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Supplemental Figures

SMAC mimetic birinapant plus radiation eradicates human head and neck cancers with genomic alterations in cell death pathways

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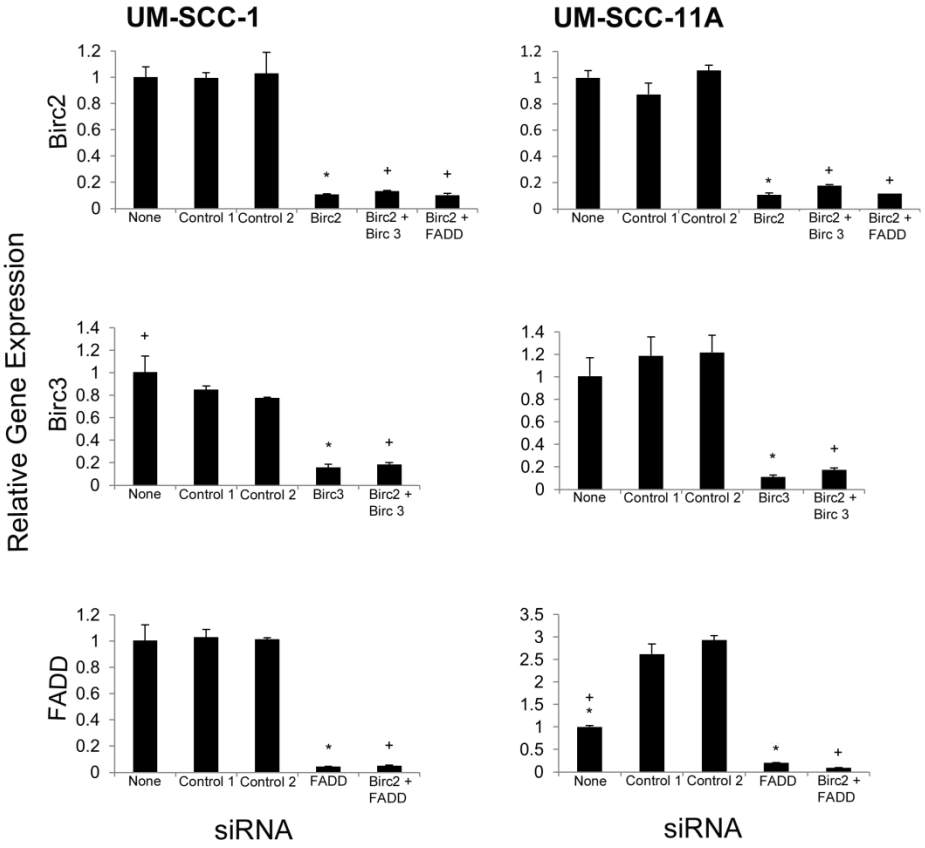
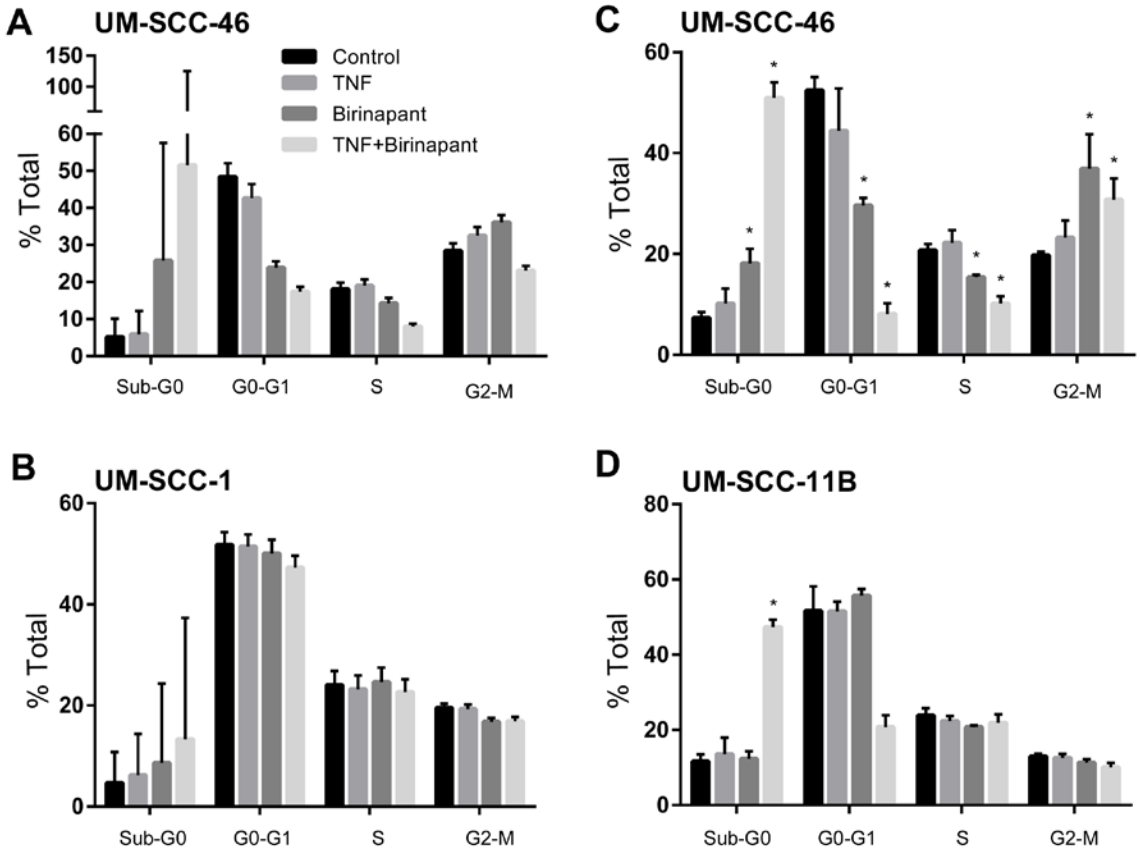
Supplemental Figure 1



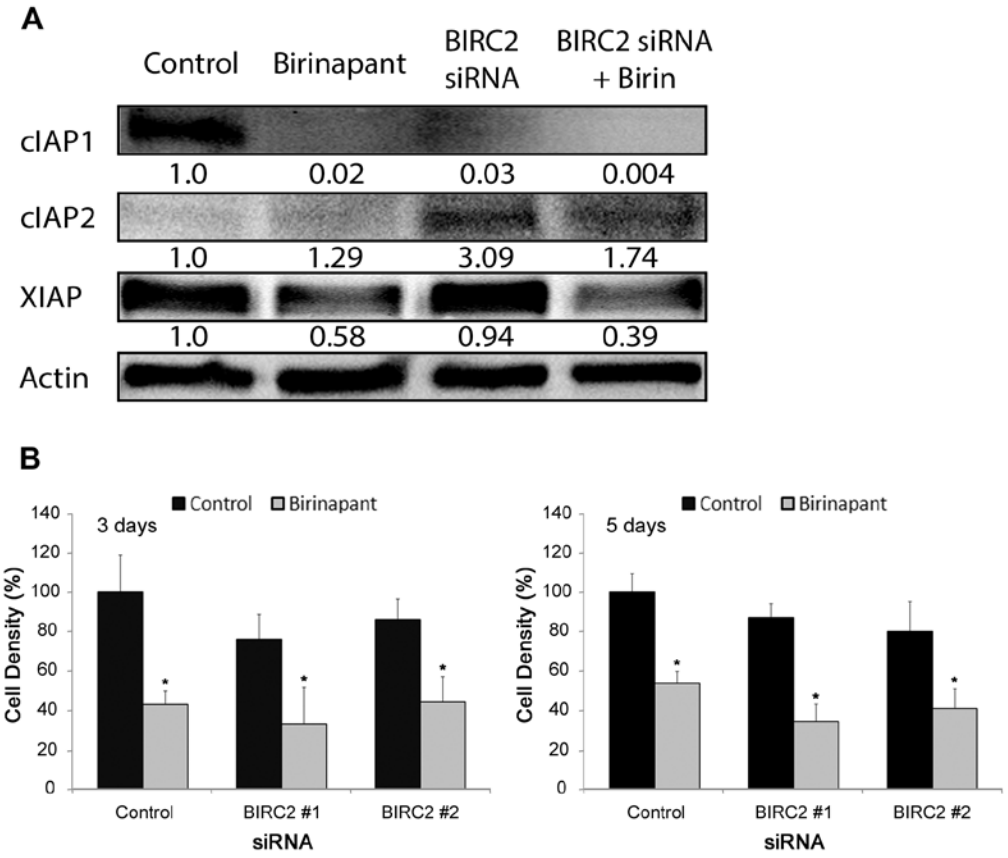
Supplemental Figure 1. Effect of *Birc2, Birc3,* and *FADD* knockdown in UM-SCC-1 and UM-SCC-11A. *Birc2, Birc3,* and *FADD* gene expression were quantified by real-time PCR at 48 hours post-transfection with 50 nM *Birc2, Birc3,* or *FADD* siRNAs alone or in combination. Two concentrations (Control 1, 50 nM and Control 2, 100 nM) of control siRNAs were used to match the siRNA concentration in the single or dual knockdown, respectively. Graphs depict gene expression relative to non-transfected cells. With siRNA transfection, expression of each target gene was decreased to <20% of levels in non-transfected cells. Statistical significance (p <0.05, Student t-test) was calculated by comparing single knockdown conditions to Control 1 and double knockdown conditions to Control 2. Statistical significance was calculated for non-transfected cells compared to Control 1 and Control 2. Error bars represent standard deviation.

Supplemental Figure 2

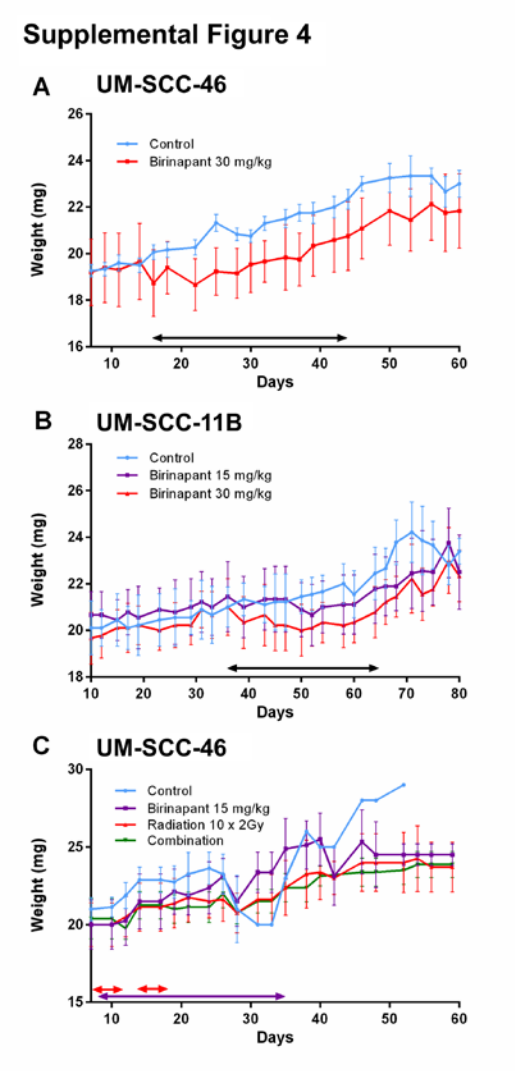


Supplemental Figure 2. Summary of DNA cell cycle cytofluorometry in UM-SCC lines treated with TNFα, birinapant and combination. UM-SCC-46 (**A**) and -1 (**B**) cells were treated with 1 μM birinapant and 20 ng/mL TNFα and were harvested 48 hours post-treatment. Error bars are the coefficient of variance. UM-SCC-46 (**C**) and -11B (**D**) were treated with 1 μM birinapant and 20 ng/mL TNFα, and were harvested 24 hours post-treatment. Samples were run in triplicate and data is presented as the mean +/- standard deviation, and statistical significance are indicated as * (student t-test, $p<0.05$).

Supplemental Figure 3

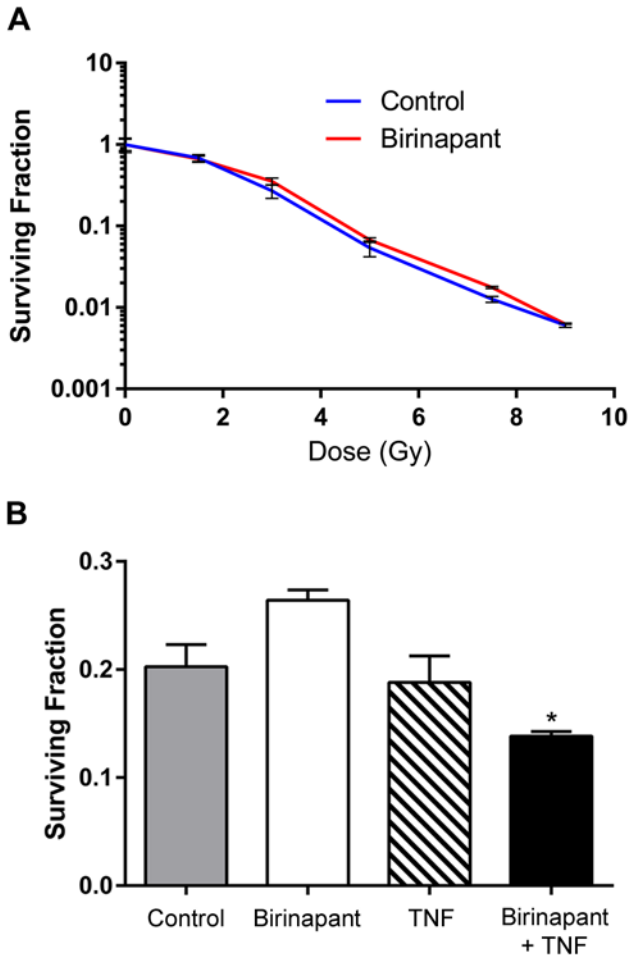


Supplemental Figure 3. Effect of birinapant treatment and/or BIRC2 siRNA knockdown on cell IAP death pathway components and cell survival. **A**, UM-SCC-46 cells were treated with 50 nM birinapant (IC50) and/or transfected with BIRC2 siRNA. Whole cell lysates were procured 48 hours after treatment and subjected to SDS-PAGE and Western blot. Expression was quantified using actin as a loading control. **B**, UM-SCC-46 cells were transfected with control and two different BIRC2 siRNAs. Subsequent treatment with 50 nM birinapant was performed. Cellular proliferation was measured via XTT assay 3 and 5 days following treatment. Student t-test, *$p<0.001$ for birinapant vs no treatment.



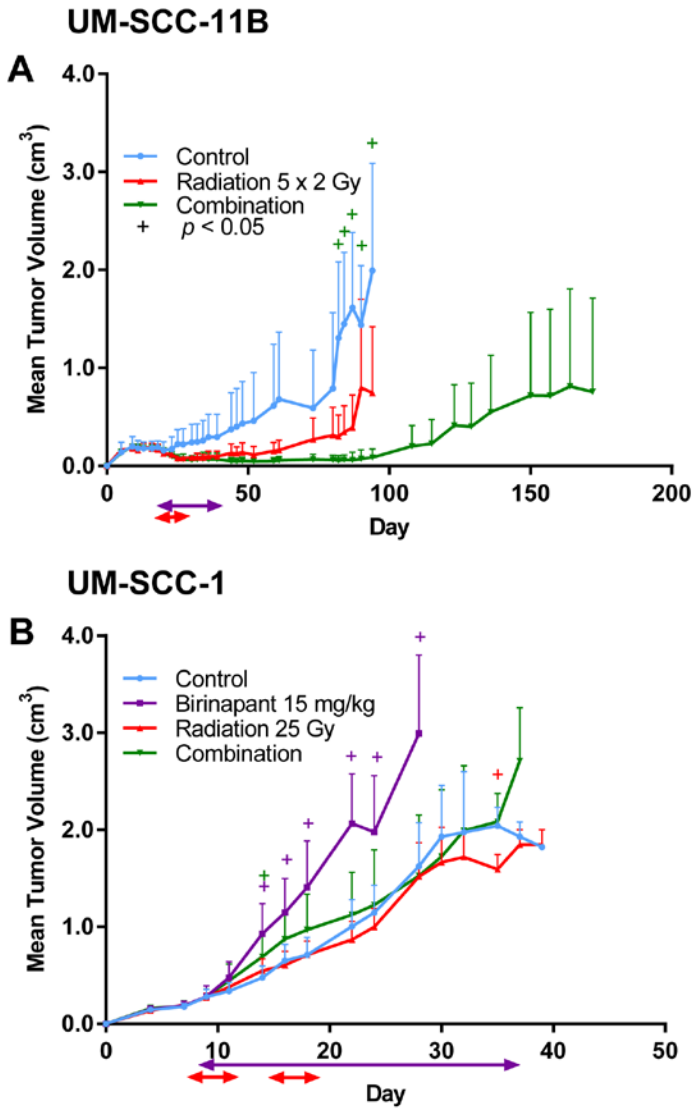
Supplemental Figure 4: The average body weight of mice in all treatment groups before, during, and after the treatment period. **A**, The body weight of mice bearing UM-SCC-46 xenografts (control and birinapant 30 mg/kg i.p.). Birinapant was given every three days from day 16 through day 43 (represented by the black arrow). **B**, The body weight of mice bearing UM-SCC-11B xenografts (control, birinapant 15 mg/kg i.p., and birinapant 30 mg/kg i.p.). Birinapant was given every three days from day 36 through day 63 (represented by the black arrow). **C**, The body weight of mice bearing UM-SCC-46 xenografts (control, birinapant 15 mg/kg i.p., fractionated radiation 10 × 2Gy/day, combination). Birinapant was given every three days from day 8 through day 35 (represented by purple arrow) and 2 Gy radiation was given days 7-11 and days 14-18 (represented by red arrows).

Supplemental Figure 5



Supplemental Figure 5. *In vitro* effect of birinapant on radiosensitivity of UM-SCC-46 cells. **A**, Radiation was administered at 6 different doses (0-9 Gy) at a single timepoint. Designated cells were treated with 30 nM birinapant immediately after radiation. Cells were re-plated 24 hours following irradiation. Graph depicts surviving fraction of colonies for control vs. birinapant treated cells. Error bars represent standard deviation of 6 replicates. D_q = 0.10 and 0.41 Gy for control and birinapant treated cells, respectively. D_{10} = 4.11 and 4.58 Gy for control and birinapant treated cells, respectively. **B**, Surviving fraction of cells receiving 3 Gy radiation followed by 25 nM birinapant, 2 ng/mL TNFα, or combination treatment. Values normalized to non-irradiated controls receiving the same drug treatment. Error bars represent standard deviation of 6 replicates. Student t-test, *$p<0.001$ for control vs. combination birinapant + TNFα treatment.

Supplemental Figure 6



Supplemental Figure 6: Combination of birinapant plus radiation induces tumor regression and regrowth delay in UM-SCC-11B xenograft model, but not in the UM-SCC-1 xenograft model. Tumorigenic inoculation of 5×10^6 UM-SCC-11B cells or 1.5×10^6 UM-SCC-1 cells were implanted into the upper portion of the right leg in athymic *nu/nu* mice. **A**, UM-SCC-11B xenograft bearing mice were randomized into 3 groups eighteen days after tumor inoculation (day 18) when average tumor volume reached ~200 mm^3 (vehicle control n=4, 5 × 2 Gy radiation n=8, or 15 mg/kg birinapant i.p. combined with radiation n=4). Radiation treatment began on day 18: 2 Gy of radiation was given Monday-Friday for one week for a total of 10 Gy

(represented by red double headed arrow). Birinapant treatment began on day 19: 15 mg/kg birinapant i.p. every three days for a total of ten doses (treatment span represented by purple double headed arrow). Mice treated with combination therapy of birinapant and radiation showed significantly decreased tumor volume compared to the control. Statistical analysis by Student t-test (+; p value < 0.05). Error bars, SEM. **B**, Mice injected with UM-SCC-1 cells were randomized seven days after tumor inoculation (day 7) when average tumor volume reached ~ 200 mm^3. Mice were randomized into four treatment groups (vehicle control n=8, 15 mg/kg birinapant i.p., n=8, 10 × 2 Gy radiation n=8, or the combination n=8). Radiation treatment began on day 7: 3 Gy of radiation was given day 7-11 and 2 Gy of radiation was given day 14-18 for a total of 25 Gy (represented by red double headed arrows). Birinapant treatment began on day 8: 15 mg/kg birinapant i.p. every three days for a total of ten doses (treatment span represented by purple double headed arrow). Combination treatment did not provide delayed tumor growth.