Expanded View Figures

Figure EV1. Transcriptional regulation of HES1 by RASSF1A.

A Analysis of TCGA RNA-seq data from clinical samples expressing highest (LAML) and lowest (ESCA) RASSF1A levels to identify transcription factors following RASSF1A expression. One of the most prominent hits was the HES1 repressor GATA1 ($P < 0.00001$).

B Top: qPCR for HES1 levels in U2OS cells transfected with non-targeting (siCTRL) or GATA1-targeting (siGATA1) siRNA. Bottom: qPCR for GATA1 levels verifying successful KD.

C qPCR for GATA1 levels in U2OS cells transfected with either siCTRL or RASSF1A-targeting (siRASSF1A) siRNA. Western blotting for RASSF1A levels demonstrates knockdown efficiency.

D qPCR for GATA1 levels in U2OS cells transfected with pCDNA3 or FLAG-RASSF1A. Western blotting demonstrates FLAG-RASSF1A induction.

Data information: Tumor type abbreviations: LAML: Acute Myeloid Leukemia; ESCA: Esophageal carcinoma. $^{**}P < 0.01$, of Student's *t*-test. Error bars indicate s.e.m. Data shown are representative of three biological replicates ($n = 3$).
Source data are available online for this figure.

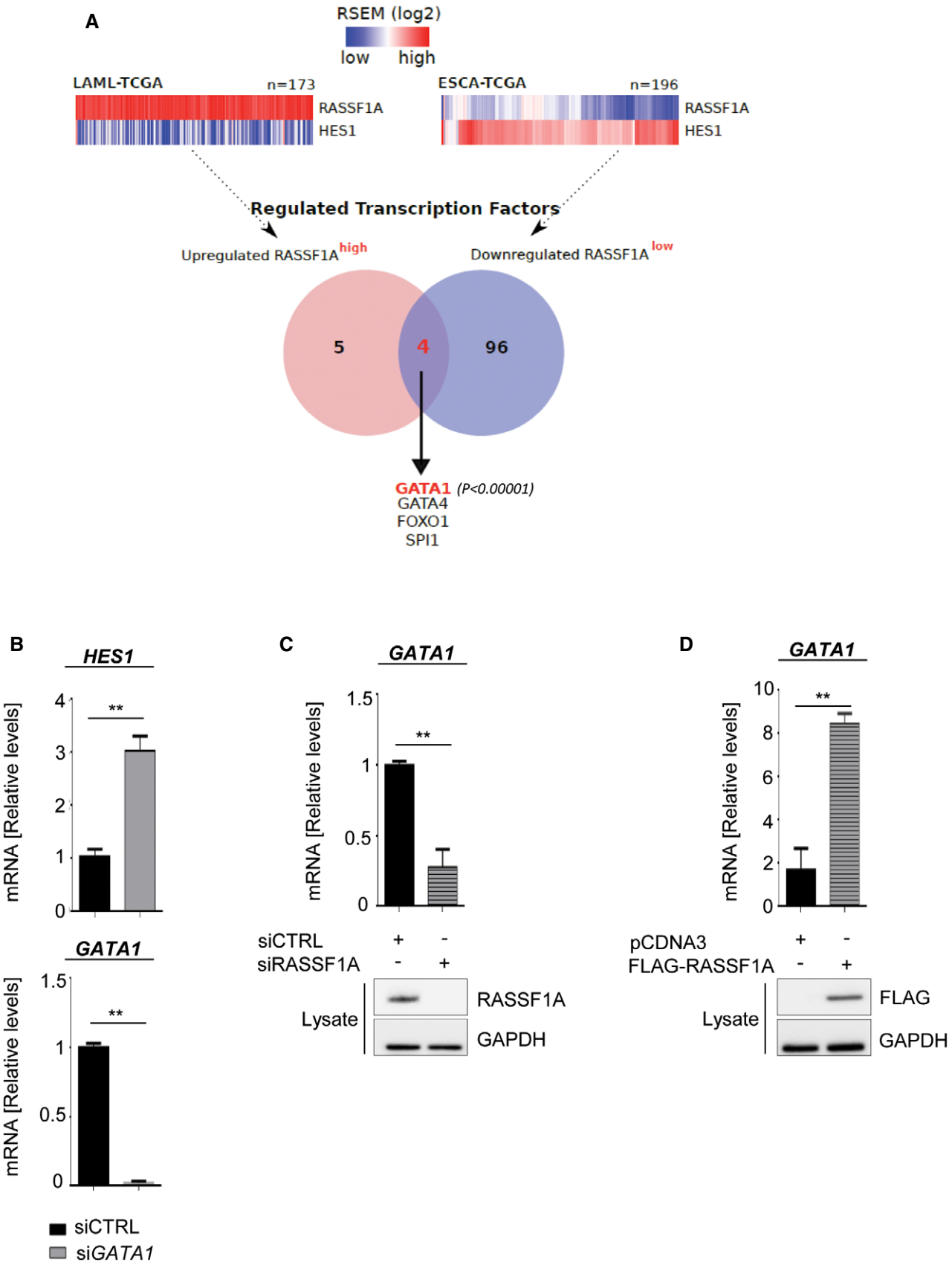
A
RSEM (log2)
low high
LAML-TCGA
n=173
RASSF1A
HES1
ESCA-TCGA
n=196
RASSF1A
HES1
Regulated Transcription Factors
Upregulated RASSF1A high
Downregulated RASSF1A low
5
4
96
GATA1 (P<0.00001)
GATA4
FOXO1
SPI1
B
HES1
mRNA [Relative levels]
**
GATA1
mRNA [Relative levels]
**
siCTRL
siGATA1
C
GATA1
mRNA [Relative levels]
**
siCTRL + -
siRASSF1A - +
Lysate
RASSF1A
GAPDH
D
GATA1
mRNA [Relative levels]
**
pCDNA3 + -
FLAG-RASSF1A - +
Lysate
FLAG
GAPDH

Figure EV1.

Figure EV2. RASSF1A-mediated ubiquitination of HES1.

A Densitometry on Fig 1G showing that only nuclear HES1 levels are significantly reduced upon RASSF1A induction, based on the nuclear HES1/LAMIN B1 ratio. Cytoplasmic HES1 levels remain unaffected based on the cytoplasmic HES1/GAPDH ratio.

B *In vivo* ubiquitination assay in HES1 immunoprecipitates from nuclear and cytoplasmic fractions of U2OS cells Tet-On inducibly expressing FLAG-RASSF1A versus Control. Doxycycline (DOX) was used at a concentration of 0.5 μg/ml for 24 h. Immunoprecipitates and Input lysates are probed with displayed antibodies.

C *In vivo* ubiquitination assay in HES1 immunoprecipitates from nuclear and cytoplasmic fractions of U2OS cells transfected with either siCTRL or siRASSF1A. Immunoprecipitates and Input lysates are probed with displayed antibodies.

Data information: $^{**}P < 0.01$, of Student's *t*-test. Error bars indicate s.e.m. Data shown are representative of three biological replicates ($n = 3$).
Source data are available online for this figure.

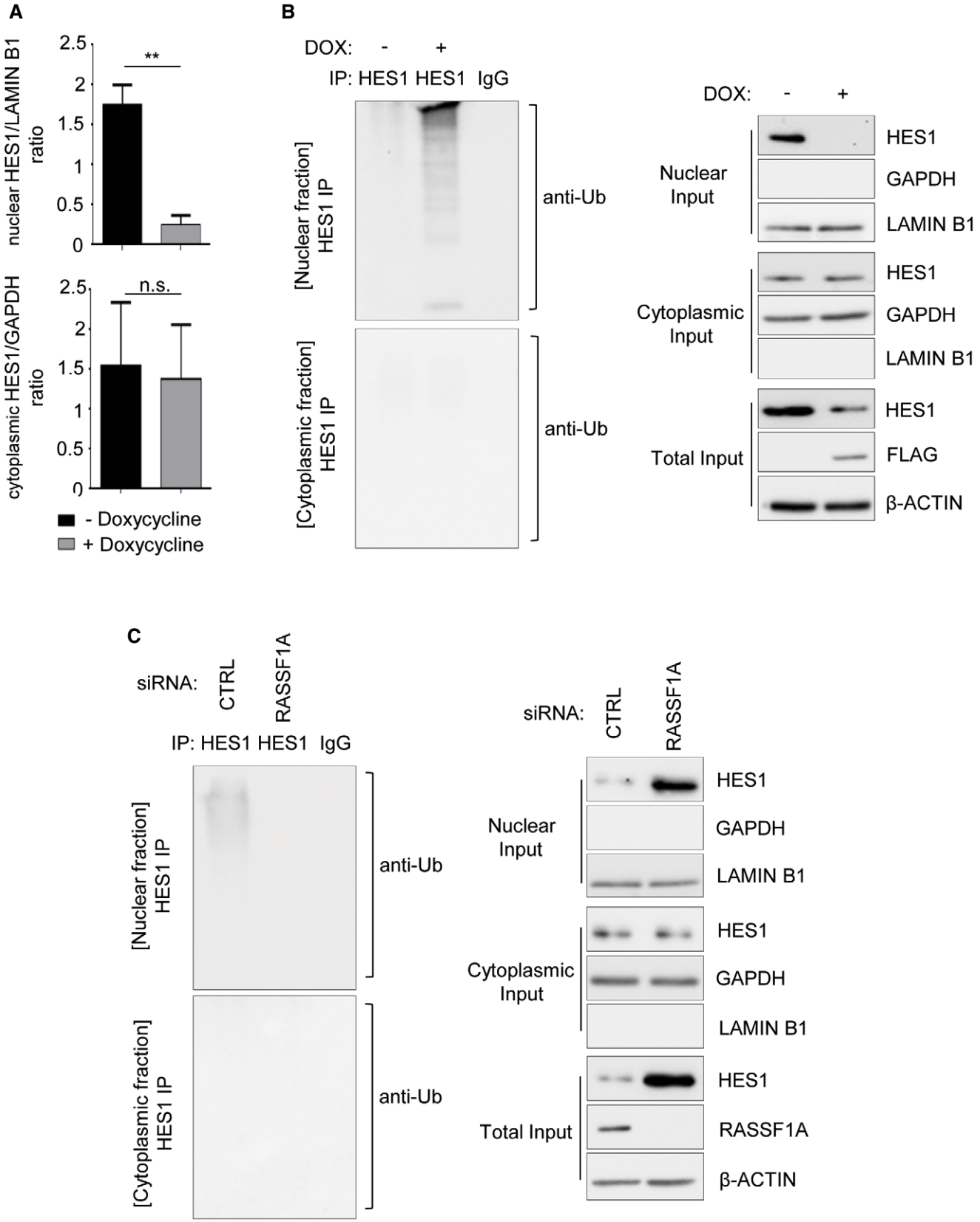

Figure EV2.

Figure EV3. RASSF1A regulates the core pluripotency markers in cancer.

A Western blotting and densitometry for core pluripotency marker expression in HeLa cells transfected with either non-targeting siRNA (siCTRL) or siRNA against RASSF1A (siRASSF1A).
B Immunofluorescence for core pluripotency marker expression in HeLa cells transfected with either siCTRL or siRASSF1A.
C qPCR for core stem cell marker levels in U2OS and HeLa cells in response to siRASSF1A versus siCTRL.
D Immunoblotting and densitometry for core pluripotency marker expression in U2OS cells transfected with either siCTRL or siRASSF1A.
E U2OS cells Tet-On inducibly expressing FLAG-RASSF1A were treated with 0.5 μg/ml of doxycycline for 24 h. Cells were subsequently lysed and lysates were immunoblotted with the indicated antibodies. Densitometry is provided for displayed Western blot images.

Data information: Scale bars: 20 μm. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$, of Student's *t*-test. Error bars indicate s.e.m. Data shown are representative of three biological replicates ($n = 3$).
Source data are available online for this figure.

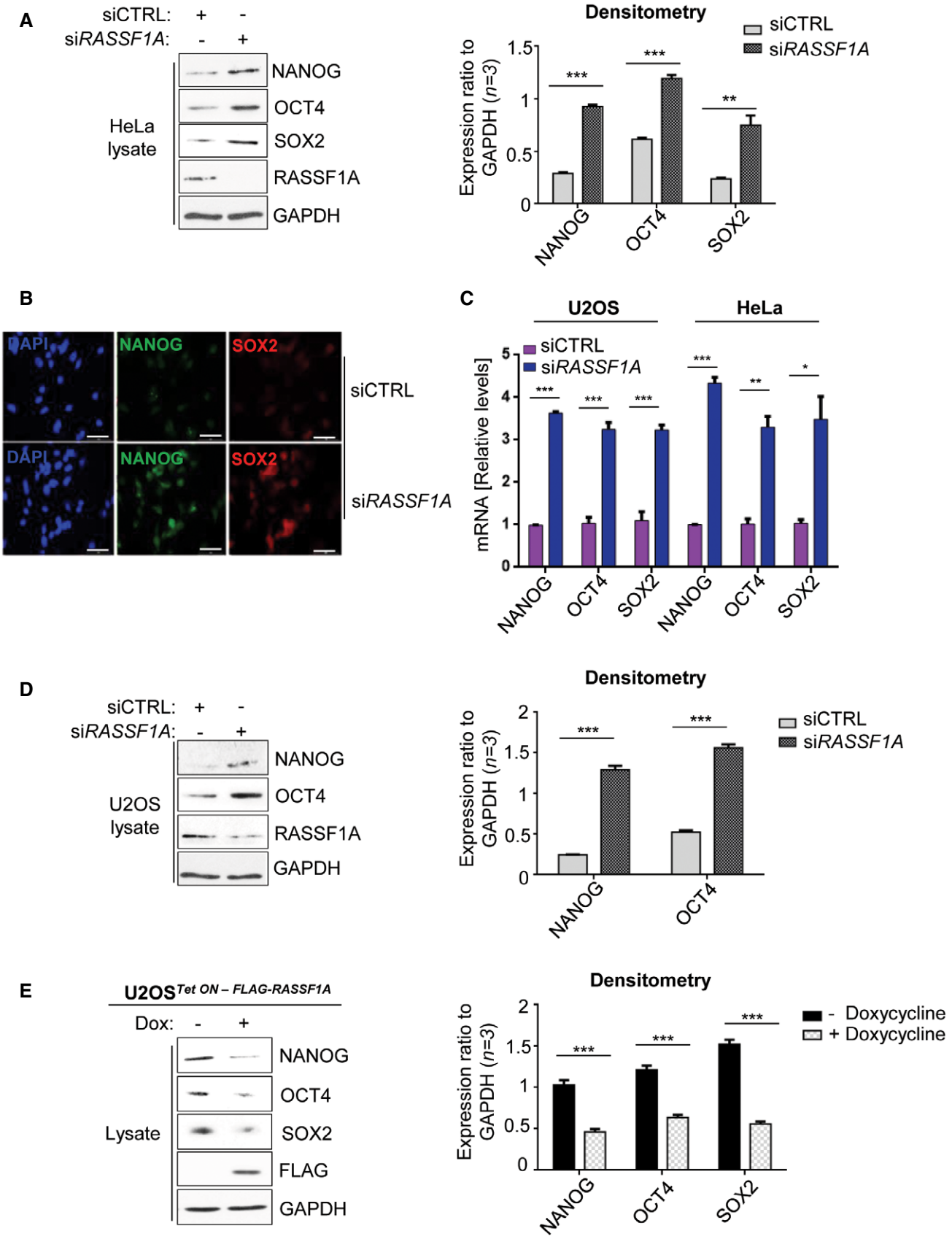
A
siCTRL: + -
siRASSF1A: - +
HeLa lysate
NANOG
OCT4
SOX2
RASSF1A
GAPDH
Densitometry
siCTRL
siRASSF1A
Expression ratio to GAPDH (n=3)
1.5
1
0.5
0
***
***
**
NANOG
OCT4
SOX2
B
DAPI
NANOG
SOX2
siCTRL
siRASSF1A
C
U2OS
HeLa
siCTRL
siRASSF1A
mRNA [Relative levels]
5
4
3
2
1
0
***
***
***
***
**
*
NANOG
OCT4
SOX2
NANOG
OCT4
SOX2
D
siCTRL: + -
siRASSF1A: - +
U2OS lysate
NANOG
OCT4
RASSF1A
GAPDH
Densitometry
siCTRL
siRASSF1A
Expression ratio to GAPDH (n=3)
2
1.5
1
0.5
0
***
***
NANOG
OCT4
E
U2OS Tet ON – FLAG-RASSF1A
Dox: - +
Lysate
NANOG
OCT4
SOX2
FLAG
GAPDH
Densitometry
- Doxycycline
+ Doxycycline
Expression ratio to GAPDH (n=3)
2
1.5
1
0.5
0
***
***
***
NANOG
OCT4
SOX2

Figure EV3.

Figure EV4. RASSF1A induction confers a differentiation phenotype accompanied by HES1 inactivation in cancer cells.

A H1299 cells transfected with either pCDNA3 or FLAG-RASSF1A and subjected to Matrigel 2D differentiation assay. RASSF1A expression induces differentiation as indicated by changes in the round shape and reduction of stem cell markers assessed by immunofluorescence.

B Quantification of NANOG and SOX2 signal intensities from (A).

C H1299 cells transfected with either pCDNA3 or FLAG-RASSF1A were lysed and probed with the indicated antibodies confirming a reciprocal HES1/RNF4 regulation in response to RASSF1A.

D HeLa cells transfected with either siCTRL or siRASSF1A were subjected or not to treatment with the GSI DBZ (100 nM) and their lysates were immunoblotted against indicated antibodies. Cleaved Notch (ICN1) levels were used as readout of DBZ treatment.

E qPCR for HES1 mRNA levels in ESC in response to the γ-secretase inhibitor DBZ (100 nM) or the Notch ligand DLL4 (1 μg/ml).

Data information: Scale bars: 50 μm. ***$P < 0.001$, of Student's *t*-test. Error bars indicate s.e.m. Data shown are representative of three biological replicates ($n = 3$).
Source data are available online for this figure.

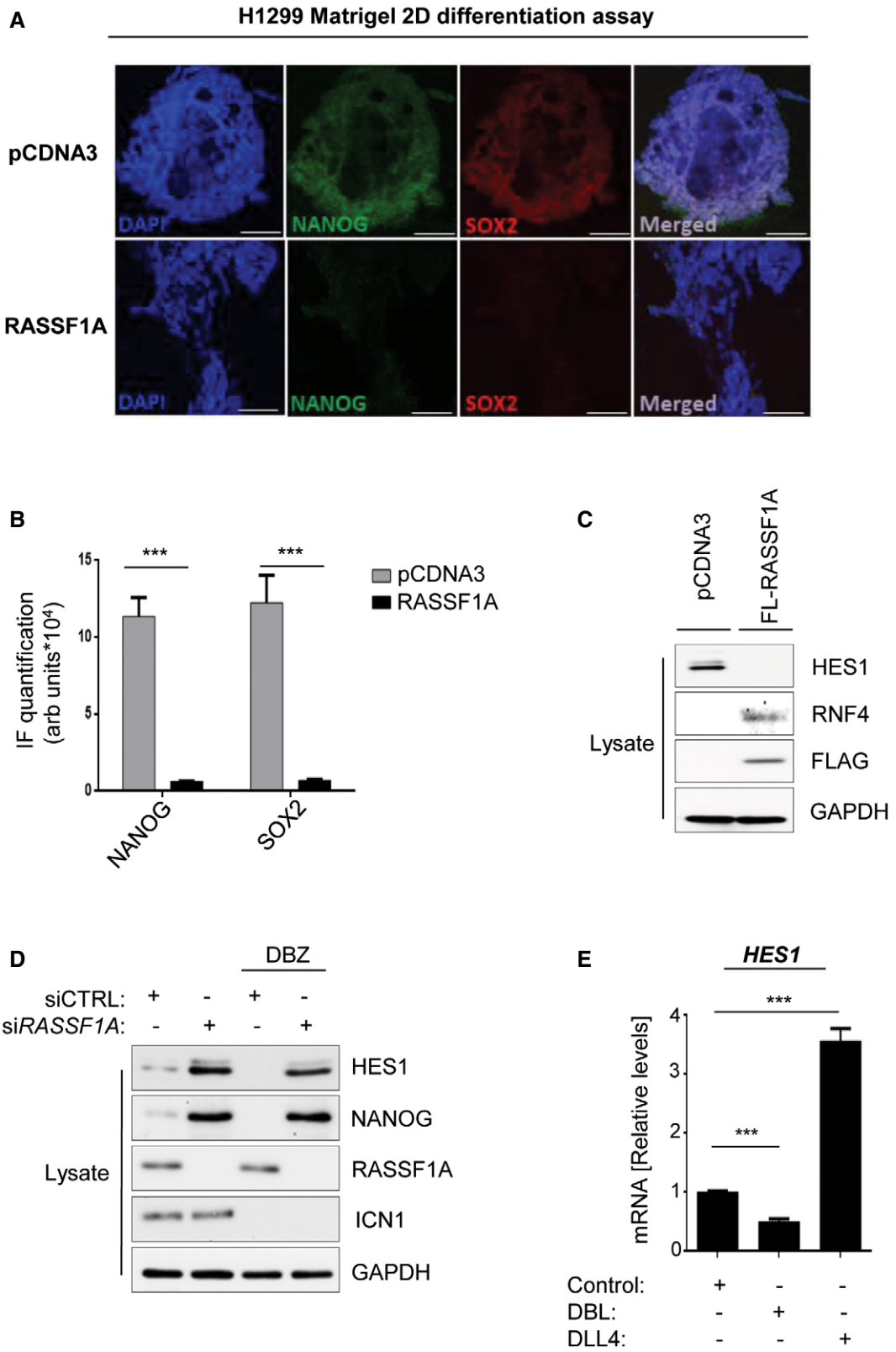
A
H1299 Matrigel 2D differentiation assay
pCDNA3
RASSF1A
DAPI
NANOG
SOX2
Merged
B
IF quantification (arb units*10⁴)
pCDNA3
RASSF1A
NANOG
SOX2
C
pCDNA3
FL-RASSF1A
Lysate
HES1
RNF4
FLAG
GAPDH
D
DBZ
siCTRL:
siRASSF1A:
Lysate
HES1
NANOG
RASSF1A
ICN1
GAPDH
E
HES1
mRNA [Relative levels]
Control:
DBL:
DLL4:

Figure EV4.

Figure EV5. HES1 activates core pluripotency marker expression in cancer cells and is regulated by RASSF1A independently of the Hippo pathway.

A U2OS cells or HeLa cells transfected with the indicated siRNAs were lysed and probed with shown antibodies. HES1 levels increase in response to RASSF1A loss regardless of YAP/TAZ signaling.

B U2OS cells or HeLa cells transfected with the indicated plasmids were lysed and Western blotted with displayed antibodies. HES1 levels decrease in response to RASSF1A induction regardless of YAP activity.

C qPCR for core pluripotency markers in U2OS and HeLa cells transfected with siCTRL, siRASSF1A, or siRASSF1A/siHES1. HES1 silencing reverses pluripotency marker increase observed upon RASSF1A loss. Immunoblotting from U2OS and HeLa cell lysates using the indicated antibodies demonstrates KD efficiencies.

D qPCR for core pluripotency markers in U2OS and HeLa cells transfected with either pCDNA3 or GFP-HES1. HES1 induction increases all core pluripotency marker levels. Western blotting from U2OS and HeLa cell lysates using the indicated antibodies demonstrates efficient HES1 induction.

Data information: ***$P < 0.001$, **$P < 0.01$, and *$P < 0.05$, of Student's t-test. Error bars indicate s.e.m. Data shown are representative of three biological replicates ($n = 3$).
Source data are available online for this figure.

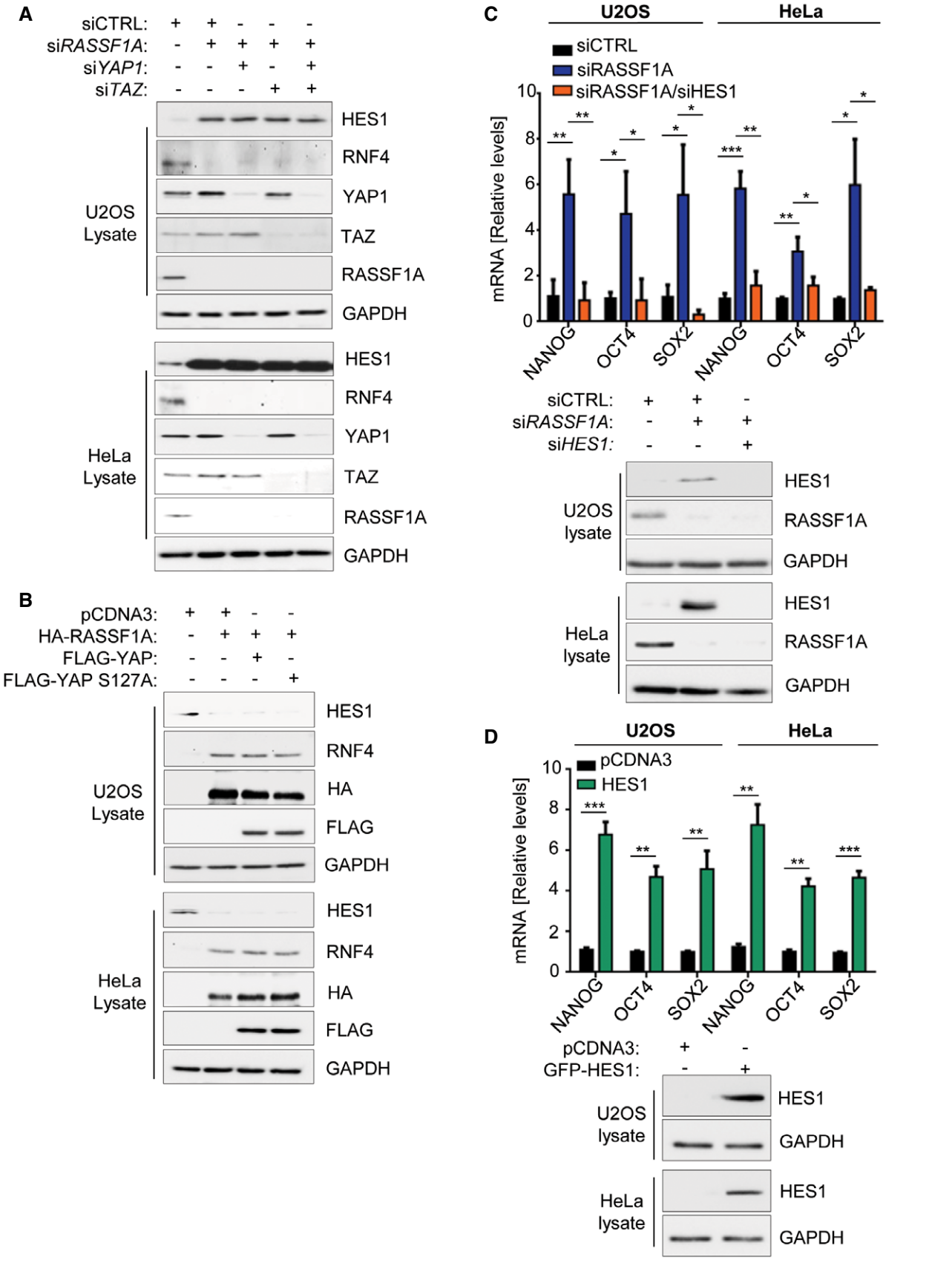
A
siCTRL:
siRASSF1A:
siYAP1:
siTAZ:
U2OS Lysate
HES1
RNF4
YAP1
TAZ
RASSF1A
GAPDH
HeLa Lysate
HES1
RNF4
YAP1
TAZ
RASSF1A
GAPDH
B
pCDNA3:
HA-RASSF1A:
FLAG-YAP:
FLAG-YAP S127A:
U2OS Lysate
HES1
RNF4
HA
FLAG
GAPDH
HeLa Lysate
HES1
RNF4
HA
FLAG
GAPDH
C
U2OS
HeLa
siCTRL
siRASSF1A
siRASSF1A/siHES1
mRNA [Relative levels]
NANOG
OCT4
SOX2
siCTRL:
siRASSF1A:
siHES1:
U2OS lysate
HES1
RASSF1A
GAPDH
HeLa lysate
HES1
RASSF1A
GAPDH
D
U2OS
HeLa
pCDNA3
HES1
mRNA [Relative levels]
NANOG
OCT4
SOX2
pCDNA3:
GFP-HES1:
U2OS lysate
HES1
GAPDH
HeLa lysate
HES1
GAPDH

Figure EV5.