Supporting Information

Frew *et al.* 10.1073/pnas.0801868105

SI Text

Mice. BALB/c mice at 6–8 weeks of age were purchased from The Walter and Eliza Hall Institute of Medical Research. BALB/c SCID mice at 6–8 weeks of age were purchased from Animal Resources Centre. All mice were maintained under specific pathogen-free conditions and used in accordance with the institutional guidelines of the Peter MacCallum Cancer Centre. Animal care was provided in accordance with the procedures outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Reagents. Agonistic mAb to mouse DR5 (MD5-1) and control hamster mAb (UC8-1B9) were prepared as described (1). For tumor therapy, 50 μg mAbs were administered i.p. 4 times every 4 d to mice. Vorinostat (Merck) was diluted in a 50:50 Polyethylene glycol 400:MilliQ water mix and administered i.p. daily at 100 mg/kg. The dose and schedule of vorinostat used was based on our published studies by using C57BL/6 mice (2).

Tumor Cell Lines. 4T1.2 mammary carcinoma, Renca renal adenocarcinoma, A20 B-cell lymphoma, and MC38 colon carcinoma cells were prepared as described (3, 4). All tumor lines were cultured in RPMI 1640, supplemented with 10% FCS, penicillin/streptomycin, and L-glutamine. 4T1.2/Bcl-2, 4T1.2/CrmA, and 4T1.2/c-FLIP cells were engineered by retroviral transduction. Retrovirus-containing supernatant was produced by transfecting 293T packaging cells with murine stem cell virus (MSCV)-IRES-GFP/Bcl-2, MSCV-IRES-GFP/CrmA, or MSCV-IRES-GFP/c-FLIPL combined with an amphotrophic helper plasmid by using standard calcium phosphate transfection methods. Viral supernatant was used to transduce 4T1.2 cells. Seventy two hours after transduction, GFP-positive cells were isolated by flow cytometry-mediated cell sorting and cultured.

DR5 and TRAIL Expression. Cell surface DR5 and TRAIL levels were assessed by using flow cytometry. For surface staining, 1×10^6 cells were stained with TRAIL-Bio (Clone N2B2, eBiosciences catalog no. 12-5951), DR5-Bio (MD5-1, eBiosciences catalog no. 13-5883), or control isotype antibody (rat IgG2a, eBiosciences catalog no. 14-4321) in the presence of 2.4G2 (Fc receptor blocking antibody), followed by allophycocyanin-conjugated streptavidin (eBiosciences catalog no. 17-4317). Cells were incubated on ice, washed, and resuspended in FACS buffer (0.5% BSA in PBS with 0.04% sodium azide) containing fluorogold. Nonviable cells were excluded via fluorogold positivity.

Preparation of Whole-Cell Extracts. Cell pellets were frozen at −80°C, then resuspended in lysis buffer (50 mM Tris·Cl pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, and 20% vol/vol glycerol), supplemented with protease inhibitors [aprotinin (1 μg/ml), PMSF (2 mM), and pepstatin/leustatin (1 μg/ml)], and then incubated on ice for 5 min. Lysates were then sonicated 3 times for 10 sec each and cleared by centrifugation (18,000 $\times$ *g* for 15 min at 4°C). Quantification of protein levels was determined by using the BCA quantification assay (Pierce catalog no. 23223, catalog no. 23224).

Western Blotting. Proteins were separated by using SDS polyacrylamide gel (15%) electrophoresis and electroblotted on PVDF membranes (Millipore). For analysis of Bcl-2, CrmA, and c-FLIP overexpression, membranes were incubated with anti-mouse Bcl-2 antibody, (BD Pharmingen, catalog no. 554218; 1:1,000 in 5% skim milk PBS/0.1% Tween-20), anti-CrmA antibody (BD Pharmingen, catalog no. 556427; 1:500), or anti-c-FLIP mAb (Clone Dave-2, Alexis Biochemicals, 804-127-C100). Pro- and cleaved forms of caspase-3 and caspase-8 were detected by using anti-mouse caspase-3/CPP32 (Clone 46, BD Transduction Laboratories, catalog no. 611048) and anti-mouse caspase-8 (Clone 1G12, Alexis Biochemicals ALX-804-447) monoclonal antibodies. Membranes were subsequently incubated with HRP-conjugated secondary antibodies (DAKO) with immunoreactive bands visualized by using ECL (Amersham). Equivalent protein loading was confirmed by using anti-mouse actin mAb (Clone AC-74, Sigma).

1. Takeda K, *et al.* (2002) Critical role for tumor necrosis factor-related apoptosis-inducing ligand in immune surveillance against tumor development. *J Exp Med* 195:161–169.
2. Lindemann RK, *et al.* (2007) Analysis of the apoptotic and therapeutic activities of histone deacetylase inhibitors by using a mouse model of B cell lymphoma. *Proc Natl Acad Sci USA* 104:8071–8076.
3. Takeda K, *et al.* (2001) Involvement of tumor necrosis factor-related apoptosis-inducing ligand in NK cell-mediated and IFN-gamma-dependent suppression of subcutaneous tumor growth. *Cell Immunol* 214:194–200.
4. Seki N, *et al.* (2003) Tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis is an important endogenous mechanism for resistance to liver metastases in murine renal cancer. *Cancer Res* 63:207–213.

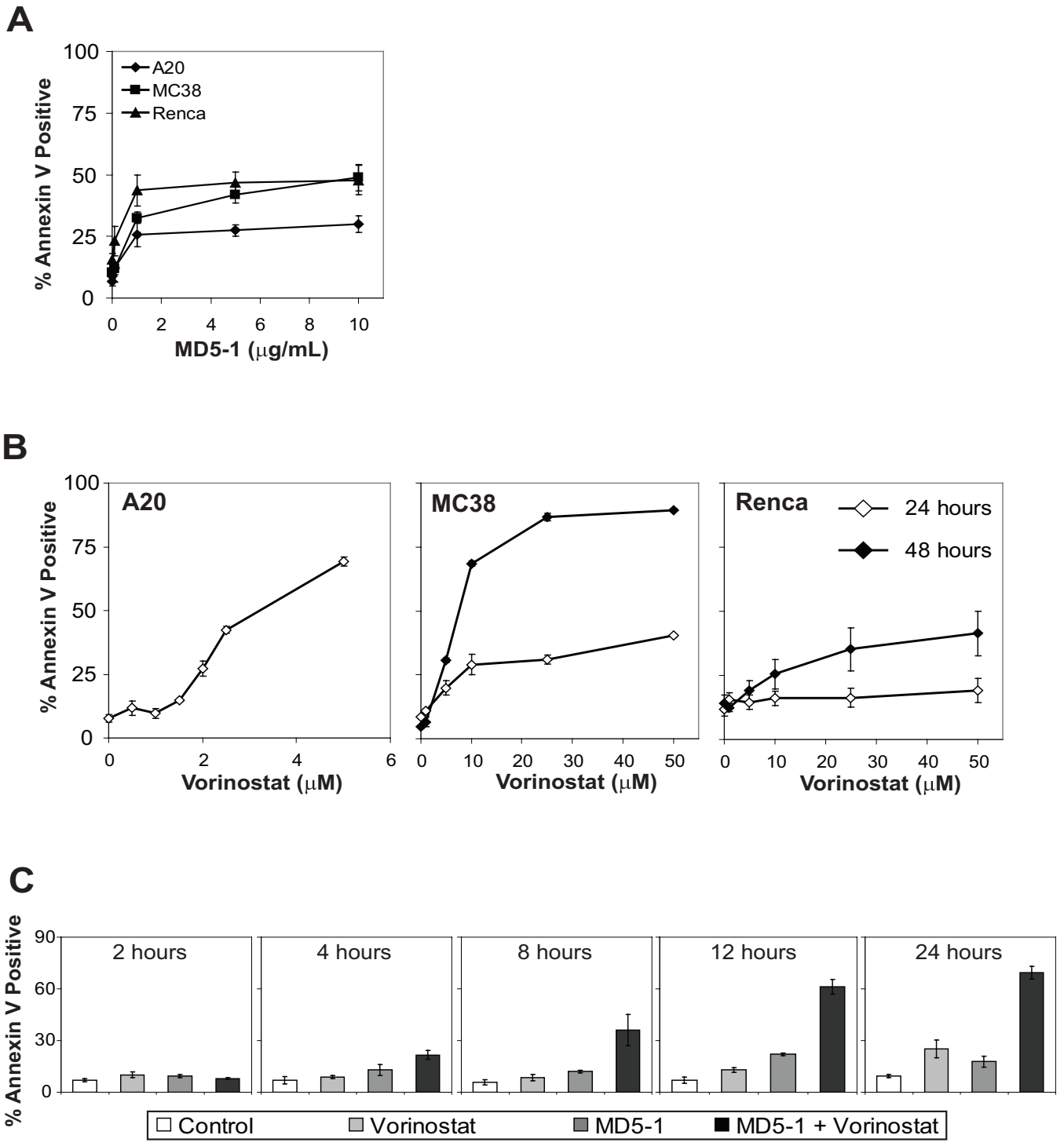

Fig. S1. (*A*) A20 lymphoma, MC38 colon adenocarcinoma, and Renca renal carcinoma were incubated with the increasing amounts of MD5-1 for 24 h. (*B*) A20, MC38, and Renca tumor lines were incubated with increasing concentrations of vorinostat for 24 h (open diamonds) and 48 h (closed diamonds). (*C*) 4T1.2 cells were treated with vorinostat (5 μM) and MD5-1 (1 μg/ml), and apoptosis was assessed at 2, 4, 8, 12, and 24 h by annexin V staining and flow cytometry. Mean ± SEM of at least three independent experiments is shown.

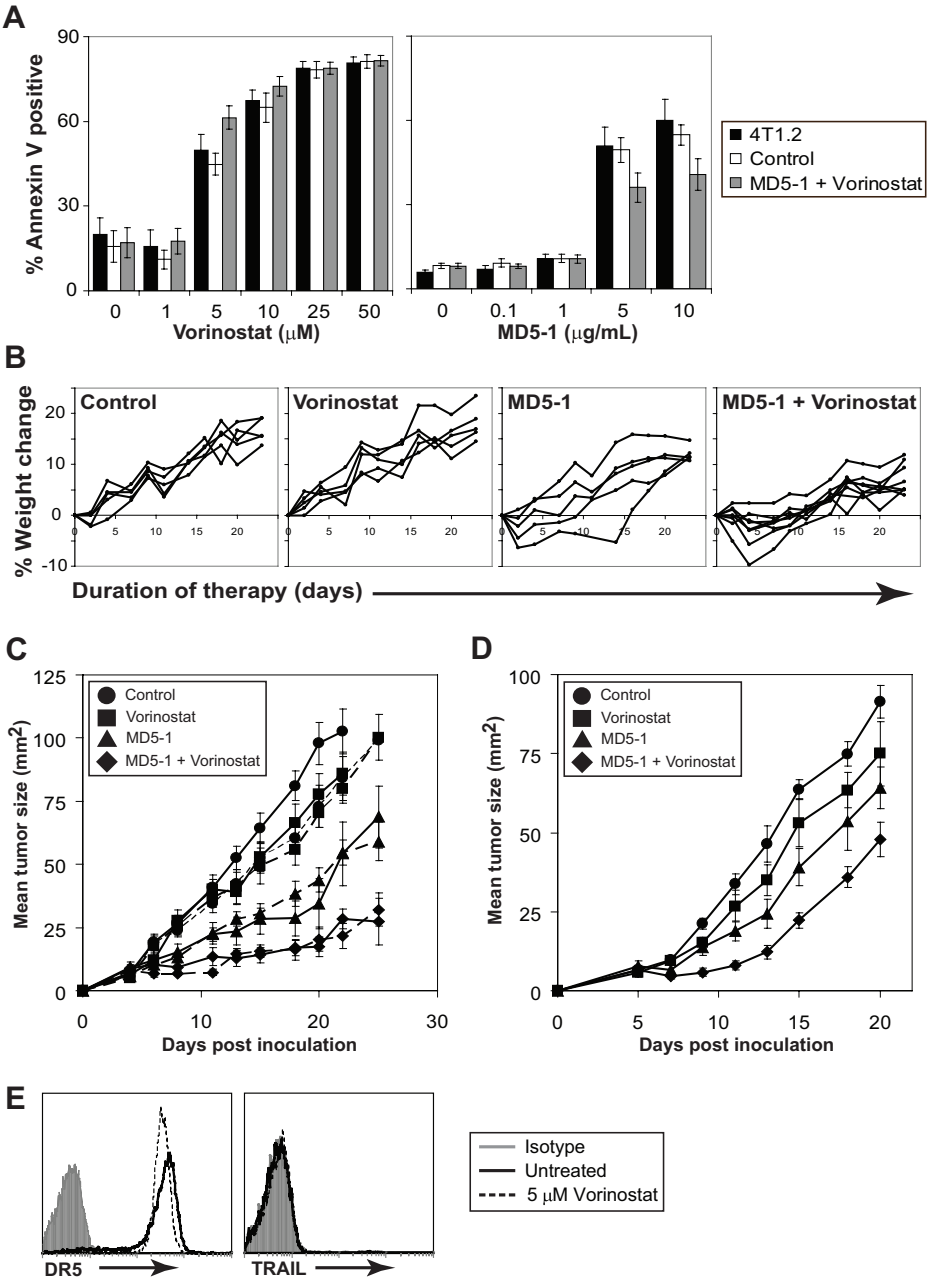

Fig. S2. (*A*) Tumor lines created from outgrown post-combination therapy tumors were assessed for *in vitro* sensitivity to MD5-1 (*Right*) and vorinostat (*Left*). Two tumor lines from each treatment group were tested. Data shown is the mean ± SEM from three independent experiments. (*B*) BALB/c mice with established s.c. 4T1.2 tumors (>9 mm^2, day 6) were treated with control mAb, vorinostat, MD5-1, or MD5-1 and vorinostat. MAbs (50 μg) were given i.p. (4 doses in total, given every 4 d), and vorinostat was injected i.p. daily at 100 mg/kg. Body weights of BALB/c mice were assessed over the course of therapy. Percentage weight changes for individual mice from a representative experiment are shown for control, vorinostat, MD5-1, and vorinostat with MD5-1. Serum aspartate aminotransferase, alanine aminotransferase, and creatinine levels did not increase in any of the treatment groups over the course of therapy (data not shown). (*C*) BALB/c (solid lines) and SCID (dashed lines) mice with established 4T1.2 tumors (>9 mm^2, day 6) were treated with control antibody, vorinostat, MD5-1, and a combination of vorinostat and MD5-1 as in *B* above. Tumor growth was assessed every 2–3 days; data shown is the mean of at least six mice per group ± SEM. (*D*) BALB/c mice with established s.c. Renca tumors (>9 mm^2, day 5) were treated with control mAb (n = 6), vorinostat (n = 6), MD5-1 (n = 6), or MD5-1 and vorinostat (n = 8). MAbs (50 μg) were given i.p. (4 doses in total, given every 4 d), and vorinostat was injected i.p. daily at 100 mg/kg. Tumor growth was assessed every 2–3 d with mean tumor size ± SEM per group shown. Data shown is representative of three independent experiments. (*E*) 4T1.2 cells were treated with vehicle or vorinostat (5 μM) for 16 h, and expression of cell surface TRAIL and DR5 were assessed by using flow cytometry.

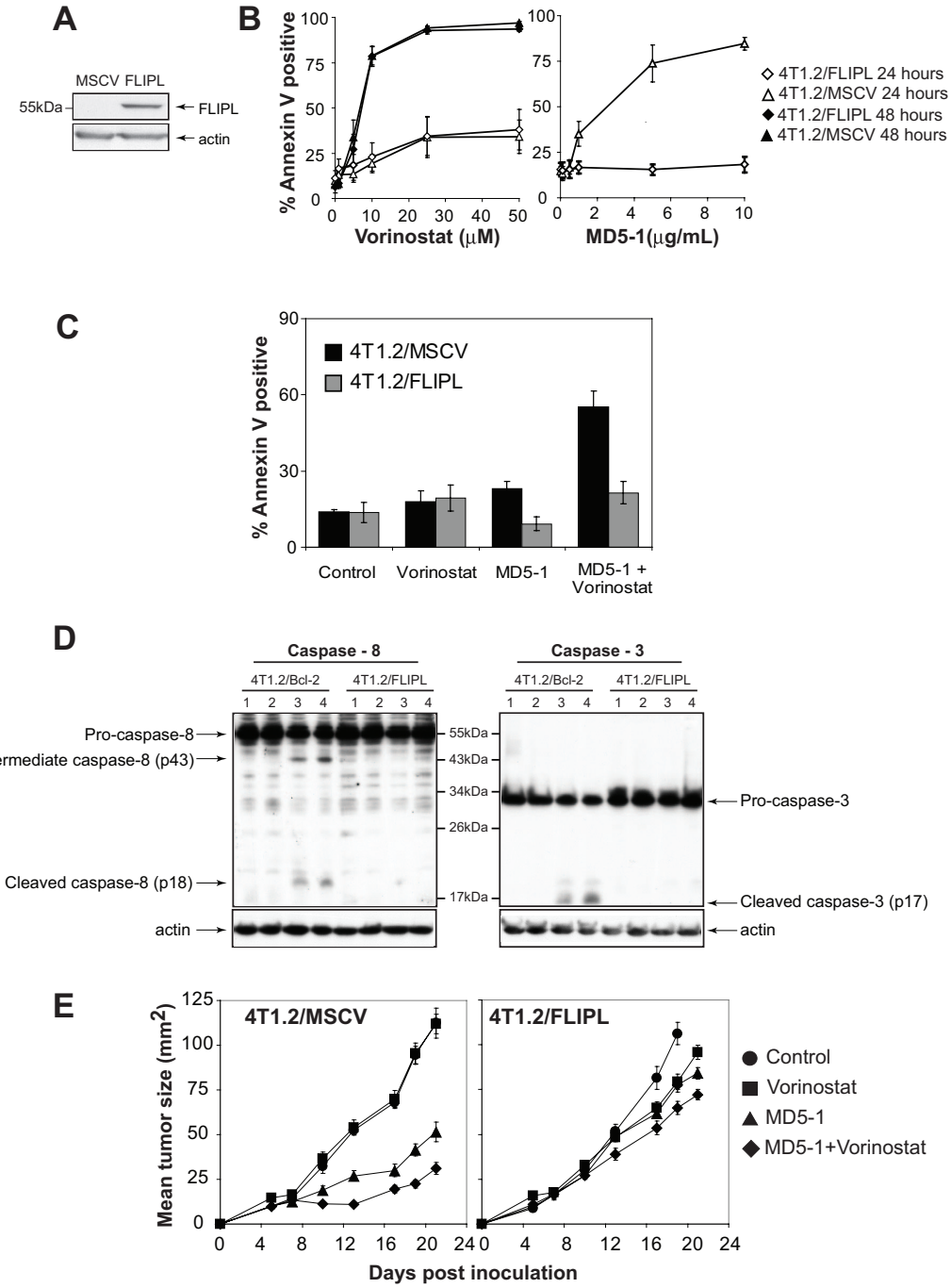

Fig. S3. (*A*) 4T1.2 cells transduced with MSCV or MSCV/c-FLIP were assessed by Western blot analysis for expression of exogenous protein by using an anti-mFLIP antibody. Equivalent protein loading was confirmed by reprobing blots with anti-actin mAb. (*B*) 4T1.2/MSCV and 4T1.2/c-FLIP mammary carcinomas were incubated with increasing concentrations of vorinostat (*Left*) and MD5-1 (*Right*) for 24 h (open diamonds and triangles) and 48 h (closed diamonds and triangles). Apoptosis was assessed by annexin V staining and flow cytometry. Mean ± SEM of three independent experiments is shown. (*C*) 4T1.2/MSCV and 4T1.2/c-FLIP cells were cultured for 24 h with control antibody (1 μg/ml), vorinostat (5 μM), MD5-1 (1 μg/ml), or a combination of vorinostat and MD5-1 (5 μM and 1 μg/ml, respectively). Apoptotic cells were stained with annexin V/PI and assessed via flow cytometry. The mean ± SEM of three independent experiments is shown. (*D*) 4T1.2/Bcl-2 and 4T1.2/c-FLIP cells were treated *in vitro* with either control (1), 5 μM vorinostat (2), 1 μg/ml plate bound MD5-1 (3), or 5 μM vorinostat with 1 μg/ml MD5-1 (4) for 4 h. Cell lysates were prepared and caspase-8 and caspase-3 proteins were visualized by using standard Western blotting techniques. Representative blots from two independent experiments are shown. (*E*) SCID mice with established 4T1.2/MSCV or 4T1.2/c-FLIP tumors (>9 mm^2, day 5) were treated with control antibody (50 μg × 4; $n = 6$), vorinostat (100 mg/kg/d; $n = 6$), MD5-1 (50 μg × 4; $n = 6$) or MD5-1 and vorinostat (50 μg × 4, 100 mg/kg/d, respectively; $n = 8$). Representative data from two independent experiments are shown.