The role of sirtuin 2 activation by nicotinamide phosphoribosyltransferase in the aberrant proliferation and survival of myeloid leukemia cells

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Online Supplementary Design and Methods

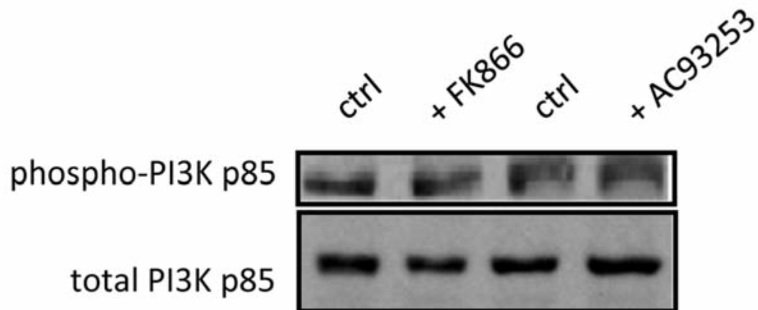
Detection of proteins and protein acetylation by in situ *proximity ligation assay (DUOLINK)*

Endogenous SIRT2, NAMPT, phospho-Ser9-GSK-3β, and phospho-Ser33/37-β-catenin proteins in the HL60 cell line, in $CD34^+$ cells from healthy volunteers (n = 6), and in blasts from patients with acute myeloid leukemia (n = 6) were detected using a proximity ligation assay (PLA) from the DUOLINK reagent series (Olink Bioscience, Uppsala, Sweden), as described in the manufacturer's protocol. DUOLINK enables the detection of endogenous proteins or protein modifications through a pair of specific oligonucleotide-conjugated antibodies. Individual proteins are visualized by fluorescent dots (red) of amplified oligonucleotides conjugated to antibodies, specific to protein/s of interest in the cell. Cytospin slides of cells (1×10^4 cells/slide) were prepared. Cells on cytospins were fixed in ice-cold methanol and permeabilized using 0.1% Triton-X100. Samples were blocked in 1× blocking stock (Olink Bioscience, Uppsala, Sweden) for 30 min at 37°C in a humidified chamber. All subsequent incubations were performed in a humidified chamber maintained at 37 °C. We used rabbit anti-SIRT2, goat anti-NAMPT/PBEF (SC-46440) (both from Santa Cruz Biotechnology), rabbit anti-phospho-GSK-3β (Ser9) (5B3), and rabbit anti-phospho-β-catenin (Ser33/37) (all from Cell Signaling Technology). Acetylation of AKT protein was analyzed by DUOLINK using rabbit anti-AKT (9272) and mouse anti-acetyl-Lys (9681S) antibody from Cell Signaling Technology. Antibodies were diluted in 1× antibody diluent stock (Olink Bioscience) and co-incubated for 1 h. Anti-mouse and anti-rabbit PLA MINUS and PLA PLUS secondary probes (Olink Bioscience) were diluted 1:5 in 1× antibody diluent stock and co-incubated for 45 min at 37°C in a humidified chamber. Oligonucleotides complementary to the proximity probe DNA extensions were incubated as 1× hybridization stock (Olink Bioscience) for 15 min at 37°C in a humidified chamber. To complete the rolling- circle priming template, T4 DNA ligase (1:40) was added to samples in 1× ligation stock (Olink Bioscience), and samples were incubated for 15 min at 37°C in a humidified chamber. PLA probes act as a primer for rolling cycle amplification reaction (RCA) using the ligated circular nucleotide as a primer. RCA of the ligated oligonucleotide template was initiated by addition of Phi29 DNA polymerase (1:80) in 1× amplification stock (Olink Bioscience) and incubated for 60 min at 37°C in a humidified chamber. Finally, Texas red–labeled oligonucleotide detection probes (Olink Bioscience) were incubated as 1× detection stock and incubated for 60 min at 37°C in a humidified chamber. Cells were washed in PBS-T, in 2x SSC, in 0.2x SSC, in 0.02x SSC, and in 70% ethanol before being mounted. All washing procedures and washing solutions are described in the manufacturer's protocol.[1] Samples were air dried and mounted with Olink mounting media containing DAPI nuclear stain.

References

1. Söderberg O, Gullberg M, Jarvius M, Ridderstråle K, Leuchowius KJ, Jarvius J, et al. Direct observation of individual endogenous protein complexes in situ by proximity ligation. Nat Methods. 2006;3(12):995-1000.

Online Supplementary Figure S1. (A, B) HL60 cells were cultured with 1 nM, 10 nM, or 100 nM of FK866 for 96 h (A) or 10 nM, 100 nM or 1000 nM of AC93253 for 48 h (B), or with DMSO as a control; the number of viable cells was assessed using a CellTiter-Glo luminescence cell proliferation assay; data represent means ± SD of triplicate measurements and are derived from three experiments (***P*<0.01); (C,D) bone marrow $CD34^+$ cells were cultured in *ex-vivo* supplemented medium in the presence of 10 nM of FK866 for 96 h, or 100 nM of AC93253 for 48 h or with DMSO for the respective time points; cell proliferation was assessed as described above.



Online Supplementary Figure S2. Representative images of phospho-PI3K p85 (Tyr 458) and total PI3K p85 protein expression in HL60 cells treated with 10 nM of FK866 or 100 nM of AC93253 or DMSO as a control.