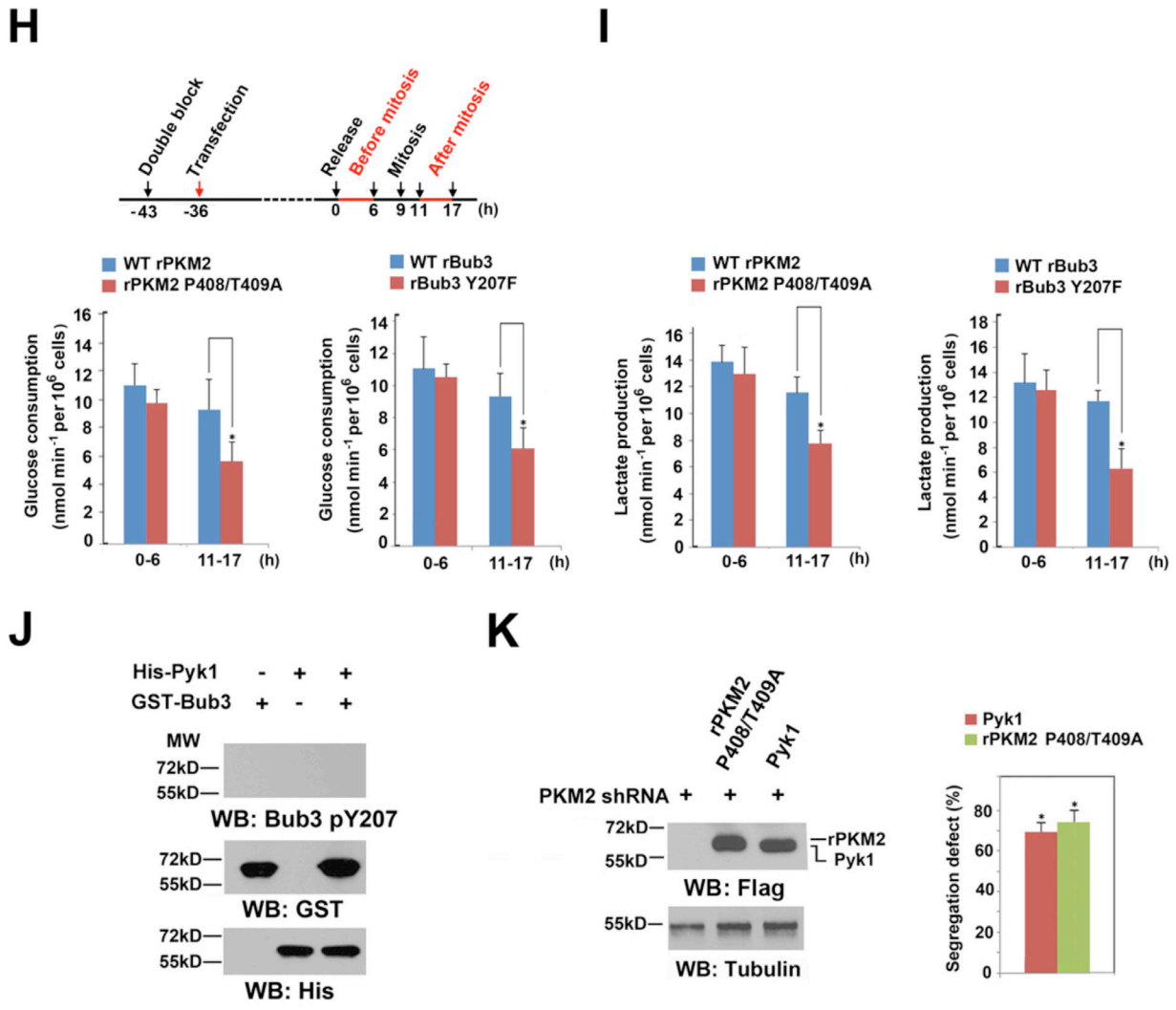

Figure S6, Related to Figure 5. MEFs expressing active EGFRvIII have enhanced PKM2 expression and increased dependence on PKM2's regulation on mitosis

(**A**) MEFs and EGFRvIII-expressing MEFs with depleted Bub3 and reconstituted expression of WT rBub3 or rBub3 Y207F or depleted PKM2 and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A were immunoblotted with the indicated antibodies.

(**B**) MEFs synchronized by thymidine double block (2 mM) were released for the indicated periods of time. Immunoblotting analyses were performed with the indicated antibodies.

(**C**) MEFs with depleted PKM2 and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A were synchronized by thymidine double block (2 mM) with or without release for 9 h. Immunoblotting analyses were performed with the indicated antibodies. I, interphase; M, mitosis.

(**D**) MEFs with depleted PKM2 and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A or with depleted Bub3 and reconstituted expression of WT rBub3 or rBub3 Y207F were synchronized by thymidine double block (2 mM) and released for 9 h. The cells were immunostained with the indicated antibodies.

(**E**) MEFs and EGFRvIII-expressing MEFs with PKM2 depletion and reconstituted expression of WT rPKM2 or rPKM2 P408/T409A or with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F were synchronized by thymidine double block (2 mM) and released for 6 h, followed by nocodazole (20 ng/ml) treatment for 36 h. Flow cytometric analyses of mitotic (left panel) and apoptotic cells (middle panel) was performed. Cells (2×10^4) were plated and counted 7 days after seeding in DMEM with 2% bovine calf serum (right panel). Data represent the mean ± SD of three independent experiments.

(**F**) Immunoblotting analyses of the indicated cell lines were performed with the indicated antibodies.

(**G**) MEFs and EGFRvIII-expressing MEFs synchronized by thymidine double block (2 mM) with or without release for 6 h were treated with nocodazole (20 ng/ml) for 12 h. Immunoprecipitation and immunoblotting analyses were performed with the indicated antibodies.

(H, I) U87/EGFRvIII cells were infected with lentivirus expressing PKM2 shRNA and WT rPKM2 or rPKM2 P408/T409A or with lentivirus Bub3 shRNA and WT rBub3 or rBub3 Y207F

48 h before release from thymidine double block (2 mM). The media were collected for analysis of glucose consumption (H) or lactate production (I), which was normalized by cell numbers (per 10^6). The G1-S phase of cell glycolysis rate was measured from the media collected during 0-6 h after release from thymidine double block; The glycolysis of cells exited from mitosis rate was measured from the media collected during 11-17 h after release from thymidine double block. Data represent the means ± SD of three independent experiments. $*P<0.05$.

(**J**) In vitro phosphorylation assays were performed by mixing purified His-Pyk1 with recombinant GST-Bub3 proteins in the presence of PEP. Immunoblotting analyses were performed with the indicated antibodies.

(**K**) U87/EGFRvIII cells with PKM2 depletion were reconstituted the expression of Flag-tagged rPKM2 P408/T409A or Pyk1. Immunoblotting analyses were performed with the indicated antibodies (left panel). These cells were synchronized by thymidine double block (2 mM) and released for 9 h. The cells were immunostained with anti-tubulin and anti-CENP-A antibodies. One hundred metaphase cells were analyzed. Data represent the mean ± SD of three independent experiments (right panel).

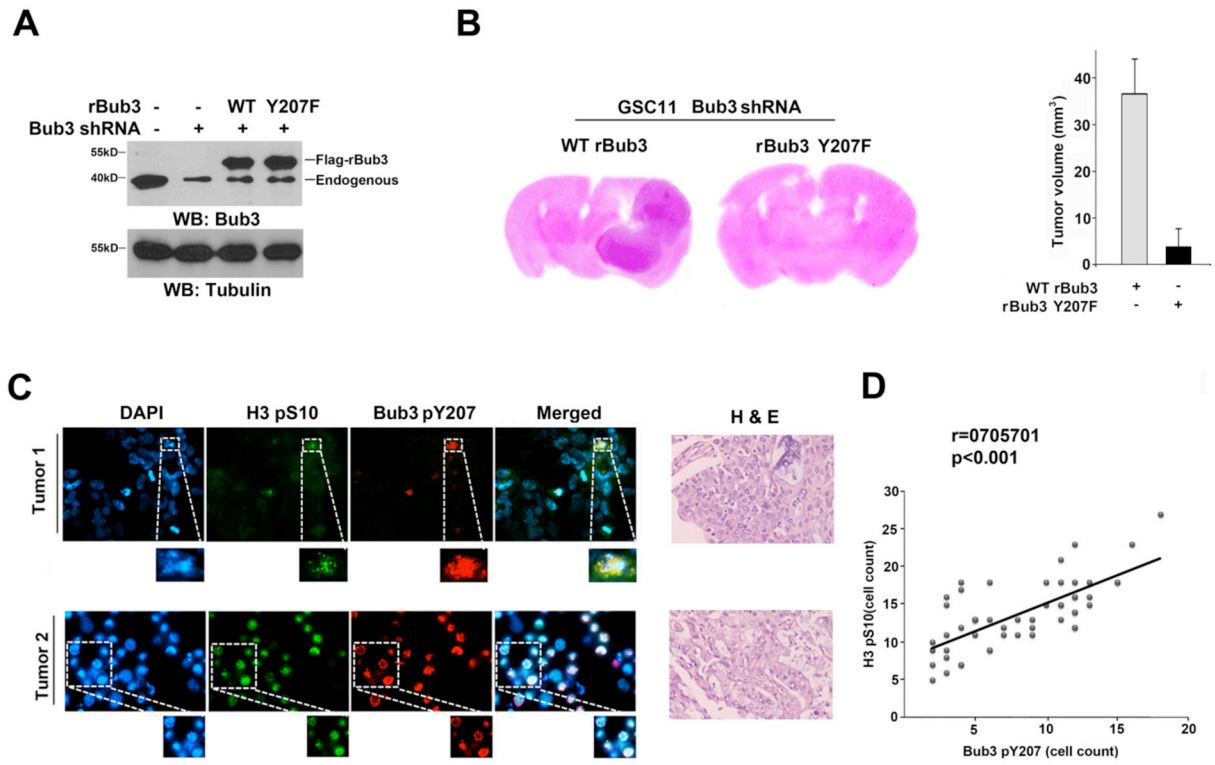

Figure S7, Related to Figure 6. PKM2-dependent Bub3 Y207 phosphorylation is required for tumorigenesis and positively correlates with the level of H3-S10 phosphorylation in human lung adenocarcinoma specimens

(**A**) GSC11 cells with Bub3 depletion were reconstituted with the expression of WT rBub3 or rBub3 Y207F. Immunoblotting analyses were performed with the indicated antibodies.

(**B**) A total of 5 × 10^5 GSC11 cells with Bub3 depletion and reconstituted expression of WT rBub3 or rBub3 Y207F were intracranially injected into athymic nude mice. The mice were sacrificed and examined for tumor growth. H&E-stained coronal brain sections show representative tumor xenografts. Tumor volumes were measured by using length "a" and width "b" and calculated using the equation: $V = ab^2/2$. Data represent the means ± SD of 7 mice.

(C, D) H&E staining and immunofluorescent staining with anti–phospho-Bub3 Y207 and anti–

phospho-H3-S10 antibodies were performed on 50 lung adenocarcinoma specimens. Representative photos of two tumors are shown (C). We quantitatively scored the tissue sections by counting positively stained cells in 10 microscopic fields. (Pearson product moment correlation test; $r = 0.71$, $p < 0.001$) (D). Note that some of the dots on the graphs represent more than one specimen (some scores overlapped).

EXPERIMENTAL PROCEDURES

Cell culture and synchronization

HeLa, U87/EGFRvIII, U251, and immortalized MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (HyClone, Logan, UT). GSC11 cancer cells were maintained in DMEM/F-12 50/50 supplemented with B27, EGF (10 ng/ml), and bFGF (10 ng/ml).

For the double-thymidine block, 30-40% confluent cells were washed twice with phosphate-buffered saline (PBS), treated with 2 mM thymidine for 17 h, washed twice in PBS again, released in complete medium containing 10 μM deoxycytidine for 9 h, treated with 2 mM thymidine for 17 h, and then released into complete medium with 10 μM deoxycytidine and assayed.

After the double-thymidine block, cells were washed twice with PBS, released in complete medium for 6 h, and then treated with 20 ng/ml nocodazole for different periods of time.

Materials

A mouse antibody recognizing Bub3 was obtained from BD Biosciences (San Jose, CA). Antibodies for CENP-A, CENP-C, di-methyl-histone H3 K4, tri-methyl-histone H3 K9, phospho-histone H3 T11, Aurora-B, and Bub1 were purchased from Abcam (Boston, MA). A polyclonal antibody against Blinkin was purchased from Bioss (Woburn, MA). A rabbit polyclonal antibody against tubulin was purchased from Cell Signaling Technology (Beverly, MA). A polyclonal antibody for acetylated histone H3 was obtained from Upstate Biotechnology (Billerica, MA). Thymidine, nocodazole, and mouse monoclonal antibodies for Flag, GST, His, and EGFR were purchased from Sigma (St. Louis, MO). Hygromycin, puromycin, DNase-free RNase A, and propidium iodide were purchased from EMD Biosciences (San Diego, CA). DAPI, Alexa Fluor 488, 594, and 633 goat anti-rabbit antibodies, and Alexa Fluor 488, 594, and 633 goat anti-mouse antibodies were from Molecular Probes (Eugene, OR). HyFect transfection reagents were from Denville Scientific (Metuchen, NJ). GelCode Blue Stain Reagent was obtained from Pierce (Rockford, IL).

DNA constructs and mutagenesis

Bub3 was cloned into pcDNA3.1/hygro (+) vector between BamHI and XhoI. pcDNA 3.1/hygro (+) Bub3 Y141F, Y194F, and Y207F and pcDNA 3.1/hygro (+) PKM2 R400/L401A, P403/I404A, D407A, P408A, D407/P408A, T409A, P408/T409A, and K433E were made using the QuikChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA). The pGIPZ controls were generated with control oligonucleotide GCTTCTAACACCGGAGGTCTT or GCCCGAAAGGGTTCCAGCTTA. pGIPZ and pTRIPZ human PKM2 shRNA was generated with CATCTACCACTTGCAATTA oligonucleotide that targeted exon 10 of the PKM2 transcript. pTRIPZ was mutated to abolish RFP expression. pGIPZ human Bub3 shRNA was

generated with oligonucleotide AAGGCCGAGTGGCAGTTGAGT. pGIPZ mouse PKM2 shRNA was generated with TCCTCGAATAGCTGCAAGT oligonucleotide. pGIPZ mouse Bub3 shRNA was generated with oligonucleotide GTTCGGATGGATTCGTCAATA. pTRIPZ scrambled shRNA were generated with TTGCAAAGGAAGCCTTTAA oligonucleotide.

In vitro kinase assays

The kinase reactions were performed as described previously (Fang et al., 2007). In brief, bacterially purified recombinant PKM2 (200 ng) was incubated with Bub3 (100 ng) in 25 μl kinase buffer (50 mM Tris-HCl [pH 7.5], 100 mM KCl, 1 mM $MnCl_2$, 50 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM dithiothreitol [DTT], 5% glycerol, 0.5 mM PEP, 0.05 mM FBP) at 25°C for 1 h. The reactions were terminated by the addition of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and heated to 100°C. The reaction mixtures were then subjected to SDS-PAGE analyses. ^{32}P-labeled PEP was prepared as described previously (Vander Heiden et al., 2010).

ChIP assay

ChIP was performed using an Upstate Biotechnology kit, as described previously (Xia et al., 2007). Quantitative real-time PCR was used to measure the amount of bound DNA, and the value of enrichment was calculated according to the relative amount of input and the ratio to IgG. The specific primers used in PCR for human centromeric Satellite α (Satα) repeats were 5'-CTGCACTACCTGAAGAGGAC-3' (forward) and 5'-GATGGTTCAACACTCTTACA-3' (reverse).

Immunoprecipitation and immunoblotting analyses

Extraction of proteins from cultured cells using a modified buffer was followed by immunoprecipitation and immunoblotting with corresponding antibodies, as described previously (Lu et al., 1998). The immunoprecipitates were washed with the cell lysis buffer 5 times (10 minutes each).

Metabolic labeling

Cells were labeled by incubating them with 1 mCi/ml of ^{32}P-phosphate (MP Biochemicals; Solon, OH) in phosphate-free DMEM containing 10% dialyzed FBS (Invitrogen; San Diego, CA) for 12 h (Ji et al., 2009). Immunoprecipitated proteins were run on SDS-PAGE and then transferred onto nitrocellulose membrane. Phosphorylated proteins were visualized by autoradiography.

Purification of recombinant proteins

Wild-type and mutant GST-PKM2, His-PKM2, His-PKM1, GST-Bub1, His-Bub1, GST–Bub3, and His-Bub3 were expressed in bacteria and purified as described previously (Xia et al., 2007).

Immunofluorescence analysis

Cells were fixed and incubated with primary antibodies, Alexa Fluor dye-conjugated secondary antibodies, and DAPI according to standard protocols. Cells were examined using a deconvolution microscope (Zeiss, Thornwood, NY) with a 63-Å oil immersion objective. Axio Vision software from Zeiss was used to deconvolute Z-series images. The relative fluorescence

intensity of the indicated proteins of the whole cell images was compared and quantified through image analysis software.

Human and mouse tumor tissue sections from paraffin-embedded human GBM and lung adenocarcinoma specimens were stained with antibodies against phospho-Bub3 Y207, phospho-histone H3-S10, or nonspecific IgG as a negative control. We quantitatively scored the tissue sections by counting positively-stained cells in 10 microscopic fields. The use of human brain tumor specimens and the database was approved by the institutional review board of The University of Texas MD Anderson Cancer Center.

H&E staining

Human and mouse tumor tissues were fixed and then stained with Mayer's hematoxylin and eosin (H&E) (Biogenex Laboratories, Fremont, CA). The slides were mounted using Fluorogel with Tris buffer (Electron Microscopy Sciences, Hatfield, PA).

The use of animal and human brain tumor specimens and the database were approved by the institutional review boards at MD Anderson Cancer Center.

Pyruvate kinase assay

The activity of bacterially purified WT PKM2 (0.1 μg) and PKM2 P408/T409A (0.1 μg) toward PEP was measured with a pyruvate kinase assay kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Data represent the mean ± SD of three independent experiments.

Mass spectrometry analysis

In vitro PKM2-phosphorylated Bub3 was reduced with TCEP, alkylated with iodoacetic acid, then acetone-precipitated at -20°C overnight and re-suspended in 50 mM ammonium bicarbonate buffer containing Rapigest (Waters Corp, MA). The sample was heated to 95°C for 10 minutes and allowed to cool before 100 ng of sequencing-grade modified trypsin and Glu-C endoproteinase (Promega, Madison, WI) was added. The digestion proceeded overnight at 37°C and was analyzed by LC-MS/MS using an Obitrap-Elite mass spectrometer (Thermo Fisher Scientific, Waltham, MA).

Proteins were identified by comparing the fragment spectra against those in the SwissProt protein database (EBI) using Mascot v.2.3 (Matrix Science, London, UK) and Sequest v.1.20 via Proteome Discoverer v.1.3 (Thermo Fisher Scientific). Phosphopeptide matches were analyzed using PhosphoRS via Proteome Discoverer and manually curated (Yang et al., 2012).

Measurements of glucose consumption and lactate production

Cells were seeded in culture dishes and the medium was changed after 6 h. Cells were then synchronized by thymidine double block (2 mM) and released for 5 h (before mitosis) or 15 h (after mitosis and cell division), and the culture medium was then collected for measurement of glucose and lactate concentrations. Glucose levels were determined using a glucose (GO) assay kit (Sigma). Glucose consumption was the difference in glucose concentration compared with control. Lactate levels were determined using a lactate assay kit (Eton Bioscience, Inc., San Diego, CA). Cells were collected and counted, and glucose consumption and lactate production were normalized by cell numbers (per 10^6).

TUNEL assay

The paraffin slides of brain tumor tissue were used to perform TUNEL staining according to the manufacturer's protocol (In Situ Cell Death Detection kit, Roche, Indianapolis, IN). The stained tissues were examined under a confocal microscope. The TUNEL positive cells were quantified. Data represent the mean ± SD of 5 mice from each group.

Intracranial injection

We intracranially injected 5×10^5 U87/EGFRvIII or GSC11 cells with depletion of PKM2 or Bub3 and reconstitution of WT rPKM2, rPKM2 P408/T409A, WT rBub3, or rBub3 Y207F expression (in 5 μl of DMEM per mouse) into 4-week-old female athymic nude mice. The intracranial injections were performed as described in a previous publication (Yang et al., 2011). Seven mice per group in each experiment were included. The mice were sacrificed 2 weeks after glioma cell injection. The brain of each mouse was harvested, fixed in 4% formaldehyde, and embedded in paraffin. Tumor formation and phenotype were determined by histological analysis of H&E-stained sections.

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