SUPPLEMENTARY DATA

Supplementary Experimental Procedures

Chemicals. Arsenic trioxide (As_2O_3), 6-aminocaproic acid, cisplatin, coenzyme Q_2 (CoQ), Coomassie Brilliant Blue G, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), cycloheximid (CHX), 2,6-dichlorophenolindophenol (DCPIP), doxorubicin, 5-(N-ethyl-N-isopropyl)amiloride (EIPA), fluorocitrate, fluoroacetate, Polyethylene glycol-catalase (PEG-catalase), malonate, menadione, n-dodecyl-B-D-maltoside, 3-nitropropionic acid (3NP), phenazine methosulfate (PMS), propidium iodide (PI), succinate, succinate dibasic ester (succinate DBE), bovine superoxide dismutase (SOD), PEG-SOD, 2-thenoyltrifluoroacetone (TTFA) and Trolox (6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) were purchased from Sigma-Aldrich. Etoposide and geneticin (G418) were from Calbiochem. Human TNFα and Z-Val-Ala-Asp-fluoromethylketone (ZVAD-fmk) were purchased from Bachem. Super Fas Ligand (FasL) was from Alexis. Human interferon γ (IFNγ) and the Complete protease inhibitor cocktail were purchased from Roche Applied Science. The pH-sensitive fluoroprobe carboxy-SNARF-1, 7-Aminoactinomycin D (7-AAD) and the superoxide-sensitive fluoroprobe dihydroethidium (DHE) and its mitochondria-targeted form MitoSOX were from Invitrogen, as well as the RNA extraction reagent TRIzol. The transfection reagents Lipofectamine 2000 and Effectene were from Invitrogen and Qiagen, respectively.

Detection of Active Caspase-3 by FACS

HeLa cells were treated with various drugs (see Supplemental Table 1) or transfected with a plasmid coding for SHDC using Effectene (Qiagen, UK). Cells were harvested and then fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. After a blocking step with 3% BSA in Tris-HCl 50 mM, NaCl 155 mM, pH 7.6 and 0.02% saponine (TBSS) for 30 minutes at 37°C, cells

were incubated with the primary antibody for 1 hour at RT. Rabbit polyclonal anti-cleaved caspase-3 antibody (Cell Signalling, UK) was used at a 1/200 dilution in TBSS. After centrifugation, cells were rinsed in TBSS and then incubated with the secondary anti-rabbit Alexa fluor 488 antibody (Molecular Probes, Invitrogen) at a dilution of 1/500 in TBSS. After washing, cells were resuspended in 300 µl of PBS and Alexa 488 emission was analysed by flow cytometry using FL1 channel.

Western blotting. The cells were lysed in RIPA buffer and the proteins separated on a 10% SDS-PAGE, electroblotted onto nitrocellulose membranes (BioRad), which were blocked with 5% milk (Sigma). Either a rabbit anti-PARP antibody (Cell signalling) at a 1/1000 dilution in 5% milk O/N or a mouse anti-actin antibody (Sigma) at a 1/10000 dilution was added, which was then detected by a goat anti-rabbit antibody (Sigma) at a dilution of 1/25 000 or an anti-mouse antibody (Jackson Lab) at a dilution of 1/2500.

Supplementary Table 1. Concentration of the various anticancer drugs and proapoptotic compounds used for the treatment of HeLa and B9 CCL16 cells.

Supplementary Table 2. ShRNA sequences used for the transient downregulation of the four complex II subunits.

Supplementary Table 3. Forward and reverse primer sequences used for PCR detection.

	HeLa	B9 CCL16
Arsenic trioxide	10 µM	-
Doxorubicin	1.4 µM	1 µM
Cisplatin	17.5 µM	-
Menadione	15 µM	-
Etoposide	250 µM	25 µM
TNFα (+ CHX 0.2 µg/mL or INFγ 1000 UI/mL)	150 ng/mL	-
FasL	125 ng/mL	-

Supplementary Table 1

	ShRNA sequences
SDHA sense	5′-GATCCCC-TGCAGGCCTGGAGATAAAG-TTCAAGAGA-CTTTATCTCCAGGCCTGCA-TTTTTGGAAA-3’
SDHB sense	5′-GATCCCC-ATGGAGGCAACACTCTAGC-TTCAAGAGA-GCTAGAGTGTTGCCTCCAT-TTTTTGGAAA-3’
SDHC sense	5′-GATCCCC-CTGATCCACACAGCTAAGT-TTCAAGAGA-ACTTAGCTGTGTGGATCAG-TTTTTGGAAA-3’
SDHD sense	5′-GATCCCC-TGGGGATGCCTTGCAGAAA-TTCAAGAGA-TTTCTGCAAGGCATCCCCA-TTTTTGGAAA-3’

Supplementary Table 2

	PCR primer sequences
SDHA (human)	*forward* 5’-TATCAGCGTGCATTTGGTG-3’ *reverse* 3’- CCTGTGGGGTGGAACTGA-5’
SDHB (human)	*forward* 5’-TACAAATCCATTGAGCC-3’ *reverse* 3’-CAGTTCATGATGGTGTG-5’
SDHC (human)	*forward* 5’-GAGATGGAGCGGTTCTGGA-3’ *reverse* 3’-CAAGTGTCGGATCCCATT-5’
SDHC (murine/hamster)	*forward* 5’-GAGATGGAGCGGTTCTGGA-3’ *reverse* 3’-CAGAGGACAACACAGCAAG-5’
SDHD (human)	*forward* 5’-GAGAGGGTTGTCAGTGTT-3’ *reverse* 3’-AGCAAAGCCCAGCAAAGG-5’
NHE1 (human)	*forward* 5’-CATCTGTGGCCTCTCTCCAC-3’ *reverse* 3’-AGGAAGAAGACGTCGGACTG-5’

Supplementary Table 3

Supplementary figure 1. Schematic representation of the two distinct complex II enzymatic activities. (**a**) Schematic representation of complex II functions. SDHA-mediated oxidation of succinate to fumarate by the SDH activity, as part of the Krebs cycle, provides electrons to complex II. They are transferred to the Fe-S clusters of SDHB and finally to the CoQ reduction site at the interface between SDHC and SDHD. The SQR reaction comprises in addition the electron transfer to CoQ, linking complex II to the rest of the respiratory chain. (**b**) Succinate dehydrogenase activity (SDH) is measured on isolated mitochondria through the SDHA/SDHB-mediated oxidation of succinate to fumarate, via the reduction of the SDHA-bound flavin adenine dinucleotide (FAD) cofactor. The electrons generated are transferred to phenazine methosulfate (PMS), and finally to dichlorophenylindophenol (DCPIP), as the final electron acceptor. Extinction of the DCPIP absorbance in response to its reduction is spectrophotometrically monitored at 610 nm. (**c**) Succinate coenzyme Q oxidoreductase activity (SQR) is measured on isolated mitochondria through the SDHA/SDHB-mediated oxidation of succinate into fumarate. The electrons are then transferred to the iron-sulfur centres of the SDHB subunit and finally to the coenzymeQ reduction site at the interface between SDHC and SDHD, the two transmembrane subunits of complex II. Coenzyme Q, supplied as an intermediate electron acceptor during this enzymatic reaction, is reduced and transfers the electrons to DCPIP, the final electron acceptor. Extinction of the DCPIP absorbance following its reduction is spectrophotometrically monitored at 610 nm, as for **b**.

Supplementary figure 2. Caspase-3 activity and PARP cleavage as specific apoptosis features induced by drugs and cytokines. HeLa cells were treated for 24h with the indicated compounds or cytokines or transfected with a *wt* SDHC expression vector as a positive control for apoptosis induction. Cells were harvested and caspase-3 activity was analysed by flow cytometry as described in *Supplementary Experimental Procedures* (*Upper*

panel). The cells were lysed in RIPA buffer and total protein extracts subjected to SDS-PAGE to detect the cleaved and uncleaved forms of Poly (ADP-ribose) polymerase (PARP). Equal gel loading and transfer efficiency were checked with an anti-actin mouse mAb (Sigma) (*Lower panel*).

Supplementary figure 3. Involvement of ROS in apoptosis induced by various apoptotic signals. (**a**) Effects of the different anticancer drugs and cytokines on ROS production. HeLa cells were treated for 8h with the indicated drugs or cytokines at the concentrations shown in Supplementary Table 1. ROS were quantified by flow cytometry through the DHE-related fluorescence. *, $p<0.05$ compared to the related control. (**b**) Effects of NHE1 overexpression on mitochondrial ROS production induced by the different drugs and cytokines. HeLa cells were transfected either with pcDNA3 or with a NHE1 expression plasmid. After 24h, cells were treated with the compounds for the indicated times and mitochondrial ROS were measured by flow cytometry using MitoSOX staining. Results are shown as the percentages of increase of MitoSOX positive cells compared to the corresponding-untreated cells. #, $p<0.05$. (**c**) Effect of the specific complex II inhibitor TTFA on mitochondrial ROS production. HeLa cells were treated for 8h with TTFA (1mM) and mitochondrial ROS were measured as in (b). *, $p<0.05$ compared to the related control. (**d**) Effect of the superoxide scavenger PEG-SOD on drug-induced mitochondrial ROS production. HeLa cells were left untreated or pre-treated for 2h with PEG-SOD (100 UI/mL) and then 1mM TTFA (1mM) or Doxorubicin (1µM) were added for 7h. Mitochondrial ROS were measured as in (b). #, $p<0.05$.

Supplementary figure 4. Downregulation of SDHA, SDHB, SDHC or SDHD proteins by specific shRNA expression. Mitochondrial fractions were isolated 72h after HeLa cells were transfected with either scrambled shRNA or the respective shRNA. The protein levels of the

four complex II subunits were analyzed by Western Blot for each shRNA construct. Equal gel loading and transfer efficiency were checked by protein hybridization with an anti-cytochrome C-oxidase IV (Cox IV) antibody. Blots are representative of three independent experiments.

Supplementary figure 5. Stable reconstitution of SDHC*wt* in SDHC negative cells. (**a**) B9 cells (SDHC-/-) were transfected either with an empty pcDNA3 vector (left) or an SDHC*wt* expression plasmid (right*)* and stable clones were selected with geneticin. mRNA levels of SDHC and pcDNA3 were quantified by RT-PCR and compared with the B1 parental cells and with B9 cells. SDHC*wt* clone 4 with the same SDHC expression level than B1 cells was chosen, as well as the pcDNA3 clone 2 which expressed pcDNA3. β-actin was analysed as a loading control. (**b**) Measurement of SQR and SDH activities in B1 and B9 cells, pcDNA3 clone 2 and SDHC*wt* clone 4. Mitochondria were isolated and SQR and SDH activities were assessed by the appropriate assay.

Supplementary figure 6. Markers for necrosis and apoptosis induced by TTFA. (**a**) Assessment of the dose-dependent effect of TTFA on apoptosis and necrosis. HeLa cells were treated for 8h or 24h with the indicated concentrations of the direct complex II inhibitor TTFA. Double-staining of cells was performed with propidium iodide (PI) and Annexin V (AV) in order to quantify necrotic (PI+/AV- cells), apoptotic cells (PI-/AV+ cells), and late-apoptotic and necrotic cells (PI+/AV+) by flow cytometry. (**b,c**) Characterization of TTFA-induced cell death by caspase inhibition. HeLa cells were treated for 24h with the different complex II inhibitors 3NP or TTFA at the indicated concentrations in the absence or presence of the pan-caspase inhibitor zVAD-fmk 100μM (ZVAD). Then, apoptotic (**b**) or necrotic and late-apoptotic cells (**c**) were quantified using flow cytometry by PI analysis of subG1-DNA content (**b**) and 7-AAD staining (**c**), respectively. #, $p<0.05$.

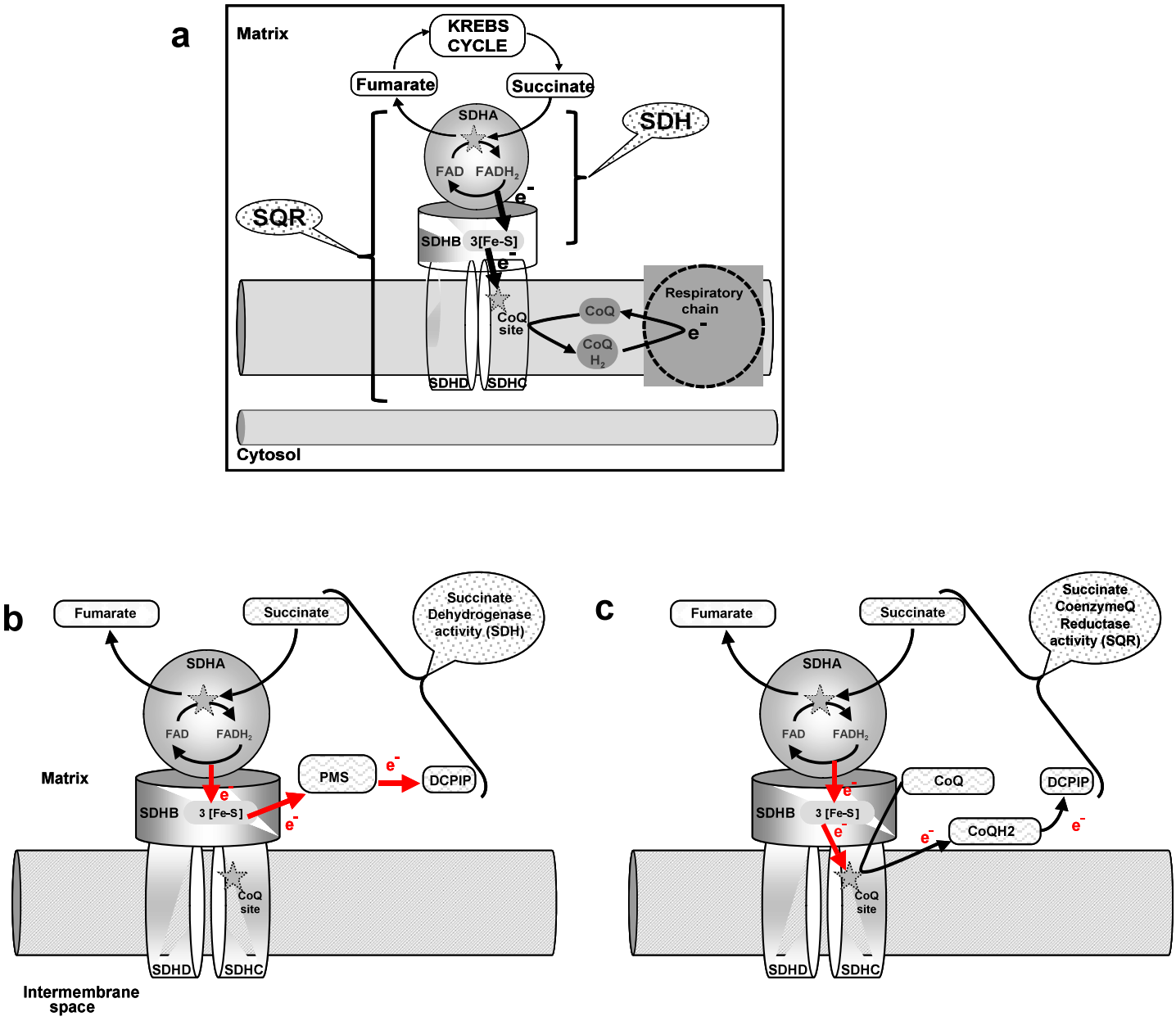
Supplementary figure 7. Schematic representation of the different complex II inhibition models following 3NP/malonate or TTFA treatment. (**a**) 3NP and malonate, two complex II inhibitors at the SDHA level, inhibit both the SDH and the SQR activity and fail to provoke electron leakage, ROS production and apoptosis induction. (**b**) TTFA, a complex II inhibitor targeting the CoQ reduction sites, inhibits partially the SQR activity without impairing the SDH reaction. This specific inhibition allows an electron leak between the two enzymatic sites of complex II, leading to superoxide production and apoptosis induction. Prior inhibition of the SDH activity by direct (3NP, malonate) or indirect (Krebs cycle inhibitors) inhibitors block TTFA-induced complex II uncoupling and subsequent apoptosis induction.

Supplementary figure 8. Integrity of complex II analysed by blue native PAGE. Human 293T cells were incubated for 7h with 10µM As_2O_3 or 1.4µM doxorubicin. Whole cell lysates were prepared and their proteins solubilized as described in *Materials and Methods*. Equal amounts of protein were loaded onto a native gel, blotted onto a membrane, which was probed for SDHA.

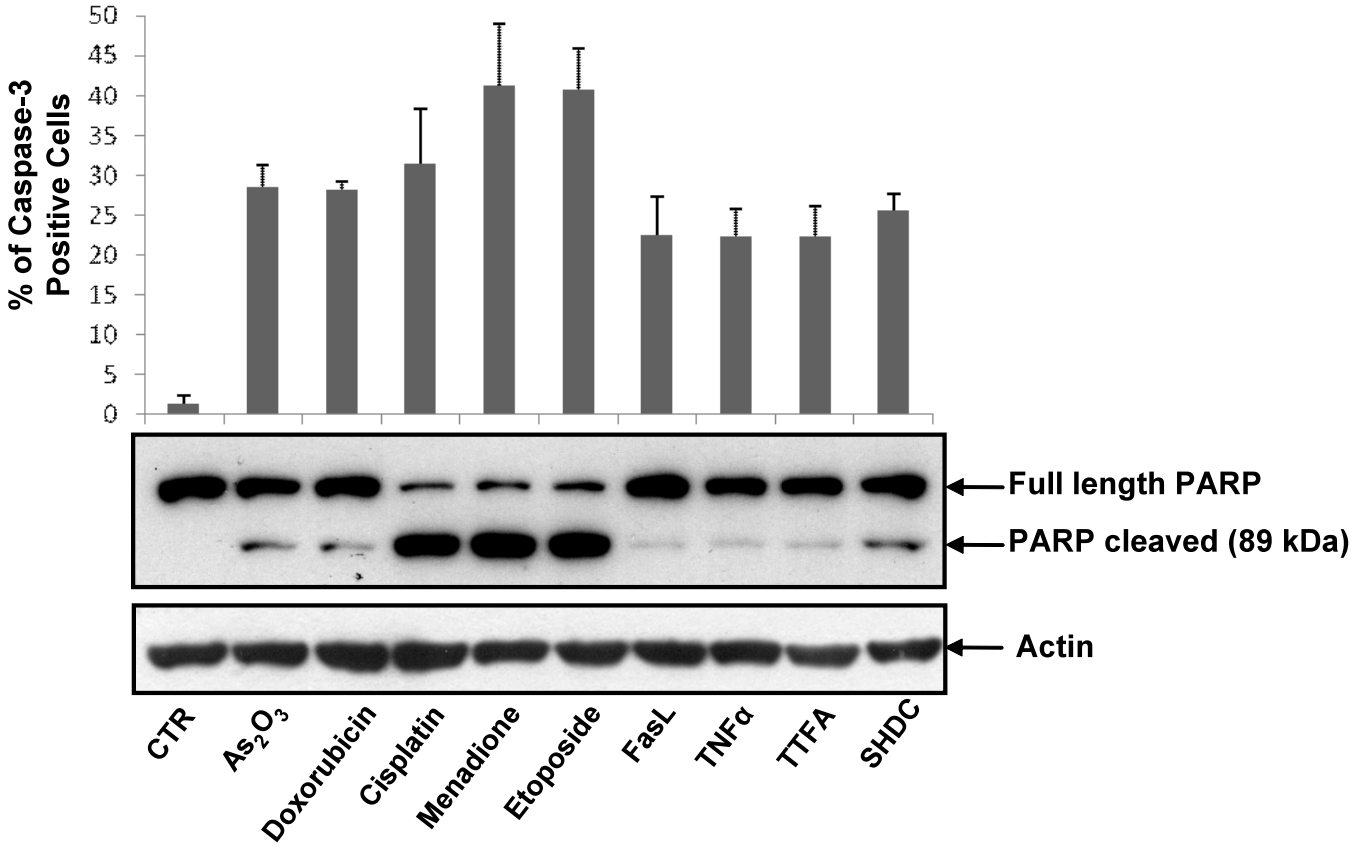
Supplementary figure 9. Flow cytometry representations of the cell population characteristics observed during pHi analysis. HeLa cells were treated for 7h in the absence (**a**) or presence of EIPA (20µM) (**b**), an inhibitor of NHE1 used as a positive control for cytosolic acidification, or treated with doxorubicin (1.4µM) (**c**). Cells were collected and incubated with the pH-sensitive fluorescent probe carboxy-SNARF-1. pHi-related fluorescence ratios FL3-H (geometric mean)/FL2-H (geometric mean) were analysed by flow cytometry and converted into pH values, as described in *Material and Methods*. For each condition, density plots showing the cell population distribution according to FL2-H and FL3-H fluorescence

intensities are represented as well as the FL2-H and FL3-H fluorescence peak distributions. Quadrants and distribution percentages were drawn on density plots. On the histograms, for each peak, the percentage of robust coefficient of variation (Robust CV) was calculated in order to statistically assess the normality of the cell population distribution (Niepel, M et al, Current Opinion in Chemical Biology, 2009).

Supplementary figure 10. Luciferase expression has no effect on the sensitivity of the cells for apoptosis. Hela cells were transfected with an expression plasmid for luciferase for 24h or left untransfected and were then treated for 24h with either Doxorubicin (1.4µM) or TTFA (1mM). After treatment, apoptotic cells were quantified by flow cytometry using PI staining of the Sub-G1 population.



Supplementary figure 1



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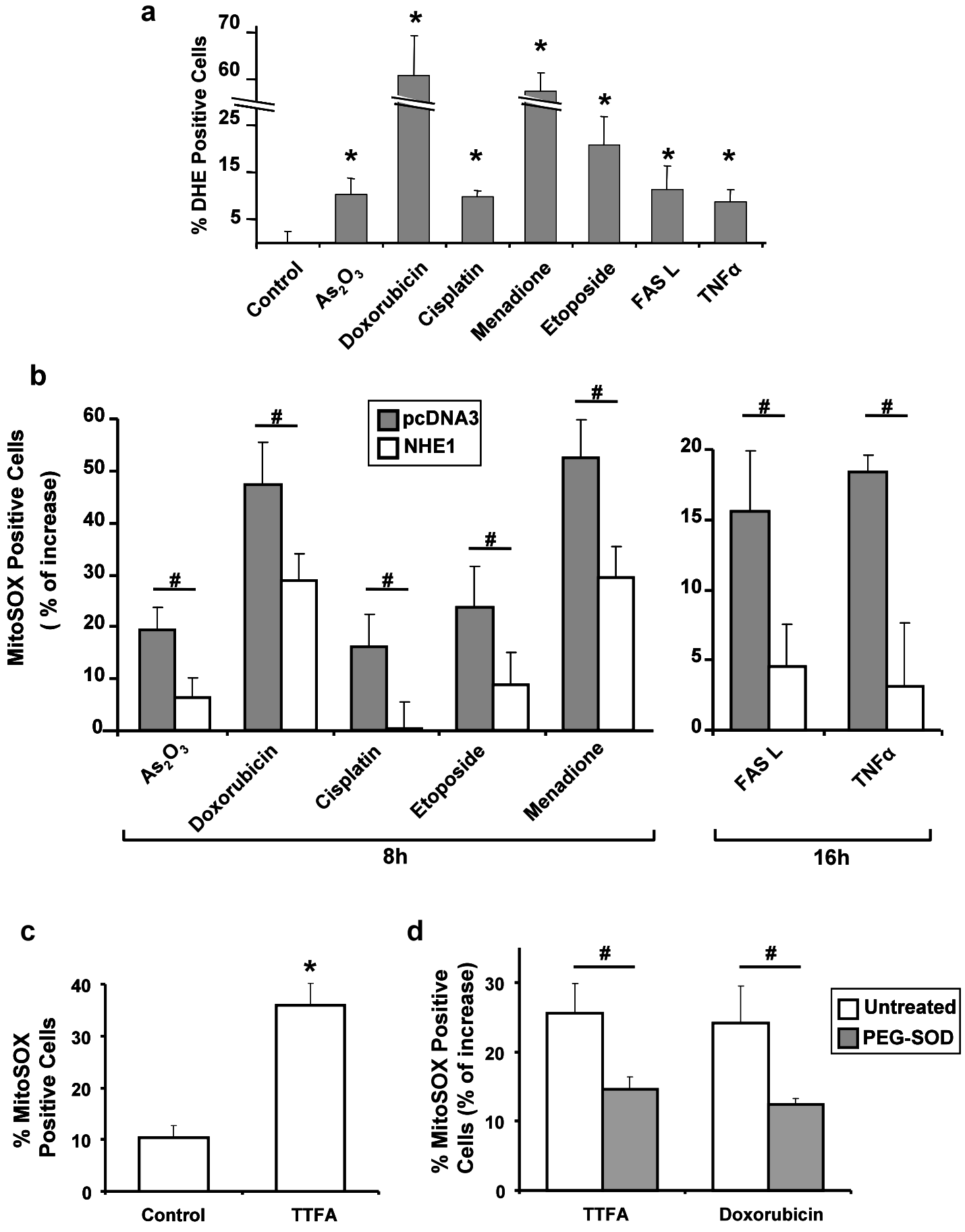

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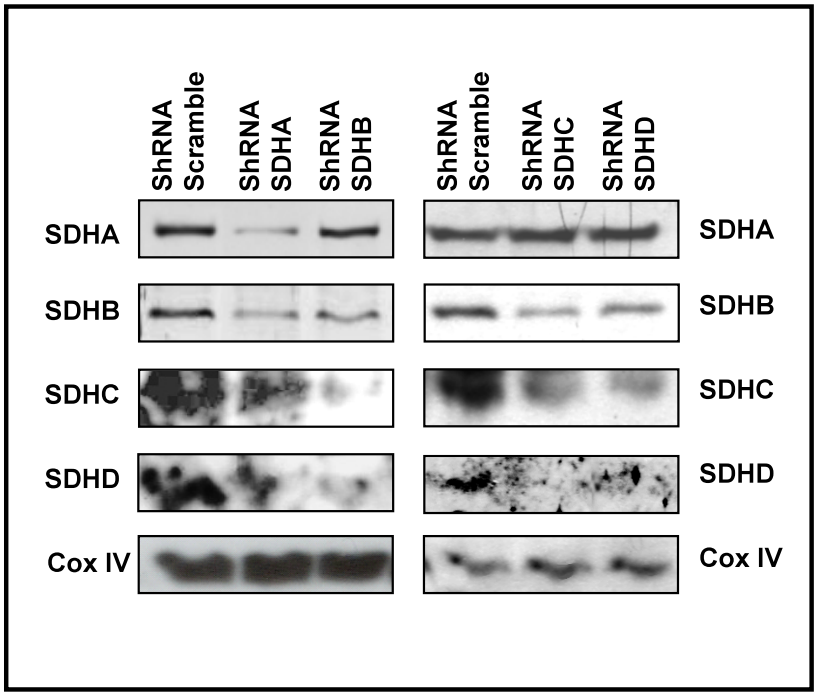

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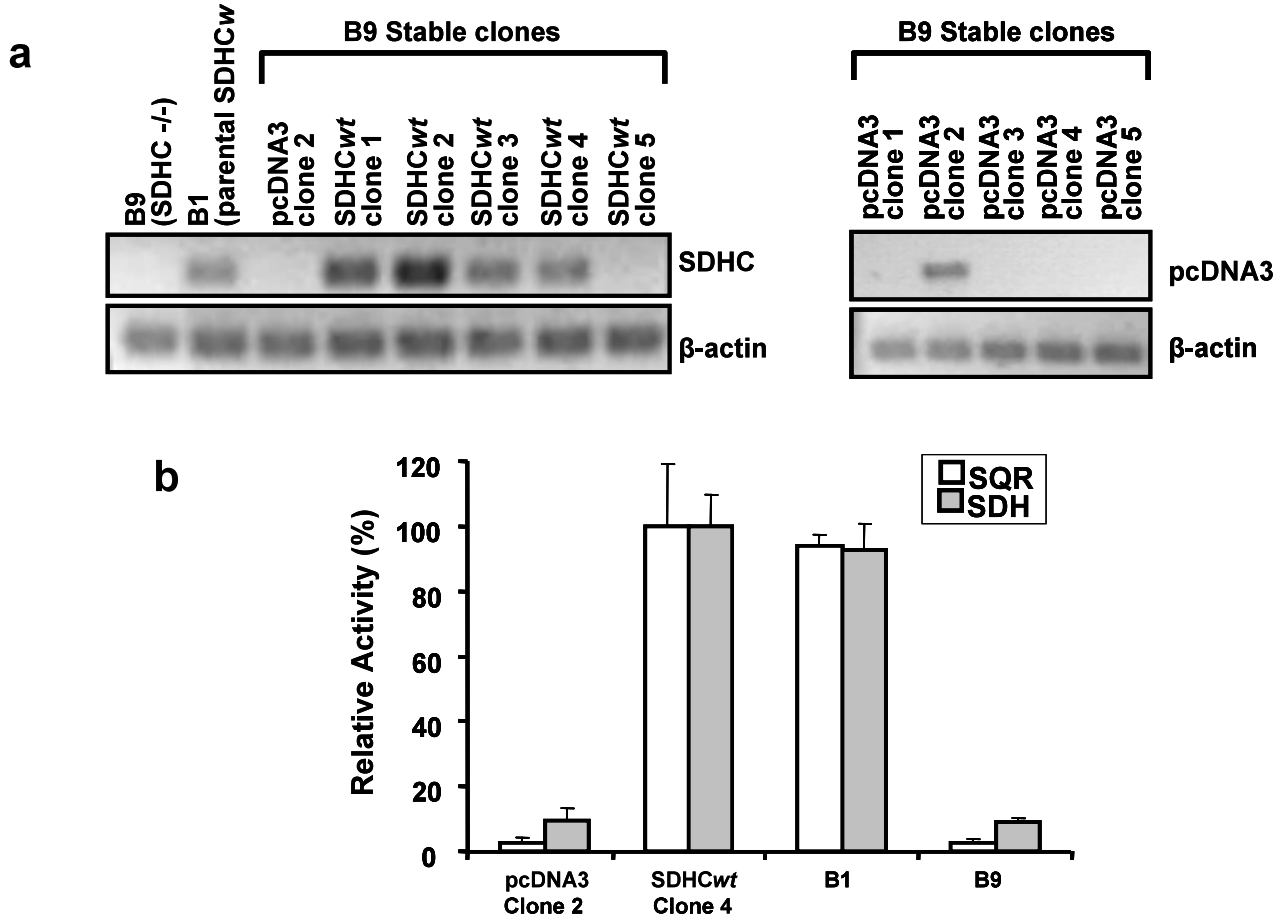

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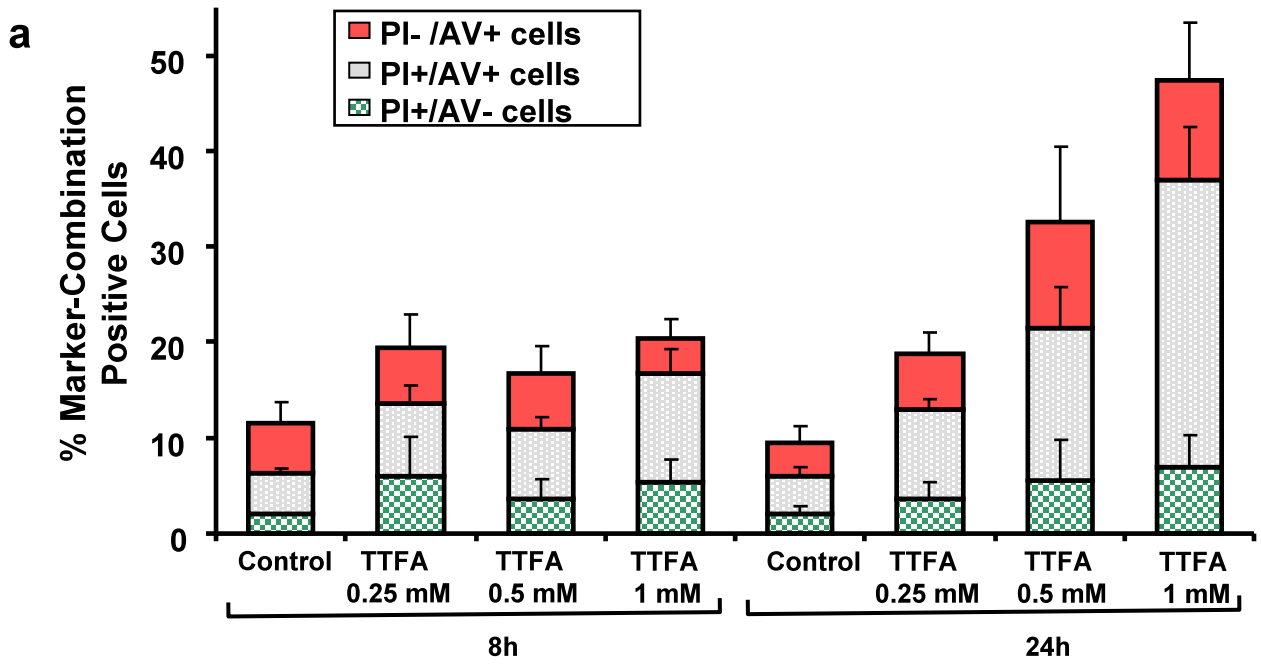

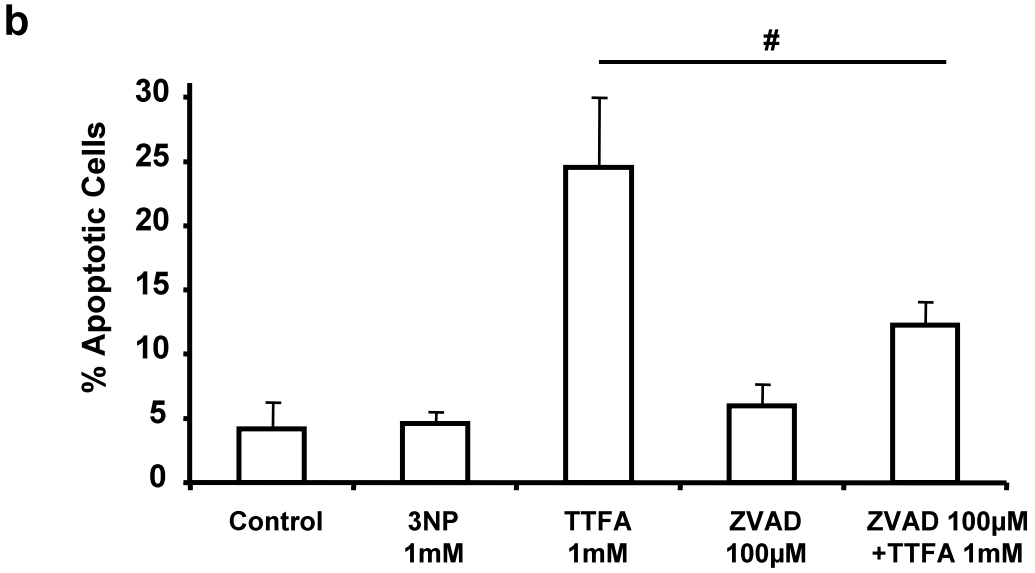

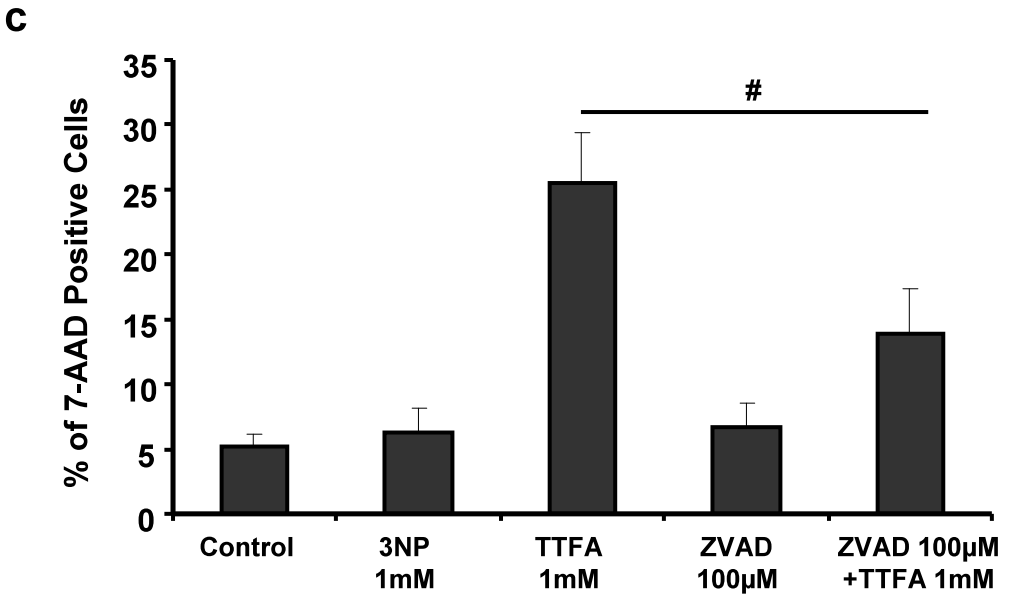

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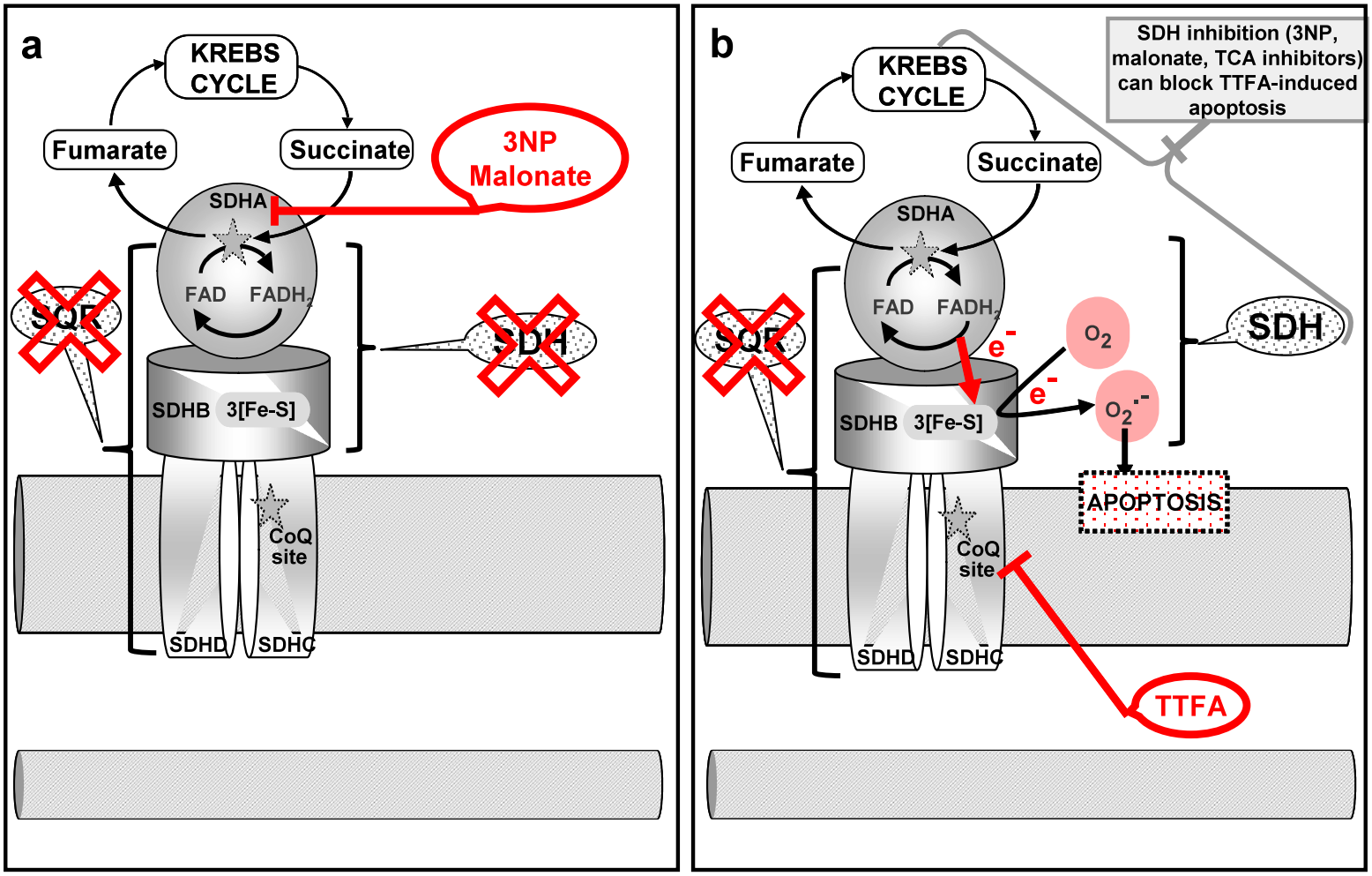

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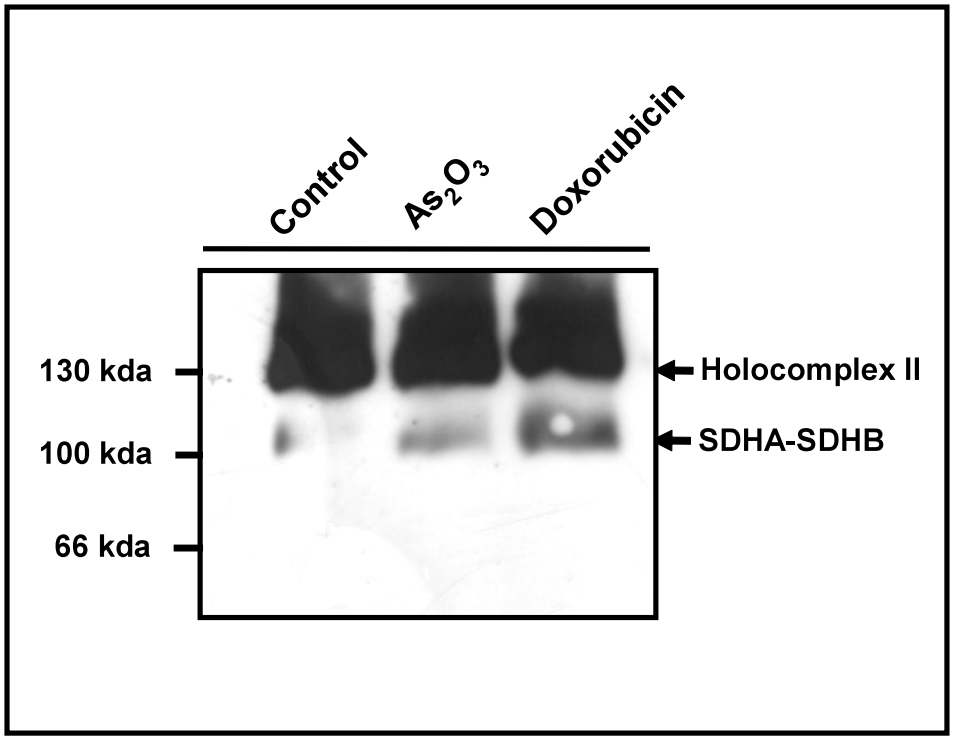
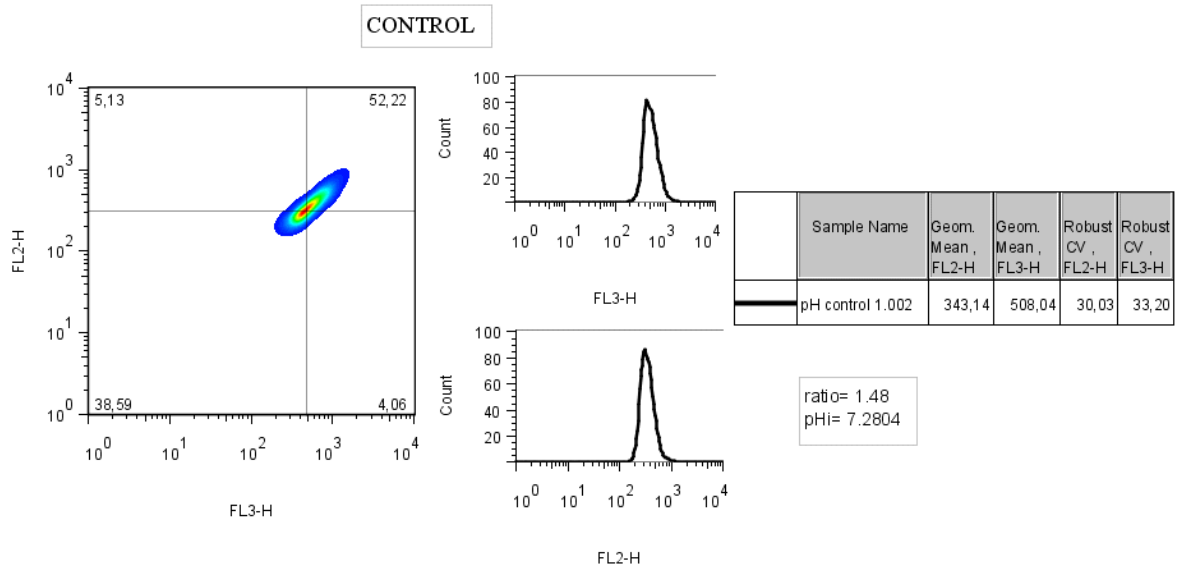


Supplementary figure 8

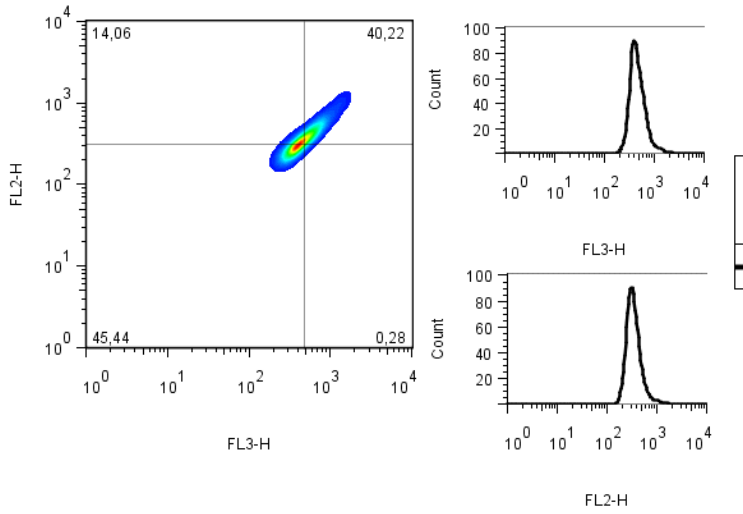
a



	Sample Name	Geom. Mean , FL2-H	Geom. Mean , FL3-H	Robust CV , FL2-H	Robust CV , FL3-H
	pH control 1.002	343,14	508,04	30,03	33,20

ratio= 1.48
pHi= 7.2804

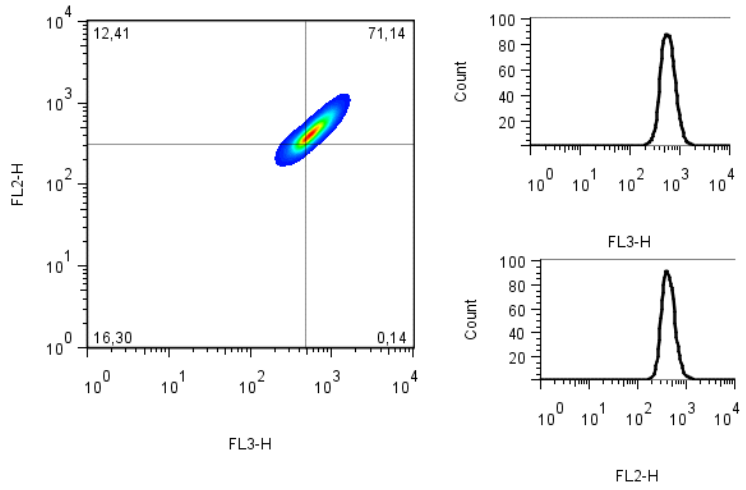
b



	Sample Name	Geom. Mean , FL2-H	Geom. Mean , FL3-H	Robust CV , FL2-H	Robust CV , FL3-H
	pH EIPA 2.007	339,82	455,12	31,27	34,64

ratio= 1.34
pHi= 7.026

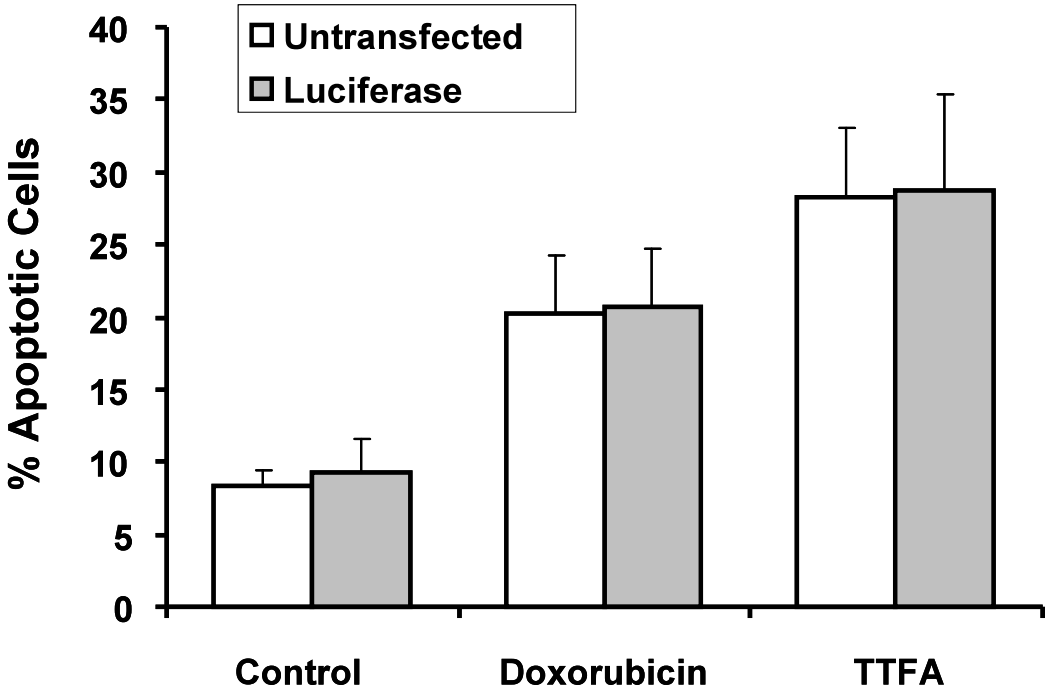
c



	Sample Name	Geom. Mean , FL2-H	Geom. Mean , FL3-H	Robust CV , FL2-H	Robust CV , FL3-H
	pH doxo 2.028	425,45	570,72	30,76	33,78

ratio= 1.34
pHi= 7.029

Supplementary figure 9



Supplementary figure 10