Supplementary Information

Cellular senescence checkpoint function determines differential Notch1-dependent oncogenic and tumor suppressor activities

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Supplementary Table S1: Changes induced by $p14^{ARF}$ or $p16^{INK4A}$ knockdown with or without ectopically expressed ICN1

Supplementary Figures (SF) S1-S15

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SF S2: ICN1 induces senescence in EPC1 and EPC1-hTERT cells

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Supplementary Table S1 Changes induced by $p14^{ARF}$ or $p16^{INK4A}$ knockdown with or without ectopically expressed ICN1

$p16^{INK4A}$ siRNA only vs. scramble siRNA only

Cells	RNAi efficiency*	ICN1	pRb	p53	p14	p16	p21	proliferation	SABG
EPC1	90%	(-)	↓↓	↑	↔	↓↓	↓	↓↓	↔
EPC2-hTERT	90%	(-)	↑↑	↑↑	↔	↓↓	↑↑	↑↑	↔
EPC2-T	70%	(-)	↑↑	↔†	↓↓	↓↓	↑	↑↑#	↔

$p16^{INK4A}$ siRNA with ICN1 vs. scramble siRNA with ICN1

Cells	ICN1	pRb	p53	p14	p16	p21	proliferation	SABG
EPC1	(+)	↑↑	↑	↑	↓↓	↔	↑↑	↓↓
EPC2-hTERT	(+)	↑↑	↔	↔	↓↓	↑↑	↑↑	↓↓
EPC2-T	(+)	↑↑	↔†	↓↓	↓↓	↔	↑↑	↓↓

$p14^{ARF}$ siRNA only vs. scramble siRNA only

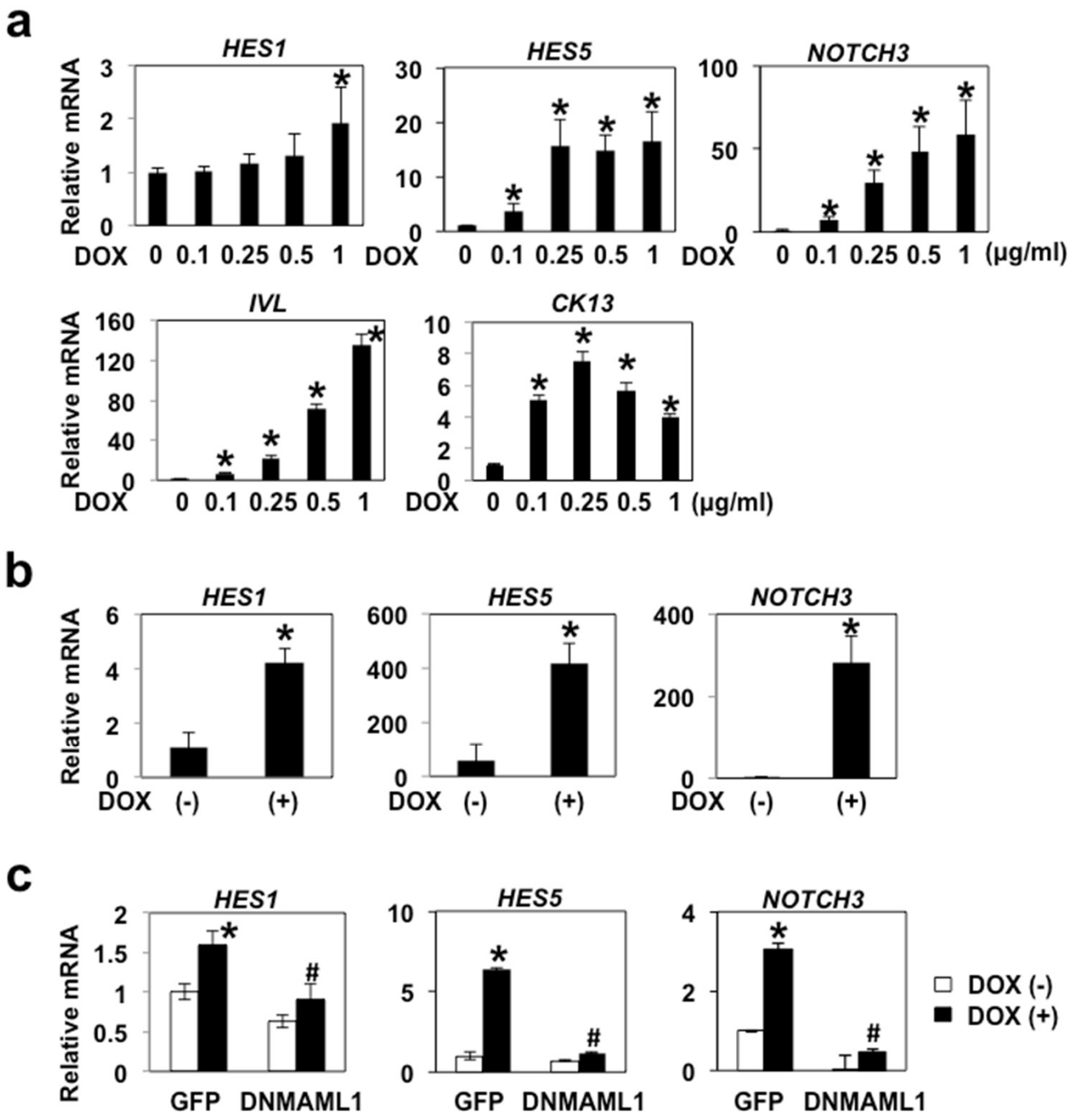
Cells	RNAi efficiency*	ICN1	pRb	p53	p14	p16	p21	proliferation	SABG
EPC1	90%	(-)	↑	↑↑	↓↓	↔	↑	↔	↔
EPC2-hTERT	50%	(-)	↑↑	↓	↓↓	↔	↓↓	↔	↔
EPC2-T	35%	(-)	↓↓	↔†	↓	↑↑	↔	↓#	↔

$p14^{ARF}$ siRNA with ICN1 vs. scramble siRNA with ICN1

Cells	ICN1	pRb	p53	p14	p16	p21	proliferation	SABG
EPC1	(+)	↓	↔	↓	↔	↓↓	↔	↔
EPC2-hTERT	(+)	*nd*	↔	*nd*	↔	*nd*	↔	↔
EPC2-T	(+)	↓↓	↔†	↓↓	↔	↔	↔	↔

The summary of densitometry of Western blotting along with cell proliferation and SABG assay data shown in **Figure 4** and **Supplementary Figures S9 and S10**. Genes targeted by RNAi was highlighted in red. *, RNAi efficiency was determined by comparing the signal intensity for either $p14^{ARF}$ or $p16^{INK4A}$ in cells transfected with specific siRNA (**Figure 4a**) compared to those transfected with scramble non-silencing siRNA in the absence of ectopically expressed ICN1. *nd*, not determined due to a very low signal intensity for cells with scramble siRNA and ectopically expressed ICN1. #, cell proliferation data compared at day 5 as cell proliferation reached plateau by day 7. All other data were compared at day 7. ↑↑ or ↓↓, > 1.5 fold-expression difference; ↑ or ↓, 1.2- to 1.5-fold-expression difference; ↔, no change of the signal intensity in Western blot densitometry data and the colorimetric data in WST1 cell proliferation assays. ↓↓, > 2-fold difference; and ↔, no change in SABG positive cell rate in SABG assays. pRb, phospho-Rb^{Ser780}. †, Dominant negative p53 ($p53^{R175H}$) is constitutively expressed in EPC2-T cells.

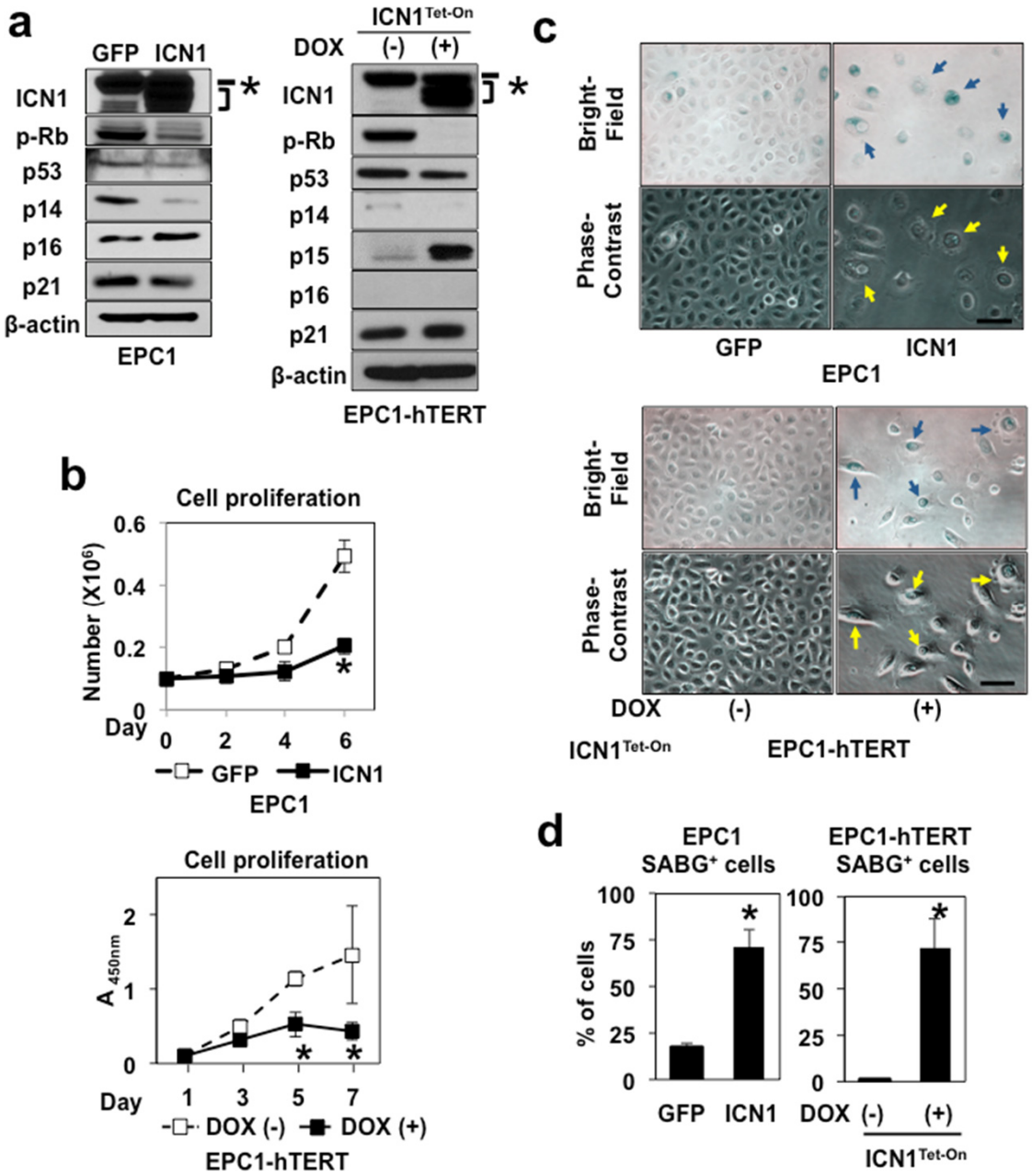
Note that molecular changes and cellular responses to RNAi directed against either *$p14^{ARF}$* or *$p16^{INK4A}$* appeared to be complex, influencing their expression mutually as well as p53 or p21 in a context-dependent manner and that were not necessarily explained by the non-specific cross-reactivity in RNAi. For example, $p16^{INK4A}$ knockdown reduced $p14^{ARF}$ expression in EPC2-T, but not EPC2-hTERT or EPC1 with or without ectopically expressed ICN1 (**Figure 4b; Supplementary Figures S9a and S10a and Supplementary Table S1**). In EPC2-T and EPC2-hTERT cells, $p16^{INK4A}$ knockdown resulted in accelerated basal cell proliferation with increased Rb phosphorylation, despite concurrent p21 upregulation in the absence of ectopically expressed ICN1 (**Figure 4b and c; Supplementary Figure S9a and b**). By contrast and unexpectedly, $p16^{INK4A}$ knockdown in EPC1 decreased basal cell proliferation and Rb phosphorylation although SABG positive cells were not significantly increased (**Supplementary Figure S10**). In the absence of ectopically expressed ICN1, $p14^{ARF}$ knockdown in EPC2-T cells resulted in decreased basal cell proliferation with concurrent Rb dephosphorylation and $p16^{INK4A}$ upregulation without inducing the SABG activity (**Figure 4**), despite limited $p14^{ARF}$ knockdown efficiency (i.e. 35%). Such changes were not induced in EPC2-hTERT and EPC1 cells by RNAi directed against $p14^{ARF}$ despite greater $p14^{ARF}$ knockdown efficiency (50-90%) was achieved (**Figure 4a; Supplementary Figures S9a and S10a; Supplementary Table S1**). $p14^{ARF}$ knockdown also augmented $p16^{INK4A}$ expression in EPC2-T cells in the presence of ectopically expressed ICN1 (**Figure 4b**), however, $p14^{ARF}$ knockdown neither affected $p16^{INK4A}$ expression nor ICN1-induced senescence in EPC2-hTERT and EPC1 cells (**Supplementary Figures S9 and S10; Supplementary Table S1**).



Supplementary Figure S1 ICN1 induces Notch target genes in EPC1-hTERT, EPC2-hTERT and EPC2-T cells

EPC2-hTERT (**a**), EPC1-hTERT (**b**), and EPC2-T with either DNMAML1 or GFP (control)(**c**), each carrying *ICN1*$^{Tet\text{-}On}$, were treated with DOX at indicated concentrations for 7 days in (**a**) or 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX for 72 h in (**b**) and (**c**). Following DOX treatment, cells were analyzed by quantitative RT-PCR for mRNA of indicated Notch1 downstream-target genes. β-actin served as an internal control. In (**a**) and (**b**), *, $P < 0.05$ vs. 0 μg/ml DOX or DOX (-); (n=3). In (**c**), *, $P < 0.05$ vs. GFP and DOX (-); #, $P < 0.05$ vs. GFP and DOX (+); (n=3).

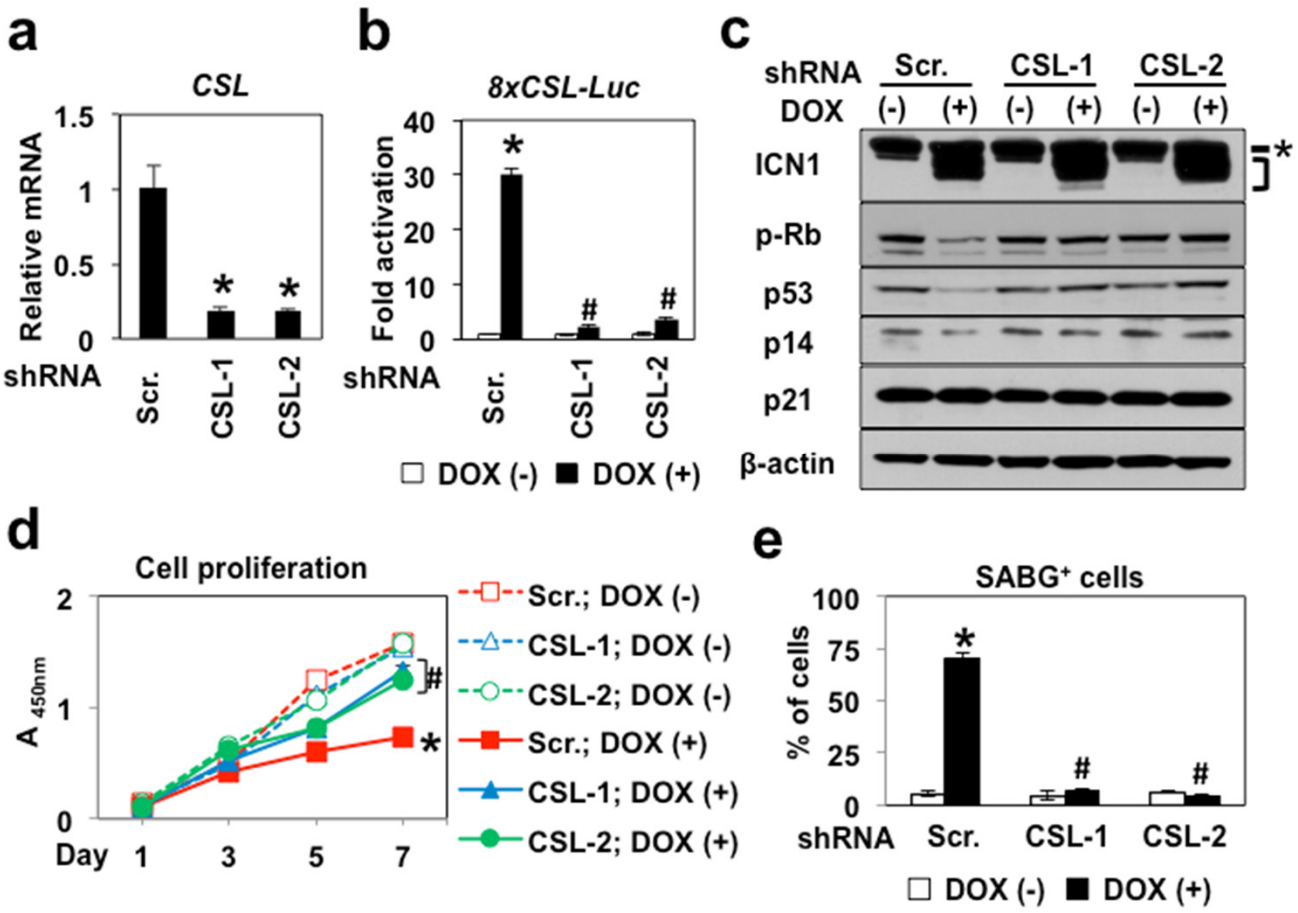
Figure S1



Supplementary Figure S2 ICN1 induces senescence in EPC1 and EPC1-hTERT cells

Non-immortalized EPC1 was transduced with a retrovirus expressing constitutively either ICN1 (co-expressed with GFP) or GFP alone (control), where GFP expression validated >90% gene transduction efficiency. EPC1-hTERT, a telomerase-immortalized derivative of EPC1, carrying *ICN1*$^{Tet\text{-}On}$ was treated with either 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX to induce ICN1. Cells were subjected to Western blotting for ICN1, phospho-Rb^{S780} (p-Rb), p53 and cell-cycle regulators in (**a**); assessment of cell proliferation in (**b**); and SABG assays in (**c**) and (**d**) 7 days after ICN1 or GFP transduction or DOX treatment. Cell proliferation was assessed by counting cells for EPC1 and WST1 assays for EPC1-hTERT, respectively, at indicated time points in (**b**). In (**a**), β-actin served as a loading control. * denotes transmembrane/intracellular region of endogenous Notch1. Bracket indicates virally expressed ICN1. In (**b**), *, $P < 0.05$ vs. DOX (-) or GFP at the indicated time points; n=3 for EPC1 and n=6 for EPC1-hTERT. In (**c**), representative bright-field and phase contrast images of SABG-positive cells with flat and enlarged cell morphology (arrows) were shown as scored in (**d**); *, $P < 0.05$ vs. DOX (-) or GFP; (n=6).

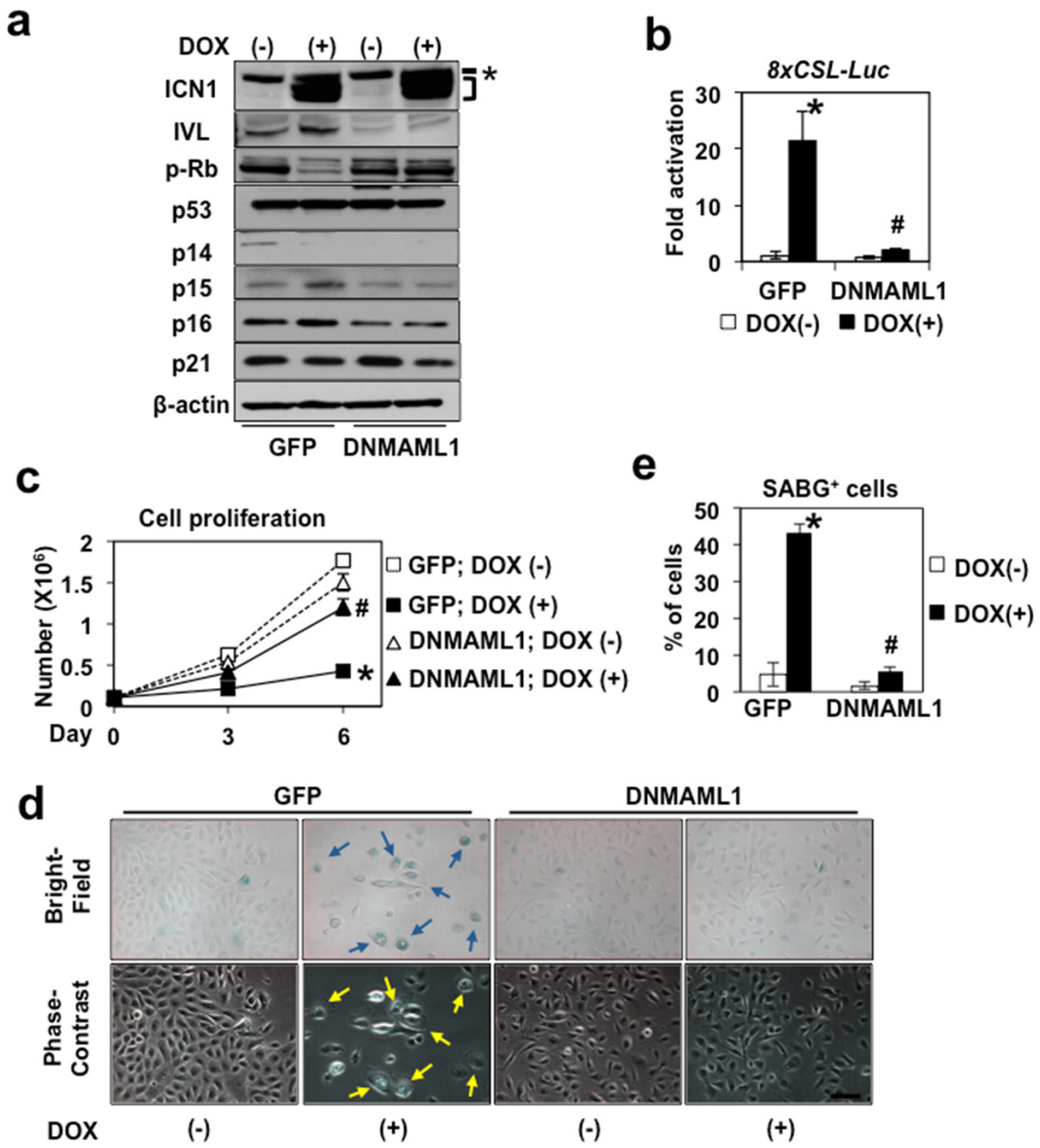
Figure S2



Supplementary Figure S3 CSL knockdown inhibits ICN1-mediated senescence in EPC1-hTERT cells

EPC1-hTERT carrying *ICN1*$^{Tet\text{-}On}$ was stably transduced with lentivirus expressing two independent shRNA sequences directed against CSL (CSL-1 and CSL-2) or a non-silencing control scramble shRNA (Scr.) sequence. In (**b**), cells were transiently transfected with 8x*CSL-luc* 24 h before DOX treatment. Cells were treated with 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX to induce ICN1 for 48 h in (**b**) and 7 days in (**c**)-(**e**). Cells were harvested at indicated time points in (**d**). Following DOX treatment, cells were subjected to quantitative RT-PCR for *CSL* mRNA in (**a**); luciferase assays for *8xCSL-luc* reporter activity in (**b**); Western blotting for ICN1, phospho-Rb^{S780} (p-Rb), p53, and cell-cycle regulators in (**c**); WST1 assays for cell proliferation in (**d**); and SABG assays in (**e**). In (**a**), β-actin served as an internal control. *, $P < 0.05$ vs. Scr. (n=3). In (**b**), *, $P < 0.05$ vs. Scr. and DOX (-); #, $P < 0.05$ vs. Scr. and DOX (+); (n=3). In (**c**), β-actin served as a loading control. * denotes transmembrane/intracellular region of endogenous Notch1. Bracket indicates lentivirally expressed ICN1 induced by DOX. In (**d**), *, $P < 0.05$ vs. Scr. and DOX (-) at day 7; #, $P < 0.05$ vs. Scr. and DOX (+) at day 7; (n=6). In (**e**), SABG-positive cells with flat and enlarged cell morphology (image not shown) were scored; *, $P < 0.05$ vs. Scr. and DOX (-); #, $P < 0.05$ vs. Scr. and DOX (+); (n=6).

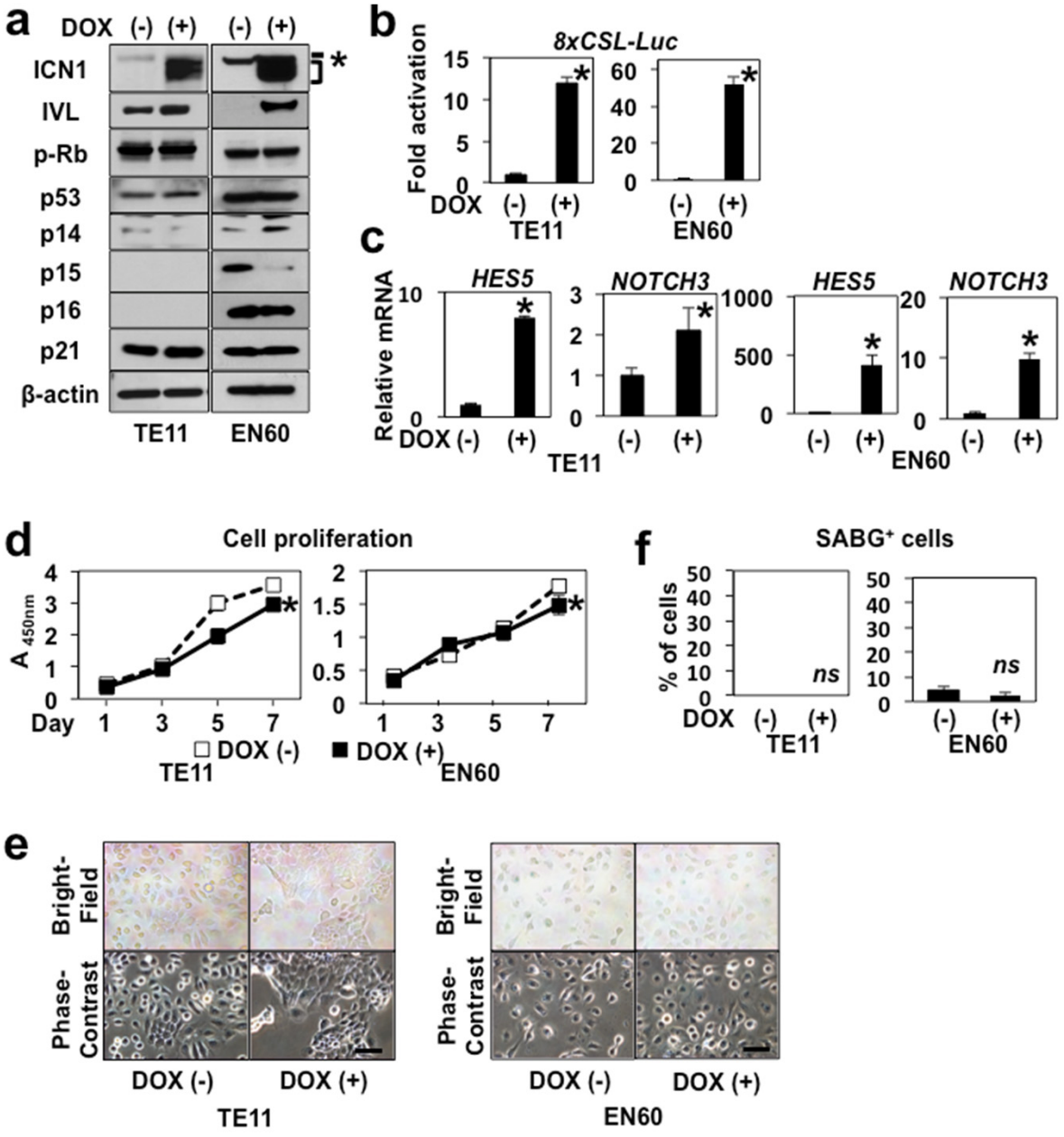
Figure S3



Supplementary Figure S4 DNMAML1 inhibits ICN1-mediated senescence in EPC2-T cells

EPC2-T, a transformed derivative of EPC2-hTERT, carrying *ICN1*$^{Tet\text{-}On}$ along with either *DNMAML1* or *GFP* (control) was treated with 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX to induce ICN1 in (**a**)-(**e**). In (**b**), Cells were transiently transfected with 8x*CSL-luc* 24 h before DOX treatment and exposed to DOX for 48 h in (**b**). In (**a**), (**d**) and (**e**), cells were exposed to DOX for 7 days. In (**c**), cells were exposed to DOX for indicated time period. Following DOX treatment, cells were subjected to Western blotting for ICN1, phospho-Rb^{S780} (p-Rb), p53, and cell-cycle regulators in (**a**); luciferase assays for *8xCSL-luc* reporter activity in (**b**); assessment of cell proliferation by counting cells in (**c**); and SABG assays in (**d**) and (**e**). In (**a**), β-actin served as a loading control. * denotes transmembrane/intracellular region of endogenous Notch1. Bracket indicates lentivirally expressed ICN1. In (**b**), *, $P < 0.05$ vs. GFP and DOX (-); #, $P < 0.05$ vs. GFP and DOX (+); (n=3). In (**c**), *, $P < 0.05$ vs. GFP and DOX (-); #, $P < 0.05$ vs. GFP and DOX (+) at indicated time point; (n=3). In (**d**), representative bright-field and phase contrast images of SABG-positive cells with flat and enlarged cell morphology (arrows) as scored in (**e**); *, $P < 0.05$ vs. GFP and DOX (-); #, $P < 0.05$ vs. GFP and DOX (+); (n=6).

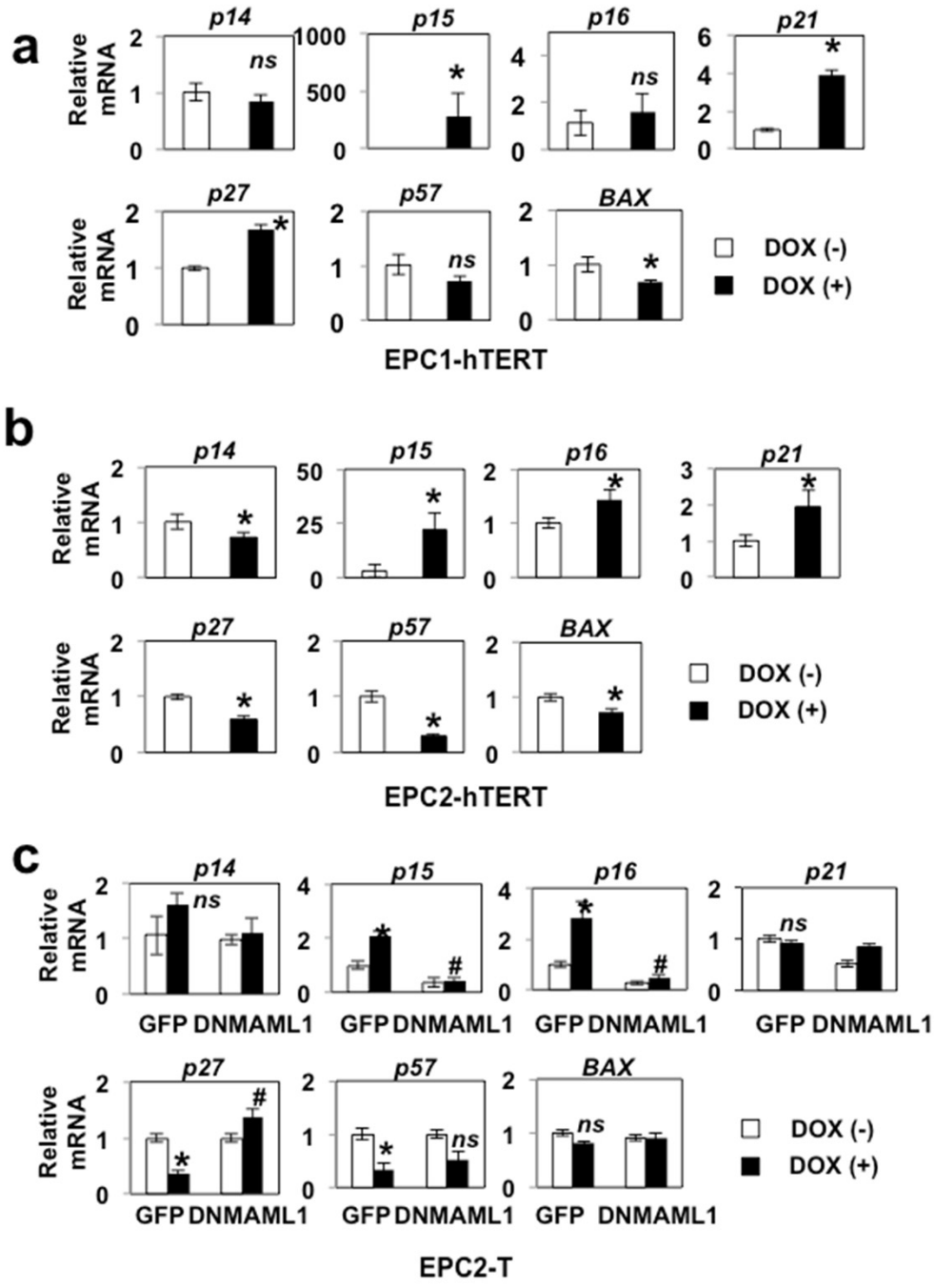
Figure S4



Supplementary Figure S5 ICN1 fails to induce senescence in EN60 and TE11 cells with dysfunctional p53 and p16^{INK4A}-Rb pathways

TE11 (biallelic *p53* inactivation and *INK4* deletion) and EN60 (HPV E6/E7), carrying *ICN1*$^{Tet\text{-}On}$, were treated with 0 µg/ml [DOX (-)] or 1 µg/ml [DOX (+)] of DOX to induce ICN1. In (**a**), (**b**) and (**c**) cells were exposed to DOX for 72 h, 48 h and 72 h, respectively. In (**d**), cells were exposed to DOX for indicated time period. In (**e**) and (**f**), cells were exposed to DOX for 7 days. In (**b**), cells were transiently transfected with 8x*CSL-luc* 24 h before DOX treatment. Following DOX treatment, cells were analyzed by Western blotting for indicated molecules in (**a**); luciferase assays for *8xCSL-luc* reporter activity in (**b**); quantitative RT-PCR for the indicated Notch1 target genes in (**c**); WST1 assays for cell proliferation in (**d**). In (**a**), β-actin served as a loading control. * denotes transmembrane/intracellular region of endogenous Notch1. Bracket indicates lentivirally expressed ICN1 induced by DOX. In (**b**), *, $P < 0.05$ DOX (-); (n=3). In (**c**), β-actin serves as an internal control. *, $P < 0.05$ vs. DOX (-); (n=3). In (**d**), *, $P < 0.05$ vs. DOX (-) at each time point (n=6). In (**e**), representative bright-field and phase contrast images document the absence of SABG-positive cells as scored in (**f**). *ns*, *not significant* vs. DOX (-); (n=6). Note that a modestly reduced cell proliferation in response to ICN1 observed in (**d**) may be accounted for by squamous-cell differentiation suggested by an induction of IVL in (**a**).

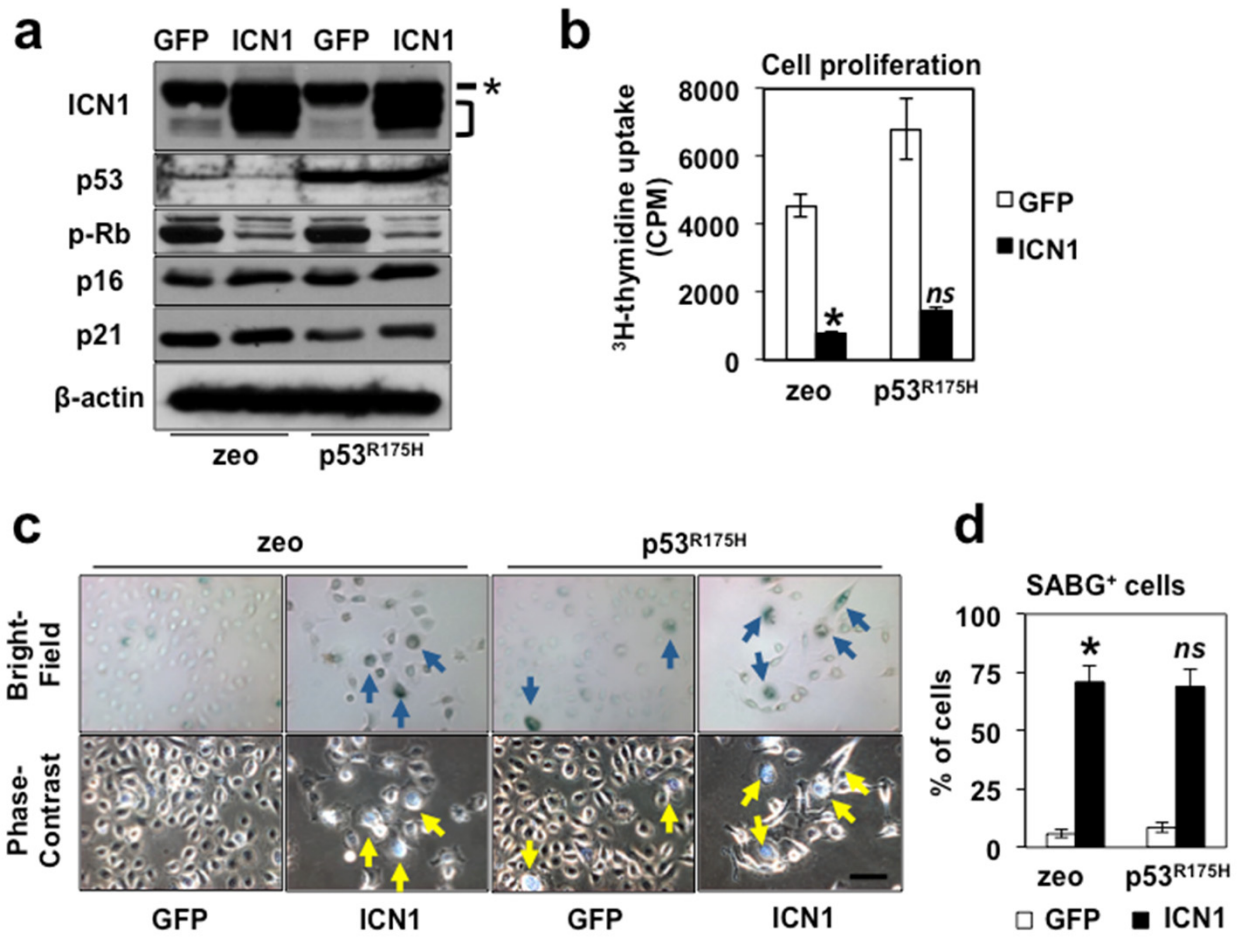
Figure S5



Supplementary Figure S6 ICN1 induces *p15*^{INK4B} and *p16*^{INK4A} in EPC1-hTERT, EPC2-hTERT and EPC2-T cells

EPC1-hTERT (**a**), EPC2-hTERT (**b**), and EPC2-T with either DNMAML1 or GFP (control)(**c**), each carrying *ICN1*$^{Tet\text{-}On}$, were treated with 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX for 72 h. Following DOX treatment, cells were analyzed by quantitative RT-PCR for indicated cell-cycle regulators and *BAX*. β-actin served as an internal control. In (**a**) and (**b**), *, $P < 0.05$ vs. DOX (-); *ns*, not significant vs. DOX (-); (n=3). In (**c**), *, $P < 0.05$ vs. GFP and DOX (-); #, $P < 0.05$ vs. GFP and DOX (+); *ns*, not significant vs. GFP and DOX (-) for *p14* and *p21*; *ns*, not significant vs. DNMAML1 and DOX (-) for *p57* and *BAX*; (n=3).

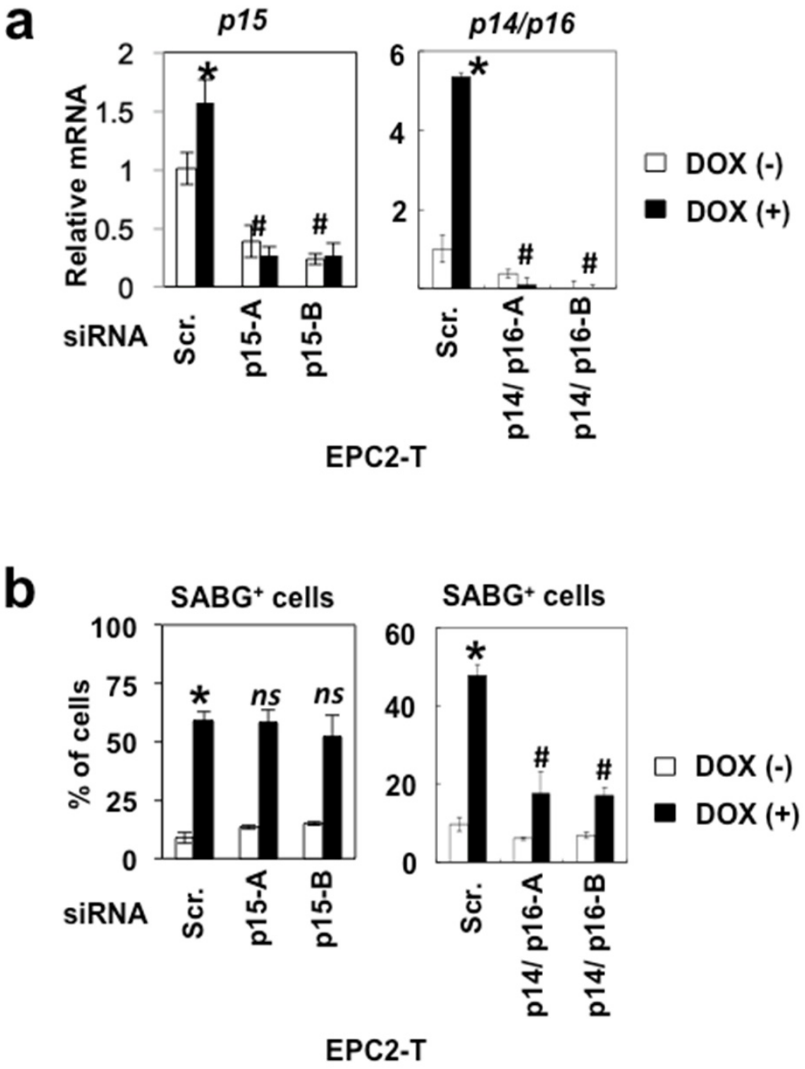
Figure S6



Supplementary Figure S7 $p53^{R175H}$ fails to prevent ICN1 from inducing senescence in EPC2-hTERT cells

EPC2-hTERT carrying either $p53^{R175H}$ or zeo (an empty vector control) was transduced with a retrovirus expressing constitutively either ICN1 (co-expressed with GFP) or GFP alone (control), where GFP expression validated >90% gene transduction efficiency. At 72 h following retrovirus-mediated ICN1 transduction, cells were subjected to Western blotting for indicated molecules in (**a**) and [^{3}H] thymidine incorporation assays to assess cell proliferation as described (Takaoka M *et al*. Oncogene 2004; 23: 6760-6768) in (**b**). SABG assays were carried out 7 days after ICN1 transduction in (**c**) and (**d**). In (**a**), β-actin served as a loading control. * denotes transmembrane/intracellular region of endogenous Notch1. Bracket indicates retrovirally expressed ICN1. In (**b**), *, *P* <0.05 vs. zeo and GFP; *ns*, not significant vs. zeo and ICN1; (n=4). In (**c**), representative bright-field and phase contrast images of SABG-positive cells with flat and enlarged cell morphology (arrows) as scored in (**d**). *, *P* <0.05 vs. zeo and GFP; *ns*, not significant vs. zeo and ICN1; (n=6). Note that dominant negative p53 ($p53^{R175H}$) did not affect Rb dephosphorylation or the SABG activity induced by ICN1.

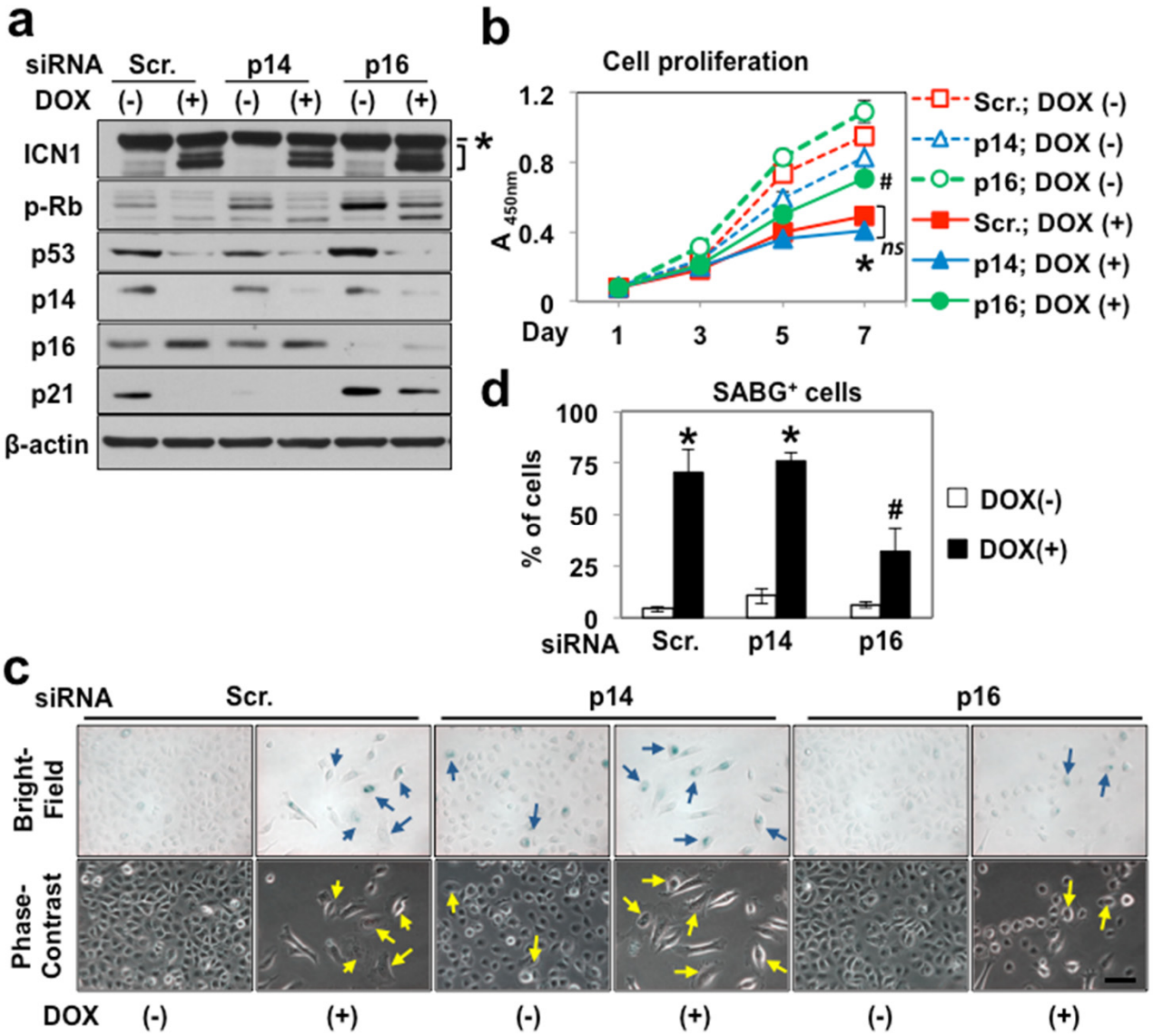
Figure S7



Supplementary Figure S8 $p15^{INK4B}$ knockdown fails to prevent ICN1 from inducing senescence in EPC2-T cells

EPC2-T carrying *ICN1*$^{Tet\text{-}On}$ was treated with 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX to induce ICN1 following transient transfection with independent siRNA sequences directed against $p15^{INK4B}$ (p15-A and p15-B), $p14^{ARF}$/$p16^{INK4A}$ (p14/p16-A and p14/p16-B)(*INK4* exon 3; **Figure 4**a), or a non-silencing control scramble short interfering RNA (Scr.). DOX treatment was done for 7 days starting 24 h after transfection. Cells were analyzed by quantitative RT-PCR to determine mRNA for *p14*^{ARF}/*p16*^{INK4A} (*p14*/*p16*) or *p15*^{INK4B} (*p15*) in (**a**); and SABG assays in (**b**). In (**a**), β-actin served as an internal control. *, $P < 0.05$ vs. Scr. and DOX (-); #, $P < 0.05$ vs. Scr. and DOX (+); (n=3). In (**b**), SABG-positive cells were scored (photomicrographs not shown). *, $P < 0.05$ vs. Scr. and DOX (-); #, $P < 0.05$ vs. Scr. and DOX (+); *ns*, not significant vs. Scr. and DOX (+); (n=6). Note that quantitative RT-PCR (TaqMan® Gene Expression Assay Hs99999189_m1) for CDKN2A/$p16^{INK4a}$ used here does not distinguish $p14^{ARF}$ and $p16^{INK4A}$, albeit validating the RNAi efficiency targeting both $p14^{ARF}$ and $p16^{INK4A}$ concurrently.

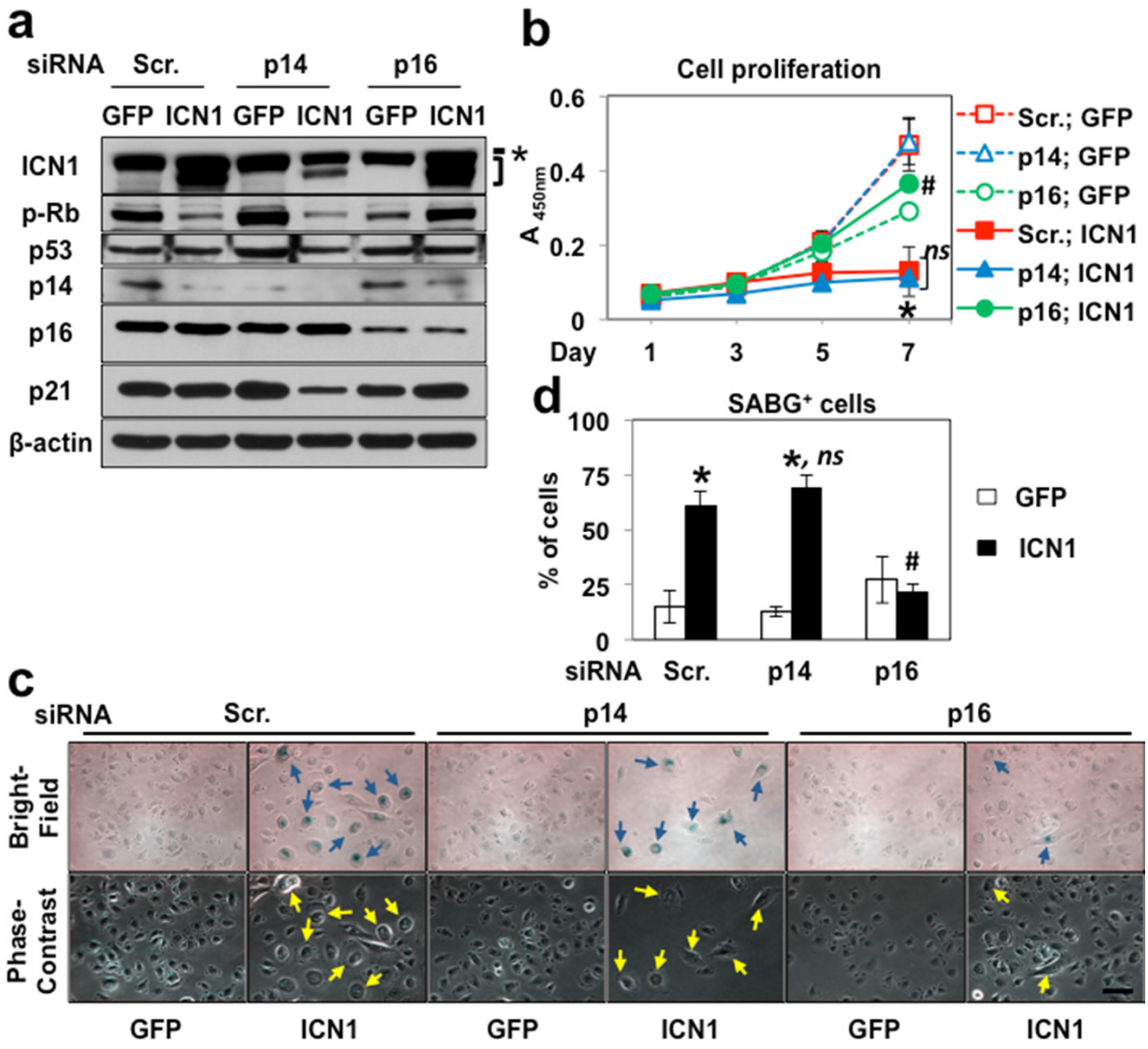
Figure S8



Supplementary Figure S9 p16^{INK4A} may contribute to ICN1-induced senescence in EPC2-hTERT cells

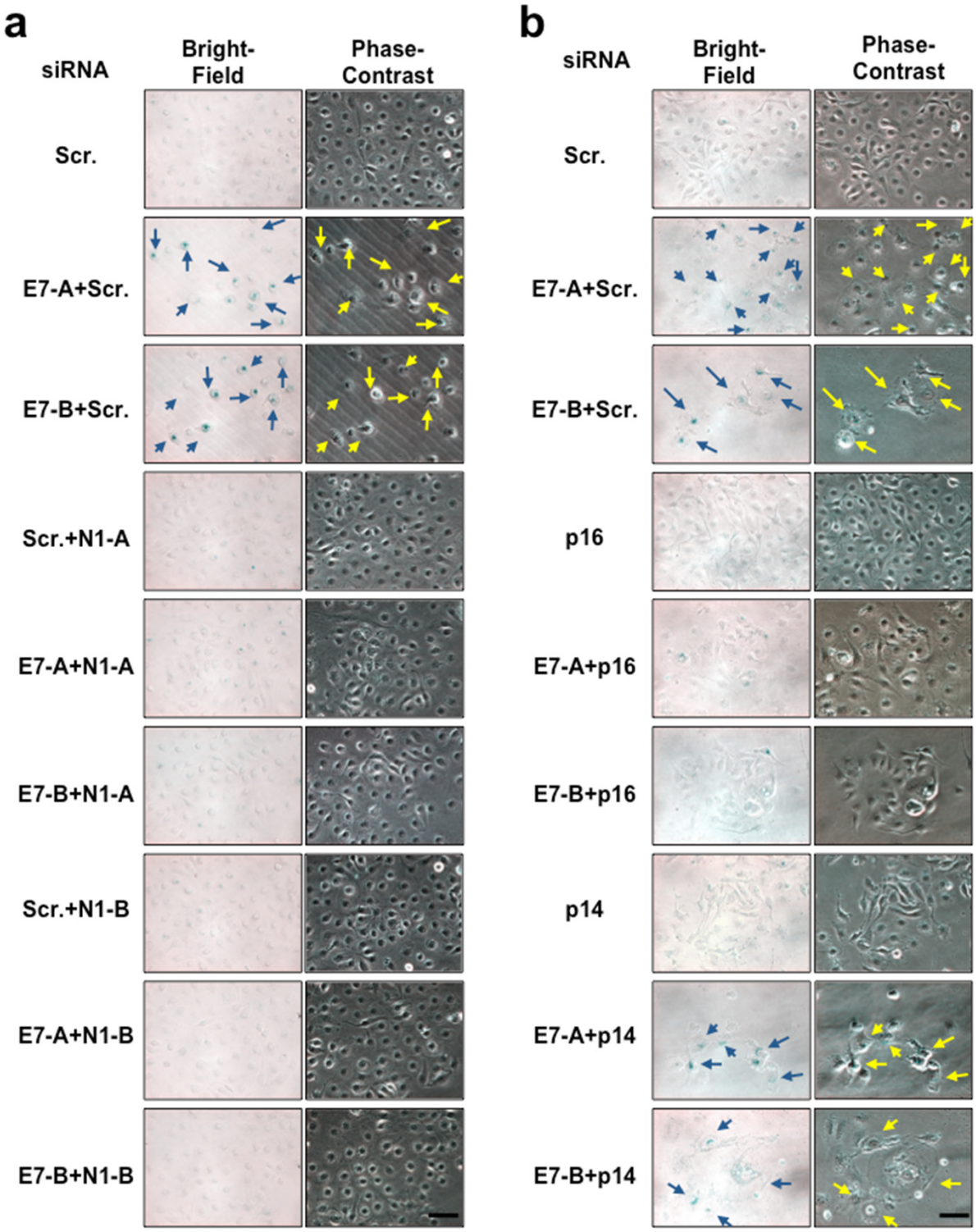
Using siRNA targeting *p14^{ARF}* (p14 siRNA), *p16^{iNK4A}* (p16 siRNA)(**Figure 4a**) or a non-silencing control scramble siRNA (Scr.), *p14^{ARF}* and *p16^{iNK4A}* were knocked down in EPC2-hTERT. In (**a**)-(**d**), EPC2-hTERT carrying *ICN1^{Tet-On}* was treated for 7 days with 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX to induce ICN1 24 h after transfection with siRNA. Cells were harvested at indicated time points in (**b**). Following DOX treatment, cells were subjected to Western blotting for ICN1, phospho-Rb^{S780} (p-Rb), p53, and cell-cycle regulators in (**a**); WST1 assays for cell proliferation in (**b**); and SABG assays in (**c**) and (**d**). In (**a**), β-actin served as a loading control. * denotes transmembrane/intracellular region of endogenous Notch1. Bracket indicates lentivirally expressed ICN1 induced by DOX. In (**b**), *, $P < 0.05$ vs. Scr. and DOX (-) at day 7; #, $P < 0.05$ vs. Scr. and DOX (+) at day 7; *ns*, not significant vs. Scr. and DOX (+) at day 7 (n=6). In (**c**), representative bright-field and phase contrast images of SABG-positive cells with flat and enlarged cell morphology (arrows) as scored in (**d**); *, $P < 0.05$ vs. Scr. and DOX (-); #, $P < 0.05$ vs. Scr. and DOX (+); *ns*, not significant vs. Scr. and DOX (+); (n=6). Note densitometry from (**a**) was summarized along with cell proliferation and SABG data from (**b**)-(**d**) in **Supplementary Table S1**.

Figure S9



Supplementary Figure S10 $p16^{INK4A}$ may contribute to ICN1-induced senescence in EPC1 cells
Using siRNA targeting *$p14^{ARF}$* (p14 siRNA), *$p16^{iNK4A}$* (p16 siRNA)(**Figure 4a**) or a non-silencing control scramble siRNA (Scr.), *$p14^{ARF}$* and *$p16^{iNK4A}$* were knocked down in EPC1. 24 h after transfection with siRNA, cells were transduced with a retrovirus expressing constitutively either ICN1 (co-expressed with GFP) or GFP alone (control), where GFP expression validated >90% gene transduction efficiency. Following retrovirus infection, cells were harvested at 7 days In (**a**), (**c**) and (**d**); and at indicated time points in (**b**). Cells were subjected to Western blotting for ICN1, phospho-Rb^{S780} (p-Rb), p53, and cell-cycle regulators in (**a**); WST1 assays for cell proliferation in (**b**); and SABG assays in (**c**) and (**d**). In (**a**), β-actin served as a loading control. * denotes transmembrane/intracellular region of endogenous Notch1. Bracket indicates retrovirally expressed ICN1. In (**b**), *, $P < 0.05$ vs. Scr. and GFP at day 7; #, $P < 0.05$ vs. Scr. and ICN1 at day 7; *ns*, not significant vs. Scr. and ICN1 at day 7 (n=6). In (**c**), representative bright-field and phase contrast images of SABG-positive cells and the corresponding cells with flat and enlarged cell morphology (arrows) as scored in (**d**); *, $P < 0.05$ vs. Scr. and GFP; #, $P < 0.05$ vs. Scr. and ICN1; *ns*, not significant vs. Scr. and ICN1; (n=6). Note densitometric data from (**a**) was summarized along with cell proliferation and SABG data from (**b**)-(**d**) in **Supplementary Table S1**.

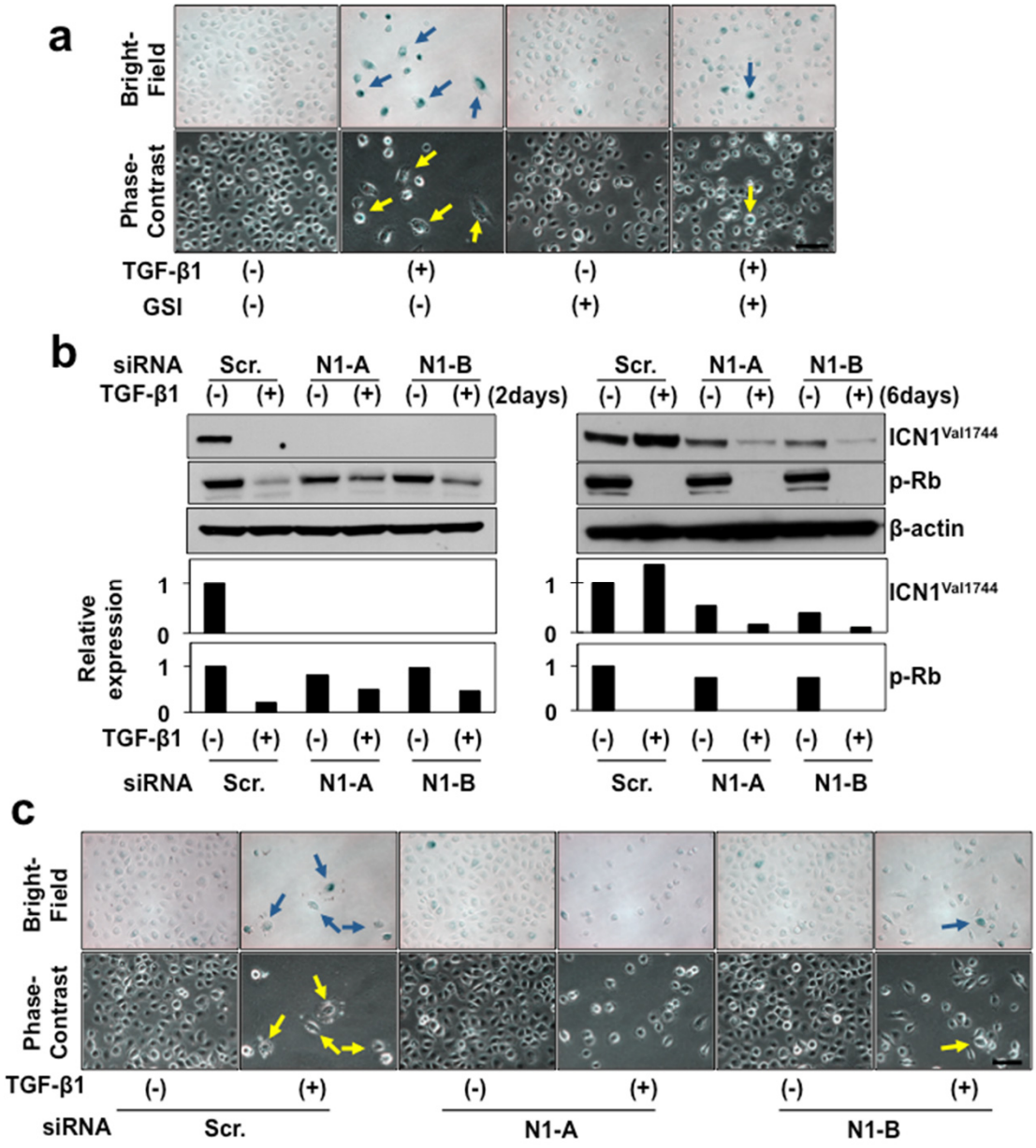
Figure S10



Supplementary Figure S11 HPV E7 knockdown leads to Notch1 and p16^{INK4A}-mediated senescence in EN60 cells

EN60 cells expressing HPV E6/E7 were transiently transfected with two independent siRNA sequences directed against either HPV E7 (E7-A and E7-B) or a non-silencing control scramble short interfering RNA (Scr.) along with or without siRNA sequences directed against Notch1 (N1-A and N1-B)(**Figure 5**) in (**a**) and either p16^{INK4A} or p14^{ARF} (**Figure 4a**) in (**b**) and subjected to SABG assays. Representative bright-field and phase contrast images document SABG-positive cells with flat and enlarged cell morphology (arrows) as scored in **Figure 5e** for (**a**) and **Figure 5f** for (**b**). Note that the induction of SABG positive cells by E7 siRNA was antagonized by co-transfected either Notch1 or p16^{INK4A}, but not p14^{ARF}, siRNA.

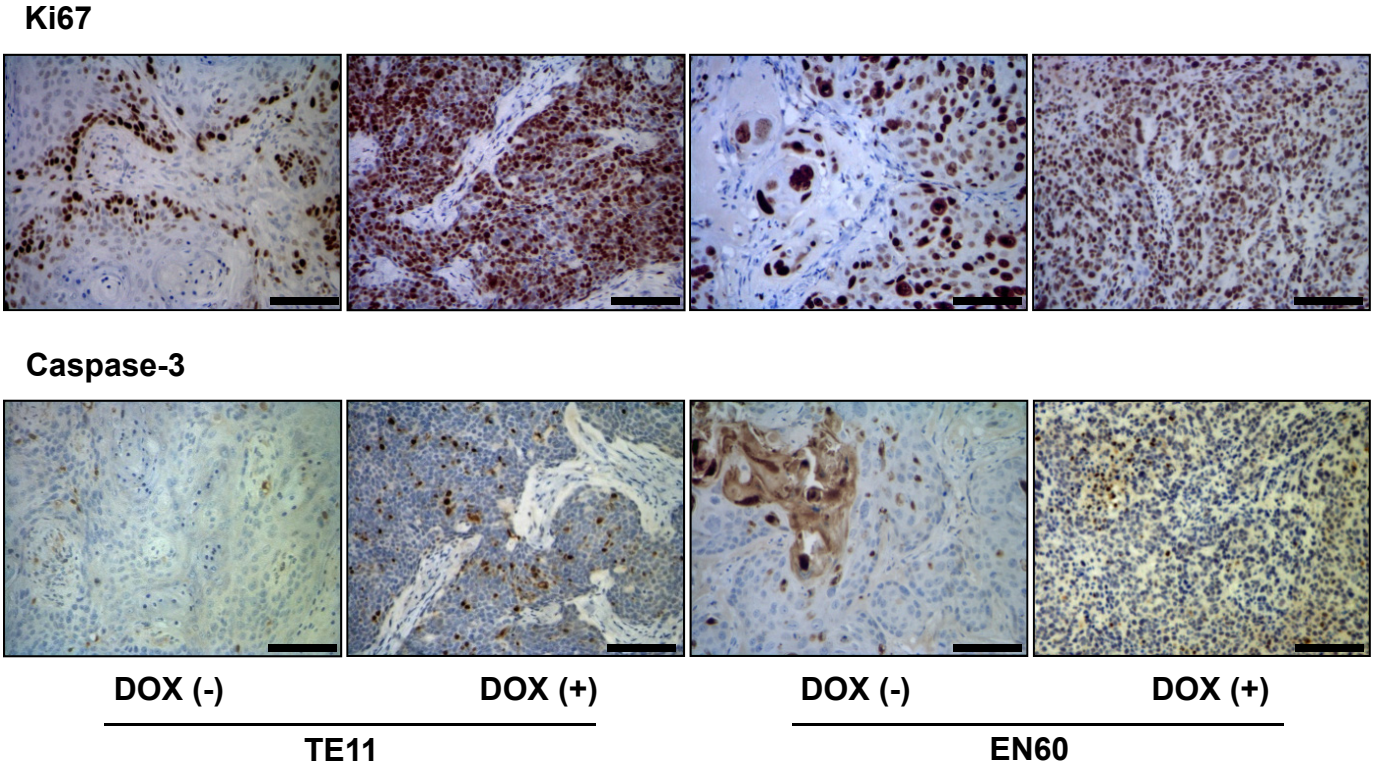
Figure S11



Supplementary Figure S12 Notch1 mediates TGF-β-induced senescence in EPC2-hTERT cells

EPC2-hTERT cells were stimulated with 5 ng/ml TGF-β1 alone or along with either GSI (1μM compound E) or DMSO (vehicle) in (**a**) and (**b**) as shown in **Figure 6**. In (**c**), cells were transiently transfected with a siRNA directed against Notch1 (N1-A and N1-B) or a non-silencing control scramble short interfering RNA (Scr.) 24 h prior to TGF-β stimulation as shown in **Figure 6g**. Cells were subjected to SABG assays 7 days after TGF-β stimulation in (**a**) and (**c**); and Western blotting for indicated molecules at indicated time points along with densitometry calibrated by the signal intensity of β-actin as a loading control in (**b**). In (**a**) and (**c**), representative bright-field and phase contrast images document the presence of SABG-positive cells (arrows) as scored in **Figure 6f and g**, respectively**.** Note that flat and enlarged cell morphology and SABG positive cells induced by TGF-β1 were greatly diminished by either GSI (**a**) or Notch1 siRNA (**c**). Note that Notch1 siRNA delayed Rb dephosphorylation induced 2 days after TGF-β1 stimulation, implying the role of Notch1 in Rb activation. The active form of Notch1 ($ICN1^{Val1744}$) was found to be transiently downregulated within 2 days after TGF-β1 stimulation compared to the TGF-β1-untreated control cells (Scr.)(see also $ICN1^{Val1744}$ at day 1 in **Figure 6a**). Notch1 siRNA reduced the $ICN1^{Val1744}$ level at 6 days following TGF-β1 stimulation; however, Notch siRNA antagonized barely Rb dephosphorylation at that time point. Therefore, TGF-β1 may induce cell cycle arrest independent of the Notch1 activity while requiring Notch1 for SABG activation.

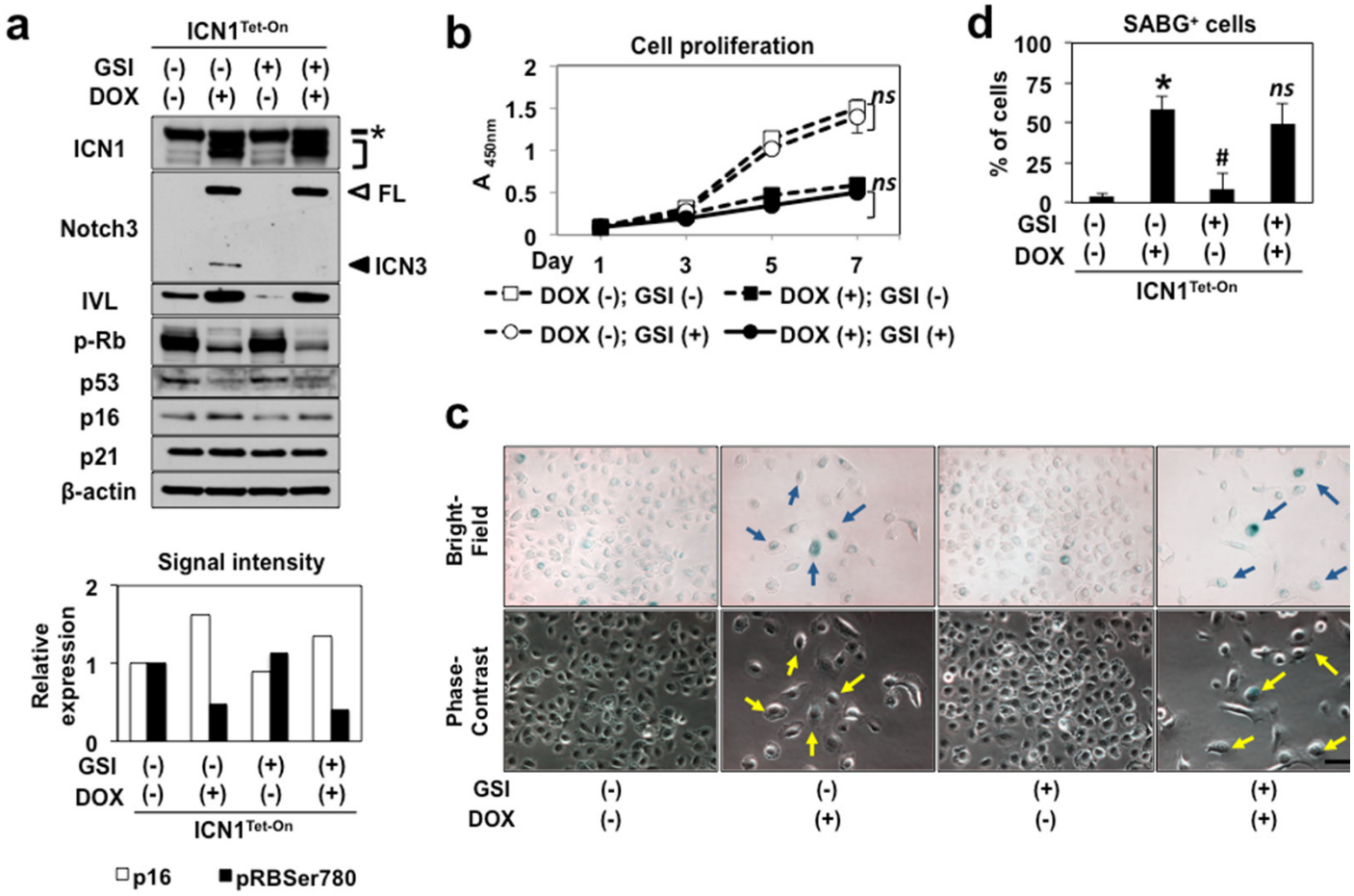
Figure S12



Supplementary Figure S13 ICN1 may promote TE11 and EN60 tumor growth by influencing cell proliferation, differentiation and cell size

TE11 and EN60 cells carrying *ICN1*$^{Tet\text{-}On}$ formed xenograft tumors in the presence or absence of DOX as shown in **Figure 7b and c**. Resulting tumors were subjected to immunohistochemistry for Ki67 and caspase-3 to assess cell proliferation and apoptosis, respectively. Scale bar, 100 μm. Note that DOX treatment changed markedly the differentiation pattern and the cell size. Less differentiated smaller ESCC cells induced by DOX appeared to contain more proliferative cells and few apoptotic cells.

Figure S13

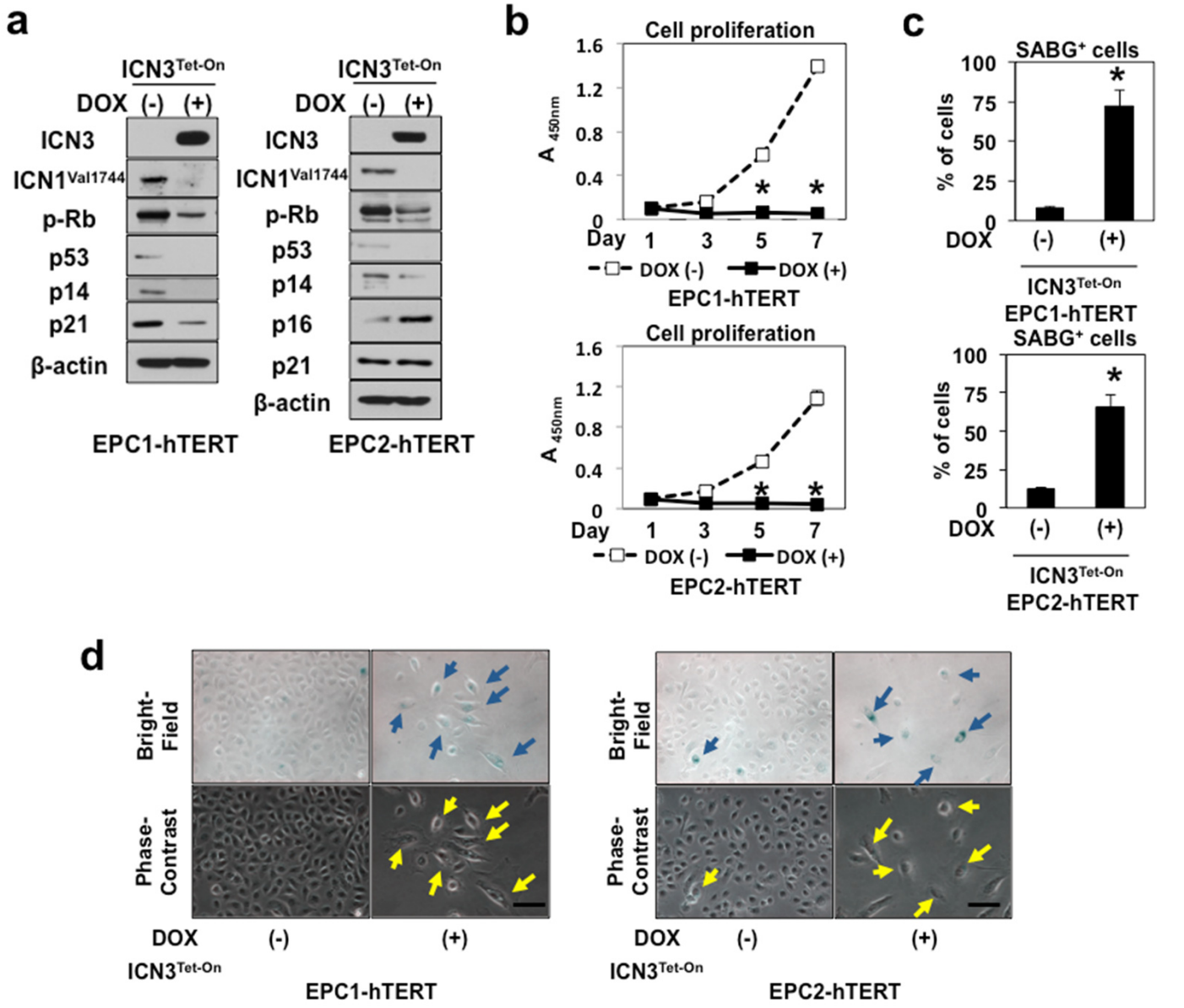


Supplementary Figure S14 ICN1 may induce senescence independent of Notch3

EPC2-hTERT carrying ICN1^{Tet-On} was treated with 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX to induce ICN1 for 7 days. In (**a**) and (**b**), cells were concurrently treated with either 1 μM compound E (GSI) or DMSO as a vehicle. Cells were subjected to Western blotting for indicated molecules along with densitometry calibrated by the signal intensity of β-actin as a loading control in (**a**); WST1 assays for cell proliferation at indicated time points in (**b**); and SABG assays (**c**) as scored in (**d**). In (**a**), * denotes transmembrane/ intracellular region of endogenous Notch1. Bracket indicates lentivirally expressed ICN1 induced by DOX. Note that ICN1 induced both full-length (FL, open arrowhead) and cleaved form of Notch3 (ICN3, solid arrowhead). GSI suppressed the cleaved form of Notch3 identifying the lower band as ICN3 in (**a**). Although densitometry shows that p16^{INK4A} was modestly decreased in the presence of GSI, the p16^{INK4A} level induced by ICN1 in the presence of GSI appeared to be sufficient for Rb dephosphorylation and senescence. In (**b**), *, *P* <0.05 vs. DOX (-) and GSI (-); *ns*, not significant vs. DOX (+) and GSI (-); (n=6). In (**c**), representative bright-field and phase contrast images of SABG-positive cells with flat and enlarged cell morphology (arrows). In (**d**), *, *P* <0.05 vs. DOX (-); (n=6).

Since Notch3 has been implicated in senescence (Cui H *et al.* Cancer Research 2013; 73: 3451-3459), we asked if Notch3 may mediate ICN1-induced senescence. In the experiments shown in **Supplementary Figure S14**, we asked whether ICN1 induces senescence even if Notch3 activation was impaired. To this end, we used γ-secretase inhibitor (GSI) because GSI inhibits the activity of endogenous Notch receptors, but not that of ectopically expressed ICN1. ICN1 induces Notch3 in EPC2-hTERT cells (Ohashi S *et al*. Gastroenterology 2010; 139: 2113-2123). As expected, GSI permitted ectopically expressed ICN1 to induce full-length endogenous Notch3 while blocking the generation of ICN3, the cleaved and activated form of Notch3 (**Supplementary Figure S14a**). ICN1 appeared to induce senescence with or without GSI to a comparable extent as corroborated by cell proliferation, Rb phosphorylation and SABG assay data (**Supplementary Figures S14, a-d**). These data suggest that ICN1 may induce senescence without requiring activation of other Notch receptor paralogs including Notch3.

Figure S14



Supplementary Figure S15 ICN3 may induce senescence independent of Notch1

EPC1-hTERT and EPC2-hTERT, carrying ICN3^{Tet-On}, were treated with 0 μg/ml [DOX (-)] or 1 μg/ml [DOX (+)] of DOX to induce ICN3 for 7 days. Cells were subjected to Western blotting for indicated molecules in (**a**); WST1 assays for cell proliferation at indicated time points in (**b**); and SABG assays (**c**) as scored in (**d**). In (**a**), β-actin served as a loading control. Note that ectopically expressed ICN3 suppressed ICN1^{Val1744}, the activated form of endogenous Notch1. p16^{INK4A} was not detectable in EPC1-hTERT cells (data not shown). In (**b**), *, *P* <0.05 vs. DOX (-); (n=6). In (**c**), representative bright-field and phase contrast images of SABG-positive cells with flat and enlarged cell morphology (arrows). In (**d**), *, *P* <0.05 vs. DOX (-); (n=6).

Ectopically expressed ICN3 inhibited cell proliferation, inducing Rb dephosphorylation and the SABG activity in EPC1-hTERT and EPC2-hTERT while suppressing ICN1^{Val1744} (**Supplementary Figures S15a**), suggesting that ICN3 may induce senescence without requiring the endogenous Notch1 activity. Thus, there may be functional redundancy amongst Notch family members. It should be noted that ICN3 induced Rb dephosphorylation in EPC1-hTERT with no detectable p16^{INK4A} expression. Although p21 has been implicated in ICN3-mediated senescence (Cui H et al. Cancer Research 2013; 73: 3451-3459), ICN3 suppressed p21 in EPC1-hTERT. In EPC2-hTERT, ICN3 induced p16^{INK4A}, but not p21. ICN3 downregulated p53 and p14^{ARF} in both cell lines tested. Therefore, it is likely that cells have redundant pathways to mediate Rb dephosphorylation and senescence in response to ICN1 as well as ICN3.

Figure S15