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Author(s)	Hassan, Mohamed K.; Watari, Hidemichi; Salah-eldin, Alaa-eldin et al.
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Histone Deacetylase Inhibitors Sensitize Lung Cancer Cells to Hyperthermia: Involvement of Ku70/SirT-1 in Thermo-Protection

Mohamed K. Hassan^{1,2*}, Hidemichi Watari^{1*}, Alaa-eldin Salah-eldin^{3a}, Ahmed S. Sultan^{4a,b}, Zainab Mohamed¹, Yoichiro Fujioka⁵, Yusuke Ohba⁵, Noriaki Sakuragi¹

1 Department of Obstetrics and Gynecology, Hokkaido University, Graduate School of Medicine, Sapporo, Japan, **2** Biotechnology program, Department of Zoology, Faculty of Science, Port Said University, Port Said, Egypt, **3** Department of Zoology, Aswan Faculty of Science, Aswan University, Aswan, Egypt, **4** Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC, United States of America, **5** Department of Cell Physiology, Hokkaido University, Graduate School of Medicine, Sapporo, Japan

Abstract

This study describes the sensitization mechanism to thermal stress by histone deacetylase inhibitors (HDACIs) in lung cancer cells and shows that Ku70, based on its acetylation status, mediates the protection of lung cancer from hyperthermia (42.5°C, 1–6 hrs). Ku70 regulates apoptosis by sequestering pro-apoptotic Bax. However, its role in thermal stress is not fully understood. The findings showed that, pre-treating lung cancer cells with HDACIs, nicotinamide (NM) or Trichostatin A (TsA) or both significantly enhanced hyperthermia-induced Bax-dependent apoptosis in PC-10 cells. We found that hyperthermia induces SirT-1, Sirtuin, upregulation but not HDAC6 or SirT-3, therefore transfection with dominant negative SirT-1 (Y/H) also eliminated the protection and resulted in more cell death by hyperthermia, in H1299 cells through Bax activation. Hyperthermia alone primed lung cancer cells to apoptosis without prominent death. After hyperthermia Bax was upregulated, Bcl-2 was downregulated, the Bax/Bcl-2 ratio was inversed and Bax/Bcl-2 heterodimer was dissociated. Although hyperthermia did not affect total Ku70 expression level, it stimulated Ku70 deacetylation, which in turn could bind more Bax in the PC-10 cells. These findings suggest an escape mechanism from hyperthermia-induced Bax activation. To verify the role of Ku70 in this protection mechanism, Ku70 was silenced by siRNA. Ku70 silencing significantly sensitized the lung cancer cells to hyperthermia. The Ku70 KD cells underwent cytotoxic G1 arrest and caspase-dependant apoptosis when compared to scrambled transfectants which showed only G2/M cytostatic arrest in the cell lines investigated, suggesting an additional cell cycle-dependent, novel, role of Ku70 in protection from hyperthermia. Taken together, our data show a Ku70-dependent protection mechanism from hyperthermia. Targeting Ku70 and/or its acetylation during hyperthermia may represent a promising therapeutic approach for lung cancer.

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* E-mail: mohamedkamel24@yahoo.com (MKH); watarih@med.hokudai.ac.jp (HW)

^a Current address: Medical Laboratories Department, College of Science in AlZulfi, Majmaah University, Majmaah, Saudi Arabia

^b Current address: Biochemistry Dept., Faculty of Science, Alexandria University, Alexandria, Egypt

Introduction

A long-standing research interest has been targeted the specific mechanisms responsible for the development of cancer cell resistance to different therapies. Targeting these mechanisms may enhance the specific destruction of cancer cells. Hyperthermia is a modality used in the clinical setting, for the treatment of many cancers; it is usually used in combination with radiotherapy and/or chemotherapy [1,2]. However, a significant obstacle to the effectiveness of hyperthermia is the development of cellular resistance, which blocks apoptotic signaling and enhances cell survival [3,4]. This resistance causes limitation of apoptosis after hyperthermia [5,6]. Thus, the identification of the mechanisms responsible for the development of thermo-resistance in cancer cells, might help improve specific targeting to enhance cellular

sensitivity treatment outcomes to hyperthermia. Resistance to apoptosis is a common characteristic of cancer cells [3,7]. Apoptosis is induced by, extrinsic and intrinsic pathways [8]. Binding of ligands to a death receptor activates the extrinsic pathway; the intrinsic pathway is activated by cell stress, such as DNA damage. The Bcl-2 protein family regulates the intrinsic pathway; it influences the permeability of the outer mitochondrial membrane [9]. Members of the Bcl-2 family are divided into proapoptotic proteins such as Bax, Bak, and Bok, and anti-apoptotic proteins including Bcl-2, Bcl-xL, Bcl-w, and Mcl-1 [10–13].

Accumulation of Bcl-2 and Bcl-xL can protect cells from apoptosis, promote cell survival and accelerate tumor growth by sequestering pro-apoptotic Bax. Ku70 is another anti-apoptotic molecule; it naturally binds Bax, sequestering it from activation or

mitochondrial translocation in unstressed cells [14,15]. Ku70 is one of the components of the Ku70/Ku80 heterodimer that is involved in DNA damage repair [16]. Acetylation of two critical lysines, on the carboxyl terminus of Ku70, regulates the binding/dissociation to Bax and this affects the subsequent sensitivity of the cell to apoptotic stimuli [14]. Only deacetylated Ku70 can bind to Bax. High expression of Ku70 in cancer cells would enhance DNA repair ability and reduce Bax-mediated apoptosis; therefore, Ku70 might play a role in treatment resistance. The apoptosis-related activity of Ku70 is independent of its role in DNA repair [17]. The Ku70 acetylation/deacetylation cycle is regulated by histone acetyltransferases and histone deacetylases (HDACs). Ku70 is a target of some members of class I/II HDAC and class III HDAC [18,19]. The HDAC family of proteins is divided into two categories: zinc-dependent enzymes (HDAC1-11), subdivided into class I and class II which are inhibited by Trichostatin A (TSA) and NAD⁺-dependent enzymes (class III; SIRT1-7) which is inhibited by nicotinamide (NAM). More precisely, SirT-1, a member of the class III HDACs, plays a crucial role in Ku70 deacetylation, which enhances the protection of cells from Bax during caloric restriction [19]. The majority of cancer cells over-express SirT1 [20]. Thus, targeting the Ku70-dependent protection from apoptosis, by HDAC inhibitors that inhibit SirT-1, could be an effective strategy for sensitizing cancer cells to different therapies. In this study, our model is that lung cancer cells are significantly killed by hyperthermia when pretreated with HDACIs compared with hyperthermia only. SirT-1 and its target, Ku70, are central to the mechanism by which lung cancer cells can escape thermal-induced death. Changes in the activity of Bax, Ku70 acetylation and the cell cycle were studied during exposure to hyperthermia. In addition, the efficiency of sequence specific targeting of Ku70, using siRNA, were also studied with regard to sensitizing lung cancer cells to hyperthermia. Ku70 appears to play a crucial role in the protection of cells from hyperthermia probably by sequestering up-regulated Bax.

Materials and Methods

Cell lines

Two human, non-small cell lung carcinoma cell lines: PC-10 [21], and H1299, were purchased (American Type Culture Collection, ATCC) and cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Sigma, St. Louis, MO, USA) (complete medium) in a humidified atmosphere of 5% CO₂ at 37°C. The cultured medium was replaced by fresh complete medium every three days.

Antibodies and reagents

Anti-human Bax rabbit polyclonal antibody (pAb) and anti-human Ku70 mouse monoclonal antibody (mAb) were purchased from BD Bioscience (Erembodegem, Belgium); another anti-human Ku70 mouse mAb for immunoprecipitation, anti-human HDAC-6 and anti-human SirT-3 rabbit (pAb) were purchased from Abcam (Cambridge, UK); anti-human Ku80 mouse mAb from Signal Transduction (USA), anti human SirT-1 and anti pan-K (Acetylated lysine) from Upstate (Upstate Biotechnology, Lake Placid, NY, USA); anti human Bcl-2 mouse mAb was purchased from DAKO (Glostrup, Denmark). Detection by immunoblotting was carried out with anti-mouse or anti-rabbit secondary HRP-conjugated Antibodies (Dako) diluted at 1: 2,000. Immunofluorescence staining was performed using anti-rabbit FITC-conjugated secondary Antibodies (Dako) diluted at 1: 50. Histone deacetylase inhibitors (HDACIs), Nicotinamide (NAM) and Trichostatin (TSA) were purchased from Wako Chemicals, Japan.

HDACI treatment optimization

Each HDACI used was screened for its sub-lethal dose in a pilot experiment. Cell viability was evaluated using the MTT assay, with or without DMSO only, and the addition of different doses of each of the HDACIs; 300 nM of TSA and 20 mM of NAM were chosen as non-toxic doses and further used in the subsequent experiments.

Heat treatment

The cells attached to the bottom of the culture dishes, were pre-culture for 48 h, and were then incubated in a humidified atmosphere of 5% CO₂ at either 37.0°C (control) or 42.5°C for 1–9 h.

Flow cytometry, cell cycle analysis and Annexin V staining

After exposure to hyperthermia, the cells were re-incubated at 37°C for 0 h, 24 h, or 48 h. Then, the cell cycle phases were analyzed. Briefly, the cells were fixed with 70% ethanol at 4°C overnight. After washing with Ca²⁺-Mg²⁺-free Dulbecco's PBS, the cells were treated with 0.1 mg/ml RNase (Type I-A; Sigma, St Louis, MO, USA) and then stained with 100 µg/ml propidium iodide (PI; Sigma), in the dark, at room temperature for 20 min. After passing through a 40 nm nylon mesh, the samples were kept on ice until measurements. The data obtained, using the FACS calibrator, were used to analyze the cell cycle phase proportions with ModFit software. A cell fraction of DNA, below the sub-G0/G1 peak, indicated apoptotic cells; DNA histograms were used for their estimation.

For Annexin V staining, the cells were directly stained with PI and Annexin-V Floures (Roche) for 10 min and then washed with incubation buffer. The cells were identified with a FACS calibrator after setting the voltage using non-treated stained control cells. The cells were analyzed using Cell Quest software and classified into four different stages: unstained living cells, early age apoptotic cells stained only with Annexin-V, middle age apoptotic cells doubly stained, and late age apoptotic and necrotic cells stained with PI only.

Immunoprecipitation

The cells (5×10⁶/dish) were washed with cold PBS, lysed on ice in RIPA lysis buffer (50 mM Tris, pH 7, 150 mM NaCl, 0.5% sodium deoxycholate and .1% NP-40)(NP-40; Nacalai Teque, Kyoto, Japan) or CHAPS lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1% CHAPS) [22] supplemented with a protease inhibitor cocktail (Sigma) for 1 h, and then centrifuged at 15,000 rpm for 10 min. The supernatant was mixed with protein A-Sepharose (for pAb) or protein G-Sepharose (for mAb) (Amersham Pharmacia Biotech, Piscataway, NJ, USA), pre-swelled in PBS and pre-coated with the desired antibody against Bax, Ku70, Acetylated lysine or SirT-1 by gently shaking for 1 h at 4°C and centrifuged for 1 min at 3000 rpm. After washing with lysis buffer, the immunocomplex was fractionated by SDS-PAGE (10–12% gels) and then underwent Western blot analysis.

Western Blot Analysis

After SDS-PAGE, the proteins were transferred to a PVDF membrane (Amersham, Buckinghamshire, UK). The membrane was blocked at 4°C overnight with blocking buffer. The membrane was incubated for 1 h at room temperature with the desired Ab. After washing three times with TPBS, the membranes were incubated for 1 h with secondary Ab, at room temperature, followed by three washes with TPBS. The membrane was

developed using ECL reagents (Amersham). The chemiluminescence was visualized with a polaroid camera (Amersham Pharmacia) and quantified using densitometry.

siRNA design and transfection

siRNA oligomers against Ku70 mRNA (Ku70-siRNA) and a control sequence that did not match any gene sequence (Cont-siRNA) were either purchased as a validated one (Ambion, USA; Ku70-siRNA-2 and cont-siRNA-2, respectively) or designed by the investigators and then synthesized by Ambion according to the following sequence: Ku-siRNA, 5_UUCUCUUGGUAACUUUCCCTdT_3 (Ku70-siRNA-1) and 3_dTdTAAAGGAACCAUUGAAAGGG_5; Cont-siRNA, 5_GCG CGC UUU GUA GGA UUC GdTdT_3 and 3_dTdTTCGCGCG AAA CAU CCU AAG C_5 (cont-siRNA-1). This sequence targeting was validated [23]. siRNA oligomers against Bax mRNA (Bax-si) was purchased from Qiagen (Cat No. SI04948202). The Bax-si, the Ku70-siRNAs or cont-siRNAs were transfected into the lung cancer cells (10^5 cells/60-mm dish) using SiPORT *Neofex* (Ambion; USA) to a final concentration of 200 nM, two times. One day after the last transfection, the cells were trypsinized and plated onto 60 mm dishes (50,000 cells per dish) in triplicate. After cell attachment, the cells were exposed to hyperthermia at the indicated time intervals according to the experimental design. Each experiment was repeated at least three independent times for reproducibility and statistical calculation.

Statistical evaluation

Statistical analyses were performed using Minitab Release (Ver.12). Data are expressed as the mean $\pm$ S.E.M. One way analysis of variance (ANOVA) was used to assess the statistical significance between means. Differences between means were considered significant at p-values less than 0.05.

Results

HDACIs significantly facilitated cell death with hyperthermia

As a fact, SirT-1, a human deacetylase, was specifically targeted by small molecules known as HDACIs, for example NAM. Moreover, Ku70 deacetylation was inhibited by inhibitor molecules that target the class I/II HDAC; for example TsA. We first pre-treated the PC-10 cells with different HDACIs, NAM (20 mM), TsA (300 nM) or both, for 4 h right before exposure to hyperthermia. This combined treatment significantly increased apoptosis, more than two folds, compared with hyperthermia treatment alone, as observed by phase contrast microscopy (Figure. 1A) and Annexin V staining (Figure. 1B). The Bax expression pattern was analyzed in whole cell lysates from treated and untreated PC-10 cells. Bax expression (21 kDa) was increased by hyperthermia. Interestingly, HDACIs (NAM and TSA) increased the production of 18 kDa Bax, which is known to be a N-terminus truncated form of Bax. Given that Bax activation is induced by either the N-terminus exposure by conformational change (reversible change) or N-terminal truncation (irreversible change), these findings suggest that sustainable enhanced apoptosis with hyperthermia and HDACIs is Bax-dependent and this apoptosis may depend on upregulation of Bax or the release of Bax from antiapoptotic protein(s) to promote apoptosis (Figure. 1C). Importantly, the treatment of lung cancer cells with HDACIs, only at selected doses, had no appreciable toxic effect. Similar effects of HDACIs were observed in the H1299 cells (Figure. S1 and S2). Unexpectedly, the triple combination of NAM, TsA and hyperthermia was less effective in H1299 than

dual combination. Although the exact molecular mechanism of this phenomenon remains obscure, one possible reason is that the triple combination may enhance the division of the surviving cells escaped the challenge of triple treatment, which can yield the production of new daughter cells within the 48 hours post treatment (before annexin V staining detection) and cause the reduction of the annexin V staining percentage finally. To verify that Bax plays the major role in hyperthermia-induced apoptosis after targeting Ku70 deacetylation, Bax was targeted by specific siRNA in PC-10 cells (see materials and methods). Bax was amenable to siRNA transfection (Bax-si) when compared with control scrambled oligo siRNA (cont-si) as detected by western blot analysis (Figure. 1D; upper panel). Again, when Bax-knocked down PC-10 cells were treated with hyperthermia for 6 h in the presence of HDACIs, the hyperthermia-induced cell death was significantly inhibited (Figure. 1D; lower panel). Meanwhile, cell death was not completely blocked under hyperthermia treatment alone (Figure. 1D; lower panel and Figure. S3). This result may indicate the involvement of other pro-apoptotic molecules in the limited cell death induced by hyperthermia.

Effect of hyperthermia on expression of apoptosis related proteins in PC-10 cells

To investigate the effect of hyperthermia on Bax and its major binding molecules, some of the apoptosis-related proteins (Bax, Bcl-2, Ku70 and Ku80) were studied by western blot analysis in a representative cell line, PC-10. Because most of the housekeeping genes are responsive to hyperthermia, thus Ponceau S staining was used to show the loading control. Hyperthermia (42.5°C) for 1–9 h induced quantitative changes in Bax and Bcl-2 expression, with no observable changes in Bcl-xL, Ku70 and Ku80 (Figure. 2A) Bax expression was slightly up-regulated while Bcl-2 expression was down-regulated in PC-10 cells. The Bax/Bcl-2 ratio gradually increased after hyperthermia treatment (Figure. 2B, upper panel). Western blot analysis of Bax and Bcl-2, with different loading amounts of protein (20 μ g, 40 μ g and 60 μ g/lane), after 0 h and 6 h hyperthermia treatment confirmed an increased Bax/Bcl-2 ratio (Figure. 2B, lower panel) as verified densitometrically (using Scion Image software). Similar observations were also obtained in H1299 cells (Figure. S4). Hence, we had much interest to answer the question why cancer cells, with wild-type Bax, which was upregulated, did not show prominent apoptosis after hyperthermia unless HDACIs are added. Noticeably, by immunocytochemistry Bax and Bcl-2 showed mainly cytosolic localization in the cell lines studied (Figure. S5). Therefore, we moved to study Bax dimerization.

Disturbance of Bax heterodimerization by hyperthermia

In unstressed cells, Bax heterodimerizes with many, anti-apoptotic, partner molecules; it homodimerizes under stress to induce apoptosis. To study the effect of hyperthermia on Bax dimerization, Bax was immunoprecipitated from the PC-10 cell lysate after 6 h exposure to hyperthermia. Figure 3A, shows decreased Bax/Bcl-2 heterodimer formation; the Bax/Bcl-xL heterodimer was not affected by hyperthermia, according to the results of the Western blot analysis. Of note, was that the amount of Ku70 bound to Bax increased with increased exposure time to hyperthermia. The Ku70 immunoprecipitation confirmed enhanced Bax/Ku70 binding after 6 h exposure to hyperthermia, while Ku70/Ku80 binding showed no change (Figure. 3B). Similar observation was detected when CHAPS buffer was used for the immunoprecipitation experiments (Figure. S6)

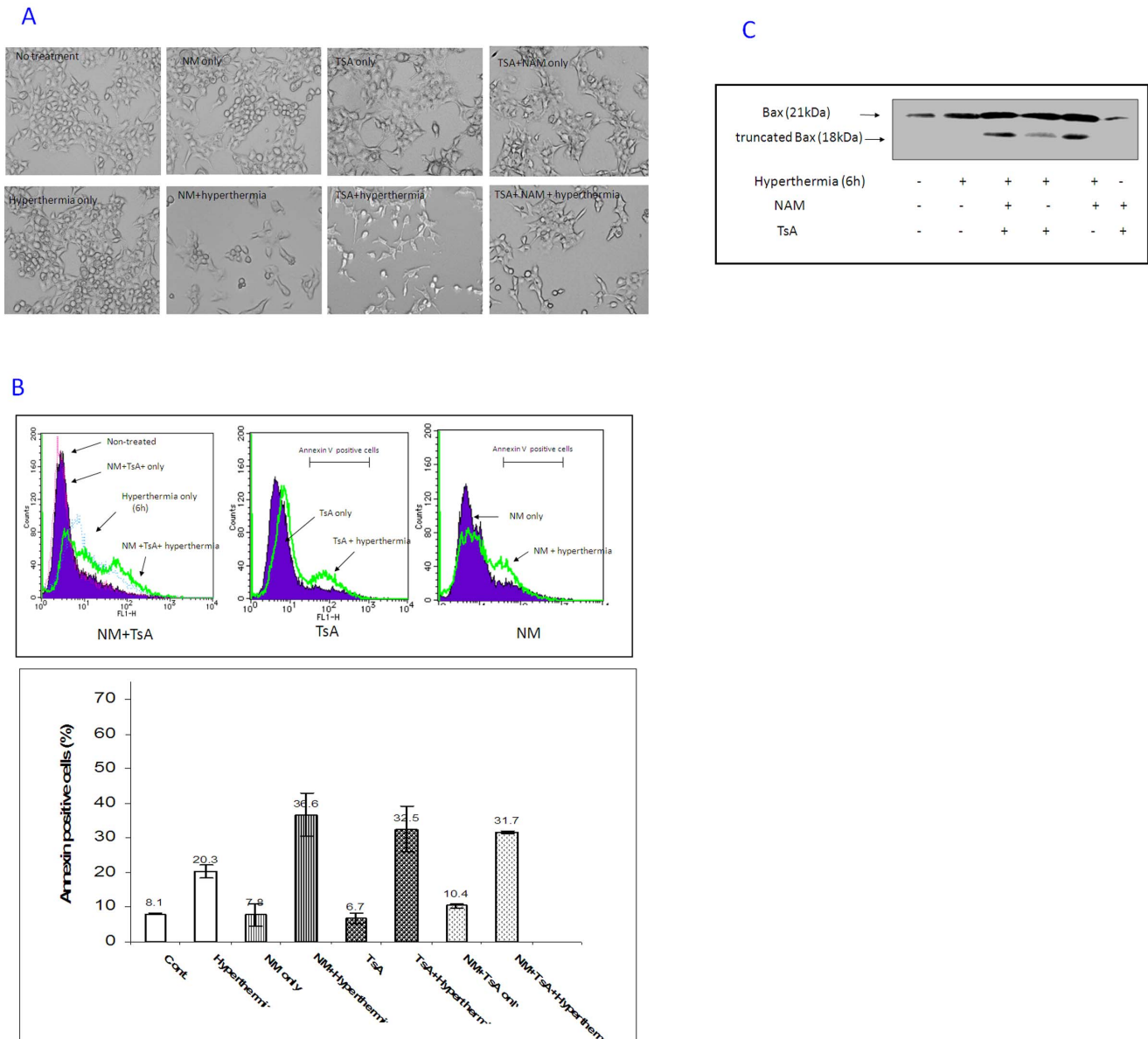


Figure 1. **A.** Phase contrast photographs of PC-10 cells, two days after treatment with HDACIs then- hyperthermia for 6 h. The image showed limited number of recovered cells as well as the rounded and floating apoptotic cells in the colonies pre-treated with HDACIs then hyperthermia. **B.** Pre-treatment of PC-10 cells with HDACIs significantly increased hyperthermia-induced apoptosis. Comparative cytograms show Annexin V staining after hyperthermia in PC-10 cells pre-treated with nictotinamide (20 mM), Trichostatin A (300 nM) or both (upper panel). Summary of average annexin V results is concluded (lower panel). **C.** Bax expression levels, by Western analysis, in PC-10 cells after hyperthermia (6 h) pre-treated with different HDACIs. Lower band (18 KDa) indicates truncated, active, Bax only increased when cells treated with combination of HDACIs and hyperthermia. **D.** A representative western blot shows the amendment of Bax for the specific siRNA used. PC-10 cells were either transfected with Bax-si or cont-si twice. Actin immunoblot was used as a loading control (upper panel). Significant reduction of annexin V staining after hyperthermia and HDACIs in Bax KD cells compared with control(s) indicating that Bax is the key proapoptotic player in the double treatment-induced apoptosis (lower panel). Each data point represents the mean of three experiments; bars denote SD; ** indicates difference from control transfectant at $P < 0.01$. doi:10.1371/journal.pone.0094213.g001

Hyperthermia reduced Ku70 acetylation in the PC-10 cells

Acetylation of either K539 or K542 at the Ku70 C-terminal linker is sufficient to completely block Ku70 suppression of Bax-mediated apoptosis [14]. The effects of hyperthermia on the Ku70 acetylation status were therefore investigated by probing the blot of Ku70 immunoprecipitant with antiacetylated lysine Ab (Figure. 4A); the results show a significant reduction in Ku70 acetylation after 6 h exposure to hyperthermia in PC-10 cells. In

addition, the amount of Ku70 detected, in the acetylated protein immunoprecipitant, decreased in a time-dependent manner (Figure. 4B).

SirT-1 mediates Ku70-dependent cytoprotection from hyperthermia

It is well known that Ku70 acetylation is specifically reversed by SirT-1, a human deacetylase, under mild stress (such as, caloric restriction); Under such conditions, Ku70 sequesters more Bax

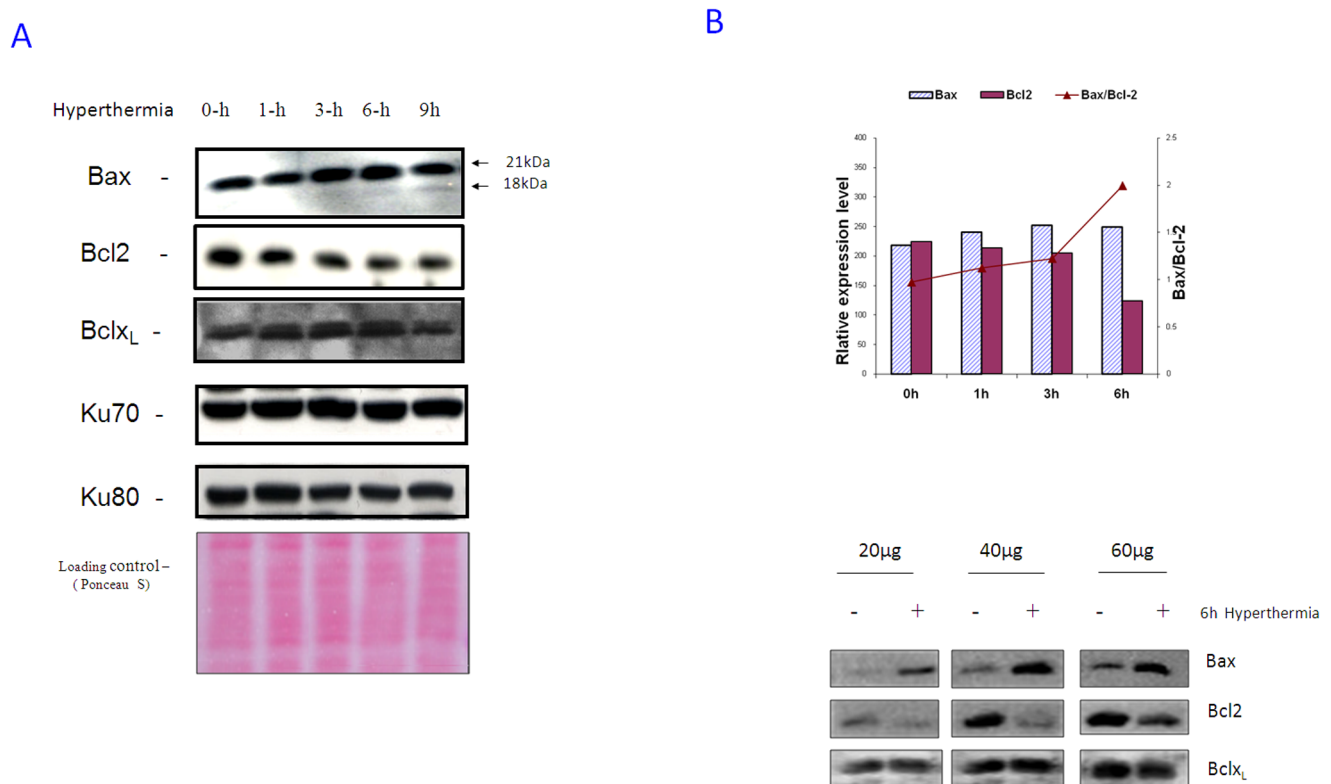


Figure 2. Hyperthermia affects expression levels of different apoptosis-related proteins. *A*. Whole cell extracts were prepared at indicated periods after 42.5°C hyperthermia and Bax, Ku70, Ku80 and Bcl-2 were analyzed by immunoblotting. Slight increase in Bax only after 6 and 9 h hyperthermia and limited Bax activation after 9 h are observable. Hyperthermia reduced Bcl-2 while Bcl-xL and Ku70 had not been clearly affected. Analysis was performed in the same blot so each protein worked as a loading control for the other. A representative Ponceau S staining of the membrane was shown to verify the normalization because most of the basic house-keeping genes (e.g: actin and tubulin) are responding to hyperthermia. *B*. Quantitative analyses of Bcl-2 and Bax protein expression during hyperthermia. Each band was quantified densitometrically. A representative set of Bax/Bcl-2 ratio is shown. Western analysis with different loading amount of PC-10 cells lysates after 6 h hyperthermia verifies the change in the Bax/Bcl-2 ratio, while Bcl-xL expression was used as a loading control from same blot.
doi:10.1371/journal.pone.0094213.g002

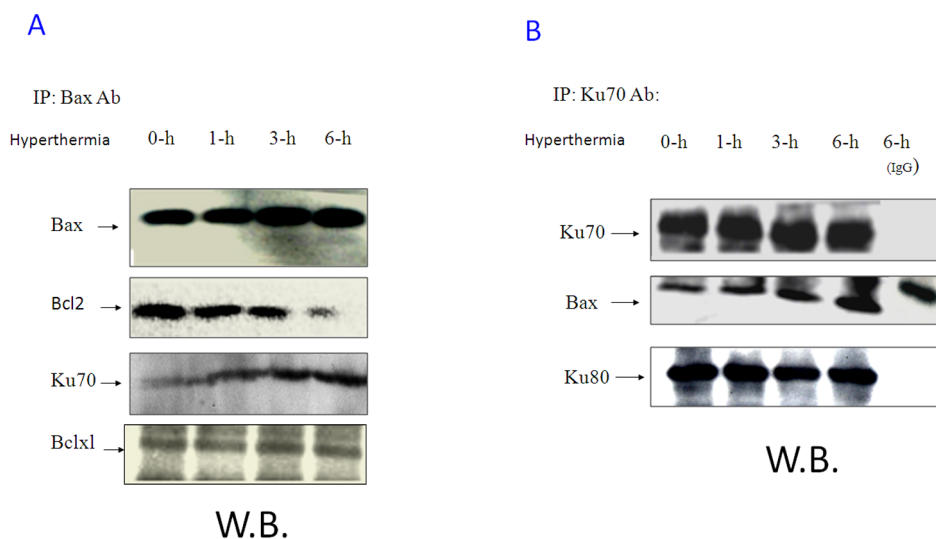


Figure 3. Hyperthermia modulates Bax association with Ku70 and Bcl-2. *A*. PC-10 cells were incubated at 42.5°C for 0, 1, 3 and 6 h. Bax was co-immunoprecipitated from 2 mg total protein and Bcl-xL, Bcl-2 and Ku70 were detected in the immunoprecipitant by western analysis. Hyperthermia induces Bax up-regulation and Bax dissociation from Bcl2 and enhances association between Bax and Ku70, while no effect on the Bax/Bcl-xL association. In contrast, Ku70 was co-immunoprecipitated from similar cell lysates. Bax and Ku80 are shown in the immunoprecipitant. *B*. After hyperthermia, total Ku70 levels showed no changes, but association between Ku70 and Bax was enhanced.
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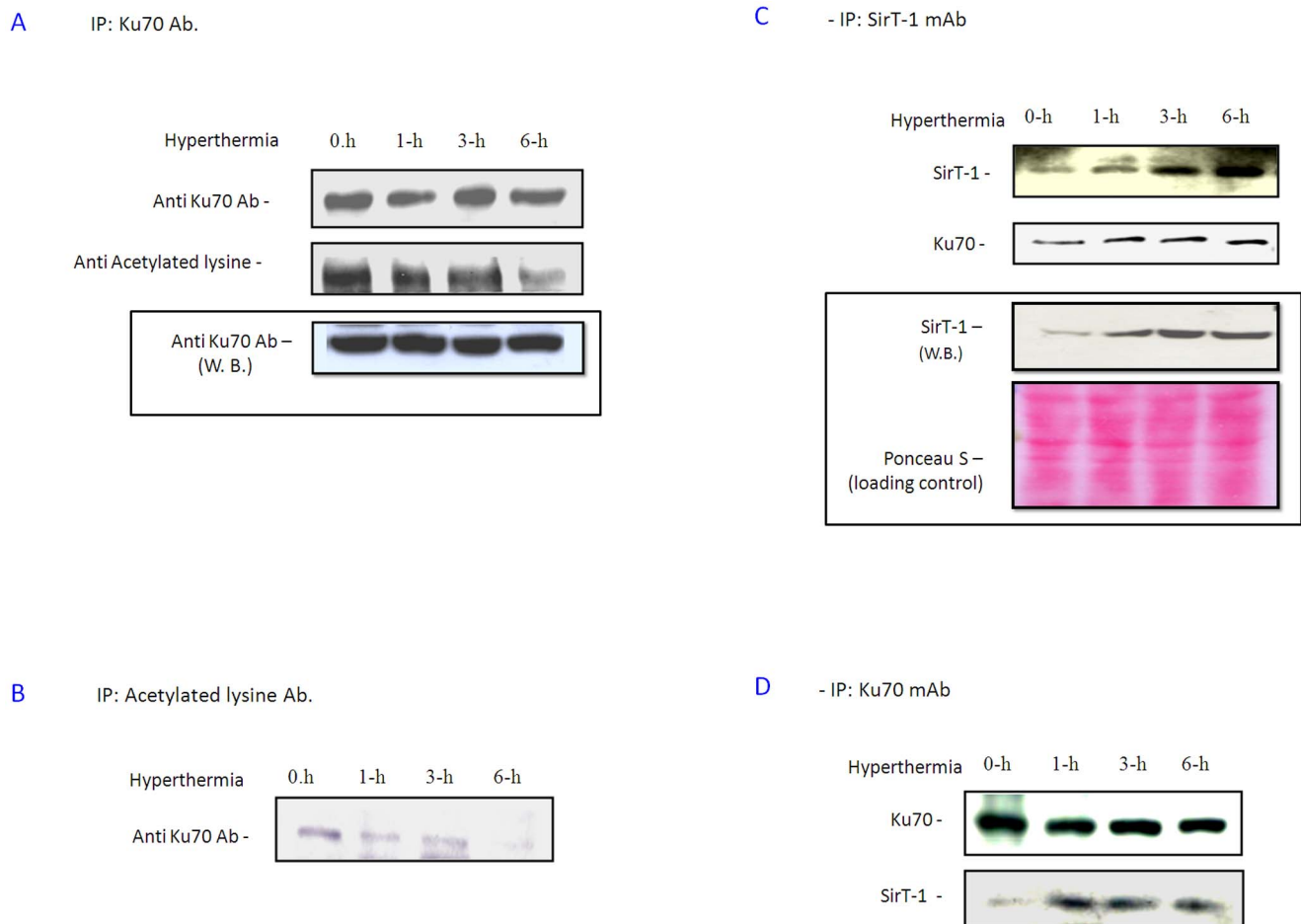


Figure 4. Hyperthermia treatment reduced Ku70 acetylation. **A.** Ku70 was immunoprecipitated from equal protein amounts (3 mg) of PC-10 cell lysat after hyperthermia at indicated time. Ku70 and acetyl Lysine were detected in the immunoprecipitant. Total Ku70 showed no change after 6 h hyperthermia (in put) but acetylated Ku70 was decreased. **B.** All acetylated proteins were immunoprecipitated, fractionated, blotted and Ku70 was detected in the blot. Bands indicated acetylated Ku70 became fainter with increasing hyperthermia time. **C.** Hyperthermia enhanced both Ku70 expression and Ku70/SirT-1 binding. SirT-1 was immunoprecipitated from PC-10 after hyperthermia (0–6 h). Blot indicates SirT-1 up-regulation (in put; lower panel) and enhanced binding to Ku70, in the same blot, was up-regulated (upper panels). **D.** Similar hyperthermia treatment in H1299. Total Ku70 was precipitated. Ku70 and SirT-1 were detected in the immunoprecipitant by Western analysis. SirT-1/Ku70 binding was increased, indicating an enhanced Ku70 deacetylation in lung cancer cells by hyperthermia.
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and protects cells from Bax-mediated apoptosis [19]. To investigate whether acetylation inhibition, with exposure to hyperthermia, was due to activation of SirT-1, we analyzed the SirT-1 expression after exposure to hyperthermia (0–6 h). Western blot analysis revealed that SirT-1 expression was induced by hyperthermia in PC-10 cells (Figure. 4C). Further, SirT-1 was immunoprecipitated from PC-10 whole cell lysates and then subjected to Western blotting analysis. The amount of Ku70 immunoprecipitated with SirT-1 was enhanced by exposure to hyperthermia (Figure. 4C). Similarly, the amount of SirT-1 immunoprecipitated with Ku70 was also enhanced by exposure to hyperthermia (0–6 h) confirming that Ku70/SirT-1 binding was enhanced by hyperthermia in PC-10 cells (Figure. 4D). We speculated that up-regulated SirT-1, under conditions of hyperthermia, binds to Ku70 and changes it from acetylated to deacetylated form, which allows Ku70 to sequester more Bax, either liberated from Bcl2 or newly induced under hyperthermia, to inhibit hyperthermia-induced apoptosis finally. These results explain, at least in part, why HDACIs, such as NAM, can enhance apoptosis by hyperthermia, probably by targeting some HDACs

like SirT-1. Notably, other histone deacetylases including HDAC6 and SirT-3, did not show significant changes in the expression profile after exposure to hyperthermia (Figure. S7). To confirm the above results, H1299 cells (with relatively high transfection efficiency, >50% as determined by Beta gal transfection) were transiently transfected either with wild-type SirT-1 or dominant negative H363Y/SirT-1. Hyperthermia-induced apoptosis was significantly enhanced in the H363Y/SirT-1-transfected cells compared to control vector transfectants (Figure. 5A; $P < 0.01$). Importantly, the wild-type SirT-1-transfected cells showed slight but significant ($P < 0.05$) protection from hyperthermia in the tested cells, indicating that the anti-apoptotic effect of the exogenous SirT-1 is significant but limited. This was likely because the endogenous SirT-1 had triggered most of the spontaneous protection from hyperthermia as indicated by the DNA content experiments (Figure. 5A). Similar results were obtained from the Annexin V staining; H363Y/SirT-1 transfection into H1299 cells significantly enhanced apoptosis after exposure to hyperthermia compared to the empty vector transfectant (Figure. 5B). Notably, neither wild-type SirT-1 nor

dominant negative H363Y/SirT-1 transfection individually showed appreciable changes in the cell cycle.

Targeting Ku70 by siRNA enhanced hyperthermia-induced cell death

To examine whether Ku70 was a key mediator in the aforementioned protection of the cells from hyperthermia, Ku70 mRNA was targeted by sequence specific Ku70-siRNA-1,-2. Ku70 mRNA was amenable to Ku7-siRNA-1 (custom design) and subsequently, the level of protein expression was significantly reduced (dose; 200 nM) in PC-10 cells (Figure. 6A). The control siRNA (cont-siRNA-1) transfection did not affect Ku70 expression. In addition, Ku70-siRNA-2 transfection (see materials and

methods) was confirmed to efficiently knockdown Ku70 (Figure. 6A, lower panel). Next, both the Ku70 knockdown (KD) and control cells were challenged with exposure to hyperthermia. The Ku70 KD PC-10 cells showed enhanced cell death after hyperthermia exposure in a time-dependent manner (Figure. 6B, C) compared to the cont-siRNA transfectant, two days after treatment, as indicated by the FACS analysis (using Ku70-siRNA-1). Similar results were obtained with the use of H1299 cells (using Ku70-siRNA-2 and cont-siRNA-2 (Figure. S8)). These results suggest that Ku70 mediates cytoprotection from hyperthermia exposure and likely plays a key role in hyperthermia-induced apoptosis.

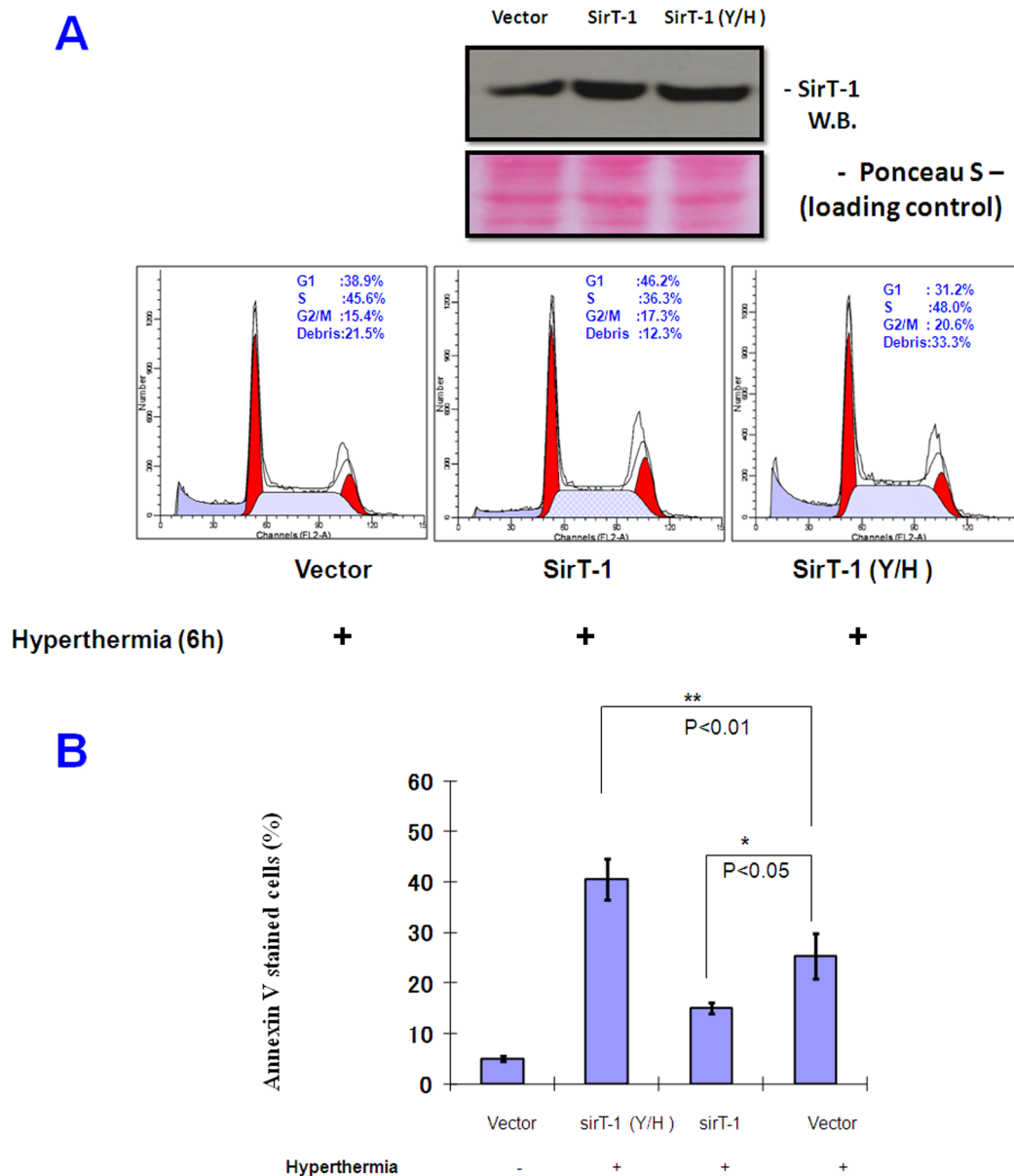


Figure 5. Dominant negative SirT-1 (H363Y) and HDAC1s enhanced hyperthermia-induced cell death. **A.** SirT-1 is involved in the cytoprotection from hyperthermia. Western blot shows the over-expressed SirT-1. FACS analysis of H1299 cells after transient transfection either with wide type SirT-1, dominant negative SirT-1 or FLAG expression vector. H363Y/SirT-1 significantly enhanced hyperthermia-induced cell death while wide type SirT-1 had no significant effect (upper panel). **B.** Results of annexin V staining of H1299 cells, from similar experiment, confirming that H363Y/SirT-1-induced cell death is apoptosis (lower panel). doi:10.1371/journal.pone.0094213.g005

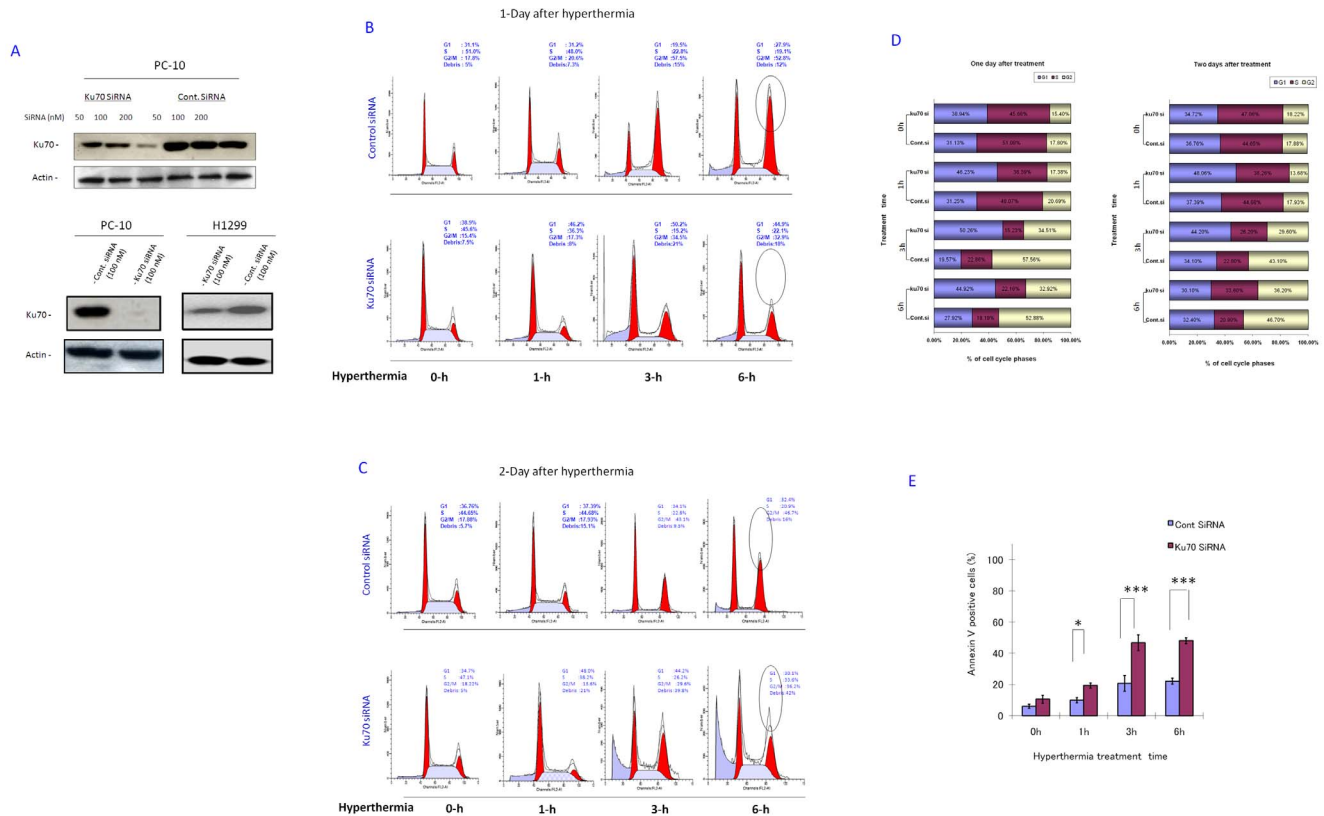


Figure 6. Ku70 knock down enhanced apoptotic cell death after hyperthermia in PC-10. **A.** A representative western blot shows the amendment of Ku70 for both siRNAs used. PC-10 cells and H1299 cells were transfected by one siRNA twice. The western result shows significant knock down by Ku70-siRNA-1,2 comparing with the cont-siRNA-1,-2, respectively. Actin immunoblot was used as a loading control. The experiment was repeated three independent times for reproducibility. PC-10 cells were transfected with Ku70-siRNA-1 or cont-siRNA-1 (200-nM) twice. 24 h after last transfection, equal cell numbers were subcultured for further 24 h, and then treated with hyperthermia for indicated time intervals, and then re-cultured at 37°C for 24 h (B) or 48 h (C). Cells were acquired by FACS analyzer for cell cycle analysis. Results shown are a representative one from, at least, three independent experiments for each time point (24 h and 48 h). **D.** Ku70 is required for cytosstatic arrest by hyperthermia. Histograms show the differential accumulation of cell populations into G2/M phases in cells with or without Ku70, one and two days after 0, 1, 3 and 6 h hyperthermia treatment. Populations in different cell cycle phases, G1, S and G2/M phases, were calculated using computer after hyperthermia treatment. Results shown are average from three independent experiments. **E.** Significant enhancement of annexin V staining after hyperthermia in Ku70 KD cells compared with control indicating that Ku70 silencing-based cell death by hyperthermia is apoptosis. Each data point represents the mean of three experiments; bars denote SD; * indicates difference from control transfectant at $P < 0.001$. doi:10.1371/journal.pone.0094213.g006

Ku70 mediated hyperthermia-induced G2/M accumulation

Pulse-labeling experiments with bromodeoxyuridine (BrdUrd; 20 μ M) in PC-10 cells indicated that exposure to hyperthermia induced cytostatic but not cytotoxic arrest (data not shown). The hyperthermia-induced cell cycle disturbance was analyzed 24 h and 48 h after 1–6 h exposures to hyperthermia. G2/M subpopulations were significantly increased 24 h after exposure to hyperthermia and then gradually decreased to normal subpopulations 48 h after exposure to hyperthermia (Figure. 6D). Simultaneously, the percent of G1 and S phase subpopulations gradually decreased 24 h after exposure to hyperthermia and recovered 48 h after the removal hyperthermia. Different from the control cells, the Ku70 KD PC-10 cells did not show G2/M accumulation 24 h after exposure to hyperthermia, but directly underwent G1 cytotoxic arrest that resulted in apoptosis without significant recovery (Figure. 6B, C, and D). These data suggest that Ku70 was required for cytoprotective cytosstatic arrest during the G2/M phase after exposure to hyperthermia.

Hyperthermia-induced cell death in Ku70 KD cells was a caspase-dependent apoptosis

Annexin V staining confirmed that Ku70 silencing-induced cell death, under conditions of hyperthermia, is apoptosis (Figure. 6E). Similar results were obtained with siRNA transfection into another lung cancer cell line, H1299 (data not shown). To investigate whether the enhanced apoptosis in the Ku70 KD cells was caspase-dependent, both Ku70 KD and control PC-10 cells were exposed to hyperthermia (6 h) in the presence or absence of a caspase inhibitor, v-DEVD-Fmk (z-VAD) and then assessed by FACS analysis to determine their apoptotic status. Figure. 7A shows a significant reduction of hyperthermia-induced apoptosis, especially in the Ku70 KD cells. Western blot analysis of some apoptosis-related proteins in the Ku70 KD and control cells, showed significant reduction of Ku70 levels in the Ku70 KD cells compared to the control cells (Figure. 7B). The Bax levels were up-regulated in both cells in response to hyperthermia whereas Bax activation (highly migrating truncated band; 18 kDa) was only observed in the Ku70 KD cells. The active level of Bax was increased with the hyperthermia treatment time. P53 expression was down-regulated after exposure to hyperthermia in both clones

(Figure. 7B; far lower panels); these results suggest that Bax up-regulation was independent of P53 as confirmed in H1299 cells which express no functional P53. Figure. 7C concludes the possible protection mechanism by Ku70 and interprets how HDACIs disturb this mechanism.

Discussion

The results of this study suggest a candidate mechanism responsible for resistance to hyperthermia-induced apoptosis in lung cancer cells. As a fact, Bcl-2 heterodimerizes with Bax to inhibit its apoptotic effects [24]. Thus, the Bax/Bcl-2 ratio reflects apoptosis susceptibility [25]. However, Bcl-2 and Bax function independently to regulate cell death [26]. Although hyperthermia can activate some caspases [27], when hyperthermia is used clinically for cancer treatment, hyperthermia-induced apoptosis has very limited effects. This is consistent with our observation that Bcl-2 was down-regulated while Bax was up-regulated, without prominent Bax activation, in the cells studied. The Bax/Bcl-2 ratio increased under conditions of hyperthermia.

It is well known that Ku70 plays a dual role in DNA double strand break (DSBs) repair and in suppressing Bax-mediated apoptosis, by interacting with Ku80 and Bax [28,29]. However, in the absence of DNA breaks, it is not known whether Ku70 inhibits apoptosis by associating only with Bax or by mediating other pathway(s) that affect Bax. The recently reported cytoprotective function of Ku70 is based on deacetylation [14,17,29], that renders cancer cells more susceptible to DNA damaging agents or to Bax activating factors and that are affected by targeting acetylation. The results of this study showed that the total amount of Ku70 did not significantly change; however, Bax/Ku70 binding was increased with exposure to hyperthermia. The Ku70 binding to Bax might be due to either increased Bax expression or Bax liberated from Bcl-2. The acetylation status of Ku70 changed with exposure to hyperthermia.

Ku70 acetylation, by both the I/II HDACs and class III/Sirtuin deacetylases, including SirT-1, has been previously reported [17,30]. The results of this study demonstrated that, SirT-1 was directly up-regulated and interacted with Ku70, under conditions of hyperthermia, resulting in deacetylation, and the subsequent ability to sequester more Bax. This scenario might be one plausible mechanism associated with the promotion of cell survival. SirT-1, was reported to deacetylates specific lysine residues of many substrate proteins including Ku70 [31]. SirT-1 was consistently found to mediate survival with exposure to stress [32]. Ku70 is acetylated by p300, PCAF, and CBP. This acetylation process accelerates Bax-mediated apoptosis [14]. Ku70 deacetylation has been shown to contribute to longevity under conditions of caloric restriction [19]. Ku70 acts to sequester Bax from mitochondria [14,15]. Here, we found that SirT-1 and Ku70 work together to modulate thermo-sensitivity by regulating Ku70 acetylation. This is consistent with the reports describing SirT-1 as a responder to environmental stress [20,32]. The results of this study demonstrated that the inhibition of Ku70 deacetylation, by specific HDACIs, NAM or TSA, attenuated the protective role of SirT-1 from hyperthermia and enhanced hyperthermia-induced apoptosis.

Lung cancer cells were sensitive to Ku70 siRNA-based inhibition. This inhibition interfered with the protective mechanism against hyperthermia, and resulted in significant apoptosis. The DNA content, annexin-V staining and morphological changes observed, all confirmed induced apoptosis. Bax and/or Bax activation is necessary for intrinsic apoptosis [33]. Bax activation is essential and sufficient for mitochondrial permeabi-

lization and cytochrome C release [33,34]. The results of this study demonstrated Bax activation after exposure to hyperthermia in Ku70 KD cells; in addition, apoptosis was blocked by treatment with the apoptosis inhibitor, z-VAD. The findings of this study showed that heat stress-induced apoptosis takes several hours in cells compared to minutes in isolated mitochondrial systems [35,36]. The antiapoptotic proteins, Bcl-2 and Bcl-xL, can sequester activator proteins and inhibit their ability to homodimerize and regulate apoptosis. Ku70 can be added to the list of antiapoptotic molecules that sequester Bax and inhibit its activation after exposure to hyperthermia *ex vivo*. Hyperthermia primed the signal for apoptosis by increasing the expression of Bax. However, the direct activation of Bax by hyperthermia may either require more exposure time or special conditions. Among these conditions are the addition of HDACIs to culture media to induce Bax/Ku70 flipping and Bax-based apoptosis. The combination (hyperthermia and HDACIs) treatment-induced apoptosis was significantly inhibited in Bax KD cells, which indicates the crucial role of Bax in this cell death. Rather than “death by default,” the emerging view is that apoptosis requires Bax activation, which can be achieved by targeting Ku70 deacetylation with HDACIs or Ku70 knockdown during exposure to hyperthermia.

Human lung cancer cells are thermo-sensitive [36]. Hyperthermia may induce double strand DNA breaks [37]; however, only limited cell death occurs with hyperthermia independent of DNA breaks [38]. Although hyperthermia destroys some cells, by an unknown mechanism, hyperthermia selectively induces apoptosis during the S-phase in lung cancer cells [39]. This is probably because the S-phase and M-phase are the most sensitive to the cell death program [40].

Many studies, including this one, have shown that hyperthermia results in temporary (cytostatic) arrest of most cancer cells in the G2/M phase [41]. A majority of such cells re-enter the cell cycle after removal of the thermal stress and limited apoptosis occurs. The results of this study showed that Ku70 silencing inhibited the cytostatic effects of hyperthermia and caused cytotoxic G1 accumulation. These findings suggest that Ku70 is essential for G2/M accumulation and subsequent protection from hyperthermia. Yamamoto *et al.*, [42], reported that cells are more susceptible to a death signal during G2/M because of Bcl-2 phosphorylation, which lowers the apoptosis threshold. The results of this study did not show Bcl-2 phosphorylation but rather only down-regulation under conditions of hyperthermia; these findings indicate that Bcl-2 does not play a role in protection from hyperthermia *ex vivo*. Instead, Ku70 was mainly involved in the protection scenario. Changing the acetylation status of cells may influence chromatin condensation and hence, DNA-repair. Deacetylation of a critical component of DNA repair machinery such as Ku70 or Ku70 KD may affect DNA repair machinery; however, again, cell death by hyperthermia had not been attributed to DNA break [39]. Even though the Ku70 KD cells, in this study, did not show abnormal cell cycle patterns without stress. These findings indicate that Ku70 plays a key role in the cell cycle progression that is essential for protection from hyperthermia. Thus, targeting Ku70, rather than inhibiting its deacetylation, may facilitate cell toxicity under conditions of hyperthermia, by a mechanism that is associated with a cell cycle-dependent disturbance.

Conclusion

In summary, the main finding of this study was the biphasic role of Ku70 during thermal stress. This finding might have

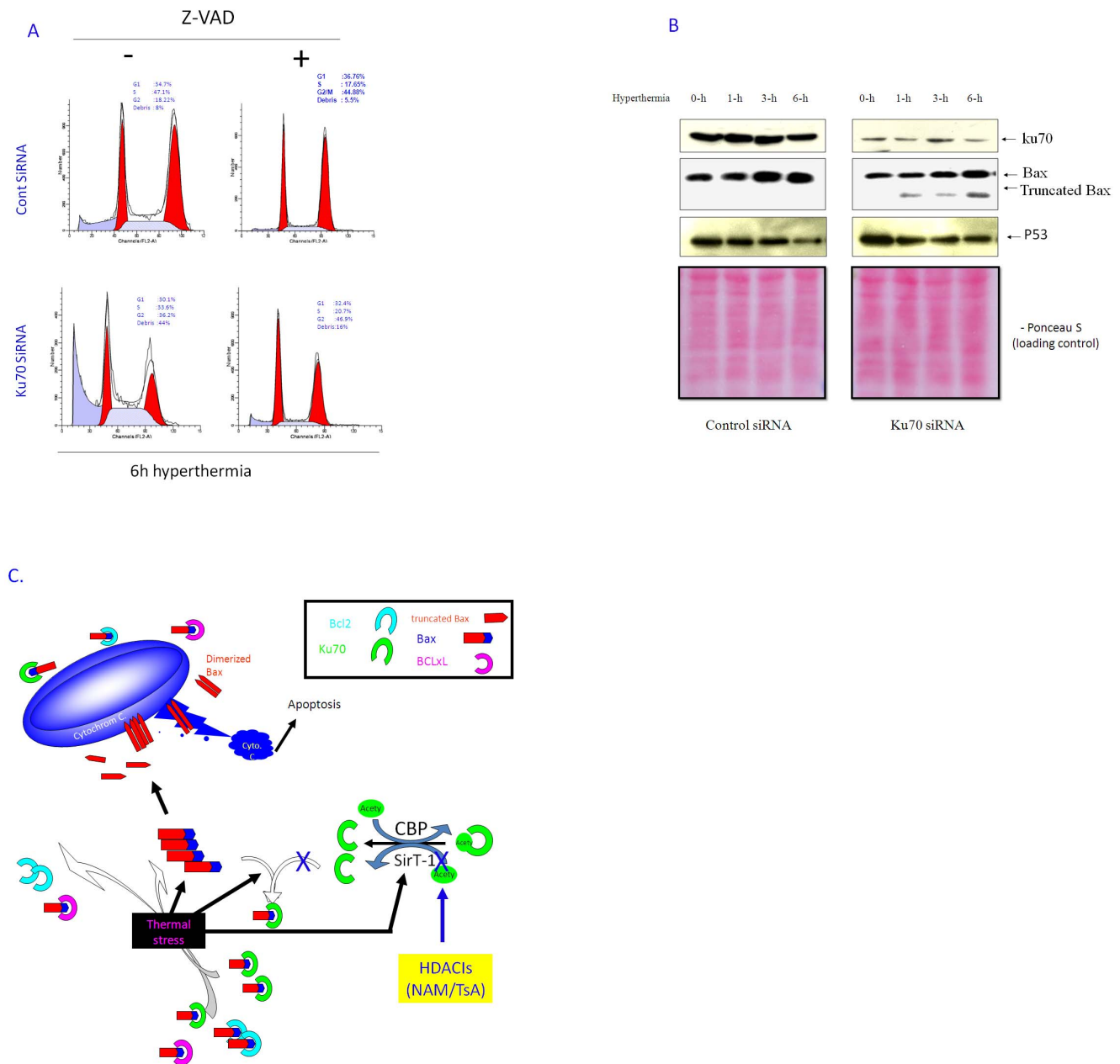


Figure 7. Enhanced cell death in Ku70 KD cells is caspase-dependent. **A.** Ku70 KD and control PC-10 cells were treated with hyperthermia (42.5°C) for 6 h in presence or absence of caspase inhibitor (Z-VAD; 50 nM). Two days later, cells were fixed and acquired by FACS analyzer. Cell death by hyperthermia in both cell clones was regressed by caspase inhibitor indicating that hyperthermia kills Ku70 KD and control cancer cells through caspase-dependent apoptosis. **B.** Proteomic profile of some apoptosis-related proteins in Ku70 KD cells. Ku70 KD and control PC-10 cells treated with hyperthermia at indicated time, lysed, fractionated and blotted. Ku70, Bax and P53 were detected in the blots. Right panel, Ku70 clearly knocked down. Bax shows extra band around 18 kDa indicating active Bax only in the Ku70 KD cell but not the control transfectant, left panel. The active band increased with increasing hyperthermia treatment time. P53 decreased by hyperthermia in both clones indicating that hyperthermia-induced Bax up-regulation and activation are P53 independent. **C.** Schematic presentation describes role of Ku70 in cellular protection from hyperthermia. Ku70, like Bcl-2 and Bcl-xL, restrains Bax from translocation into mitochondria in cells without stress. Under hyperthermia application, Bcl-2 is down-regulated and some Bax become free. In addition, more Bax is overexpressed by hyperthermia. Despite Bax regulation, no Bax is activated because Ku70 bind with this free Bax. Total Ku70 has change but acetylated Ku70 transformed into deacetylated due to SirT-1 activation. Addition of HDACIs during hyperthermia sensitized lung cancer cells to hyperthermia. HDACIs inhibited SirT-1 function and subsequently increase the chance of Bax activation and translocation to mitochondria inducing apoptosis under hyperthermia. doi:10.1371/journal.pone.0094213.g007

therapeutic relevance, with regard to the interplay between Ku70 acetylation and/or expression as a modulator of subsequent Bax activation. The results of this study add to the understanding of apoptosis under thermal stress as well as the identification of

potential targets to improve hyperthermia related treatment of lung cancer; the targeting of Ku70 might have therapeutic relevance in combination with siRNAs and/or specific HDACIs.

Supporting Information

Figure S1 Phase contrast photographs of H1299 cells two days after treatment with HDACIs (NAM at 20 mM or TsA at 300 nM final conc. or both for 4 h) then hyperthermia for 6 h. The image showed limited number of recovered cells as well as the rounded and floating apoptotic cells mainly in the colonies pre-treated with HDACIs then hyperthermia. HDACIs themselves did not induce significant cell death.

(TIF)

Figure S2 Hyperthermia-induced apoptosis was enhanced by HDACIs. Pre-treatment of H1299 cells with DHACIs (NAM at 20 mM or TsA at 300 nM final conc. for four hours) significantly increased hyperthermia-induced apoptosis. Comparative cytograms show Annexin V staining after HDACIs and hyperthermia in H1299 cells.

(TIF)

Figure S3 In PC-10 cells, Hyperthermia and HDACIs combination-induced apoptosis was attenuated by Bax siRNA. PC-10 cells were either transfected with Bax siRNA or cont siRNA. When these cells were pre-treated with HDACIs (NAM at 20 mM or TsA at 300 nM final conc. for four hours) followed by hyperthermia (6 h), the apoptotic outcome was significantly decreased in the Bax KD cells compared with control ones. Comparative cytograms show Annexin V staining after HDACIs and hyperthermia in Bax KD and control PC-10 cells.

(TIF)

Figure S4 Proteomic analysis of some apoptosis -related proteins in H1299 cells. Whole cell extracts were prepared after 0 h or 6 h hyperthermia (42.5°C). Bax, Ku70 and Bcl-2 were analyzed by immunoblotting. Six hours hyperthermia induced slight increase in Bax expression level and reduced Bcl-2 while Bcl-xL and Ku70 had not been affected. Analysis was performed in the same blot so each protein worked as a loading control for the other. A representative Coomassie Brilliant Blue (CBB) staining of the membrane was shown to act as a loading control.

(TIF)

Figure S5 Representative images show localization of Bcl-2 and Bax in lung cancer cell lines. Bax (green), Bcl-2 (green), and nuclei (red) were stained. Bax localization: cytosol in PC-10 cells (a) and in the cytoplasm and the nucleus in H1299 cells (b). Bcl-2 is localized in the cytoplasm in all cells tested (c,d).

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Figure S6 Hyperthermia modulates Bax association with Ku70 in CHAPS buffer. PC-10 cells were incubated at 42.5°C for 0, 1, 3 and 6 h, then lysed in CHAPS buffer. Ku70 was co-immunoprecipitated from 2 mg total protein and Bax was detected in the immunoprecipitant by western analysis (upper panel). Total Ku70 levels showed no changes. Hyperthermia induced Bax up-regulation and enhanced association between Bax and Ku70. On the other hand, Bax was co-immunoprecipitated from similar cell lysates. Ku70 was detected in the immunoprecipitant. Again, after hyperthermia association between Ku70 and Bax was enhanced.

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Figure S7 Hyperthermia did not change expression of HDAC6 or SirT-3. PC-10 was treated with hyperthermia (0–6 h) and cells were lysed and fractionated and blotted. Both HDAC-6 and SirT-3 expression was evaluated by immunoblotting analysis. Immunodetection indicated that hyperthermia did not induce significant changes in the expression of both proteins in PC-10.

(TIF)

Figure S8 Ku70 is required for cytostatic arrest by hyperthermia. H1299 cells were transfected with Ku70-siRNA-2 or cont-siRNA-2 (100 nM) twice. 24 h after last transfection, equal cell numbers were subcultured for further 24 h, and then treated with hyperthermia for indicated time periods and then re-cultured at 37°C for 24 h (a) or 48 h (b). Cells were acquired by FACS analyzer for cell cycle analysis. A representative results is shown at each time point (24 h and 48 h).

(TIF)

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Author Contributions

Conceived and designed the experiments: MH HW. Performed the experiments: MH AS.S ZM. Analyzed the data: MH AE.S YF. Contributed reagents/materials/analysis tools: YO NS. Wrote the paper: MH YO.

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