Supporting Information

Materials and methods

Cell culture and drug treatments

Human non-transformed mammary epithelial MCF10A cells were cultured in DMEM/F-12 1:1medium (GIBCO, Rockville, MD, USA), supplemented with 20 ng/mL epidermal growth factor (Invitrogen, Carlsbad, CA, USA), 100 ng/mL cholera toxin (Sigma, St Louis, MO),10 μg/mL insulin (Sigma), 500 ng/mL (95%) hydrocortisone (Sigma) and 5% of fetal bovine serum (FBS; Hyclone, Logan, UT, USA). HCC1806, Hs578T, FaDu and HEK 293T cells were cultured in DMEM medium (GIBCO) supplemented with 10% FBS (Hyclone). Cells were grown in media supplemented 100 units/mL penicillin (GIBCO) and 100 µg/mL streptomycin (GIBCO). Cells were maintained in a humidified 37°C incubator under a 5% CO_2 atmosphere. Cells at 75-85% confluence were treated with an indicated chemical compound(s). Rapamycin (37094), metformin (PHR1084), cycloheximide (CHX) (C7698), AMP (01930) and 2-deoxy-D-glucose (2-DG, D8375) were purchased from Sigma. AICAR (S1802), Lapatinib (S2111), LY294002 (S1105) and MK2206 (S1078) were purchased from Selleck (Houston, USA).

Plasmids transfection, lentiviral infection and RNA interference

Cells at 80% confluence were transfected using Lipofectamine 2000 (Invitrogen). Expression plasmids including human E-cadherin, p110α^{H1047R}, HER2^{V659E}, H-Ras^{G12V}, AMPKα1-WT, AMPKα1-CA, AMPKα1-Dn, TAp63α, TAp63γ, ΔNp63 γ, ΔNp63 β, ΔNp63α and or ΔNp63α^{C306R}. Lentiviruses were amplified by transfection of HEK 293T cells with

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23 psPAX2 and pMD2.G packaging plasmids and Lentivirus expression plasmid using
24 Lipofectamine 2000. Viruses were collected at 60 hours after transfection. Cells at 50%
25 confluence in the presence of 10 μg/mL polybrene were infected with recombinant
26 Lentivirus encoding or an empty vector, followed by 12 hours incubation at 37°C with 5%
27 CO_2. Lentiviral-based shRNAs specific for green fluorescent protein (GFP), AMPKα1,
28 Twsit1 or p63 were constructed as described (1). (The primer sequences were listed in SI
29 Appendix, Table S1).
30

31 **Western blot, Immunofluorescence and IHC**

32 Cells were collected, washed with cold PBS, and resuspended in EBC_{250} lysis buffer
33 (250 mM NaCl, 50 mM Tris pH 8.0, 0.5% Nonidet P-40, 50 mM NaF, 0.5 mM Na_3VO_4, 1 mM
34 phenylmethylsulfonyl fluoride (PMSF), 2 μg/mL aprotinin, and 2 μg/mL leupeptin). Equal
35 amounts of protein were loaded, separated by SDS-PAGE, transferred to PVDF
36 membranes (Millipore, Darmstadt, Germany), hybridized to an appropriate primary antibody
37 and HRP-conjugated secondary antibody for subsequent detection by enhanced
38 chemiluminescence. Antibody for p63 (sc-8431), AMPKα2 (sc-19129), actin (sc-1615) and
39 Twist1 (sc-15393) were purchased from Santa Cruz Biotech (CA, USA). Antibodies for
40 phospho-AMPK (Thr172) (2535), phospho-S6 (2211), S6 (2217), phospho-S6K (9205),
41 S6K (2708), Vimentin (5741), ZEB1 (3396), Integrin β4 (4707), Integrin α5 (4705), p110α
42 (4149), Ras (3965), phospho-AKT (4508), AKT (9272) and Slug (9585) were purchased
43 from Cell Signaling Technology (Danvers, MA, USA). Antibody for E-cadherin (ab40772)
44 and AMPKα1 (ab32047) were purchased from Abcam (Cambridge, MA, USA). Flag (F1804)

45 was purchased from Sigma. HER2 (AH210) was purchased from Beyotime (Shanghai,
46 China). For Immunofluorescent analyses, cells grown on coverslips were fixed with 4%
47 polyformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, blocked with 4%
48 bovine serum albumin in PBS, hybridized to an appropriate primary antibody, and
49 FITC-conjugated secondary antibody (Jackson ImmunoRsearch, Westgrove, Pennsylvania,
50 USA) and counter-staining using DAPI (Beyotime) for subsequent detection. Coverslips
51 were mounted with ProLong Gold antifade reagent (Invitrogen). Images were acquired
52 using Leica TCS SP5 II system. IHC analyses were performed as previously described (2).
53 For quantitative analyses, slides were scanned through NanoZoomer (Hamamatsu, Japan).
54 Scanned images were then subjected to integrated optical density (IOD) measurements
55 using Image-Pro Plus 6.0 to calculate average optical density (AOD) using the formula:
56 AOD=IOD/Area [(3)] . Human breast tumor tissue microarrays (TMA) consisting of breast
57 cancer specimens from different patients were purchased from Shanghai Outdo Co., LTD
58 (Shang Hai, China).

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60 **Quantitative PCR**

61 Total RNA was extracted from cells using RNeasy plus Mini Kit (QIAGEN) and
62 reverse-transcribed. qPCR was carried out for AMPKα1, E-cadherin, Twist1, slug and
63 GAPDH (The primer sequences used in the reactions were listed in Table S1). The qPCR
64 reactions were performed in CFX-960 Real time PCR System (Bio-Rad) and using Bio-Rad
65 SoFast Eva-Green Supermix Kit (Bio-Rad) according to the manufacturer’s instructions.
66 Relative quantitation values were calculated using the ΔΔCt method.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed in MCF10A cells with ChIP-IT Kit (Active Motif, USA) using antibodies specific for p63 (ab97865, Abcam) or normal rabbit IgG (Invitrogen), as described previously (4). ChIP samples were subjected to PCR experiments to amplify fragments of the AMPKα1 promoter elements using indicated primers as listed in Table S1. To examine the strength of p63 for binding to AMPKα1 promoter elements, ChIP samples were subjected to qPCR or reverse transcriptional PCR using primers as indicated. The value of each ChIP sample was normalized to its corresponding input.

Invasion transwell assay

Cell invasion was measured using Matrigel-coated inserts (BD Biosciences). Cells (5.0×10^4) were suspended in serum-free DMEM (for Hs578T and HCC1806 cells) or DMEM:F12 (for MCF10A cells) media and seeded into the inner chamber. The outer chamber was filled with normal growth media (as described previously for each cell type). Cells were incubated for 24 h. Non-migrating cells were carefully removed with a cotton swab. Migrating cells were stained with 0.1% crystal violet for 10 min at room temperature, and photographed under a Nikon light microscope. At least 100 cells from five random fields were counted.

Luciferase reporter assays

Luciferase reporter assays were performed with Secrete-Pair™ Dual Luminescence

89 Assay Kit (GeneCopoeia, USA) according to the manufacturer's instructions. Briefly, cells
90 were co-transfected with 500 ng of AMPKα1-Gluc-SEAP reporter and 750 ng of p63
91 expression plasmid. 36 hours post-transfection, cell culture media were collected and
92 AMPKα1-Gluc and SEAP activities were measured. The AMPKα-Gluc activity was
93 normalized to SEAP activity.
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95 **Cell-cell adhesion assay**

96 To assess cell-cell adhesion, 150 cells/well were seeded in 6-well plates and grown for
97 6 to 10 days to allow colony formation. Cells were then fixed with methanol for 15 min,
98 stained with 0.1% crystal violet for 10 min at room temperature, and photographed under a
99 Nikon light microscope. Micro-colonies were defined to contain at least four cells clustered
100 together. The number of micro-colonies of each well were counted, normalized and
101 presented to reflect cell-cell adhesion ability.
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103 **In vivo metastasis assay**

104 Animal care and experiments in this study were carried out according the principles
105 outlined in Guide for the Care and Use of Laboratory Animals published by the USA
106 National Institutes of Health (NIH). Cells suspended in 100 μL PBS were injected into the
107 lateral tail veins of 5-week old female nude mice. Mice were observed daily and euthanized
108 at designated day. The lungs were dissected, fixed overnight in 4% polyformaldehyde,
109 embedded in paraffin and sectioned onto microscope slides for hematoxylin and eosin
110 (H&E) staining prior to histological analysis. The numbers of metastatic nodules in the lungs

111 per mouse were counted. To examine the effects of pharmacological activators of AMPK on
112 breast tumor metastasis in vivo, seven-week old MMTV-PyMT female FVB mice (n=6/group)
113 were intraperitoneally injected daily for 46 days with metformin (200 mg/kg) or PBS.
114 Alternatively, twelve-week old MMTV-PyMT female C57BL/6 mice (n=4/group) were
115 intraperitoneally injected every other day for 20 days with AICAR (400 mg/kg) or PBS. Mice
116 were sacrificed and the primary mammary tumors and lungs were dissected, fixed and
117 sectioned.
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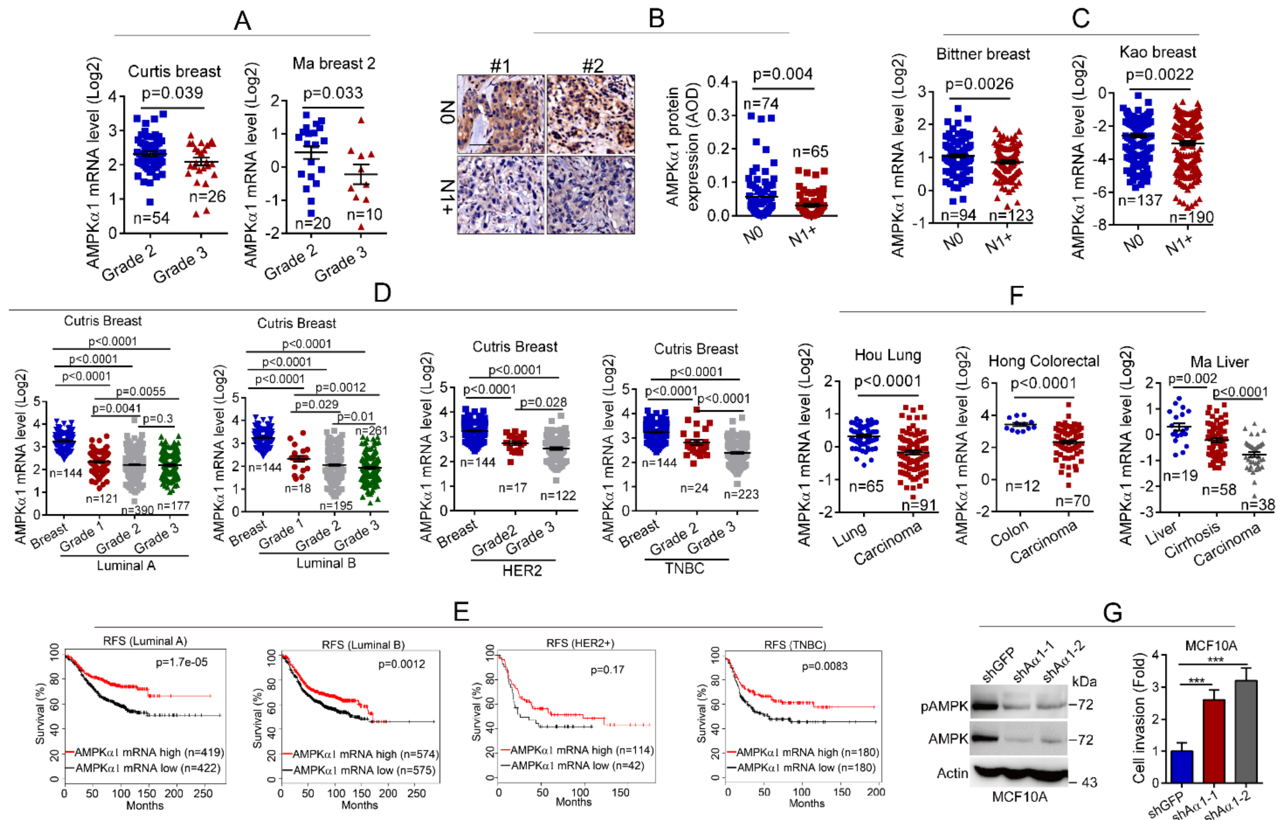

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120 **Fig. S1. AMPKα1 mRNA expression is reduced in advanced human breast cancer. (A)** The AMPKα1 mRNA levels in human breast cancer at different stages were analyzed using Oncomine Curtis breast dataset and Ma breast 2 dataset. **(B)** Human breast cancer tissue microarray consisting of lymph node-negative (N0, n=74) or lymph node-positive (N1+, n=65) breast cancer samples were subjected to IHC staining for AMPKα1 (Left panel) with quantitative analyses using average optical density (AOD) (Right panel). **(C)** The AMPKα1 mRNA levels in N0 or N1+ human breast cancer were analyzed using Oncomine Bitter breast dataset and Kao breast dataset. **(D)** The AMPKα1 mRNA levels in luminal A, luminal B, HER2 positive (HER2+) or triple negative breast cancer (TNBC) at different stages were analyzed using Oncomine Curtis breast dataset.**(E)** The correlation between the AMPKα1 mRNA levels and recurrence free survival (RFS) in luminal A, luminal B, HER2+ or TNBC breast cancer patients were analyzed using Kaplan-Meier Plotter database. Split patients by median was used to analyze Luminal A, Luminal B and TNBC breast cancers. Split patients by best cutoff was used to analyze HER2+ breast cancers. **(F)** The AMPKα1 mRNA levels in human colon cancer, lung cancer and liver cancer were analyzed using the Oncomine Hou lung dataset, Hong Colorectal dataset and Ma liver dataset. **(G) Ablation of AMPK α1 expression promotes cell invasion.** MCF10A cells stably expressing shGFP, shAMPKα1-1 (shAα1-1) or shAMPKα1-2 (shAα1-2) were subjected to western bot analyses (Left panel) or transwell assays for cell invasion (Right panel). Data are presented as means ± SEM. ***, p< 0.001.

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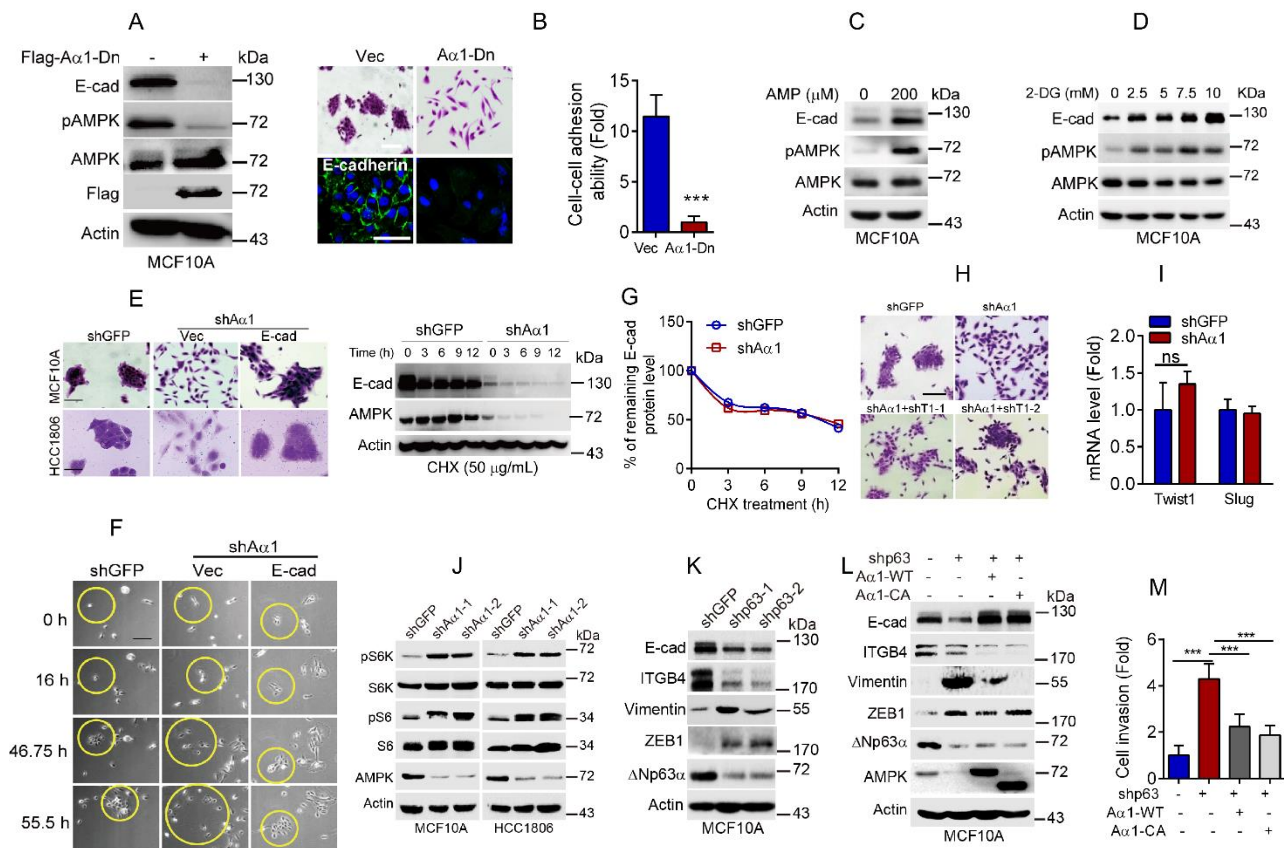
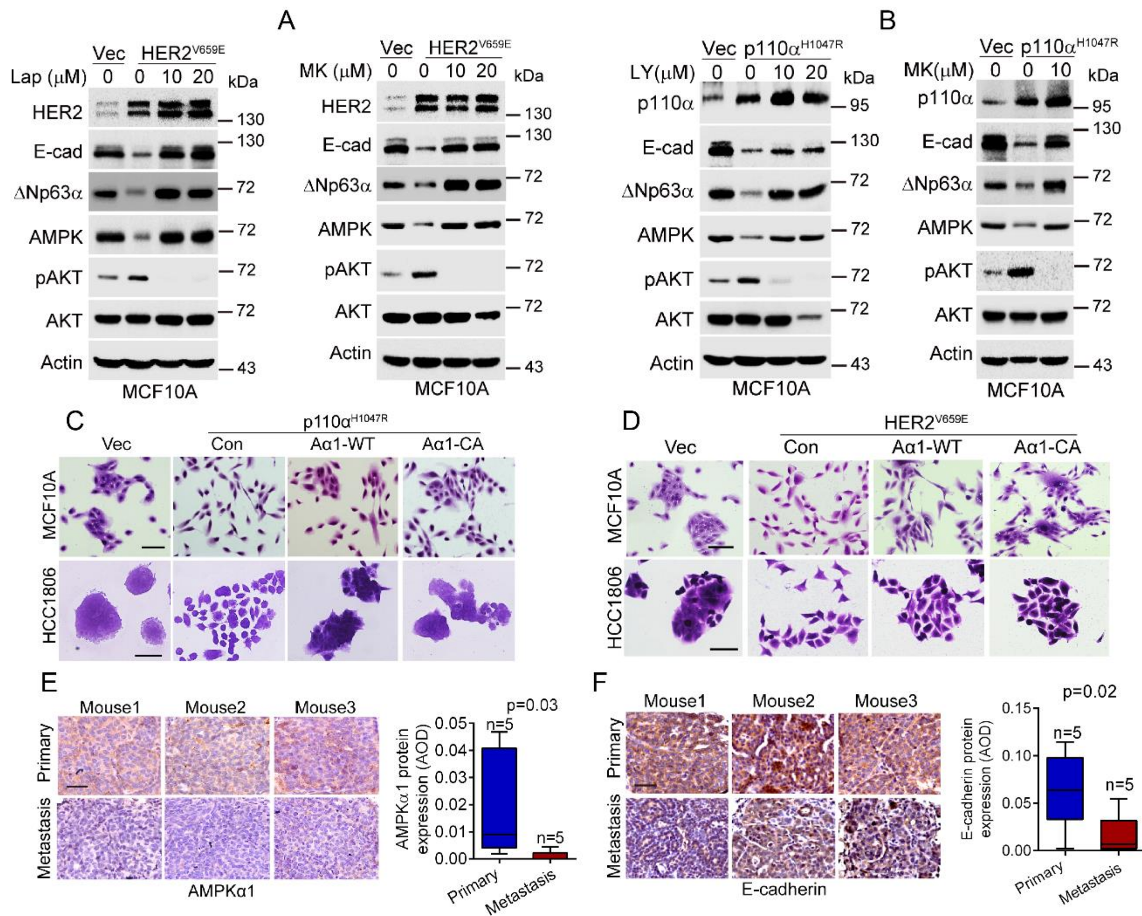
139 **Fig. S2. (A) Activated Ras inhibits AMPKα1 expression.** MCF10A cells stably expressing
140 H-Ras^{G12V} or vector control (Vec) were subjected to western blot analyses (Left panel) or Q-PCR
141 assays for AMPKα1 mRNA levels (Right panel). **(B) Knockdown of ΔNp63α reduces AMPKα1**
142 **expression.** FaDu cells stably expressing shp63-1, shp63-2 or shGFP were subjected to western
143 blot analyses. **(C) Ectopic expression of ΔNp63α upregulates AMPKα1 expression.** MCF10A
144 or FaDu cells stably expressing ΔNp63α were subjected to western blot analyses. **(D)** Whole cell
145 lysates derived from MCF10A, HCC1806 cells and 293T cells transiently transfected with TAp63α,
146 ΔNp63α, ΔNp63β, TAp63γ, ΔNp63γ or a vector control (Vec) were subjected to western blot
147 analyses. **(E)** 293T cells transiently transfected with ΔNp63α, ΔNp63β, ΔNp63γ, TAp63α, TAp63γ or
148 a vector control (Vec) were subjected to western blot analyses (Left panel) or Q-PCR assays (Right
149 panel). **(F)** Four putative p63-binding elements (CNNGNNNNNNCNNG) on AMPKα1 gene
150 promoter was shown. **(G)** Whole cell lysates derived from MCF10A, MCF7, HCC1806, Hs578T,
151 MDA-MB-231 cells and mouse liver tissue were subjected to western blot analyses. Data are
152 presented as means ± SEM. ***, p< 0.001.

153 

154 **Fig.S3. Alteration of AMPK activity affects E-cadherin protein expression and cell-cell**
155 **adhesion. (A-B)** MCF10A cells stably expressing dominant negative mutant flag-AMPKα1-D139A
156 (Aα1-Dn) were subjected to western blot analyses (A), staining with 0.1% crystal violet or
157 immunofluorescent staining for E-cadherin. Representative micrographs and cell-cell adhesion
158 ability, as defined in the Materials and Methods, were presented (B). **(C-D)** MCF10A cells were
159 treated with AMP or an indicated dose of 2-Deoxy-D-glucose (2-DG) for 24 h prior to western blot
160 analyses. **(E-F)** MCF10A-shAα1-1 or HCC1806-shAα1-1 cells stably expressing E-cadherin were
161 stained with crystal violet and representative micrographs were shown (E). To visualize the dynamic
162 cell-cell adhesion during cell proliferation, MCF10A-shAα1 cells with or without restoration of
163 E-cadherin expression were subjected to monitoring cell adhesion under the Living Cell Imaging
164 System. Representative snap-shots at the indicated time intervals were shown. Circles pointed to
165 dynamics of cell adhesion during the time course (F). **(G-J) Silencing of AMPKα1 results in down**
166 **regulation of E-cadherin expression through a mTOR-Twist1-dependent pathway.**
167 MCF10A-shAα1 or MCF10A-shGFP cells were subjected to protein half-life assays upon
168 cycloheximide (CHX) treatment (G). MCF10A-shAα 1 cells stably expressing shTwist1-1 (shT1-1)
169 or shTwist1-2 (shT1-2) were stained with crystal violet and representative micrographs were shown
170 (H). MCF10A-shAα1 or MCF10A-shGFP cells were subjected to Q-PCR analyses for Twist1 or Slug
171 (I). MCF10A-shAα1-1, MCF10A-shAα1-2 or MCF10A-shGFP cells were subjected to western blot
172 analyses (J). **(K-M) Ectopic expression of AMPK inhibits silencing of p63-induced cell**
173 **invasion.** MCF10A cells stably expressing shp63-1 or shp63-2 were subjected to western blot
174 analyses (K). MCF10A-shp63 cells stably expressing wild type flag-AMPKα1 (Aα1-WT) or
175 constitutively active flag-AMPKα1-T172D (Aα1-CA) were subjected to western blot analyses (L) or
176 transwell assays for cell invasion (M). Data are presented as means ± SEM. ***, $p < 0.001$; ns, no

177 significance. Scale bar=50 μm.



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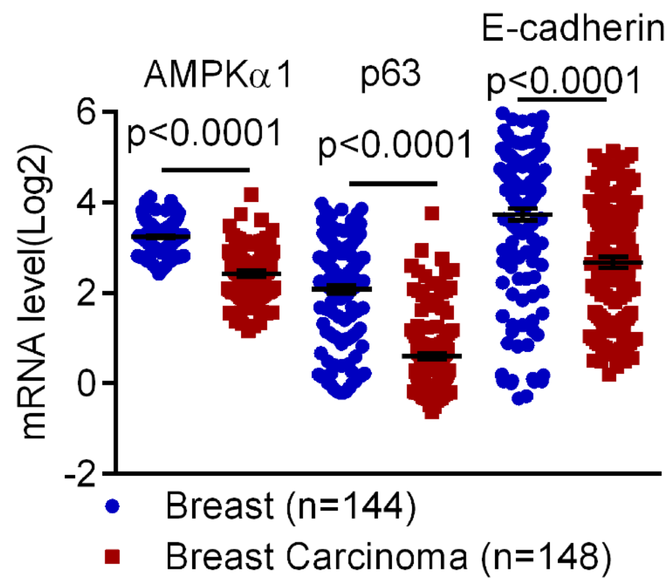
179 **Fig. S4. Pharmacological inhibiting HER2 or PI3K signaling significantly rescues activated**
180 **HER2 or PI3K-induced downregulation of ΔNp63α, AMPK and E-cadherin expression.** (A-B)
181 MCF10A-HER2^{V659E} cells were treated with an indicated concentration lapatinib (Lap, HER2
182 inhibitor) or MK2206 (MK, AKT inhibitor) for 24 h (A) or MCF10A-p110α^{H1047R} cells were treated with
183 an indicated concentration LY294002 (LY, PI3K inhibitor) or MK2206 for 24 h (B). Whole cell lysates
184 were subjected to western blot analyses. **(C-D) Ectopic expression of AMPK markedly inhibits**
185 **PI3K/HER2-induced disruption of cell-cell adhesion.** MCF10A-p110α^{H1047R},
186 HCC1806-p110α^{H1047R}, MCF10A-HER2^{V659E} or HCC1806-HER2^{V659E} cells stably expressing
187 Aα1-WT or Aα1-CA were stained with crystal violet and representative micrographs were shown.
188 Scale bar=50 μm. **(E-F)** The sections of primary mammary tumors or lung metastasized tumors
189 derived from the same MMTV-PyMT female FVB mouse (n=5, 95 days old) were subjected to IHC
190 staining for protein expression of AMPKα1 (E) or E-cadherin (F). Average optical density (AOD) was
191 presented. Scale bar=50 μm.



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193 **Fig. S5. Expression of AMPKα1, p63 and E-cadherin in human breast cancer.** The Oncomine
194 Curtis breast dataset was analyzed for mRNA levels of AMPKα1, p63 or E-cadherin in human
195 normal breast or breast carcinoma.

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197 **Table S1. Primers were used in this study.**

Target	Application	Sequence
GFP	shRNA	GAAGCAGCACGACTTCTTC
P63	shRNA-1	GAGTGGAATGACTTCAACTTT
P63	shRNA-2	CCGTTTCGTCAGAACACACAT
AMPKα1	shRNA-1	GTATGATGTCAGATGGTGAATT
AMPKα1	shRNA-2	GTTGCCTACCATCTCATAATA
Twist1	shRNA-1	GCTGAGCAAGATTCAGACC
Twist1	shRNA-2	GTACATCGACTTCCTCTAC
Twist1 F	Q-PCR	TGTCCGCGTCCCACTAGC
Twist1 R	Q-PCR	TGTCCATTTTCTCCTTCTCTGGA
Slug F	Q-PCR	CTGGGCTGGCCAAACATAAG
Slug R	Q-PCR	CCTTGTCACAGTATTTACAGCTGAAAG
AMPKα1 F	Q-PCR	TGCGTGTACGAAGGAAGAATCC
AMPKα1 R	Q-PCR	TGTGACTTCCAGGTCTTGGAGTT
E-cadherin F	Q-PCR	GGATGTGCTGGATGTGAATG
E-cadherin R	Q-PCR	CACATCAGACAGGATCAGCAGAA
GAPDH F	Q-PCR	GGGGAGCCAAAAGGGTCATCATCT
GAPDH R	Q-PCR	GAGGGGCCATCCACAGTCTTCT
P1 F	CHIP	GTACTGGTGATTCTCCTGGCAG
P1 R	CHIP	GGCGCTGCGGGAGGGGGCGGA
P2 F	CHIP	CAGATGTGCATCACTCAAGCGG

P2 R	CHIP	CCGCCTCCTCGCCCCGCATCCT
P3 F	CHIP	AGGATCACCTCCAGAAGAAAACAC
P3 R	CHIP	ATCTTTCCTTCTCGGATGGAATC
P4 F	CHIP	TTGCCCTTCAAACAATGTGCAGTCCTC
P4 R	CHIP	TCTGAAATCAAAGTAACATGC
ITGA6 F	CHIP	GCAAAAAGAAACACCTACCTCATAGGAC
ITGA6 R	CHIP	GGGACTACAGCTGTGTAGACTGTTCTG
LAMC2 F	CHIP	GGTTACTTTATGAGTTGCTAACCCTGGTG
LAMC2 R	CHIP	CCCAATCTTAAGAGCGCTAACTCAGAAA

SI References

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