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Supplemental information

Irreversible cell cycle exit associated with senescence is mediated by constitutive MYC degradation

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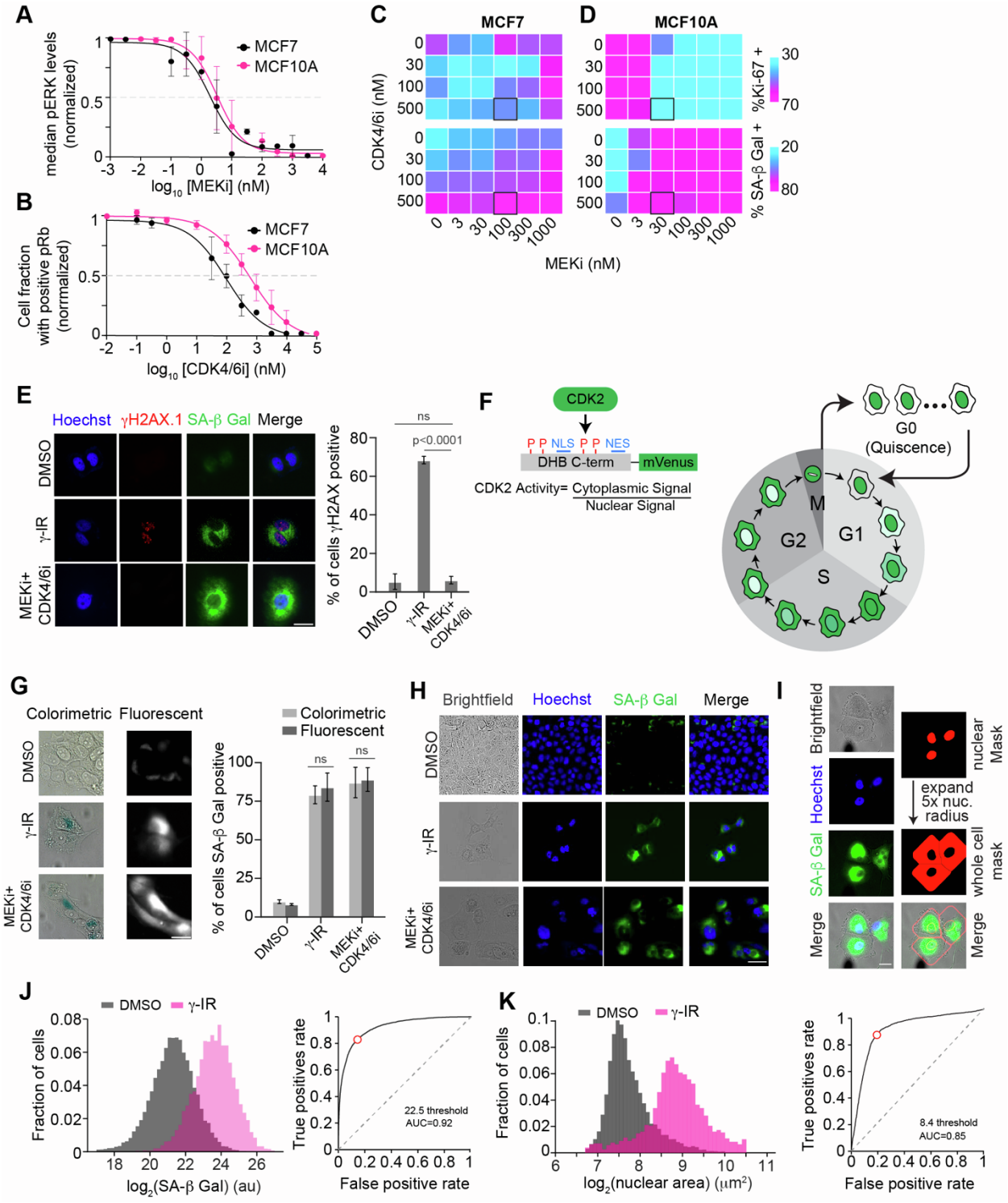

Figure S1. Optimization of MEKi and CDK4/6i treatments to induce senescence, related to Figure 1

(**A**) Median phospho-ERK (pERK) levels from single MCF7 and MCF10A cells as a function of MEKi concentration. Data are represented as mean ± SD of three independent experiments. Dashed line denotes the IC50.

(**B**) Percentage of MCF7 and MCF10A cells expressing high levels of pRb (positive pRb) as a function of

CDK4/6i concentration. Data are represented as mean ± SD of three independent experiments. Dashed line denotes the IC50.
(**C** and **D**) Drug combination matrix to determine optimal concentration of MEKi (x-axis) and CDK4/6i (y-axis) in MCF7 (C) and MCF10A (D) cells to induce cellular senescence. Heatmaps convey percentage of Ki67 positive cells (top) and percentage of SA-β Gal positive cells (bottom). Black boxes denote the best drug concentration combination in each heatmap.
(**E**) Left: Representative images of MCF7 cells stained for SA-β Gal (green) and γH2AX.1 (red). Right: Percentage of γH2AX.1 positive cells after the indicated treatment. Data are represented as mean ± SD of three independent experiments. p values were obtained from one-way ANOVA with multiple comparison test. ns, not significant. Scale bar is 10 μm.
(**F**) Schematic explaining the live-cell CDK2 reporter. CDK2-mediated phosphorylation of the reporter causes a translocation from the nucleus to the cytoplasm. Quantifying the ratio of fluorescence in the cytoplasm and the nucleus yields relative CDK2 activity. Interphase cells have progressively more reporter fluorescence in the cytoplasm, yielding a high cyto:nuc ratio while non-cycling/quiescent cells have more reporter fluorescence in the nucleus, yielding a low cyto:nuc ratio.
(**G**) Left: Representative images of MCF7 cells stained using both colorimetric and fluorescent SA-β Gal activity. Cells were treated with DMSO or 100 nM MEKi and 500 nM CDK4/6i for 7 days or were exposed to 10 Gy γ-IR and allowed to grow in complete medium for 7 days. Scale bar is100 μm. Right: Percentage of SA-β Gal positive cells. Data are represented as mean ± SD of two independent experiments. p values were obtained from one-way ANOVA with multiple comparisons tests. ns, not significant.
(**H**) Example images of MCF7 cells treated with DMSO or 100 nM MEKi and 500 nM CDK4/6i for 7 days or exposed to 10 Gy γ-IR and allowed to grow in complete medium for 7 days. Scale bar is 10 μm.
(**I**) Automated image analysis pipeline for quantification of fluorescent SA-β Gal staining. A nuclear mask generated from the Hoechst signal was expanded using a water-shed algorithm up to 5 times the nuclear radius to yield a whole cell mask. The relative fluorescence in the GFP channel (SA-β Gal staining) in the whole cell mask is reported as SA-β Gal activity. Scale bar is 10 μm.
(**J**) MCF7 cells were treated with either DMSO or 10 Gy γ-IR for 7 days, then fixed and stained for fluorescent SA-β Gal activity. Left: histograms of single-cell SA-β Gal activity. Right: Receiver operating characteristic (ROC) curve to determine the optimal threshold to distinguish SA-β Gal positive cells from SA-β Gal negative cells.
(**K**) Single-cell nuclear area measurements from the same cells in (J). Left: histograms of nuclear area. Right: Receiver operating characteristic (ROC) curve to determine the optimal threshold to distinguish cells with karyomegaly, which is associated with cellular senescence.

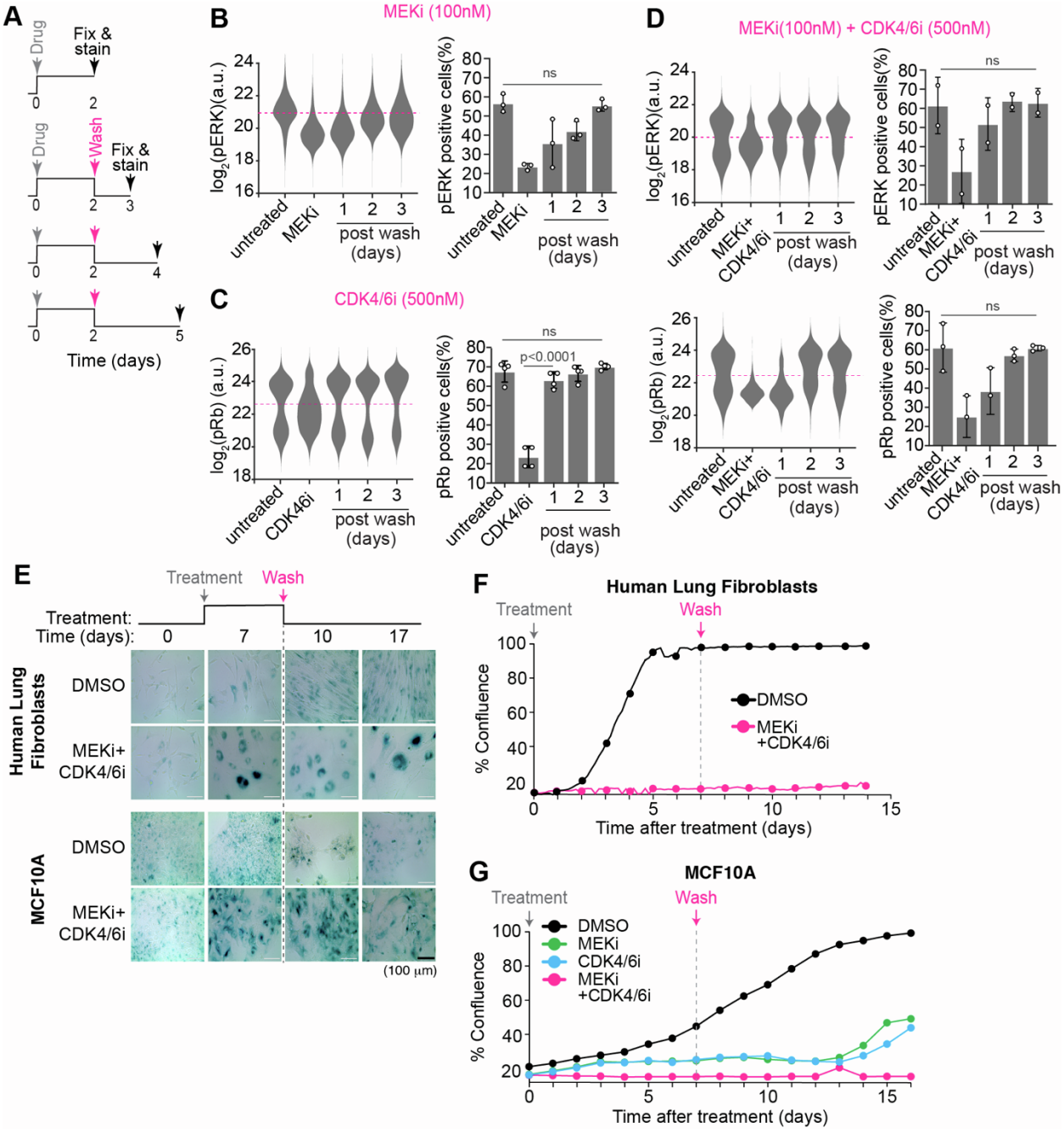

Figure S2. Downstream signal recovery after washing off the drugs as single agents or combination, related to Figure 2

(**A**) Schematic diagram of the experimental set up for (B-D). MCF7 cells were treated with either DMSO, 100 nM MEKi (B), 500 nM CDK4/6i (C), or a combination of MEKi and CDK4/6i (D) for 2 days. We chose 2 days of drug treatment to make a technical demonstration that our washout procedure successfully removes enough drug to reactivate the relevant signaling pathways. As indicated, the treatment conditions were then washed off and cells were allowed to grow in complete growth medium until day 3, 4, or 5. Cells were then fixed and stained for either pERK or pRb.

(**B**) Left: Violin plot of pERK levels in cells treated as described in (A) from one representative experiment.

Pink dashed line represents median pERK levels in untreated cells. Right: Percentage of pERK positive cells. Data are represented as mean ± SD of three independent experiments. p values were obtained from one-way ANOVA with multiple comparison test. ns, not significant.
(**C**) Left: Violin plot of pRb levels in cells treated as described in (A) from one representative experiment. Pink dashed line represents the threshold above which cells were called pRb positive. Right: Percentage of pRb positive cells. Data are represented as mean ± SD of four independent experiments. p values were obtained from one-way ANOVA with multiple comparison test. ns, not significant.
(**D**) Left: Violin plots of pERK levels (top) or pRb levels (bottom) in cells treated as described in (A) from one representative experiment. Right: Bar graph showing percentage of pERK positive cells (top) or pRb positive cells (bottom). Error bars are SD from at least two independent experiments. p values were obtained from one-way ANOVA with multiple comparison test. ns, not significant.
(**E**) Representative images of human lung fibroblasts and MCF10A cells treated with DMSO, or a combination of MEKi and CDK4/6i for 7 days at which the treatment conditions were washed off and the cells were allowed to grow in complete culture medium until day 17. Cells were stained for SA-β Gal activity at day 0, 7, 10, and 17. Scale bar is 100 μm.
(**F** and **G**) Percentage of confluence of human lung fibroblasts (F) and MCF10A (G) cells treated with the indicated conditions as a function of time after treatment. Vertical line denotes time at which drugs were washed off.

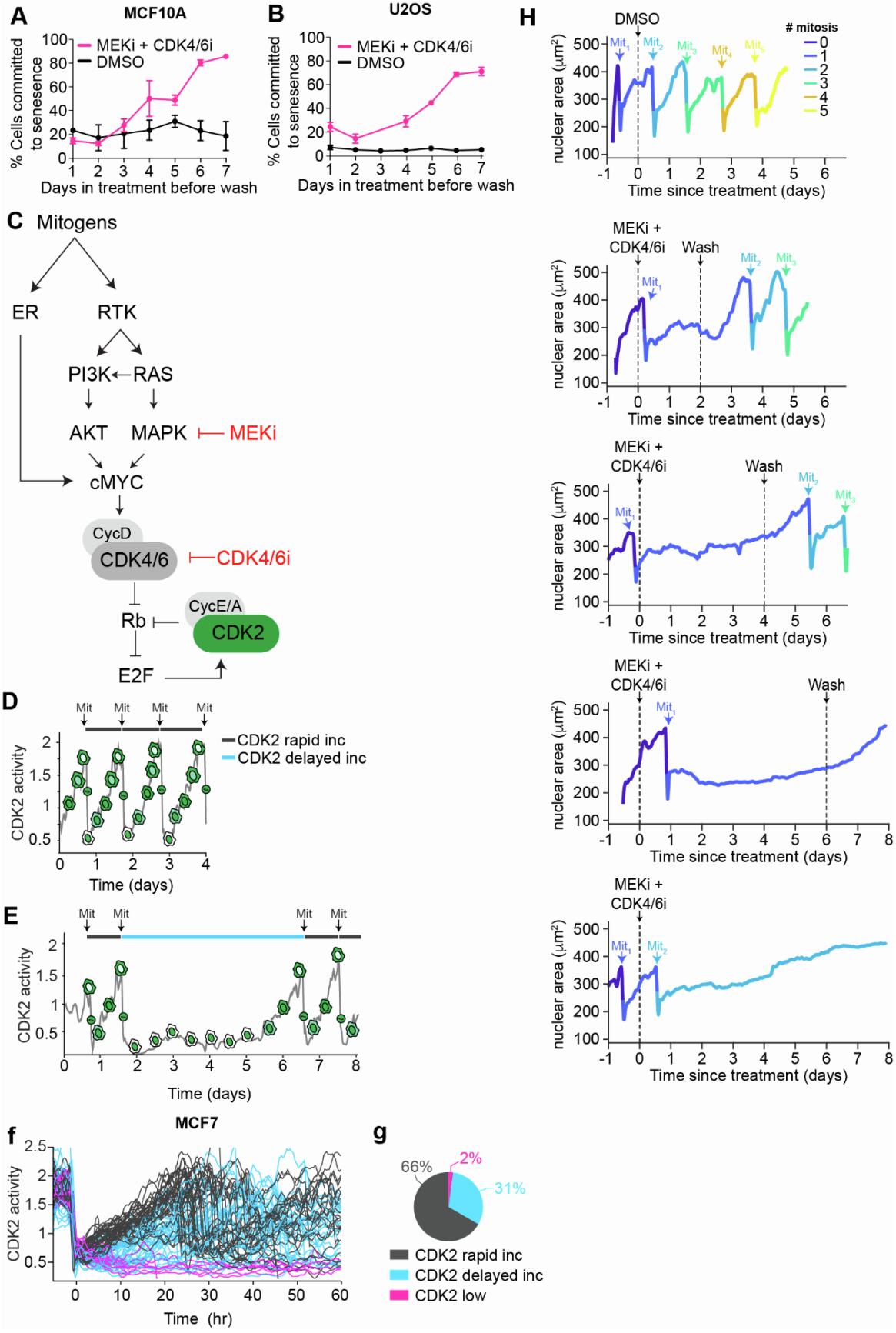
A
MCF10A
MEKi + CDK4/6i
DMSO
% Cells committed to senescence
Days in treatment before wash
B
U2OS
MEKi + CDK4/6i
DMSO
% Cells committed to senescence
Days in treatment before wash
C
Mitogens
ER
RTK
PI3K
RAS
AKT
MAPK
MEKi
cMYC
CycD
CDK4/6
CDK4/6i
Rb
CycE/A
CDK2
E2F
D
Mit
CDK2 rapid inc
CDK2 delayed inc
CDK2 activity
Time (days)
E
Mit
CDK2 activity
Time (days)
f
MCF7
CDK2 activity
Time (hr)
g
66%
2%
31%
CDK2 rapid inc
CDK2 delayed inc
CDK2 low
H
DMSO
mitosis
nuclear area (μm²)
Time since treatment (days)
MEKi + CDK4/6i
Wash

Figure S3. CDK2 reporter as a proxy for cell cycle progression, related to Figure 3

(**A** and **B**) MCF10A (A) or U2OS (B) cells were treated as described in Figure 3A. Graph represents quantification of SA-β Gal positive cells as a function of time in treatment before washing off the drugs. Data are represented as mean ± SD of three independent experiments.

(**C**) Signaling diagram showing CDK2 activity as good candidate to follow in response to MEK and CDK4/6 inhibitors treatment.

(**D** and **E**) Single-cell trace of CDK2 activity as a function of time in days. (D) an example of a cycling cell where CDK2 activity rapidly increases above the 0.6 threshold within 10 h after mitosis (Mit). Note that a given cell can divide 4 times over the span of 4 days. (E) A single-cell trace showing CDK2 activity in a transiently arrested cell where CDK2 activity doesn't increase above the 0.6 threshold for multiple days after mitosis, CDK2 delayed inc (cyan).

(**F**) Single-cell CDK2 activity traces over time of asynchronous cells. Cells were colored grey if CDK2 activity increased above 0.6 within 10 h after mitosis, indicating the cycling population (CDK2 rapid inc). Cells were colored cyan if CDK2 activity was below 0.6 for the first 10 h after mitosis and then increased above 1 any time after that (CDK2 delayed inc). Cells were colored pink if CDK2 activity remained below 0.6 throughout the entire imaging period (CDK2 low).

(**G**) Percentage of untreated MCF7 cells with CDK2 rapid inc (grey), CDK2 delayed inc (cyan), CDK2 low (pink).

(**H**) Change in nuclear area over time for single, MCF7 cells exposed to the indicated treatments. Traces are colored based on the number of mitoses that cell has gone through during the imaging period. These example traces were used to make the heatmaps in Figure 3F.

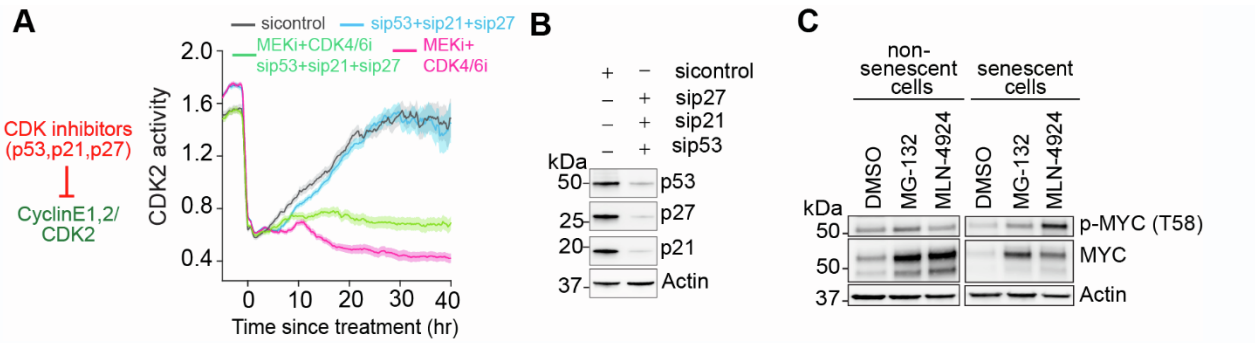

Figure S4. MYC inactivation during senescence entry and commitment, related to Figure 4

(**A**) Median CDK2 activity in MCF7 cells treated with DMSO, sip53+sip21+sip27, MEKi+CDK4/6i, sip53+sip21+sip27+ MEKi+CDK4/6i as a function of time since treatment. Solid line is median trace and shaded region is SEM.

(**B**) Western blot validation of p53, p21, and p27 knockdown using siRNA.

(**C**) Immunoblotting of MYC, and p-MYC (T58). Cells were initially treated with MEKi + CDK4/6i for 4 days to induce senescence. Cells were then treated with DMSO, MG132 (10 μM), or MLN-4924 (3 μM) for 8 h before collection of whole cell lysates. Representative blot from n=3 experiments. Actin was used as the loading control.

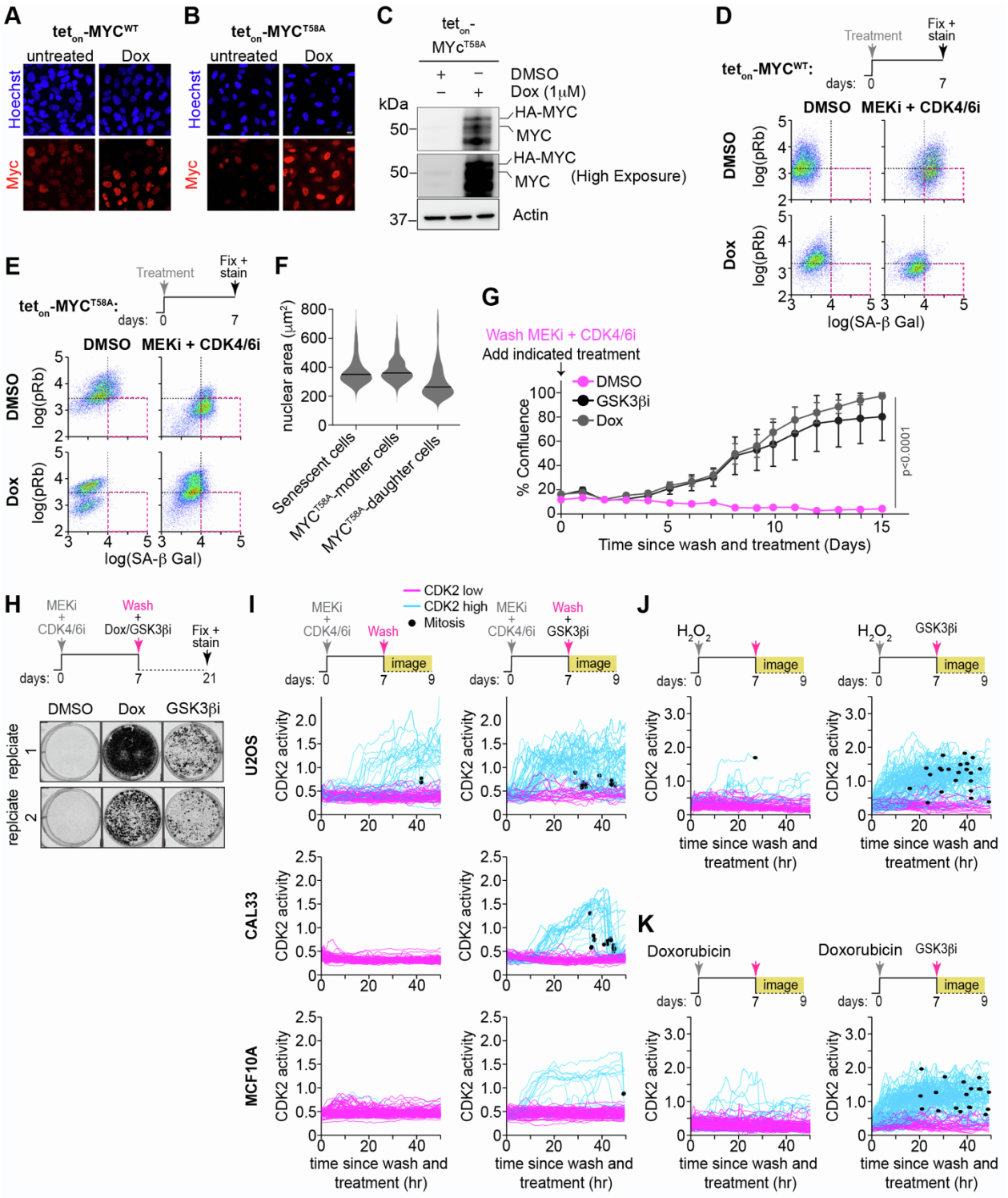

Figure S5. GSK3b-mediated MYC degradation is necessary for senescence, related to Figure 5.

(**A**-**B**) Representative images of MCF7 cells transduced with tet$_{on}$-MYC^{WT} (A) or tet$_{on}$-MYC^{T58A} (B). Cells were either untreated or treated with doxycycline (Dox; 1 μM) for 48 h and then fixed and immunostained for MYC protein. Scale bar is 10 μm.

(**C**) Immunoblotting of MYC in MCF7 cells transduced with tet$_{on}$-MYC^{T58A} treated with and without Dox as indicated. Actin was used as a loading control.

(**D** and **E**) Scatterplot of SA-β Gal staining versus pRb levels in MCF7 cells transduced with tet_{on}-MYC^{WT} (D) or tet_{on}-MYC^{T58A} (E). Cells were treated with DMSO, MEKi+CDK4/6i, Dox (1 μM), or MEKi+CDK4/6i + Dox for 7 days before fixing and staining.
(**F**) Violin plots of nuclear area from cells treated as in Figures 5C and D. Nuclear area of senescent cells refer to the nuclear area of cells treated with MEKi and CDK4/6i for 7 days, then drugs were washed off and the nuclear area was measured 48 h later (see Figure 5C). Nuclear area of MYC^{T58A} -mother cells refer to the nuclear area of cells expressing tet_{on}-MYC^{T58A} treated with MEKi and CDK4/6i for 7 days, then the drugs were washed off and cells were treated with 1 μM Dox, and the nuclear area was measured 48 h later (see Figure 5D). Nuclear area of MYC^{T58A}-daughter cells refers to the nuclear area of cells expressing tet_{on}-Myc^{T58A} treated with MEKi and CDK4/6i for 7 days, then the drugs were washed off and cells were treated with 1 μM Dox, and the nuclear area was measured 48 h after the cells went through mitosis (see Figure 5D).
(**G**) Cells were initially treated with a combination of MEK inhibitor and CDK4/6 inhibitor for 7 days to render them senescent. After 7 days (time 0h), cells were washed and given the indicated treatment. Percent confluence over time was measured using an Incucyte microscope. Data are represented as mean ± SD of three independent experiments. p values were obtained from one-way ANOVA with multiple comparison test.
(**H**) Cells were initially treated with a combination of MEK inhibitor and CDK4/6 inhibitor for 7 days to render them senescent. Cells were then washed, given the indicated treatment, and allowed to grow until day 21 when they were fixed and stained with crystal violet. Representative images from n=4 independent experiments with two technical replicates each.
(**I**) Single-cell CDK2 activity traces from the indicated cell line. Cells were treated with 100 nM MEKi and 500 nM CDK4/6i for 7 days. At day 7, cells were washed to remove drugs and then were treated with either DMSO or 1 μM GSK3β inhibitor (GSK3βi) when time lapse imaging was started. Trace color denotes long-term fate of each cell: Cyan, started in the CDK2 low state before eventually building up CDK2 activity (CDK2 high); Pink, remained in CDK2 low state for the entire imaging period (CDK2 low). Black dot denotes when a cell went through mitosis.
(**J** and **K**) Single-cell CDK2 activity traces in MCF7 cells treated with either 100 μM hydrogen peroxide (H_2O_2; J) or 50 nM doxorubicin (K). At day 7, cells were washed to remove the drugs and then were treated with either DMSO or 1 μM GSK3βi when time lapse imaging was started. Trace color denotes long-term fate of each cell: Cyan, started in the CDK2 low state before eventually building up CDK2 activity (CDK2 high); Pink, remained in CDK2 low state for the entire imaging period (CDK2 low). Black dot denotes when a cell went through mitosis.

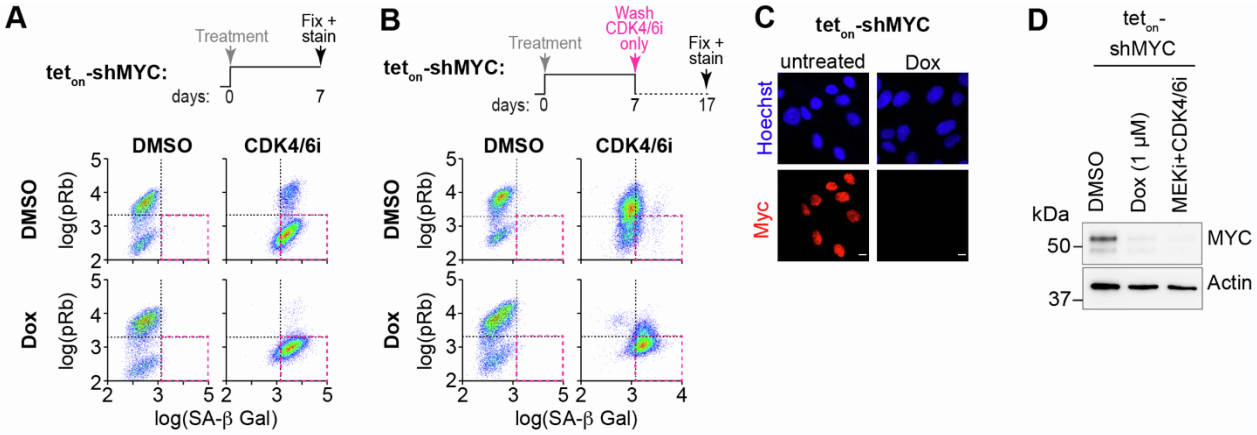

Figure S6. MYC knockdown converts quiescent cells to senescence, related to Figure 5

(**A**) Scatterplot of SA-β Gal staining versus pRb levels in MCF7 cells transduced with tet$_{on}$-shMYC. Cells were treated with DMSO, CDK4/6i (500 nM), Dox (1 μM), CDK4/6i (500 nM) + Dox (1 μM) for 7 days before fixing and staining.

(**B**) Scatterplot of SA-β Gal staining versus pRb levels in MCF7 cells transduced with tet$_{on}$-shMYC. Cells were treated with DMSO, CDK4/6i (500 nM), Dox (1 μM), CDK4/6i (500 nM) + Dox (1 μM) for 7 days before washing off CDK4/6i or DMSO. Cells that were treated with Dox at the beginning of the experiment were maintained in Dox-containing media after the wash step. Cells were fixed and stained 10 days after wash.

(**C**) Representative images of MCF7 cells transduced with tet$_{on}$-shMYC. Cells were either untreated or treated with Dox (1 μM) for 48 h and then fixed and immune stained for MYC protein. Scale bar is 10 μm.

(**D**) Immunoblotting of MYC in MCF7 cells transduced with tet$_{on}$-shMYC treated with and without Dox as indicated. Actin was used as a loading control.

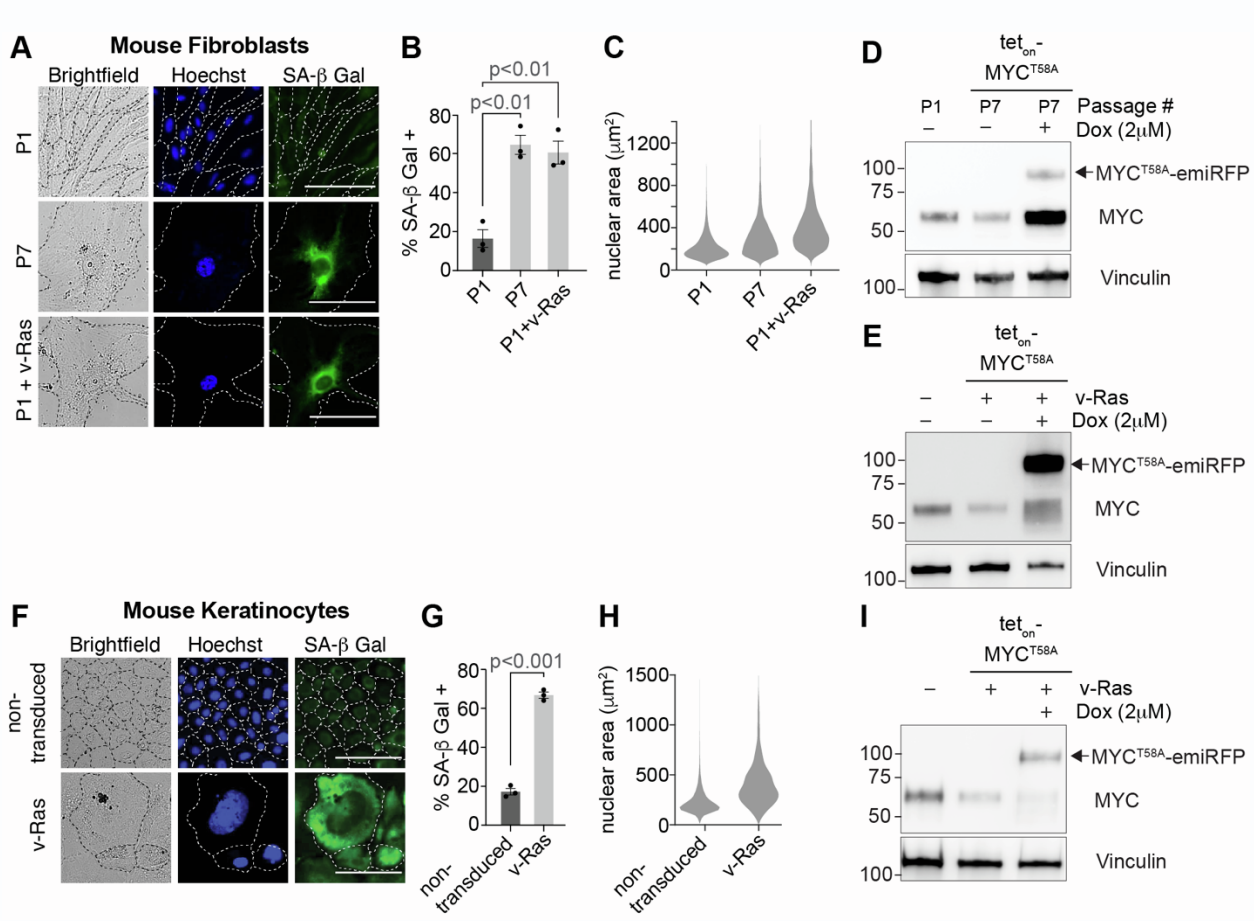

Figure S7. OIS and replicative senescence in primary mouse cells, related to Figure 6

(**A**) Representative images of primary mouse fibroblasts, untreated (P1), passaged 7 times (P7), and transduced with oncogenic Ras (P1 + v-Ras). Cells were fixed and stained for SA-β Gal activity (green). Nuclei were stained with Hoechst (blue). Scale bar is 50 μm.

(**B**) Quantification of SA-β Gal positive cells from (A). Data are represented as mean ± SD of three independent experiments. p values were obtained from one-way ANOVA with multiple comparison test.

(**C**) Violin plots of nuclear area (μm^2) in the indicated conditions from (A).

(**D**) Western blot of the indicated conditions. Lane 1 is early passage cycling cells. Vinculin is the loading control.

(**E**) Western blot analysis of the indicated conditions. Lane 1 is non-transduced cells. Vinculin is the loading control.

(**F**) Representative images of primary mouse keratinocytes, non-transduced, and transduced with oncogenic Ras (v-Ras). Cells were fixed and stained for SA-β Gal activity (green). Nuclei were stained with Hoechst (blue). Scale bar is 50 μm.

(**G**) Quantification of SA-β Gal positive cells from (F). Data are represented as mean ± SD of three independent experiments. p values were obtained from one-way ANOVA with multiple comparison test.

(**H**) Violin plots of nuclear area (μm^2) in the indicated conditions from (F).

(**I**) Western blot analysis of the indicated conditions. Lane 1 are non-transduced cells. Vinculin is the loading control.

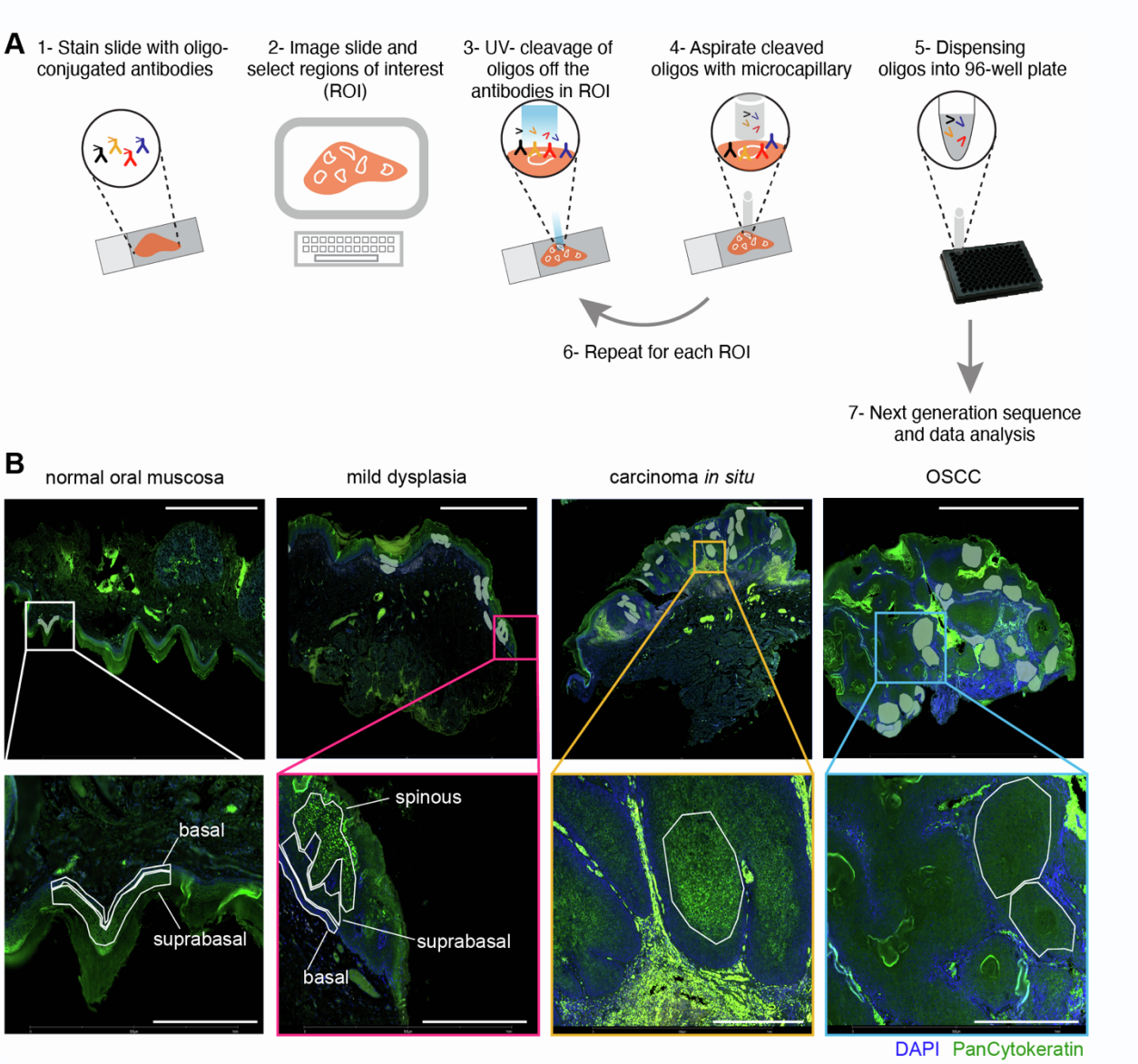

Figure S8. Workflow for digital spatial profiling, related to Figure 7

(**A**) Schematic workflow for the GeoMX digital spatial profiling.

(**B**) Tissue sections from normal oral mucosa, mild oral dysplasia, carcinoma *in situ*, and oral squamous cell carcinoma (OSCC) were stained for DAPI (blue) and PanCytokeratin (green), imaged to select regions of interest (ROI). A total of 381 regions of interest (ROI) were selected which were variably sized while accounting for the characteristic stratification of the oral surface epithelium. ROIs included basal, suprabasal, and spinous cell layers as well as epithelial cell nests in the underlying connective tissue. Selected ROIs were then stained with oligo-conjugated antibodies targeting Ki67 and phospho-p38. Scale bar is 2.5 mm (Top) and 500 μm (Bottom).

Table S1. Patients clinical data, related to Figure 7

Age	Gender	Clinical presentation	Site	Duration before diagnosis
48	M	White plaque lesion	Buccal mucosa	N/A
67	M	Exophytic mass	Lip	1 month
42	F	Exophytic warty lesion	Infero-lateral surface of tongue	2 months
53	M	3x4 cm firm ulcer	Retromolar area	10 days
19	M	3x3 cm ulcer	Lateral surface of tongue	N/A
53	M	3x5 cm exophytic mass	Lateral surface of tongue	7 months
40	F	5x5 mm leukoplakic/erythroplakic lesion	Buccal mucosa	1 year
58	M	Fungating mass	Tip of the tongue	N/A
62	F	4x3 cm erythroplakic lesion	Lateral surface of tongue	N/A
59	M	3x3 cm intrabony lesion	Retromolar area	1 year
63	F	4x5 cm ulcer	Posterior third of tongue	1 year
54	M	Keratotic lesion	Left buccal mucosa	6 months
38	F	Mandibular lesion	Lower left wisdom tooth	6 months
62	M	White keratotic lesion	Buccal mucosa	Recurrent lesion since 2 months
75	M	White lesion	Retromolar area	N/A
55	M	White patchy lesion	Buccal mucosa	1 year
32	F	Ulcer	Lateral surface of tongue	N/A
75	F	Ulcer	Vermillion border of the lip	4 months
42	M	White keratotic lesion	Lateral surface of tongue	N/A
35	F	White lesion	Retromolar area	N/A
79	M	White lesion	Buccal mucosa	20 years
49	M	White lesion	Lateral surface of tongue	N/A
23	M	Firm white plaque	Buccal mucosa	N/A
52	M	White lesion	Floor of the mouth	N/A
66	M	Ulcer	Buccal mucosa	N/A
53	F	Swelling	Palate	N/A
45	M	Ulcer	Tip of the tongue	Recurrent lesion
38	M	Ulcer	Lower lip	7 months
66	M	White lesion	Buccal mucosa	1 year
62	M	Intra-bony lesion	mandible	N/A
65	F	Ulcer	Buccal mucosa extended to palate and alveolus	N/A
66	M	Leukoplakic/erythroplakic lesion	Buccal mucosa	Accidently observed

Table S2. Oligonucleotides for RT-PCR

Name	Sequence	Source
C/EBPA forward	ACCCACGTGTAACTGTCAGCC	IDT
C/EBPA reverse	TCAACAGCAACAAGCCCGT	IDT
c-Jun forward	GAGCTGGAGCGCCTGATAA	IDT
c-Jun reverse	CCCTCCTGCTCATCTGTCAC	IDT
c-Fos forward	CGTCTCCAGTGCCAACTTCA	IDT
c-Fos reverse	GGTCCGGACTGGTCGAGAT	IDT
c-MYC forward	CTTCTCTCCGTCCTCGGATTCT	IDT
c-MYC reverse	GAAGGTGATCCAGACTCTGACCTT	IDT
GAPDH forward	GTGAAGGTCGGAGTCAACG	IDT
GAPDH reverse	TGAGGTCAATGAAGGGGTC	IDT