Published in final edited form as:

Nature. 2014 February 27; 506(7489): E4–E6. doi:10.1038/nature13024.

Is SIRT2 required for necroptosis?

Kim Newton¹, Joanne M. Hildebrand^{2,3}, Zhirong Shen⁴, Diego Rodriguez⁷, Silvia Alvarez-Diaz^{2,3}, Sean Petersen¹, Saumil Shah⁵, Debra L. Dugger¹, Chunzi Huang⁶, Johan Auwerx⁸, Peter Vandenabeele^{9,10,11,*}, Douglas R. Green^{7,*}, Avi Ashkenazi^{1,*}, Vishva M. Dixit^{1,*}, William J. Kaiser^{6,*}, Andreas Strasser^{2,3,*}, Alexei Degterev^{5,*}, and John Silke^{2,3,*}

¹Genentech, Inc., South San Francisco, CA 94080, USA ²The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia ³Department of Medical Biology, University of Melbourne, Parkville, Victoria 3050, Australia ⁴National Institute of Biological Sciences, Zhongguancun Life Science Park, Beijing 102206, China ⁵Department of Biochemistry, Tufts University, Boston, Massachusetts 02111, USA ⁶Department of Microbiology and Immunology, Emory Vaccine Center, Emory University School of Medicine, Atlanta, GA 30322, USA ⁷Department of Immunology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105, USA ⁸Laboratory of Integrative and Systems Physiology, EPFL, CH-1015 Lausanne, Switzerland ⁹Molecular Signaling and Cell Death Unit, Inflammation Research Center, VIB, 9052 Gent, Belgium ¹⁰Department of Biomedical Molecular Biology, Ghent University, 9052 Gent, Belgium ¹¹Methusalem BOF09/01M00709, Ghent University, 9052 Gent, Belgium

Abstract

Sirtuins can promote deacetylation of a wide range of substrates in diverse cellular compartments and regulate many cellular processes^{1,2}. Recently Narayan *et al.*, reported that SIRT2 was required for necroptosis based on their findings that SIRT2 inhibition, knock-down or knock-out prevented necroptosis. We sought to confirm and explore the role of SIRT2 in necroptosis and tested four different sources of the SIRT2 inhibitor AGK2, three independent siRNAs against SIRT2, and cells from two independently generated *Sirt2*^{-/-} mouse strains, however we were unable to show that inhibiting or depleting SIRT2 protected cells from necroptosis. Furthermore, *Sirt2*^{-/-} mice succumbed to TNF induced Systemic Inflammatory Response Syndrome (SIRS) more rapidly than wild type mice while *Ripk3*^{-/-} mice were resistant. Our results therefore question the importance of SIRT2 in the necroptosis cell death pathway.

There are seven mammalian sirtuins and work has focused on SIRT1, the closest homolog to the founding member of the Sirtuin family, *Saccharomyces cerevisiae* Sir2. Mammalian SIRT2 has been reported to regulate cellular metabolism, division and differentiation^{1,2}. A recent study suggested an additional role, namely that SIRT2 is required for programmed necrosis, otherwise known as necroptosis³. Using co-immunoprecipitation followed by mass-spectrometric analysis, Narayan *et al* found that SIRT2 interacted with RIPK3, a kinase that is essential for tumor necrosis factor (TNF)-induced necroptosis^{4–6}. Using a SIRT2 inhibitor, AGK2⁷, they observed that SIRT2 activity was required for formation of

*These authors contributed equally

the RIPK1/RIPK3 complex in response to the necroptosis inducing stimulus of TNF, the caspase inhibitor, z-VAD-fmk and the translation inhibitor cycloheximide. Narayan *et al.* proposed that SIRT2-mediated deacetylation of a lysine near the RIPK3-interacting RHIM motif of RIPK1 was required for RIPK1/RIPK3 association and necroptosis induction. Consistent with this hypothesis, they showed that knock-down, knock-out or inhibition of *Sirt2*/SIRT2 prevented TNF-induced necroptosis *in vitro*³.

The necroptotic cell death pathway has attracted interest because inhibiting it, particularly in conjunction with blocking apoptosis, has the potential to ameliorate diverse inflammatory and possibly also degenerative diseases^{8–11}. Importantly, Narayan *et al.* reported that genetic ablation or pharmacological blockade of *Sirt2*/SIRT2 afforded mice significant protection from injury caused by cardiac ischemia-reperfusion.

The promise of these findings prompted us to confirm and explore the role of SIRT2 in necroptosis. We tested the ability of four different sources of the SIRT2 inhibitor, AGK2, at a broad range of concentrations, to inhibit necroptosis triggered by TNF+Smac-mimetic+the caspase inhibitor, Q-VD-Oph (TSQ) or TNF+z-VAD-fmk (TZ). In contrast to the findings of Narayan *et al.*, AGK2 failed to inhibit TNF-induced necroptosis in our experiments (Figure 1a & Appendix Figure). As previously described⁷, AGK2 (SIGMA) increased levels of acetylated-tubulin in NIH-3T3 cells, validating its ability to inhibit SIRT2 at the doses used (Appendix Figure). Narayan *et al.* found that two independent shRNAs used to knock down *Sirt2* expression prevented TZ-induced necroptosis in L929 cells. In our experiments, three independent siRNAs, which reduced *Sirt2* expression by greater than 80%, failed to inhibit TZ-induced necroptosis in L929 cells (Figure 1b & Appendix Figure). We also tested mouse embryonic fibroblasts (MEFs) and bone marrow derived macrophages (BMDMs) from two independently generated *Sirt2* knock-out mouse strains (Appendix Figure), but found neither cell type to be protected from TNF-induced necroptotic death (Figure 1c & d).

Finally Narayan *et al.* observed that *Sirt2*-deficient mice were protected from cardiac ischaemia-reperfusion injury and concluded that transient inhibition of SIRT2 might provide protection from diseases involving necroptosis. Because RIPK3 deficient mice have not been tested for their ability to resist cardiac ischemia-reperfusion injury, we chose to test the response of *Sirt2*^{-/-} mice to a classic RIPK3-dependent necroptotic insult: we injected wild-type, *Ripk3*^{-/-}, and *Sirt2*^{-/-} mice with TNF and monitored their body temperature. As previously described⁸, the core body temperature of wild-type mice dropped rapidly following injection of 300 µg/kg of TNF, and some of these animals died within the indicated time-frame. In contrast, *Ripk3*^{-/-} mice maintained normal body temperature and all survived. Far from being protected against this necroptosis-inducing insult, *Sirt2*^{-/-} mice were more sensitive than their wild-type counterparts, and all died within the indicated time frame (Fig. 1e & Appendix Figure).

These experiments, performed with SIRT2 inhibitors, *Sirt2* knock-down cells, *Sirt2*^{-/-} mice and cells from two independently derived *Sirt2*^{-/-} mice, raise concerns about the claimed role for SIRT2 in necroptotic cell death.

Methods

Data were generated by nine independent labs. We have endeavored to provide key experimental information, and detailed experimental methods are available on request. AGK2 was sourced from TOCRIS Bioscience (3233), Sigma (A8231), Biovision and the Kazatsev lab⁷. Cell survival was measured using propidium iodide exclusion and flow cytometric analysis, Cell Titer Glo assay (Promega) and IncuCyte system counting of SYTOX® green positive cells in real time. *Sirt2*^{-/-} mice were provided by the Donmez and Auwerx labs^{12,13}. TNF was sourced from Peprotech or made in house. Published protocols for siRNA, BMDM maturation and SIRS experiments were followed^{8, x, x}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Houtkooper RH, Pirinen E, Auwerx J. Sirtuins as regulators of metabolism and healthspan. *Nature reviews. Molecular cell biology*. 2012; 13:225–238.10.1038/nrm3293
2. Sebastian C, Satterstrom FK, Haigis MC, Mostoslavsky R. From sirtuin biology to human diseases: an update. *J Biol Chem*. 2012; 287:42444–42452.10.1074/jbc.R112.402768 [PubMed: 23086954]
3. Narayan N, et al. The NAD-dependent deacetylase SIRT2 is required for programmed necrosis. *Nature*. 2012; 492:199–204.10.1038/nature11700 [PubMed: 23201684]
4. Cho YS, et al. Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation. *Cell*. 2009; 137:1112–1123.10.1016/j.cell.2009.05.037 [PubMed: 19524513]
5. He S, et al. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF- α . *Cell*. 2009; 137:1100–1111.10.1016/j.cell.2009.05.021 [PubMed: 19524512]
6. Zhang DW, et al. RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. *Science*. 2009; 325:332–336.10.1126/science.1172308 [PubMed: 19498109]
7. Outeiro TF, et al. Sirtuin 2 inhibitors rescue α -synuclein-mediated toxicity in models of Parkinson's disease. *Science*. 2007; 317:516–519.10.1126/science.1143780 [PubMed: 17588900]
8. Duprez L, et al. RIP kinase-dependent necrosis drives lethal systemic inflammatory response syndrome. *Immunity*. 2011; 35:908–918.10.1016/j.immuni.2011.09.020 [PubMed: 22195746]
9. Gunther C, et al. Caspase-8 regulates TNF- α -induced epithelial necroptosis and terminal ileitis. *Nature*. 2011; 477:335–339.10.1038/nature10400 [PubMed: 21921917]
10. Piao X, et al. c-FLIP maintains tissue homeostasis by preventing apoptosis and programmed necrosis. *Science signaling*. 2012; 5:ra93.10.1126/scisignal.2003558 [PubMed: 23250397]
11. Welz PS, et al. FADD prevents RIP3-mediated epithelial cell necrosis and chronic intestinal inflammation. *Nature*. 2011; 477:330–334.10.1038/nature10273 [PubMed: 21804564]
12. Beirowski B, et al. Sir-two-homolog 2 (*Sirt2*) modulates peripheral myelination through polarity protein Par-3/atypical protein kinase C (α PKC) signaling. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:E952–961.10.1073/pnas.1104969108 [PubMed: 21949390]
13. Bobrowska A, Donmez G, Weiss A, Guarente L, Bates G. SIRT2 ablation has no effect on tubulin acetylation in brain, cholesterol biosynthesis or the progression of Huntington's disease phenotypes in vivo. *PLoS One*. 2012; 7:e34805.10.1371/journal.pone.0034805 [PubMed: 22511966]

5 sentences; first sentence sets the scene, the second summarises the results of the Nature paper under discussion, the third & 4th presents your contradictory view/results, and the fifth states the implications.

Figure 1. Neither chemical inhibition nor genetic depletion of SIRT2 inhibits TNF-induced Necroptosis

a, Indicated cell lines were treated with the indicated necroptotic stimuli in the presence or absence of AGK2. **b**, L929 cells were transfected with siRNAs targeting SIRT2 or a control siRNA before treatment with TZ. **c**, MEFs derived from *Sirt2*^{-/-} and wild-type mice were treated with TCZ $\pm$ inhibitors. Plots in panels **a–c** represent the mean $\pm$ standard deviation of two biological repeats (MEFs, MDFs) or the mean $\pm$ standard deviation of three independent repeats (L929, NIH 3T3). **d**, BMDMs isolated from *Sirt2*^{+/+}, *Sirt2*^{-/-}, *Ripk3*^{+/+} or *Ripk3*^{-/-} mice (n=3 per genotype) were treated with TSZ. **e**, Core body temperature of *Sirt2*^{+/+}, *Sirt2*^{-/-}, *Ripk3*^{+/+} or *Ripk3*^{-/-} mice (n=10 per genotype) injected with 300 μ g/kg TNF. An unpaired students T test was used to determine P values in **d**, and **e**.