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Supplemental Information

Negative Selection and Chromosome Instability Induced by Mad2 Overexpression Delay Breast Cancer but Facilitate Oncogene-Independent Outgrowth

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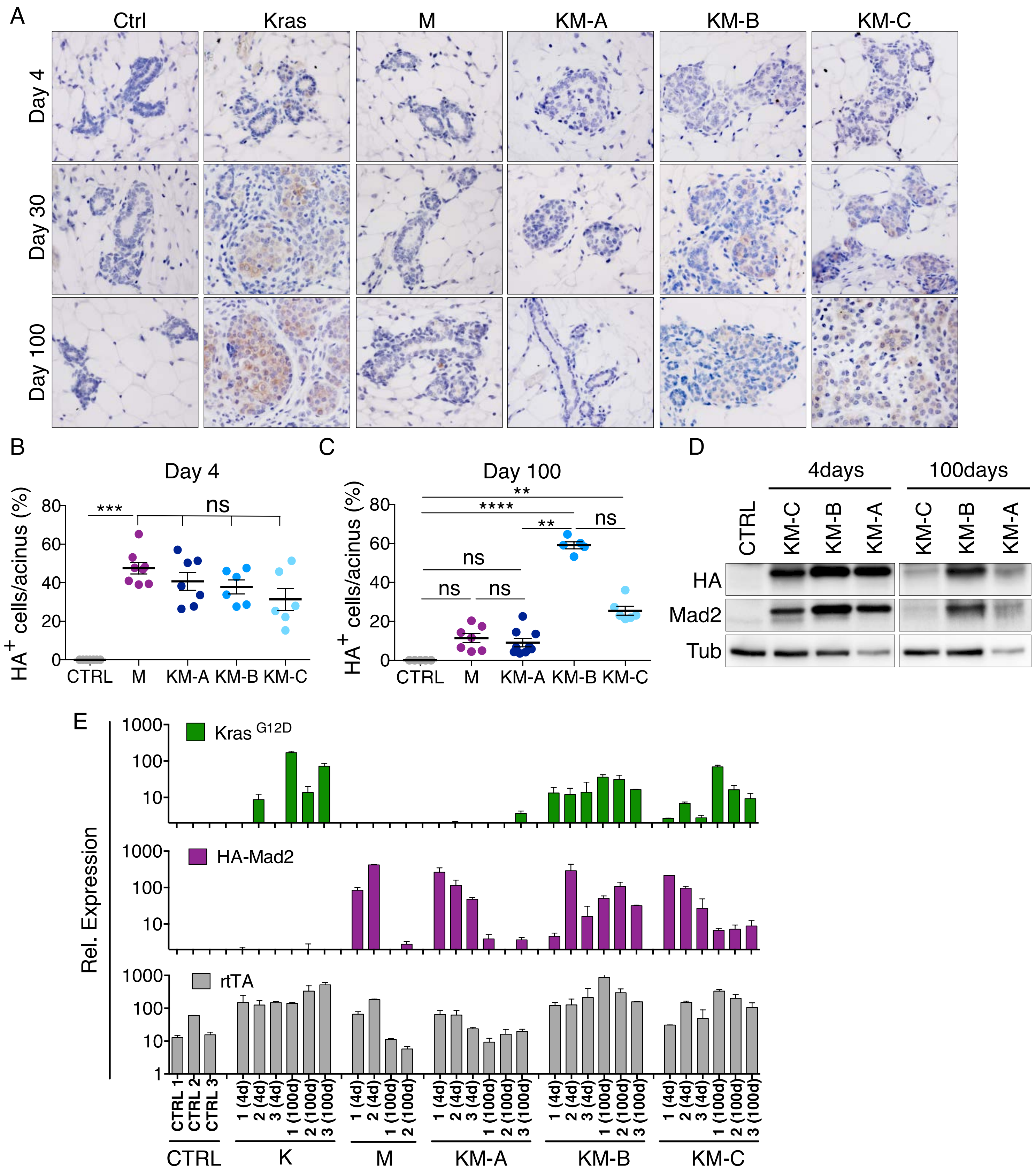

Figure S1 (Related to Figures 1 and 2). Differential transgene expression and mammary architecture in transgenic animals. (A) $Kras_{G12D}$ staining on paraffin sections of mammary tissue after 4, 30 and 100 days on doxycycline; scale bar 50µm. (B-C) Percentage of HA positive cells per acinus after 4 days (CTRL: n=7, M: n=7; KM-A: n=7; KM-B: n=6; KM-C: n=6; $p=0.0005$) and 100 days on doxycycline (CTRL: n=5, M: n=7; KM-A: n=9; KM-B: n=5; KM-C: n=6; $p<0.0001$); Kruskal-Wallis test; Dunn's multiple comparisons test; points represent average per animal. (D) HA and Mad2 western blots of mammary glands after 4 and 100 days on doxycycline. Tubulin serves as loading control. (E) Quantitative RT-PCR analysis of $Kras_{G12D}$, HA-Mad2 and rtTA transgene expression in mammary samples after 4 and 100 days on doxycycline; KM animals without rtTA on doxycycline were used as reference and KM and K animals on normal diet are displayed as controls. Error bars represent SEM.

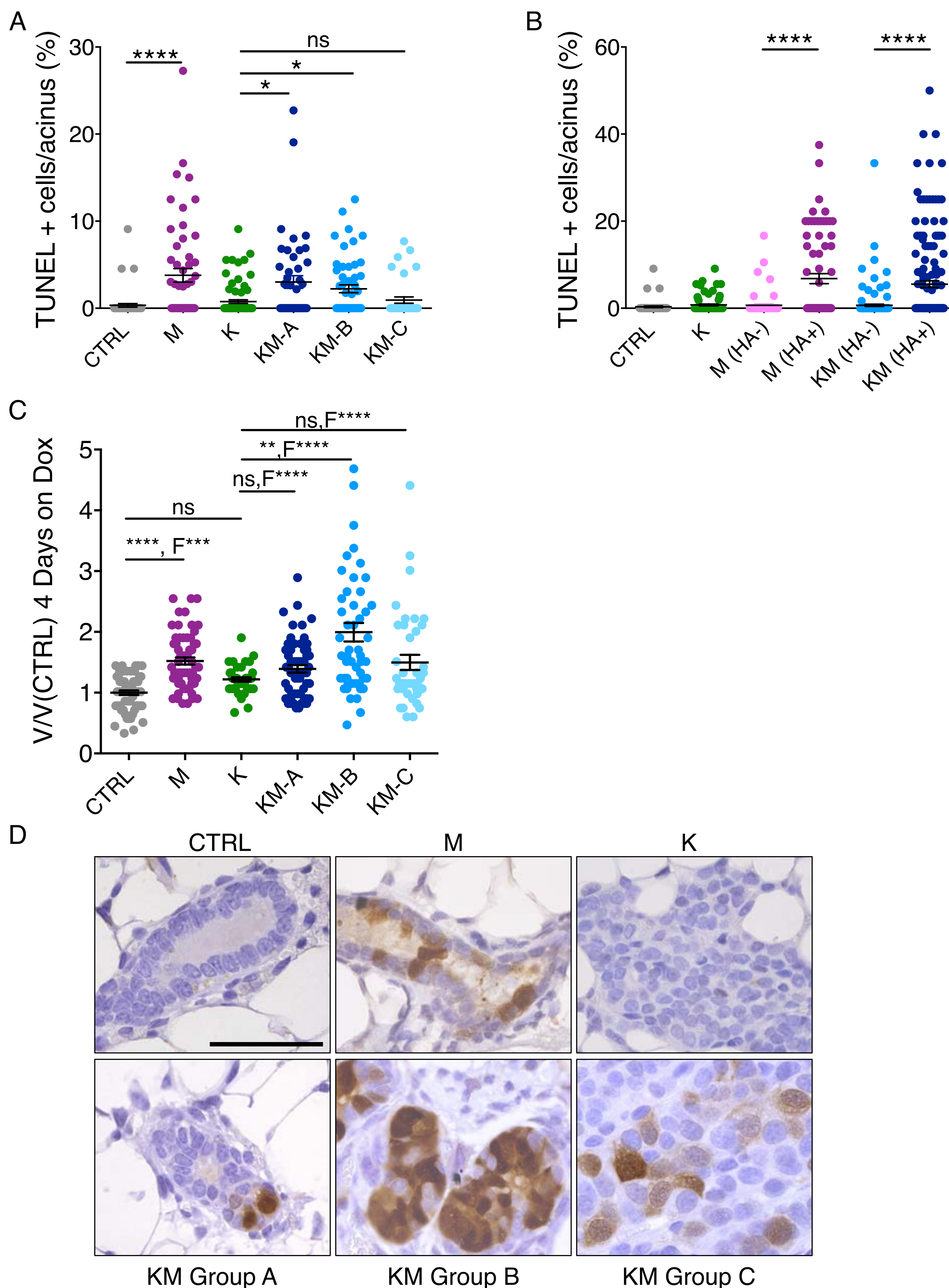

Figure S2 (Related to Figures 1 and 2). TUNEL staining and nuclear size of mammary epithelial cells in transgenic animals.
(A-B) Percentage of TUNEL positive cells per acinus after 4 days in all cohorts (CTRL: n=6, M: n=6, K: n=9, KM-A: n=5, KM-B: n=6, KM-C: n=4; p-values n.s.) and separated into HA positive and negative cells. Points represent single acinar measurements. (C) Nuclear volume relative to control cells after 4 days on doxycycline (CTRL: n=6; M: n=6; K: n=4; KM-A: n=6, KM-B: n=5, KM-C: n=4); points represent single nuclear measurements. (D) Immunohistochemistry against HA-Mad2 in paraffin sections of mammary tissue after 100 days on doxycycline; scale bar 40µm. Kruskal-Wallis test, $p<0.0001$; Dunn's multiple comparisons test. Error bars represent the SEM.

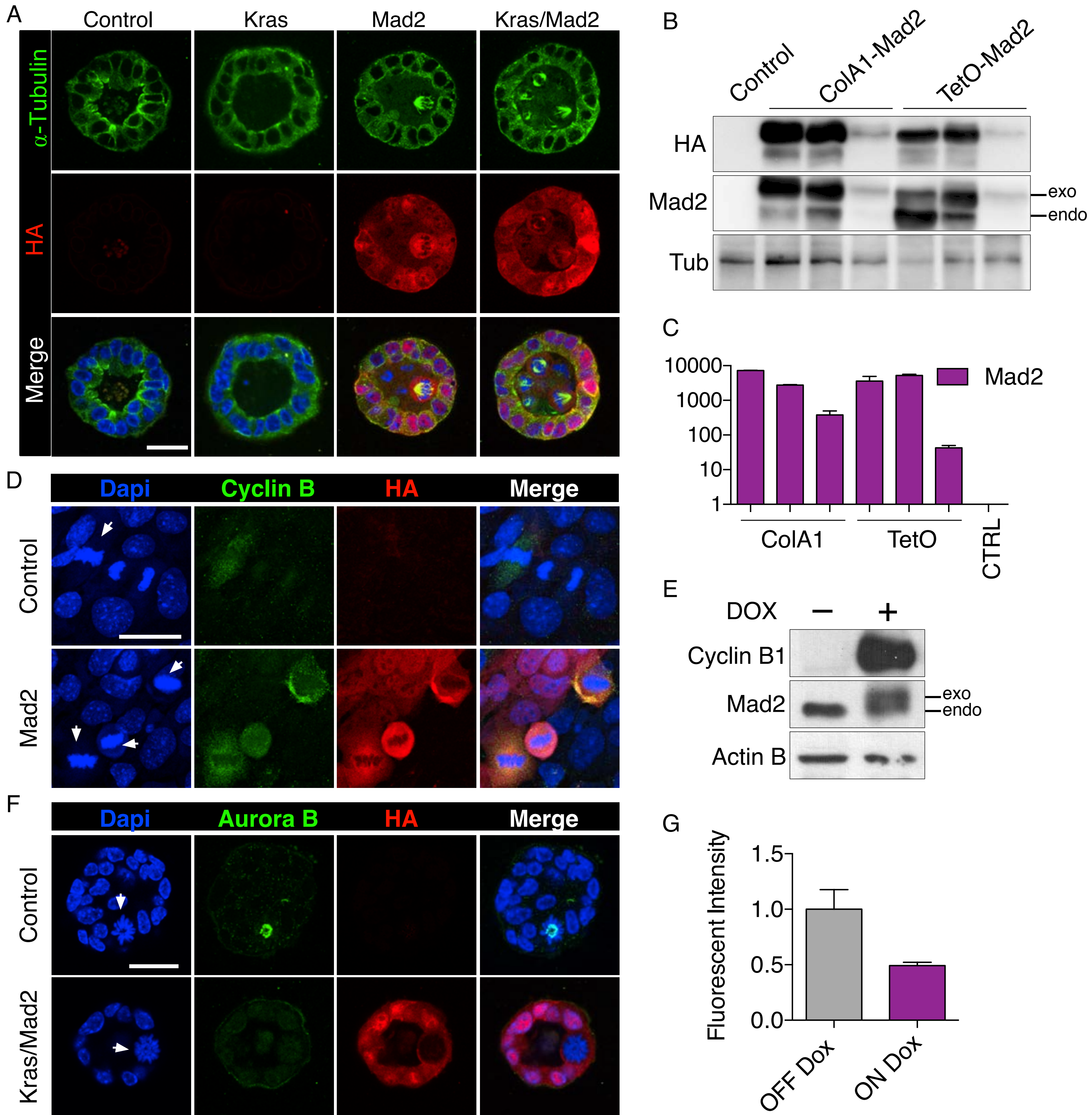

Figure S3 (Related to Figure 3). Spheroid cultures of primary mammary cells 36h after doxycycline administration.

(A) Immunofluorescence in 3D cultures of primary mammary acini 36 hours after transgene induction; α-Tubulin (green), HA-Mad2 (red), merge contains dapi (blue), scale bar 20µm. (B) HA and Mad2 western blot of protein extracts obtained from 3 ColA1-Mad2/MMTV-rtTA and 3 TetO-Mad2/MMTV-rtTA spheroid cultures on doxycycline. Tubulin serves as loading control, culture without doxycycline serves as control. (C) Quantitative RT-PCR analysis for exogenous Mad2 of 3 ColA1-Mad2 and 3 TetO-Mad2 spheroid cultures on doxycycline; cultures without doxycycline were used as control. (D) Immunofluorescence in primary mammary cells 2 days after doxycycline administration; Dapi (blue), Cyclin B (green), HA-Mad2 (red), scale bar 20µm. (E) Cyclin B and Mad2 western blot of protein extracts obtained from TetO-Mad2/MMTV-rtTA spheroid cultures maintained with (+) or without (–) doxycycline. Actin serves as loading control. (F) Immunofluorescence in spheroid cultures 2 days after doxycycline administration. Arrows indicate mitotic cells; Dapi (blue), Aurora B (green), HA-Mad2 (red), scale bar 20µm. (G) Fluorescent intensities of Aurora B stained KM 3D cultures off doxcycline (n=19 cells from 4 animals) and after 2 days on (n=60 cells from 4 animals); only cells in metaphase were analyzed; intensities were calculated via CTCF method. Error bars represent the SEM.

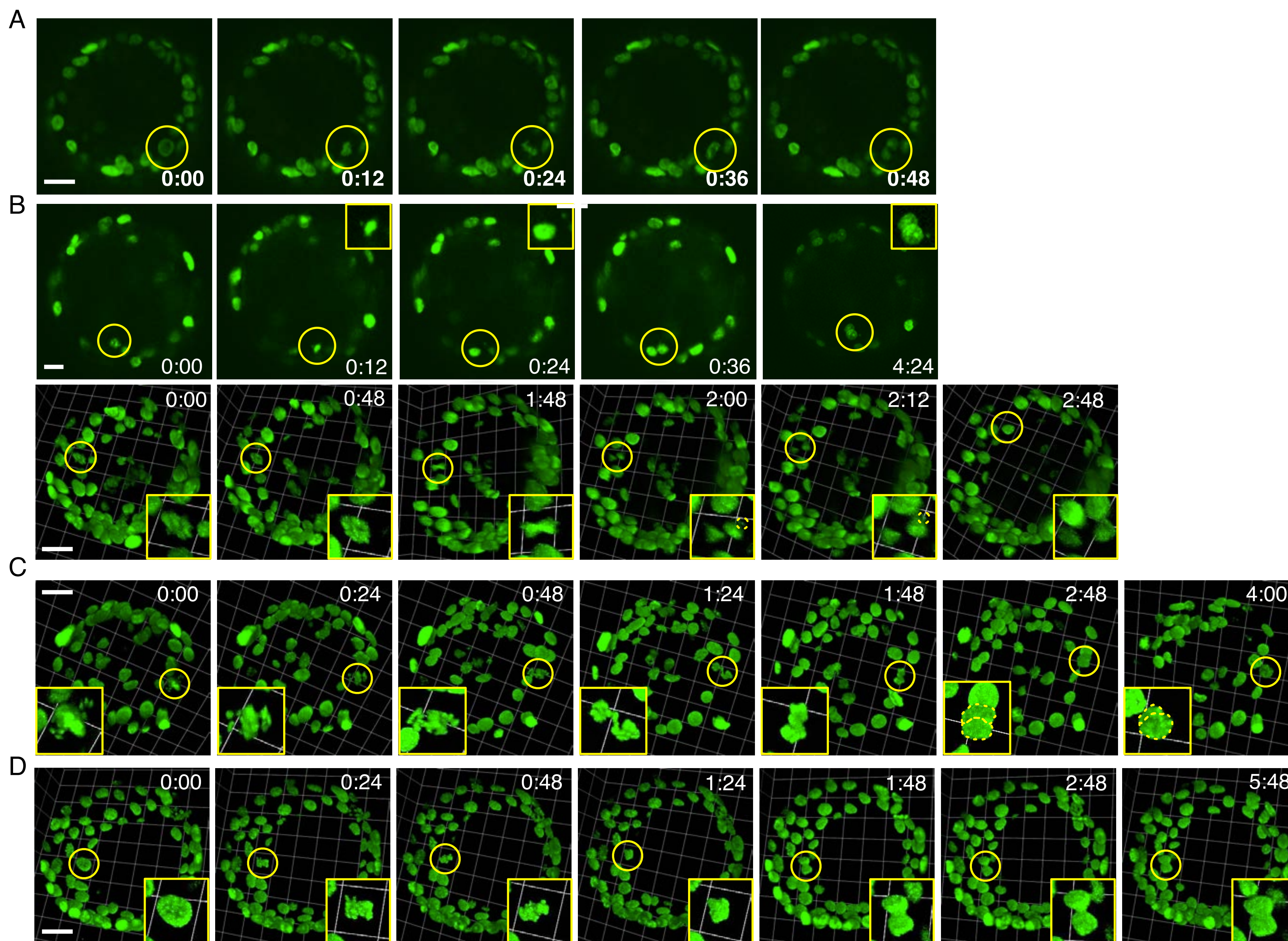

Figure S4 (Related to Figure 4). Time-lapse imaging of three dimensional mammary cultures.
(A-D) Time-lapse micrographs of Kras (A) and KM (B-D) acinar spheres grown in vitro starting at 30 hours after transgene induction, t=0. Yellow circle indicate mitotic cells; H2B-GFP (green), scale bar 25μm. (A) Representative mitotic cell division in a control culture oriented perpendicular to the apical and basal membrane within the epithelial layer. (B) Examples of KM acini showing a cell that divides and forms micronuclei. (C) Chromosome misalignment and cytokinesis failure resulting in a binucleated cell. (D) Furrow regression.

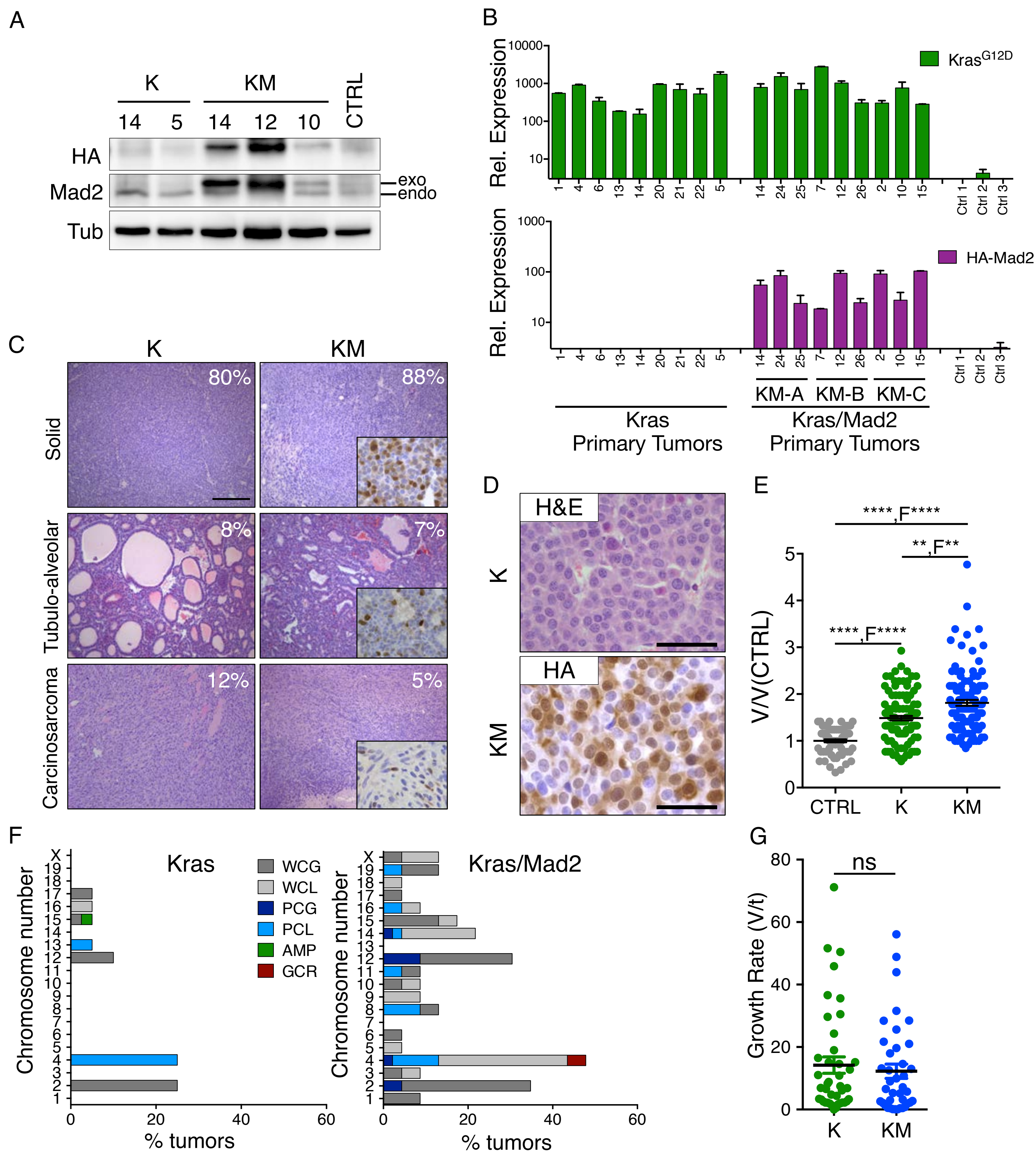

Figure S5 (Related to Figure 5). Phenotypic and Karyotypic evaluation of K and KM primary tumors.

(A) HA and Mad2 western blot of protein extracts from 2 K and 3 KM tumors. Numbers refer to the tumor sample which are represented in B. Tubulin serves as loading control. (B) Quantitative RT-PCR analysis of KrasG12D and HA-Mad2 transgene expression in a representative number of primary tumors from K and KM cohorts; KM animals without rtTA on doxycycline were used as reference and KM and K animals on normal diet are displayed as controls. (C) H&E staining of paraffin sections from different histological tumor subtypes of K and KM. Inserts show anti-HA immunohistochemistry; scale bar 200µm. (D) Histological sections from a K (H&E stained) and a KM tumor (stained for HA-tagged Mad2); scale bar 40µm. (E) Quantification of nuclear volume relative to wild-type control cells (CTRL: n=7; K: n=11; KM: n=11); size variability via F-test; points represent single nuclear measurements; Kruskal-Wallis test, $p<0.0001$; Dunn's multiple comparisons test. (F) Structural variants per individual chromosome in primary tumors of K and KM cohort. (F) Tumor growth rate calculated with final tumor volume and time from tumor onset; unpaired t test, p-value n.s. Error bars represent the SEM.

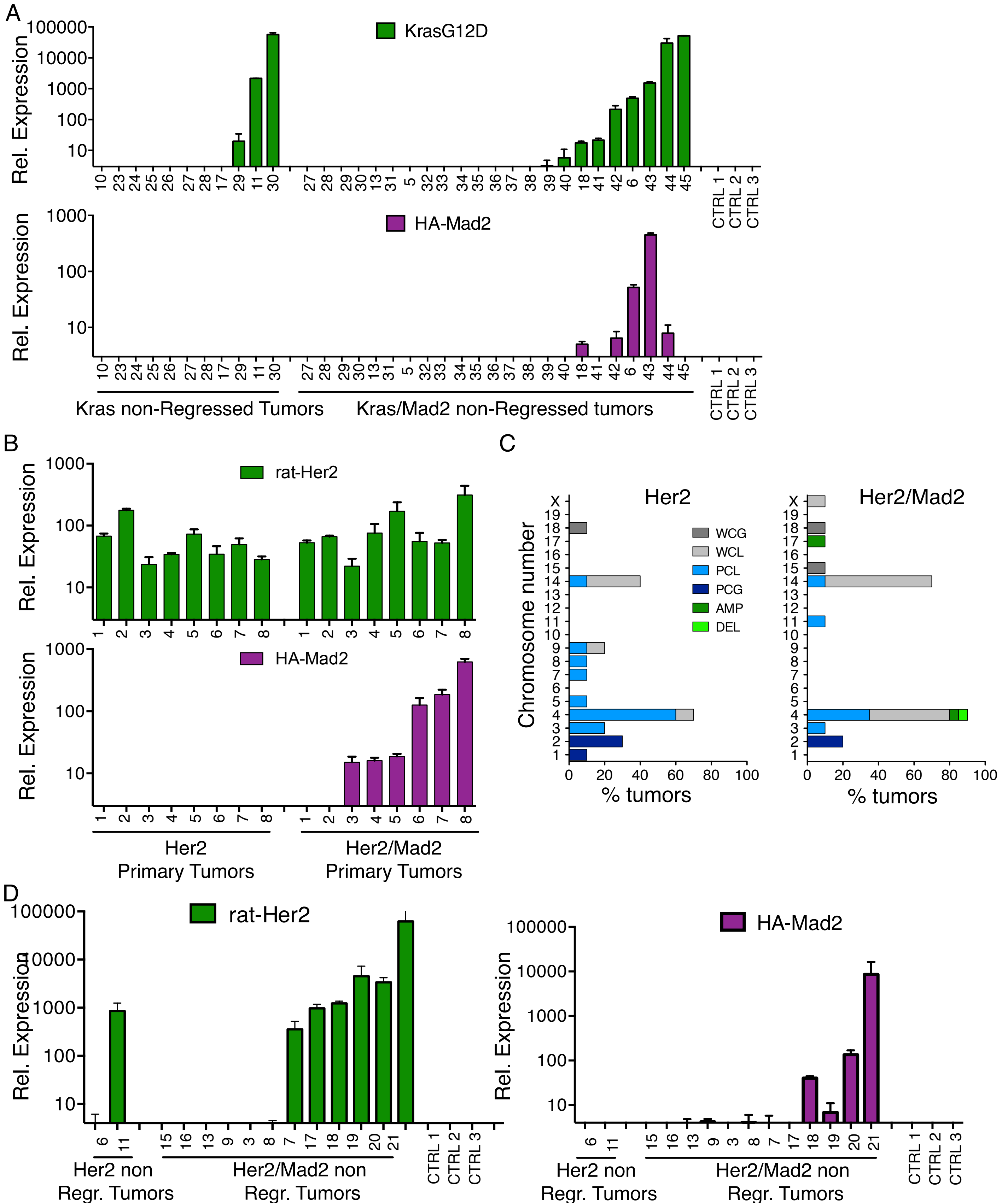

Figure S6 (Related to Figures 5 and 6). Phenotypic evaluation of H and HM primary tumors and K, KM, H,HM non regressed tumors.

(A) Quantitative RT-PCR analysis of Kras and Mad2 transgene expression in non-regressing tumors from K and KM cohorts. (B) Quantitative RT-PCR analysis of Her2 and HA-Mad2 transgene expression in a representative number of primary tumors from Her2 and Her2/Mad2 cohorts. (C) Percentage of primary tumors of H and HM containing specific structural variants per individual chromosome. (D) Quantitative RT-PCR analysis of Her2 and Mad2 transgene expression in 2 Her2 and 12 Her2/Mad2 non-regressing tumors. HM and/or KM animals without rtTA on doxycycline were used as reference and H, HM, K and KM animals on normal diet as controls. Error bars represent the SEM.

Supplemental information

Supplemental Experimental Procedures

Generation of ColA1-HA-Mad2 animals

ColA1-HA-Mad2 transgenic mice were generated using KH2 ES cells (ThermoScientific) according to a previously described method (Beard et al., 2006). Murine Mad2 cDNA was amplified with specific primers containing the HA epitope tag and ligated into the EcoRI site of Flp-in vector pBS31'. Electroporation of pBS31'-mHA-Mad2 together with pCAGGS-FLPe vector into KH2 ES cells resulted in the targeted integration into the ColA1 locus. KH2 ES positive clones expressing HA-Mad2 were injected into 8 cell blastocysts by the Transgenic facility at EMBL-Monterotondo. Four chimaeras from two independent ColA1-HA-Mad2 clones achieved germline transmission. Animals were backcrossed to FVB. To exclude the Rosa26-M2rtTA, crosses with the MMTV-rtTA line followed.

Animal Experimentation

For *in vivo* transgene induction, doxycycline was administered via impregnated food pellets (625 mg/kg; Harlan-Teklad) to female mice from an age of 8-9 weeks. Disease progression was monitored regularly. Animals were sacrificed when total tumor volume reached a diameter of 2cm. Surgical procedures were preformed under isoflurane inhalation (2.5% in 0.8 L/min, Esteve) and in accordance with local disinfection and sterilization guidelines.

Genotyping

Isolation of tail-DNA was performed via incubation in 200µl 0.05M NaOH at 98 degrees for 1.5 hours and subsequent neutralization with 20µl 1M Tris HCl ph7.5. TetO-HA–Mad2, TetO–Kras, TetO–Her2, MMTV–rtTA, H2B-GFP transgenic mice and p53 knock out mice were genotyped as described (Fisher et al., 2001; Gunther et al., 2002; Hadjantonakis and Papaioannou, 2004; Moody et al., 2002; Sotillo et al., 2007). Additional primers for ColA1-HA-Mad2 were: KH2-Mad2 A – GCACAGCATTGCGGACATGC, KH2-Mad2 B – CCCTCCATGTGTGACCAAGG, KH2-Mad2 C – GCAGAAGCGCGGCCGTCTGG. For all transgenes the following PCR program was applied: 94°C for 2', 30x [95°C for 30'', 60°C for 30'', 72°C for 30''], 72°C for 1'].

Quantitative PCR

Snap frozen tissue was grinded with mortar and pestle on dry ice. For RNA extraction 30mg tissue was used. Further steps were performed using the RNeasy Mini Kit (Qiagen) according to technical specifications. For cDNA synthesis the reagents and protocol of the QuantiTect Reverse Transcription Kit (Qiagen) were applied to 400ng RNA. Real-time Quantification was performed on a starting material of 8ng cDNA with SYBR Green PCR Master Mix (2x) (Applied Biosystems) in a LightCycler® 480 (Roche). Primers were: Actin B (F: GCTTCTTTGCAGCTCCTTCGT, R: ACCAGCCGCAGCGATATCG), 18S (F: AAGGAGACTCTGGCATGCTAAC, R: CAGACATCTAAGGGCATCACAGAC), HA-Mad2 (F: GGCTTACCCATACGATGTTCC, R: CGACGGATAAATGCCACG), $Kras^{G12D}$ (F: AAGGACAAGGTGTACAGTTATGTGA, R: CTCCGTCTGCGACATCTTC), rtTA (F: CGCGTTATATGCACTCAGCG, R: TAAGAAGGCTGGCTCTGCAC), Her2 (F: TGTACCTTGGGACCAGCTCT, R: GGAGCAGGGCCTGATGTGGGTT). All reactions were performed in triplicates. The following program was applied: 95°C for 5', 45x [95°C for 10'', 60°C for 15'', 72°C for 15''], 95°C for 5', 65°C for 1']. The following formulas served for calculation of the relative gene expression: ΔCt = Ct (gene of interest) – Ct (reference gene); $\Delta\Delta Ct = \Delta Ct - \Delta Ct$ (reference sample); Relative Expression = $2^{(-\Delta\Delta Ct)}$.

Immunohistochemistry and immunofluorescence

The following primary antibodies were used: HA (1:200, Roche, 11867423001), HA (1:1000, Covance, MMS-101R), pH3 (1:200, Cell Signaling, 9701), $Kras^{G12D}$ (1:50, Cell Signaling, 14429), TUNEL (In Situ Cell Death Detection Kit, TMR red, Roche, 12156792910). Species-specific Alexa fluorophore-labeled

goat IgG secondary antibodies were used (1:800, Invitrogen). Images were acquired with a Leica LMD7000 and Leica TCS SP5 microscope via Leica LAS 4.5 software and analyzed with ImageJ software.

Immunofluorescence of monolayer cultures

Mammary glands were harvested from 8-9week old female mice and prepared according to published records (Jechlinger et al., 2009). Cells were cultured on 8 well chambered slides (Thermo Scientific, 154941) in serum-free MEBM media with supplements (Lonza, CC-3150) and treated with 1 mg/mL doxycycline after complete cell attachment. Cultures were washed with PBS and fixed for 7min in 4% PFA and permeabilized with 0.15% Triton X in PBS. After fixation all further washing and staining steps were performed with PBS plus 0.03% Triton X. Cultures were blocked for 1h with 5% goat serum (Jackson ImmunoResearch). Primary antibodies were incubated for 1h and species-specific Alexa fluorophore-labeled goat IgG secondary antibodies (1:800, Invitrogen) for 30min at room temperature. After removal of the chambers cultures were mounted with Mowiol (Merck, 475904). The following primary antibodies were used: HA (1:1000, BioLegend, 901511) and Cyclin B1 (BD Pharmingen, 554179). Images were acquired with the Leica LAS 4.5 software on a Leica SP5 confocal microscope. Image analysis was performed with ImageJ software.

Western Blot

Protein expression was assessed by immunoblotting using 40µg of total cell lysates obtained from 3D cultures of primary mammary cells. Blots were probed with antibodies directed against Mad2 (1:2000, 610679 BD Transduction Laboratories), HA (1:2000, H9658, Sigma), Cyclin B (1:200, SC-245, Santa Cruz), α-Tubulin (1:6000, T6199, Sigma) and Actin (1:3000, A2066 Sigma).

Supplemental References

Beard, C., Hochedlinger, K., Plath, K., Wutz, A., and Jaenisch, R. (2006). Efficient method to generate single-copy transgenic mice by site-specific integration in embryonic stem cells. Genesis *44*, 23-28.

Movie S1: Related to Figure 4. Cell division in non-induced spheroid culture

Time-lapse imaging of a cell division (red circle) in a control culture oriented perpendicular to the apical and basal membrane within the epithelial layer; H2B-GFP (green).

Movie S2: Related to Figure 4. Cell division in Kras^{G12D} positive spheroid culture

Time-lapse imaging of cell (red circle) entering mitosis and completing cell division inside the epithelial rim in a Kras^{G12D} positive spheroid *in vitro*, t=0 after 30h on doxycycline; H2B-GFP (green).

Movie S3: Related to Figure 4. Micronucleus formation in Kras^{G12D}/Mad2 positive spheroid culture

Time-lapse imaging of a Kras^{G12D}/Mad2 positive acinus showing a mitotic cell (red circle) that divides and forms a micronucleus, t=0 after 30h on doxycycline; H2B-GFP (green).

Movie S4: Related to Figure 4. Chromosome misalignment in Kras^{G12D}/Mad2 positive spheroid culture

Time-lapse imaging of Kras^{G12D}/Mad2 positive acinus with a mitotic cell (red circle) undergoing apoptosis after severe chromosome misalignments, t=0 after 30h on doxycycline; H2B-GFP (green).

Movie S5: Related to Figure 4. Cytokinesis failure in Kras^{G12D}/Mad2 positive spheroid culture

Time-lapse imaging of Kras^{G12D}/Mad2 positive spheroid with a mitotic cell (red circle) failing to complete nuclear division, t=0 after 30h on doxycycline; H2B-GFP (green).