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# Supplemental Material to:

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# SUPPLEMENTARY INFORMATION

**Dynamics of histone H3.3 deposition in proliferating and senescent cells reveals a DAXX-dependent targeting to PML-NBs important for pericentromeric heterochromatin organization**

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## Supplementary Materials and Methods

*Immunofluorescence microscopy*

For senescence-associated Beta-Gal assay, we followed protocols published in [1,2]. For detection of replicating cells 8 to 12 days after infection, we pulse-labeled cells in vivo with 10μM of BrdU (SIGMA) for 2 hours **(Fig. S2A)**. Alternatively, we pulse-labeled cells in vivo with 40μM of BrdU during the TMR-Star pulse labeling in the last 20 minutes **(Fig. S1C)**. In order to detect BrdU, we denatured fixed cells for 10 minutes in 4N HCl, followed by extensive washes in PBS and we then detected BrdU by standard immunofluorescence as described in main text.

*Microscopy Analysis and Quantification of TMR Signal*

We carried out confocal microscopy with a Leica SP5 confocal laser scanning microscope (Leica, Heerbrugg, Switzerland) equipped with a Plan-Apochromat 63x NA 1.4 oil immersion objective **(Fig. S3C)**. We acquired dual confocal images with standard settings using laser lines 488nm and 561nm for excitation of Alexa Fluor 488 and TMR-Star dyes respectively. We used band pass filters 498-552nm and 588-647nm to collect the emitted fluorescence signals. We imaged nuclear DAPI using excitation by 405nm laser and band pass filter 410-452nm. Images from **Fig. S3C** represent single confocal planes.

*RNA extraction and Quantitative RT-PCR*

We used the RNeasy mini kit (QIAGEN) for total RNA extraction. After treating 1μg of RNA with Turbo DNAse (AMBION, Life Technologies) according to the manufacturer's instructions. We then performed reverse transcription using MMLV reverse transcriptase (Promega) with 100ng of random primers (ABI, Life Technologies) per

reaction, respectively. For quantitative PCR analysis, we used the 96-well plate LightCycler 480 Real-time PCR system from Roche and the SYBR Green PCR Master mix (Roche). We checked the efficiency of each primer pair (sequences below) with four cDNA dilutions of a standart cDNA. We measured duplicates (ChIP) or triplicates (quantiative RT-PCR) in all experiments and we normalized the quantity of mRNA to the quantity of mRNA corresponding to the human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). As a control when measuring SatIII RNA levels, all quantitative RT-PCRs were also performed in control samples lacking reverse transcriptase. Primer pairs for the quantitative RT-PCRs were the following: H3.1-F 5'-GGTGCGAGAAATAGCTCAGG-3'; H3.1-R 5'- CAAAACAATTCACGCCCTCT-3'; H3.3A-F 5'-CCAGGAAGCAACTGGCTACA-3'; H3.3A-R 5'- ACCAGGCCTGTAACGATGAG-3'; H3.3B-F 5'-GGATTTCAAAACCGACCTGA-3'; H3.3B-R 5'-AGCCAACTGGATGTCTTTGG-3'; GAPDH-F 5'-GAGTCAACGGATTTGGTCGT-3'; GAPDH-R 5'- TTGATTTTGGAGGGATCTCG-3'; SatIII-F 5'-AATCAACCCGAGTGCAATCGAATGGAATCG-3'; SatIII-R 5'-TCCATTCCATTCCTGTACTCGG-3' [3].

For ChIP analysis, we used the following primer pairs: Satα-F 5'-TAGACAGAAGCATTCTCAGAAACT-3'; Satα-R 5'-TCCCGCTTCCAACGAAA TCCTCCAAAC-3' [4]; SatIII : same primer pair as for RT-PCR.

## Supplementary References

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## Supplementary Figures

**Figure S1: In vivo labeling assays for H3-SNAP-HAx3 in MRC5 human primary cells**

**A.** Scheme for the assays of in vivo labeling of H3-SNAP-HAx3 histones in Pulse, Quench-Pulse and Quench-Chase-Pulse experiments in vivo. The Pulse labels pre-existing H3-SNAP with red fluorescent TMR-Star, the Quench-Pulse quenches pre-existing H3-SNAP with non-fluorescent Block preventing their subsequent labeling with TMR-Star and the Quench-Chase-Pulse labels new H3-SNAP synthesized during the 3h30 chase. HA labels total histone H3. Adapted from [5].

**B.** Fluorescent microscopy visualization of total e-H3.1 and e-H3.3 (HA, green) colocalization with mitotic chromosomes in MRC5 cells. New H3.1 or H3.3 (TMR, red) were also labeled in vivo in a quench-chase-pulse experiment. During the chase period, new H3.3, but not H3.1, is incorporated in G2 phase cells which then subsequently entered into mitosis allowing detection of new H3.3 on mitotic chromosomes. DAPI stains nuclei. Scale bar is 10 μm.

**C.** Newly H3.1 is deposited during S-phase while H3.3 is deposited throughout the cell cycle. Fluorescent microscopy visualization of replication sites (BrdU, green) and of new H3.1 and H3.3 (TMR, red) after in vivo labeling in a quench-chase-pulse experiment. White and green arrowheads indicate typical negative and positive BrdU cells, respectively. DAPI stains nuclei. Scale bar is 10 μm.

**D.** Histogram shows the percentage of H3.1 and H3.3 cells negative (BrdU –) or positive (BrdU +) for BrdU staining which are labeled with TMR-Star. Error bars indicate the standard deviation of two independent experiments (n=200 nuclei counted per experiment).

**Figure S2: New H3.3 is incorporated in oncogene-induced senescent MRC5 cells**

**A.** Fluorescent microscopy visualization of proliferating (empty) or senescent (Ras) MRC5 cells labeled with BrdU (green). We fixed cells at day 8 post-infection. DAPI stains nuclei and is a marker of SAHF formation. Scale bar is 10 μm.

**B.** Conventional light microscopy vizualisation of a senescence associated Beta-Gal assay in MRC5 cells treated as in (A). Scale bar is 20μm.

**C.** Histograms show quantitative analysis of senescence induction in MRC5 cells. BrdU positive cells are a marker of cell proliferation (left panel), while cells positive for SAHF (middle panel) or SA-β-Gal (right panel) are markers of senescence entry. Numbers represent the mean of 2-3 independent experiments ± s.d.

**D.** Fluorescent microscopy visualization of new H3.1 and H3.3 (TMR, red) after *in vivo* labelling of MRC5 e-H3.1 and e-H3.3, transduced with an empty retroviral vector (empty) or with a vector expressing H-RasV12 (Ras) for 10 days, in a quench-chase-pulse experiment. Prior to fixation, soluble proteins were pre-extracted by detergent treatment (Triton) allowing detection of the incorporated histones only. HA (green) stains total H3 histones and DAPI stains nuclei. Of note, SAHF appear more fuzzy upon pre-extraction but H3.3 exclusion from SAHF is clearer. Scale bar is 10 μm.

**Figure S3: H3.3 does not accumulate in SAHF in oncogene-induced senescent cells.**

**A.** Quantitative RT-PCR analysis of H3.1, H3.3A and H3.3B mRNA levels in proliferating (empty) and senescent (Ras) MRC5 cells. We normalized levels to the reference gene GAPDH and set levels in proliferating cells to 100%. Error bars represent s.d. from 2 independent experiments.

**B.** Western blot analysis of fractionated cell extracts from MRC5 e-H3.1/e-H3.3, either proliferating (empty) or induced into senescence (Ras) for 9 days. 15μg of cytosolic

(Cyt.) and nuclear salt-extractable (Nuc.) protein extracts and 25 μg of the chromatin pellet (Pellet) were loaded. Mcm7 was used as a marker for cell proliferation and p16 as a marker for proliferation arrest. Membranes were probed for ATRX, DAXX, HIRA, ASF1a, H3.3 and Ras. Smc-1 and α-Tubulin served as loading controls. M: molecular weight marker.

**C.** Fluorescent confocal microscopy visualization of new H3.3 (TMR) after in vivo labeling of MRC5 e-H3.3 cells proliferating (empty) or induced into senescence with H-RasV12 overexpression (Ras) for 9 days in a quench-chase-pulse experiment. H3K9me3 and DAPI were used as markers for SAHF formation. Scale bar is 10 μm.

**D.** Fluorescent microscopy visualization of endogenous H3.3 (green) in IMR90 ER:Ras cells, non-induced (no 4-OHT) or induced into senescence with 4-OHT (+ 4-OHT) for 6 days. DAPI stains nuclei. Insets representing enlarged images (3X) of selected area underscore absence of H3.3 accumulation in SAHF. Scale bar is 10 μm.

**Figure S4: H3.3 and its associated histone chaperones localize together in PML-NBs in proliferating and senescent cells**

**A.** Fluorescent microscopy visualization of endogenous H3.3 (green) in IMR90 ER:Ras cells, non-induced (no 4-OHT) or induced into senescence with 4-OHT (+ 4-OHT) for 6 days. One day prior to addition of 4-OHT, cells were transfected with control siRNA (siLuc) or siRNAs targeting H3.3A and H3.3B (siH3.3). siRNA transfection was performed a second time after 3 days of 4-OHT. Absence of H3.3 staining in siH3.3 shows specificity of the antibody. PML (red) stains PML-NBs and DAPI stains nuclei. Insets representing enlarged images (3X) of selected area underscore localization of endogenous H3.3 in PML-NBs. Scale bar is 10 μm.

**B.** Fluorescent microscopy visualization of new H3.3 (TMR, red) after in vivo labeling of MRC5 e-H3.3 cells either proliferating (empty) or induced into senescence (Ras) in a quench-chase-pulse experiment. Co-staining with ASF1a (green) and HIRA (cyan, pseudo-color) shows colocalization of new H3.3 with its chaperone ASF1a and HIRA in PML-NBs (white arrowheads). DAPI stains nuclei. Scale bar is 10 μm.

**C.** Fluorescent microscopy visualization showing colocalization of histone H3.3 chaperones ATRX, DAXX, ASF1a or HIRA (red) together with PML (green) in normal MRC5 cells, proliferating (empty) or induced into senescence (Ras) for 12 days. Numbers indicate the mean percentage of cells with colocalization of the histone H3.3 chaperone within PML-NBs ± s.d. from 2 independent experiments. DAPI stains nuclei. Scale bar is 10 μm.

**Figure S5: DAXX-dependent localization of ATRX at PML-NBs**

**A.** Western blot analysis of total cell extracts from MRC5 treated for 48h with the indicated siRNAs. Membranes were probed for ATRX, DAXX, HIRA (left panel) and H3.3 (right panel). α-Tubulin and Smc-1 served as loading controls. M: molecular weight marker.

**B.** Fluorescent microscopy visualization of ATRX (red, left panel) or DAXX (red, right panel) in MRC5 cells treated as in (A) showing loss of ATRX from PML-NBs (green) upon DAXX depletion. Insets represent enlarged images of selected area. DAPI stains nuclei. Scale bar is 10 μm.

**Figure S6: ATRX localization to SAHF is increased in absence of DAXX**

**A.** Fluorescent microscopy visualization of ATRX (green) in MRC5 e-H3.3 cells treated as in **Fig. 4A**. Western blot analysis is shown in **Fig. 4B**. DAPI stains nuclei and is a marker of SAHF. Scale bar is 10 μm.

**B.** Graphics show fluorescent intensity profiles quantified using Image J along lines drawn through nuclei as shown in panel (A). Comparison of profiles in proliferating (empty) cells treated with siLuc (i) or siDAXX1 (ii) shows loss of ATRX localization at PML-NBs upon DAXX depletion (see also **Fig. S5B**). In oncogene-induced senescent cells (Ras), ATRX localization at PML-NBs increases (compare (iii) with (i)). Upon DAXX knockdown, ATRX localization to SAHF is increased (iv), since it is no longer targeted to PML-NBs.

**Figure S7: H3.3 localization to PML-NBs in absence of PML or HIRA**

**A.** Western blot analysis of total cell extracts from MRC5 e-H3.3 cells either proliferating (empty) or induced into senescence by H-RasV12 overexpression (Ras) for 7 days and treated with the indicated siRNAs during the last 48h. Membranes were probed for PML and Ras. Smc1 served as a loading control. M: molecular weight marker.

**B.** Fluorescent microscopy visualization of new H3.3 (TMR, red) after in vivo labeling of proliferating MRC5 e-H3.3 cells treated as in (A) in a quench-chase-pulse experiment. New H3.3 localization at PML-NBs is lost in absence of PML (green). Insets represent enlarged images (3x) of selected area. DAPI stains nuclei. Scale bar is 10 μm.

**C.** Histogram shows quantitative analysis of the proportion of cells showing new H3.3 localization at PML-NBs. Numbers represent the mean of 2 independent experiments ± s.d.

**D.** Western blot analysis of total cell extracts from MRC5 e-H3.3 cells treated as in (A). Membranes were probed for HIRA, Ras and HA. α-Tubulin served as a loading control. M: molecular weight marker.

**E.** Fluorescent microscopy visualization of new H3.3 (TMR, red) after in vivo labeling of MRC5 e-H3.3 cells treated as in (A) in a quench-chase-pulse experiment. New H3.3 localization at PML-NBs is not impaired in absence of HIRA. Insets represent enlarged images of selected area. DAPI stains nuclei. Scale bar is 10 μm.

**F.** Histogram shows quantitative analysis of the proportion of cells showing new H3.3 localization at PML-NBs. Numbers represent the mean of 2 independent experiments ± s.d.

**Figure S8: Depletion of DAXX or ATRX or overexpression of DAXX in MRC5 cells does not impair senescence entry upon overexpression of H-RasV12**

**A.** Fluorescent microscopy visualization of MRC5 cells cotransduced with a virus encoding HRas-V12 (Ras, green) and a virus encoding a control shRNA (pRetro empty) or a virus encoding a specific shRNA targeting DAXX (shDAXX1) or ATRX (shATRX1) for 8 days. DAPI stains nuclei and is a marker of SAHF. DAXX (left panel) or ATRX (right panel) are stained in red. Scale bar is 10 μm.

**B.** Histogram shows quantification of the mean number of positive cells for SAHF formation for cells treated as in (A). Error bars represent s.d. from 2 independent experiments.

**C.** Western blot analysis of total cell extracts from MRC5 treated as in (A). Membranes were probed for DAXX and Ras to verify expression of these proteins. Cyclin A was used as a marker for cell proliferation and p16 as a marker for proliferation arrest. α-Tubulin

served as a loading control. The vertical bar indicates that a lane was cut from the gel. M: molecular weight marker.

**D.** Western blot analysis of total cell extracts from MRC5 empty cells or MRC5 overexpressing Myc-DAXX, transduced with an empty vector or with a vector expressing oncogenic Ras for 8 days. Membranes were probed for Myc, DAXX, and Ras to verify expression of the transduced proteins. Cyclin A was used as a marker for cell proliferation and p16 as a marker for proliferation arrest. α-Tubulin served as a loading control. M: molecular weight marker.

**E.** Fluorescent microscopy visualization of MRC5 cells treated as in (D). Myc (red) stains the epitope-tagged Myc-DAXX. DAPI stains nuclei and is a marker of SAHF. Scale bar is 10 μm.

**Figure S9: Depletion of DAXX, ATRX or H3.3 in IMR90 ER:Ras does not impair senescence entry upon overexpression of H-RasV12**

**A.** Scheme for the assay of induction into senescence in IMR90 ER:Ras. Cells were first transduced with a virus encoding a control shRNA (empty) or a virus encoding an shRNA against DAXX (shDAXX1) or ATRX (shATRX1). 5 days later, IMR90 ER:Ras were induced into senescence by addition of 4-OHT (100nM) and senescence was assessed 6 days after.

**B.** Immunofluorescence analysis of IMR90 ER:Ras treated as in (A). DAXX (red, left panel) or ATRX (red, right panel) proteins are depleted in the specific shRNAs. DAPI stains nuclei and is a marker of SAHF. Scale bar is 10 μm.

**C.** Histogram shows quantification of the mean number of positive cells for SAHF formation for cells treated as in (A). Error bars represent s.d. from 2 independent experiments.

**D.** Conventional light microscopy vizualisation of a senescence associated Beta-Gal assay in IMR90 ER:Ras cells treated as in (A). Scale bar is 20μm.

**E.** Western blot analysis of total cell extracts from IMR90 ER:Ras cells treated as in (A). In addition, IMR90 ER:Ras transduced with an empty virus (pLNCX2 empty) or a virus encoding Myc-DAXX (Myc-DAXX) were also included in the analysis of senescence induction with 4-OHT for 6 days. Membranes were probed for Myc, DAXX, and Ras to verify expression of the transduced proteins. Cyclin A was used as a marker for cell proliferation and p16 as a marker for proliferation arrest. α-Tubulin served as a loading control. M: molecular weight marker. The vertical bar indicates that a lane was cut from the gel. As shown on MRC5 cells in **Fig. S8**, depletion or overexpression of DAXX does not impair senescence entry.

**F.** Western blot analysis of total cell extracts from IMR90 ER:Ras cells, non-induced (no 4-OHT) or induced into senescence with 4-OHT (+ 4-OHT) for 6 days. One day prior to addition of 4-OHT, cells were transfected with control siRNA (siLuc) or siRNAs targeting DAXX (siDAXX1 or siDAXX3), ATRX (siATRX1) or H3.3A and H3.3B (siH3.3). siRNA transfection was performed a second time after 3 days of 4-OHT. Membranes were probed for ATRX, DAXX, H3.3 and Ras to verify expression of these proteins. Cyclin A was used as a marker for cell proliferation and p16 as a marker for proliferation arrest. α-Tubulin served as a loading control. M: molecular weight marker.

**G.** Histogram shows quantification of the mean number of positive cells for SAHF formation for cells treated as in (F). Error bars represent s.d. from 2 independent experiments.

**H.** Fluorescent microscopy visualization of IMR90 ER:Ras cells treated as in (F). Addition of 4-OHT (+4-OHT) triggers accumulation of HRas-V12 (green) and senescence induction. DAPI stains nuclei and is a marker of SAHF. DAXX or ATRX are stained in red.

Arrowheads indicate ATRX localization at SAHF. Scale bar is 10 μm. See also **Fig. S4A** for immunofluorescence analysis of siH3.3 treated cells.

**Figure S10: Overexpression of DAXX triggers accumulation of new H3.3 in PML-NBs**

**A.** (Left panel) Fluorescent microscopy visualization of new H3.3 (TMR, red) after *in vivo* labelling of MRC5 e-H3.3 cells transduced with an empty vector (Empty) or a vector expressing Myc-DAXX (Myc-DAXX) and co-stained with Myc (green) and DAXX (cyan, pseudo-color). Scale bar is 10 μm. (Right panel) Graphics show distribution of new H3.3 (TMR) signal in MRC5 e-H3.3. TMR fluorescence intensity of each pixel delimited within the nuclei is plotted in a 3D-surface plot shows increase of targeting of H3.3 at PML-NBs upon DAXX overexpression.

**B.** Fluorescent microscopy visualization of new H3.3 (TMR, red) after *in vivo* labelling of proliferating (empty) or senescent (Ras) MRC5 e-H3.3 cells in a quench-chase-pulse experiment. Prior to fixation, soluble proteins were pre-extracted by detergent treatment (Triton) allowing detection of the triton-insoluble proteins only. Co-staining with H3K9me3 (green) and PML (cyan, pseudo-color) shows colocalization of new triton-insoluble H3.3 within the PML-NBs and exclusion from SAHF, marked as H3K9me3 dense regions. Scale bar is 10 μm.

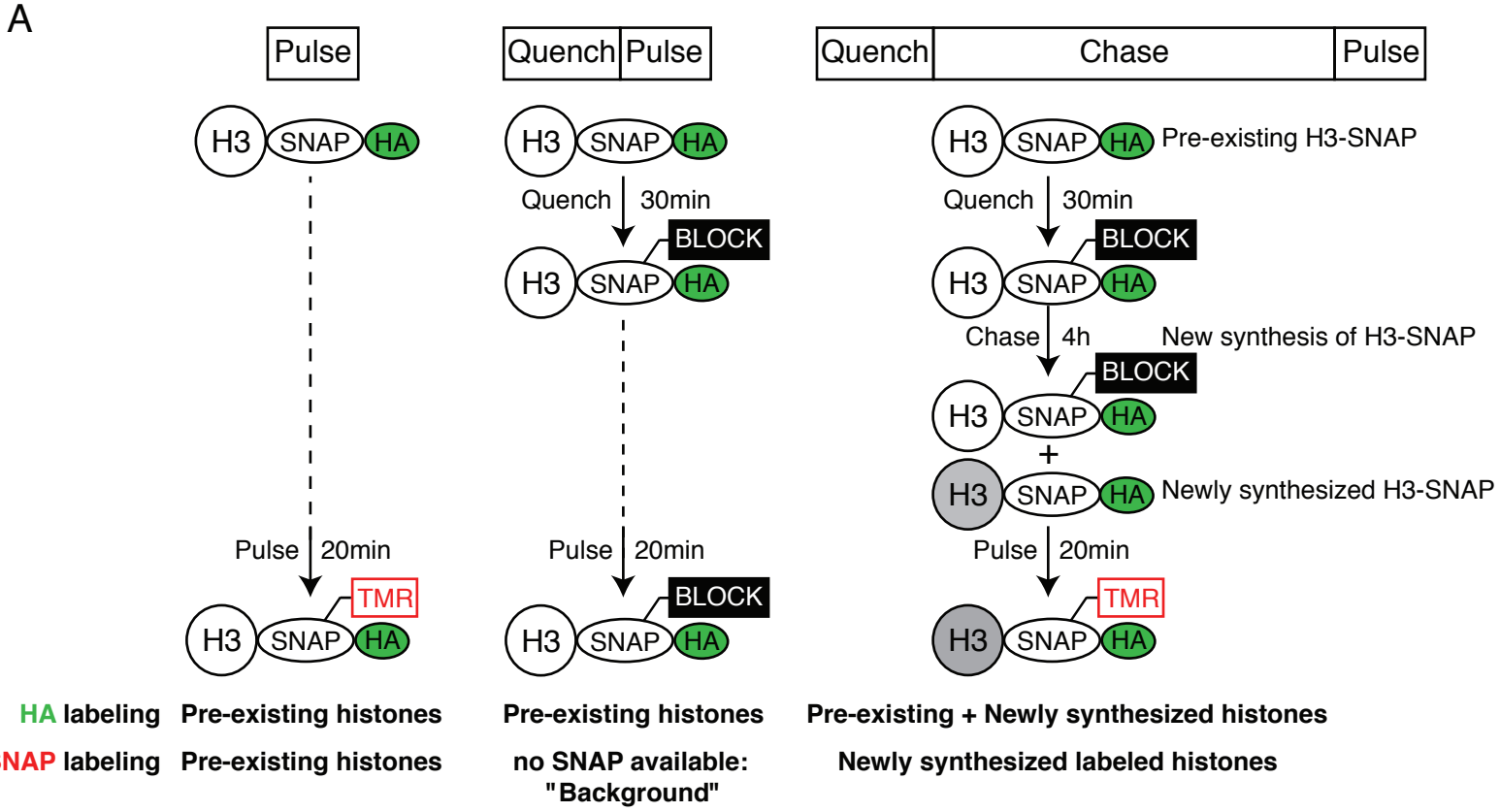
**Figure S11: New H3.3 localizes in PML-NBs in nucleolar caps upon transcription inhibition.**

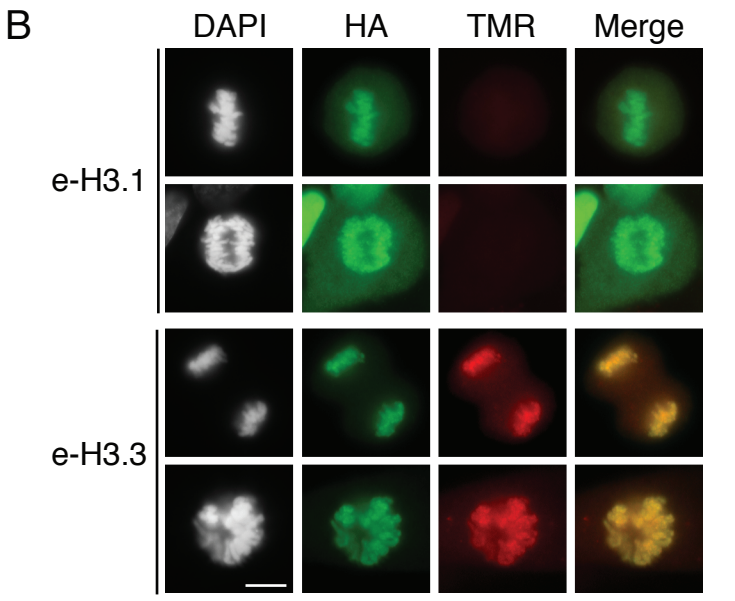
**A.** Fluorescent microscopy visualization of new H3.3 (TMR, red) after *in vivo* labelling of MRC5 e-H3.3 in a quench-chase-pulse experiment. Cells were treated with DMSO or with Actinomycin D (ActD) at a dose of 2μg/mL for the total lenght of the chase (3h30) to

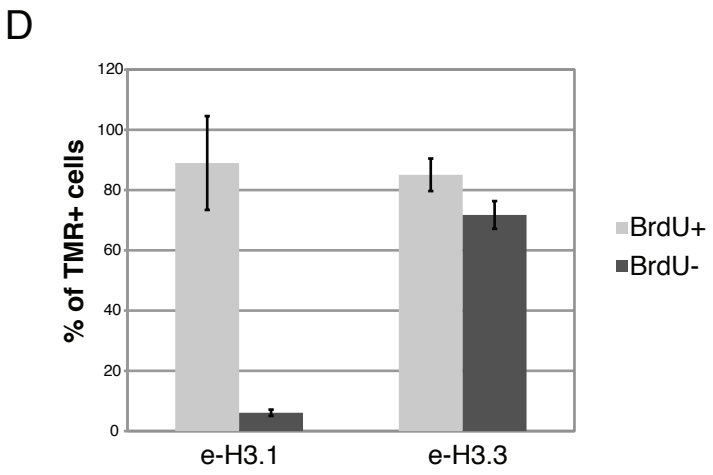
prevent transcription of new H3.3 (control, no TMR signal), or for the end of the chase (1h30) to allow synthesis of new H3.3 before transcription inhibition. In this latter case, new H3.3 relocalizes together with PML in nucleolar caps as shown previously for PML upon transcription inhibition [6]. Insets represent enlarged images (3X) of selected area. PML (green) stains PML-NBs and DAPI stains nuclei. Scale bar is 10 μm.

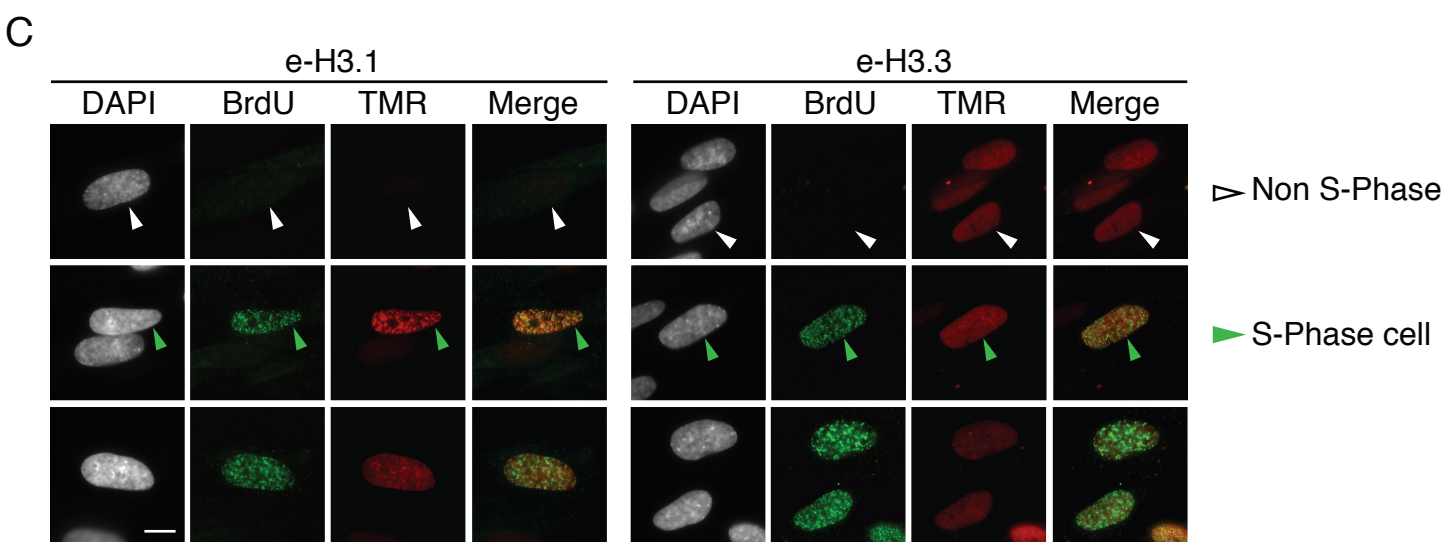
**B.** Fluorescent microscopy visualization of new H3.3 (TMR, red) as in **A**. MRC5 e-H3.3 were transduced with an empty retroviral vector (empty) or with a vector expressing H-RasV12 (Ras) for 7 days. Actinomycin D (ActD) at a dose of 2μg/mL was added during the end of the chase (1h30). Insets represent enlarged images (3X) of selected area. PML (green) stains PML-NBs and DAPI stains nuclei. Scale bar is 10 μm.

# Supplementary Figure S1

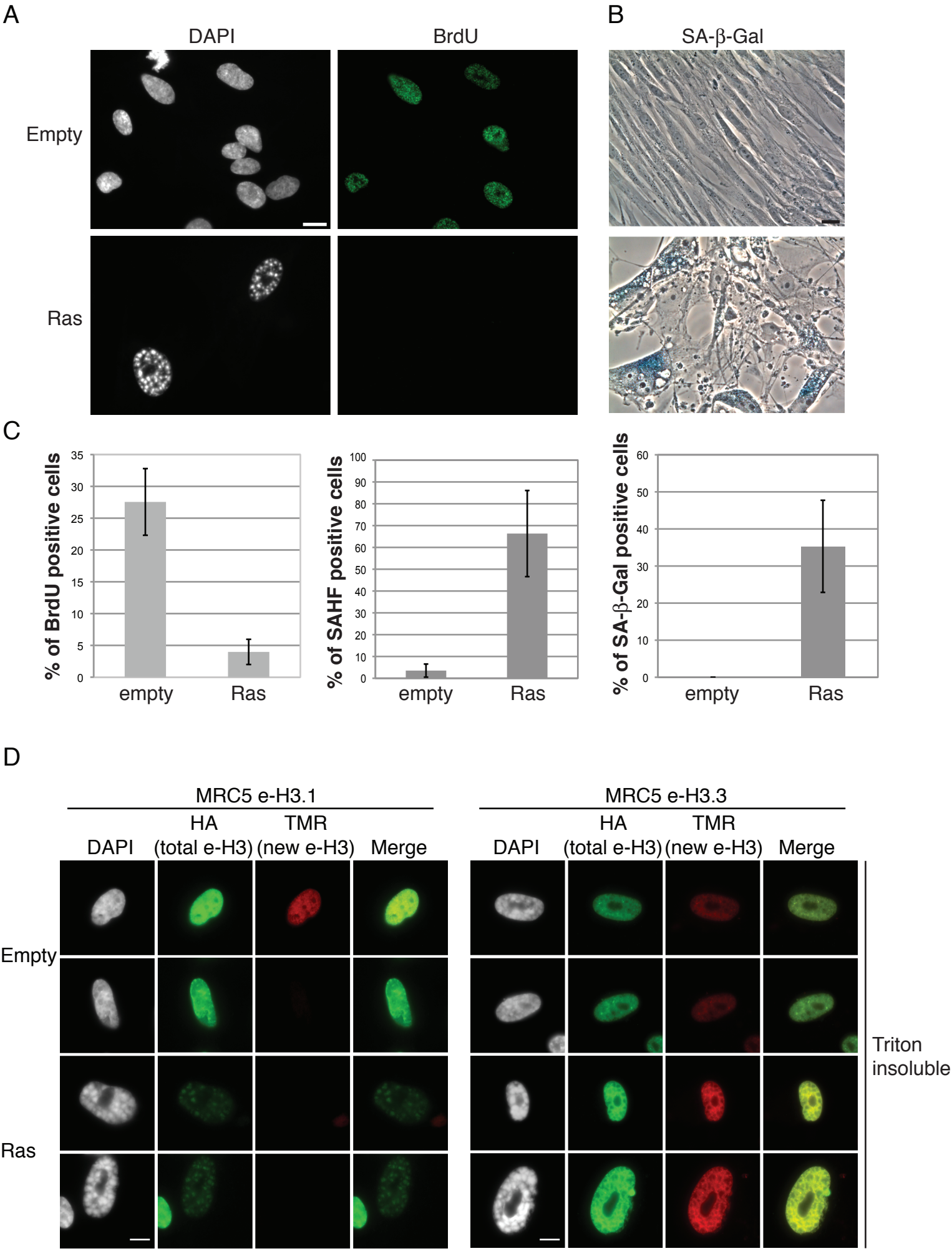


# Supplementary Figure S2



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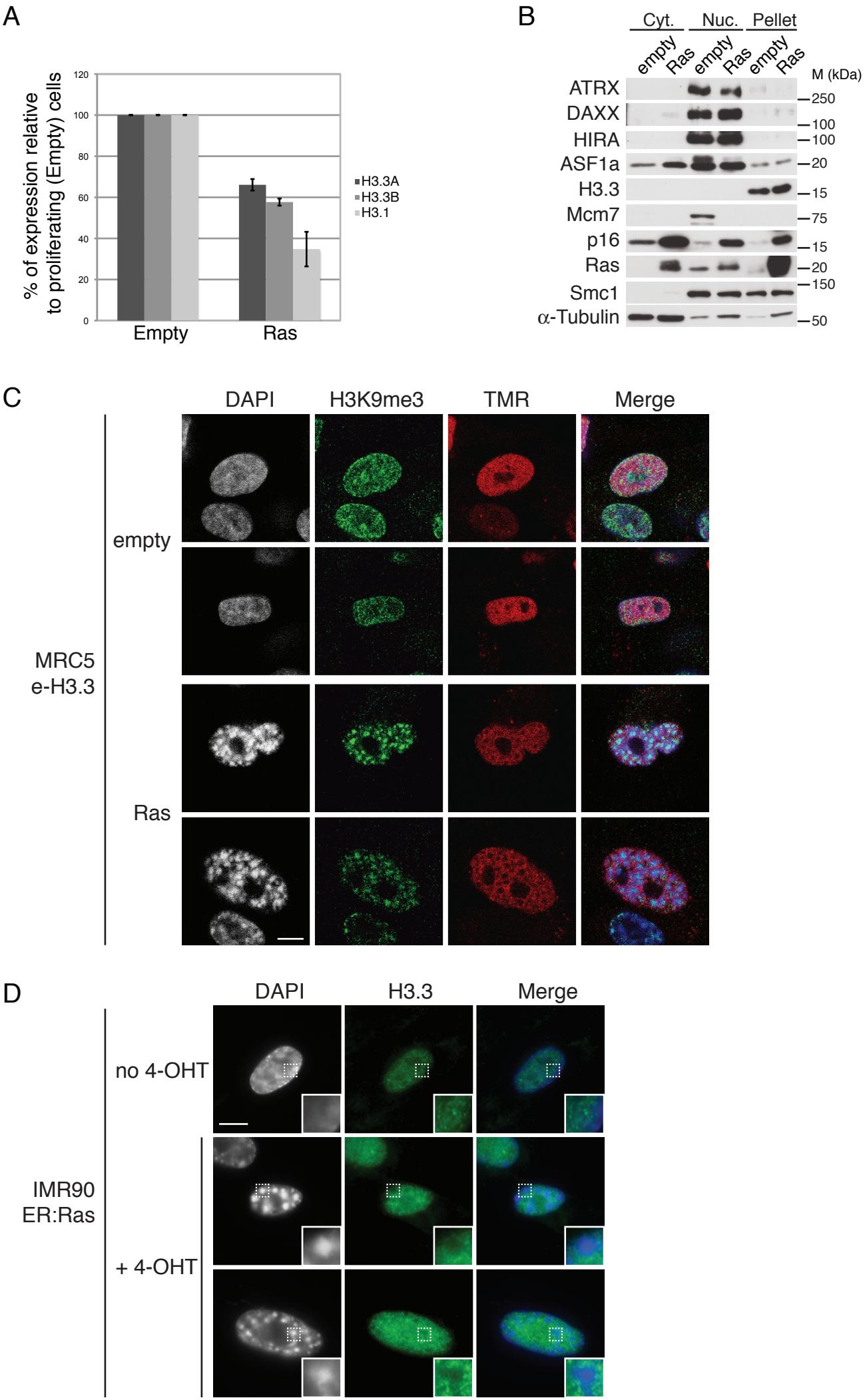

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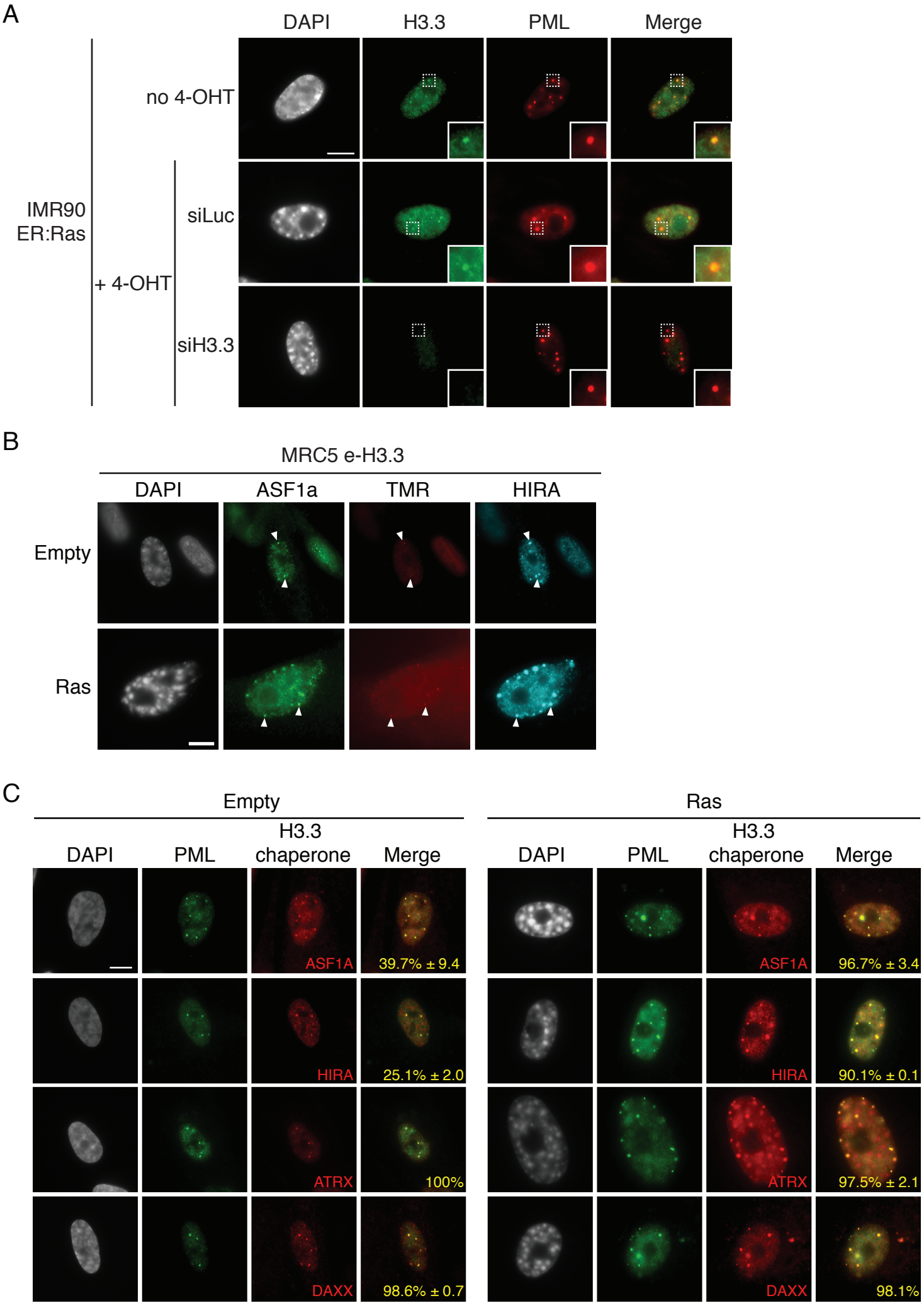

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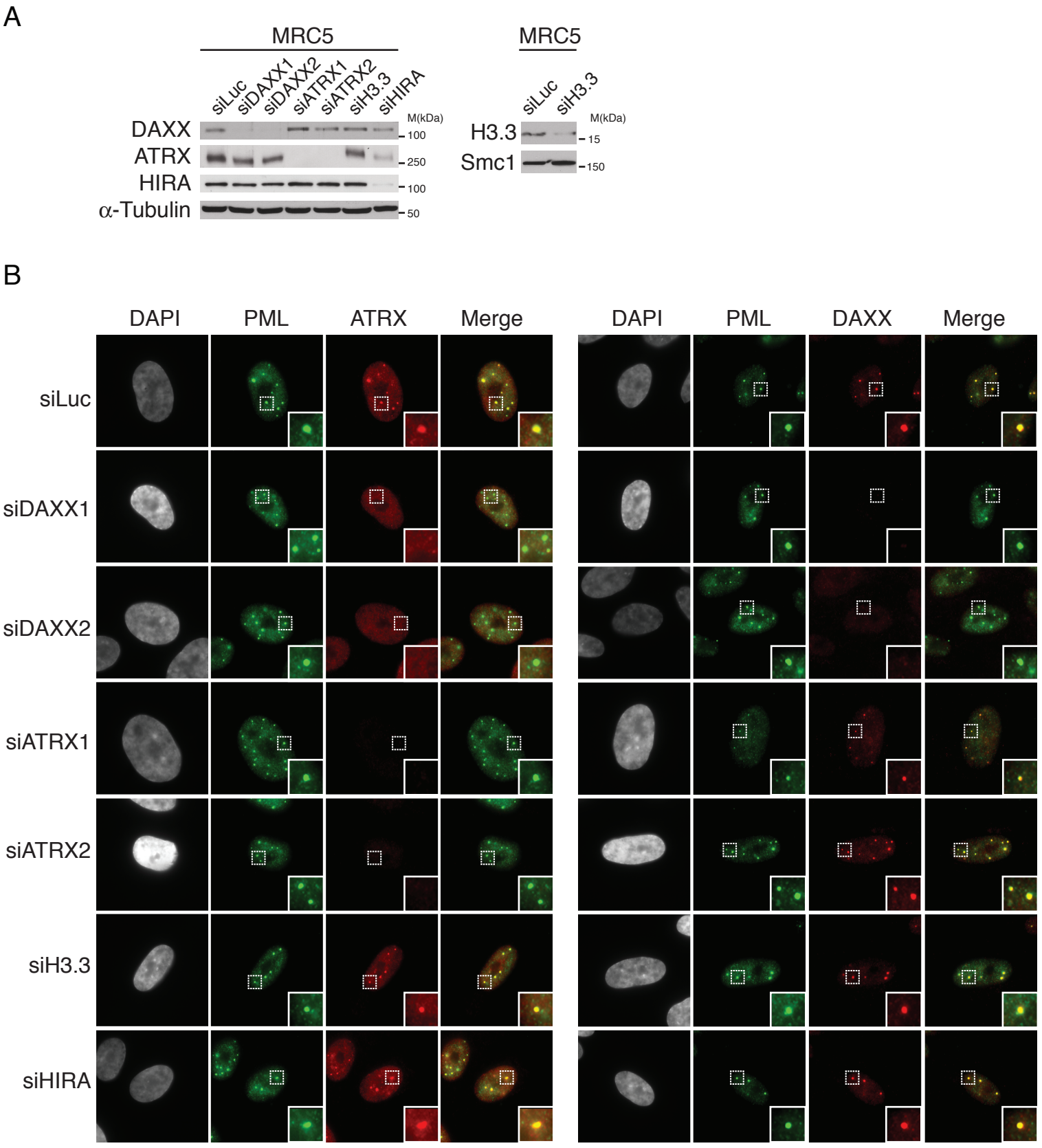

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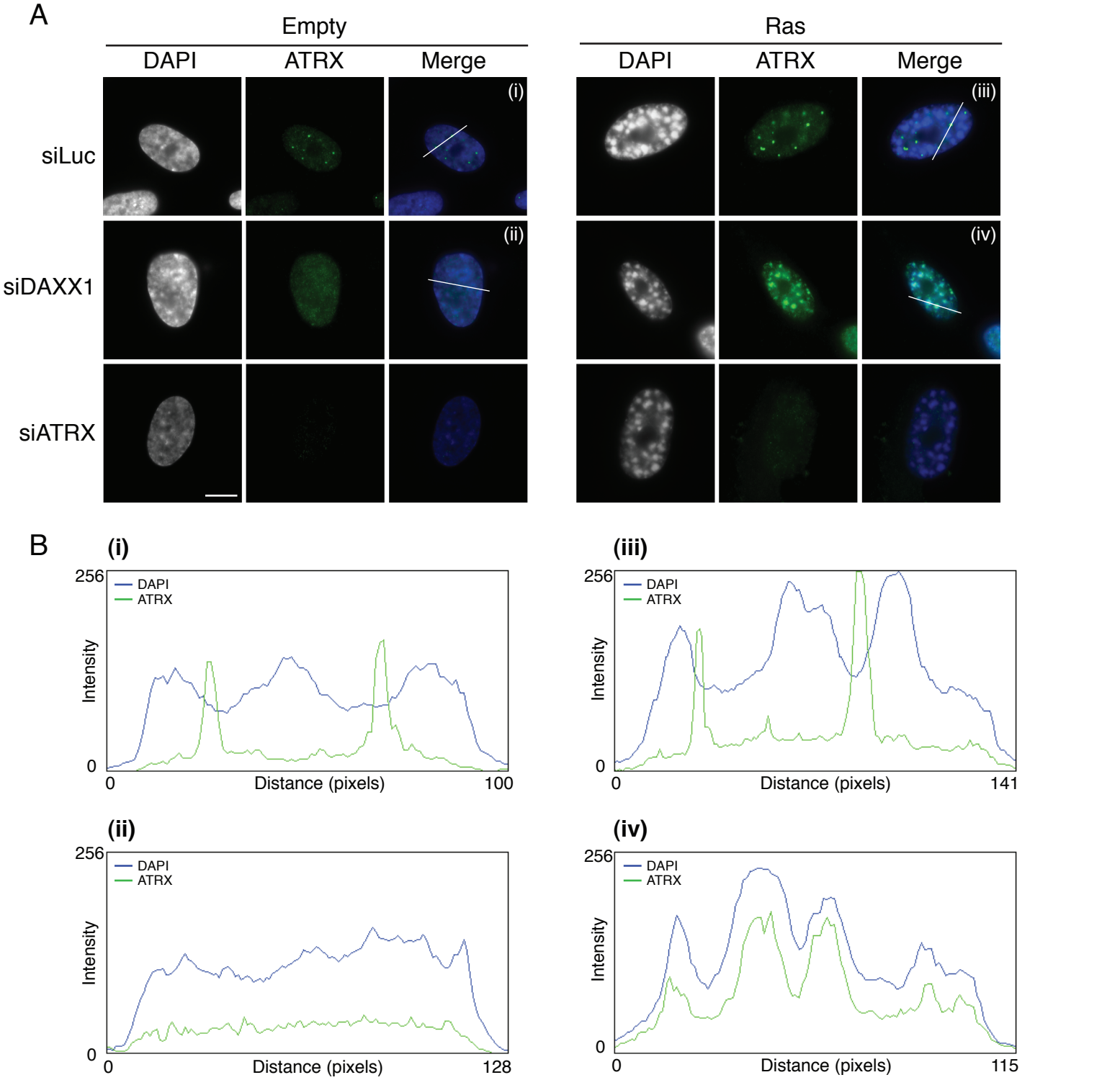

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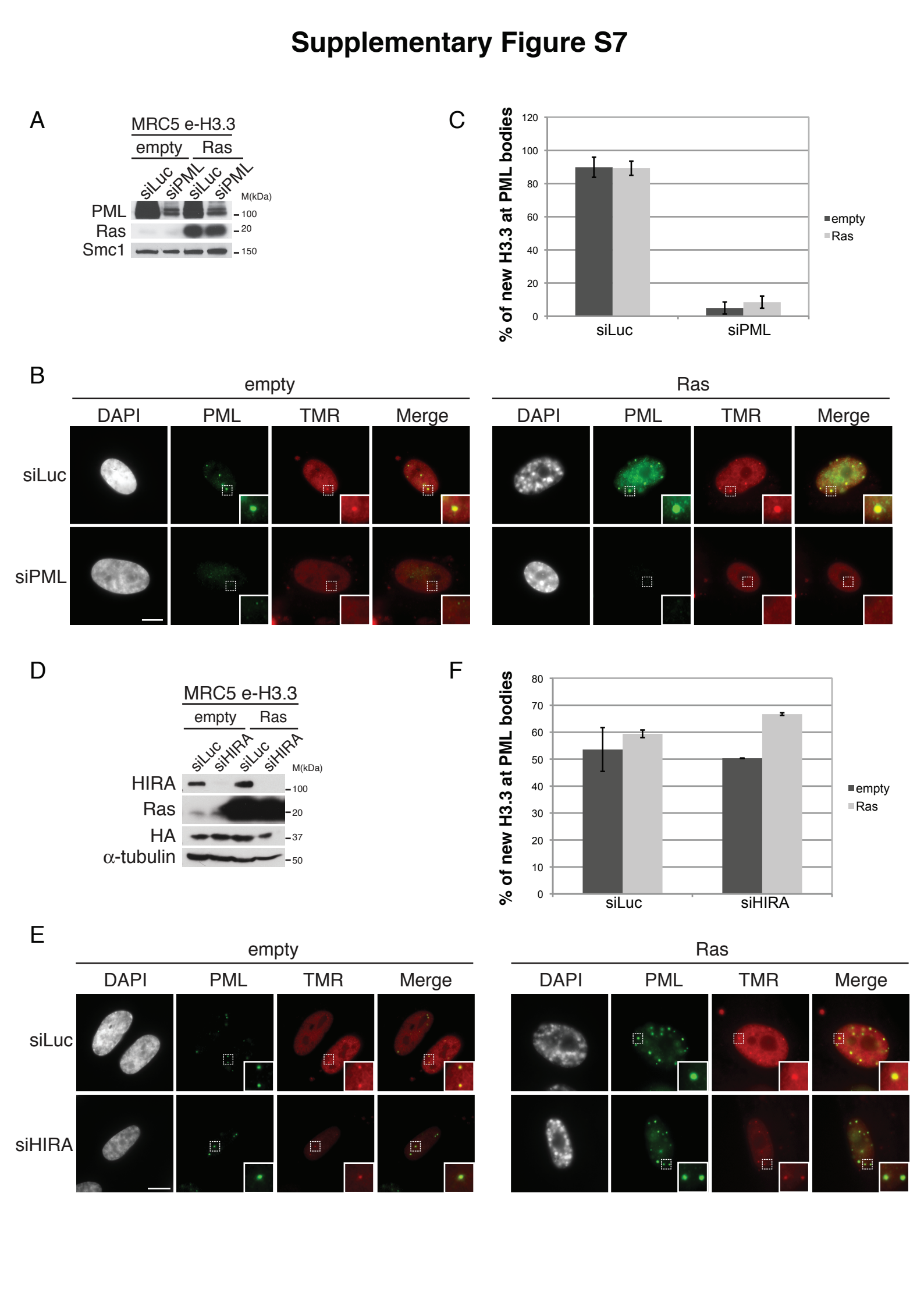
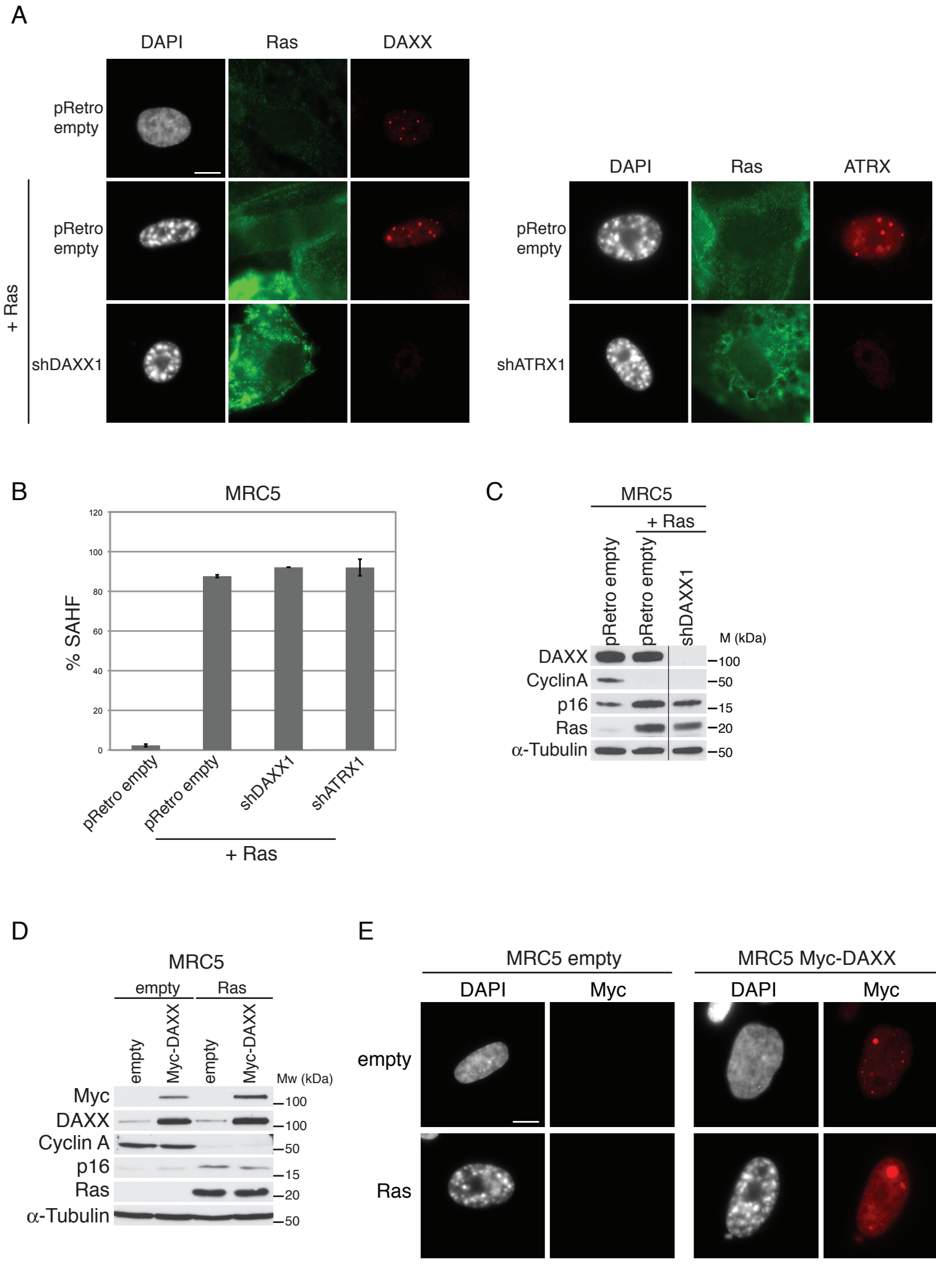


# Supplementary Figure S8



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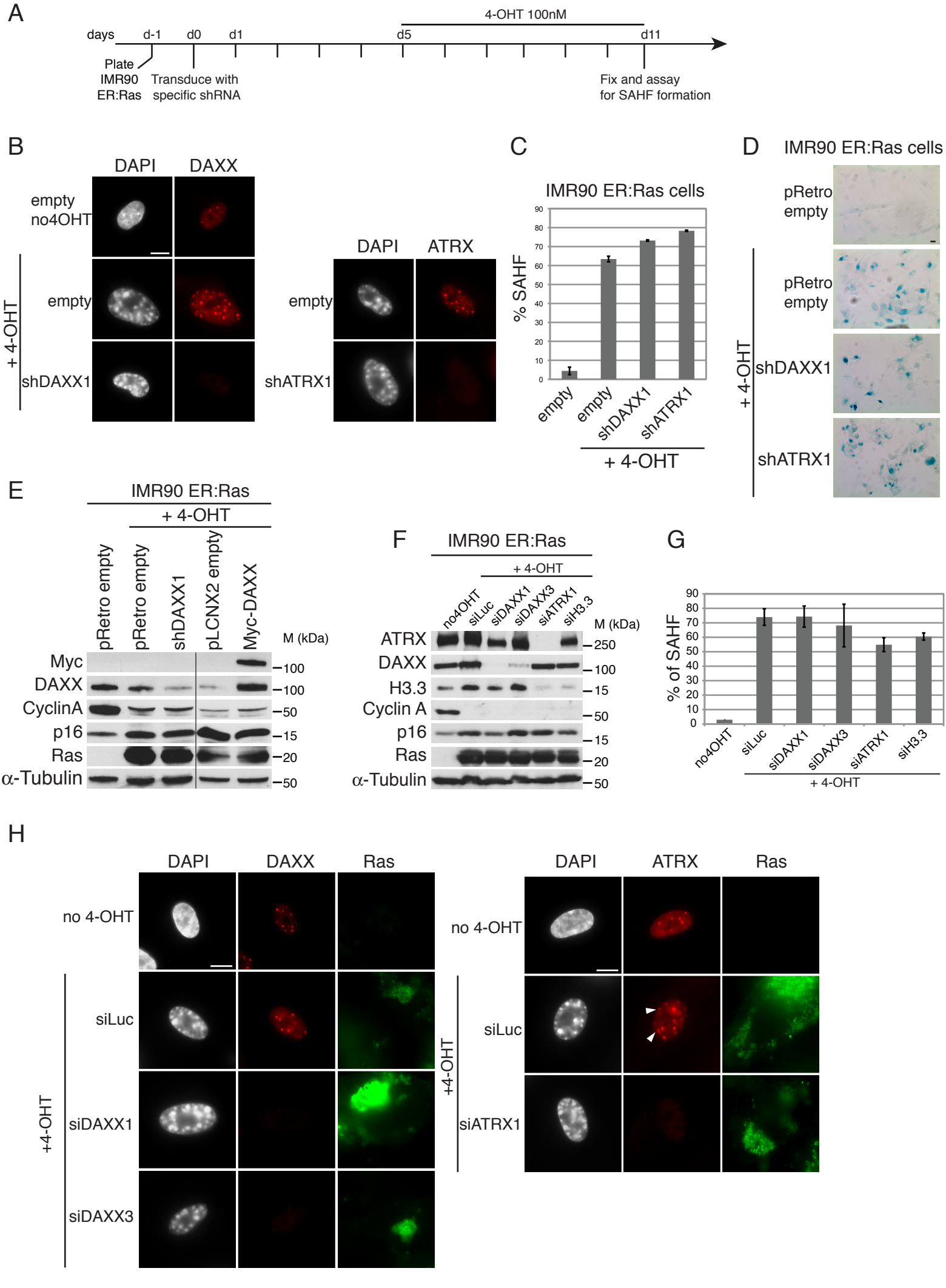

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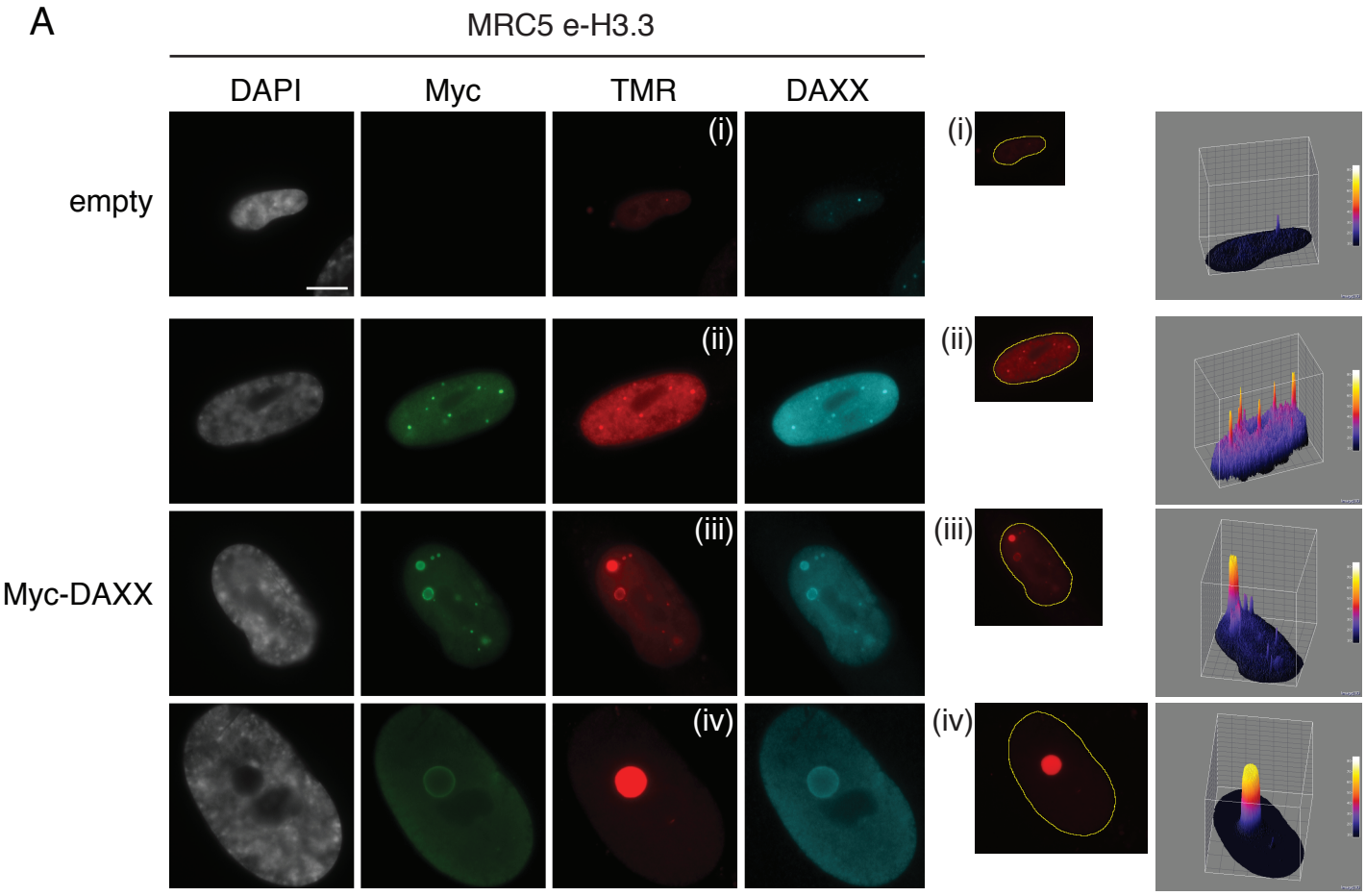
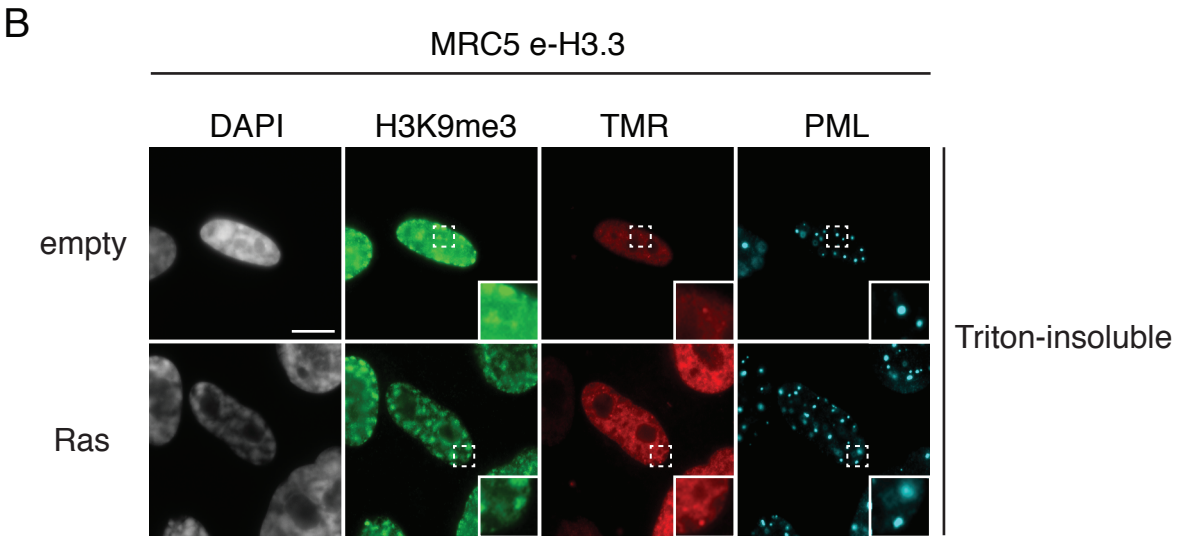
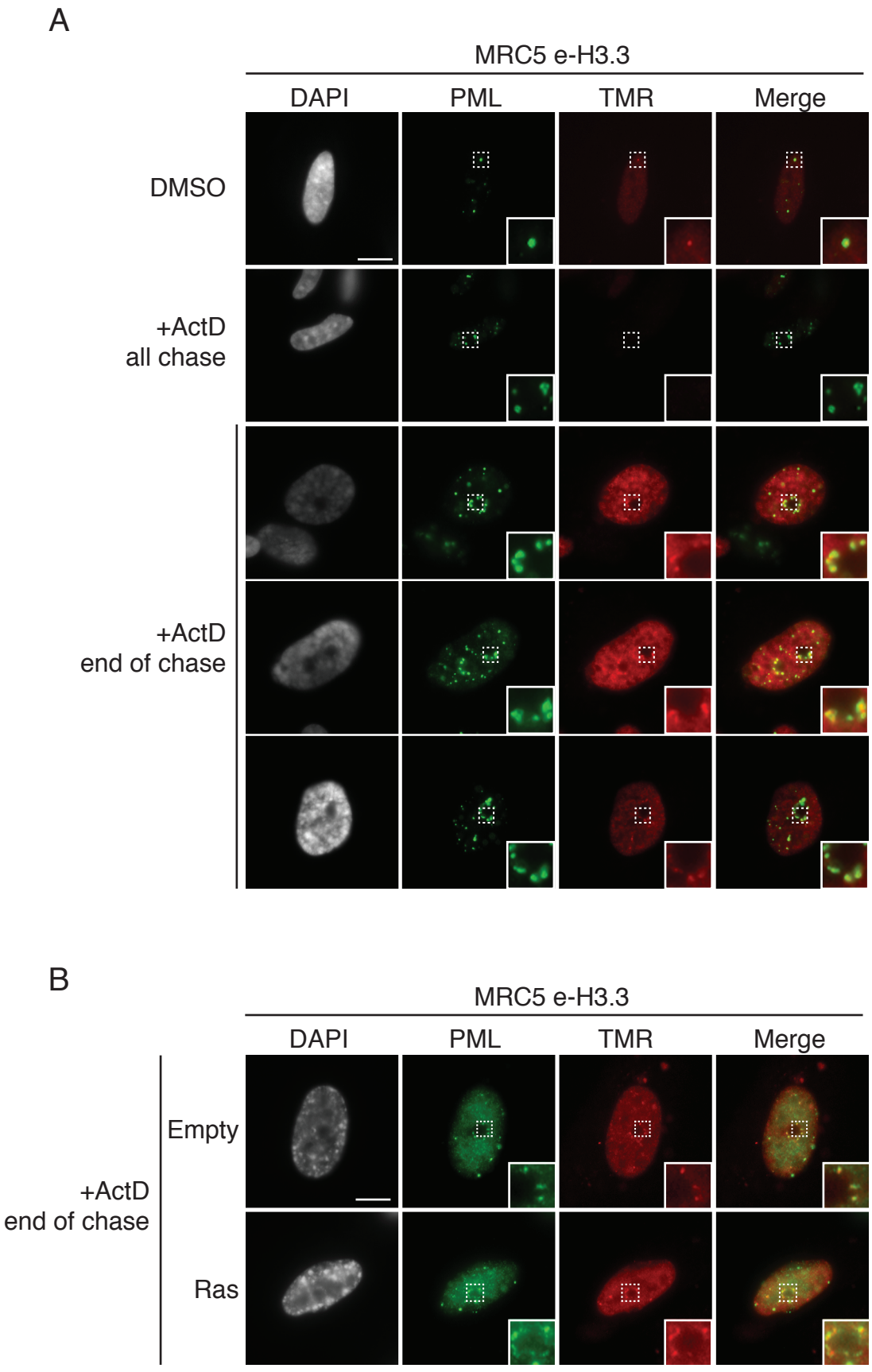


# Supplementary Figure S11



## Supplementary Table SI: List of primary antibodies

| Antibody | Company/Reference | Order number | Lot number | Species | WB dilution | IF dilution |
|---|---|---|---|---|---|---|
| **ASF1a** | Mello et al., 2002 | #28134 | - | Rabbit polyclonal | 1/1000 | 1/1000 |
| **α-Tubulin** | SIGMA | T6199 | - | Mouse monoclonal | 1/200 | - |
| **ATRX** | Santa Cruz Biotechnology | sc-15408 (clone H-300) | F0211 | Rabbit polyclonal | 1/1000 | 1/250 |
| **Cyclin A** | Santa Cruz Biotechnology | sc-751 (clone H-432) | G0811 | Rabbit polyclonal | 1/1000 | - |
| **DAXX** | Santa Cruz Biotechnology | sc-7152 (clone M-112) | E1412 | Rabbit polyclonal | 1/1000 | 1/250 |
| **HA** | Abcam | ab9110 | - | Rabbit polyclonal | 1/6000 | 1/1000 |
| **HIRA** | Active Motif | 39558 (clone WC119.2H11) | 909001 | Mouse monoclonal | 1/1000 | 1/100 |
| **HP1γ** | Pierce - Thermo Scientific | PA5-17445 | OB1666806 | Rabbit polyclonal | - | 1/200 |
| **H3.3** | Abnova | H00003021-M01 (clone 2D7-H1) | 12264-S1 | Mouse monoclonal | 1/100 | 1/100 |
| **MCM7** | Santa Cruz Biotechnology | sc-65469 (clone DCS-141) | H0210 | Mouse monoclonal | 1/500 | - |
| **Myc** | GeneTex | GTX80249 | - | Mouse monoclonal | 1/2000 | 1/250 |
| **PML** | Santa Cruz Biotechnology | sc-5621 (clone H-238) | K2912 | Rabbit polyclonal | 1/500 | 1/100 |
| **PML** | Santa Cruz Biotechnology | sc-966 (clone PG-M3) | F1511 | Mouse monoclonal | - | 1/250 |
| **p16** | Santa Cruz Biotechnology | sc-56330 (clone JC8) | I2512 | Mouse monoclonal | 1/200 | - |
| **Ras** | BD Transduction Laboratories | 610001 | 82593 | Mouse monoclonal | 1/1000 | 1/500 |
| **Smc1** | Abcam | ab9262 | GR1468-4 | Rabbit polyclonal | 1/1000 | - |
| **IgG** | Santa Cruz Biotechnology | sc-2027 | C0411 | Rabbit Polyclonal | 1μg for IP | - |