

Fig. S1. Loss of contact inhibition in HC11 cells cultured in the presence of AlCl₃. HC11 cells cultured for 71 weeks in the presence of AlCl₃ 10 μM (B), AlCl₃ 100 μM (C) or the same dilution (1/1000) of solvent (H₂O) alone (A) were photographed under phase contrast. Bar = 100 μm.

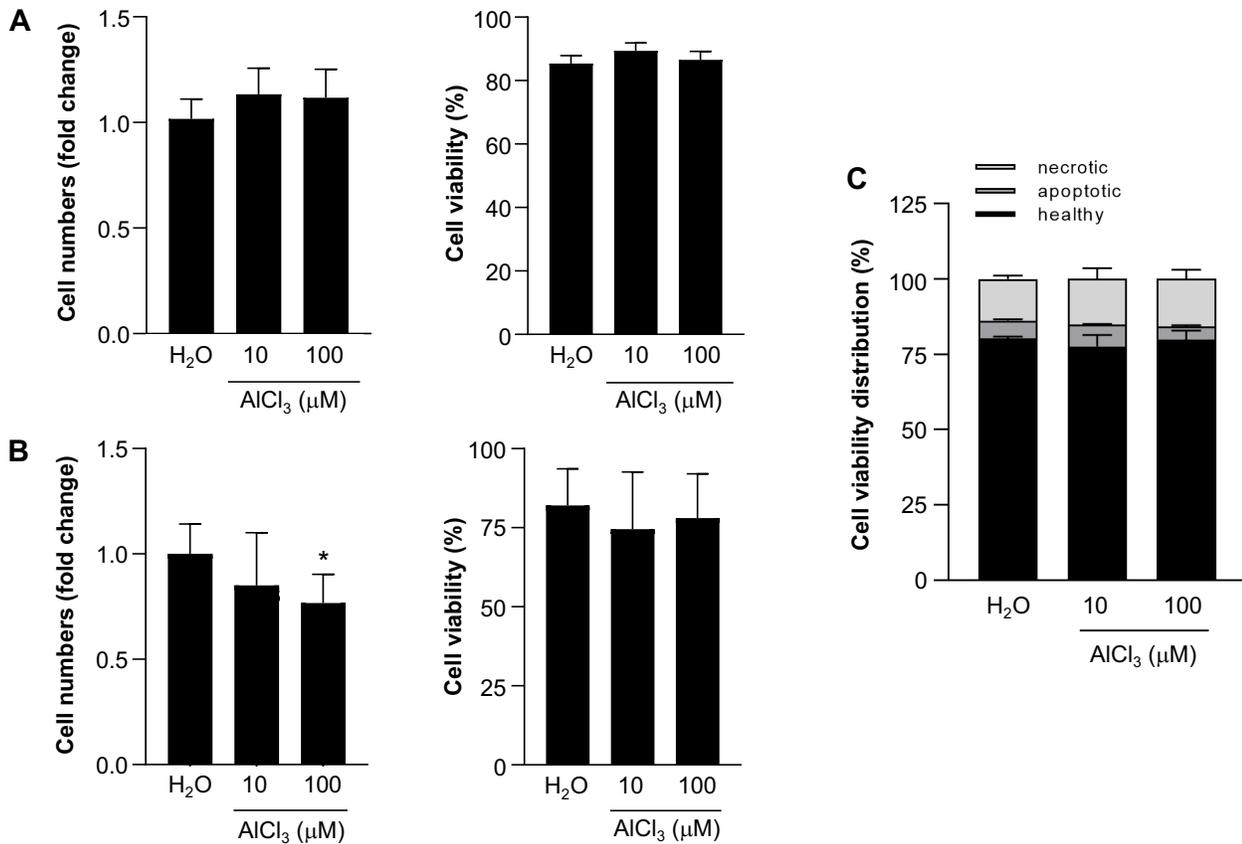


Fig. S2. AlCl₃ is not a mitogen for HC11 cells. **A)** Left panel: HC11 cells were seeded in 6-wells culture plates at the density of 6×10^3 cells/well and cultured in the presence of the indicated concentrations of AlCl₃, or same dilution (1/1000) of solvent (H₂O) alone, for four days. At the end of the incubation, cells were counted using an automated cell counter. The values reported in the graph are means \pm SEM from three independent experiments performed in triplicate. The number of cells in controls (H₂O) was set to 1. Right panel: in the same samples, cell viability was assessed by trypan blue exclusion with an automated cell counter. **B)** Same as A, except that incubation was for seven days. Medium and treatments were renewed 4 days after seeding. Left: * p AlCl₃ 100μM vs H₂O = 0.049 (Dunnnett's multiple comparison test). **C)** HC11 cells were seeded in 6-wells culture plates at the density of 6×10^3 cells/well and cultured in the presence of the indicated concentrations of AlCl₃, or same dilution (1/1000) of solvent (H₂O) alone, for four days. At the end of the incubation, cell viability was assessed by annexin V/PI staining in flow cytometry. The values reported in the graph are means \pm SEM from two independent experiments performed in triplicate.

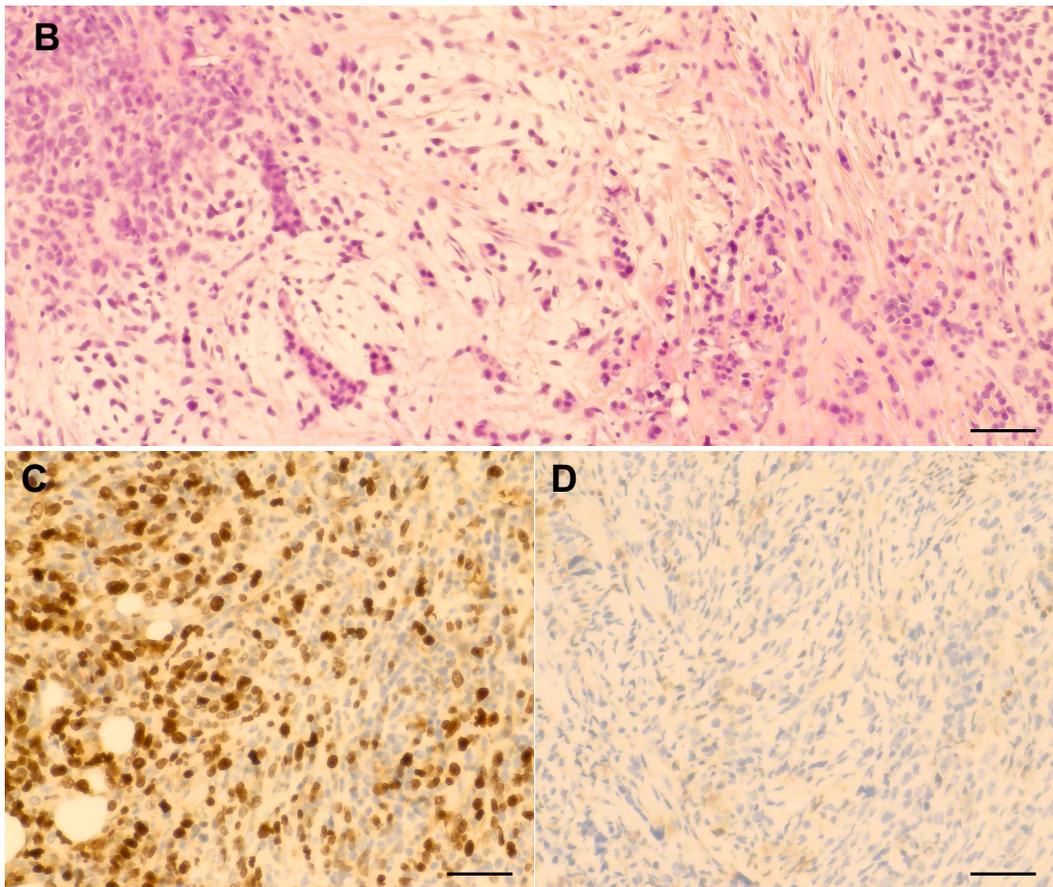
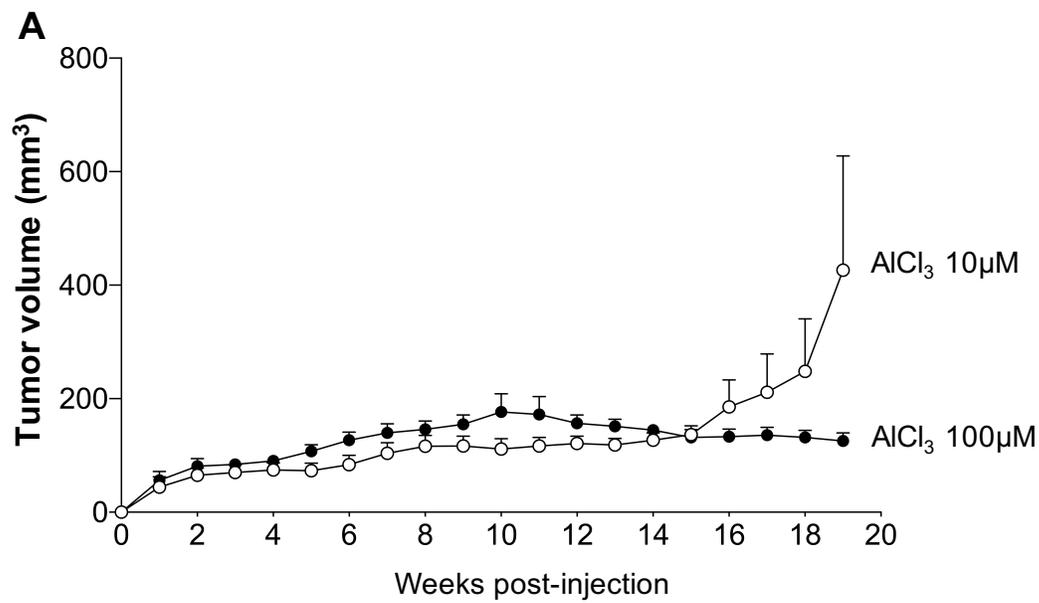


Fig. S3. Kinetics and histology of tumors formed by AlCl₃-transformed HC11 cells. **A)** The graph shows the growth, over time, of the tumors reported in Fig. 1B. Tumor size was calculated according to the formula: $V \text{ (mm}^3\text{)} = d^2 \text{ (mm}^2\text{)} \times D \text{ (mm)}/2$, where d and D are the smallest and largest tumor diameters, respectively, at the indicated number of weeks after sub-cutaneous injection. Results were obtained from 2 independent experiments. Values displayed are means \pm SEM. **B)** Hematoxylin/eosin staining or **C)** representative Ki67 staining or **D)** irrelevant polyclonal rabbit IgG staining of a tumor formed by HC11 cells transformed *in vitro* by AlCl₃ 10 μM, after subcutaneous injection into a 6-weeks old BALB/cByJ female mouse. Bar = 50 μm in B-D.

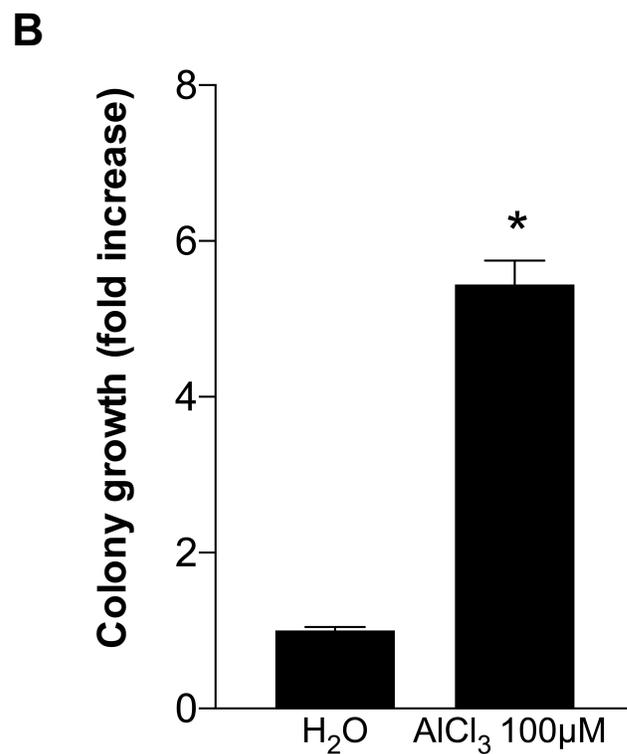
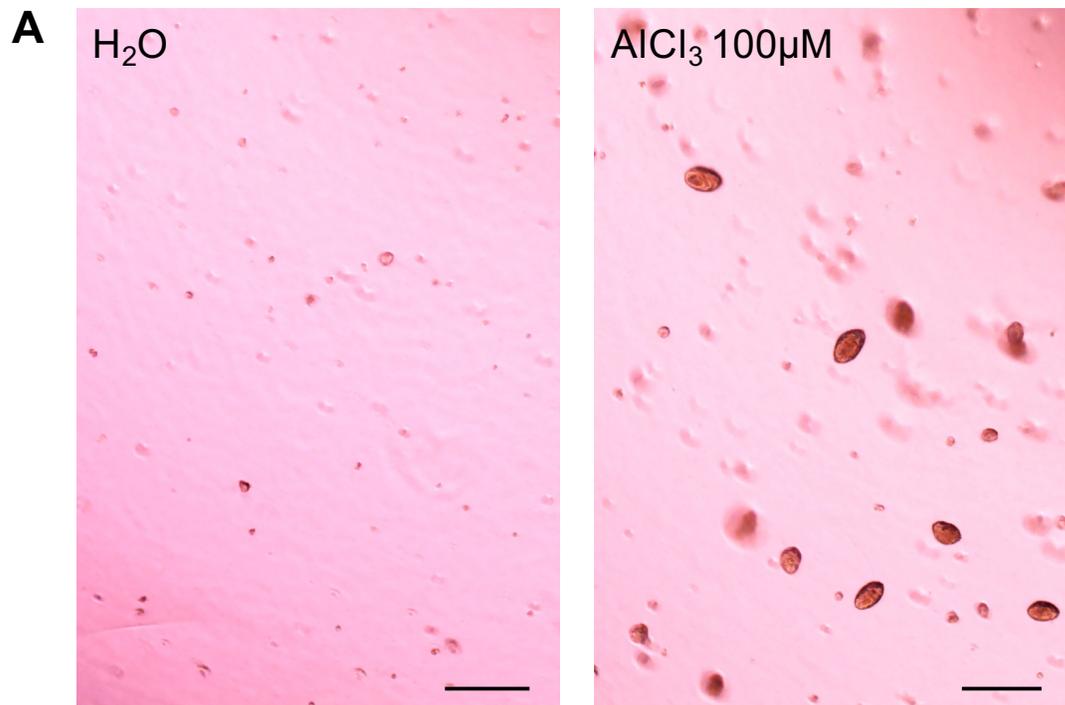


Fig. S4. A) AlCl₃ transforms NMuMG cells *in vitro*. NMuMG cells cultured for 40 weeks in the presence of AlCl₃ 100µM or the same dilution (1/1000) of solvent (H₂O) alone were grown in soft agar for 14 days in the absence of AlCl₃. Bar = 200µm. **B)** The growth in agarose gels was quantified by measuring the total area of colonies having an area of at least 100 pixels (corresponding to a colony of approximately ten cells). Growth of controls (H₂O) was set to 1. Colony number and area were measured in at least 17 fields in 6 different wells from 2 independent experiments per condition. The values shown are means +/- SEM. **p* AlCl₃ 100µM vs H₂O < 0,0001 (unpaired two-tailed t-test).

Excel File S1 is provided separately.

Table S1. A) *Sulf2*, *Enpp5* or *Hsd11b1* mRNA levels in AlCl_3 -transformed HC11 or NMuMG cells. mRNA levels were assessed by cDNA microarray in HC11 cells or NMuMG cells of Series I (S. I) or Series III (S. III) transformed *in vitro* by AlCl_3 10 μM or 100 μM or parallel control cultures incubated in the presence of the same dilution (1/1000) of solvent (H_2O) alone, as indicated. FDR p-values refer to Anova.

| Condition | Cell line | <i>Sulf2</i> | | <i>Enpp5</i> | | <i>Hsd11b1</i> | |
|---|--------------|--------------|-------------|--------------|-------------|----------------|-------------|
| | | Fold change | FDR p-value | Fold change | FDR p-value | Fold change | FDR p-value |
| AlCl_3 100 μM vs H_2O | NMuMG S. I | + 5.84 | <0.000001 | - 7.44 | <0.000001 | - 4.06 | 0.0055 |
| | NMuMG S. III | + 106.34 | <0.000001 | - 11.39 | <0.000001 | - 6.72 | 0.0005 |
| | HC11 | + 24.86 | <0.000001 | - 26.48 | <0.000001 | - 65.14 | <0.000001 |
| AlCl_3 10 μM vs H_2O | HC11 | + 21.90 | <0.000001 | - 35.74 | <0.000001 | - 45.68 | 0.00003 |

B) Same as A, except that mRNA levels were assessed by Real-Time quantitative PCR. *nd*: undetectable. p-values refer to Dunnett's multiple comparisons test.

| Condition | Cell line | <i>Sulf2</i> | | <i>Enpp5</i> | | <i>Hsd11b1</i> | |
|---|--------------|-------------------------------|-----------|-------------------------------|-----------|-------------------------------|-----------|
| | | Relative mRNA level \pm SEM | p-value | Relative mRNA level \pm SEM | p-value | Relative mRNA level \pm SEM | p-value |
| AlCl_3 100 μM vs H_2O | NMuMG S. I | 6.85 \pm 0.38 | 0.0001 | 0.25 \pm 0.02 | 0.000006 | 0.29 \pm 0.11 | 0.0031 |
| | NMuMG S. III | 552.1 \pm 7.39 | <0.000001 | 0.15 \pm 0.02 | 0.000005 | 0.24 \pm 0.01 | <0.000001 |
| | HC11 | 20.10 \pm 3.69 | 0.0067 | 0.05 \pm 0.02 | <0.000001 | 0.12 \pm 0.05 | 0.00006 |
| AlCl_3 10 μM vs H_2O | HC11 | 13.05 \pm 3.45 | 0.0249 | <i>nd</i> | - | 0.10 \pm 0.06 | 0.00009 |

Excel File S2 is provided separately.

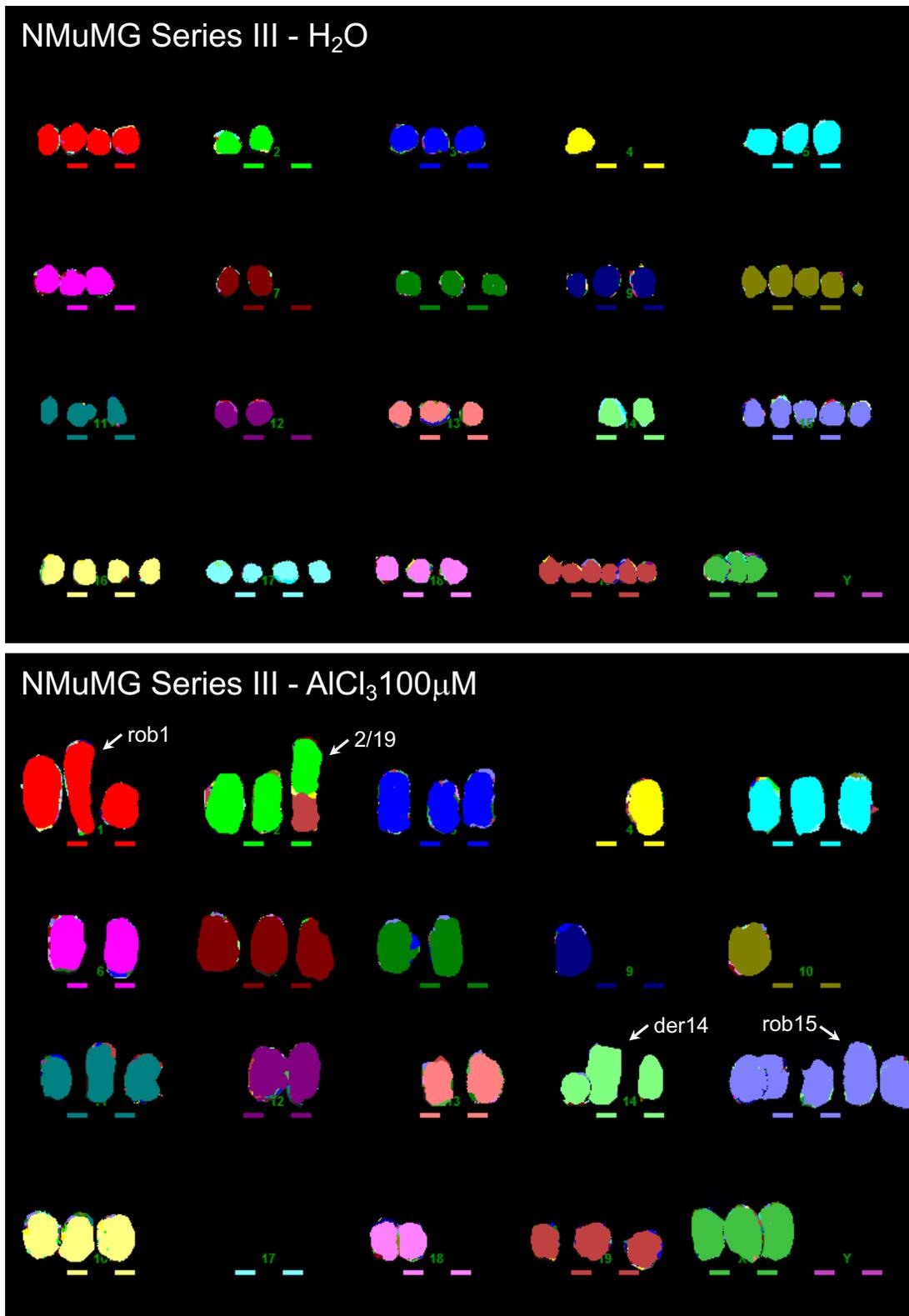


Fig. S5. MFISH analysis of NMuMG Series III. NMuMG cells cultured for 40 weeks in the presence of AlCl₃ 100 μM or the same volume (1/1000) of solvent (H₂O) alone (NMuMG Series III, see text) were analyzed for the presence of chromosomal rearrangements by MFISH. The photos shown are representative examples of the results obtained. Arrows indicate chromosomal rearrangements observed uniquely in the specific condition considered.

gDNA fold change

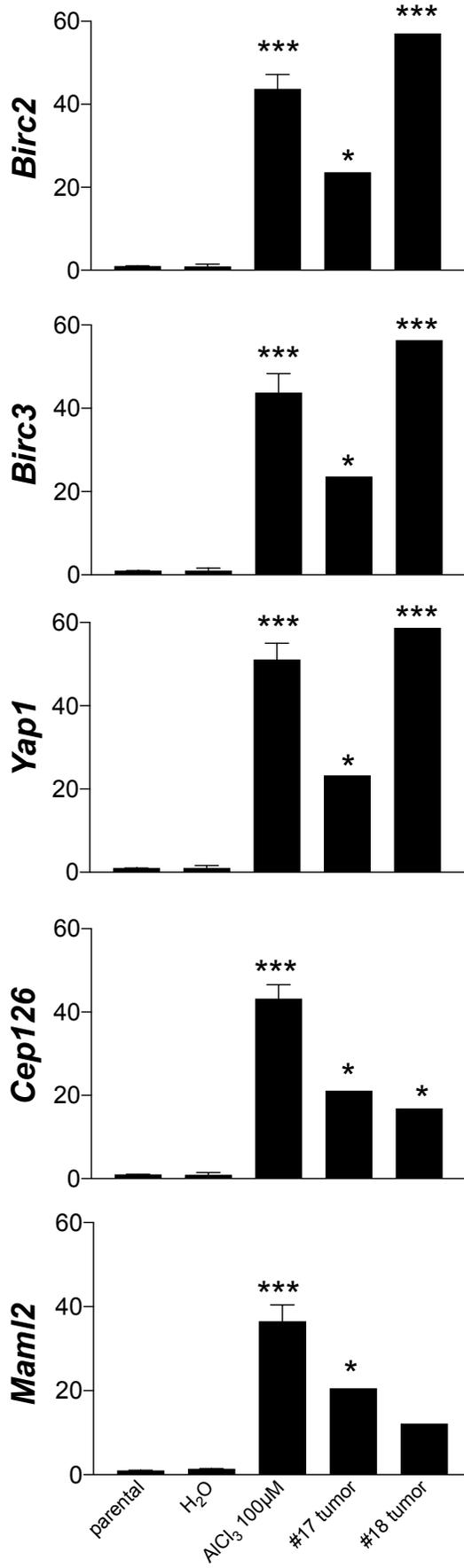
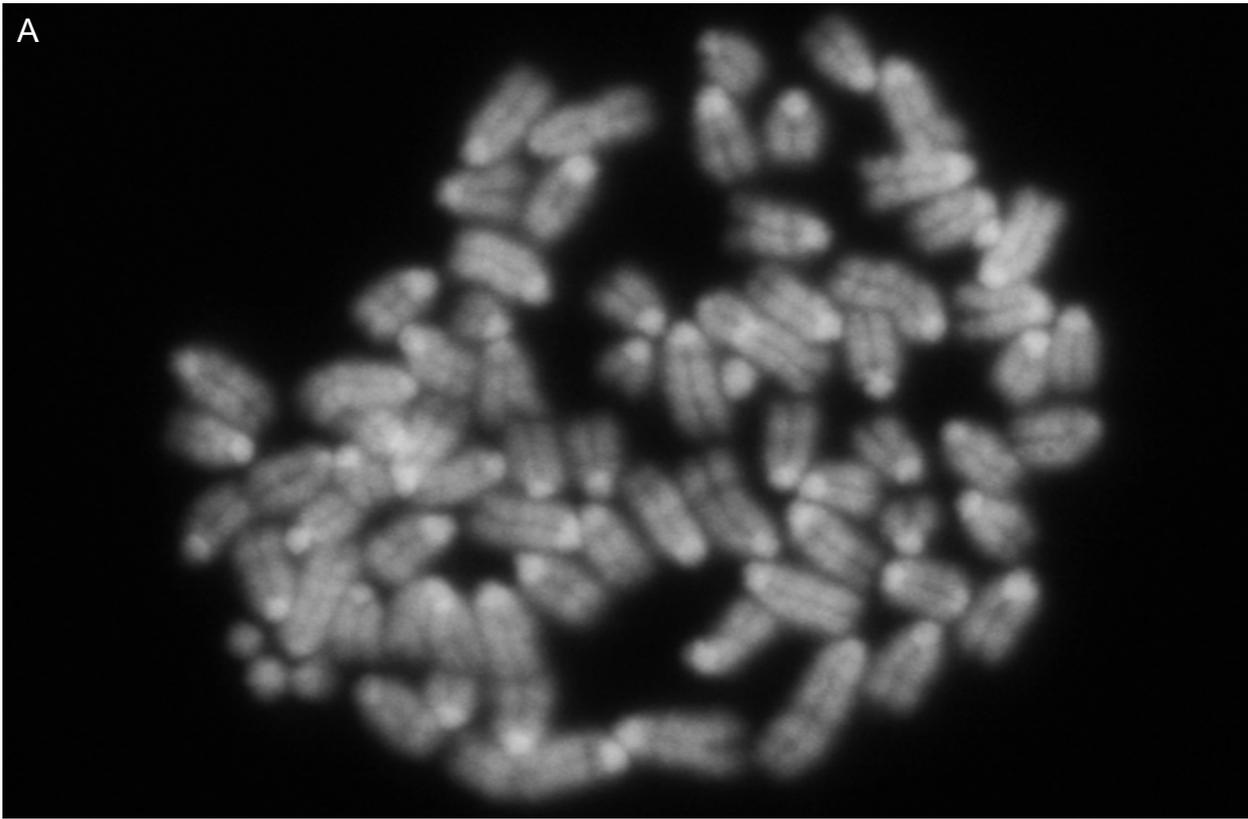
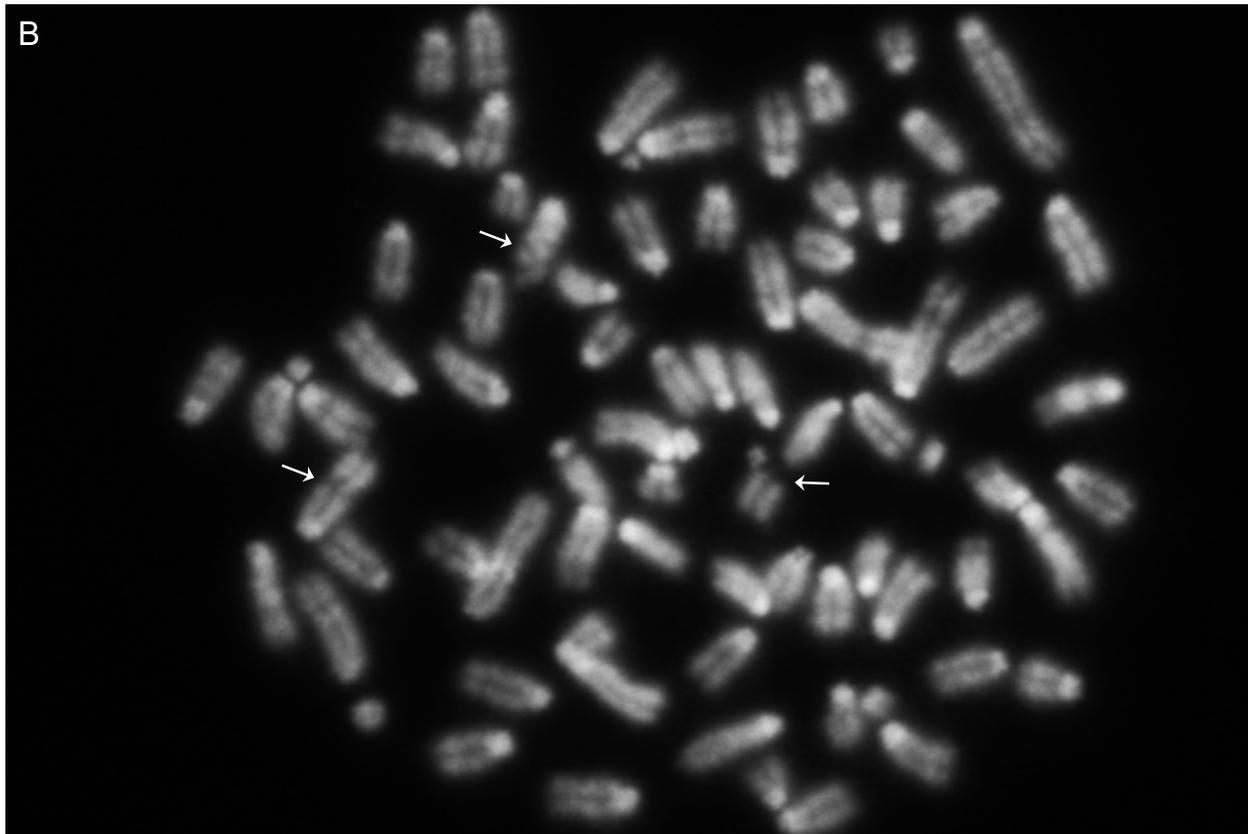


Fig. S6. Chromosome 9 amplification in NMuMG cells transformed *in vitro* by AlCl₃. Genomic DNA purified from parental NMuMG cells (parental), from NMuMG cells cultured for 38 weeks in the presence of AlCl₃ 100μM (AlCl₃ 100μM) or the same dilution of solvent (H₂O) alone (NMuMG Series I, see text), or from fresh whole cell isolates from two different tumors (#17, #18) formed by AlCl₃-transformed cells of the same series in NOD-SCID gamma mice [8] were analysed for *Yap1*, *Birc2*, *Birc3*, *Cep126* or *Mam12* gDNA quantities by Real-Time quantitative PCR. The graphs show the average values +/- SEM from three independent biological replicates (parental, H₂O, AlCl₃ 100μM) or from the two individual tumors. ****p*<0.001; **p*<0.05 (Dunnett's multiple comparisons test).

A



B



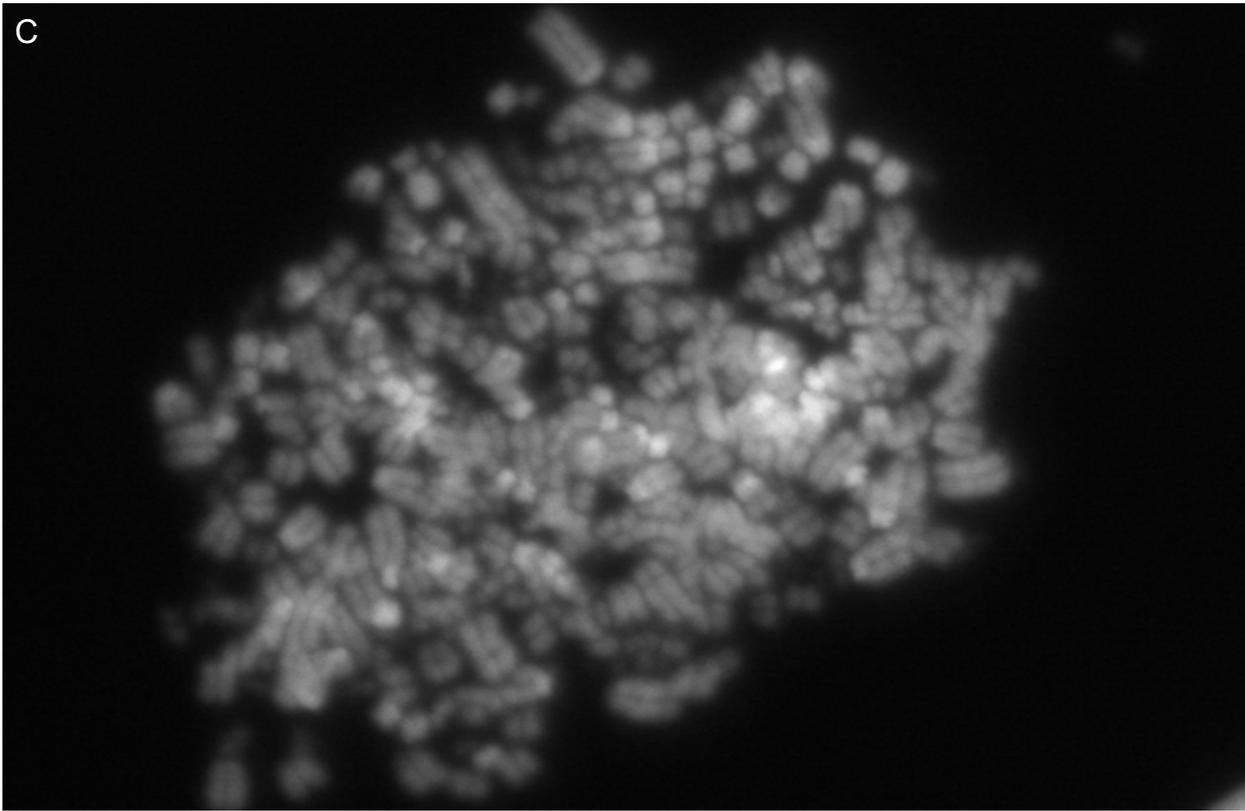


Fig. S7. Short AlCl_3 exposure induces chromosomal structural abnormalities in HC11 cells. DAPI-stained metaphases of HC11 cells incubated for 24 hours in the presence of AlCl_3 100 μM (**B, C**) or the same dilution (1/1000) of solvent (H_2O) alone (**A**). Arrows in B indicate DSB. C shows chromosome fragmentation.

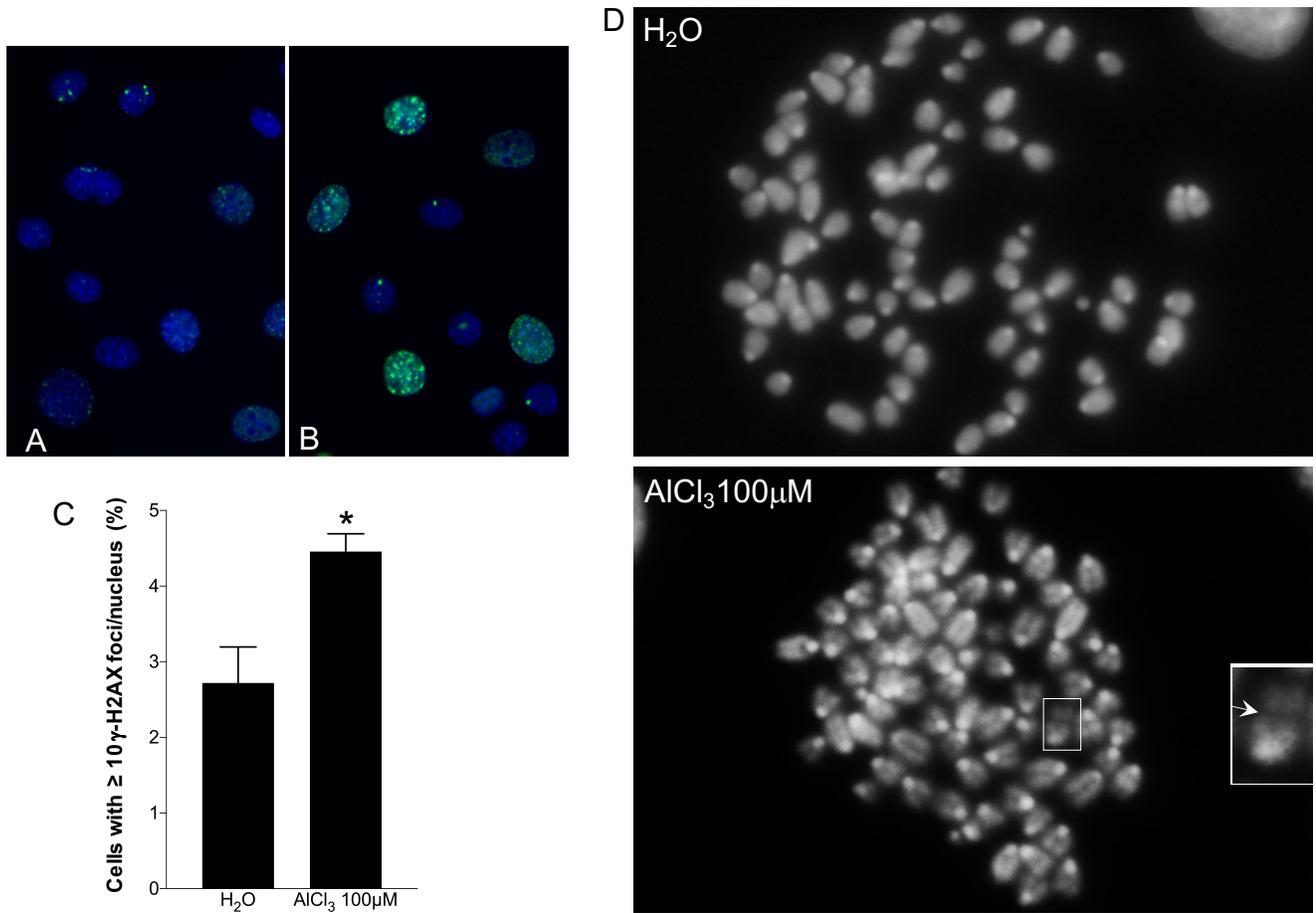


Fig. S8. Short AlCl₃ exposure induces DNA damage in HC11 and NMuMG cells. **A, B**) Examples of γ -H2AX immunostaining in HC11 parental cells incubated for 24 hours in the presence AlCl₃ 100 μ M (**B**) or the same dilution (1/1000) of solvent (H₂O) alone (**A**), whose quantification is reported in Fig. 5B. Nuclei were counterstained with DAPI. **C**) Quantification of γ -H2AX immunostaining in NMuMG cells incubated for 24 hours in the presence AlCl₃ 100 μ M or the same dilution (1/1000) of solvent (H₂O) alone. The graph shows the percentage of cells with at least 10 γ -H2AX nuclear foci/nucleus. Data (mean +/- SEM) are from three independent experiments. $p = \text{AlCl}_3 \text{ vs H}_2\text{O} < 0.0001$ (chi-square test). **D**) DAPI-stained metaphases of NMuMG cells incubated for 48 hours in the presence of AlCl₃ 100 μ M or the same dilution (1/1000) of solvent (H₂O) alone. Arrows indicate DSB (inset).

Supplementary Materials and Methods.

Cell proliferation assay

Cells were seeded in 6-well plates in triplicate at the density of 6×10^3 cells/well. The indicated concentrations of AlCl_3 or the same dilution (1/1000) of solvent (H_2O) alone were added at the time of seeding. Medium and treatments were renewed after 3 days for the 7-day proliferation experiments. Four or seven days after seeding, cells were counted with an automated cell counter that simultaneously measures trypan blue exclusion as a measure of cell viability.

Flow cytometry

Cell viability was measured by flow cytometry using an Annexin V/Propidium Iodide apoptosis detection kit (cat. No. 88-8005-72, Thermo Fisher Scientific). Stained cells were analyzed on an Attune Nxt flow cytometer (Thermo Fisher Scientific). Data analysis was performed using FlowJo software (version 10.6.1) (BD Life Sciences, Franklin Lakes, NJ, USA).

Soft agar assay

Soft agar assay was as described [7, 8]. After 14 days, colonies were photographed using an EVOS XL Core Cell Imaging System (Thermo Fisher Scientific) at 4x magnification. Quantification of the cell growth was performed by ImageJ software (version 8) (National Institute of Health, Bethesda, MD, USA) as described [29]. AlCl_3 was not added to the soft agar assay.

Genomic DNA extraction

Genomic DNA was extracted using PureLink™ Genomic DNA Mini Kit (cat. No. K182000, Thermo Fisher Scientific).

Real-Time quantitative PCR

Primer sequences were as follows (5' to 3'):

For mouse cDNA:

Hprt_F: GCCGAGGATTTGGAAAAAGTG; Hprt_R: TGTAATCCAGCAGGTCAGCA;
Gapdh_F: AGGTCGGTGTGAACGGATTTG; Gapdh_R: TGTAGACCATGTAGTTGAGGTCA;
Igfbp4_F: GCAAGATGAAGATCGTGGGGA; Igfbp4_R: GTTGCGGTCACAGTTTGGAA;
Enpp5_F: GTAGCTGCCATCCTGCCAAA; Enpp5_R: GGCCACAGCTACGATTGGT;
Hsd11b1_F: TCCAGAAGGTAGTGTCTCGCT; Hsd11b1_R: CGAGGTCTGAGTGATGTGGTT;
Sulf2_F: GTGGAGGGGACTACAACTGG; Sulf2_R: AGTGCGGCTTGCTAAGGTTG.

For mouse gDNA:

Hprt_F: GCTCGAGATGTCATGAAGGAGAT; Hprt_R: AAAGAACTTATAGCCCCCTTGA;
Gapdh_F: TCCATGACAACCTTTGGCATTG; Gapdh_R: CAGTCTTCTGGGTGGCAGTGA;
Birc2_F: CTATTACGTGGATCGCAATGATGA; Birc2_R: CCACCATCACAACAAAAGCACTT;
Birc3_F: TGAGAATACTGGCTATTTCAAGTGGC; Birc3_R: GGGTCTGAGGGAAAGCTCG;
Yap1_F: AGCATGTTGAGCTCACTCCTC; Yap1_R: AAACGGCACCCAGCTGC;
Cep126_F: TTTTCAAATCCAGTTCCTCCATT; Cep126_R: TCTTGAATTTGTTTTAGGGCCTCT;
Maml2_F: CTGGCACCTACAACGTCACCTC; Maml2_R: TTTCTCTGTTGTGTCAGCTGGTTC.