RIP1-HAT1-SirT complex identification and targeting in treatment and prevention of cancer

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Abstract

Purpose: Alteration in cell death is a hallmark of cancer. A functional role regulating survival, apoptosis and necroptosis has been attributed to RIP1/3 complexes.

Experimental design: We have investigated the role of RIP1 and the effects of MC2494 in cell death induction, using different methods as flow cytometry, transcriptome analysis, immunoprecipitation, enzymatic assays, transfections, mutagenesis and *in vivo* studies with different mice models.

Results: Here, we show that RIP1 is highly expressed in cancer and we define a novel RIP1/3-SIRT1/2-HAT1/4 complex. Mass Spectrometry identified 5 acetylations in the kinase and death domain of RIP1. The novel characterised pan-SirT inhibitor, MC2494, increases RIP1 acetylation at 2 additional sites in the death domain. Mutagenesis of the acetylated lysine decreases RIP1-dependent cell death suggesting a role for acetylation of the RIP1 complex in cell death modulation. Accordingly, MC2494 displays tumour-selective potential *in vitro*, in leukemic blasts *ex vivo*, and *in vivo* in both xenograft and allograft cancer models. Mechanistically, MC2494 induces *bona fide* tumour-restricted acetylated RIP1/caspase-8-mediated apoptosis. Excitingly, MC2494 displays tumour-preventive activity by blocking DMBA-induced mammary gland hyper-proliferation *in vivo*.

Conclusions: These preventive features might prove useful in patients who may benefit from a recurrence-preventive approach with low toxicity during follow-up phases and in cases of established cancer predisposition. Thus, targeting the newly identified RIP1 complex may represent an attractive novel paradigm in cancer treatment and prevention.

Translational Relevance

It is becoming increasingly clear that cancer is not only a genetic but also an epigenetic disease. Here, we identified a novel RIP1-SirT/HAT1 complex controlling survival and death via regulation of RIP1 acetylation. Notwithstanding the increasing interest for sirtuins modulation in tumorigenesis, very little known is on their involvement in programmed cell death programs. A major goal of epi-drug development is to increase the therapeutic index and limit development of resistance. One attractive option is to combine anticancer effects with drugs able to prevent neoplasia. Here, we have developed and report on a novel pan-SirT inhibitor that alters HAT1/SirT equilibrium in the RIP1 complex, showing *bona fide* anticancer-selective and cancer-preventive activities *in vitro*, *ex vivo* and *in vivo*. Our work expands the current views in the drug discovery and might prove useful in patients who may benefit from a recurrence-preventive approach with low toxicity during follow-up phases and in cases of established cancer predisposition.

Introduction

Cell death is a normal process responsible of tissue homeostasis. Different pathways of cell death have been described (1) and recently classified (2,3). Both the classical apoptosis, autophagy and necroptosis (a new form of programmed cell death) (4) have been causally connected to cancer. The RIP1 kinase has been reported displaying a functional role in either regulation of survival or apoptosis and necroptosis (5) and is a key regulator of many signalling pathways such as inflammation (6), oxidative stress, plasma membrane permeabilization and cytosolic ATP reduction (7). RIP1, initially identified as a Fas-interacting protein (8,9), is also named 'a death domain kinase', having a 112 amino acid death domain (DD) at its C-terminus. RIP1 is the founding member of the RIP family (10). RIP1 kinase activity is responsible for RIP3 phosphorylation (11), and subsequently, for MLKL phosphorylation and trimerization, which is necessary to activate the necroptotic death pathway (12,13). The fact that other RIP family members do not compensate for RIP1-deficiency suggests a unique role for RIP1(10,14). The central deregulation of cell death in tumourigenesis has become clear, yet, very little is known of RIP1 and cancer. RIP1 and RIP3, as well as necroptosis, are deregulated in many types of cancers thus presenting a potential therapeutic targets in treatment of cancers resistant to chemotherapeutic agents or to apoptosis inducers (15,16). Here, we show that RIP1 is highly expressed in cancer and we define a novel RIP1/3-SIRT1/2-HAT1/4 complex in which RIP1 is subject to regulation by acetylation. Excitingly, mutation of the acetylated lysine decreases RIP1-dependent cell death, suggesting a role for acetylation of the RIP1 complex in cell death deregulation and function. Increasing RIP1 acetylation with a novel multi-acting SirT inhibitor, MC2494, displays tumor-selective therapeutic potential *in vitro*, *ex vivo*, and *in vivo* inducing tumour-restricted apoptosis. Extraordinarily, MC2494 shows tumour-preventive activity *in vivo*. Thus, targeting the newly identified RIP1 complex and its acetylation may represent a feasible and attractive novel paradigm for therapeutic purposes in cancer treatment and prevention.

Materials and Methods

Cell lines, primary cells and ligands. U937, NB4, HL-60, K562, U266, JURKAT, MCF7, MDA-MB231, LnCap, NIH3T3 and HCT116 cells, were purchased from DSMZ. MCF10A was purchased from ATCC. HEK293FT and HACAT cells, were ordered from Thermo Fisher Scientific. EPN, hMSC and Primary normal amniocytes cells, were obtained from University of Campania Luigi Vanvitelli, IT. All cell lines and primary cells were grown following standard protocols. A more detailed description is reported in Supplementary Material and Methods. Mycoplasma contamination was regularly examined using EZ-PCR Mycoplasma kit (Biological Industries), prior to freezing working stabs. All cell lines were tested and authenticated. Cells were used for experiments within 10-20 passages and then discarded. AGK2 (Sigma), MC2494 (and derivatives) were dissolved in DMSO and used at $5x10^{-5}$ M. MC2494 was synthetized by University Sapienza, Rome IT. For MC2494 synthesis details, see Supplementary notes. EX527 (Alexis) was dissolved in DMSO (Sigma) and used at $5x10^{-6}$ M, unless otherwise specified. Staurosporine (Alexis) was dissolved in DMSO and used at $2x10^{-6}$ M. Suramin (Bio Mol) was used at $5x10^{-5}$ M.

Antibodies. H3K9-14ac, H3K9ac, H3K56ac and RIP1 were from Diagenode and BD. H4ac and p53ac were from Upstate. H3, Acetyl-lysine, H4 H2A.X (phS139), CPS1, MLYCD, SirT1, SirT2, PCAF and KAT1/HAT1 were from Abcam. ATM (phS1981) and ATR were from R&D. Acetyl-tubulin was from Sigma. ERK1/2, IAP, FLIP-L, PARP and RIP3 were from Santa-Cruz. Bax, t-Bid, caspase 3, caspase 8, CYLD, IKKγ, FADD, FAS, BCL2 were from Cell Signaling. HAT4 was from myBiosurce.

Morphological analysis. For U937, MCF7 and MDA-MB-231 cancer cell lines morphological analysis was performed, using bright field light microscopy (20X).

Reagents. Z-VAD, Z-IETD, Z-LEHD (R&D) were used at 50 μM. N-Acetyl cysteine (NAC) and Nec-1 (Sigma) were used at 50 and 100 μM, respectively. 1 μg of RIP1, $RIP^{(K596/599R)}$ were transfected. H_2O_2 and PIETC (Sigma) were used at 1 mM and 10 μM, respectively. C646 (Sigma) was used at 50 μM.

Cell vitality, Cell cycle, differentiation, death and apoptosis. To study cell vitality, experiments were performed in triplicate. Cells were diluted 1:1 in Trypan blue (Sigma) and counted. Cell cycle and differentiation analysis, was performed in triplicate as reported in (17)**.** Apoptosis was measured by caspase 3-7, 8 and 9 (R&D) and quantified by FACS (BD). Apoptosis was measured as pre-G1 DNA fragmentation or by Annexin V detection as in (17). Apoptosis vs necrosis was measured using apoptosis/necrosis kit as suggested by the supplier (Enzo life sciences).

S**irTs, HDACs & PCAF assays.** SirT1, -2 and -3 assays experiments were performed as suggested by the supplier (BioMol). Moreover, for SirT1, additional assays were performed: i) HRTF assay; ii) BioMol assay with a different excitation/emission range; iii) SIRTainty assay (Millipore). SirT3 and 6 assays were performed *in vitro* following the supplier's instructions (ENZO life and Cayman, respectively). To evaluate the action of MC2494 on SirT4 and 5, we IPed CPS1 and MLYCD (reported as substrates for SirT4 and SirT5, respectively) and they used in a radiolabelling assay. Finally, both HDAC and PCAF radioactive assays were performed according to supplier's instructions (Upstate). A detailed description of *in vitro* assays is reported in Supplementary Material and Methods.

Proliferation and migration analysis in real time. Proliferation and migration analyses were evaluated in MDA-MB231 cells and performed according to the supplier instruction (xCELLigence, Roche). A detailed description is in Supplementary Material and Methods.

RNA, RT-PCR and Chromatin immunoprecipitation (ChIP). Total RNA was extracted with Trizol (Invitrogen) and converted into cDNA using VILO (Invitrogen). RNA extraction and RT-PCR are detailed in

the Supplementary Methods. ChIP was carried out as previously reported (18) using H3K9-14ac. More detailes are in Supplementary Material and Methods.

Protein extraction. After PBS wash, cell pellets were suspended in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP40, 10 mM NaF, 1 mM PMSF and protease inhibitor cocktail). The lysis reaction was carried out for 15 min at 4°C. Finally, the samples were centrifuged at 13000 rpm for 30 min at 4°C and protein concentration quantified by Bradford assay (Bio-Rad).

Histone extraction. After stimulation with the indicated compounds cells were collected and washed two times with PBS. Pellets were resuspended in Triton Extraction Buffer (TEB) (PBS containing 0.5% Triton X 100 (v/v), 2 mM PMSF, 0.02% (w/v) NaN3) and the lysis procedure was performed for 10 min at 4°C. The samples were centrifuged at 2000 rpm for 10 min at 4°C and pellets washed in TEB (half volume). Samples were then resuspended in 0.2 N HCl and acid histone extraction was carried out overnight at 4°C. The next day the supernatant was recovered and protein concentration quantified by Bradford assay (Bio-Rad).

Nucleus/Cytosol extraction. Pellet were resuspended in 2-2.5 volumes of NP-40 Buffer (10mM Tris-HCl pH 7.0, 10mM NaCl, 3mM MgCl2, 30mM Sucrose, 0.5 % NP-40) and incubate for 10' on a rotating platform at 4°C. After centrifugation at 1000 rpm for 10 min at 4°, the supernatant were transfer in a new tube (cytosolic fraction). Pellet (nuclei) were suspended in 2 mL of NP-40 Buffer and centrifuged at 3000 rpm for 10 min. The supernatant were removed and the pellet of nuclei was washed in Lysis Buffer (20mM Tris-HCl pH8, 137mM NaCl, 10% glycerol, 1% NP-40, 2mM EDTA) for 10 min in ice. The suspension was sonicated (4x 30"off/ 30"on high power). Spin out debris 14.000 rpm for 5 min at 4°and the nuclear proteins were transfer in a new tube.

Western Blot. 50 μg of proteins were loaded on 10-15% polyacrylamide gels. 5-10 μg of histone extract was loaded on 15% polyacrylamide gel. The nitrocellulose filters were stained with Ponceau red (Sigma) as additional control for equal loading. The antibody used, are listed in Supplementary Material and Methods.

Immunoprecipitation assay. MDA-MB-231 and HEK293FT cells were lysed in NP-40 (0.5%), Tris-HCl pH 8.0 (20 mM), NaCl, (150 mM), PMSF (1 mM), 10% glycerol, EDTA (1 mM) and 1X protease inhibitor mix (Sigma) for 20' on ice. Cell debris was removed and the protein soluble fraction was incubated with antibody overnight at 4°C. The immune complexes were immunoprecipitated with Sepharose-protein A/G Plus (Santa-Cruz) or with GFP -TRAP_A beads (Cromotech) for 2h at 4°C. Proteins were then eluted, resuspended and analyzed by western blot.

CETSA assay. HEK293FT cells were harvested and washed with PBS after treatment with MC2494 (50 μM) and an equal amount of DMSO, as control, for 1 h. The respective samples were

suspended in PBS (1.5 mL), divided into aliquots (100 µl), and heated at different temperatures (25°-37°-44°-53°-57°) for 3 min by Thermo Mixer (Eppendorf, Milan, Italy), followed by cooling for 3 min at 4C. After incubation, lysis buffer (100 µl) was added to the samples and incubated for 15 min. The samples were then centrifuged at 13,000 rpm for 30 min at 4°C, the supernatant was removed and protein concentration was determined using a Bradford assay (Bio-Rad). Of the total protein extract, 20 µg was loaded on 10% SDS-PAGE and western blott analysis was performed. The antibody used were SirT1, SirT2 and SirT3 (Abcam).

Transfections. RIP1-GFP, RIP-GFP$^{(K596/599R)}$ were transfected in HEK293FT cells with Lipofectamine 2000 (Invitrogen). TRAIL promoter mutants were used as previously described (17).

RNA interference

To silence RIP1 and Caspase8 were used specific pre-designed SiRNA for RIP1 (SI00288092) and CASP8 (SI02661946) purchased from Qiagen. U937 cells were transfected with Nucleofector™ Technology and siRNA were used at 1 µM. To silence SIRT1 gene expression, HEK293 cells were transfected with a specific SirT1 siRNA directed against human SirT1 (On-Target plus SMART pool), which was purchased from Dharmacon, and with a specific pre-designed SiRNA for SirT1 purchased from Ambion. A scrambled siRNA was used as a control. Cells were transfected with the indicated siRNAs at 50 nM of concentration, using DharmaFECT1 Transfection reagent (Dharmacon).

Allograft experiments. All animal procedures were conducted according to national and international guidelines. The breast cancer 4T1–Luc model was a gift from Dr. P. Steeg, (NIH, Bethesda, USA). Approval was obtained from the Institutional Animal Care and Ethical Committee at CEINGE and "Federico II" University of Naples (Protocol #29, 01/09/2009), and the Italian Ministry of Health, Dipartimento Sanità Pubblica Veterinaria D.L. 116/92, confirming that all of the experiments conform to the relevant regulatory standards. A detailed description of procedures is in Supplementary Material and Methods.

Cancer prevention & mito-mice. MC2494 was dissolved in DMSO and diluted 1:5 in vehicle (corn oil). 7,12-dimethylbenz(α)anthracene (DMBA, Sigma) was dissolved in acetone (8 mM). 8 female MITO-Luc mice (repTOP™ mitoIRE, Italy) (19) 2 months of age were housed in plastic cages, fed *ad libitum* with a standard diet (4RF21 standard diet, Mucedola, Italy). Room temperature was within 22-25°C and humidity of 50 ± 10%. Animals were divided into two groups and s.c. treated with 50 mg/kg MC2494 or placebo (corn oil) every day at 02.00 PM for 9 days. At day 6, mice were subjected to a single s.c. intra-glandular injection of DMBA (left) or vehicle (right gland); at day 9, after the last *in vivo* imaging acquisition, mice were sacrificed, mammary glands explanted for *ex vivo* imaging and fixed for analysis. A detailed description is in Supplementary Material and Methods. Imaging *in vivo* procedure was as in (20,21) and is detailed in Supplementary Material and Methods.

Xenograft experiments and Pulse generator *in vivo*. All procedures involving animals and their care were conducted in conformity with institutional guidelines, which are in compliance with national (D.L. No. 116, G.U., Suppl. 40, Feb. 18, 1992; Circolare No. 8, G.U., July 1994) and international (EEC Council Directive 86/609, O.J. L 358. 1, Dec 12, 1987; Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996) laws. A detailed description of procedures and histology, immunohistochemistry and TUNEL assay *in vivo* are explained in Supplementary Material and Methods.
High resolution nanoLC−Tandem Mass Spectrometry Mass spectrometry analysis was performed on a Q Exactive Orbitrap mass spectrometer equipped with an EASY-Spray nano-electrospray ion source (Thermo Fisher Scientific, Bremen, Germany) and coupled to a Thermo Scientific Dionex UltiMate 3000RSLC nano system (Thermo Fisher Scientific). A detailed description and data processing are in Supplementary Material and Methods.

IP-Mass spectrometry. For mass spectrometry analysis RIP1 was overexpressed using a GFP-tagged vector and immunoprecipitated with GFP-trap_A beads. The data dependent mass spectra were acquired with the LTQ-Orbitrap mass spectrometer (Thermo Scientific). A detail description of these procedures is in Supplementary Material and Methods.

Results

RIP1 is highly expressed in cancer cells and interacts with HAT1 and SirT1/2

Programmed necrosis or necroptosis is an alternative form of programmed cell death in which the RIP1-RIP3 complex displays a functional role. To explore RIP1 function in cancer, RIP1 protein levels were evaluated in different cancer cells (Fig. 1A), showing a generally high expression of RIP1. Mass spectrometry analysis performed after RIP1 immunoprecipitation (ProteomeXchange, PRIDE database, dataset identifier PXD007198), identified a new RIP1-HAT1-SirT1 complex (Fig. 1B). RIP1-HAT1-SirT1 complex was validated by western blot. Both RIP3 (with a weak signal) and HAT4, known interactors of RIP1 and HAT1 respectively (22-24), were found in the complex (Fig. 1C). The fact that both SirT1 and HAT1 immunoprecipitations were reciprocally detecting RIP1, strongly corroborated and strengthen the existence of the RIP1-HAT1-SirT1 single complex (Supplementary Fig. S1). The observation that both HAT1 and SirT1 (and 2) were detectable within the RIP1-IP, prompted us to investigate whether these acetyltransferases/deacetylases might regulate RIP1 by (de)acetylation. By probing RIP1 immunoprecipitated cells with anti-acetyl-lysine antibody, we observed the presence of an acetylated form of RIP1 (Fig. 1D). Given that RIP1 acetylation has been very debated (25) and never proven in living cells, to investigate the location of RIP1 acetylation sites, high-resolution tandem mass spectrometry was applied. To this end, peptides resulting after digestion with trypsin were separated and analysed by nano-HPLC coupled to an Orbitrap Q-Exactive mass spectrometer. Data-dependent HCD spectra were obtained on the five most intense mass peaks generated in each scan at 17500 resolution. Amino acid sequences of high confidence

peptides obtained by high-resolution tandem mass spectrometry are reported in Supplementary Fig. S2; S3; S4 and Supplementary Table S1. Five acetylated lysine residues were identified (Fig. 1E); of these, K115 was localized within the RIP1 kinase domain, whereas K625, K627, K642, K648 were mapped within the RIP1 death domain, suggesting a potential role for acetylation in RIP1 kinase and cell death-regulating functions. Given that RIP1 is localised in the cytoplasm (26), HAT1 and SirT1 location in the different cellular compartments was studied. Interestingly, while RIP1 confirmed exclusive cytosolic localization, HAT1 and SirT1 were found in both nuclear and cytosolic compartments, with a higher expression in the cytosol (Fig. 1F).

RIP1 acetylation module is enhanced by SirT inhibition at sites K596-K599

To assess RIP1, HAT1 and SirT1/2 relationship, we measured RIP1 acetylation using a newly synthesised pan-SirT inhibitor, MC2494 (Fig. 2A; Supplementary Table S2 and Supplementary note) an AGK-2-related molecule (27). This compound displays inhibitory actions against SirT1, SirT2 and SirT3/4/5/6, revealing unique features when compared to SirT1- and 2-selective inhibitors (27-29) (Fig. 2B). The IC50 values for SirT1 and 2 inhibition were 38.5μM and 58.6μM, respectively (Supplementary Fig. S5A). While MC2494-SirT1 modulation was corroborated on a panel of orthogonal *in vitro* assays (Supplementary Fig. S5B), MC2494 was inactive against both HATs (PCAF) and HDACs (Supplementary Fig. S5C and D), suggesting high pan-SirT specificity. Direct binding between Sirtuins and MC2494, was corroborated by CETSA assay (30) (Supplementary Fig. S5E). Interestingly, while the presence of MC2494 protected SirTs from thermal degradation, SirT1/2/3 mRNA and protein levels were reduced in three different cancer systems by MC2494, differently from normal cells (Fig. 2C and D), assuming features of cancer-selectivity. In agreement with the pan inhibitory action, MC2494-induced levels of p53K382 and tubulin acetylation were highly increased, as were H3 (K9-14ac and K56ac) and H4 acetylations (Supplementary Fig. S5F). Excitingly, a strong increase of RIP1 acetylation was observed (Fig. 2E) upon MC2494 stimulation strengthening a possible role of SirTs in modulating RIP1 (de)acetylation. Mass spectrometry analyses were applied to corroborate this data. Under MC2494 treatment conditions, no qualitative differences were detected in RIP1 acetylation with the exception of the di-acetylated peptide 592-603 ($[M+2H]^+$ at m/z 777.88), containing two additional acetylation sites (K596-K599), only detected following MC2494 treatment (Fig. 2F). Interestingly, this region was also identified as the top score deacetylation site (P-Value: 0.015) by the Predict(S) algorithm of the ASEB web server for lysine acetylation/deacetylation site prediction by selecting the database of 129 known deacetylation sites of SirT1. Finally, MC2494 regulated the newly identified RIP1 interactome, decreasing SirT1 expression in the complex, thus unbalancing SirT1 in favour of HAT1. Also HAT4 and RIP3 were still detectable with a slight increase of RIP3 (Fig. 2G). Interestingly when the HAT inhibitor C646 was used, this drug was able to abrogate RIP1 acetylation, corroborating the involvement of the acetyl transferases in RIP1 acetylation (Supplementary Fig. S6A). This evidence is also supported by the presence of PCAF in the complex. (Supplementary Fig. S6B). Moreover, a siRNA approach was performed to corroborate and strengthen this data. Upon SirT1 silencing, RIP1 acetylation increased, clarifying the SirT1-

mediated action mechanism of MC2494. (Fig. 2H). These results indicate that RIP1 acetylation at K596-K599 is governed by SirT inhibition using this novel pan-SirT inhibitor. To gain more insights into the role of MC2494 in RIP1 acetylation, immunoprecipitation assay was carried out in cells stimulated with one inactive derivative of MC2494, MC2582 (Supplementary Table S2). As expected, MC2582 has no effect on RIP1 acetylation. (Supplementary Fig. S7).

Pan SirT inhibition induces caspase 8 dependent cancer-selective cell death

To define the potential biomedical effects of pan-SirT inhibition and RIP1 complex acetylation the action of MC2494 was investigated on both leukaemia and breast (BC) cancer cells. In contrast to EX527 (SirT1i) and AGK-2 (SirT2i), MC2494 induced strong proliferation arrest (Fig. 3A and Supplementary Fig S8-S9C) and importantly, did not affect cell cycle or differentiation (Supplementary Fig. S9A and B). When quantified in real time, MC2494 reduced cell proliferation and migration rate measured by cell index and slope at early time points (Fig. 3B). These data strongly imply wide-ranging anti-proliferative effects. Given the hypothesized tumour-selective action, we evaluated the cytotoxic effect of MC2494 by comparing its effect on a broad panel of cancer cells and normal (or immortalized, non-cancer) cells. Notably, MC2494 induced cell death in cancer without displaying significant cytotoxicity in normal cells (Fig. 3C). These findings strongly suggest that cell death induction by MC2494 is tumour-specific. Caspase-3/7, -8, -9 activation (Fig. 3D) and dissipation of mitochondrial membrane potential (MMD) (Fig. 3E) were induced by MC2494 in cancer cells as was DNA damage measured as increased expression of ATM, ATR and γH2AX (Fig. 3F). Both players of the extrinsic and intrinsic apoptotic pathways were modulated (Supplementary Fig. S9D and E). To gain mechanistic insights the caspase-8 Z-IETD, caspase-9 Z-LEHD and pan-caspase Z-VAD inhibitors were tested for their ability to block MC2494 action. Only Z-IETD and ZVAD completely blocked MC2494-induced programmed cell death (PCD), whereas cell death was unaltered in presence of Z-LEHD suggesting that caspase-9 activation is dispensable (Fig. 3G). Interestingly, an increment of ROS production occurred upon MC2494 treatment indicating a possible link between caspase activation, ROS production and RIP1 expression (Fig. 3H) (31).Therefore, we tested the effect of N-acetyl-cysteine (NAC) (32), a known ROS scavenger. Remarkably, in U937 cells, NAC abolished MC2494-induced PCD demonstrating a causal relevance for ROS production (Fig. 3H).

RIP1 and its acetylation causally activate cancer-selective cell death pathways

To gain insights into PCD mechanism(s), we analysed the transcriptome of U937 cells treated with MC2494 in comparison with cells treated with the lead SirT1 inhibitor EX527 (Supplementary Fig. S10A). The 1245 MC2494 specifically modulated probes were characterized for their relative abundance of Gene Ontology Biological Processes using DAVID (Supplementary Fig. S10B -11). MC2494 deregulated 116 annotated genes related to PCD (fdr=0,0029) suggesting that this modulation might account for its apoptotic action (or part of it). Strikingly, among the PCD-related targets, DR5 was selectively up-regulated by MC2494 (Supplementary Fig. S10C, $p < 0,001$), suggesting that its modulation may account for a potential cancer-

selective PCD. Corroborating the transcriptome, PCR confirmed DR5 induction and supported TRAIL mRNA up-regulation by MC2494 already after 24h of induction. Interestingly, H3ac ChIP followed by qPCR revealed strong acetylation at TRAIL and DR5 promoters after the treatment (Fig. 4A). To elucidate the acetylation modulation at TRAIL and DR5 promoters, SirT1 ChIP followed by qPCR was performed. Interestingly, SirT1 occupancy was found at those promoters and was decreased after MC2494 treatment, suggesting an active role for SirT1 at TRAIL and DR5 promoters (Fig. 4B). We investigated TRAILp by a deletion mapping approach (Fig. 4C). Whereas HDACi selectively target the proximal GC-box and complexes binding to it (17), MC2494-dependent TRAIL activation was strongly reduced by mutations of promoter-distal areas containing the distal GC-box, AP-1 and ISRE. Conversely, mutation of promoter-proximal GC-box led to over 2.5-fold increase, highlighting a potential repressive role of MC2494 on chromatin at this region. Thus, MC2494-driven TRAIL activation causally activates the tumour-selective TRAIL/DR5 pathway. To extend the hypothesis that both caspase-8 and RIP1 causally act during MC2494 PCD, we performed loss-of-function experiments in U937 cells. Upon silencing of either caspase-8 or RIP1, MC2494-mediated PCD was abolished, suggesting a potentially crucial link between caspase-8 activation and RIP1 function (33) in promoting PCD by MC2494 (Fig. 4C). In support, a similar PCD suppression was observed when pharmacologically blocked RIP1 function after the co-administered with its inhibitor Necrostatine-1 (Nec-1) (34) (Fig. 4D). Since RIP1 regulates different types of cell death and survival, we aimed to further distinguish between PCD and necrosis. Data with double Annexin V-propidium iodide (PI) staining strongly indicated that MC2494, like staurosporine, only induced PCD (Fig. 4E). Intriguingly, expression of the E3 ubiquitin ligases IAPs is fully abrogated (35) (Fig. 4E), whereas the expression of IKKγ (NEMO)(36) is induced in absence of a principal deubiquitinating enzyme, CYLD (37,38) (Fig. 4F). Collectively, the data suggest a scenario in which, following SirT inhibition by MC2494 treatment, a PCD pathway is activated, with RIP1 and caspase-8 mediating death and having a causal role. Since RIP1 is acetylated and in presence of MC2494 the new acetylation occurs at site 596-599 in the RIP1 death domain, we applied a mutational approach. Upon transfection in cancer cells, RIP1-mediated cell death was reduced of about 40% with the mutant K596-599R, suggesting that these newly acetylated sites may regulate RIP1 apoptotic function (Fig. 4G). To strength this data, we investigated the effect of MC2494 on RIP1$^{K596\text{-}599R}$ (Fig. 4H). While the point mutation has no impact on the binding between HAT1 and RIP1, cell death induction and RIP1 acetylation undergo to a reduction, suggesting the important role of these K residues in driving anticancer activity of MC2494 (Fig. 4I).

MC2494 exerts cancer cell-selective action *ex vivo* and *in vivo*

For primary cancers, acute leukaemia blasts were treated *ex vivo* with MC2494 showing a strong increment in pre-G1 phase (Fig. 5A). MC2494-mediated PCD was clearly detectable at 24 and 48 hours in all 9 primary acute myeloid leukaemia (AML) blasts and one acute lymphoid leukaemia (ALL) blast, underscoring that in primary cancer, MC2494 induces apoptosis (Fig. 5A and Supplementary Table S3). Pharmacokinetic and *in vitro* half-life studies strengthened a possible use of MC2494 in vitro and *in vivo* (Supplementary Fig. 12A-

D). *In vivo*, MC2494 strongly reduced tumour growth in allograft models as quantified by direct photon emission of luciferase-expressing immuno-competent mice (Fig. 5B and Supplementary Table S4). Moreover, the MDA-MB-231 breast cancer-based xenograft model treated with MC2494 displayed a strong anticancer effect visible as decreased tumour mass. When MC2494 was inoculated by *in vivo* chemo-electroporation, the anticancer effect increased, suggesting its potential *in vivo* also in superficial tumours (as melanoma or other skin tumours in rapid proliferation) (Fig. 5C). Following MC2494, apoptosis (tunnel assay), histone and non-histone SirT targeting, SirT1, SirT2, RIP1 and KAT1/HAT1 were observed in tumours (Fig. 5D) extending results obtained in cell lines.

MC2494 exerts cancer-preventive action *in vivo*

It is a widely shared opinion that cancer prevention is a better approach than treatment. A strategy might include prevention of cancer recurrences and/or action in genetically predisposed patients. Thus, we evaluated the ability of MC2494 to prevent early proliferation occurring in mammary glands after carcinogen exposition. Two groups of MITO-Luc reporter mice (19) (on line Methods) were subcutaneously treated with a daily dose of 50 mg/kg MC2494 or placebo (corn oil) for 9 days (9D); at D6, right and left mammary glands were exposed to 7,12-Dimethylbenz[a] anthracen (DMBA) or vehicle, respectively. As expected, 3D after DMBA injection in the left mammary gland, bioluminescent emission was detectable in the mice pre-treated with placebo (Fig. 6A, placebo). Excitingly, no bioluminescence was detected in the MC2494-pre-treated animals, suggesting that the compound was able to fully abrogate DMBA-induced malignant proliferation in vivo (Fig. 6A-C). In keeping with these data, Ki-67 proliferation marker was highly expressed in the mammary glands treated with DMBA (Fig. 6D, lower panel, left), but to a much lower extent in glands explanted from MC2494-pre-treated mice (Fig. 6D lower panel, right, and Fig. 6E). Increased acetylation of H3K9 in tissues obtained from MC2494-treated mice confirmed that the MC2494 was inhibiting this and likely other epi-targets (Fig. 6F and Supplementary Fig. S11). These experiments suggest that MC2494 counteracts hyper-proliferation occurring during the early steps of carcinogenesis, strongly supporting that MC2494 is not only active against an ‘on-going’ cancer, but also acts in a cancer-preventive manner *in vivo*.

Discussion

Our study identifies a new role for Sirtuins and HATs (mainly HAT1) (39) in modulating programmed death pathways. This occurs via a newly identified RIP1-SirT1/2/HAT1-containing complex and via regulation of RIP1 acetylation. RIP1 (and its acetylation) might represent a key regulatory restriction point between survival, stress and death. In full agreement MC2494, a novel pan-SirT inhibitor (SirTi) (40), alters the HAT1/SirT equilibrium in the RIP1 complex. The fact that our MS approach combined with affinity-based chromatography enrichment reveals not only the existence of the RIP1/KAT1/SirT axes, but also the presence of seven RIP1 acetylation sites (the majority of which are in the death domain) adds to a possible regulatory role for acetylation of RIP1-mediated death. In support, mutation of K596-599 to arginine alters

RIP1 apoptotic function, suggesting its complex regulation by post-translational modifications.

The finding that RIP1 is a main player in PCD represents a paradigm shift, identifying (acetylated) RIP1 as an apoptotic player altered in cancer and a potential target of intervention. Based on our findings, we hypothesized that Sirtuins play a key role in restricting RIP1-caspase8 apoptosis in cancer. The importance of caspase8 is demonstrated by its causal role, since pharmacological inhibition and silencing fully block SirTi-driven death. Pan-SirT inhibition-mediated anticancer activity is also causally linked to ROS and RIP1, given that treatment with both NAC and Nec-1, and RIP1 silencing, abolish PCD. Whether this action also accounts for the tumour-specificity of the pan-SirTi remains to be established. Evidence that both TRAIL and DR5 are up-regulated upon treatment is reminiscent of findings that we (and others) (41) reported for HDACi (17). The contribution of TRAIL tumour-selective PCD by SirTi is unlikely attributable to HDAC inhibition as MC2494 does not affect the activity of HDACs. Secondly, cis-acting elements responsible for MC2494 TRAIL transcriptional activation are distinct from those reported for HDACi (17). While our findings strongly suggest that anticancer action of pan-SirTi can be linked to RIP1 acetylation and TRAIL-DR5 axis activation in cancer (42), the possible tumour-selective activity of pan-SirTi via TRAIL is a key element since normal cells of diverse origin are all insensitive to pan-SirT inhibition. Although the role of oxidative stress in TRAIL-mediated apoptosis has been reported (43), our data show that impairment of either caspase-8 or RIP1 fully blocks SirTi action and that RIP1 acetylation mutant is blandly able to regulate cell death. Possibly, the presence of 7 different RIP1 acetylation sites (only 2 of which, are modulated in these settings by SirTi) suggests that acetylation might control RIP1 cell death functions in a very defined manner. These evidences propose the existence of a caspase-8/RIP1ac-dependent death paradigm of cell death, bound to epigenetic players such as (but not restricted to) Sirtuins and type-B HATs (HAT1) (44). Interestingly, SirT1 interferes with apoptosis induced by oxidative stress, deacetylating and activating FOXO1(45) FOXO3a (46), and FOXO4 (47), inducing GADD45 and the mitochondrial antioxidant manganese superoxide dismutase. Each of these factors might contribute to ROS tolerance by SirT1 alteration in cancer. Deregulation of ROS production, oxidative stress, and FOXO activity are essential steps in cancer development and progression. Moreover, SirTi induction of apoptosis is accompanied by a marked reduction of IAP and FLICE-inhibitory protein (c-FLIP). Interestingly, IAP reduction (and DR5 activation co-occurrence) was recently reported combining IAP inhibitors with lexatumumab (48). This combination results in apoptosis and vanguish cancer resistance by caspase-8/RIP1ac activation. Thus new therapeutic regimens may involve SirTi and remodulation of the RIP1-containing complexes in cancer bypassing resistance to conventional drugs. Consistently, both xenograft and allograft *in vivo* cancer models responded to SirT inhibition with arrest of progression and disease regression along with increase in apoptotic markers, block of proliferation, SirT and HAT1 expression resetting and acetylation within tumours. *Ex vivo* leukaemia blasts also undergo PCD upon pan-SirT inhibition, suggesting a broad range of anticancer actions by MC2494. Though a number of anticancer studies involving SirTi *in vivo* have been reported (49,50), to our knowledge, cancer-prevention *in vivo* has never been described. The ability of pan-SirT inhibition to fully prevent chemically-induced breast carcinogenesis, concomitantly

increasing acetylation *in vivo*, *ex vivo* and during quantitative measurements, shows its potential use in cancer-preventive settings, which has not been demonstrated before *in vivo*. These preventive features might prove useful in patients who may benefit from a recurrence-preventive approach with low toxicity during follow-up phases and in cases of established cancer predisposition.

Disclosure of Potential Conflict of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

V.C. performed the main experiments and wrote the manuscript, F.C., A.N., G.C. performed some cell-based and contributed to the figures assembling. D.R., D.T., A.M. chemistry. R.R., A.P. molecular modelling. P.B. immunohistochemistry, A.B., E.S., G.C. xenograft data. A.C., R.R., M.R. acetylated sites by MS/MS data; J.S., H.G.S. MS/MS data RIP1 interactome, P.C., L.M., E.R. MITO-mice data, P.de A., M.Z. allograft data. L.A. conceived the study and wrote the manuscript.

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FIGURE LEGENDS

Figure 1. RIP1 expression, interactome, acetylation and location. **A,** RIP1 protein expression in different cancer cell lines. **B**, Mass spectroscopy analysis of RIP1 interactome performed in HEK293FT RIP1-GFP cells. **C**, Western blot analysis of RIP1-GFP immunoprecipitated in HEK293FT cells shows the presence of RIP1, SirT1, KAT1/HAT1, SirT2, HAT4 and RIP3 proteins. **D,** Western blot analysis of RIP1-GFP immunoprecipitated with GFP Trap_A beads in HEK293FT cells. *Top panel*: WB with ac-lysines antibody reveals RIP1 acetylation levels, *Lower panel:* WB for RIP1 and KAT1/HAT1. **E,** Schematic representation of acetylated lysines and their localization within the RIP1 subdomains. **F,** Nucleo/Cytosol localisation of RIP1, SirT1and HAT1 performed in HEK293FT cells.

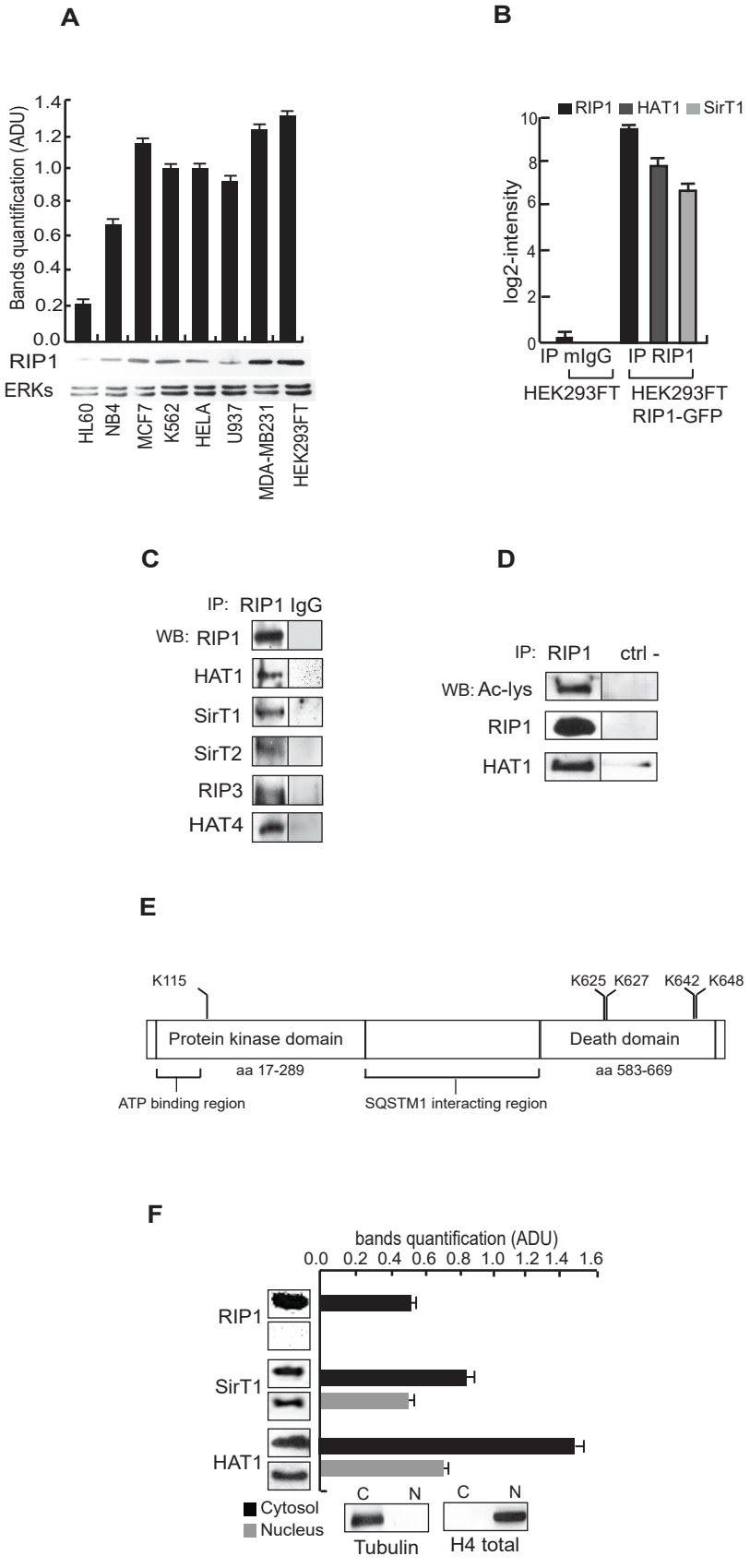
Figure 2. The novel pan-SirT inhibitor MC2494 affects RIP1 acetylation. **A,** Structure of MC2494. **B,** *in vitro* inhibition of SirT1/2/3/4/5/6 by MC2494. Enzymatic assays carried out with MC2494 (50 μM) and Suramin (100 μM), EX527 (5 μM), AGK2 (50 μM) were used as controls. **C,** mRNA evaluation of SirT1, SirT2 and SirT3 in HEK293FT, MDA-MB231 and U937 cancer cells. **D,** mRNA evaluation of SirT1, SirT2 and SirT3 in HACAT "normal" cells. **E,** Western blot analysis of RIP1-GFP immunoprecipitated in HEK293FT. *Top panel*: WB with ac-lysines antibody reveals a strong increase of RIP1 acetylation levels after MC2494 treatment, *Lower panel:* WB for RIP1. **F,** Schematic representation of the acetylated lysines upon MC2494 and their localization within the RIP1 subdomains. Extracted ion chromatogram corresponding to the monoacetylated RIP1 peptides. **G,** Molecular complex analysis after MC2494 treatment. Western blot analysis of RIP1-GFP immunoprecipitates in HEK293FT cells confirms the presence of SirT1, HAT1, RIP1, HAT4 and RIP3 proteins. After MC2494 treatment a reduction of SirT1 and a slight increase of HAT1 is observed. **H,** Evaluation of acetyl RIP1 in SirT1 loss of function condition. Graph showed the mean of three independent experiments with error bars indicating standard deviation.

Figure 3. MC2494 affects proliferation and induces caspase8-dependent apoptosis. **A,** Proliferation curve in U937 cells in presence of the indicated compounds and analysis of cell death as evaluation of preG1 phase. MC2494 and AGK2 were used at 50 μM, EX527 at 5 μM. **B,** Left: MDA-MB-231 cell growth following MC2494, monitored in real time. Right: Migration rate measure as slope (1/h) monitored within the 24 h from the indicated treatments. MC2494 was used at 50 μM. **C,** Induction of preG1 phase upon MC2494 treatment in cancer and normal cell lines. **D, E,** FACS analysis of caspase 8, 9 and 3/7 activation and MMD. The experiments were carry out in U937 cells upon treatment with MC2494 (50 μM). **F,** Western blot analysis of DNA damage evaluation performed in U937 cells upon treatment with MC2494 (50 μM). **G,** apoptosis induced by MC2494 (50 μM and 24 h) in U937 cells is selectively blocked by pan- and caspase 8 inhibitors (ZVAD and IETD), but not by caspase 9 (LEHD). **H,** ROS production following MC2494 (50 μM), PIETC (10 μM) and H_2O_2 (1 mM) for 24 h, MC2494-mediated apoptosis was blocked by NAC (50 μM). Curves and graph presented showed the mean of at least three different experiments with an error bars indicating standard deviation.

Figure 4. The TRAIL-DR5 axis mediates MC2494 cell death. **A,** TRAIL and DR5 expression upon MC2494 (50 μM) for 24-30 h (left), H3K9ac ChIP assay of DR5, TRAIL promoters followed by qPCR (right). **B,** SirT1 ChIP assay at DR5 and TRAIL promoters followed by qPCR. **C,** Schematic representation of TRAIL promoter and deletion mapping (*upper*). Transient transfection assay reveals that TRAIL promoter distal boxes (containing GC-box, AP-1 and ISRE) are essential for activation by MC2494 (*lower).* D, siRNA silencing of caspase 8 or RIP1 and RIP1 pharmacological inhibition with Necrostatine-1 (Nec-1 100 μM) abolishes MC2494 apoptosis in U937 cells. **E,** Apoptosis (left) and necrosis (Annexin/PI) (right) assays following treatment with MC2494 (50 μM) or vehicle for 24 h in U937 cells. **F,** Western blot analysis in U937 cells shows a reduction of IAP expression, a no-modulation of CYLD and an increment of IKKγ protein levels following MC2494 treatment (50 μM and 24 h). ERKs are loading controls. **G,** Evaluation of RIP1-death impairment in HEK293FT cells transfected with RIP1wt or with the $RIP^{(K596/599R)}$ mutant. **H,** Evaluation of cell death induced by MC2494 in HEK293FT cells transfected with RIP1wt or with the $RIP1^{(K596/599R)}$ mutant. **I,** Protein expression evaluation by western blot of acetyl lysine (upper) and RIP1 (lower) in HEK293FT cells transfected with RIP1wt and $RIP^{(K596/599R)}$ mutant. Graph showed the mean of three independent experiments with error bars indicating standard deviation.

Figure 5. MC2494 displays broad anticancer action *in vitro, ex vivo* and *in vivo*. **A,** Induction of apoptosis by MC2494 (50 μM) for 24 and 48 h in 10 *ex vivo* primary AML and ALL blasts. **B,** Representative images of two orthotopic allografted mice with 4T1-luc cells into mammary fat pad. Bioluminescence imaging levels of vehicle- (DMSO) and MC2494-treated mice were acquired at 0 (T0), and after 7 (T7) days of daily treatment, and quantified as photon/s. See (Supplementary information Table S4) for BLI data in photon/sec. Results are Σ ± SEM. from 7 mice each in the vehicle- and MC2494-treated groups. **C,** MC2494 reduces subcutaneous tumor growth of MDA-MB-231 xenograft using s.c. injection of $5X10^6$ cells into the left leg of nude mice by and its combination with electrochemotherapy. *In vivo* growth in volume of tumors induced. Two controls (vehicle alone and electroporation of vehicle) are reported to exclude possible effects of the vehicle and electroporation *in vivo*. In the control groups (animals treated with vehicle or electroporation), tumor volumes strongly increased over a 28-day period whereas MC2494 caused a significant reduction (p value 0.005 and 0.001, respectively). An even greater growth inhibition was obtained by the combination of MC2494/electrochemotherapy, (p value < 0.001) compared to both controls. The weight of all mice assigned to the various groups fell within the same range, providing no immediate evidence for overt toxic effects. Ki67 and TUNEL scores were performed at the end of treatment. The proliferation index was significantly lower in tumours of treated mice compared to controls (p=0.002). Apoptotic index was significantly higher in tumours of MC2494-treated mice (p=0.002). **D,** Immunohistochemical analysis for the apo-index, Ki67, H3K56ac, ac-tubulin, SirT1, SirT2, RIP1 and KAT1/HAT1 levels in tumours). Curves and graph presented showed the mean of at least two different experiments with an error bars indicating standard deviation.

Figure 6. MC2494 exerts cancer preventive effects *in vivo*. **A,** Optical imaging analysis of bioluminescence from MITO-Luc reporter mice injected in the mammary fat pad with DMBA (left) or vehicle (right gland); bioluminescent emission is represented using a pseudo-colour scale. Magnified inserts highlight details of photon emission from mammary glands. **B,** Quantitation of (A). Bars represent average photon emission ± SEM from 4 animals/group measured within the mouse areas shown in the magnified inserts in (A); bioluminescent emission is completely prevented in MC2494 group as compared to placebo. *** $p < 0.001$ DMBA (n=4) vs vehicle-treated (n=4) mammary glands calculated using unpaired t-test. **C,** *Ex vivo* imaging of mammary glands from 2 representative mice from each group. **D,** Immunohistochemistry of breast slices obtained from each group stained for Ki67 antibody reveals a marked reduction of cell proliferation in the DMBA + MC2494 as compared to DMBA. **E,** Quantitation of immunohistochemistry in (d). Bars represent the average ± SEM of the Ki67 index expressed as the ratio between Ki67-positive cells vs total. *** $p < 0.001$ DMBA vs vehicle-treated mammary glands calculated using 2-way ANOVA followed by the Bonferroni *post-hoc* test. **F,** Immunohistochemistry and relative quantization of H3K9 acetylation on breast slices obtained from each group; increased H3K9ac is visible in MC2494-treated animals corroborating SirT1/2 inhibition *in vivo*. Graph showed the mean of three independent experiments with error bars indicating standard deviation.



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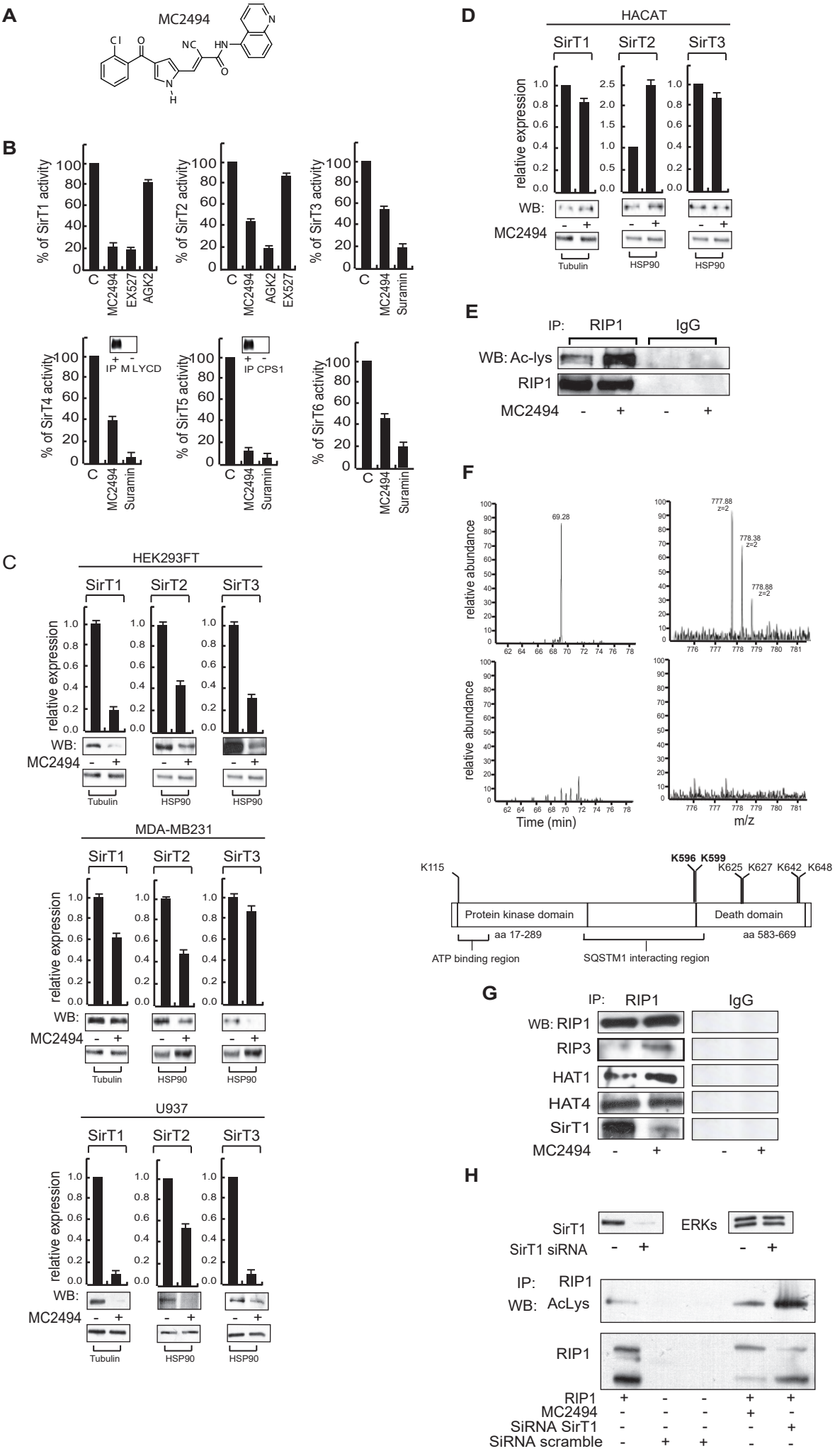

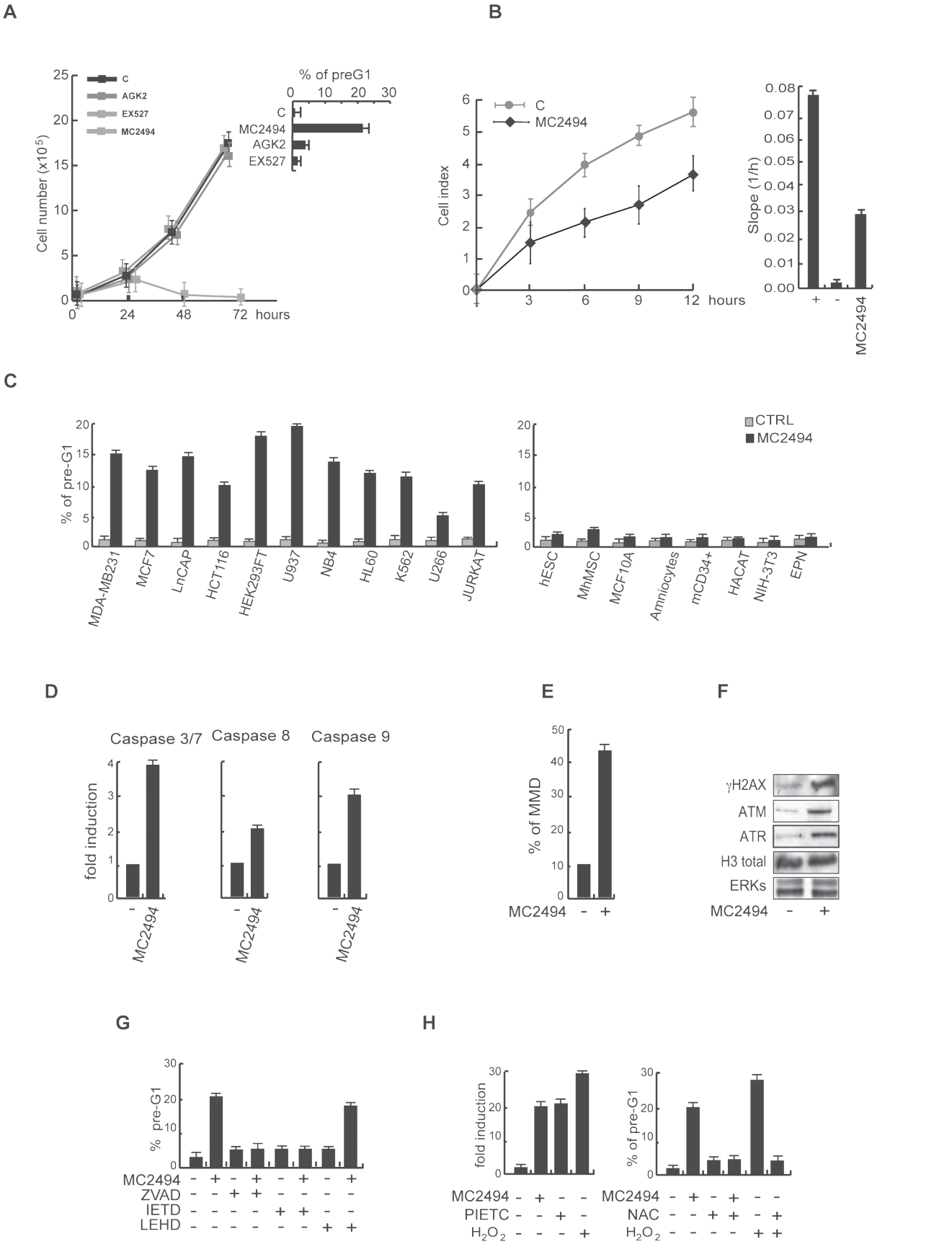

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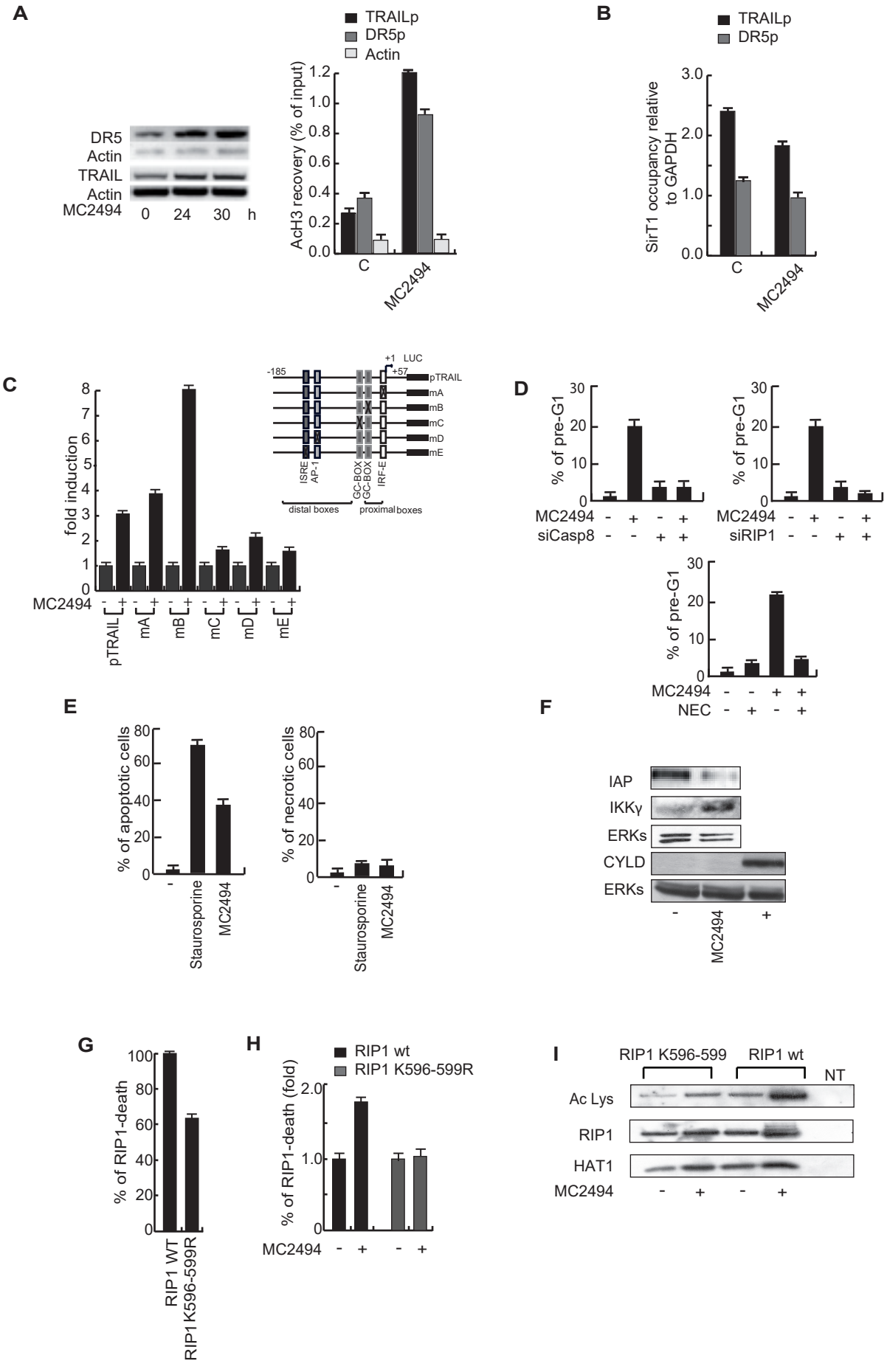
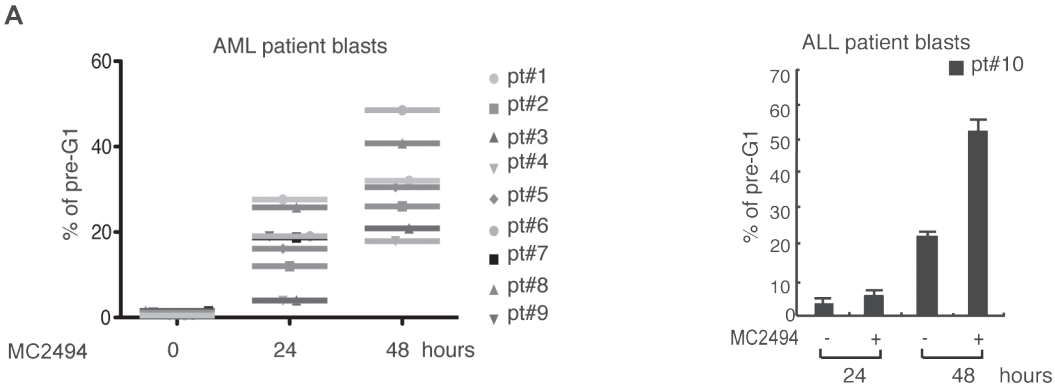
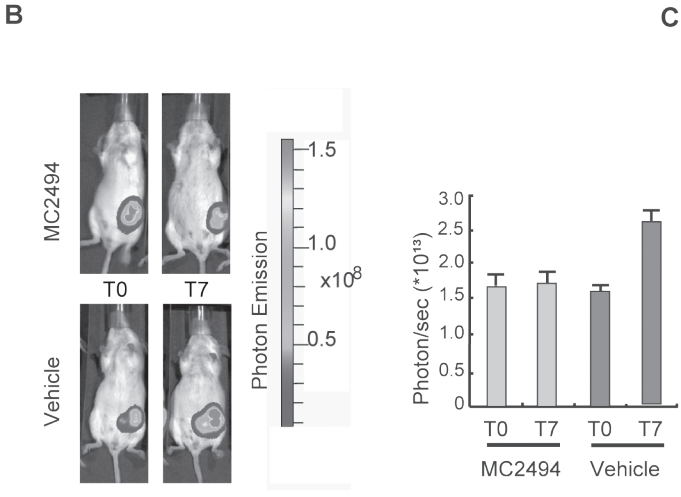
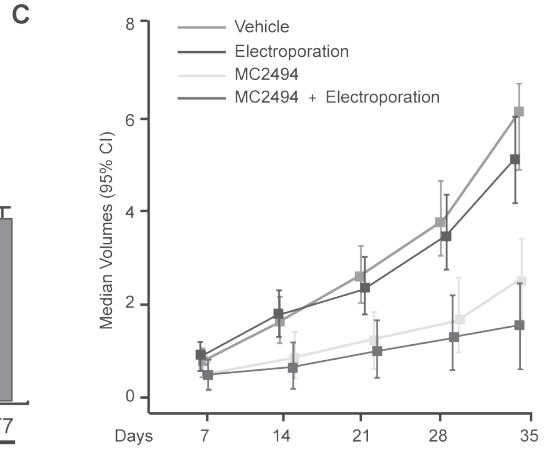


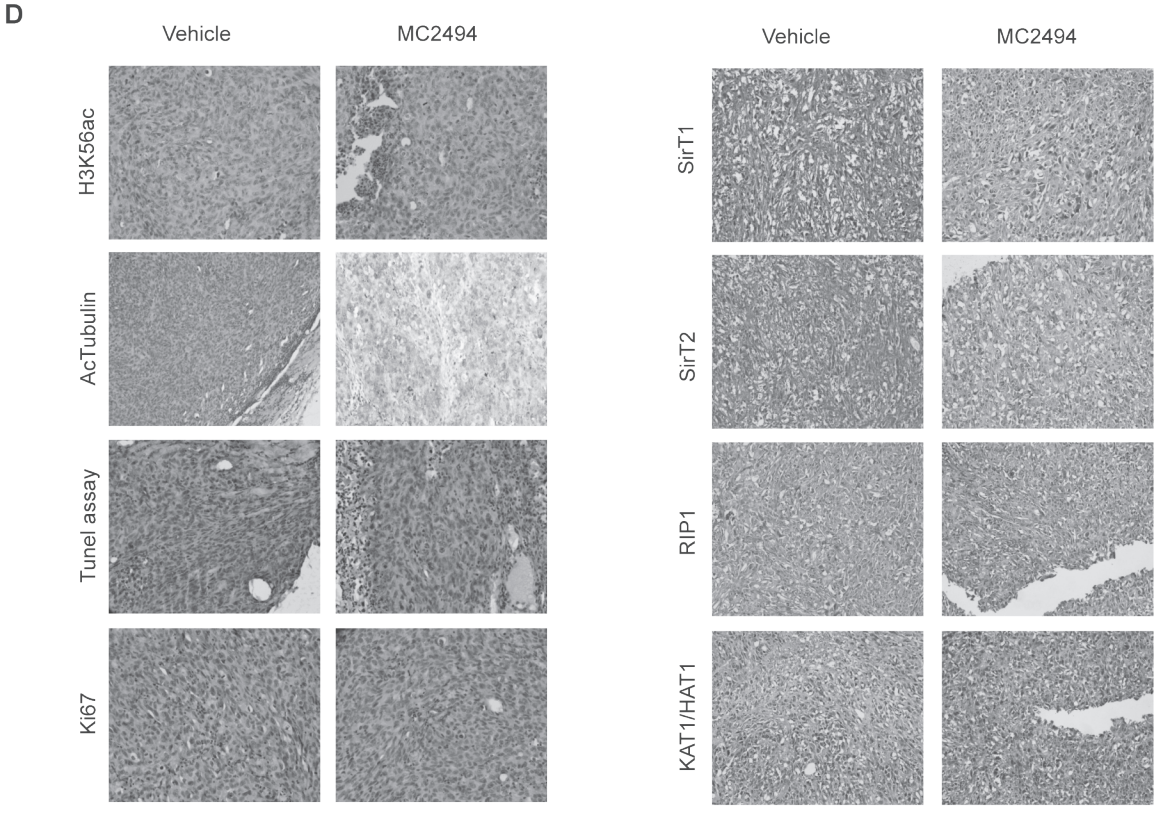
Carafa et al. Figure 4

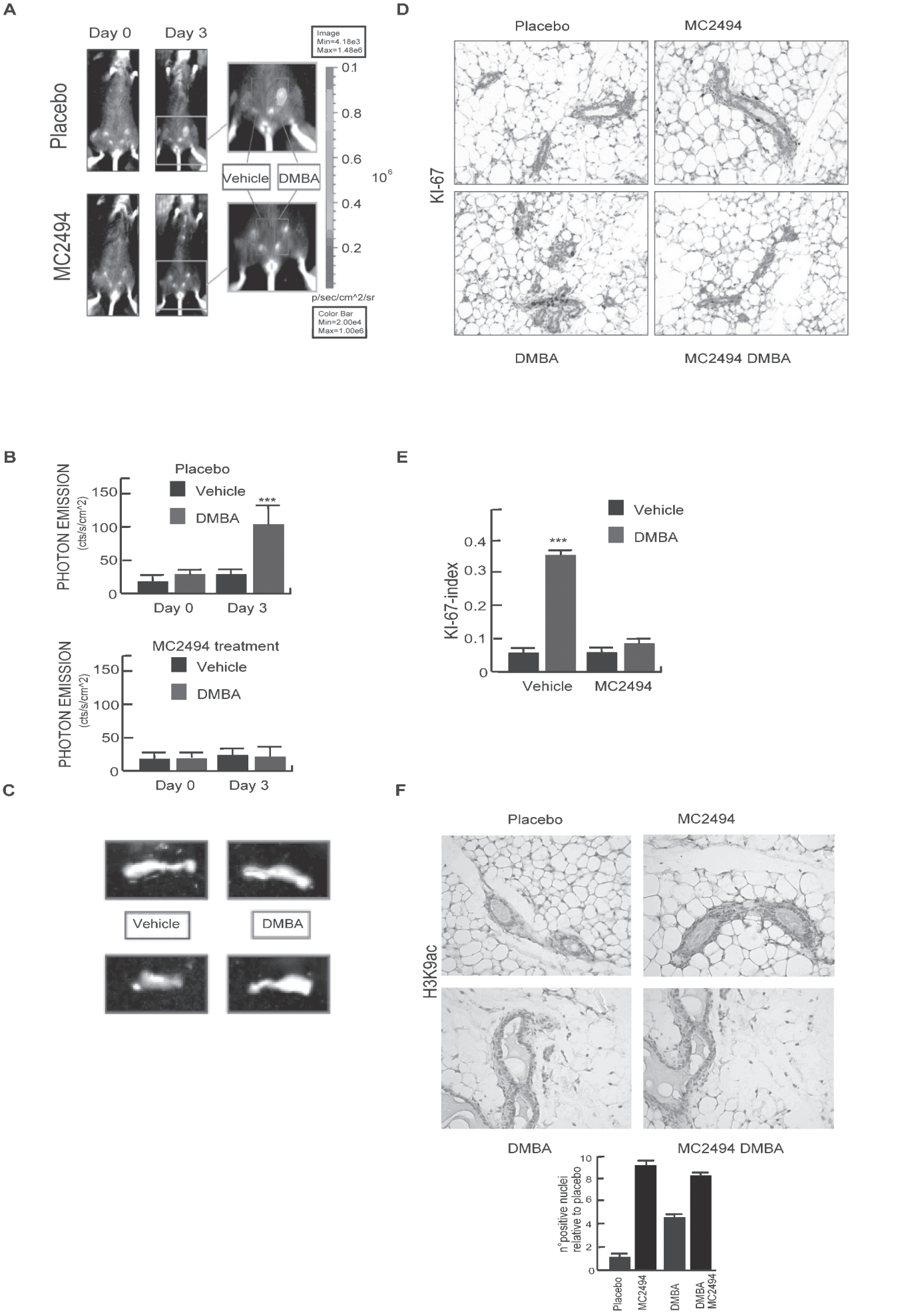






	proliferation index	apoptotic index	K56ac	AcTubulin
Vehicle	35%	7 ± 2	5%	8%
MC2494	15%	15 ± 3	25%	30%





AACR American Association for Cancer Research

Clinical Cancer Research

RIP1-HAT1-SirT complex identification and targeting in treatment and prevention of cancer

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