Inactivation of Capicua drives cancer metastasis

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The supplemental data includes 15 figures and legends, primer sequences, and four supplemental tables. Supplemental table 1 is provided here, while supplemental tables 2, 3a, 3b, and 4 are provided as separate excel files.

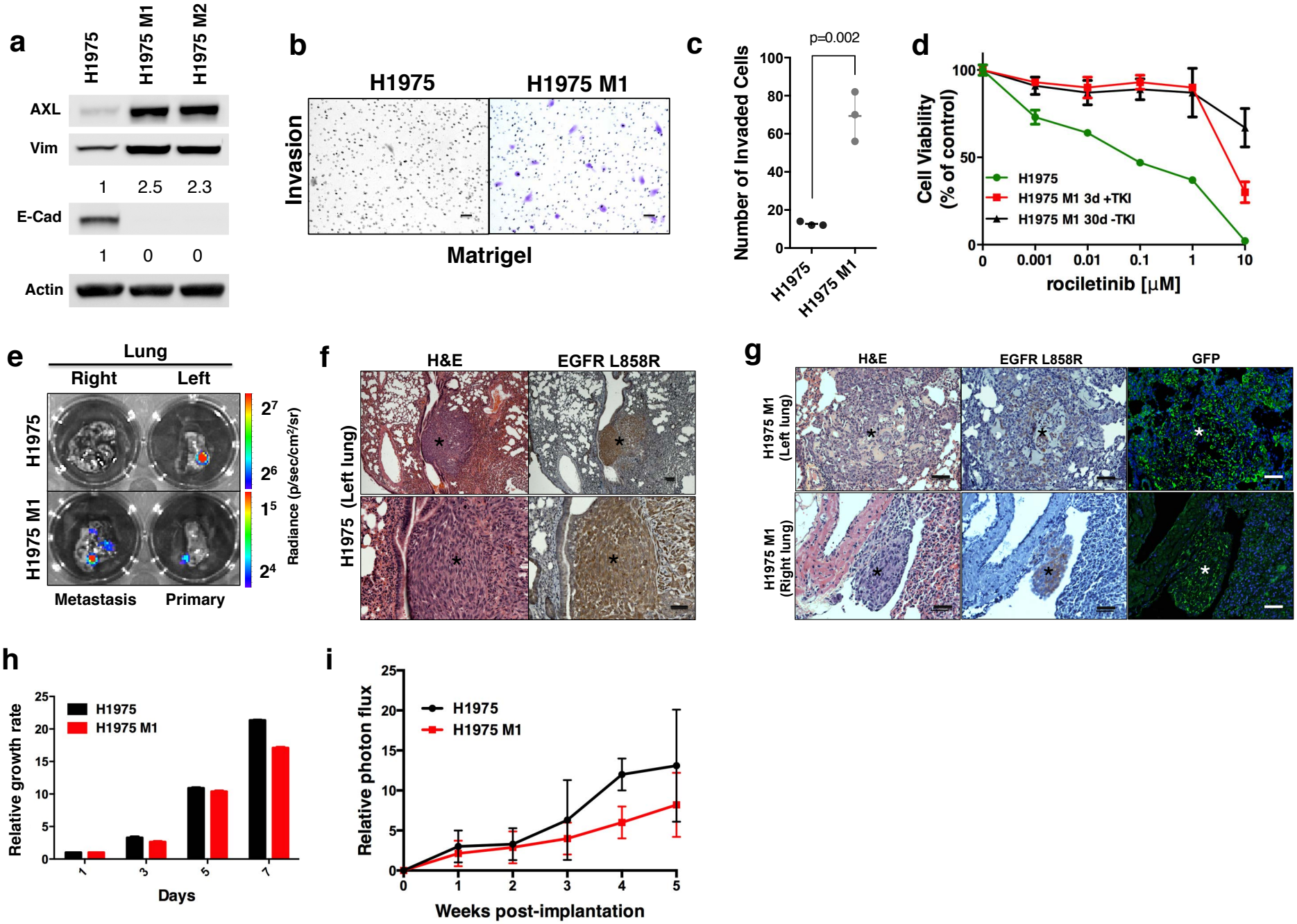

a
H1975
H1975 M1
H1975 M2
AXL
Vim
1
2.5
2.3
E-Cad
1
0
0
Actin
b
H1975
H1975 M1
Invasion
Matrigel
c
p=0.002
Number of Invaded Cells
100
80
60
40
20
0
H1975
H1975 M1
d
Cell Viability (% of control)
100
50
0
H1975
H1975 M1 3d +TKI
H1975 M1 30d -TKI
0
0.001
0.01
0.1
1
10
rociletinib [μM]
e
Lung
Right
Left
H1975
H1975 M1
2^7
2^6
1^5
2^4
Radiance (p/sec/cm²/sr)
Metastasis
Primary
f
H&E
EGFR L858R
H1975 (Left lung)
g
H&E
EGFR L858R
GFP
H1975 M1 (Left lung)
H1975 M1 (Right lung)
h
Relative growth rate
25
20
15
10
5
0
H1975
H1975 M1
1
3
5
7
Days
i
Relative photon flux
25
20
15
10
5
0
H1975
H1975 M1
0
1
2
3
4
5
Weeks post-implantation

Figure S1. Development of an *in vivo* orthotopic lung cancer metastasis model. (a) Immunoblots for epithelial (E-Cadherin) and mesenchymal (Vimentin and AXL) markers in H1975, H1975 M1 and M2. (b) Invasion of H1975 and H1975 M1 cells. Scale bars, 100 μm. (c) Number of cells invaded through Matrigel. Results of two independent experiments in triplicate are shown. p-value, Student's t-test; error bars represent SEM. (d) Cell viability of H1975, H1975 M1 continuously treated with rociletinib, and H1975 M1 grown in the absence of rociletinib for 30 days then treated with rociletinib for 3 days prior to CTG assay. Performed in triplicate. Error bars represent SEM. (e) Representative *ex vivo* BLI of the right and left lung of H1975 and H1975 M1 mice. (f) H&E and EGFR L858R IHC staining of an orthotopically implanted primary (left) lung tumor from a mouse bearing H1975 cells. Scale bars, 100 μm (top panels) and 50 μm (bottom panels). *Tumor. (g) H&E, EGFR L858R, and GFP staining of an orthotopically implanted primary lung tumor (left lung) and metastasis (right lung) from a mouse bearing H1975 M1 cells. Scale bar,50 μm. *Tumor**.** (h) *In vitro* growth rate comparing H1975 and H1975 M1 cells by CTG assay, relative to day 1. Performed in triplicate. Error bars represent SEM. (i) Normalized mean photon flux over 5 weeks in mice bearing H1975 or H1975 M1 cells. Error bars represent SEM.

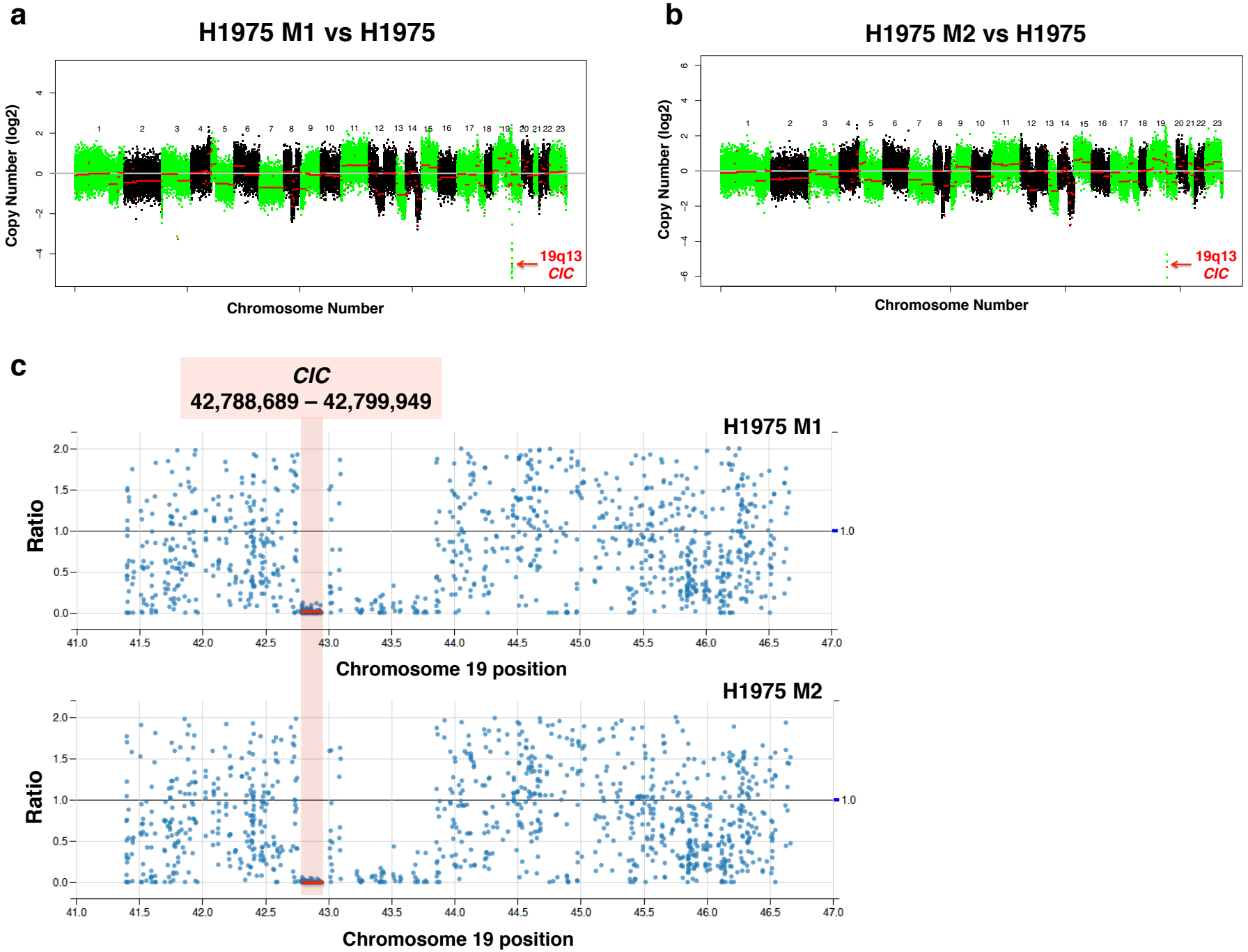

a
H1975 M1 vs H1975
Copy Number (log2)
Chromosome Number
19q13
CIC
b
H1975 M2 vs H1975
Copy Number (log2)
Chromosome Number
19q13
CIC
c
CIC
42,788,689 – 42,799,949
H1975 M1
Ratio
Chromosome 19 position
H1975 M2
Ratio
Chromosome 19 position

Figure S2. Copy number analysis identifies a focal deletion in hypermetastatic lung cancer cells. (a-b) Whole exome CNV profile comparing H1975 M1 (a) and H1975 M2 (b) to parental H1975 cells. (c) Predicted copy number alterations from exome sequencing analysis at the CIC locus (Chromosome 19 position 42,772,689 – 42,799,949 in human GRCH37/hg19) in H1975 M1 and H1975 M2 using Control-FREEC ratios and the Shiny application. The CN ratio (y-axis) between tumor DNA (H1975 M1 or H1975 M2 cells) and normal germline DNA at the genomic region containing CIC (highlighted in the pink) is depicted. Mean CN ratio demarcated with red horizontal line. Chromosome 19 coordinates are indicated on the x-axis.

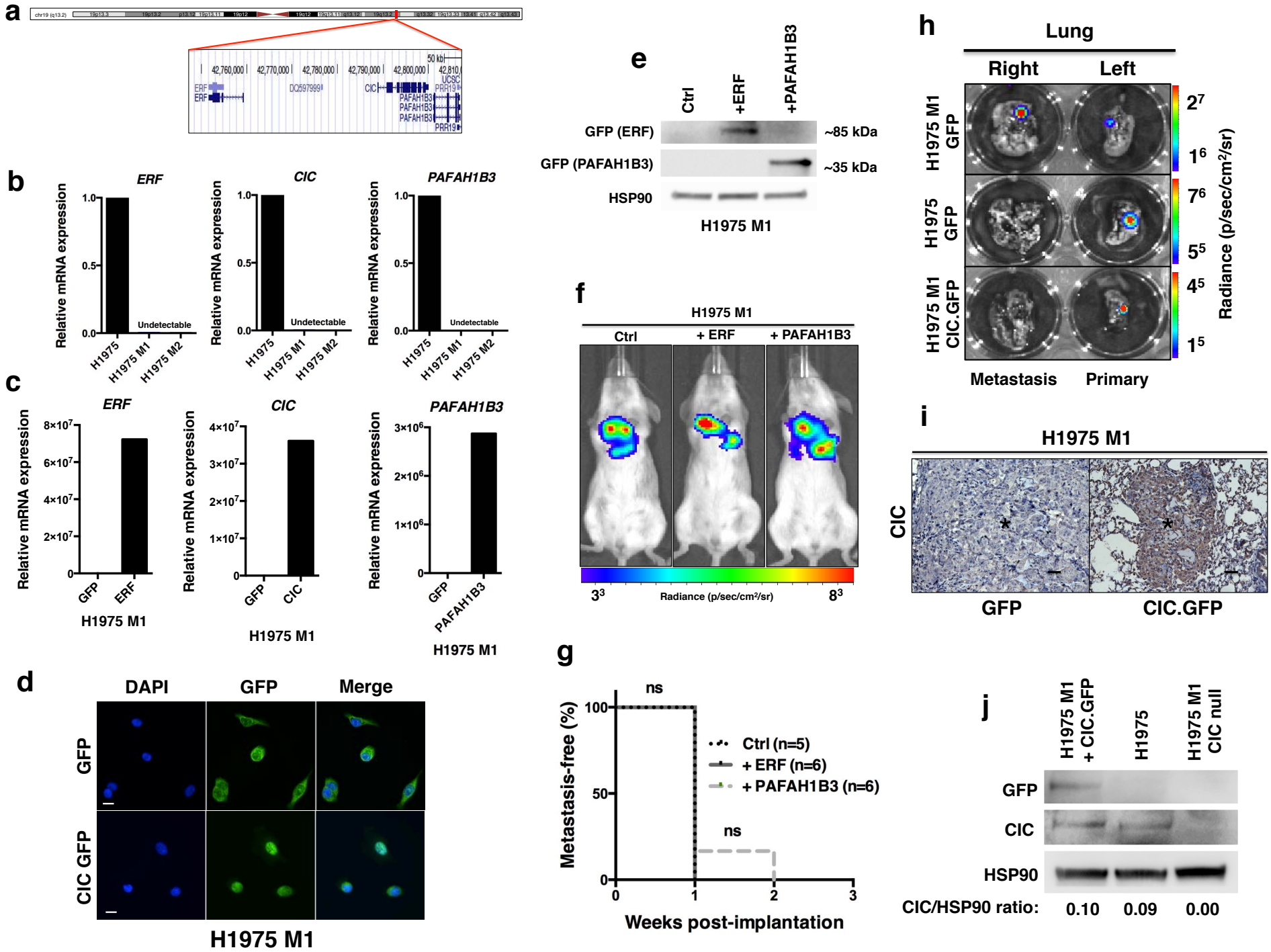
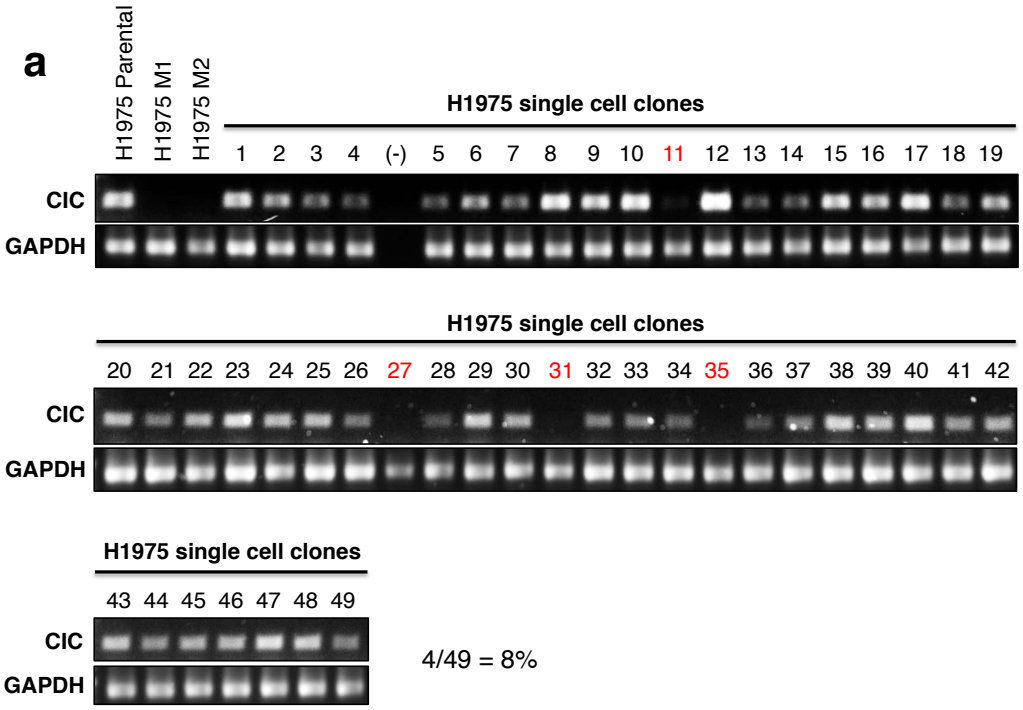
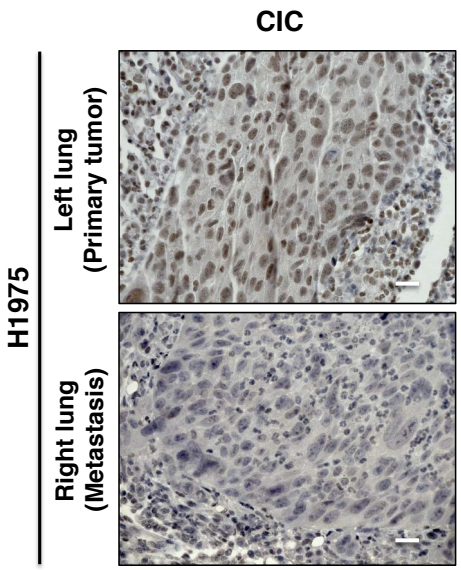

a
chr19 (q13.2)
50 kb
42,760,000
42,770,000
42,780,000
42,790,000
42,800,000
42,810,000
UCSC
ERF
ERF
DQ597999
CIC
PRR19
PAFAH1B3
PAFAH1B3
PAFAH1B3
PRR19
b
ERF
CIC
PAFAH1B3
Relative mRNA expression
1.0
0.5
0.0
Undetectable
H1975
H1975 M1
H1975 M2
c
ERF
CIC
PAFAH1B3
Relative mRNA expression
8×10^7
6×10^7
4×10^7
2×10^7
0
4×10^7
3×10^7
2×10^7
1×10^7
3×10^6
2×10^6
1×10^6
GFP
ERF
CIC
PAFAH1B3
H1975 M1
d
DAPI
GFP
Merge
GFP
CIC GFP
H1975 M1
e
Ctrl
+ERF
+PAFAH1B3
GFP (ERF)
GFP (PAFAH1B3)
HSP90
~85 kDa
~35 kDa
H1975 M1
f
H1975 M1
Ctrl
+ ERF
+ PAFAH1B3
3^3
Radiance (p/sec/cm²/sr)
8^3
g
Metastasis-free (%)
100
50
0
ns
ns
Ctrl (n=5)
+ ERF (n=6)
+ PAFAH1B3 (n=6)
0
1
2
3
Weeks post-implantation
h
Lung
Right
Left
H1975 M1 GFP
H1975 GFP
H1975 M1 CIC.GFP
2^7
1^6
7^6
5^5
4^5
1^5
Radiance (p/sec/cm²/sr)
Metastasis
Primary
i
H1975 M1
CIC
GFP
CIC.GFP
j
H1975 M1 + CIC.GFP
H1975
H1975 M1 CIC null
GFP
CIC
HSP90
CIC/HSP90 ratio:
0.10
0.09
0.00

Figure S3. In vivo orthotopic model identifies CIC as a metastasis suppressor. (a) Region of genomic loss on chromosome 19. (b) Relative ERF, CIC, PAFAH1B3 mRNA expression from RNAseq analysis between H1975 and H1975 M1 or M2. (c) Relative ERF, CIC, PAFAH1B3 mRNA expression by qRT-PCR in H1975 and H1975 M1 cells following genetic reconstitution. Results of two independent experiments in triplicate are shown. Error bars represent SEM. (d) H1975 M1 cells expressing either GFP control or GFP-tagged CIC. DAPI = blue, GFP = green. Experiments were performed in triplicate with a total of 9 high-power fields (HPFs) imaged from the GFP (control) and 9 HPFs from the CIC.GFP groups. All cells expressing GFP-tagged CIC demonstrated nuclear GFP staining. Scale bars, 20 μm. (e) Immunoblot of H1975 M1 cells expressing either luciferase control, GFP-tagged ERF, or GFP-tagged PAFAH1B3. (f) BLI images of mice bearing H1975 M1 cells expressing either luciferase control, GFP-tagged ERF, or GFP-tagged PAFAH1B3. (g) Metastasis-free survival of luciferase control (n=5), GFP-tagged ERF (n=6), or GFP-tagged PAFAH1B3 (n=6) expressing H1975 M1 mice in (f). Representative ex vivo BLI of the right and left lungs of H1975 M1 GFP-Luc, H1975 GFP-Luc, or H1975 M1 cells expressing GFP-tagged CIC. (i) CIC staining of orthotopically implanted tumors from H1975 M1 mice expressing either GFP-Luc or GFP-tagged CIC. Scale bars, 50 μm. (j) Immunoblot of H1975 M1 cells with reconstituted CIC.GFP compared to H1975 endogenous CIC and H1975 M1 (CIC null). Representative of two independent experiments.


a
H1975 Parental
H1975 M1
H1975 M2
H1975 single cell clones
1 2 3 4 (-) 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
CIC
GAPDH
H1975 single cell clones
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42
CIC
GAPDH
H1975 single cell clones
43 44 45 46 47 48 49
CIC
GAPDH
4/49 = 8%


b
CIC
H1975
Left lung
(Primary tumor)
Right lung
(Metastasis)

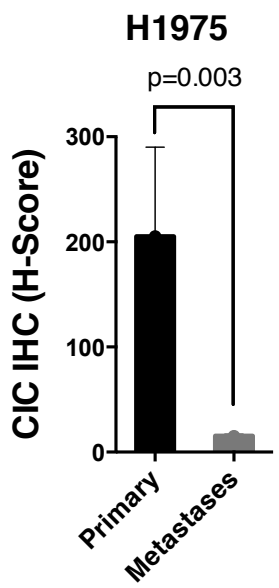
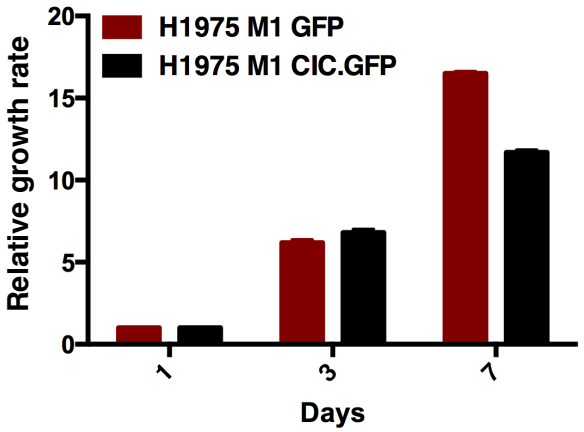
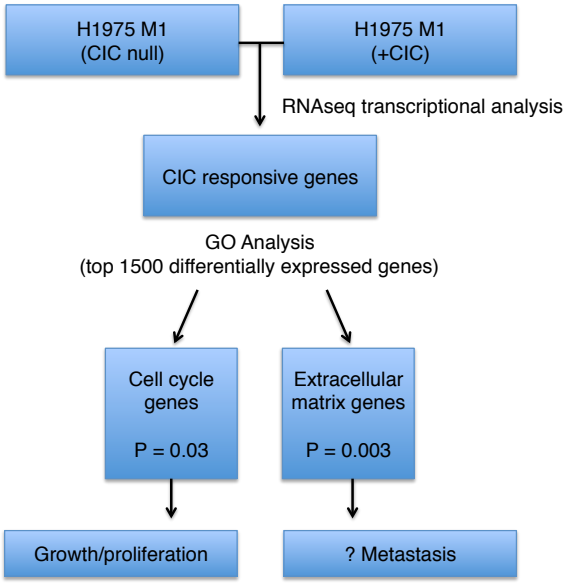

c
H1975
p=0.003
CIC IHC (H-Score)
300
200
100
0
Primary
Metastases

Figure S4. Decreased CIC expression correlates with metastasis. (a) Genomic PCR amplification (exon four and flanking introns) of *CIC* in H1975, H1975 M1, H1975 M2, and 49 H1975 single cell derived subclones. (b) Representative IHC images of the primary tumor and paired metastasis from a H1975 mouse that developed metastasis. Scale bar, 20 μm. (c) Nuclear CIC IHC scoring of primary tumors and metastasis derived from the H1975 mice who developed metastasis (n=3 total mice). p-value, student's t-test. Error bars represent SEM.

a



b



c

Downregulated CIC responsive cell cycle genes

GENE	FOLD CHANGE	ADJUSTED P-VALUE
CCNB2	0.71	1.31E-31
CCNA1	0.61	3.96E-28
CCNG2	0.66	1.09E-26
CCNB1	0.77	2.19E-18
CCNI	0.79	2.30E-17
CCNG1	0.70	4.91E-15
CDC25C	0.70	5.64E-22
CDCA2	0.75	3.03E-21
CDC40	0.81	7.31E-12
ANAPC15	0.64	5.41E-40
ANAPC16	0.75	8.73E-22

Figure S5. A subset of CIC responsive genes regulates the cell cycle in lung cancer cells. (a) In vitro growth rate comparing H1975 M1 GFP and H1975 M1 CIC.GFP cells by CTG assay, relative to day 1. Performed in triplicate. Error bars represent SEM. (b) Algorithm to identify and functionally cluster CIC responsive genes. (c) Downregulated CIC responsive cell cycle genes.

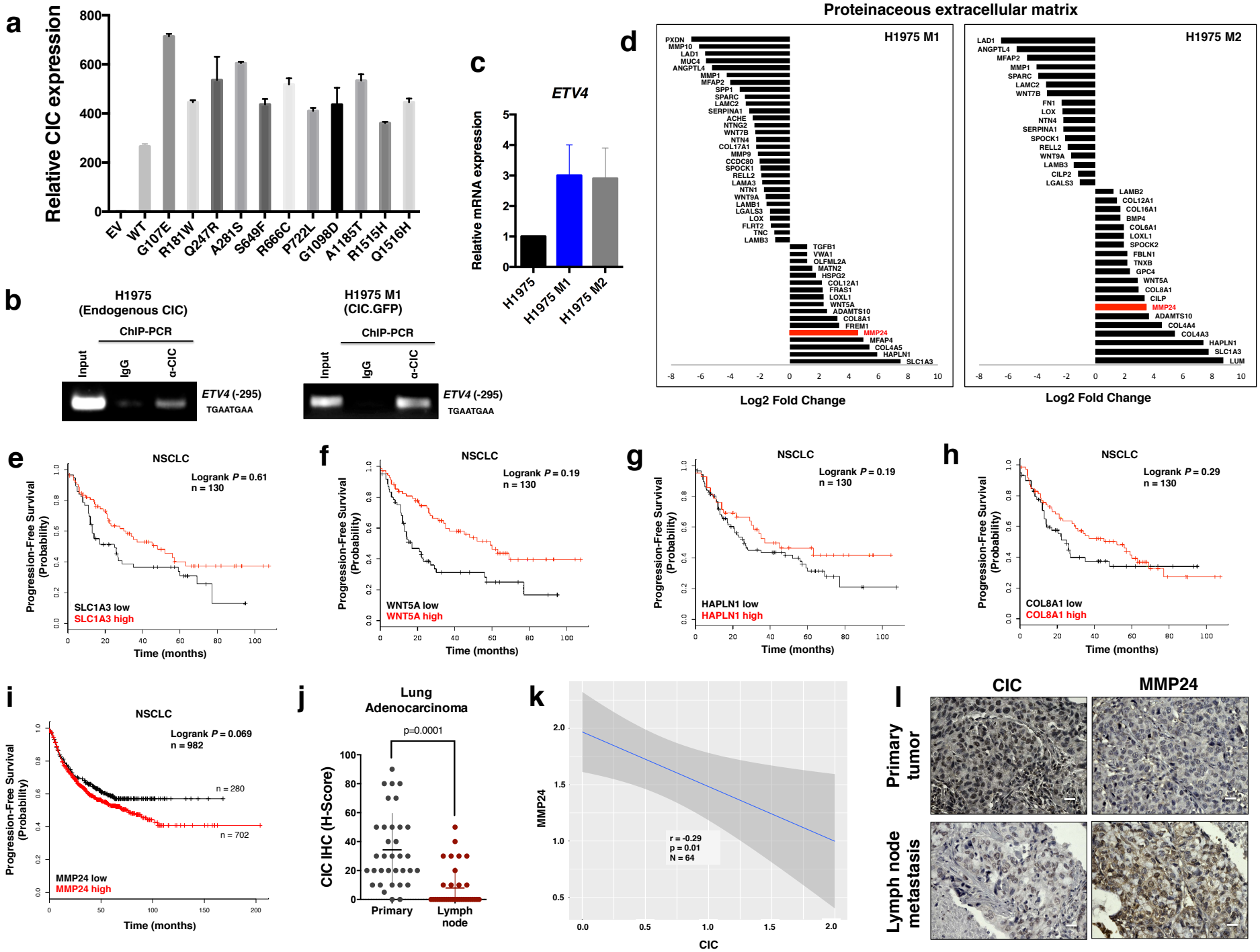

a
Relative CIC expression
800
600
400
200
0
EV
WT
G107E
R181W
Q247R
A281S
S649F
R666C
P722L
G1098D
A1185T
R1515H
Q1516H
b
H1975
(Endogenous CIC)
ChIP-PCR
Input
IgG
α-CIC
ETV4 (-295)
TGAATGAA
H1975 M1
(CIC.GFP)
ChIP-PCR
Input
IgG
α-CIC
ETV4 (-295)
TGAATGAA
c
ETV4
Relative mRNA expression
5
4
3
2
1
0
H1975
H1975 M1
H1975 M2
d
Proteinaceous extracellular matrix
H1975 M1
PXDN
MMP10
LAD1
MUC4
ANGPTL4
MMP1
MFAP2
SPP1
SPARC
LAMC2
SERPINA1
ACHE
NTNG2
WNT7B
NTN4
COL17A1
MMP9
CCDC80
SPOCK1
RELL2
LAMA3
NTN1
WNT9A
LAMB1
LGALS3
LOX
FLRT2
TNC
LAMB3
TGFB1
VWA1
OLFML2A
MATN2
HSPG2
COL12A1
FRAS1
LOXL1
WNT5A
ADAMTS10
COL8A1
FREM1
MMP24
MFAP4
COL4A5
HAPLN1
SLC1A3
-8 -6 -4 -2 0 2 4 6 8 10
Log2 Fold Change
H1975 M2
LAD1
ANGPTL4
MFAP2
MMP1
SPARC
LAMC2
WNT7B
FN1
LOX
NTN4
SERPINA1
SPOCK1
RELL2
WNT9A
LAMB3
CILP2
LGALS3
LAMB2
COL12A1
COL16A1
BMP4
COL6A1
LOXL1
SPOCK2
FBLN1
TNXB
GPC4
WNT5A
COL8A1
CILP
MMP24
ADAMTS10
COL4A4
COL4A3
HAPLN1
SLC1A3
LUM
-8 -6 -4 -2 0 2 4 6 8 10
Log2 Fold Change
e
NSCLC
Logrank P = 0.61
n = 130
Progression-Free Survival (Probability)
SLC1A3 low
SLC1A3 high
Time (months)
f
NSCLC
Logrank P = 0.19
n = 130
Progression-Free Survival (Probability)
WNT5A low
WNT5A high
Time (months)
g
NSCLC
Logrank P = 0.19
n = 130
Progression-Free Survival (Probability)
HAPLN1 low
HAPLN1 high
Time (months)
h
NSCLC
Logrank P = 0.29
n = 130
Progression-Free Survival (Probability)
COL8A1 low
COL8A1 high
Time (months)
i
NSCLC
Logrank P = 0.069
n = 982
Progression-Free Survival (Probability)
n = 280
n = 702
MMP24 low
MMP24 high
Time (months)
j
Lung
Adenocarcinoma
p=0.0001
CIC IHC (H-Score)
Primary
Lymph node
k
MMP24
r = -0.29
p = 0.01
N = 64
CIC
l
CIC
MMP24
Primary tumor
Lymph node metastasis

Figure S6. Identification of MMP24 as a metastasis effector downstream of CIC.

(a) Relative CIC protein expression from WT and CIC mutants. Obtained from two independent experiments. Error bars, SEM. (b) ChIP-PCR in H1975 and H1975 M1 CIC.GFP expressing cells. Representative of two independent experiments. (c) Relative ETV4 mRNA expression in H1975, H1975 M1, and H1975 M2 cells. Representative of two independent experiments and performed in triplicate. Error bars, SEM. (d) Differential expression of Proteinaceous ECM genes between H1975 versus H1975 M1 or H1975 M2. (e-h) PFS for patients with LN positive (N1) NSCLC with either high or low *SLC1A3* mRNA expression. n=130; p=0.61. Probe 202800_at. (e), high or low *WNT5A* mRNA expression. n=130; p=0.19. Probe 213425_at. (f), high or low *HAPLN1* mRNA expression. n=130; p=0.19. Probe 205523_at. (g), high or low *COL8A1* mRNA expression. n=130; p=0.29. Probe 214587_at. (h). (i) PFS for NSCLC patients with either *MMP24* high or *MMP24* low mRNA expression. n=982, p=0.069. Probe 213171_s_at. (j) CIC score of matched primary lung tumors and LN metastases. Mean +/- SEM, 33 +/- 4.3 (normal, n=32) and 7.1 +/- 2.3 (tumor, n=32). p value, Student's t-test. (k) Correlation between CIC and MMP24 protein expression (r = -0.29, p=0.01, n=64). (l) MMP24 and CIC staining of primary and LN metastasis. Scale bar, 20 μm.

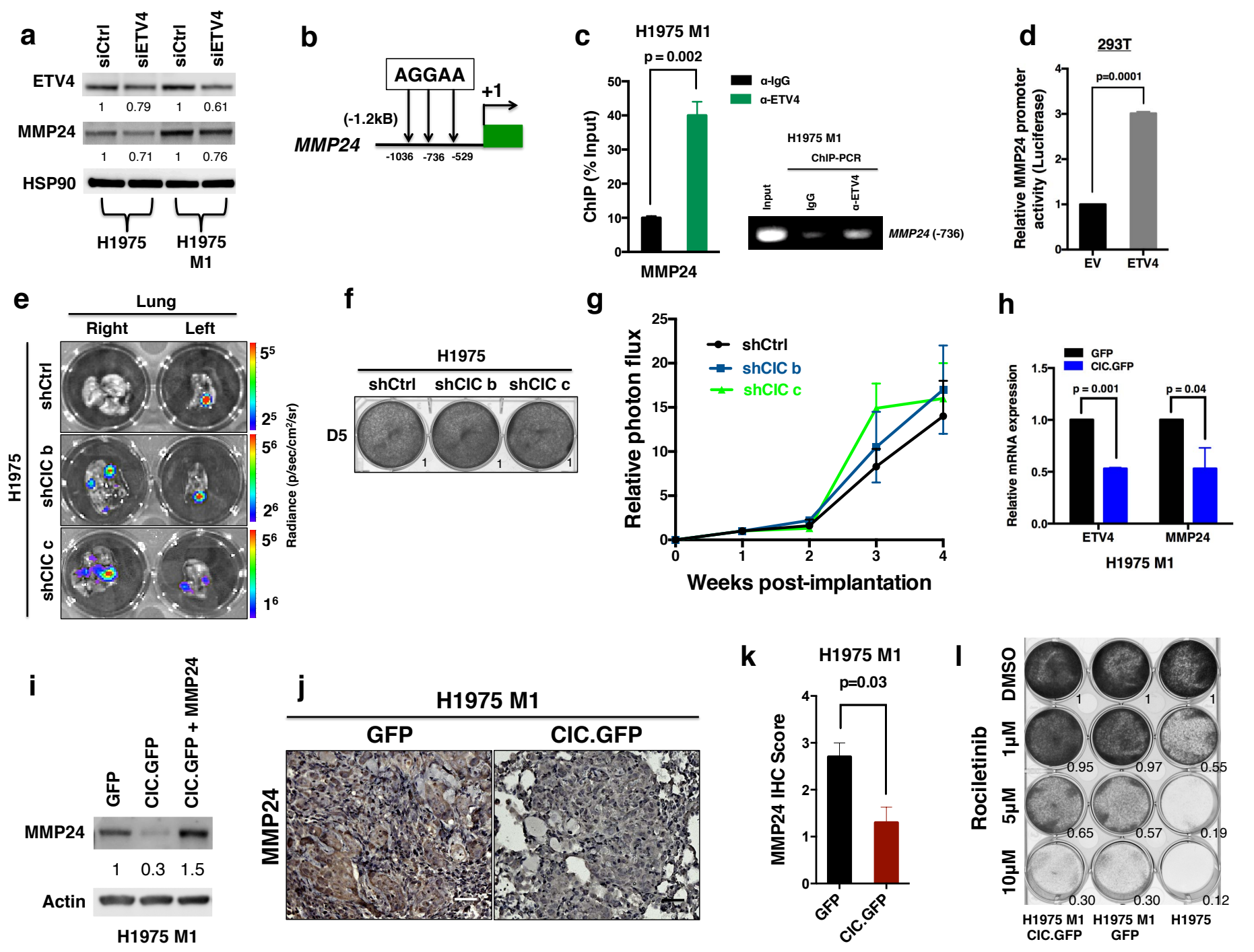

a
siCtrl
siETV4
siCtrl
siETV4
ETV4
1
0.79
1
0.61
MMP24
1
0.71
1
0.76
HSP90
H1975
H1975 M1
b
AGGAA
+1
(-1.2kB)
MMP24
-1036
-736
-529
c
H1975 M1
p = 0.002
α-IgG
α-ETV4
ChIP (% Input)
MMP24
H1975 M1
ChIP-PCR
Input
IgG
α-ETV4
MMP24 (-736)
d
293T
p=0.0001
Relative MMP24 promoter activity (Luciferase)
EV
ETV4
e
Lung
Right
Left
H1975
shCtrl
shCIC b
shCIC c
Radiance (p/sec/cm²/sr)
f
H1975
shCtrl
shCIC b
shCIC c
D5
g
shCtrl
shCIC b
shCIC c
Relative photon flux
Weeks post-implantation
h
GFP
CIC.GFP
p = 0.001
p = 0.04
Relative mRNA expression
ETV4
MMP24
H1975 M1
i
GFP
CIC.GFP
CIC.GFP + MMP24
MMP24
1
0.3
1.5
Actin
H1975 M1
j
H1975 M1
GFP
CIC.GFP
MMP24
k
H1975 M1
p=0.03
MMP24 IHC Score
GFP
CIC.GFP
l
Rociletinib
DMSO
1μM
5μM
10μM
1
1
1
0.95
0.97
0.55
0.65
0.57
0.19
0.30
0.30
0.12
H1975 M1 CIC.GFP
H1975 M1 GFP
H1975

Figure S7. MMP24 is a metastasis effector downstream of CIC.

(a) Immunoblot comparing ETV4 and MMP24 expression with siETV4 or control (siCtrl). (b) *MMP24* promoter region. (c) ChIP-PCR in H1975 M1 cells demonstrating *MMP24* promoter occupancy by ETV4. p-value, Student's t-test. Error bars, SEM. Representative of two independent experiments. (d) Relative MMP24 luciferase promoter activity with ETV4 overexpression compared to empty vector. p value, Student's t-test. Error bars represent SEM, n=5. (e) *Ex vivo* BLI of lungs from mice bearing H1975 cells expressing either shCtrl, shCIC b, or shCIC c. (f) CV assay comparing H1975 shCtrl, shCICb, or shCICc expressing cells. Representative of 2 independent experiments. (g) Relative photon flux comparing mice bearing H1975 cells expressing either shCtrl, shCIC b, or shCIC c. (h) *ETV4* and *MMP24* qRT-PCR in H1975 M1 cells expressing GFP or GFP-tagged CIC. Results of two independent experiments in triplicate are shown. p value, Student's t-test. Error bars represent SEM. (i) Immunoblot of H1975 M1 cells with GFP, CIC.GFP, or CIC.GFP +MMP24. Representative of two independent experiments. (j) MMP24 staining of orthotopic tumors from H1975 M1 mice expressing either GFP-Luc or GFP-tagged CIC. Scale bars, 50 μm. (k) Mean MMP24 IHC score from orthotopically implanted tumors bearing GFP control (n = 3) or CIC.GFP (n = 3). p-value, Student's t-test. Error bars, SEM. (l) CV assay of H1975, H1975 M1, or H1975 M1 cells expressing GFP-tagged CIC +/- rociletinib treatment. Representative of 2 independent experiments.

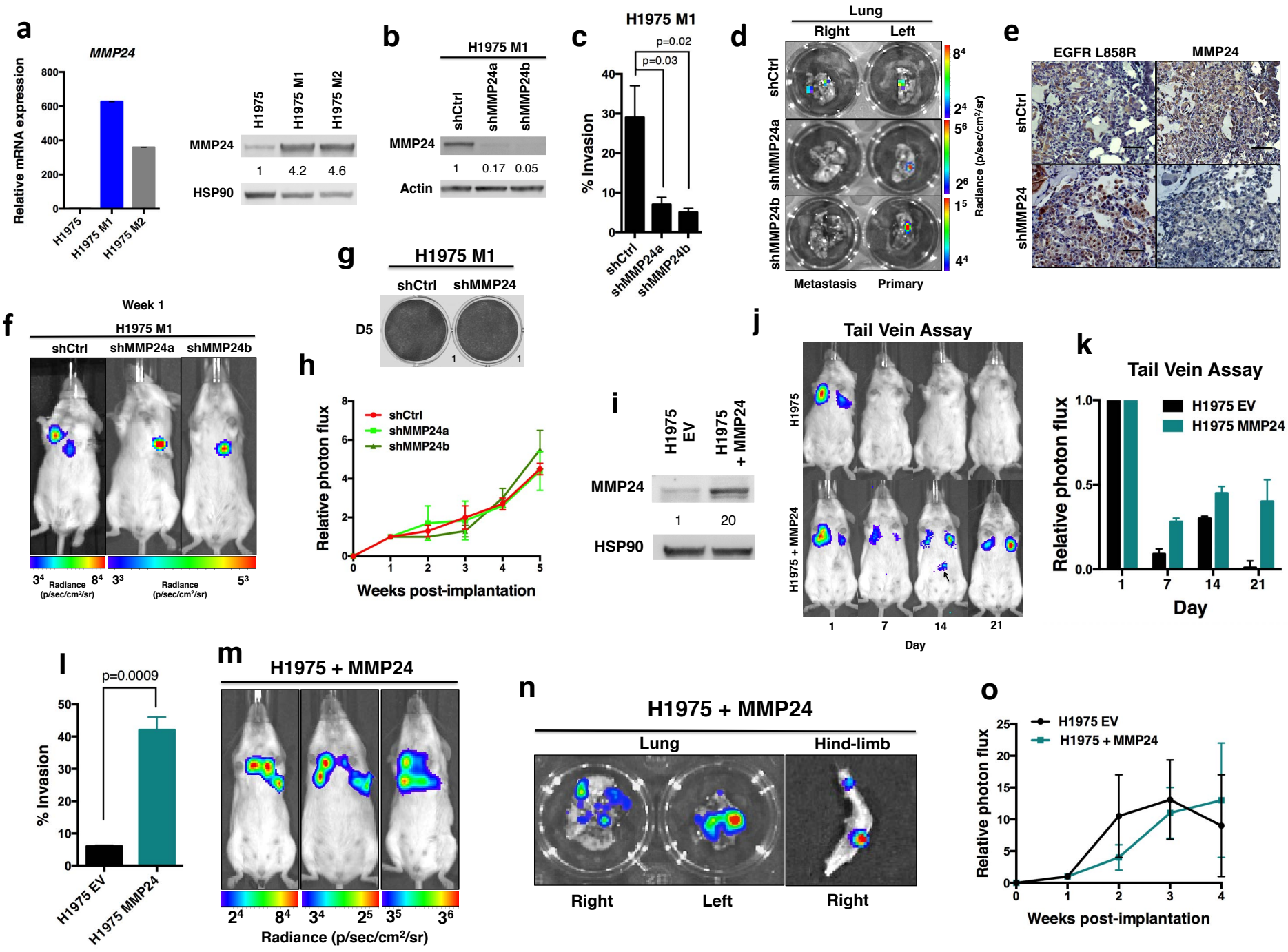
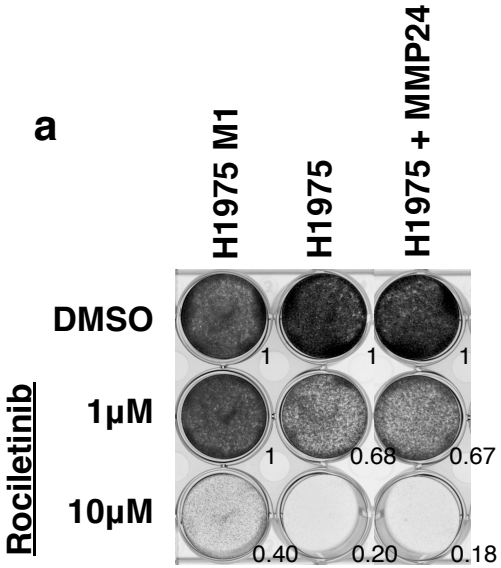
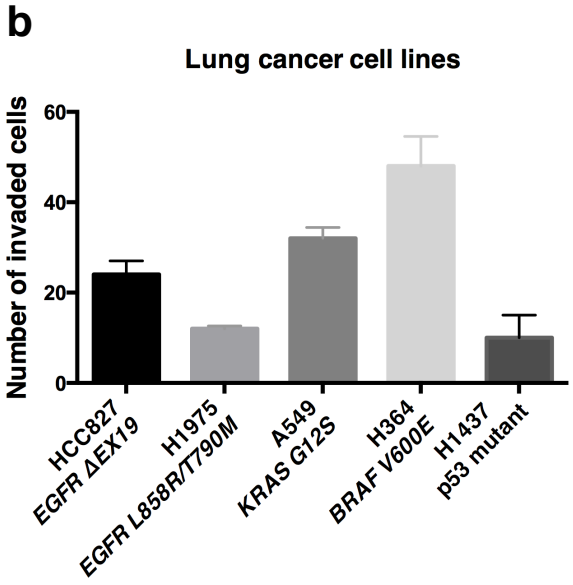

a
MMP24
Relative mRNA expression
800
600
400
200
0
H1975
H1975 M1
H1975 M2
H1975
H1975 M1
H1975 M2
MMP24
1
4.2
4.6
HSP90
b
H1975 M1
shCtrl
shMMP24a
shMMP24b
MMP24
1
0.17
0.05
Actin
c
H1975 M1
p=0.02
p=0.03
% Invasion
40
30
20
10
0
shCtrl
shMMP24a
shMMP24b
d
Lung
Right
Left
shCtrl
shMMP24a
shMMP24b
Radiance (p/sec/cm²/sr)
Metastasis
Primary
e
EGFR L858R
MMP24
shCtrl
shMMP24
f
Week 1
H1975 M1
shCtrl
shMMP24a
shMMP24b
Radiance (p/sec/cm²/sr)
Radiance (p/sec/cm²/sr)
g
H1975 M1
shCtrl
shMMP24
D5
1
1
h
Relative photon flux
shCtrl
shMMP24a
shMMP24b
Weeks post-implantation
i
H1975 EV
H1975 + MMP24
MMP24
1
20
HSP90
j
Tail Vein Assay
H1975
H1975 + MMP24
1
7
14
21
Day
k
Tail Vein Assay
Relative photon flux
H1975 EV
H1975 MMP24
Day
l
p=0.0009
% Invasion
H1975 EV
H1975 MMP24
m
H1975 + MMP24
Radiance (p/sec/cm²/sr)
n
H1975 + MMP24
Lung
Hind-limb
Right
Left
Right
o
Relative photon flux
H1975 EV
H1975 + MMP24
Weeks post-implantation

Figure S8. CIC effector, MMP24, is a multistep mediator of lung cancer metastasis. (a) MMP24 qRT-PCR and immunoblot from H1975, H1975 M1, and M2. qRT-PCR results obtained from two independent experiments in triplicate. Error bars, SEM. (b) Immunoblots for H1975 M1 shCtrl, shMMP24a, or shMMP24b. (c) H1975 M1 shCtrl, shMMP24a, or shMMP24b invasion. Results of two independent experiments in triplicate. p-value, Student's t-test. Error bars, SEM. (d) *Ex vivo* BLI of lungs from shCtrl, shMMP24a, or shMMP24b H1975 M1 GFP-Luc mice. (e) $EGFR^{L858R}$ and MMP24 staining of lung tumors from H1975 M1 shCtrl and shMMP24a. Scale bars, 50 μm. (f) BLI of shCtrl, shMMP24a, or shMMP24b H1975 M1 GFP-Luc mice one week post-implantation. (g) CV of shCtrl or shMMP24 H1975 M1 cells. Results representative of 2 independent experiments. (h) Normalized mean photon flux from H1975 M1 shCtrl, shMMP24a and shMMP24b mice over 5 weeks. Error bars, SEM. (i) MMP24 Immunoblot from H1975 +EV or +MMP24. (j) Tail vein assay comparing H1975 GFP-Luc (n=4) and H1975 GFP-luc +MMP24 (n=4) mice. Arrow (luciferin injection site). (k) Relative photon flux (normalized to day 1) of H1975 GFP-Luc (n=4) and H1975 GFP-Luc +MMP24 (n=4) cohorts. (l) Invasion of H1975 cells +EV or +MMP24. p-value, Student's t-test. Error bars, SEM. (m) BLI of mice bearing H1975 GFP-Luc +MMP24 cells. (n) *Ex vivo* BLI of right and left lungs and right hindlimb of a H1975 mouse overexpressing MMP24. (o) Normalized mean photon flux of H1975 EV and H1975 +MMP24 mice over 4 weeks. Error bars, SEM.


a
H1975 M1
H1975
H1975 + MMP24
DMSO
1μM
10μM
Rociletinib
1
1
1
1
0.68
0.67
0.40
0.20
0.18


b
Lung cancer cell lines
Number of invaded cells
60
40
20
0
HCC827
EGFR ΔEX19
H1975
EGFR L858R/T790M
A549
KRAS G12S
H364
BRAF V600E
H1437
p53 mutant

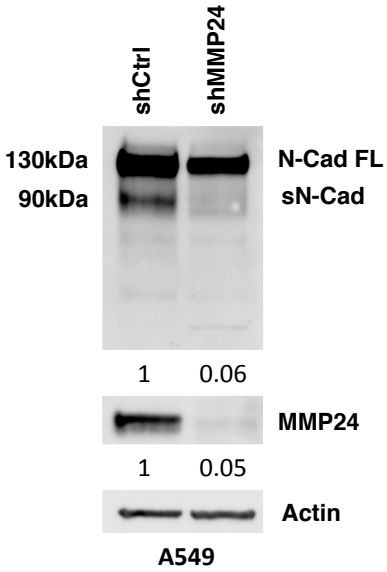

c
shCtrl
shMMP24
130kDa
90kDa
N-Cad FL
sN-Cad
1
0.06
MMP24
1
0.05
Actin
A549

Figure S9. MMP24 correlates with increased invasion in treatment naïve lung cancer cells. (a) Crystal violet assay of H1975 M1, H1975, and H1975 +MMP24 +/- rociletinib. Results representative of 2 independent experiments. (b) Bar graph depicting the number of cells invaded cells in a panel of lung cancer cell lines. Error bars, SEM. (c) Immunoblots for N-Cadherin in A549 MMP24 KD cells compared to shCtrl. FL = full length and S = soluble.

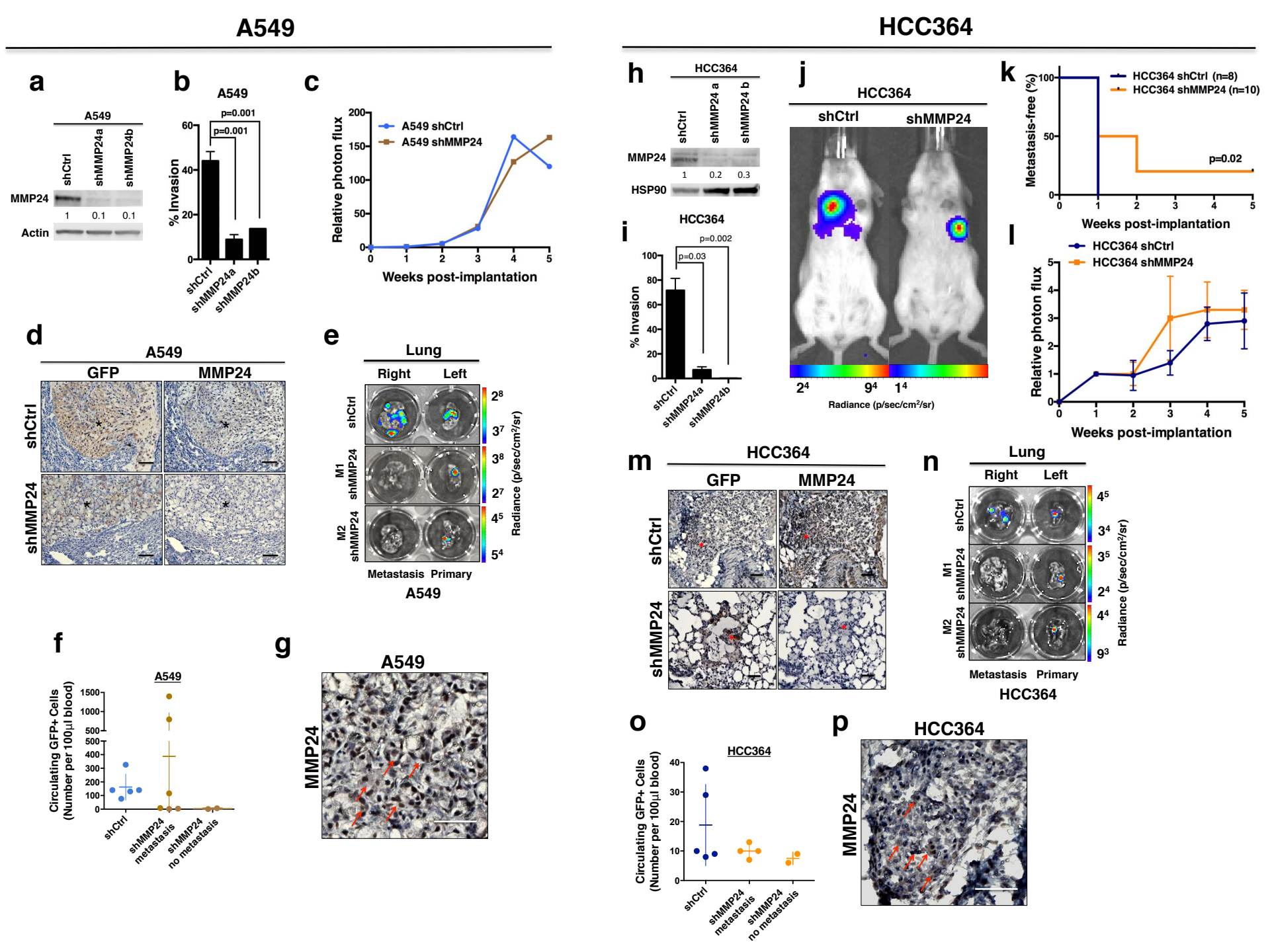
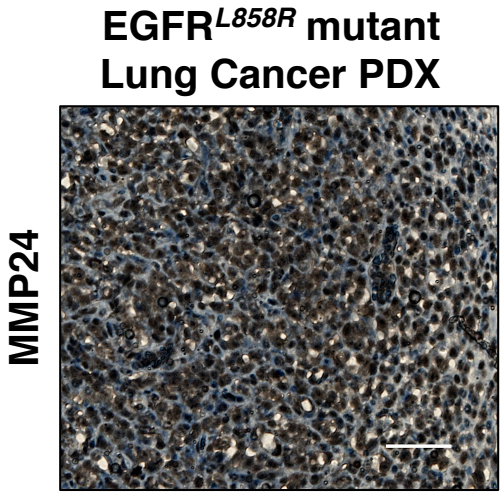
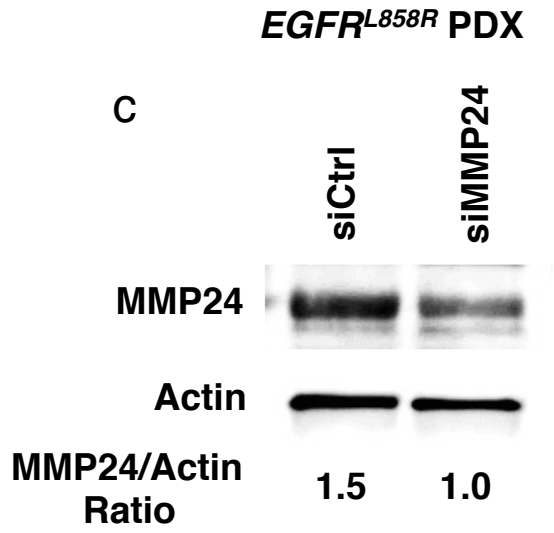

A549
HCC364
a
A549
shCtrl
shMMP24a
shMMP24b
MMP24
1
0.1
0.1
Actin
b
A549
p=0.001
p=0.001
% Invasion
shCtrl
shMMP24a
shMMP24b
c
A549 shCtrl
A549 shMMP24
Relative photon flux
Weeks post-implantation
d
A549
GFP
MMP24
shCtrl
shMMP24
e
Lung
Right
Left
shCtrl
M1 shMMP24
M2 shMMP24
Radiance (p/sec/cm²/sr)
Metastasis
Primary
A549
f
A549
Circulating GFP+ Cells (Number per 100µl blood)
shCtrl
shMMP24 metastasis
shMMP24 no metastasis
g
A549
MMP24
h
HCC364
shCtrl
shMMP24 a
shMMP24 b
MMP24
1
0.2
0.3
HSP90
i
HCC364
p=0.002
p=0.03
% Invasion
shCtrl
shMMP24a
shMMP24b
j
HCC364
shCtrl
shMMP24
Radiance (p/sec/cm²/sr)
k
HCC364 shCtrl (n=8)
HCC364 shMMP24 (n=10)
Metastasis-free (%)
p=0.02
Weeks post-implantation
l
HCC364 shCtrl
HCC364 shMMP24
Relative photon flux
Weeks post-implantation
m
HCC364
GFP
MMP24
shCtrl
shMMP24
n
Lung
Right
Left
shCtrl
M1 shMMP24
M2 shMMP24
Radiance (p/sec/cm²/sr)
Metastasis
Primary
HCC364
o
HCC364
Circulating GFP+ Cells (Number per 100µl blood)
shCtrl
shMMP24 metastasis
shMMP24 no metastasis
p
HCC364
MMP24

Figure S10. MMP24 promotes metastasis across molecular subsets. (a) Immunoblot for shCtrl, shMMP24a, and shMMP24b A549 cells. (b) A549 invasion with shCtrl, shMMP24a, or shMMP24b. Two independent experiments in triplicate. p-value, Student's t-test. Error bars, SEM. (c) Normalized mean photon flux of shCtrl and shMMP24a A549 mice over 5 weeks. Error bars, SEM. (d) MMP24 staining of A549 shCtrl and shMMP24 tumors. Scale bars, 50 μm. (e) *Ex vivo* BLI of lungs from A549 shCtrl and non-metastatic shMMP24a mice. (f) Circulating GFP+ cells/100 μl of blood at 5 weeks post-implantation. Mean +/- SEM, 162 +/- 42 (shCtrl, n=5), 387 +/- 238 (shMMP24 metastasis, n=6), 4 +/- 3 (shMMP24 no metastasis, n=2). (g) MMP24+ tumor cells (arrows) in a shMMP24 A549 mouse with metastasis. (h) Immunoblots for shCtrl, shMMP24a, and shMMP24b HCC364 cells. (i) HCC364 invasion with shCtrl, shMMP24a, or shMMP24b. Two independent experiments in triplicate. p-value, Student's t-test. Error bars, SEM. (j) BLI of shCtrl or shMMP24a HCC364 GFP-Luc mice. (k) Metastasis-free survival comparing HCC364 shCtrl and shMMP24a mice; p values, log-rank. (l) Normalized mean photon flux of HCC364 shCtrl and shMMP24a mice over 5 weeks. Error bars, SEM. (m) MMP24 staining of HCC364 shCtrl or shMMP24 tumors. Scale bars, 50 μm. (n) *Ex vivo* BLI of lungs from HCC364 shCtrl and non-metastatic shMMP24a mice (o) Circulating GFP+ cells/100 μl of blood at 5 weeks post-implantation. Mean +/- SEM, 19 +/- 6.0 (shCtrl, n=5), 10 +/- 1.2 (shMMP24 metastasis, n=4), 7.5 +/- 1.5 (shMMP24 no metastasis, n=2). Error bars, SEM. (p) MMP24+ tumor cells (arrows) in a shMMP24 HCC364 mouse with metastasis.

a



c



b

Score	Expect	Identities	Gaps	Strand	Frame
291 bits(157)	2e-82()	159/160(99%)	0/160(0%)	Plus/Plus	

Features:

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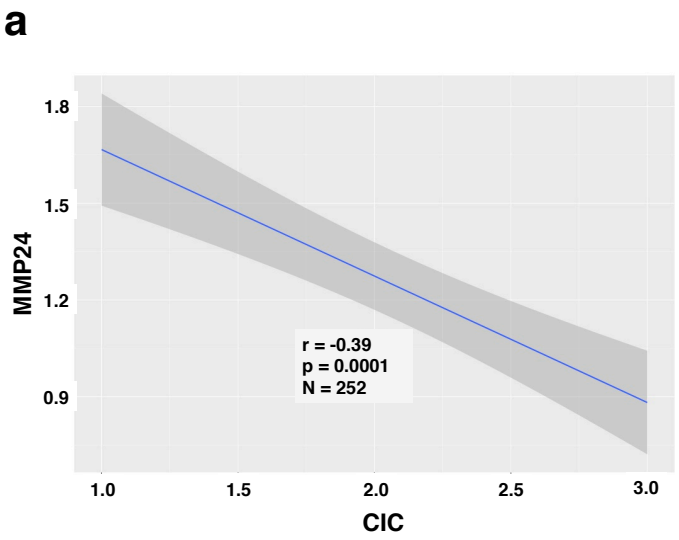
Query  2468  AGGGCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACG  2527
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  42    AGGGCATGAACTACTTGGAGGACCGTCGCTTGGTGCACCGCGACCTGGCAGCCAGGAACG  101

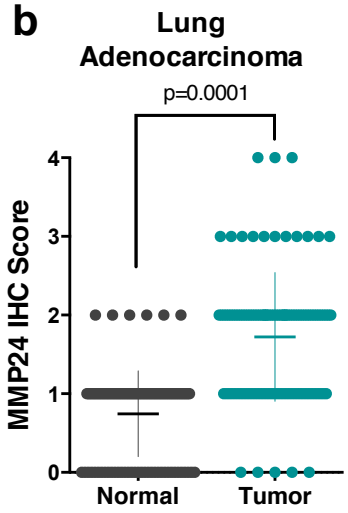
Query  2528  TACTGGTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCTGGCCAAACTGCTGG  2587  EGFR CDS
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102   TACTGGTGAAAACACCGCAGCATGTCAAGATCACAGATTTTGGGCGGGCCAAACTGCTGG  161   EGFR PDX

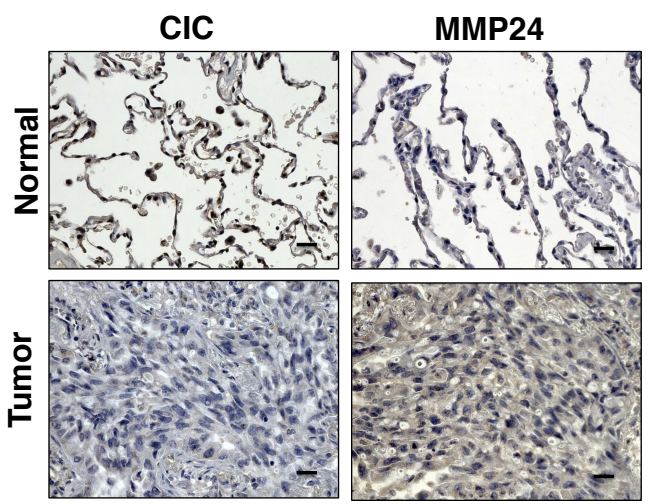
Query  2588  GTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGT  2627  2573 T>G_EGFR L858R
             ||||||||||||||||||||||||||||||||||||||||
Sbjct  162   GTGCGGAAGAGAAAGAATACCATGCAGAAGGAGGCAAAGT  201
```

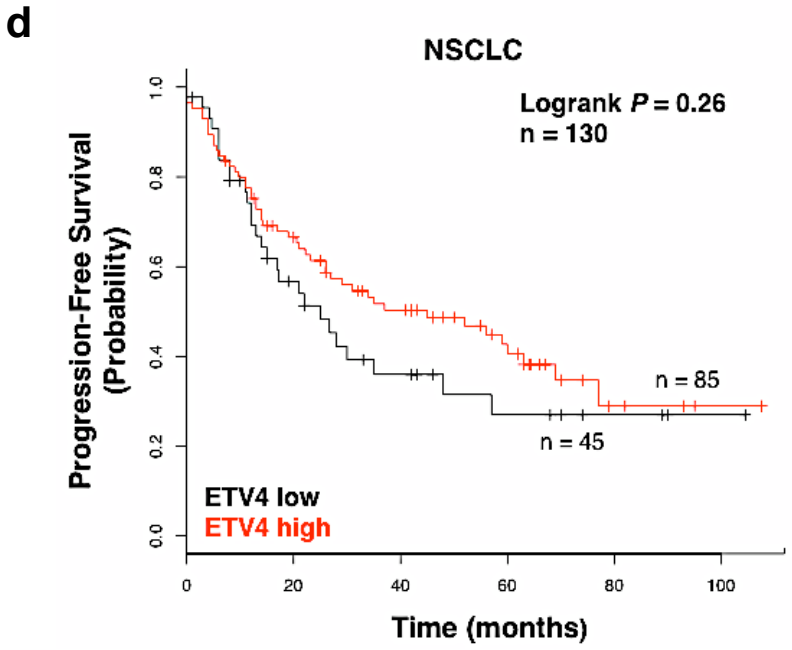
Figure S11. MMP24 is necessary for invasion in an EGFR^{L858R} PDX model.

(a) MMP24 IHC staining of an explanted EGFR^{L858R} PDX tumor. Scale bar, 50 μm. (b) EGFR exon 21 sequence alignment comparing tumor DNA derived from the EGFR^{L858R} PDX sphere culture to wildtype EGFR sequence. (c) Immunoblot comparing MMP24 knockdown to control in the EGFR^{L858R} PDX derived sphere culture cells.


a
MMP24
CIC
r = -0.39
p = 0.0001
N = 252
1.8
1.5
1.2
0.9
1.0
1.5
2.0
2.5
3.0


b
Lung Adenocarcinoma
p=0.0001
MMP24 IHC Score
Normal
Tumor
0
1
2
3
4


c
CIC
MMP24
Normal
Tumor


d
NSCLC
Logrank P = 0.26
n = 130
n = 85
n = 45
ETV4 low
ETV4 high
Progression-Free Survival (Probability)
Time (months)
0.0
0.2
0.4
0.6
0.8
1.0
0
20
40
60
80
100

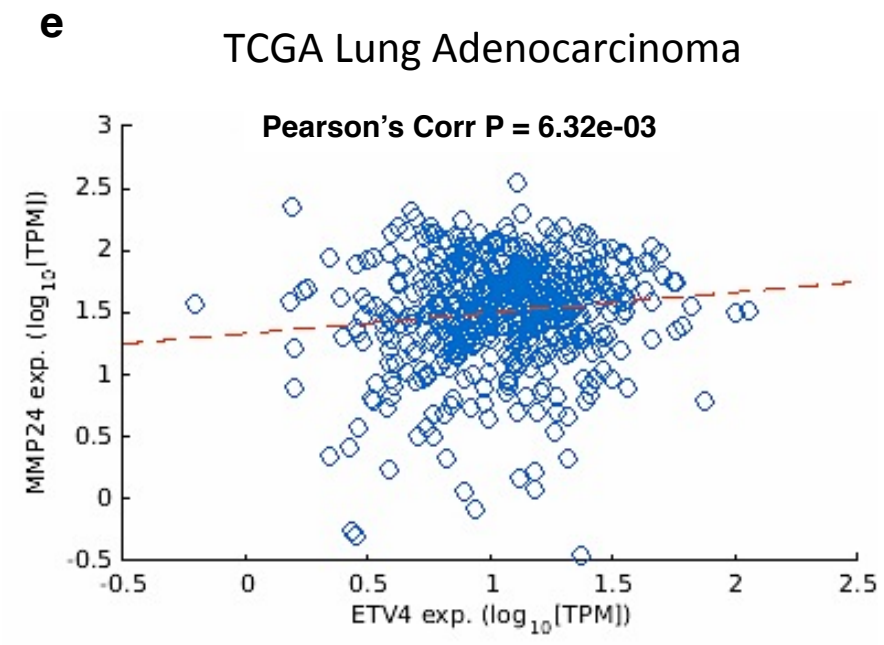

e
TCGA Lung Adenocarcinoma
Pearson's Corr P = 6.32e-03
MMP24 exp. (log10[TPM])
ETV4 exp. (log10[TPM])
-0.5
0
0.5
1
1.5
2
2.5
3

Figure S12. Decreased CIC expression in lung adenocarcinoma correlates with increased MMP24 expression. (a) Correlation plot between CIC and MMP24 protein expression from lung adenocarcinoma tumors and adjacent normal tissue (r = -0.39, p=0.0001, n=252). (b) MMP24 staining in lung adenocarcinoma tumor specimens (n =130) compared to normal adjacent tissue (n = 126). Mean +/- SEM, 0.75 +/- 0.05 (normal, n=126) and 1.73 +/- 0.08 (tumor, n=130). p-value, Student's t-test. (c) Representative images of MMP24 and CIC stained tissue sections of lung adenocarcinoma tissue and normal adjacent tissue. Scale bar, 20 μm. (d) PFS for NSCLC patients with AJCC N1 disease with either *ETV4* high or *ETV4* low mRNA expression. n=130, p=0.26. Probe 211603_s_at. (e) Correlation plot between ETV4 and MMP24 mRNA expression from the lung adenocarcinoma TCGA dataset (n=275).

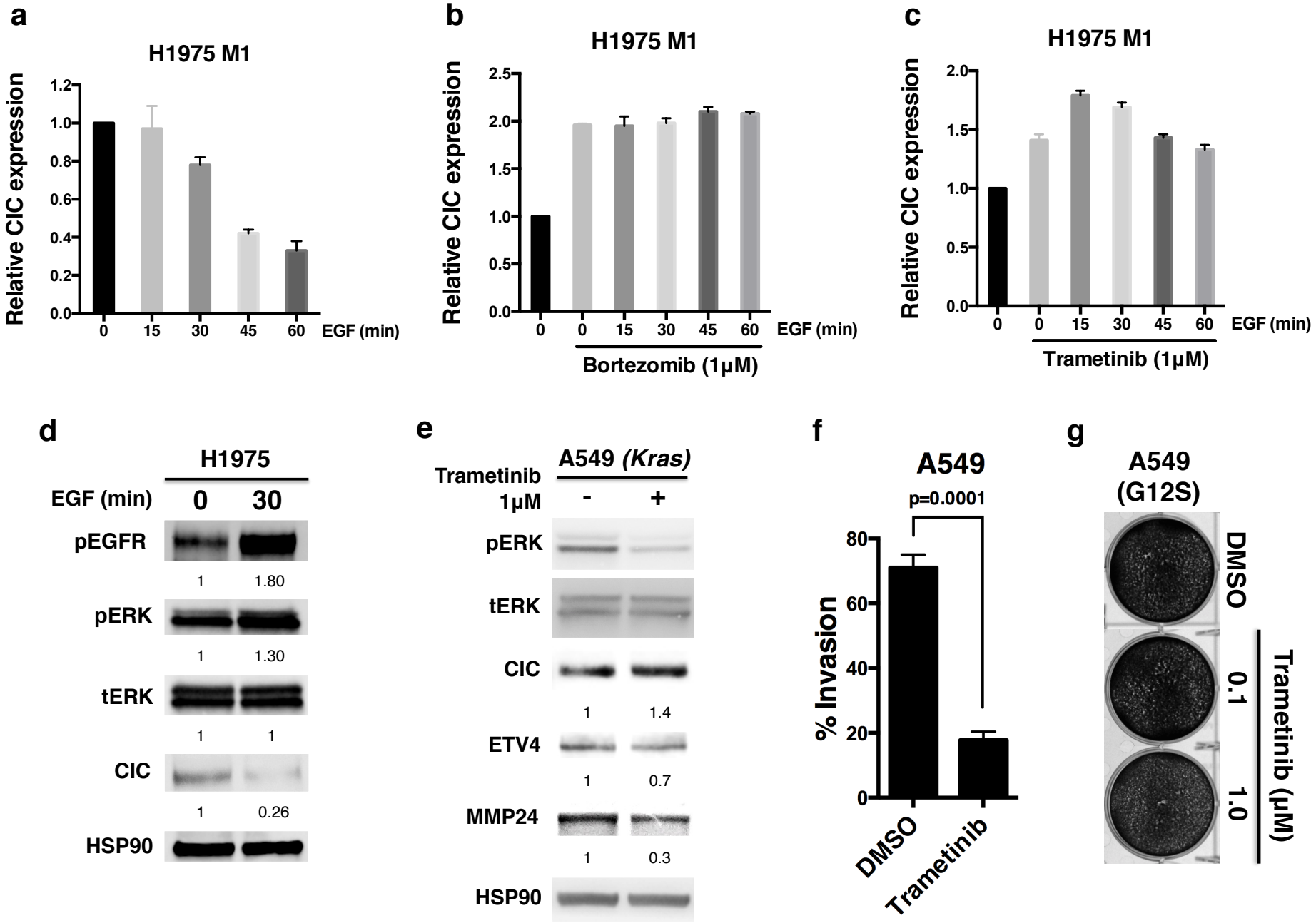
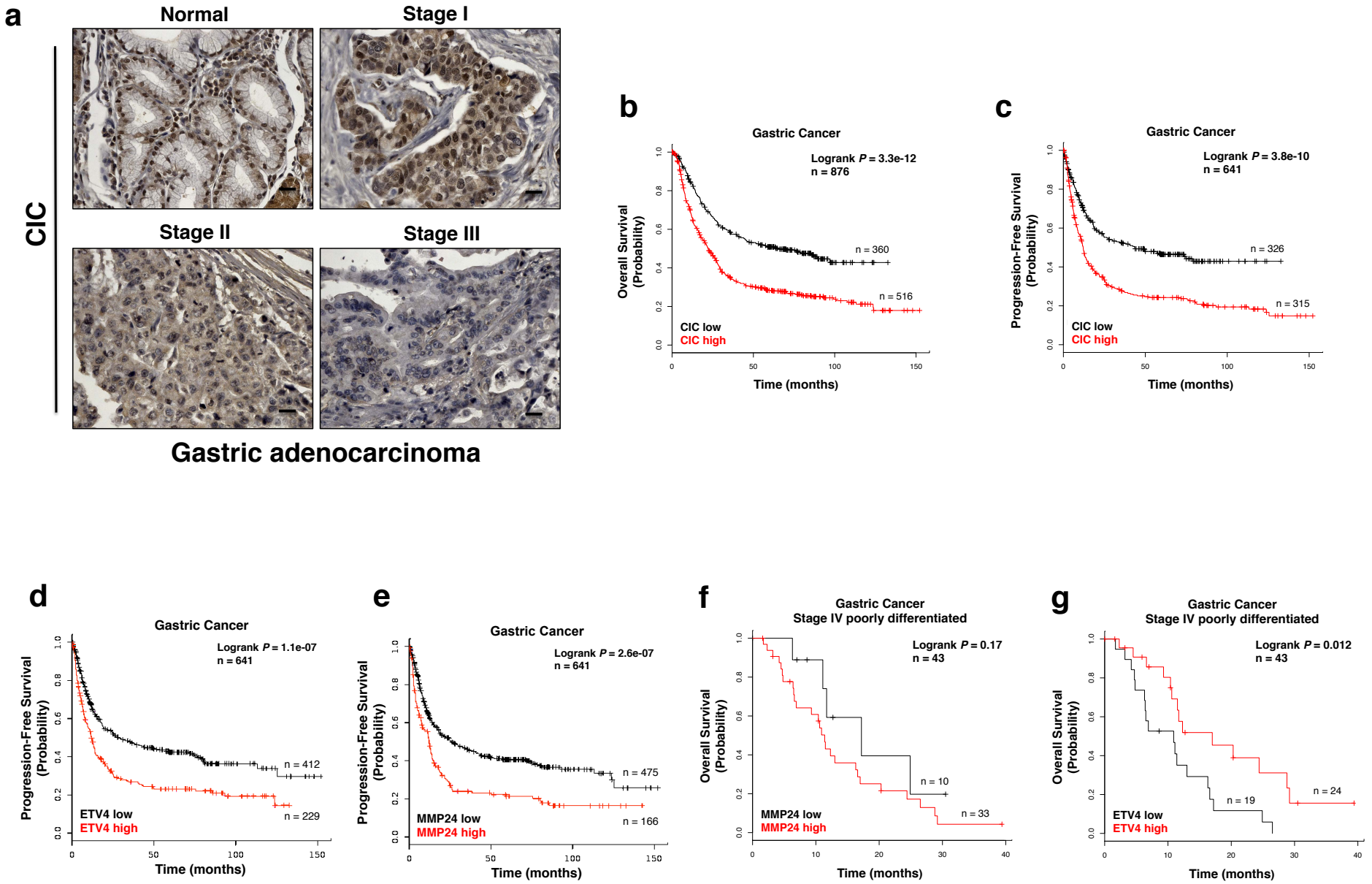

a
H1975 M1
Relative CIC expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
15
30
45
60
EGF (min)
b
H1975 M1
Relative CIC expression
2.5
2.0
1.5
1.0
0.5
0.0
0
0
15
30
45
60
EGF (min)
Bortezomib (1μM)
c
H1975 M1
Relative CIC expression
2.0
1.5
1.0
0.5
0.0
0
0
15
30
45
60
EGF (min)
Trametinib (1μM)
d
H1975
EGF (min)
0
30
pEGFR
1
1.80
pERK
1
1.30
tERK
1
1
CIC
1
0.26
HSP90
e
A549 (Kras)
Trametinib 1μM
-
+
pERK
tERK
CIC
1
1.4
ETV4
1
0.7
MMP24
1
0.3
HSP90
f
A549
p=0.0001
% Invasion
80
60
40
20
0
DMSO
Trametinib
g
A549 (G12S)
DMSO
0.1
1.0
Trametinib (μM)

Figure S13. MAPK pathway regulates CIC expression. (a) Bar graph representing relative CIC protein expression in response to EGF stimulation over 60 minutes. Data obtained from two independent experiments (quantified with ImageJ). Error bars represent SEM. (b) Bar graph representing relative CIC protein expression in H1975 M1 cells pre-treated with Bortezomib and stimulated with EGF over 60 minutes. Data obtained from two independent experiments (quantified with ImageJ). Error bars represent SEM. (c) Bar graph representing relative CIC protein expression in H1975 M1 cells pre-treated with Trametinib and stimulated with EGF over 60 minutes. Data obtained from two independent experiments (quantified with ImageJ). Error bars represent SEM. (d) Immunoblot of phospho-ERK and endogenous CIC expression in H1975 cells after serum starvation and EGF stimulation for 30 minutes. Representative of two independent experiments. (e) Immunoblot comparing CIC, ETV4, and MMP24 expression in Trametinib treated A549 cells. Representative of two independent experiments. (f) Invasion comparing DMSO and Trametinib treated A549 cells. Representative of two independent experiments performed in triplicate. (g) Crystal violet viability assay comparing A549 cells treated with DMSO and Trametinib 0.1 μM, and Trametinib 1.0 μM. Representative of two independent experiments.


a
Normal
Stage I
Stage II
Stage III
CIC
Gastric adenocarcinoma
b
Gastric Cancer
Logrank P = 3.3e-12
n = 876
Overall Survival (Probability)
n = 360
n = 516
CIC low
CIC high
Time (months)
c
Gastric Cancer
Logrank P = 3.8e-10
n = 641
Progression-Free Survival (Probability)
n = 326
n = 315
CIC low
CIC high
Time (months)
d
Gastric Cancer
Logrank P = 1.1e-07
n = 641
Progression-Free Survival (Probability)
n = 412
n = 229
ETV4 low
ETV4 high
Time (months)
e
Gastric Cancer
Logrank P = 2.6e-07
n = 641
Progression-Free Survival (Probability)
n = 475
n = 166
MMP24 low
MMP24 high
Time (months)
f
Gastric Cancer
Stage IV poorly differentiated
Logrank P = 0.17
n = 43
Overall Survival (Probability)
n = 10
n = 33
MMP24 low
MMP24 high
Time (months)
g
Gastric Cancer
Stage IV poorly differentiated
Logrank P = 0.012
n = 43
Overall Survival (Probability)
n = 19
n = 24
ETV4 low
ETV4 high
Time (months)

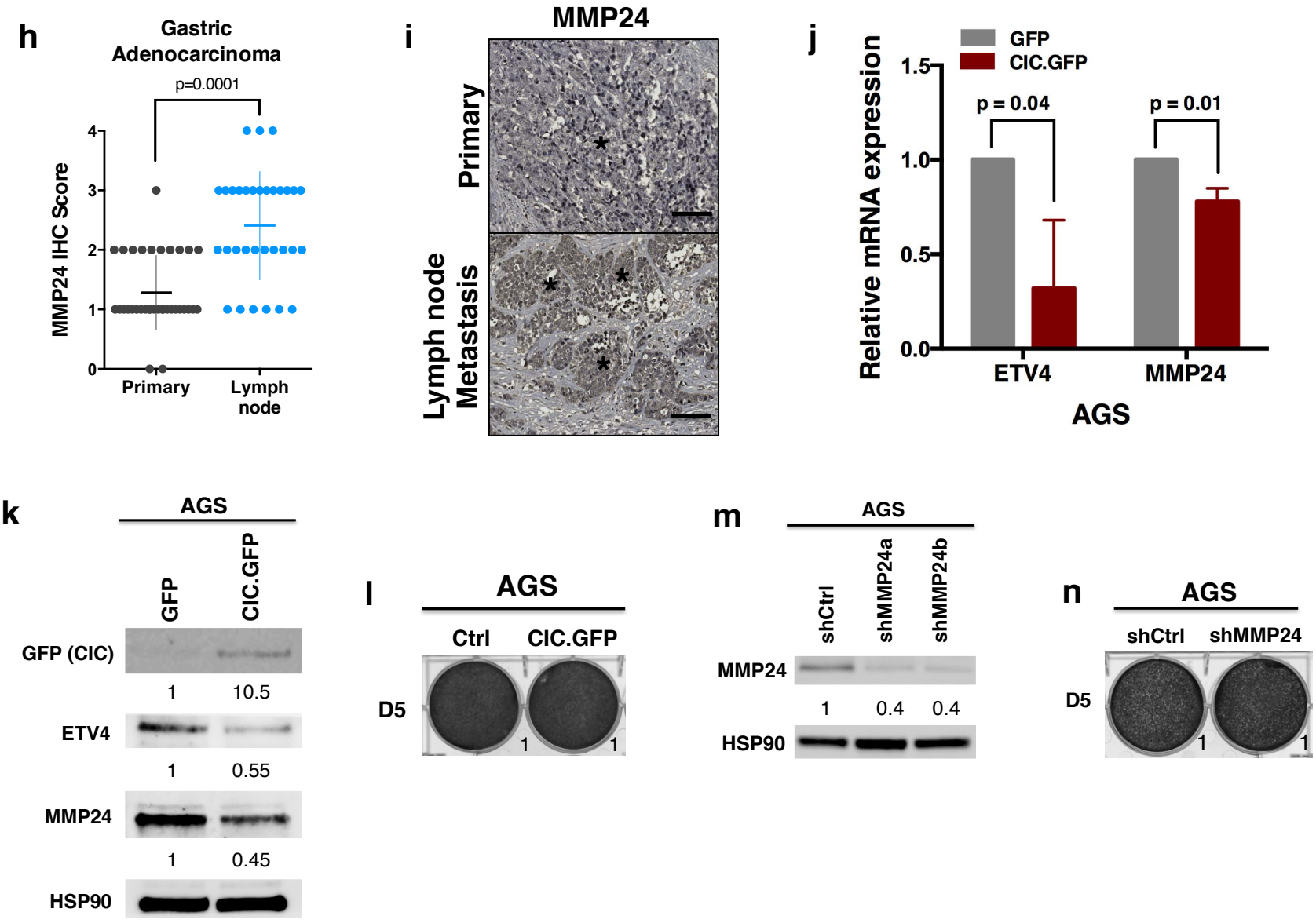

h
Gastric Adenocarcinoma
p=0.0001
MMP24 IHC Score
0
1
2
3
4
Primary
Lymph node
i
MMP24
Primary
Lymph node Metastasis
j
GFP
CIC.GFP
Relative mRNA expression
0.0
0.5
1.0
1.5
p = 0.04
p = 0.01
ETV4
MMP24
AGS
k
AGS
GFP
CIC.GFP
GFP (CIC)
1
10.5
ETV4
1
0.55
MMP24
1
0.45
HSP90
l
AGS
Ctrl
CIC.GFP
D5
1
1
m
AGS
shCtrl
shMMP24a
shMMP24b
MMP24
1
0.4
0.4
HSP90
n
AGS
shCtrl
shMMP24
D5
1
1

Figure S14. CIC effectors ETV4 and MMP24 enhance invasion and correlate with poor patient outcomes in gastric cancer. (a) Nuclear CIC staining in gastric adenocarcinoma by stage (I-III). Scale bar, 20 μm. (b) OS for gastric cancer patients with either *CIC* high or *CIC* low mRNA expression. n=876, p=3.3e-12. Probe 212784_s_at. (c) PFS for gastric cancer patients with either *CIC* high or *CIC* low mRNA expression. n=641, p=3.8e-10. Probe 212784_s_at. (d-e) PFS for gastric cancer patients with either *ETV4* high or *ETV4* low mRNA expression. n=641, p=1.1e-07. Probe 211603_s_at. (d), *MMP24* high or *MMP24* low mRNA expression. n = 641, p = 2.6e-07. Probe 213171_s_at. (e). (f-g) OS for stage IV poorly differentiated gastric cancer patients with either *MMP24* high or *MMP24* low mRNA expression. n=43, p=0.17. Probe 213171_s_at. (f), *ETV4* high or *ETV4* low mRNA expression. n=43, p=0.012. Probe 211603_s_at. (g). (h) MMP24 staining in primary GA tumors and LN metastases. Mean +/- SEM, 1.3 +/- 0.1 (primary, n=27) and 2.4 +/- 0.2 (LN metastasis, n=27). p-value, Student's t-test; error bars, SEM. (i) MMP24 staining of primary and LN metastasis. Scale bar, 50 μm. *Tumor. (j) ETV4 and MMP24 mRNA expression in GFP or GFP-tagged CIC AGS cells. Results of two independent experiments in triplicate. Error bars, SEM. (k) Immunoblots of AGS GFP and CIC-GFP cells. (l) CV assay of GFP or CIC-GFP AGS cells. Results representative of 2 independent experiments. (m) Immunoblots for AGS shCtrl, shMMP24a, shMMP24b. Representative of 2 independent experiments. (n) CV assay of shCtrl or shMMP24a AGS cells. Results representative of 2 independent experiments.

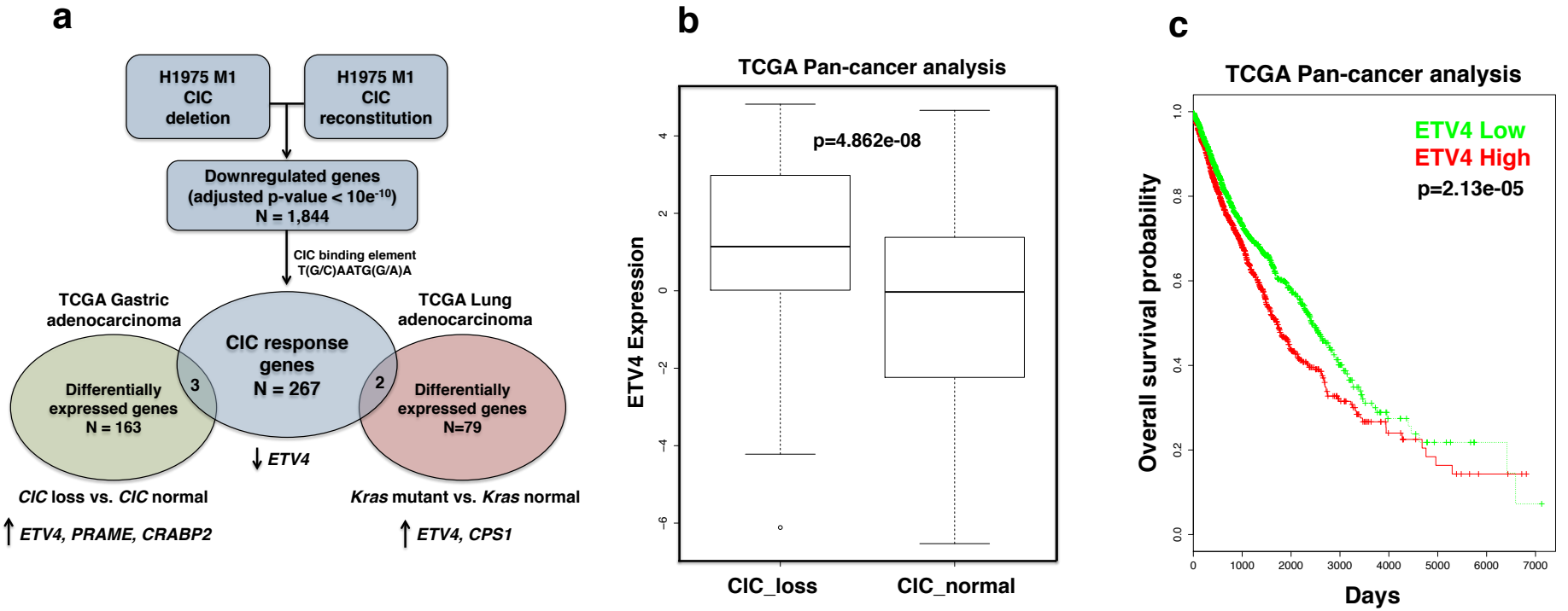

a
H1975 M1 CIC deletion
H1975 M1 CIC reconstitution
Downregulated genes (adjusted p-value < 10e-10) N = 1,844
CIC binding element T(G/C)AATG(G/A)A
TCGA Gastric adenocarcinoma
CIC response genes N = 267
TCGA Lung adenocarcinoma
Differentially expressed genes N = 163
3
2
Differentially expressed genes N=79
ETV4
CIC loss vs. CIC normal
Kras mutant vs. Kras normal
ETV4, PRAME, CRABP2
ETV4, CPS1
b
TCGA Pan-cancer analysis
p=4.862e-08
ETV4 Expression
CIC_loss
CIC_normal
c
TCGA Pan-cancer analysis
ETV4 Low
ETV4 High
p=2.13e-05
Overall survival probability
Days

Figure S15. ETV4 is broadly upregulated in tumors with CIC loss and correlates with worse survival in a pan-cancer analysis. (a) Schematic algorithm to identify differentially expressed CIC response genes in gastric adenocarcinoma tumors with genetic CIC loss and K-Ras mutant lung adenocarcinoma with functional CIC loss (b) Boxplots indicating median (gray bar) and interquartile range (box) of *ETV4* mRNA expression levels in the TCGA pan-cancer tumors with CIC copy number loss (n=82) or normal CIC (p=4.862e-08, n=2949). (c) Pan-cancer OS survival for patients with either *ETV4* high (n=1636) or *ETV4* low (n=1639) mRNA expression.

CIC mutations in Lung Adenocarcinoma

Lung Adenocarcinoma Dataset	Early Stage (I-II)	Advanced Stage (III-IV)
TCGA LUAD Provisional, 2016 (n = 488)	2/381 (0.5%)	3/107 (2.8%)
Rizvi et al. *Science*, 2015 (n = 34)	NA	2/34 (5.9%)
Total (n = 522), *p*-value = 0.02	**2/381 (0.5%)**	**5/141 (3.5%)**

Lung Adenocarinoma Dataset	Case	CIC Mutation	Stage	PolyPhen-2 (score)
TCGA LUAD Provisional, 2016	TCGA-69-7980-01	E236*	IA	Nonsense
	TCGA-97-7547-01	S1409L	IB	Probably Damaging (1.0)
	TCGA-64-5779-01	V1189L	IIIA	Probably Damaging (0.99)
	TCGA-75-6214-01	E1405*	IIIA	Nonsense
	TCGA-86-8056-01	R1412L	IIIA	Probably Damaging (0.99)
Rizvi et al. *Science*, 2015	JB112852	V672D	IV	Probably Damaging (0.99)
	ZA6965	A206V	IV	Probably Damaging (0.99)

Table S1. Non-synonymous *CIC* mutations in the TCGA lung adenocarcinoma provisional and Rizvi et al. metastatic lung adenocarcinoma datasets.

Table S2. Top 1500 differentially expressed genes between H1975 M1 (CIC null) and H1975 M1 with reconstituted CIC expression.

Table S3a. Top 1000 differentially expressed genes between H1975 and H1975 M1 cells. Ranked by p-value. Fold change and log2 fold change are also provided. The table is provided as an Excel File.

Table S3b. Top 1000 differentially expressed genes between H1975 and H1975 M2 cells. Ranked by p-value. Fold change and log2 fold change are also provided. The table is provided as an Excel File.

Table S4. 267 putative CIC response genes.

Supplementary table: primer sequences

Target gene	Sequence	Catalog #	Type
shMMP24a	CCGGCTGGTTAAAGTCCTATGGCTACTCGAGTAGCCATAGGAC TTTAACCAGTTTTTG	TRCN0000052245	shRNA
shMMP24b	CCGGCCTCAGCCTGTCACCTACTATCTCGAGATAGTAGGTGACAGGCTGAGGTTTTTG	TRCN0000052247	shRNA
shCICa	CCGGCCTGGGCTCTTACCGCAAGAACTCGAGTTCTTGCGGTAAGAGCCCAGGTTTTT	TRCN0000018924	shRNA
shCICb	CCGGCTTAGTGTATTCGGACAAGAACTCGAGTTCTTGTCCGAATACACTAAGTTTTTG	TRCN0000329967	shRNA
shCICc	CCGGCTTAGTGTATTCGGACAAGAACTCGAGTTCTTGTCCGAATACACTAAGTTTTT	TRCN0000018920	shRNA
Primer	**Sequence**	**Catalog #**	**Type**
CIC forward	5'-ACCATGATGATGCGTGAGTT-3'	N/A	Genomic DNA
CIC reverse	5'-CTTCTCCCGCTGCATTAAAC-3'	N/A	Genomic DNA
GAPDH forward	5'-CCACAGTCCATGCCATCACTGCC-3'	N/A	Genomic DNA
GAPDH reverse	5'-GAGCTTGACAAAGTGGTCGTTG-3'	N/A	Genomic DNA
ETV4 forward	5'-CGCATCAGACCCAAGACCGTGG -3'	N/A	ChIP-PCR
ETV4 reverse	5'-CCGGAGAGTCGTCCGGCCTGG -3'	N/A	ChIP-PCR
ETV5 forward	5'-GTGCTTCACTGACTCAGCTAAG -3'	N/A	ChIP-PCR
ETV5 reverse	5'-GCCATTGGCCAATCAGCACAGG -3'	N/A	ChIP-PCR
MMP24 forward	5'- GACACGTGGTGTTTGCCATTC-3'	N/A	ChIP-PCR
MMP24 reverse	5'-GTAGGGCTCTGGGCTACTTGG-3'	N/A	ChIP-PCR
EGFR exon 21 forward	5'-TGACCCTGAATTCGGATGCA-3'	N/A	Genomic DNA
EGFR exon 21 reverse	5'-ATACAGCTAGTGGGAAGGCA-3'	N/A	Genomic DNA
Primer	**Sequence**	**Catalog #**	**Type**
G107E, G to A at 320	F: 5'-ctgagagcccagaacccggaccccc-3'	N/A	Mutagenesis
G107E, G to A at 320	R: 5'-gggggtccgggttctgggctctcag-3'	N/A	Mutagenesis
R181W, C to T at 541	F: 5'-gaagatgagtcccattccttgggtagggcact-3'	N/A	Mutagenesis
R181W, C to T at 541	R: 5'-agtgccctacccaaggaatgggactcatcttc-3'	N/A	Mutagenesis
Q247R, A to G at 740	5'-gcccaaggagaagcggaagtaccacgacc-3'	N/A	Mutagenesis
Q247R, A to G at 740	5'-ggtcgtggtacttccgcttctccttgggc-3'	N/A	Mutagenesis
A281S, G to T at 841	5'-ggctcgtgggcttgctctctgagctggact-3'	N/A	Mutagenesis
A281S, G to T at 841	5'-agtccagctcagagagcaagcccacgagcc-3'	N/A	Mutagenesis
S649F, C to T at 1946	5'-cgggggcgaagggcactctggctc-3'	N/A	Mutagenesis
S649F, C to T at 1946	5'-gagccagagtgcccttcgcccccg-3'	N/A	Mutagenesis
R666C, C to T at 1996	5'-ggcggcaggacaggacaggggag-3'	N/A	Mutagenesis
R666C, C to T at 1996	5'-ctcccctgtcctgtcctgccgcc-3'	N/A	Mutagenesis
P722L, C to T at 2165	5'-ctgacacccagcagggagcccccag-3'	N/A	Mutagenesis
P722L, C to T at 2165	5'-ctgggggctccctgctgggtgtcag-3'	N/A	Mutagenesis
G1098D, G to A at 3293	5'-gtgggtgcagggctgtcagagtaaaatgctgtta-3'	N/A	Mutagenesis
G1098D, G to A at 3293	5'-taacagcattttactctgacagccctgcacccac-3'	N/A	Mutagenesis
A1185T, G to A at 3553	F: 5'-cacgggaatgctggtgatggctgccttca-3'	N/A	Mutagenesis
A1185T, G to A at 3553	R: 5'-tgaaggcagccatcaccagcattcccgtg-3'	N/A	Mutagenesis
R1515H, G to A at 4544	5'-ctgcatgatcttctggtgcacctcacggatctt-3'	N/A	Mutagenesis
R1515H, G to A at 4544	5'-aagatccgtgaggtgcaccagaagatcatgcag-3'	N/A	Mutagenesis
Q1516H, G to T at 4548	5'-gcctgcatgatcttatggcgcacctcacg-3'	N/A	Mutagenesis
Q1516H, G to T at 4548	5'-cgtgaggtgcgccataagatcatgcaggc-3'	N/A	Mutagenesis