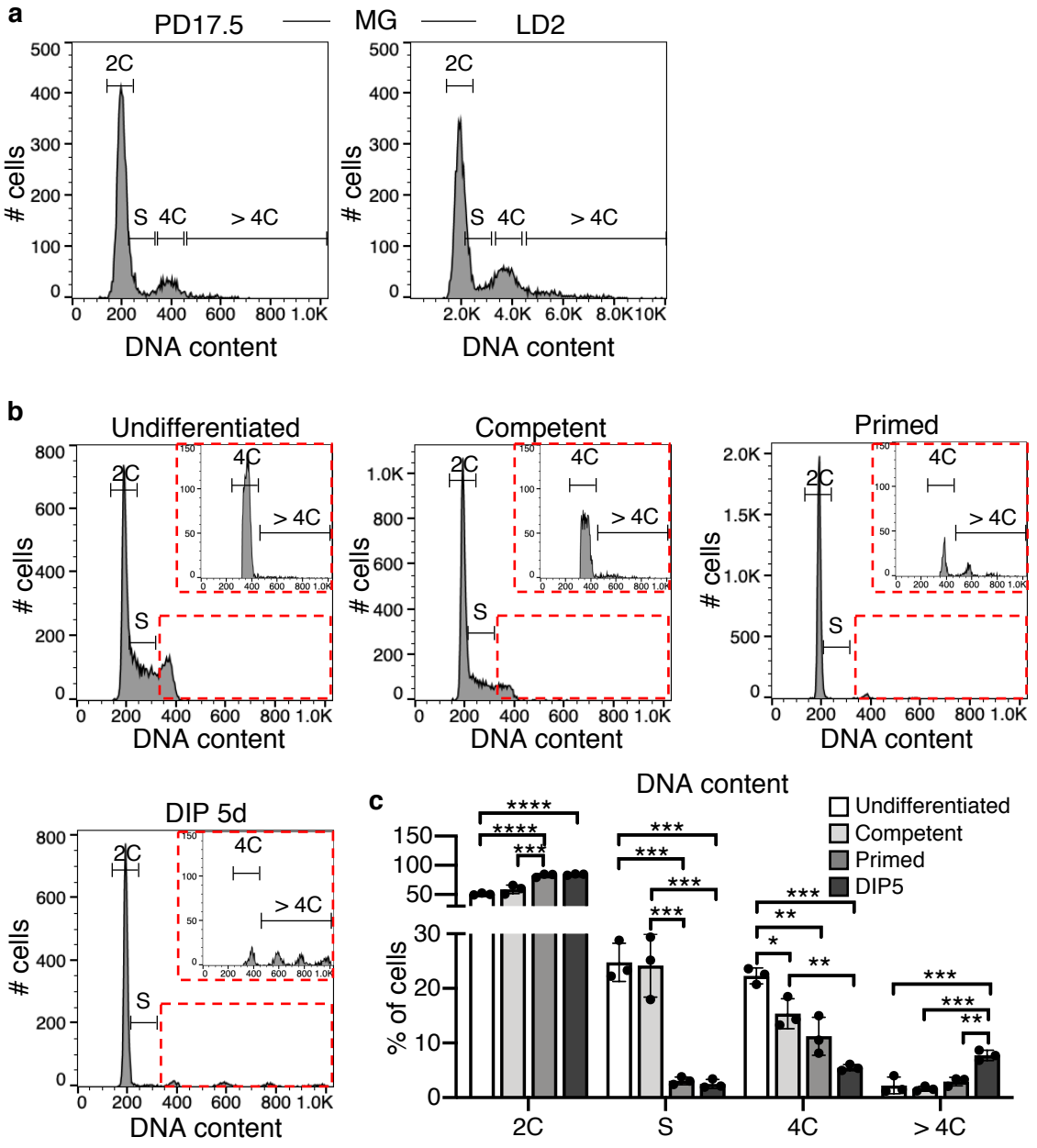
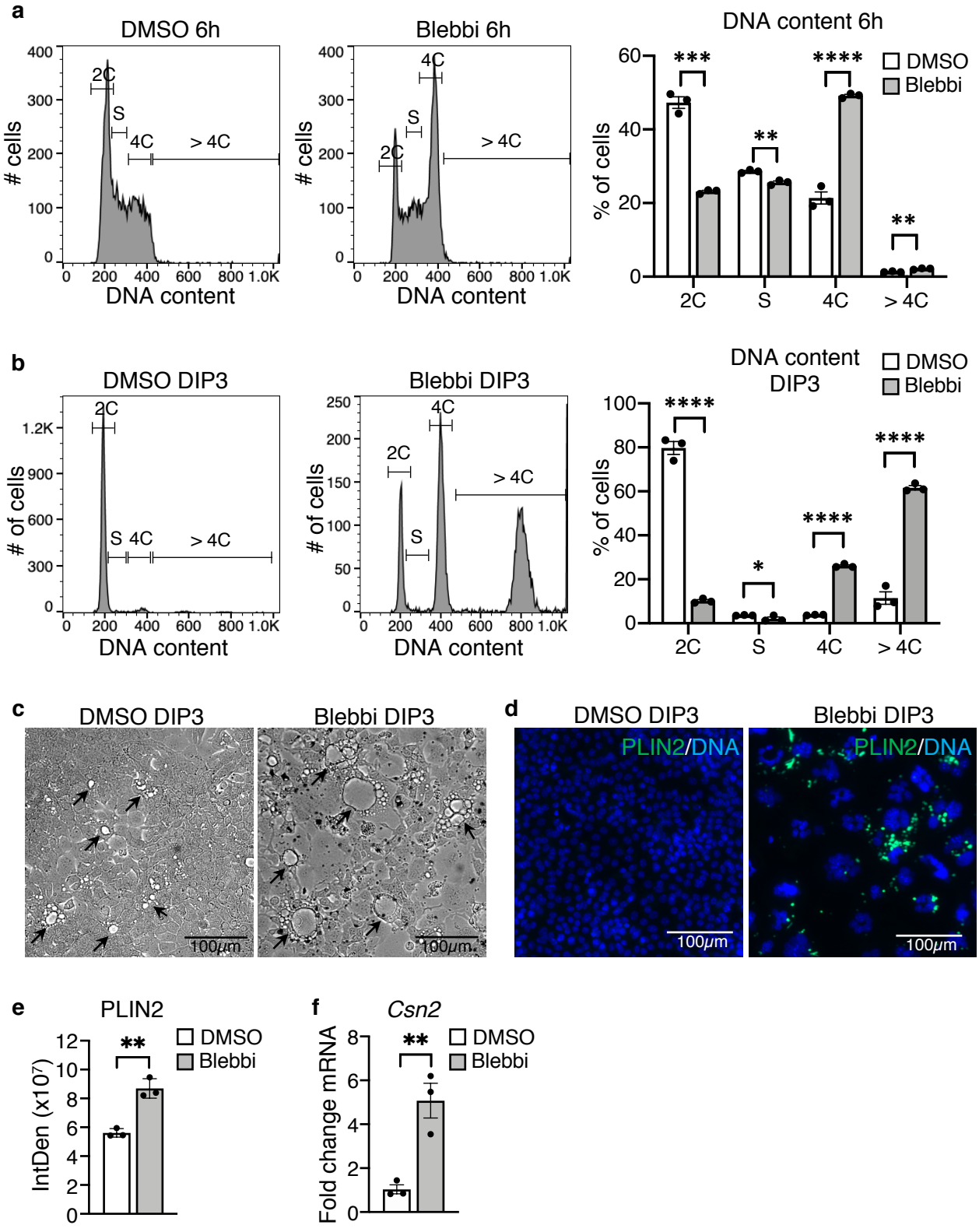
Supplementary Figures

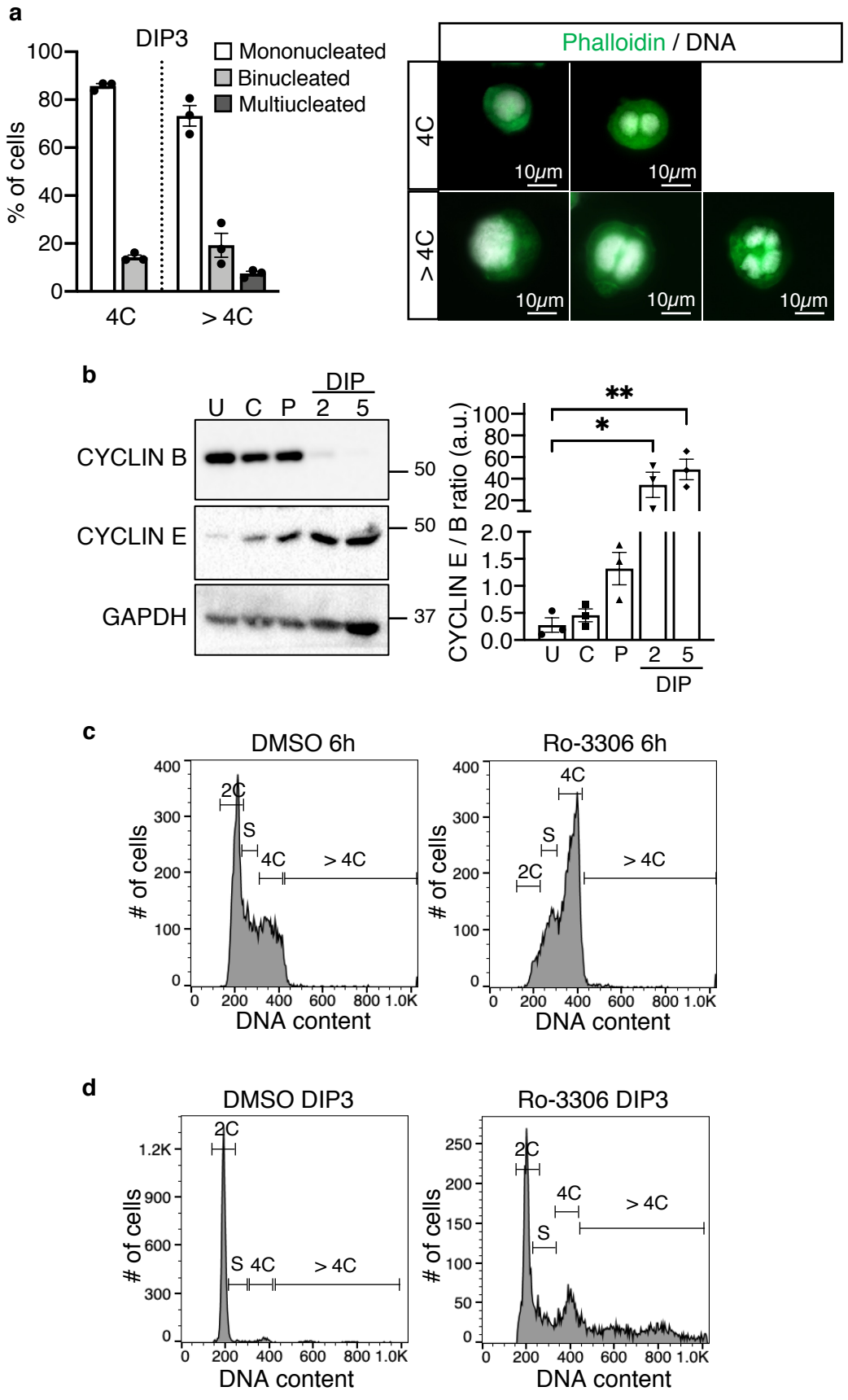
Physiological DNA damage Promotes Functional Polyploidization of Mammary Gland Alveolar Cells During Lactation.



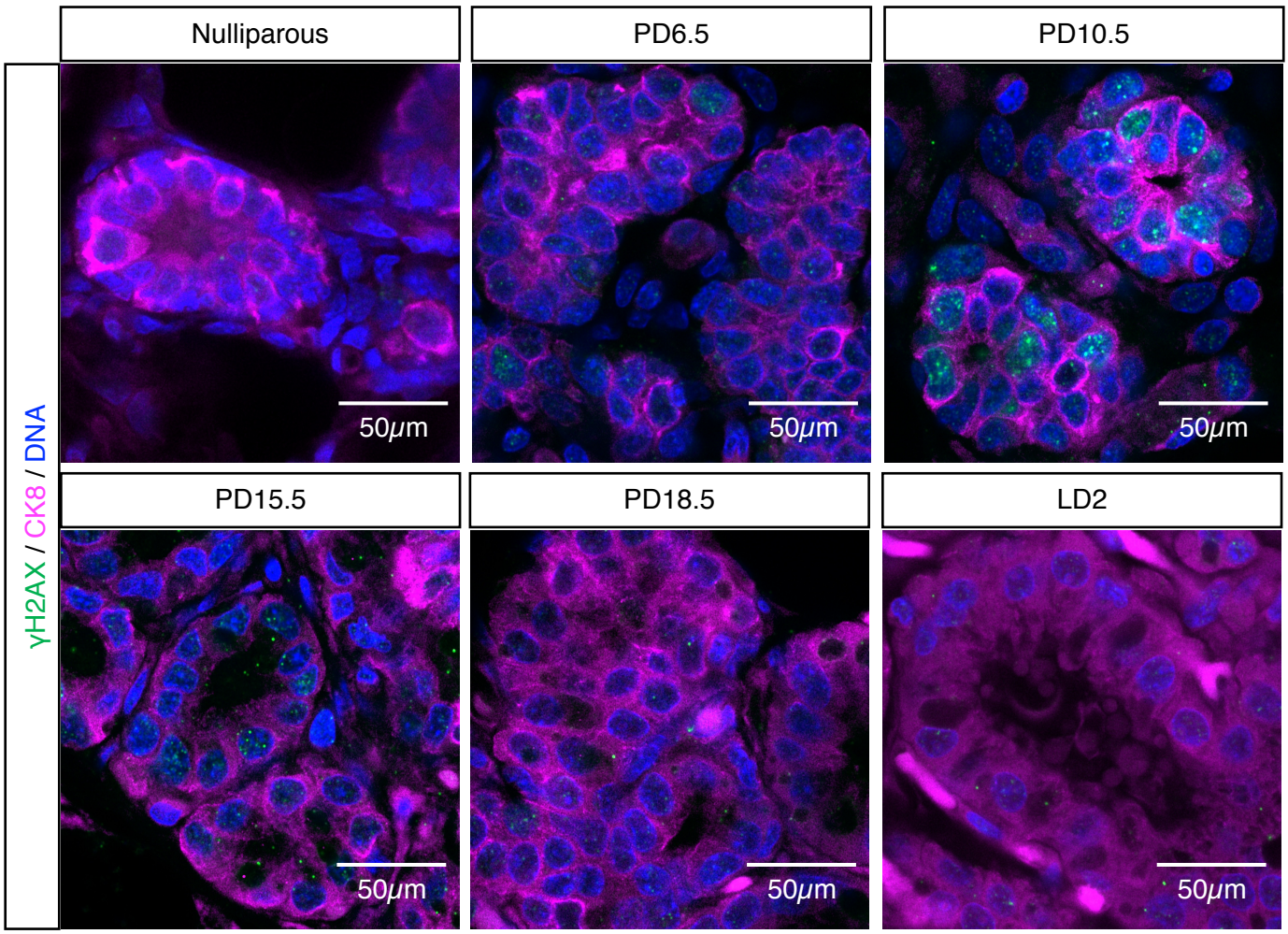
Supp. Figure 1



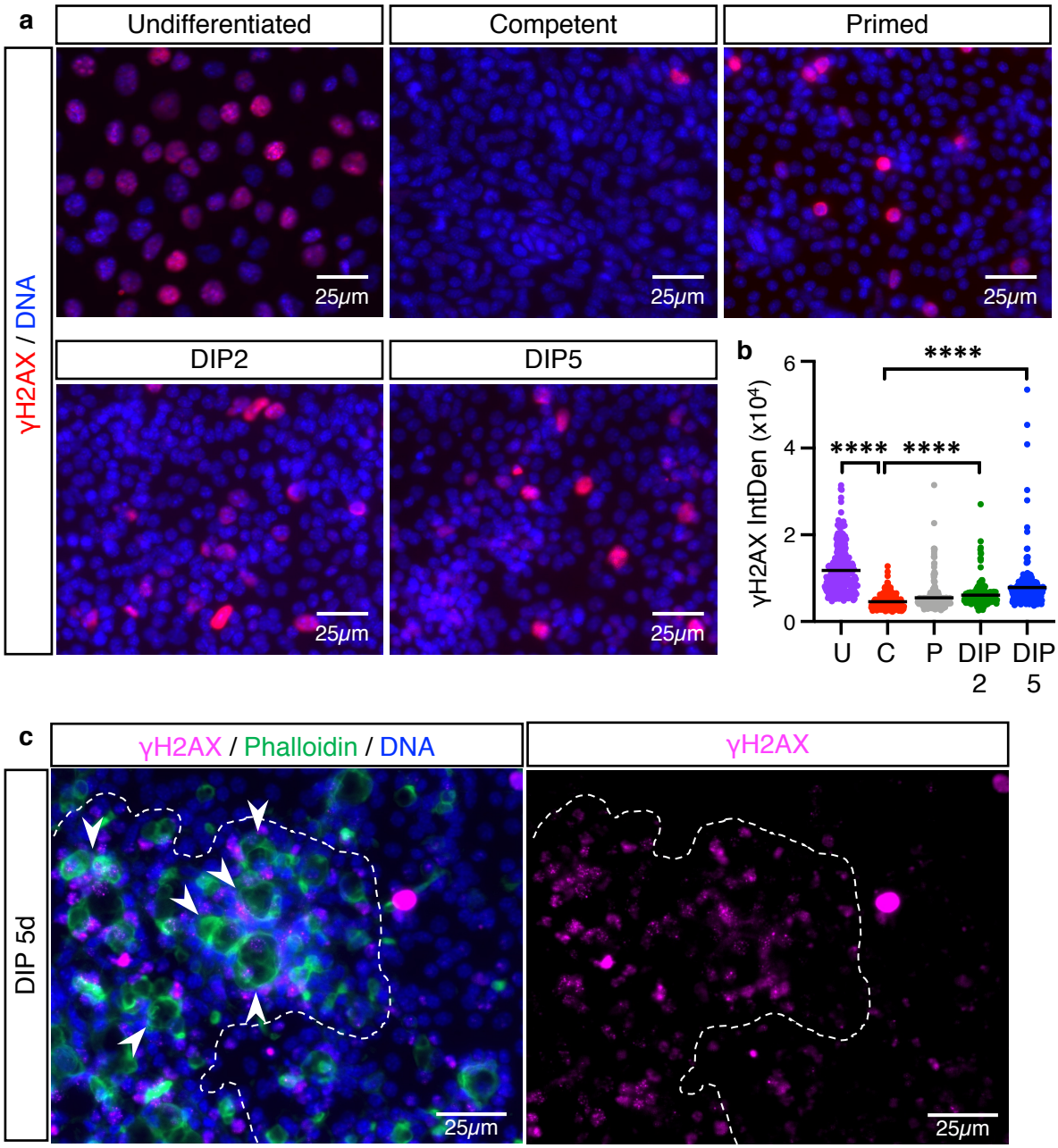
Supp. Figure 2



Supp. Figure 3



Suppl Figure 4



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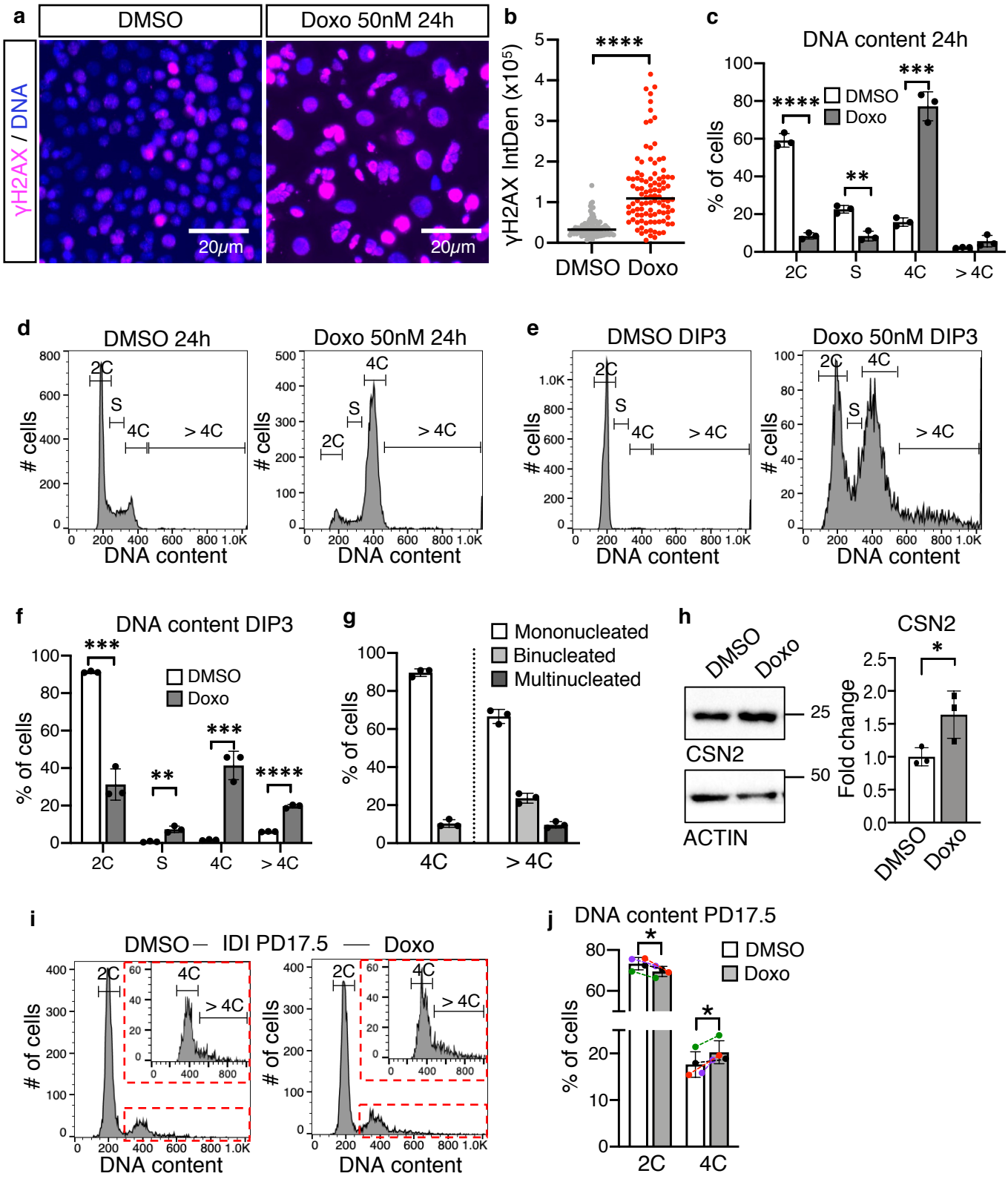
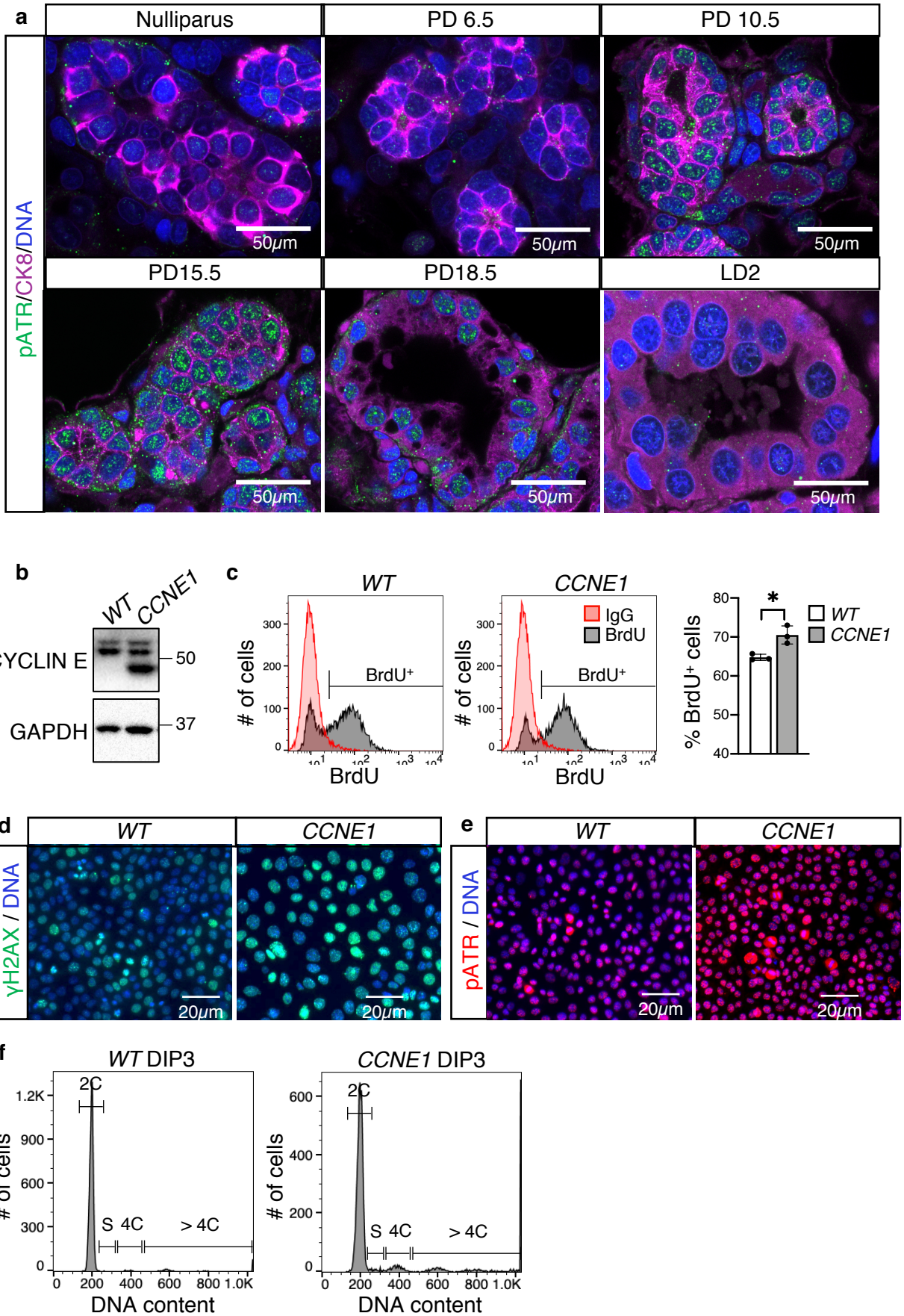
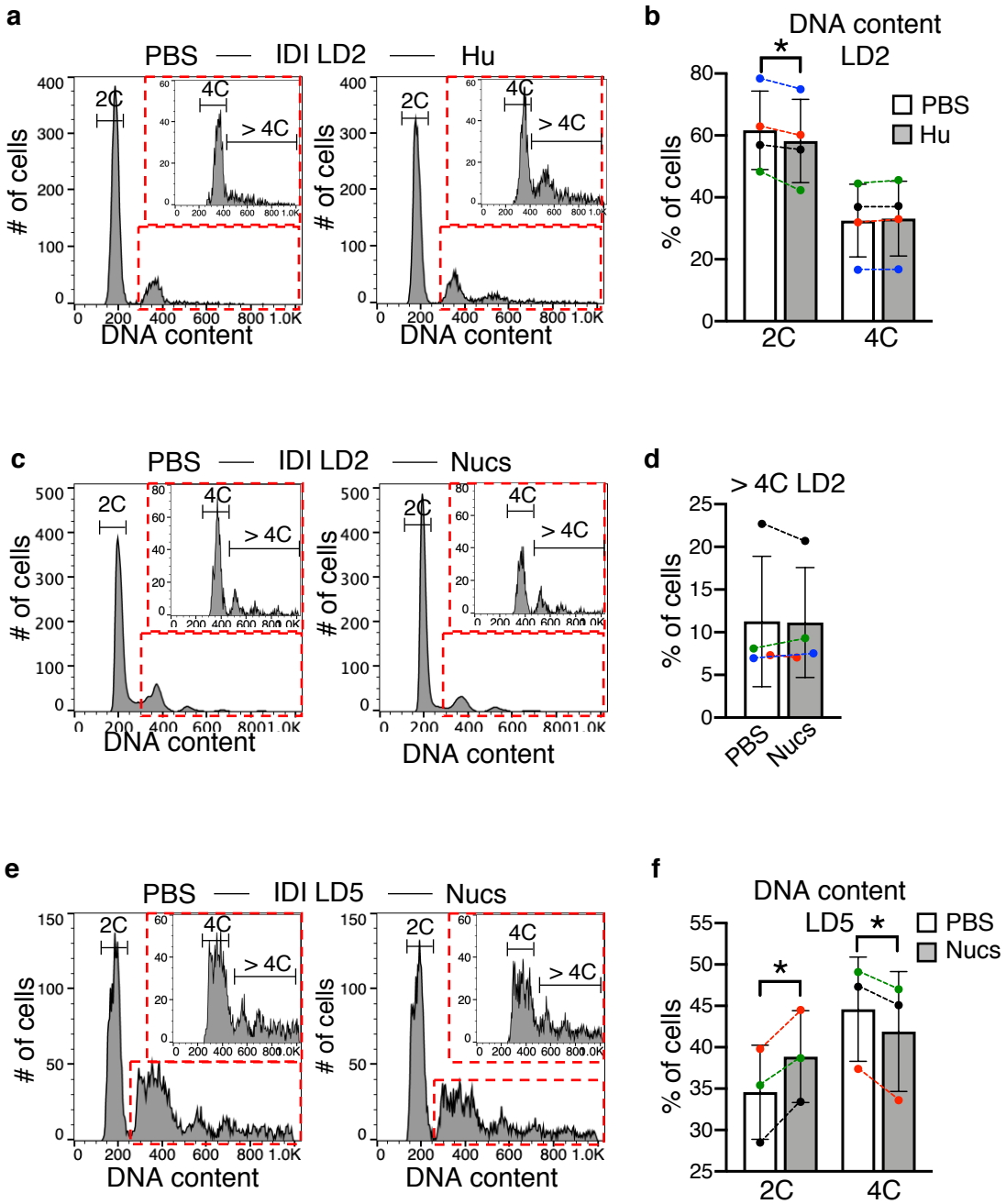


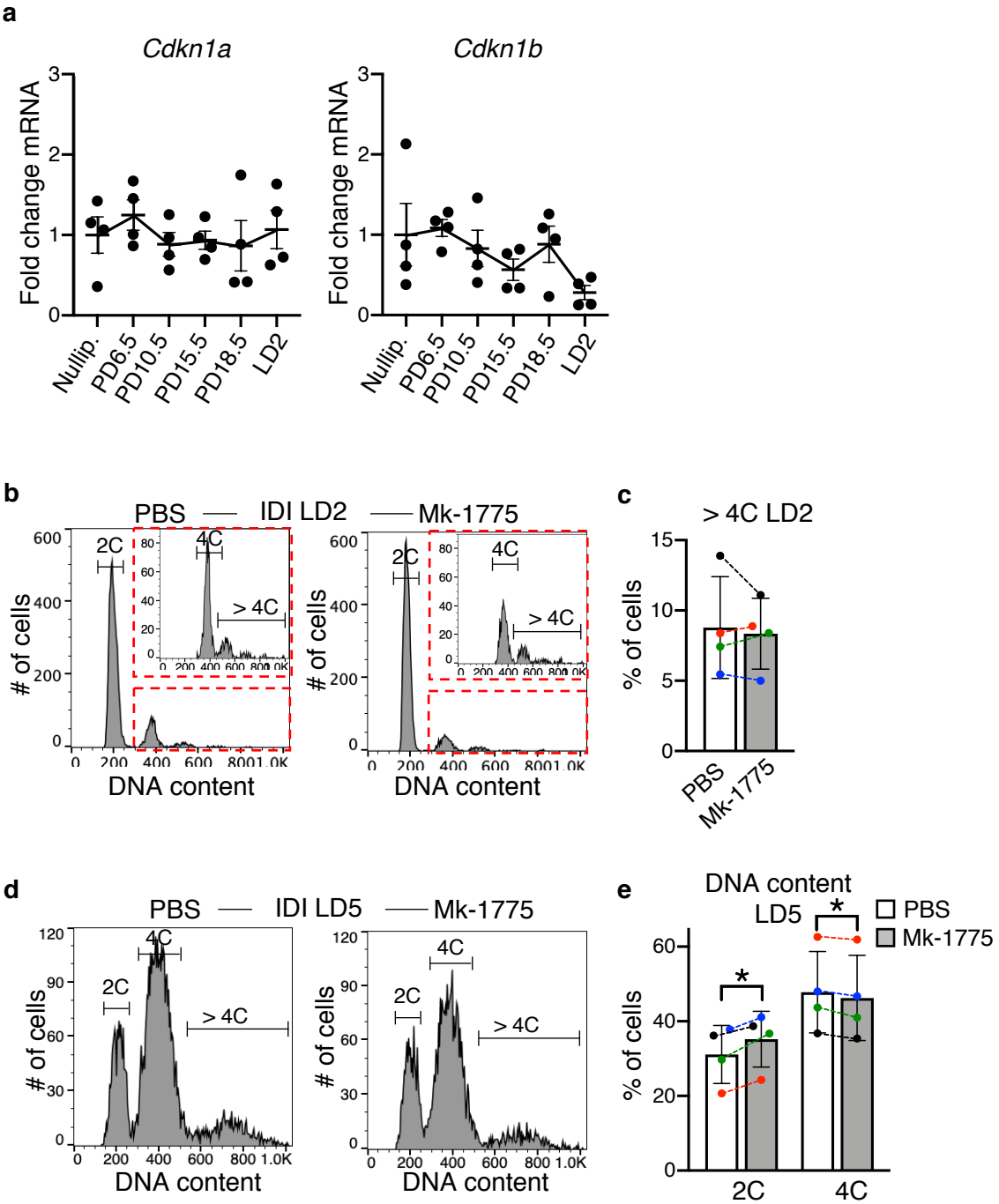
Suppl Figure 6



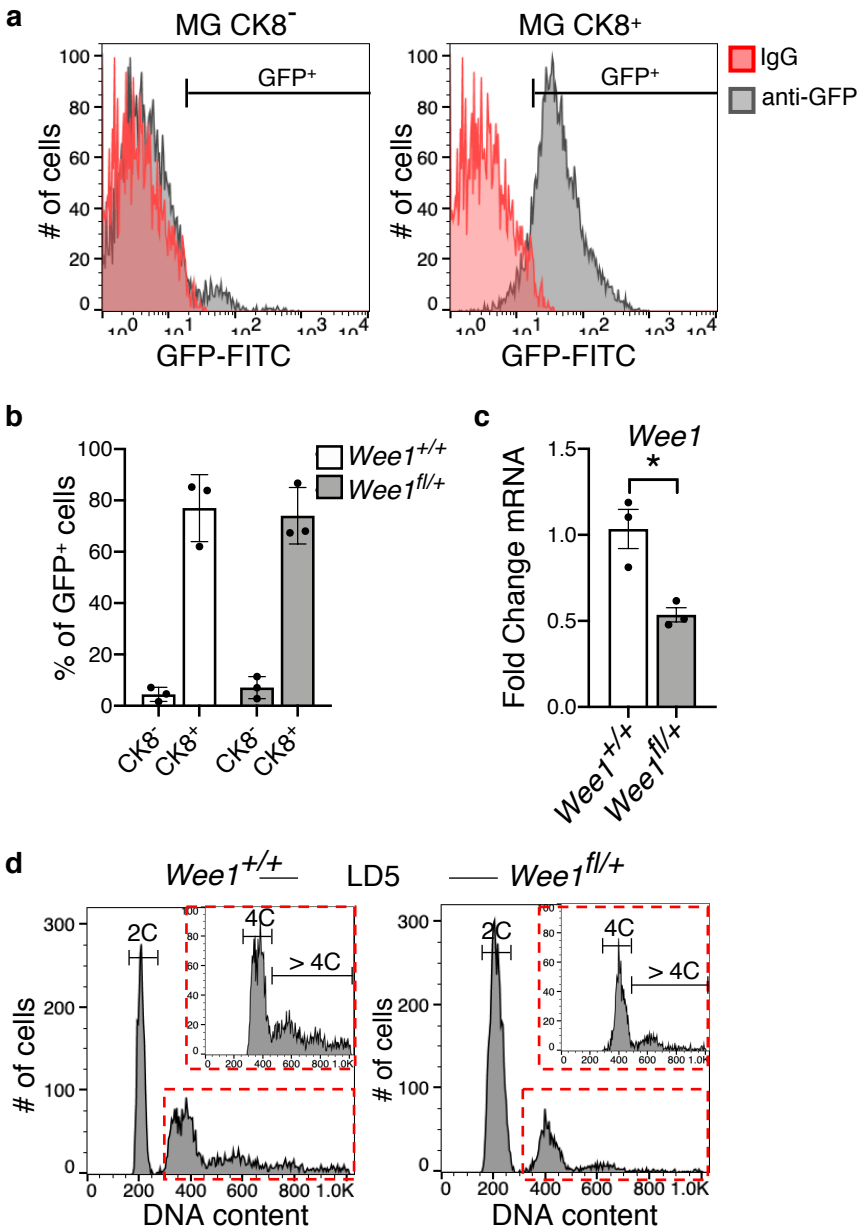
Suppl. Figure 7



Suppl. Figure 8



Suppl. Figure 9



Suppl. Figure 10

Supplementary Figure Legends

Supplementary Figure 1. Endoreplication occurs during lactogenic differentiation. **a)** Representative FACS histograms for DNA content analysis of the $CK8^+$ population in PD17.5 (left) and LD2 MGs (right). **b)** Representative FACS histograms for DNA content analysis of undifferentiated, competent, primed and differentiated (DIP5) HC11 cells. Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Histograms are representative of n=3 biological replicates. **c)** Quantification of FACS DNA content analysis showing the percentage of undifferentiated, competent, primed and differentiated (DIP5) HC11 cells with 2C, 4C or > 4C DNA content or in the S phase. Data shown as the mean ± SD; n=3 biological replicates; one-way ANOVA Tukey's test. p values: * < 0.05, **< 0.001, *** < 0.0005, **** < 0.0001.

Supplementary Figure 2. Cytokinesis failure results in endoreplication and increased milk production. a) Representative FACS histograms for DNA content analysis (left) and quantification (right) of undifferentiated HC11 treated with DMSO or blebbistatin (Blebbi) for 6 hours. **b)** Representative FACS histograms for DNA content analysis (left) and quantification (right) of differentiated HC11 (DIP3) treated with DMSO or Blebbi. **c)** Phase contrast images of showing milk domes in differentiated (DIP3) HC11, treated with DMSO or Blebbi. **d)** Detection of Perilipin2 (PLIN2, green) in differentiated (DIP3) HC11, treated with DMSO or Blebbi. Nuclear DNA by Hoechst. Images are representative of n=3 biological replicates. **e)** Quantification of PLIN2 expression in differentiated (DIP3) HC11, treated with DMSO or Blebbi. **f)** Quantification of *Csn2* expression in differentiated (DIP3) HC11, treated with DMSO or Blebbi, as detected by RT-qPCR. Data shown as the mean ± SD except for **(f)** data shown as mean ± S.E.M; n=3 biological replicates; two-tailed Student's t-test. p values: * < 0.05, **< 0.001, *** < 0.0005, **** < 0.0001.

Supplementary Figure 3. HC11 undergo endoreplication through an early mitotic arrest involving Cdk1 inactivation. a) Representative images (left) and quantification (right) of mono-, bi- and multinucleated (more than 2 nuclei) differentiated (DIP3) HC11, sorted based on DNA content (4C or >4C). Nuclear DNA by propidium iodide. Phalloidin shown in green. Data shown as the mean ± S.E.M; n=3 biological replicates. **b)** Representative western blot showing the expression of CYCLIN E and CYCLIN B in undifferentiated (U), competent (C), primed (P) and differentiated (DIP 2 and 5) HC11. Right histogram shows the quantification of the CYCLIN E: CYCLIN B ratio during HC11 differentiation. Data shown as the mean ± S.E.M; n=3 biological replicates; two-tailed un-paired Student's t-test. **c)** Representative FACS histograms for DNA content analysis of undifferentiated HC11 treated with DMSO or Ro-3306 for 6 hours. **d)** Representative FACS histograms for DNA content analysis of differentiated HC11 (DIP3) treated with DMSO or Ro-3306. p values: * < 0.05, **< 0.001.

Supplementary Figure 4. Physiological DNA damage occurs during pregnancy in the MG *in vivo*. Detection of γH2AX (green) in optical-cleared MG sections in nulliparous, PD6.5, PD10.5, PD15.5, PD18.5 and LD2. CDK8 shown in magenta. Nuclear DNA by propidium iodide. Images are representative of n=3 biological replicates.

Supplementary Figure 5. Physiological DNA damage occurs during HC11 lactogenic differentiation. a) Detection of γH2AX (red) in undifferentiated, competent, primed and differentiated (DIP 2 and 5) HC11. Nuclear DNA by Hoechst. Images are representative of n=3 biological replicates. **b)** Quantification of γH2AX Integrated Density (IntDen) detected in HC11 throughout differentiation. Data show values from 100 cells per time-point and are representative of n=3 biological replicates. Black horizontal bars represent the mean. Dunn's multiple comparison test. **c)** Representative images of differentiated HC11 (DIP5) showing that γH2AX (magenta) is high in areas with dome formation (white broken line, Phalloidin, green) Domes are indicated by arrows. Nuclear DNA by Hoechst. p values: **** < 0.0001.

Supplementary Figure 6. DNA damage increases endoreplication and milk production *in vitro*. a) Detection of γH2AX (magenta) in undifferentiated HC11 treated with DMSO or doxorubicin (Doxo) for 24 hours. Nuclear DNA by Hoechst. Images are representative of n=3 biological replicates. **b)** Quantification of γH2AX Integrated Density (IntDen) 24 hours after treatment with DMSO or Doxo. Data show values from 100 cells per treatment and are representative of n=3 biological replicates. Black horizontal bars represent the mean. Mann-Whitney test. **c)** Quantification of FACS DNA content analysis showing the percentage of undifferentiated HC11 cells with 2C, 4C or > 4C DNA content, or in the S phase, 24 hours after treatment with DMSO or Doxo. Data shown as the mean ± SD; n=3 biological replicates; two-tailed unpaired Student's t-test. **d and e)** Representative FACS histograms for DNA content analysis of undifferentiated **(d)** and DIP3 **(e)** HC11 treated with DMSO or Doxo. **f)** Quantification of FACS DNA content analysis showing the percentage of differentiated (DIP3) HC11 cells with 2C, 4C or > 4C DNA content, or in the S phase, after treatment with DMSO or Doxo. Data shown as the mean ± SD; n=3 biological replicates; two-tailed unpaired Student's t-test. **g)** Quantification of differentiated HC11 (DIP3) treated with DMSO or Doxo, and sorted based on DNA content (4C or > 4C), that are mono-, bi- or multinucleated (more than 2 nuclei). Data shown as the mean ± S.E.M; n=3 biological replicates. **h)** Representative western Blot (left) and quantification (right) of CSN2 expression in differentiated HC11 (DIP3) after DMSO or Doxo treatment. Data shown as the mean ± SD; n=3 biological replicates; two-tailed un-paired Student's t-test. **i)** Representative FACS DNA content analysis histograms from $CK8^+$ PD17.5 MGs after contralateral IDI with DMSO or doxorubicin (Doxo). Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Data are representative of n=3 biological replicates. **j)** Quantification of the percentage of $CK8^+$ cells with 2C and 4C DNA content, detected by FACS analysis, after contralateral IDI with DMSO or Doxo in PD17.5 MGs. Data shown as the mean ± SD; n=4 biological replicates; two-tailed paired Student's t-test**.** p values: * < 0.05, **< 0.001, *** < 0.0005, **** < 0.0001.

Supplementary Figure 7. Replication stress activates the DNA damage response and results in increased endoreplication and milk production. a) Detection of pATR (green) in optical-cleared MG sections in nulliparous, PD6.5, PD10.5, PD15.5, PD18.5 and LD2. CK8 shown in magenta. Nuclear DNA by propidium iodide. Images are representative of n=3 biological replicates. **b)** Detection of CYCLIN E by Western Blot in wild-type (*WT*) or Cyclin E overexpressing (*CCNE1*) HC11. **c)** Representative FACS histograms showing BrdU incorporation in *WT* and *CCNE1* undifferentiated HC11. Red histogram represents the negative isotype control (IgG). Black gate represents the identification of the $BrdU^+$ cells according to negative isotype control. Right histogram shows the quantification of *WT* or *CCNE1* HC11 that

are $BrdU^+$. Data shown as the mean ± SD; n=3 biological replicates; two-tailed un-paired Student's t-test. **d)** Detection of γH2AX (green) in undifferentiated WT or *CCNE1* HC11. Nuclear DNA by Hoechst. Images are representative of n=3 biological replicates. **e)** Detection of pATR (red) in undifferentiated *WT* or *CCNE1* HC11. Nuclear DNA by Hoechst. Images are representative of n=3 biological replicates. **f)** Representative FACS histograms for DNA content analysis of *WT* or *CCNE1* differentiated (DIP3) HC11. p values: * < 0.05.

Supplementary Figure 8. The DNA damage response to replication stress regulates endoreplication *in vivo*. a) Representative FACS DNA content analysis histograms from $CK8^+$ LD2 MGs after contralateral IDI with PBS or hydroxyurea (Hu). Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Histograms are representative of n=3 biological replicates. **b)** Quantification of the percentage of $CK8^+$ cells with 2C or 4C DNA content, detected by FACS analysis, after contralateral IDI with PBS or Hu in LD2 MGs. Data shown as the mean ± SD; n=4 biological replicates; two-tailed paired Student's t-test**. c)** Representative FACS DNA content analysis histograms from $CK8^+$ LD2 MGs after contralateral IDI with PBS or nucleosides (Nucs). Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Histograms are representative of n=4 biological replicates. **d)** Quantification of the percentage of $CK8^+$ cells with > 4C DNA content, detected by FACS analysis, after contralateral IDI with PBS or Nucs in LD2 MGs. Data shown as the mean ± SD; n=4 biological replicates; two-tailed paired Student's t-test**. e)** Representative FACS DNA content analysis histograms from $CK8^+$ LD5 MGs after contralateral IDI with PBS or nucleosides (Nucs). Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Histograms are representative of n=3 biological replicates. **f)** Quantification of the percentage of $CK8^+$ cells with 2C or 4C DNA content, detected by FACS analysis, after contralateral IDI with PBS or Nucs in LD5 MGs. Data shown as the mean ± SD; n=3 biological replicates; two-tailed paired Student's t-test**.** p values: * < 0.05.

Supplementary Figure 9. The DNA damage response to replication stress regulates endoreplication through WEE1. a) Quantification of *Cdkn1a* (left) and *Cdkn1b* (right) expression in whole tissue from nulliparous, PD6.5, PD10.5, PD15.5, PD18.5 and LD2 MGs, as detected by RT-qPCR. Data shown as the mean ± S.E.M; n=4 for biological replicates. one-way ANOVA Tukey's test. **b)** Representative FACS DNA content analysis histograms from $CK8^+$ LD2 MGs after contralateral IDI with DMSO or Mk-1775. Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Histograms are representative of n=4 biological replicates. **c)** Quantification of the percentage of $CK8^+$ cells with > 4C DNA content, detected by FACS analysis, after contralateral IDI with DMSO or Mk-1775 in LD2 MGs. Data shown as the mean ± SD; n=4 biological replicates; two-tailed paired Student's t-test**. d)** Representative FACS DNA content analysis histograms from $CK8^+$ LD5 MGs after contralateral IDI with DMSO or nucleosides Mk-1775. Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Histograms are representative of n=3 biological replicates. **e)** Quantification of the percentage of $CK8^+$ cells with 2C or 4C DNA content, detected by FACS analysis, after contralateral IDI with DMSO or Mk-1775 in LD5 MGs. Data shown as the mean ± SD; n=4 biological replicates; two-tailed paired Student's t-test**.** p values: * < 0.05.

Supplementary Figure 10. WEE1 regulates alveolar endoreplication and milk production during lactation. a) FACS analysis of mammary gland (MG) single cell preparation for the identification of GFP^+ cells in the $CK8^-$ (left) and $CK8^+$ (right) populations. Red histogram represents the negative isotype control (IgG). Gate indicates GFP^+ cells based on negative isotype control. **b)** Quantification of the % of GFP^+ cells in the $CK8^-$ and $CK8^+$ populations from LD5 MGs from *Ck8-CreER/mTmG/Wee1*$^{+/+}$ (*Wee1*$^{+/+}$) or *Ck8-CreER/mTmG/Wee1*$^{fl/+}$ (*Wee1*$^{fl/+}$) mice. Data shown as the mean ± SD; n=3 biological replicates; two-tailed unpaired Student's t-test. **c)** Quantification of *Wee1* expression in whole tissue from LD5 MGs from *Wee1*$^{+/+}$ or *Wee1*$^{fl/+}$ mice, as detected by RT-qPCR. Data shown as the mean ± S.E.M; n=3 biological replicates. two-tailed unpaired Student's t-test. **d)** Representative FACS DNA content analysis histograms from $CK8^+$ LD5 MGs from *Wee1*$^{+/+}$ or *Wee1*$^{fl/+}$ mice. Red broken lines show a magnification of the histogram corresponding to the 4C and >4C populations. Histograms are representative from n=3 biological replicates. p values: * < 0.05.