Fig. S1

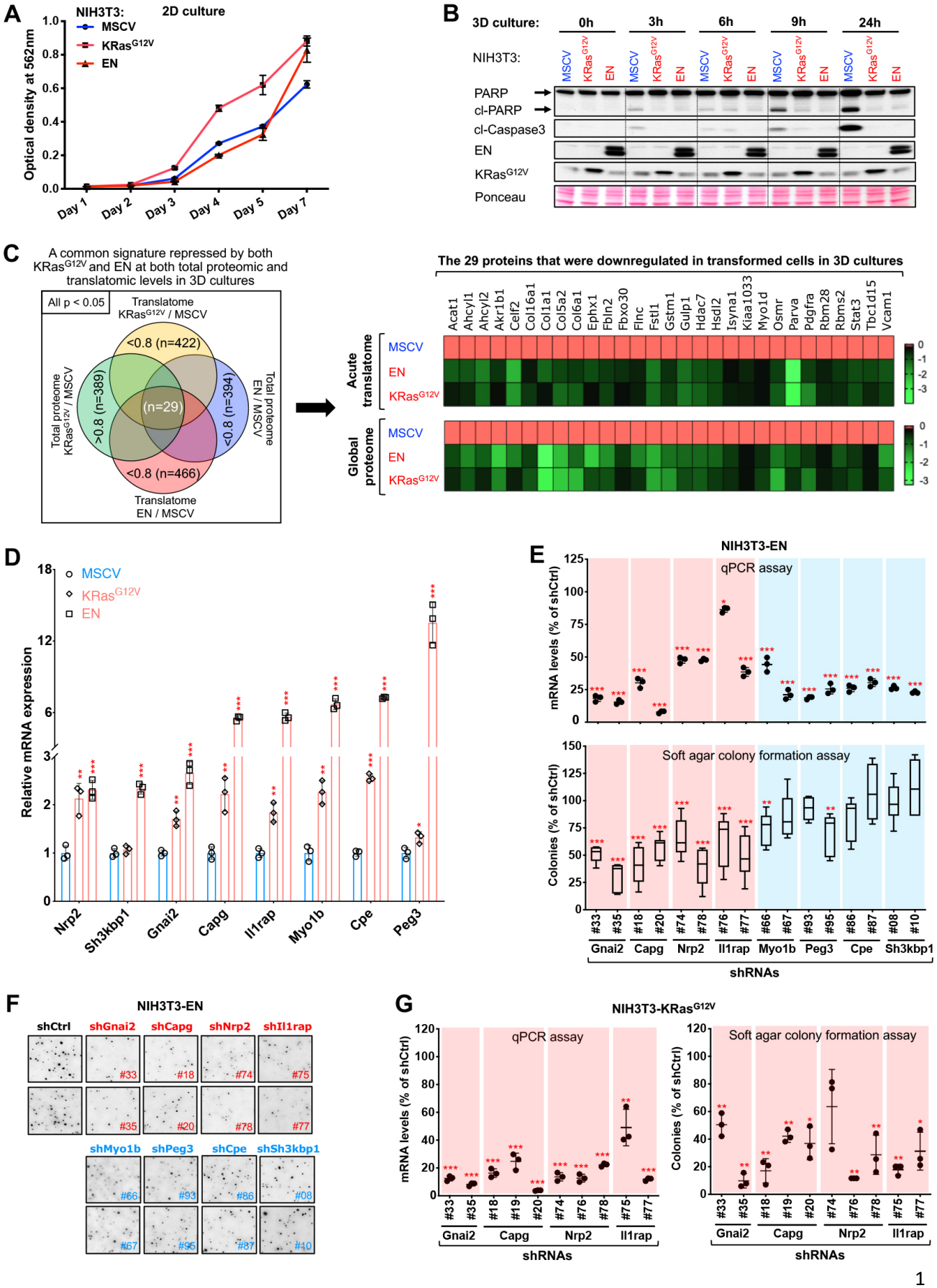
A
NIH3T3:
2D culture
MSCV
KRasG12V
EN
Optical density at 562nm
Day 1
Day 2
Day 3
Day 4
Day 5
Day 7
B
3D culture:
0h
3h
6h
9h
24h
NIH3T3:
PARP
cl-PARP
cl-Caspase3
EN
KRasG12V
Ponceau
C
A common signature repressed by both KRasG12V and EN at both total proteomic and translatomic levels in 3D cultures
All p < 0.05
Translatome KRasG12V / MSCV
<0.8 (n=422)
Total proteome KRasG12V / MSCV
>0.8 (n=389)
(n=29)
<0.8 (n=394)
Total proteome EN / MSCV
<0.8 (n=466)
Translatome EN / MSCV
The 29 proteins that were downregulated in transformed cells in 3D cultures
Acat1
Ahcyl1
Ahcyl2
Akr1b1
Celf2
Col16a1
Col1a1
Col5a2
Col6a1
Ephx1
Fbln2
Fbxo30
Flnc
Fstl1
Gstm1
Gulp1
Hdac7
Hsdl2
Isyna1
Kiaa1033
Myo1d
Osmr
Parva
Pdgfra
Rbm28
Rbms2
Stat3
Tbc1d15
Vcam1
Acute translatome
Global proteome
MSCV
EN
KRasG12V
D
MSCV
KRasG12V
EN
Relative mRNA expression
Nrp2
Sh3kbp1
Gnai2
Capg
Il1rap
Myo1b
Cpe
Peg3
E
NIH3T3-EN
qPCR assay
mRNA levels (% of shCtrl)
Soft agar colony formation assay
Colonies (% of shCtrl)
#33
#35
#18
#20
#74
#78
#76
#77
#66
#67
#93
#95
#86
#87
#08
#10
Gnai2
Capg
Nrp2
Il1rap
Myo1b
Peg3
Cpe
Sh3kbp1
shRNAs
F
NIH3T3-EN
shCtrl
shGnai2
shCapg
shNrp2
shIl1rap
shMyo1b
shPeg3
shCpe
shSh3kbp1
G
NIH3T3-KRasG12V
qPCR assay
mRNA levels (% of shCtrl)
Soft agar colony formation assay
Colonies (% of shCtrl)
#33
#35
#18
#19
#20
#74
#76
#78
#75
#77
Gnai2
Capg
Nrp2
Il1rap
shRNAs

Fig. S1. Identification of novel *anoikis* suppressors in oncogene-transformed mesenchymal cells.

A, Cell growth of the indicated cells in 2D cultures was measured by MTT assay (n=4 biologically independent samples). **B,** Immunoblot analysis of the indicated samples. The experiment was repeated twice with similar results. **C,** Integration of global proteomic and acute translatomic analyses identified 29 downregulated proteins in response to detachment that were shared between $KRas^{G12V}$ and EN transformed cells compared to MSCV controls. **D,** qPCR analysis in NIH3T3 cells expressing MSCV control, $KRas^{G12V}$ or EN. (n = 3 biologically independent samples). **E,** Upper panel: qPCR analysis of mRNA expression to determine gene depletion efficiency in cells stably expressing the indicated shRNAs (n=3 biologically independent samples). Lower panel: Soft agar colony formation assay was performed to determine the effects of each gene knockdown on anoikis resistance (n=3-6 biologically independent samples). **F,** Representative images showing the impact of each gene knockdown on soft agar colony formation in NIH3T3-EN cells (corresponding to **E,** lower panel). **G,** Left panel: qPCR analysis of mRNA expression to determine gene depletion efficiency in cells stably expressing the indicated shRNAs (n=3 biologically independent samples). Right panel: Soft agar colony formation assay was performed to determine the effects of each gene knockdown on anoikis resistance (n=3-6 biologically independent samples). For **A, D, E,** and **G**, data are shown as the mean ± SD except for **E-**lower panel where the box plots show the mean and the 5-95 percentile. Statistical significance was determined using unpaired two-tailed Student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Fig. S2

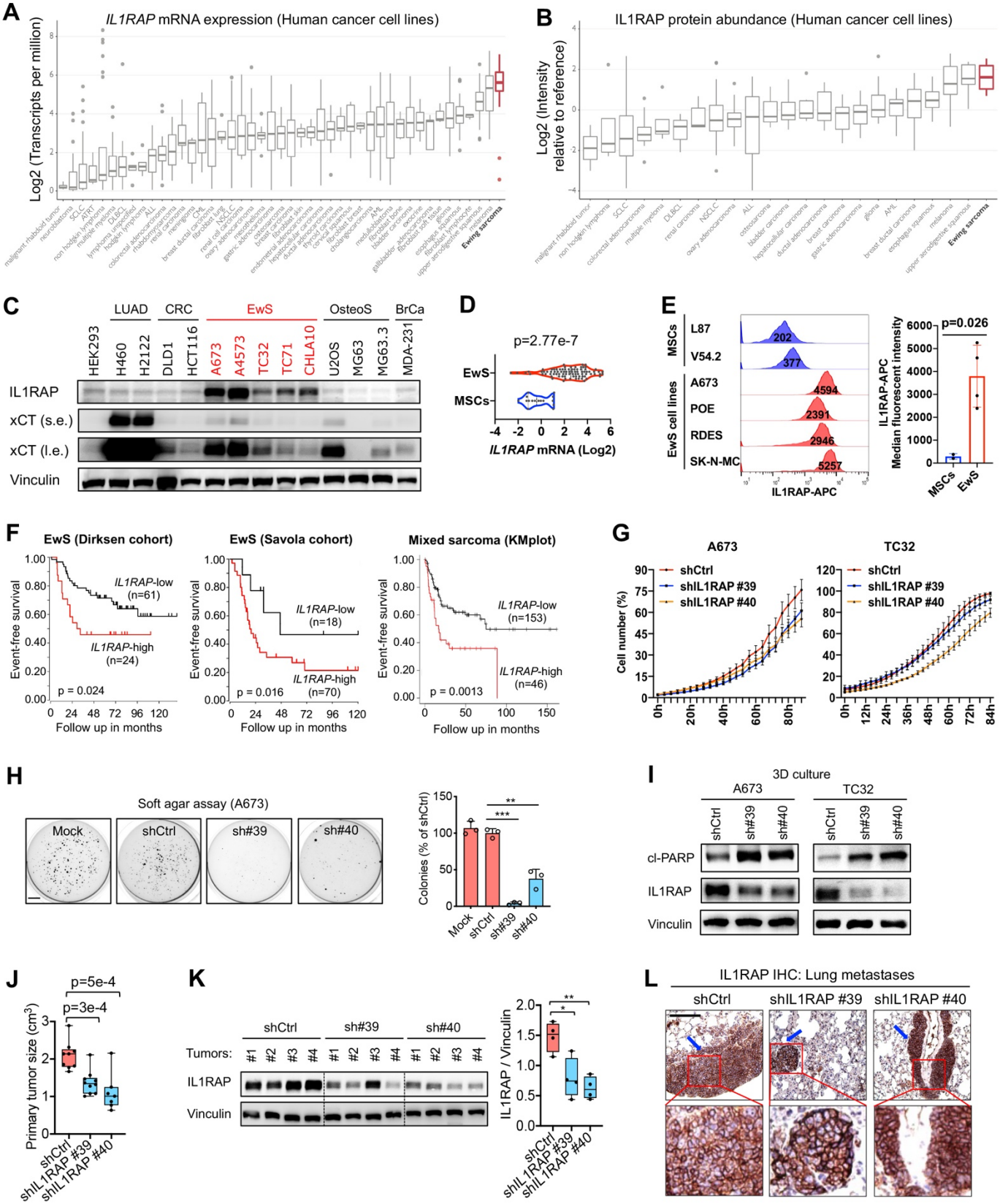
A
IL1RAP mRNA expression (Human cancer cell lines)
Log2 (Transcripts per million)
Ewing sarcoma
B
IL1RAP protein abundance (Human cancer cell lines)
Log2 (Intensity relative to reference)
Ewing sarcoma
C
LUAD
CRC
EwS
OsteoS
BrCa
HEK293
H460
H2122
DLD1
HCT116
A673
A4573
TC32
TC71
CHLA10
U2OS
MG63
MG63.3
MDA-231
IL1RAP
xCT (s.e.)
xCT (l.e.)
Vinculin
D
p=2.77e-7
EwS
MSCs
IL1RAP mRNA (Log2)
E
MSCs
L87
V54.2
EwS cell lines
A673
POE
RDES
SK-N-MC
202
377
4594
2391
2946
5257
IL1RAP-APC
IL1RAP-APC Median fluorescent intensity
p=0.026
MSCs
EwS
F
EwS (Dirksen cohort)
EwS (Savola cohort)
Mixed sarcoma (KMplot)
Event-free survival
IL1RAP-low (n=61)
IL1RAP-high (n=24)
p = 0.024
IL1RAP-low (n=18)
IL1RAP-high (n=70)
p = 0.016
IL1RAP-low (n=153)
IL1RAP-high (n=46)
p = 0.0013
Follow up in months
G
A673
TC32
shCtrl
shIL1RAP #39
shIL1RAP #40
Cell number (%)
H
Soft agar assay (A673)
Mock
shCtrl
sh#39
sh#40
Colonies (% of shCtrl)
I
3D culture
A673
TC32
shCtrl
sh#39
sh#40
cl-PARP
IL1RAP
Vinculin
J
p=5e-4
p=3e-4
Primary tumor size (cm3)
shCtrl
shIL1RAP #39
shIL1RAP #40
K
shCtrl
sh#39
sh#40
Tumors:
#1
#2
#3
#4
IL1RAP
Vinculin
IL1RAP / Vinculin
L
IL1RAP IHC: Lung metastases
shCtrl
shIL1RAP #39
shIL1RAP #40

Fig. S2. IL1RAP is highly expressed in Ewing sarcoma and promotes *anoikis* resistance and *in vivo* metastasis.

A and **B,** The expression of *IL1RAP* mRNA (**A**) and protein (**B**) in various human cancer cell lines. The graphs were generated based on the data from the DepMap project (https://depmap.org/portal/). **C,** Immunoblots showing the expression of the indicated proteins in a panel of human cell lines. The experiment was repeated twice with similar results. LUAD, lung adenocarcinoma; CRC, colorectal cancer; EwS, Ewing sarcoma; OsteoS, osteosarcoma; BrCa, breast cancer. l.e., long exposure; s.e., short exposure. **D,** Comparison of *IL1RAP* mRNA expression between human EwS tumors and MSCs (mesenchymal stem cells). **E,** Surface IL1RAP expression measured by direct IL1RAP-APC staining and Flow cytometry in EwS cell lines (n=4) and MSCs (n=2). **F,** Kaplan-Meier curves based on *IL1RAP* mRNA levels in different cohorts of sarcoma patients. Details are described in the methods. **G,** Cell growth of the indicated cells by Incucyte analysis (n = 3-4 biologically independent samples). **H,** Soft agar colony formation of A673 with no treatment (mock), shCtrl, or shIL1RAP (#39 and #40) (n=3 biologically independent samples). Scale bar: 5mm. **I,** Representative Immunoblots in the indicated EwS cells grown in 3D cultures for 5 days. The experiment was repeated twice with similar results. **J,** Volumes of TC32-derived tumours in the renal subcapsular space of mice (n = 7-10). **K,** Immunoblot analysis in lysates of TC32-derived EwS xenograft tumours in mice, and densitometry analysis of the indicated proteins. n = 4 independent tumours. **L,** IHC staining of IL1RAP in mouse lung sections. Blue arrows indicate EwS metastases in the lungs. Scale bar, 100μm. In **A, B** and **D,** data are presented as median values and the interquartile range. In **E, G**, and **H,** data presented are means ± SD. In **J,** data presented are means ± SEM. In **K,** data presented are mean values and 10-90 percentile. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, p values were determined by two-tailed unpaired Student's t-test.

Fig. S3

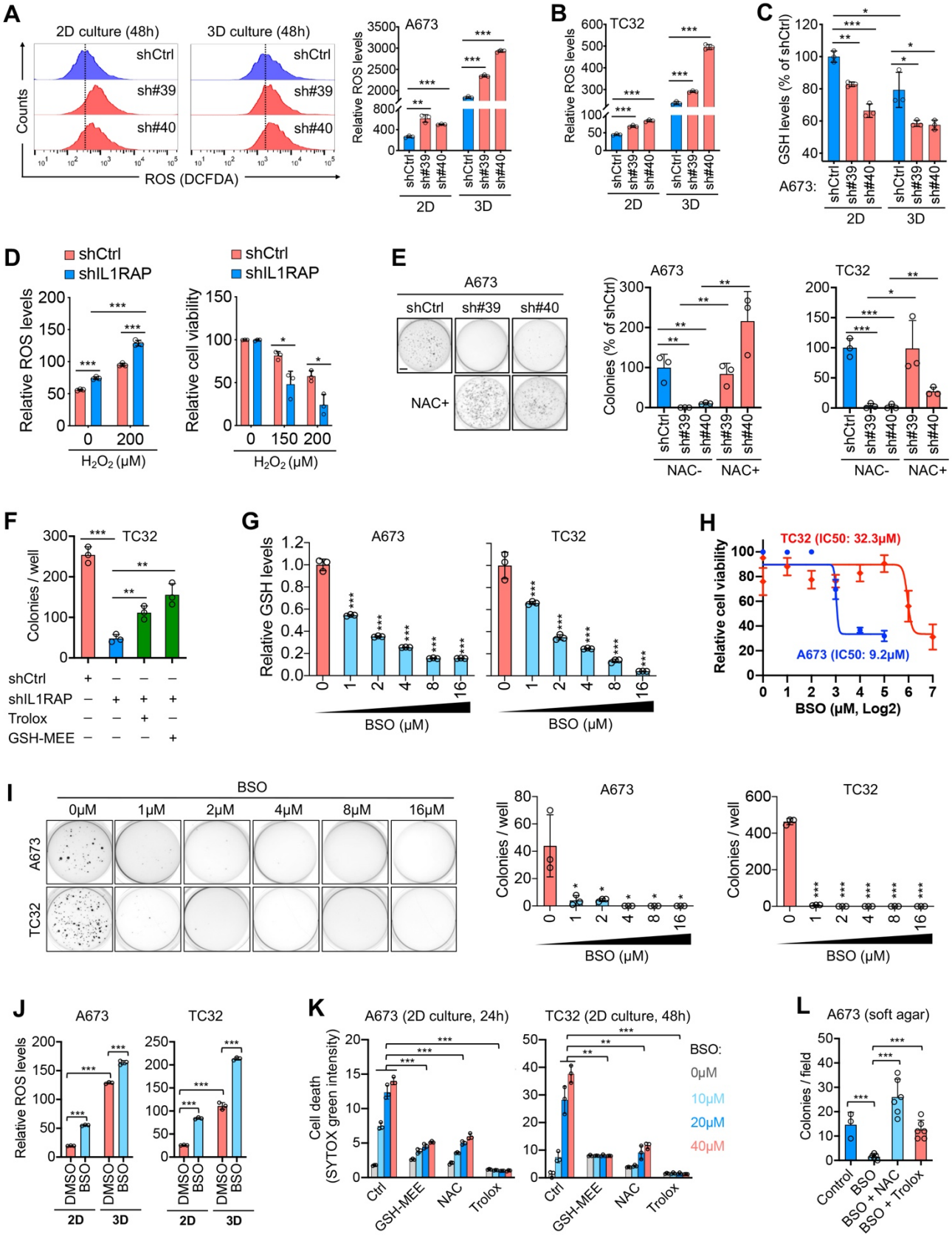
A
2D culture (48h)
3D culture (48h)
shCtrl
sh#39
sh#40
Counts
ROS (DCFDA)
A673
Relative ROS levels
2D
3D
B
TC32
C
GSH levels (% of shCtrl)
A673:
D
shIL1RAP
Relative cell viability
H2O2 (μM)
E
NAC+
NAC-
Colonies (% of shCtrl)
F
Colonies / well
Trolox
GSH-MEE
G
Relative GSH levels
BSO (μM)
H
TC32 (IC50: 32.3μM)
A673 (IC50: 9.2μM)
Relative cell viability
BSO (μM, Log2)
I
BSO
0μM
1μM
2μM
4μM
8μM
16μM
J
DMSO
BSO
K
A673 (2D culture, 24h)
TC32 (2D culture, 48h)
Cell death (SYTOX green intensity)
BSO:
0μM
10μM
20μM
40μM
Ctrl
GSH-MEE
NAC
Trolox
L
A673 (soft agar)
Colonies / field
Control
BSO
BSO + NAC
BSO + Trolox

Fig. S3. IL1RAP controls redox homeostasis and GSH pools that are vital for *anoikis* resistance in EwS.

A and **B,** Flow cytometry-based quantification of ROS (H2DCFDA) in EwS cells cultured in 2D or 3D conditions for 48 hr (n = 3 biologically independent samples). **C,** Glutathione (GSH) levels in A673 cells with shCtrl or shIL1RAP (#39 and #40) (n=3 biologically independent samples). **D,** Flow cytometry-based quantification of ROS after 24 hr treatment (left panel), and cell growth of the indicated cells by Incucyte analysis after 96 hr treatment (right panel). All panels, n = 3 biologically independent samples. **E** and **F,** Soft agar colony formation of EwS cells with shCtrl or shIL1RAP (#39 and #40) treated with/without 5mM NAC or 25μM Trolox (n=3 biologically independent samples). **G,** GSH levels were measured after 48 hr treatment with BSO (Buthionine sulfoximine). **H** and **I,** The effects of BSO on cell growth in 2D cultures (**H**) or soft agar colony formation (**I**). **J,** ROS (H2DCFDA) levels were measured after 48 hr treatment with BSO in 2D or 3D cultures. **K,** Cell death was measured by SYTOX green staining and Incucyte analysis. Cells were either pretreated with 5mM GSH-MEE for 1 hr before BSO treatment or simultaneously treated with 1mM NAC or 100μM Trolox and BSO. **L**, Soft agar assay was performed in cells treated with/without 2μM BSO +/- 0.5mM NAC, or 50μM Trolox. All data presented are means ± SD. P values were determined by two-tailed unpaired Student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Fig. S4

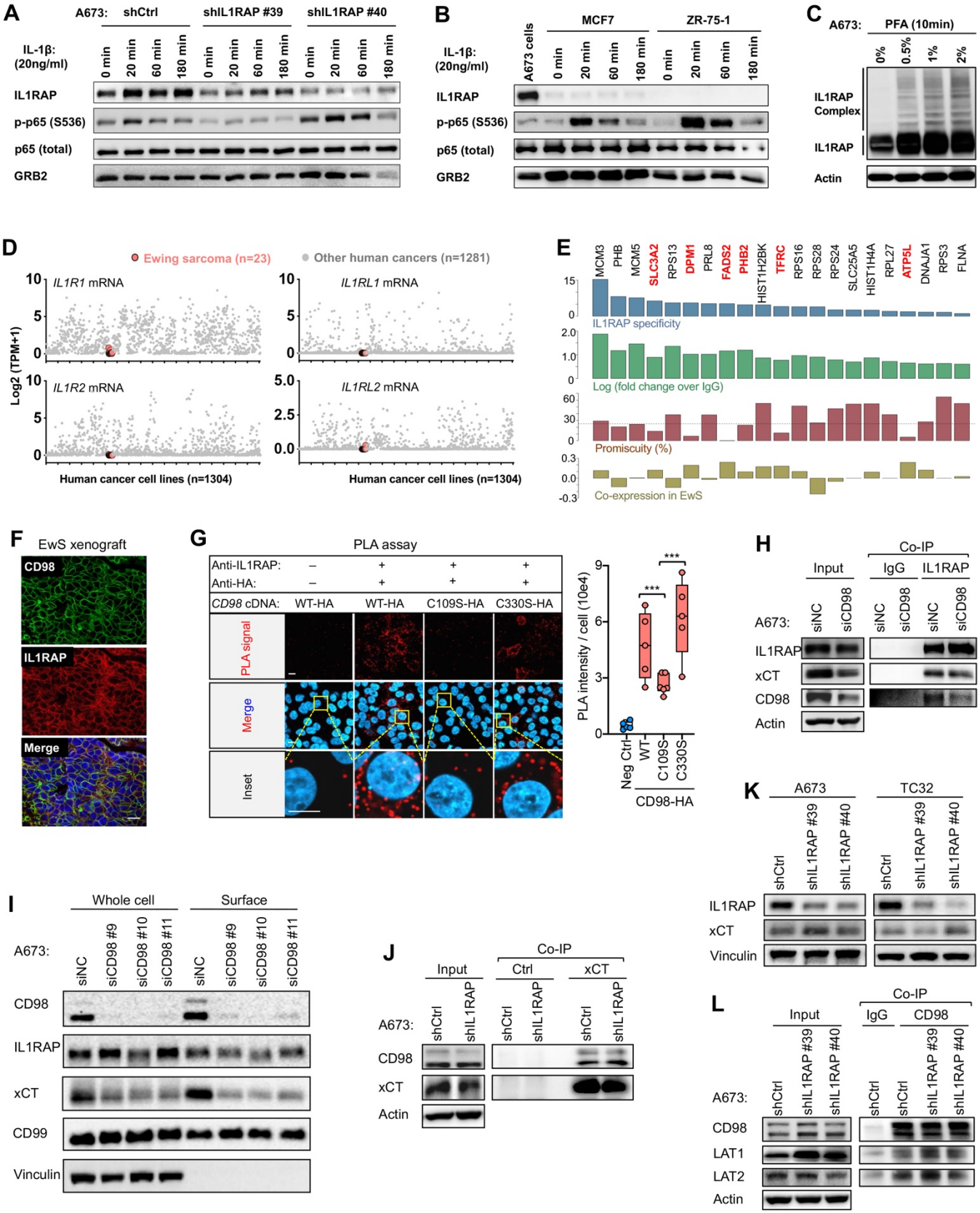
A
A673:
shCtrl
shIL1RAP #39
shIL1RAP #40
IL-1β: (20ng/ml)
0 min
20 min
60 min
180 min
IL1RAP
p-p65 (S536)
p65 (total)
GRB2
B
A673 cells
MCF7
ZR-75-1
C
PFA (10min)
0%
0.5%
1%
2%
IL1RAP Complex
IL1RAP
Actin
D
Ewing sarcoma (n=23)
Other human cancers (n=1281)
IL1R1 mRNA
IL1RL1 mRNA
IL1R2 mRNA
IL1RL2 mRNA
Log2 (TPM+1)
Human cancer cell lines (n=1304)
E
MCM3
PHB
MCM5
SLC3A2
RPS13
DPM1
PRL8
FADS2
PHB2
HIST1H2BK
TFRC
RPS16
RPS28
RPS24
SLC25A5
HIST1H4A
RPL27
ATP5L
DNAJA1
RPS3
FLNA
IL1RAP specificity
Log (fold change over IgG)
Promiscuity (%)
Co-expression in EwS
F
EwS xenograft
CD98
IL1RAP
Merge
G
PLA assay
Anti-IL1RAP:
Anti-HA:
CD98 cDNA:
WT-HA
WT-HA
C109S-HA
C330S-HA
PLA signal
Merge
Inset
PLA intensity / cell (10e4)
***
***
Neg Ctrl
WT
C109S
C330S
CD98-HA
H
Co-IP
Input
IgG
IL1RAP
A673:
siNC
siCD98
IL1RAP
xCT
CD98
Actin
K
A673
TC32
shCtrl
shIL1RAP #39
shIL1RAP #40
IL1RAP
xCT
Vinculin
I
Whole cell
Surface
A673:
siNC
siCD98 #9
siCD98 #10
siCD98 #11
CD98
IL1RAP
xCT
CD99
Vinculin
J
Co-IP
Input
Ctrl
xCT
A673:
shCtrl
shIL1RAP
CD98
xCT
Actin
L
Co-IP
Input
IgG
CD98
A673:
shCtrl
shIL1RAP #39
shIL1RAP #40
CD98
LAT1
LAT2
Actin

Fig. S4. IL1RAP forms a complex with CD98 and xCT of the system X_c^- cystine transporter: an IL-1 signaling independent function of IL1RAP.

A and **B,** Representative immunoblots of the indicated samples. The experiments were repeated twice or more with similar results. **C,** Representative immunoblots of A673 cells treated with different concentration of formaldehyde (PFA) for 10min. Note the crosslinked IL1RAP protein complexes induced by PFA treatment. The experiments were repeated twice with similar results. **D,** The expression of *IL1RAP* mRNA in human cancer cell lines. The graphs were generated based on the data from the DepMap project (https://depmap.org/portal/). **E,** The 21 proteins that had fold-changes >1.5 and ≥ 2 peptides identified in the IL1RAP interactome analysis (shown in Fig. 3a) were further analyzed based on the four parameters indicated in the graphs. The proteins labeled red were considered high-quality candidates based on low promiscuity and co-expression with IL1RAP in EwS tumors. Details are described in the Methods section. **F,** IF staining of IL1RAP and CD98 in TC32-derived EwS xenografts in the murine renal subcapsular implantation model. Scale bar, 20µm. **G,** PLA assay of IL1RAP and HA in A673 cells transfected with HA-tagged WT or mutant CD98 (n=5-6, >100 cells were analyzed for each condition). Scale bar, 10µm. **H-L,** Representative Immunoblots of the indicated samples. The experiments were repeated at least twice with similar results. *$p < 0.05$, ***$p < 0.001$, p values were determined by two-tailed unpaired Student's t-test.

Fig. S5

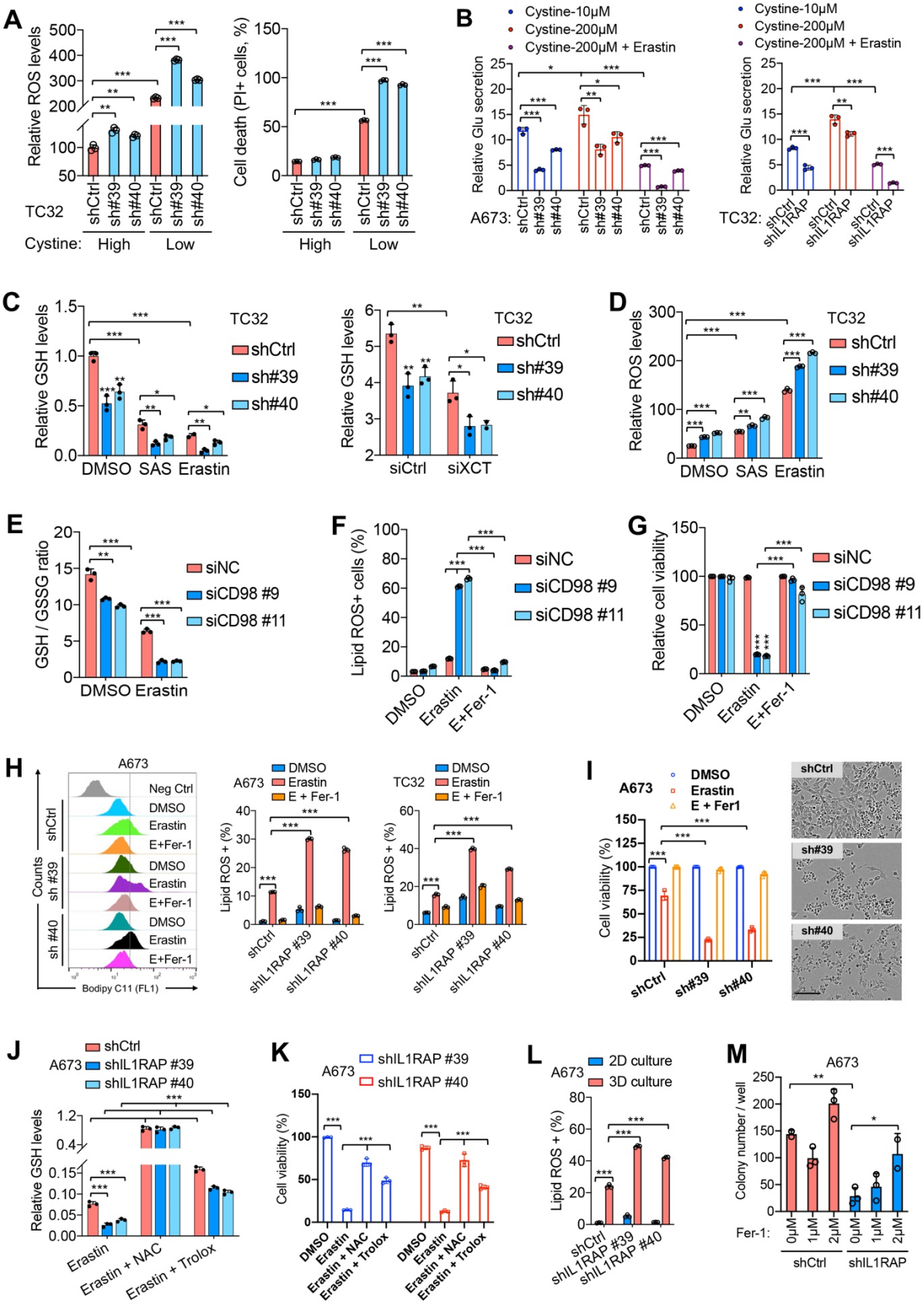
A
Relative ROS levels
Cell death (PI+ cells, %)
TC32
shCtrl
sh#39
sh#40
Cystine:
High
Low
B
Cystine-10μM
Cystine-200μM
Cystine-200μM + Erastin
Relative Glu secretion
A673:
TC32:
shCtrl
shIL1RAP
C
Relative GSH levels
TC32
DMSO
SAS
Erastin
siCtrl
siXCT
D
Relative ROS levels
E
GSH / GSSG ratio
siNC
siCD98 #9
siCD98 #11
F
Lipid ROS+ cells (%)
G
Relative cell viability
H
A673
Neg Ctrl
Counts
Bodipy C11 (FL1)
Lipid ROS + (%)
I
Cell viability (%)
J
Relative GSH levels
shIL1RAP #39
shIL1RAP #40
Erastin + NAC
Erastin + Trolox
K
Cell viability (%)
L
2D culture
3D culture
Lipid ROS + (%)
M
Colony number / well
Fer-1:
0μM
1μM
2μM

Fig. S5. The IL1RAP-system X_c^- complex maintains intracellular GSH pools and protect EwS cells from ferroptosis.

A, Flow cytometry-based quantification of ROS by H2DCFDA staining (left panel), and cell death by PI staining (right panel) in cells cultured with high (200μM) or low (10μM) levels of extracellular cystine (n=3 biologically independent samples). **B,** Glutamate secretion was measured in the conditioned media (12 hrs) from the indicated cells cultured under the indicated conditions. **C,** GSH levels in the indicated cells treated with DMSO, 500μM Sulfasalazine (SAS), or 5μM Erastin for 24 hrs (left panel), or siRNA transfection for 72 hrs (right panel). For both panels, n = 3 biologically independent samples. **D,** Flow cytometry-based quantification of ROS by H2DCFDA staining in the indicated cells treated with DMSO, 500μM Sulfasalazine (SAS), or 5μM Erastin for 18 hrs (n = 3 biologically independent samples). **E,** GSH and GSSG ratios in the indicated cells treated with DMSO or 2μM Erastin for 20 hr (n = 3 biologically independent samples). **F,** Flow cytometry-based quantification of lipid ROS in the indicated cells treated with DMSO, 5μM Erastin, and/or 2μM Fer-1 for 22 hrs. **G,** Relative cell viability was analyzed by Incucyte in cells treated with 2μM Erastin and/or 2μM Fer-1 for 72 hrs. n = 3-4 biologically independent samples. **H,** Flow cytometry-based quantification of lipid ROS 36 hr after treatment with DMSO, or 10μM Erastin +/- 2μM Fer-1 (n = 3 biologically independent samples). **I,** The indicated cells were treated with DMSO, or 10μM Erastin +/- 2μM Fer-1 for 96 hr, and cell number was analyzed by Incucyte (left). n = 3-4 biologically independent samples. Representative cell images in the Erastin group are shown on the right panel. Scale bar, 100 μm. **J,** GSH levels in the indicated cells treated with 2μM Erastin +/- 0.5mM NAC or 25μM Trolox for 16 hr. **K,** Incucyte analysis of relative cell viability (n = 3-4 biologically independent samples). **L,** The indicated cells were grown in 2D or 3D culture for 72 hr, and lipid ROS was measured by Flow cytometry (n = 3 biologically independent samples). **M,** Soft agar colony formation assay in the presence of different concentrations of Fer-1 (n = 3 biologically independent samples). All data presented are means ± SD, p values were determined by two-tailed unpaired Student's t-test, *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$.

Fig. S6

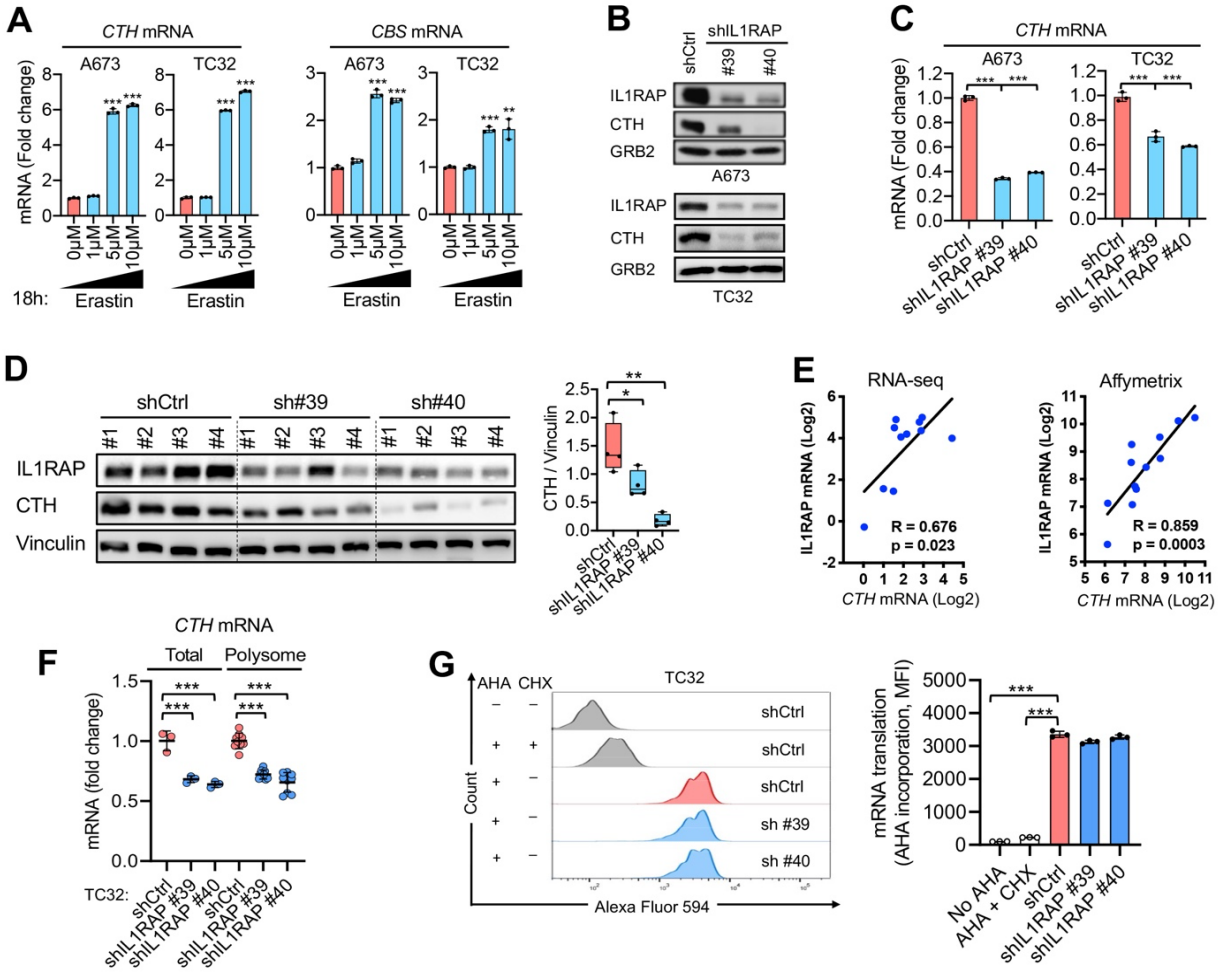
A
CTH mRNA
CBS mRNA
A673
TC32
mRNA (Fold change)
0μM 1μM 5μM 10μM
18h:
Erastin
B
shCtrl
shIL1RAP
#39
#40
IL1RAP
CTH
GRB2
A673
TC32
C
CTH mRNA
shCtrl
shIL1RAP #39
shIL1RAP #40
D
shCtrl
sh#39
sh#40
IL1RAP
CTH
Vinculin
CTH / Vinculin
E
RNA-seq
Affymetrix
IL1RAP mRNA (Log2)
CTH mRNA (Log2)
R = 0.676
p = 0.023
R = 0.859
p = 0.0003
F
CTH mRNA
Total
Polysome
mRNA (fold change)
TC32:
G
AHA CHX
TC32
shCtrl
sh #39
sh #40
Count
Alexa Fluor 594
mRNA translation (AHA incorporation, MFI)
No AHA
AHA + CHX
H
Dirksen cohort (n=85)
Savola cohort (n=88)
CTH-Low (n=64)
CTH-High (n=21)
CTH-Low (n=36)
CTH-High (n=52)
p = 0.0017
p = 0.016
Event-free survival
Follow up in months
I
TC32
n.s.
Cell death (SYTOX Green intensity)
siNC
siCTH
Hcy:
Cystine:
10 μM
0 μM
Cell growth (confluence, %)
siNC
siCTH
siNC (+Hcy)
siCTH (+Hcy)
Cystine-0μM
J
Hcy
Cysteine
γ-Glu-Cys
GSH/GSSG
Hcy (nmol / mg)
Cysteine (nmol / mg)
γ-Glu-Cys (nmol / mg)
GSH / GSSG ratio
Cystine:
Hcy:

Fig. S6. The IL1RAP-CTH axis in Ewing sarcoma.

A, qPCR analysis of the indicated genes in EwS cells (n=3 biologically independent samples). **B,** Representative immunoblots showing the impact of IL1RAP knockdown by different shRNAs (#39 and #40) on CTH expression *in vitro*. The experiments were repeated at least twice with similar results. **C,** qPCR analysis of the indicated genes in EwS cells (n=3 biologically independent samples). **D,** Representative immunoblots showing the impact of IL1RAP knockdown by different shRNAs (#39 and #40) on CTH expression in the indicated EwS xenografts derived from TC32 cells. **E,** Correlation analysis between *IL1RAP* and *CTH* mRNA expression in a panel of EwS cell lines (n=11-12). The graphs were generated based on the data from the CCLE database (https://portals.broadinstitute.org/ccle). **F,** *CTH* mRNA expression changes upon *IL1RAP* knockdown in total or polysome-bound mRNA fractions isolated from the indicated cells. **G,** Global protein synthesis was determined by flow cytometry upon incorporation of azidohomoalanine (AHA) and cell-based Click chemistry. **H,** Kaplan-Meier curves based on *CTH* mRNA levels in EwS tumours. The graphs were generated based on the data from the R2 database (https://hgserver1.amc.nl/cgi-bin/r2/main.cgi). **I,** Cell death and cell growth were measured by Incucyte in the indicated cells cultured in media containing different concentrations of cystine supplemented with/without 0.4mM homocysteine (Hcy). **J,** Intracellular levels of cystathionine and GSH were determined in cells cultured in media +/- 200µM cystine or 0.4mM Hcy for 12 hours. All data presented are means ± SD, p values were determined by two-tailed unpaired Student's t-test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. not significant.

Fig. S7

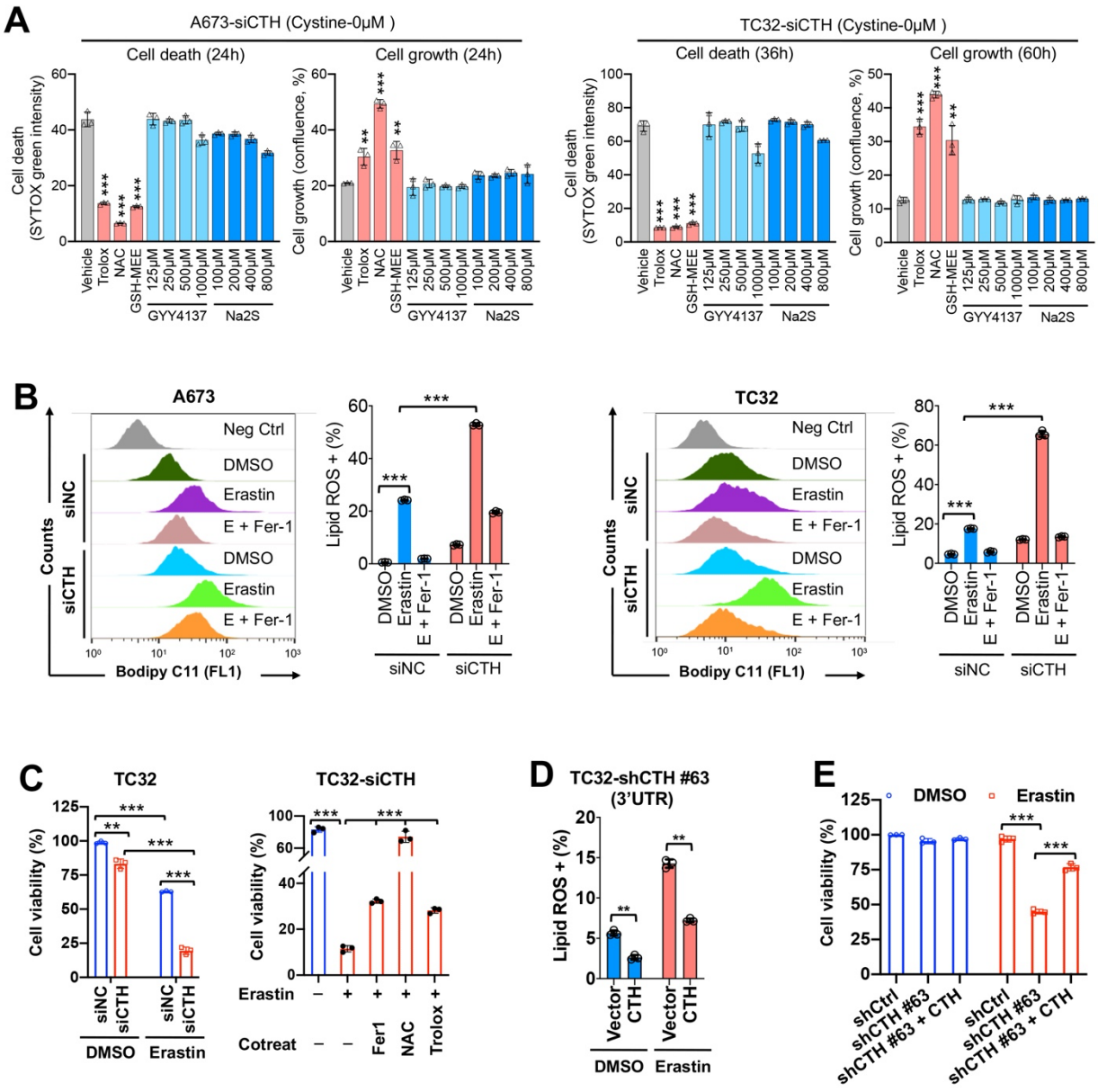
A
A673-siCTH (Cystine-0μM)
Cell death (24h)
Cell growth (24h)
TC32-siCTH (Cystine-0μM)
Cell death (36h)
Cell growth (60h)
Cell death (SYTOX green intensity)
Cell growth (confluence, %)
Vehicle
Trolox
NAC
GSH-MEE
125μM
250μM
500μM
1000μM
100μM
200μM
400μM
800μM
GYY4137
Na2S
B
A673
TC32
Neg Ctrl
DMSO
Erastin
E + Fer-1
siNC
siCTH
Counts
Bodipy C11 (FL1)
Lipid ROS + (%)
C
TC32
TC32-siCTH
Cell viability (%)
Erastin
Cotreat
Fer1
NAC
Trolox
D
TC32-shCTH #63
(3'UTR)
Vector
CTH
E
shCtrl
shCTH #63
shCTH #63 + CTH

Fig. S7. The IL1RAP-CTH axis protects EwS from ferroptosis upon extracellular cystine depletion. **A,** Cell death and cell growth were measured by Incucyte in the indicated cells cultured in cystine-free media supplemented with 50μM Trolox, 1mM NAC, 4mM GSH-MEE or the indicated concentrations of GYY4137 or Na2S. **B,** Flow cytometry-based quantification of lipid ROS in cells treated with 5μM Erastin and/or 2μM Fer-1 for 22 hr (A673) or 36 hr (TC32). n=3 biologically independent samples. **C,** Relative cell viability was analyzed by Incucyte for 96 hr (left panel: 4μM Erastin and 2μM Fer-1; right panel: 8μM Erastin, 0.5mM NAC, and 25μM Trolox). n=3 biologically independent samples. **D,** Flow cytometry-based quantification of lipid ROS in cells treated with 10μM Erastin +/- 2μM Fer-1 for 20 hr. **E,** Relative cell viability was measured by Incucyte analysis in cells treated with 5μM Erastin +/- 2μM Fer-1 for 96 hr. All data presented are means ± SD, p values were determined by two-tailed unpaired Student's t-test, **p < 0.01; ***p < 0.001.

Fig. S8

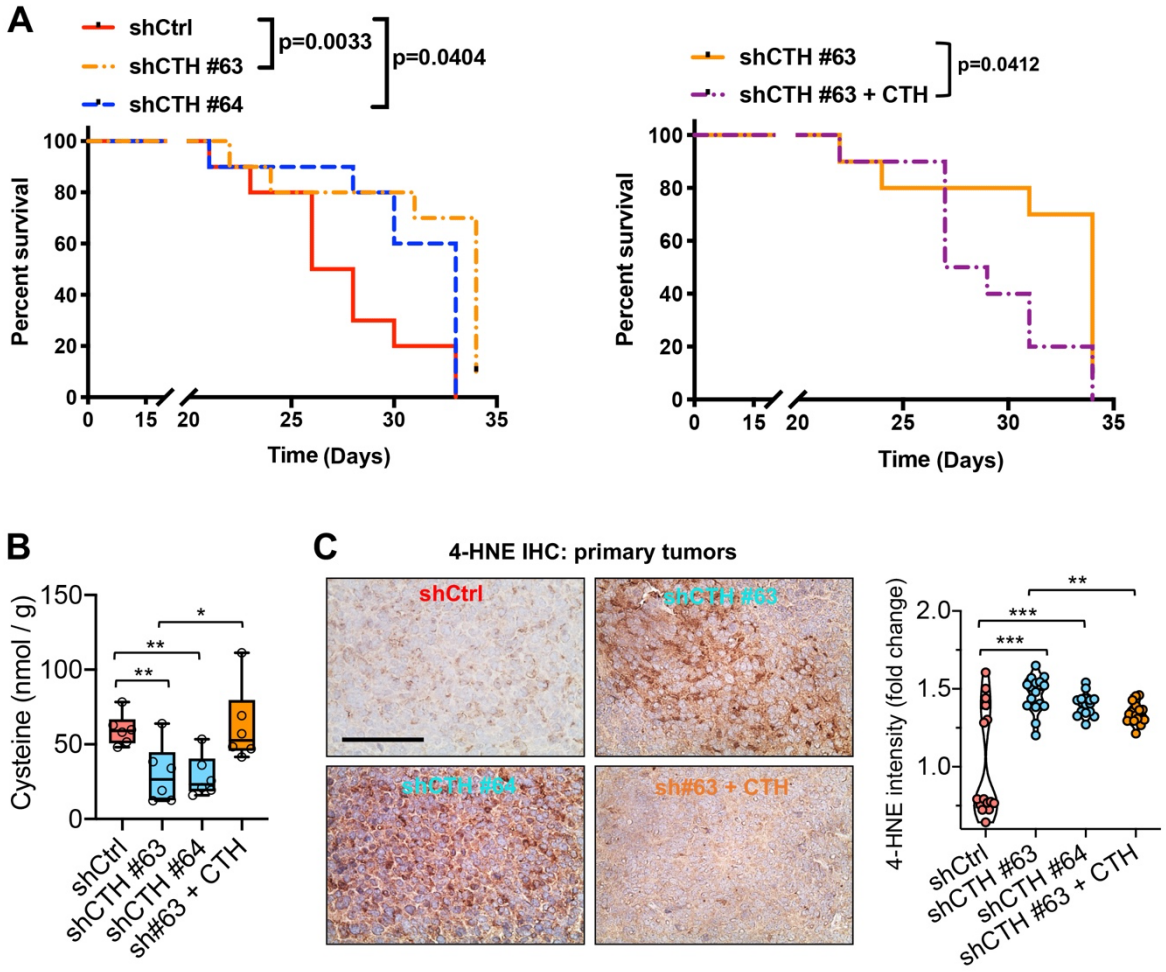
A
shCtrl
shCTH #63
shCTH #64
p=0.0033
p=0.0404
Percent survival
Time (Days)
shCTH #63
shCTH #63 + CTH
p=0.0412
Percent survival
Time (Days)
B
Cysteine (nmol / g)
shCtrl
shCTH #63
shCTH #64
sh#63 + CTH
C
4-HNE IHC: primary tumors
shCtrl
shCTH #63
shCTH #64
sh#63 + CTH
4-HNE intensity (fold change)
shCtrl
shCTH #63
shCTH #64
shCTH #63 + CTH

Fig. S8. CTH promotes EwS tumour progression *in vivo*.

A, Kaplan-Meier curves showing the tumour-free survival in mice implanted with the indicated TC32 cells in the renal subcapsular space (n=10). **B,** Intracellular cysteine pools were measured in the indicated EwS xenografts derived from TC32 (n=6). **C,** IHC staining (left) and quantification (right) of 4-HNE intensities in TC32-derived tumours in mice (n=16 fields). Scale bar, 100μm. In **A,** Log rank test was performed. In **B-C,** median and interquartile range are presented, and p values were determined by two-tailed unpaired Student's t-test, *$p < 0.05$, **$p < 0.01$; ***$p < 0.001$.

Fig. S9

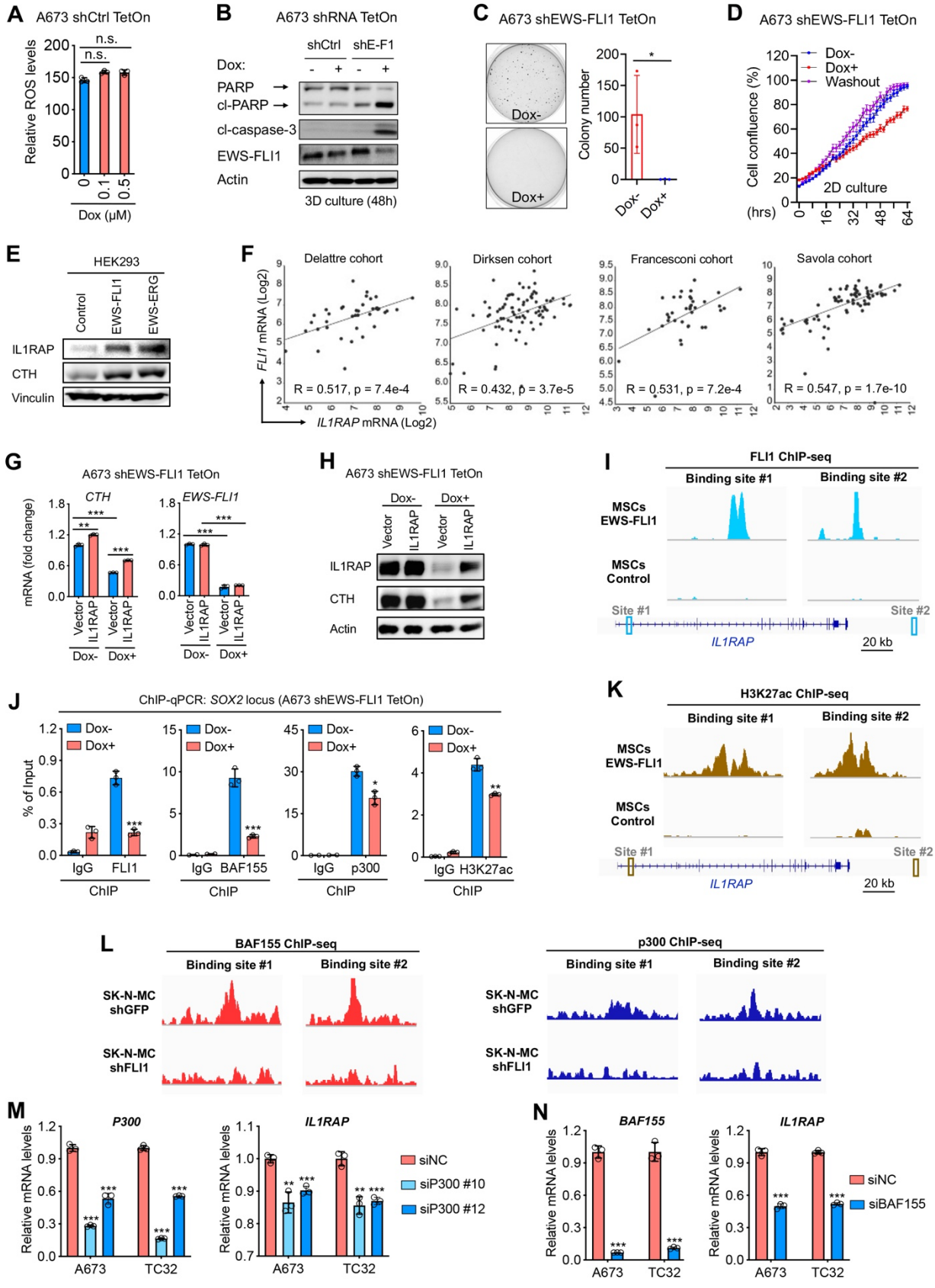
A
A673 shCtrl TetOn
Relative ROS levels
n.s.
n.s.
Dox (μM)
B
A673 shRNA TetOn
shCtrl
shE-F1
Dox:
PARP
cl-PARP
cl-caspase-3
EWS-FLI1
Actin
3D culture (48h)
C
A673 shEWS-FLI1 TetOn
Dox-
Dox+
Colony number
D
A673 shEWS-FLI1 TetOn
Dox-
Dox+
Washout
Cell confluence (%)
2D culture
(hrs)
E
HEK293
Control
EWS-FLI1
EWS-ERG
IL1RAP
CTH
Vinculin
F
Delattre cohort
Dirksen cohort
Francesconi cohort
Savola cohort
FLI1 mRNA (Log2)
IL1RAP mRNA (Log2)
R = 0.517, p = 7.4e-4
R = 0.432, p = 3.7e-5
R = 0.531, p = 7.2e-4
R = 0.547, p = 1.7e-10
G
A673 shEWS-FLI1 TetOn
CTH
EWS-FLI1
mRNA (fold change)
Vector
IL1RAP
Dox-
Dox+
H
A673 shEWS-FLI1 TetOn
Dox-
Dox+
Vector
IL1RAP
IL1RAP
CTH
Actin
I
FLI1 ChIP-seq
Binding site #1
Binding site #2
MSCs EWS-FLI1
MSCs Control
Site #1
Site #2
IL1RAP
20 kb
J
ChIP-qPCR: SOX2 locus (A673 shEWS-FLI1 TetOn)
Dox-
Dox+
% of Input
IgG
FLI1
BAF155
p300
H3K27ac
ChIP
K
H3K27ac ChIP-seq
Binding site #1
Binding site #2
MSCs EWS-FLI1
MSCs Control
Site #1
Site #2
IL1RAP
20 kb
L
BAF155 ChIP-seq
p300 ChIP-seq
Binding site #1
Binding site #2
SK-N-MC shGFP
SK-N-MC shFLI1
M
P300
IL1RAP
Relative mRNA levels
siNC
siP300 #10
siP300 #12
A673
TC32
N
BAF155
IL1RAP
Relative mRNA levels
siNC
siBAF155
A673
TC32

Fig. S9. EWS-FLI1 directly regulates *IL1RAP* expression via enhancer activation.

A, Flow cytometry-based quantification of ROS (CellROX Deep Red) 96 hr after Dox treatment. n=3 biologically independent samples. **B,** Immunoblotting analysis of the indicated proteins. The experiment was repeated three time with similar results. **C,** Soft agar analysis of the indicated cells. n=3 biologically independent samples. **D,** Cell growth in 2D culture was measured by Incucyte. Washout cells were treated with 0.5μM Dox for 4 days before Dox removal for 16 days. n>3 biologically independent samples. **E,** Immunoblotting analysis 72 hr after transfection of the indicated cDNA constructs. The experiment was repeated twice with similar results. **F,** Correlation between IL1RAP and FLI1 mRNA in different cohorts of EwS tumours. See methods for details. **G,** mRNA expression of the indicated genes was determined by qPCR analysis (n=3 biologically independent samples). Cells were transfected with empty vector or *IL1RAP* cDNA and treated with/without 0.5μM Dox for 72 hr. **H,** Immunoblot analysis in samples that were treated the same as described for (**G**). l.e. and s.e. stand for long exposure and short exposure, respectively. **I,** FLI1 ChIP-seq in MSCs expressing a control vector or *EWS-FLI1* cDNA (GEO: GSE94278). **J,** ChIP-qPCR validation of the occupancy of EWS-FLI1, BAF155, p300, and H3K27ac at the *SOX2* locus (0.5μM Dox, 72 hr, n = 3 biologically independent samples). **K** and **L,** ChIP-seq analyses of the indicated factors in MSCs expressing a control vector or *EWS-FLI1* (GEO: GSE94278), or SK-N-MC cells with shGFP control knockdown or shFLI1 knockdown (96 hr) (GEO: GSE61953). **M** and **N,** qPCR analysis in A673 and TC32 EwS cells with siRNA transfections (n = 3 biologically independent samples). Data presented are means ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, p values were determined by two-tailed unpaired Student's t-test. In **F,** Pearson correlation analysis was performed.

Fig. S10

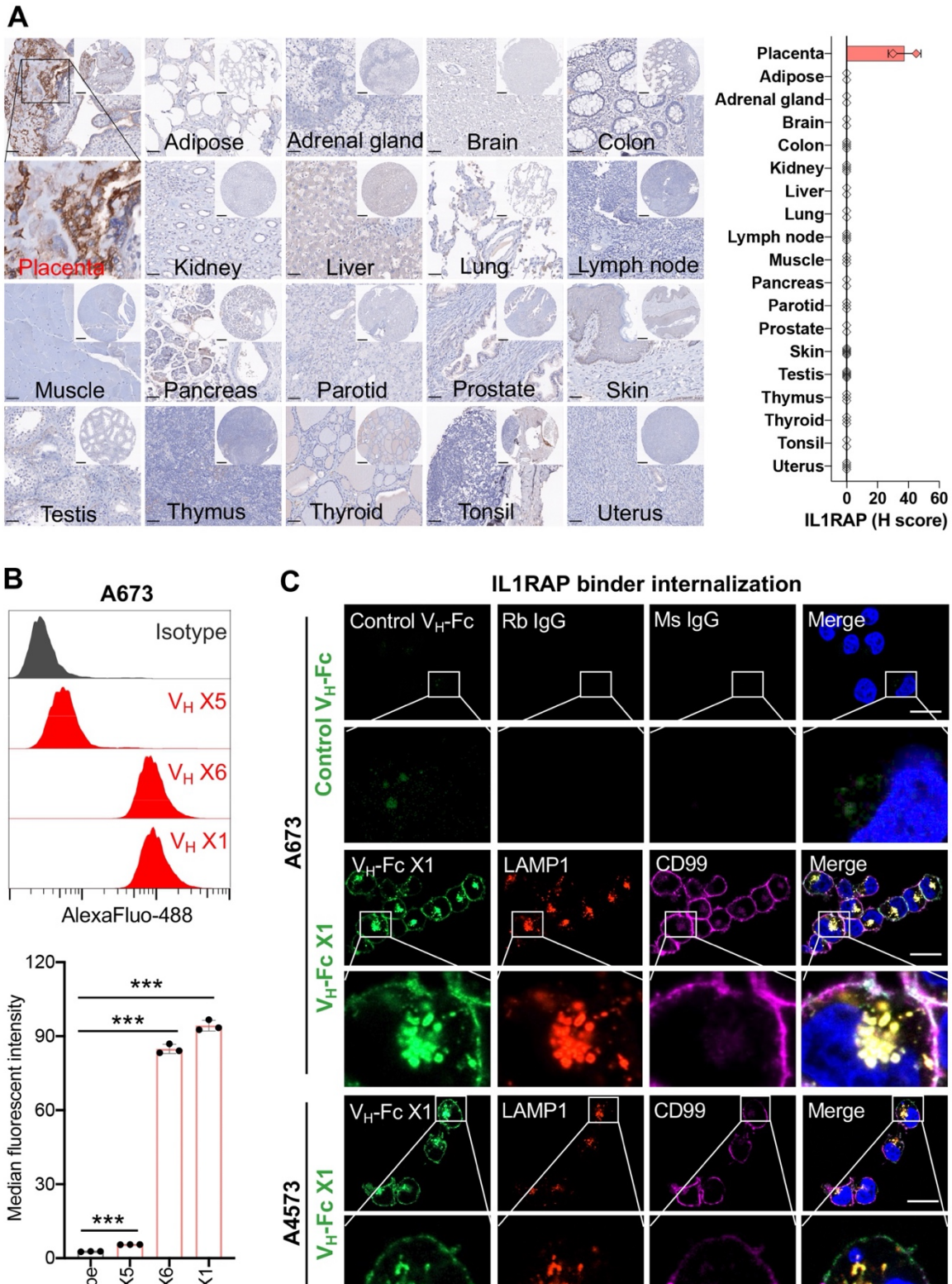
A
Adipose
Adrenal gland
Brain
Colon
Placenta
Kidney
Liver
Lung
Lymph node
Muscle
Pancreas
Parotid
Prostate
Skin
Testis
Thymus
Thyroid
Tonsil
Uterus
Placenta
Adipose
Adrenal gland
Brain
Colon
Kidney
Liver
Lung
Lymph node
Muscle
Pancreas
Parotid
Prostate
Skin
Testis
Thymus
Thyroid
Tonsil
Uterus
0 20 40 60
IL1RAP (H score)
B
A673
Isotype
VH X5
VH X6
VH X1
AlexaFluo-488
Median fluorescent intensity
120
90
60
30
0
***
***
***
Isotype
VH X5
VH X6
VH X1
C
IL1RAP binder internalization
Control VH-Fc
Rb IgG
Ms IgG
Merge
A673
Control VH-Fc
VH-Fc X1
LAMP1
CD99
Merge
A4573
VH-Fc X1

Fig. S10. IL1RAP is a potential cell surface therapeutic target in Ewing sarcoma.

A, IHC analysis of IL1RAP protein expression in adult normal organ tissue microarrays (TMAs). Scale bars, 50μm in high power images and 200μm in low power images. **B,** Flow cytometry was performed to validate the three V_H binders of IL1RAP identified by human antibody domain (V_H) phage-displayed library (n=3). **C,** IF analysis of the internalization (1 hr) of the IL1RAP binder V_H-Fc X1and a negative control V_H-Fc binder in EwS cells. Lysosome marker LAMP1 and cell surface marker CD99 was included to determine the localization of the binders. Scale bar, 10μm. All data presented are means ± SD, and two-tailed unpaired Student's t-test was performed, ***$p < 0.001$.