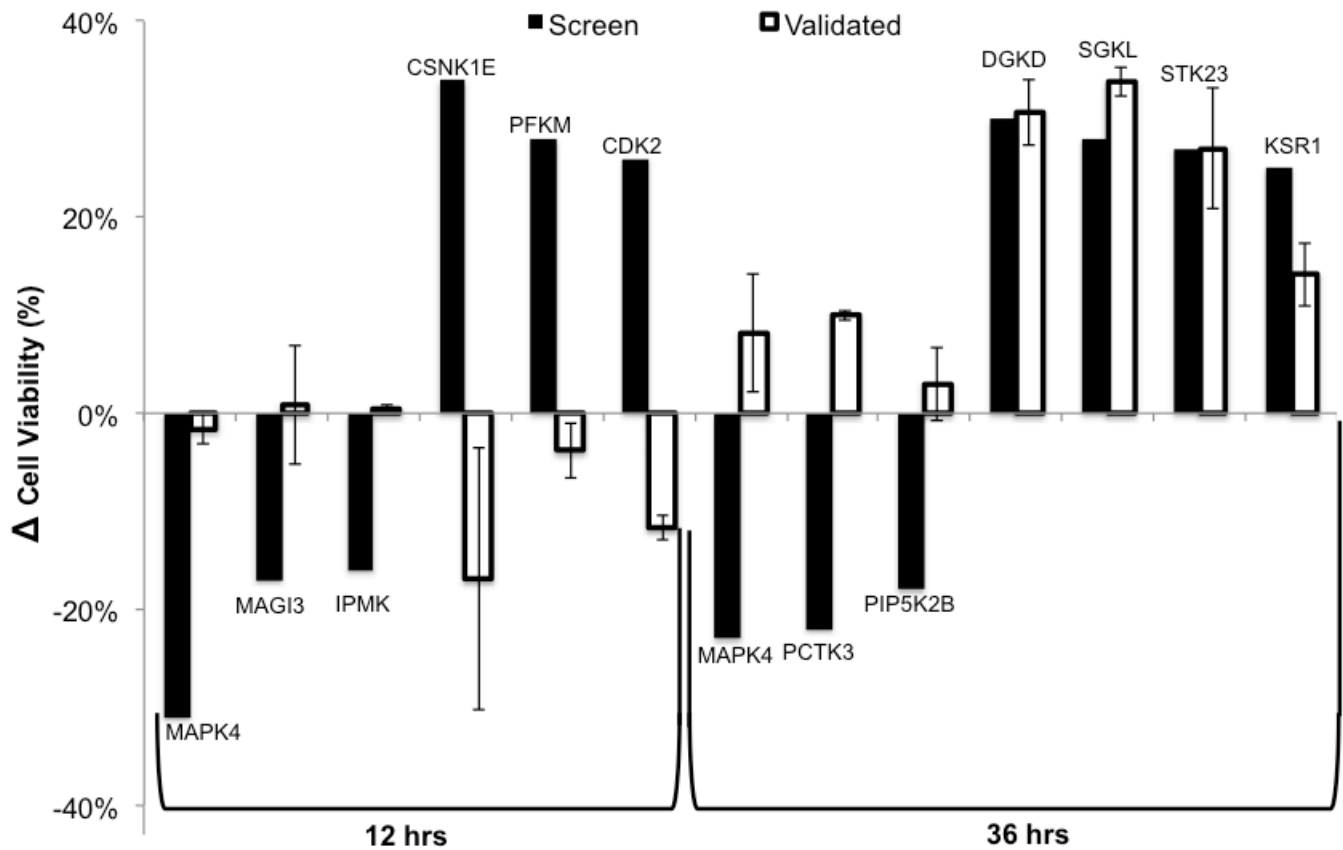# Supplementary Figures



**Figure S1. Validation of kinase regulators of ONC201 sensitivity.** Validation and screen results for changes in cell viability associated with the combination of ONC201 treatment (1 μM) and siRNA knockdown (n=3).
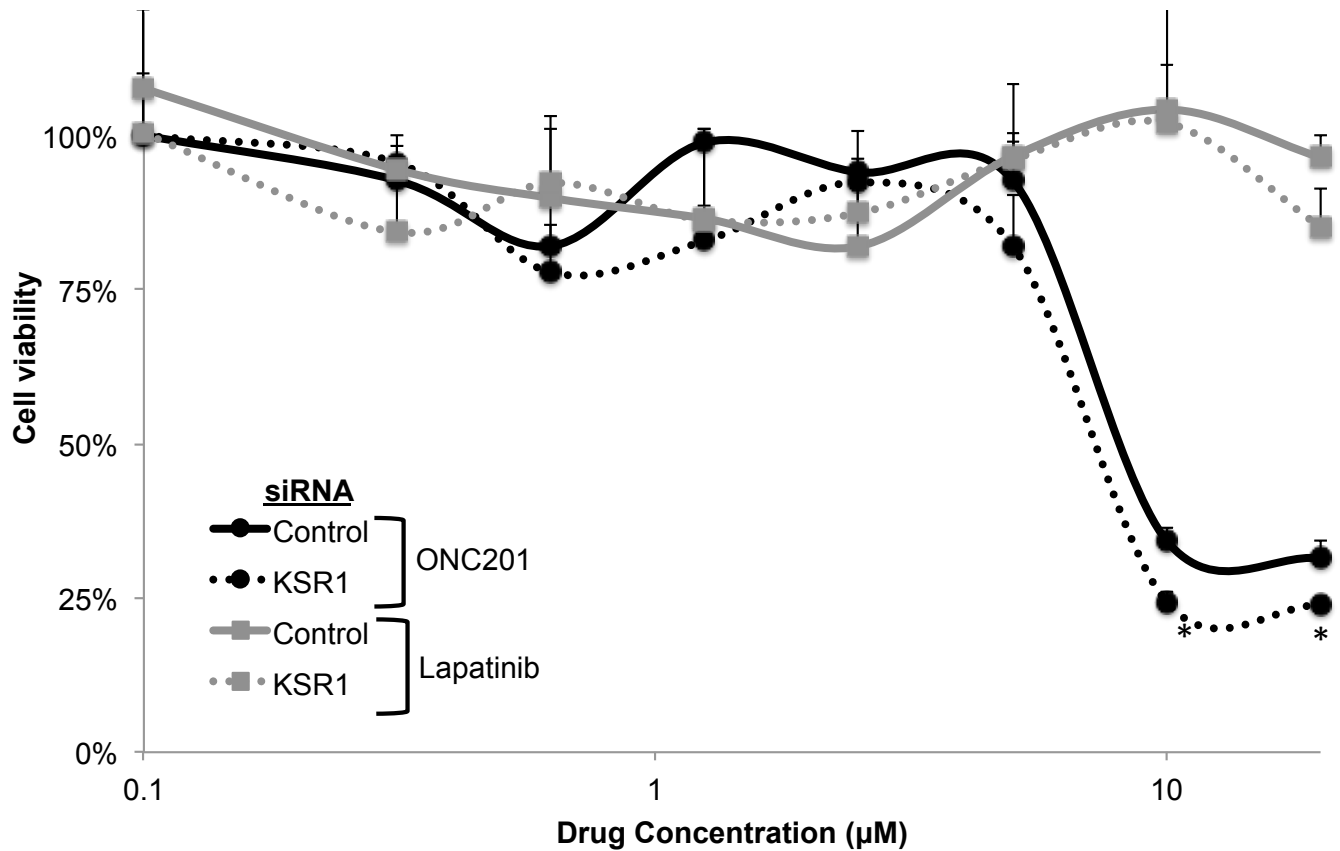
**Figure S2. KSR1 shifts the ONC201, but not lapatinib, dose-response curve downward.** Cell viability assay in HCT116 cells following siRNA knockdown and treatment with lapatinib (48 hours, n=3). *$P < 0.05$ compared to control siRNA under the same drug treatment by Student's two-tailed *t* test.
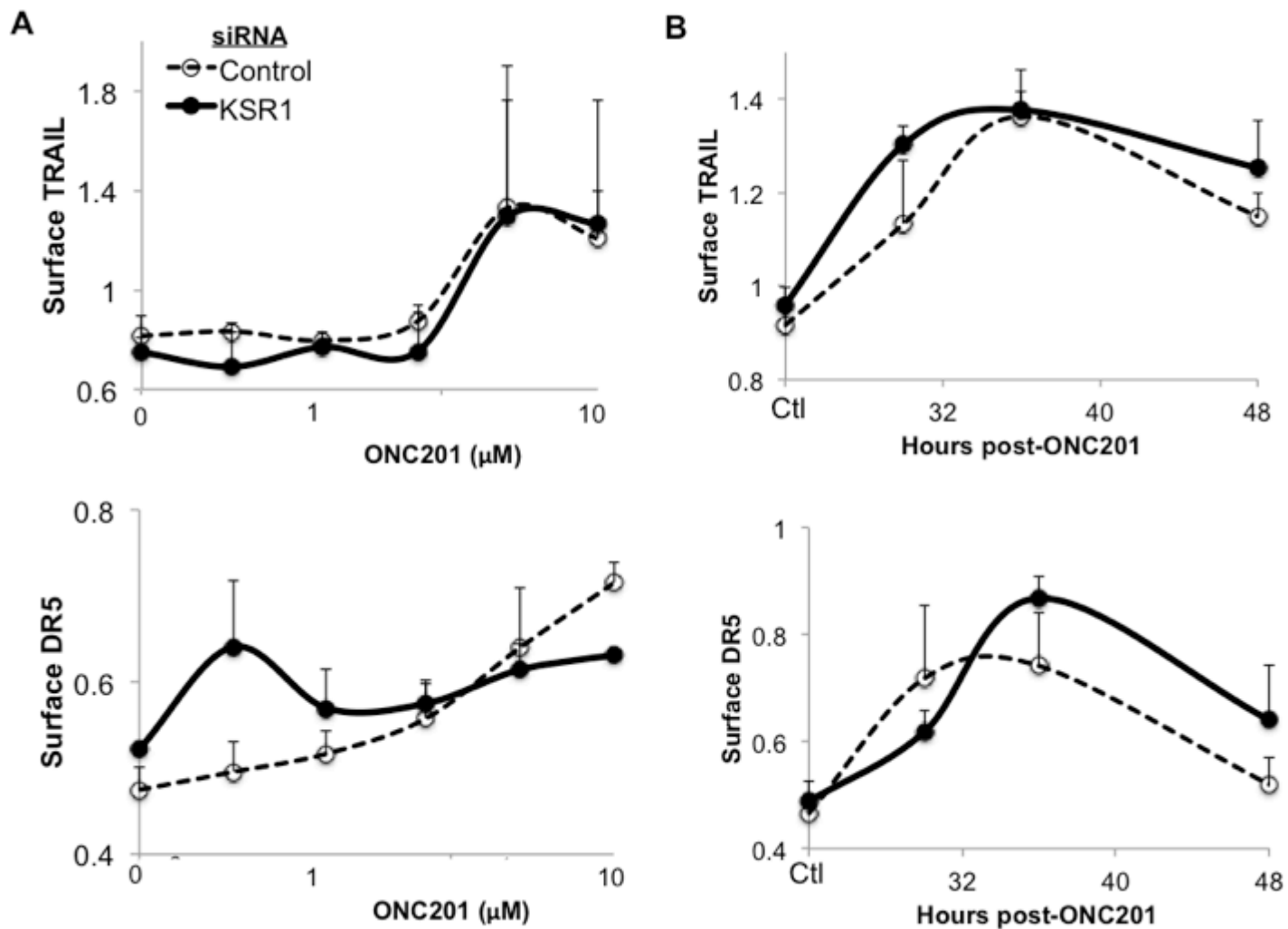
**Figure S3. KSR1 does not regulate ONC201-induced TRAIL or DR5.** (A) Surface TRAIL and DR5 assays in HCT116 cell treated with ONC201 at indicated concentrations (72 hours, n=3). (B) Time course analysis of surface TRAIL and DR5 following treatment with ONC201 in HCT116 cells (5 µM, n=3).
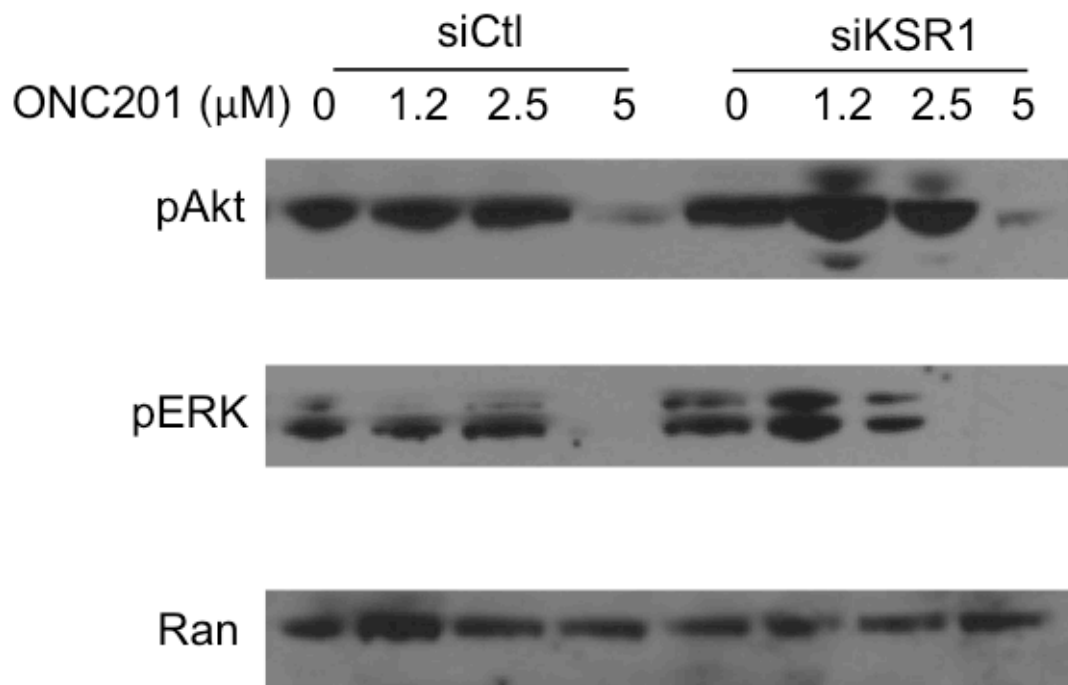
**Figure S4. KSR1 does not affect ONC201-mediated inhibition of MAPK or Akt inhibition.** Western blot analysis of ONC201-induced signaling events in HCT116 cells following ONC201 treatment in the presence or absence of KSR1 siRNA-mediated knockdown (60 hours).
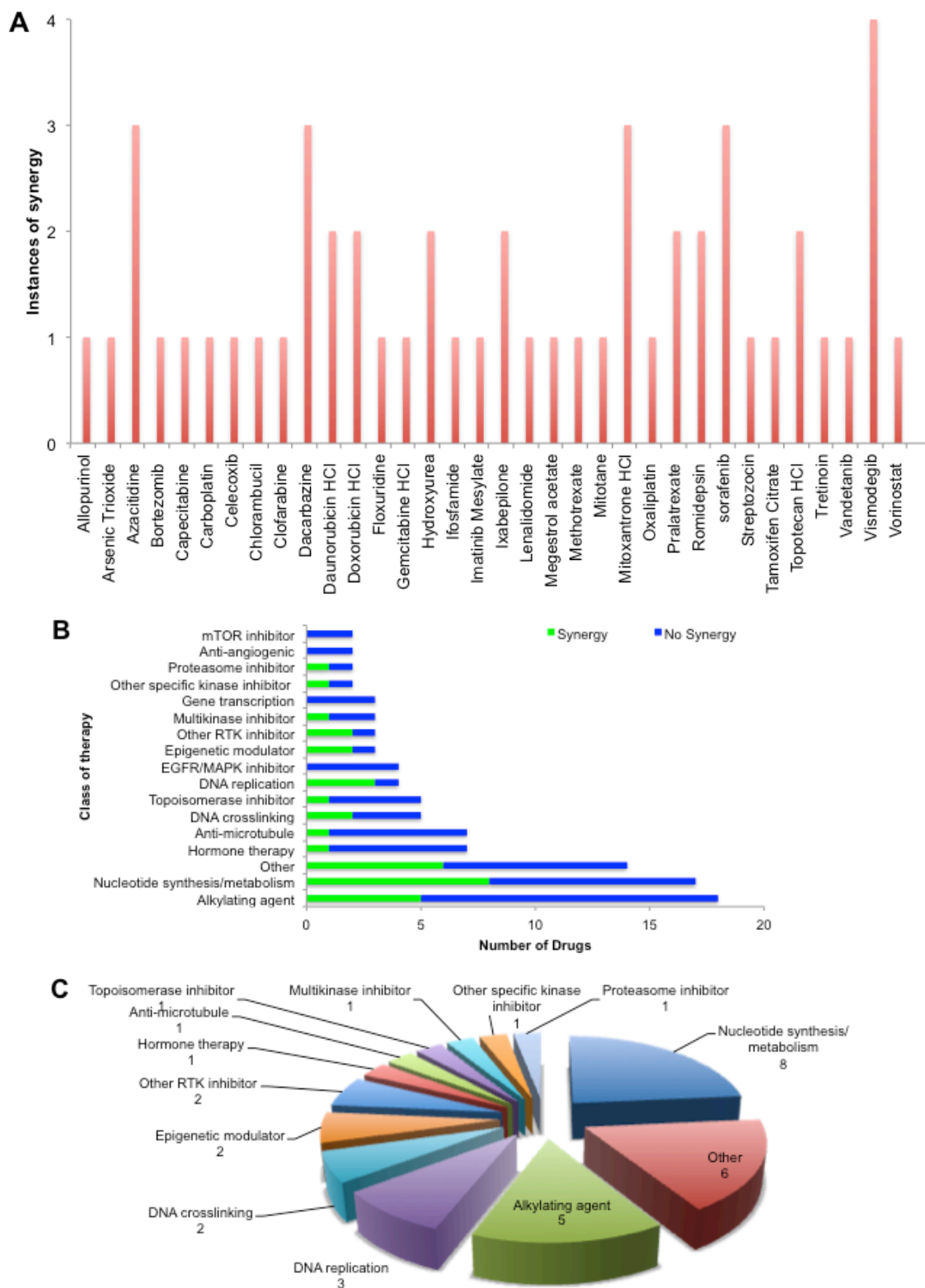
**Figure S5. Distribution of ONC201 synergy instances by drug.** (A) Number of data points for each drug in the small molecule synergy screen that exhibited synergy. Analysis of classes of agents that

synergize with ONC201. Data represented as (B) number of drugs within its class of tested agents or (C) as a fraction of the total hits.
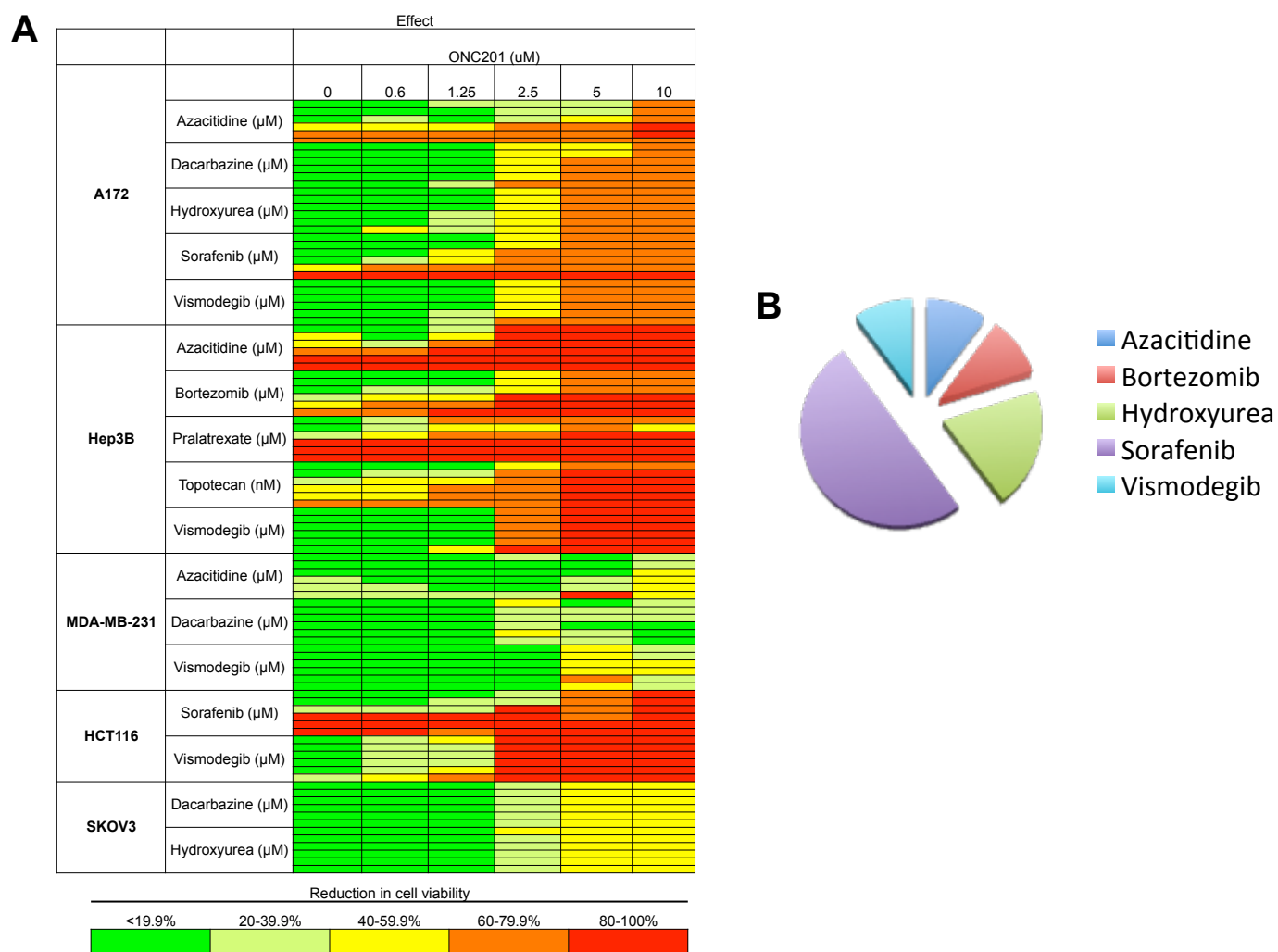


**Figure S6. Validation of ONC201 combinatorial activity with approved anti-cancer small molecules.** (A) Multi-dose evaluation of top synergy hits in a panel of cancer cell lines (72 hours, n=2). (B) Distribution of data points that meet the prioritization criteria.
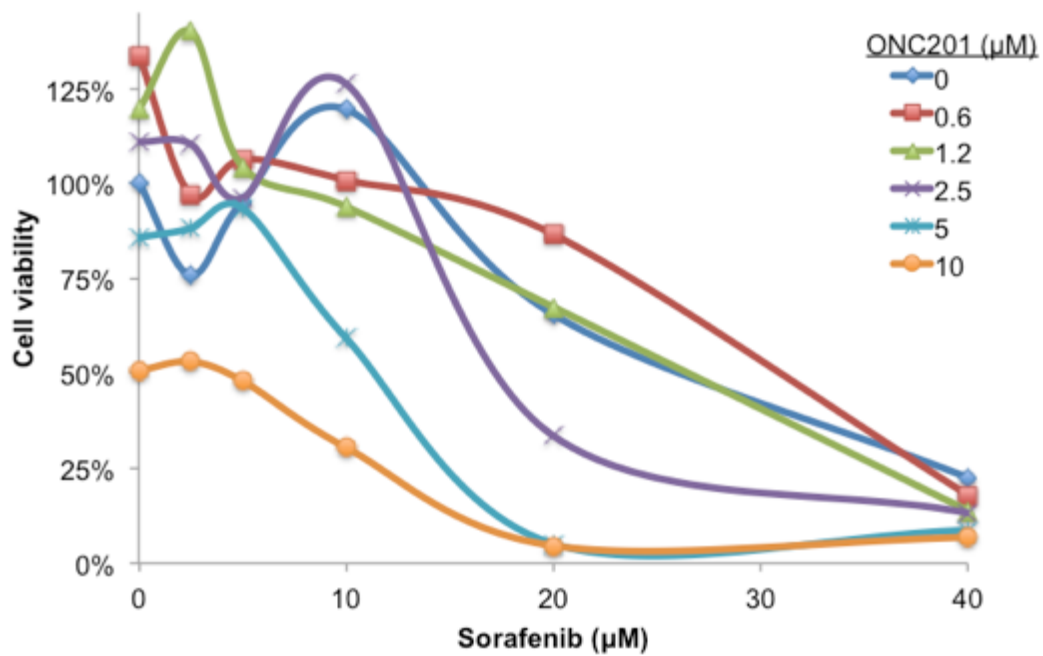
**Figure S7. ONC201 synergizes with sorafenib in HepG2 cells.** Cell viability in HepG2 cells following treatment with ONC201 and/or sorafenib at indicated concentrations (72 hours, n=2).
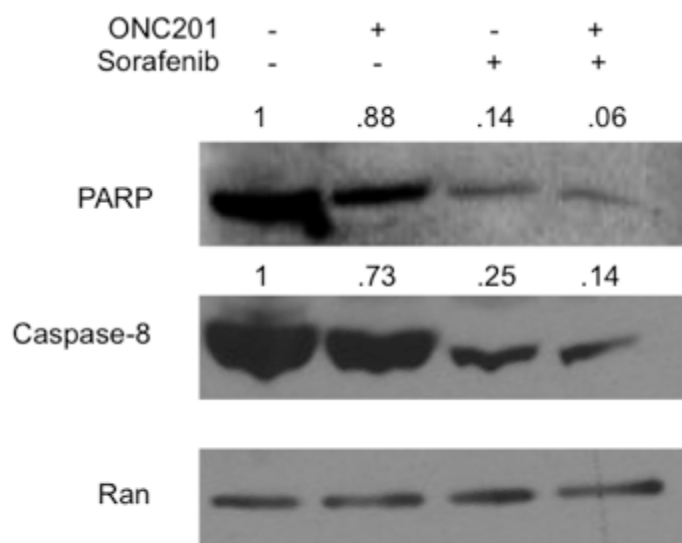
|         | ONC201 | -  | +    | -    | +    |
|         | Sorafenib | - | -   | +    | +    |
|         |        | 1  | .88  | .14  | .06  |
| PARP    |        |    |      |      |      |
|         |        | 1  | .73  | .25  | .14  |
| Caspase-8 |      |    |      |      |      |
| Ran     |        |    |      |      |      |

**Figure S8. ONC201 and sorafenib cooperatively induce apoptosis in HepG2 cells.** Western blot analysis of HepG2 cells treated with ONC201 (5 µM), sorafenib (40 µM), or the combination (72 hours).
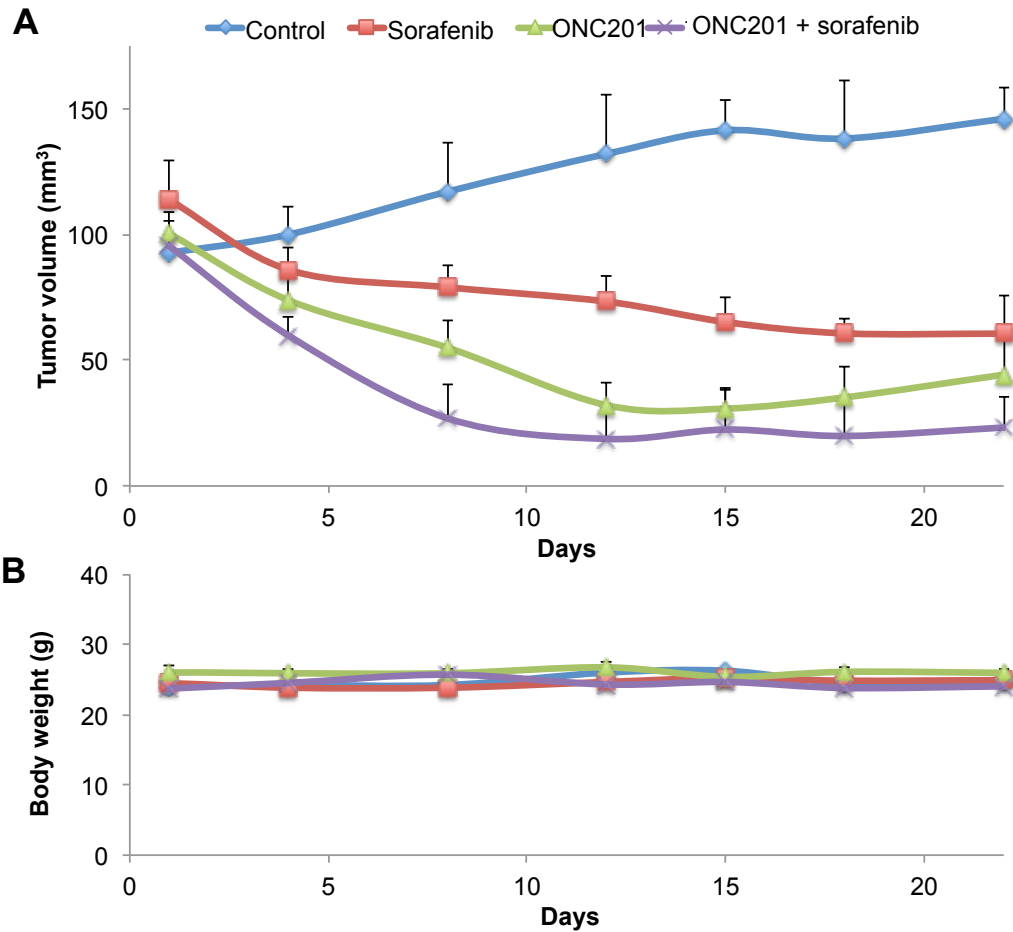
**Figure S9. ONC201 and sorafenib combination therapy is well tolerated and effective in vivo.**

(A) Tumor volume of HepG2 xenografts and (B) body weight in athymic nude mice. Treatments were ONC201 (25 mg/kg PO on day 0, 7, 14) and sorafenib (40 mg/kg PO on days 0-14) ($n{\geq}8$).
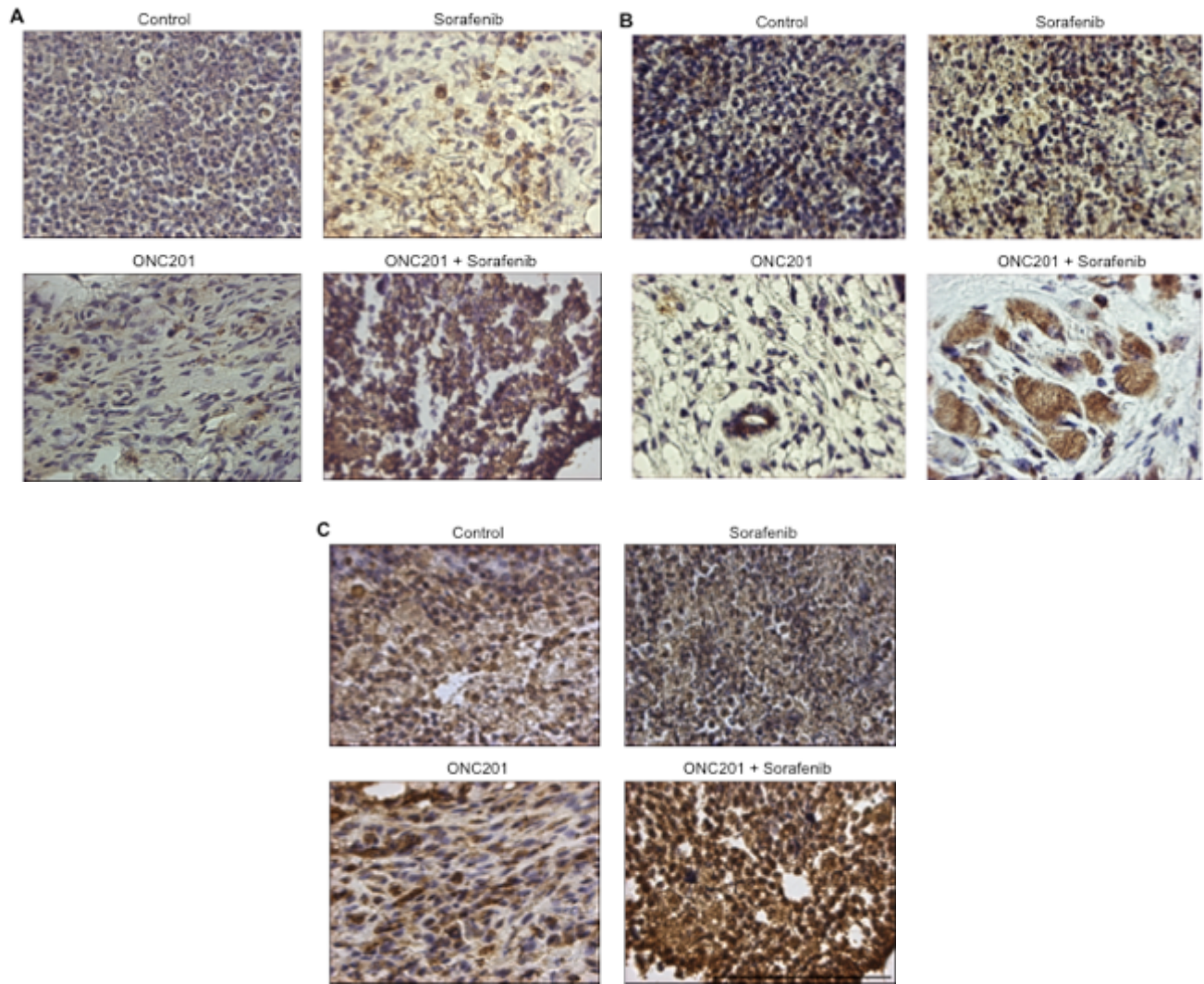
**Figure S10. ONC201 and sorafenib cooperatively induce apoptosis, TRAIL, and DR5 in vivo.**
(A) TUNEL, (B) TRAIL, and (C) DR5 IHC analysis of HepG2 tumor xenografts harvested 3 days following treatment with initiation with ONC201, sorafenib, or the combination as described in Figure 4D-E. The scale bar in panel C represent 100 μm and applies to all images in the figure.
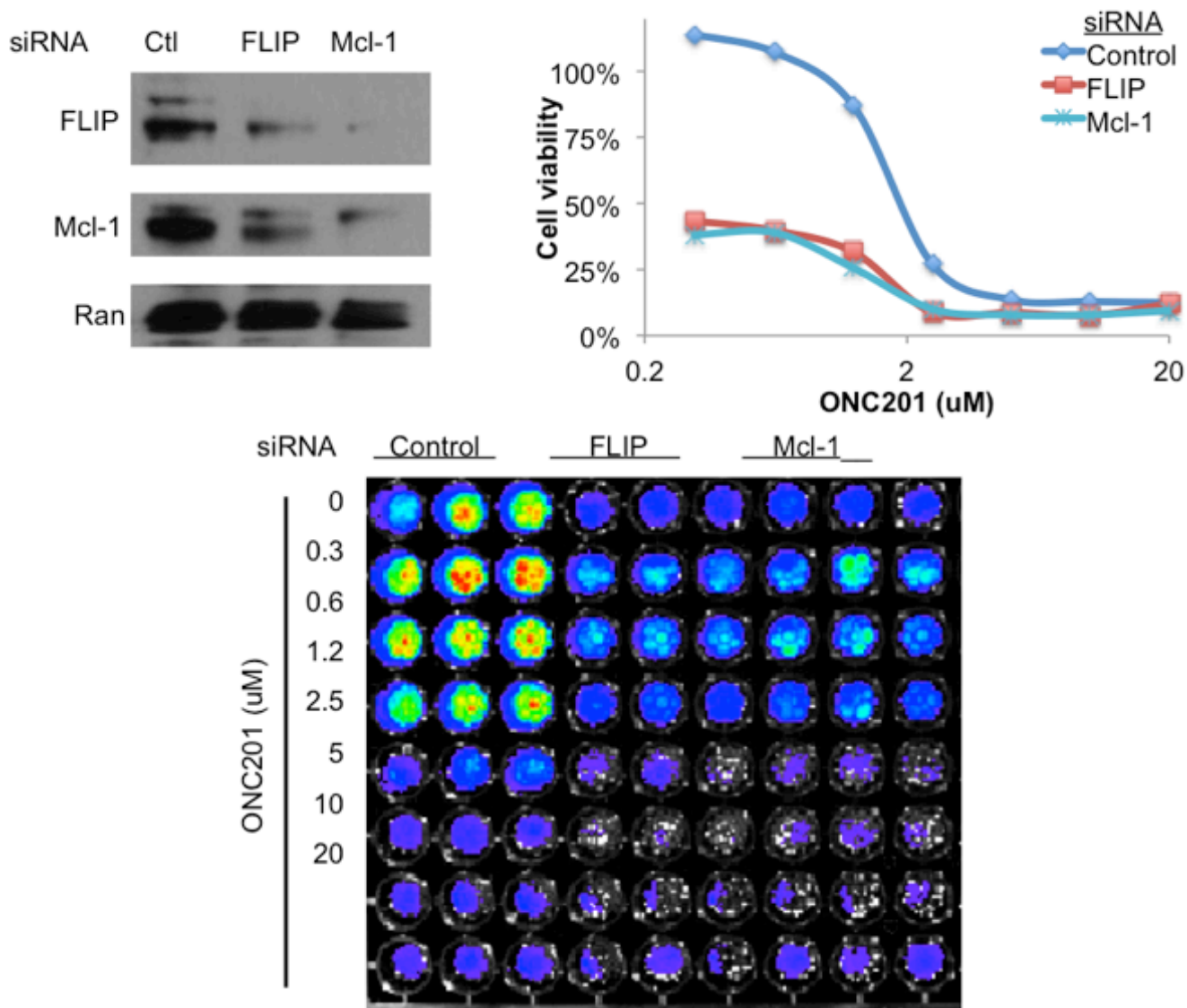
**Figure S11. FLIP, Mcl-1, and KSR-1 knockdown enhances ONC201 response in HepG2 cells.** Effect of FLIP or Mcl-1 siRNA-mediated knockdown of sensitivity to ONC201 in HepG2 cells (72 hours, n=3).
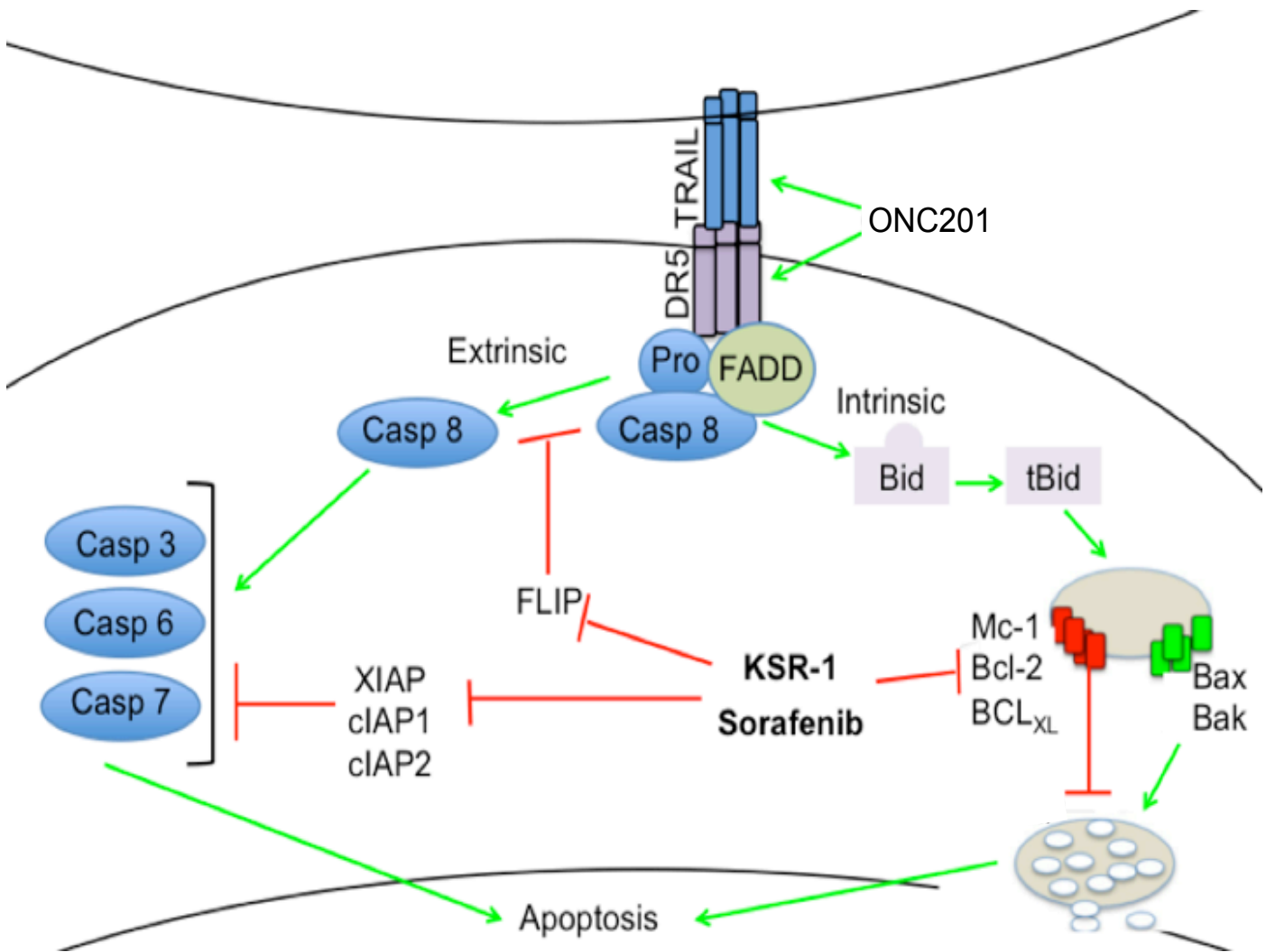
**Figure S12. Model of ONC201-induced TRAIL signaling and sensitivity factors.** ONC201 causes dual induction of TRAIL and DR5 in cancer cells, leading to a receptor-ligand interaction that results in homotrimerization of death receptors. This clusters death receptor intracellular death domains, which induces the formation of the death-inducing signaling complex (DISC) that is comprised of pro-caspase-8 and Fas-associated death domain (FADD). DISC formation causes activation of caspase-8 to trigger cell death via the extrinsic (left) or intrinsic (right) cell death pathways. The effector caspases are negatively regulated by a family of proteins called inhibitors of apoptosis (IAPs) that include survivin, XIAP, cIAP1, and cIAP2. To activate effector caspases the intrinsic cell death pathway involves permeabilization of mitochondria, which is regulated by the Bcl-2 family of proteins. The Bcl-2 family includes pro-apoptotic proteins (Bax, Bak) and anti-apoptotic proteins (Mcl-1, Bcl-2,

Bcl$_{XL}$) that directly interact with each other to form outer mitochondrial membrane pores. KSR1 appears to be a positive regulator of some IAPs, including survivin, cIAP1, and cIAP2