The elimination of miR-23a in heat-stressed cells promotes NOXA-induced cell death and is prevented by HSP70

R Roufayel1, DS Johnston1 and DD Mosser*1

Protein-damaging stress stimulates cell destruction through apoptosis; however, non-lethal proteotoxic stress induces an adaptive response leading to the increased synthesis of heat shock proteins, which inhibit apoptosis. In this study, we sought to determine the mechanism responsible for the accumulation of the BH3-only protein NOXA in heat-stressed cells and its prevention by the heat shock protein HSP70. Analysis of transcript levels by RT-qPCR revealed that miR-23a levels decreased in heat-stressed cells and that this was correlated with an increased abundance of NOXA mRNA, which contains a miR-23a binding site in its 3’ untranslated region. Cells overexpressing HSP70 had higher levels of miR-23a, maintained these levels after heat shock and accumulated lower levels of NOXA mRNA and protein. The enhanced abundance of miR-23a in these HSP70-expressing cells is primarily due to its increased stability although higher levels of pri/pre-miR-23a expression, nuclear export and maturation were also contributing factors. Stable overexpression of miR-23a in the acute lymphoblastic T-cell line PEER resulted in reduced basal and heat-induced levels of NOXA mRNA and significantly inhibited heat-induced apoptosis. Additionally, stable overexpression of an shRNA targeting miR-23a in U937 lymphoma cells produced stable knockdown of miR-23a and resulted in increased NOXA mRNA and an increased sensitivity to heat-induced apoptosis. These results demonstrate the novel finding that hyperthermia affects the abundance of a microRNA that targets the expression of a pro-apoptotic protein and that HSP70 protects cells from heat-induced apoptosis by regulating the abundance of this microRNA. We speculate that the inhibition of miRNA transcription in heat-stressed cells could represent a general mechanism for apoptosis induction that is regulated by the molecular chaperone protein HSP70. Furthermore, we propose that HSP70 could be beneficial to tumor cells by helping to maintain the expression of oncogenic miRNAs under conditions of cellular stress.

Cell Death and Disease (2014) 5, e1546; doi:10.1038/cddis.2014.484; published online 27 November 2014

Protein-damaging stress, such as exposure to elevated temperature, can activate a process of cellular destruction known as apoptosis. Exposure to hyperthermia also induces the synthesis of heat shock proteins including HSP70 (HSPA1A), which can protect cells from stress-induced apoptosis.1,2 Cell death is regulated by pro- and anti-apoptotic members of the BCL2 family.3,4 The pro-apoptotic members BAX and BAK oligomerize and form channels in the mitochondrial outer membrane of stressed cells permitting the release of pro-apoptotic factors that result in caspase activation leading to the proteolytic dismantling of the dying cell. Anti-apoptotic members of the BCL2 family, BCL2, MCL1, BCL-XL, BCL2A1 and BCL-W, prevent BAX/BAK oligomerization through direct physical interactions. The pro-apoptotic BH3-only (BCL2 homology domain 3) proteins, BIM, BAD, BID, NOXA (PMAIP1) and PUMA, ultimately control cell fate by interacting with the anti-apoptotic members and releasing their ability to suppress BAX/BAK oligomerization or, in the case of some (BIM, BID), by directly stimulating BAX/BAK activation. Although some BH3-only proteins can inhibit all anti-apoptotic BCL2 proteins, NOXA is only able to interact with MCL1 and A1.5 Both NOXA and MCL1 have short half-lives and therefore their abundance can be rapidly altered in stressed cells.6,7 NOXA, which is bound to the mitochondrial outer membrane, binds cytosolic MCL1 leading to its phosphorylation, ubiquitination and proteasomal degradation.8,9 This in turn frees BIM from MCL1 sequestration enabling BAX/BAK oligomerization.10

Apoptosis in heat-stressed cells occurs through BAX activation.11,12 This is mediated in part by a NOXA-dependent depletion of MCL1 protein that is regulated by HSP70.13 Although NOXA protein levels initially drop after cells are exposed to hyperthermia, they subsequently increase to levels greater than that of non-stressed cells.13 In this study, we sought to investigate whether this increase in NOXA protein levels is regulated posttranscriptionally by miRNA-mediated suppression. As microRNAs are generally transcribed by RNA polymerase II, which is inhibited by hyperthermia,14,15 we reasoned that the increased expression of NOXA in heat-stressed cells could be the result of decreased abundance of a microRNA targeting the NOXA mRNA. We demonstrate that miR-23a targets NOXA mRNA and that exposure to hyperthermia causes the depletion of

1Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, N1G 2W1, Canada  
*Corresponding author: D D Mosser, Department of Molecular and Cellular Biology, University of Guelph, 50 Stone Road East, Guelph, Ontario, N1G 2W1, Canada. Tel: +519 824 4120 x58059; Fax: +519 837 2075; E-mail: rmosser@uoguelph.ca  
Abbreviations: RT-qPCR, reverse transcription-quantitative real-time polymerase chain reaction; UTR, untranslated region; pri/pre-miRNA, primary/precursor microRNA; shRNA, short-hairpin RNA; DEVDase, proteolytic activity cleaving after aspartic acid in the peptide DEVD  
Received 23.6.14; revised 01.10.14; accepted 08.10.14; Edited by G Amarante-Mendes
miR-23a resulting in an increased abundance of NOXA mRNA and protein leading to cell death. Cells overexpressing HSP70 have elevated levels of miR-23a and its loss is less severe in heat-stressed cells. As a result, these cells accumulated less NOXA mRNA and protein and consequently resisted heat-induced apoptosis.

Results

We examined the expression of NOXA protein by western blotting in an acute lymphoblastic T-cell line (PEER) with tetracycline-regulated expression of HSP70 (PErT A70). As described previously, NOXA protein levels are initially reduced in non-induced cells exposed to hyperthermia (43 °C for 1 h), most likely due to the inhibition of transcription and translation in the heat-stressed cells and the short half-life of the protein. However, these levels increase when the cells are incubated at 37 °C reaching a level 2.4-fold greater than that of the control cells (Figure 1a and Supplementary Figure 1). The increase in NOXA protein levels corresponds to the time that the cells undergo apoptosis. In cells overexpressing HSP70, the level of NOXA that was regained during incubation at 37 °C did not exceed the basal level and these cells had much less caspase-3 cleavage (Figure 1a and Supplementary Figure 1) as well as other indicators of apoptosis.

We next performed semi-quantitative RT-PCR to determine whether the accumulation of NOXA protein was due to increased mRNA expression (Figure 1b). Cells that were not induced to express HSP70 appeared to accumulate more NOXA mRNA than did the HSP70-expressing cells.

Figure 1 Exposure to hyperthermia results in increased NOXA protein and mRNA levels and is correlated with a decrease in the abundance of miR-23a. (a) Immunoblots of non-induced (-HSP70) and HSP70-expressing (+ HSP70) PErT A70 cells exposed to 43 °C for 1 h and then incubated at 37 °C for 0 to 9 h. Quantitation of the western blot data for \( n = 4 \) repeats is shown in Supplementary Figure 1. (b) Semi-quantitative RT-PCR (quantification of \( n = 3 \) repeats is shown in Supplementary Figure 2) and RT-qPCR analysis of (c) NOXA mRNA, (d) pri/pre-miR-23a and (e) mature miR-23a transcript levels in cells exposed to hyperthermia (mean ± S.E.M, \( n = 3 \)). (f) As a control for the potential effects of doxycycline treatment on transcript levels, RT-qPCR was also performed using PErTA cells (the parental cell line that expresses the reverse tetracycline-controlled transactivator protein rtTA) that were either maintained at 37 °C or exposed to 43 °C for 1 h and then incubated at 37 °C for 9 h (HS).
Quantification of these results revealed a 2.5-fold increase in NOXA mRNA levels in the non-induced cells compared with a 1.4-fold increase in the HSP70-expressing cells (Supplementary Figure 2). We next used the more quantitative RT-qPCR technique to more accurately gauge the changes in NOXA mRNA expression (Figure 1c). Levels of NOXA mRNA increased approximately 30-fold in the non-induced cells but only about 2-fold in the HSP70-expressing cells. We considered the possibility that the increased levels of NOXA in heat-shocked cells could be due to the loss of an miRNA targeting the NOXA mRNA 3′UTR. A potential binding site for miR-23a was identified within the NOXA 3′ UTR sequence (position 100-107 relative to the stop codon) using the miRNA-target prediction database Targetscan. Analysis of miR-23a expression by RT-PCR and RT-qPCR revealed that levels were inversely correlated with that of NOXA mRNA in the heat-shocked cells (Figures 1b and d). Surprisingly, HSP70-expressing cells had elevated levels of miR-23a and maintained higher levels after heat shock exposure than did the non-expressing cells.

As the RT-qPCR analysis shown in Figure 1d measured levels of the pri- and pre-miR-23a transcripts, we also examined whether a similar pattern was observed for the mature miR-23a (Figure 1e). The relative levels of mature miR-23a were nearly identical to that of the pri/pre-miR-23a transcripts. The percentage of mature relative to total (pri/pre +mature) was approximately 36% for both the non-induced and the HSP70-expressing cells prior to heat shock treatment. By 6 h after heat shock, the percentage of mature transcripts was reduced to 26% in the non-induced cells but was not changed in the cells expressing HSP70. This suggests that hyperthermia has a modest affect on the maturation of miR-23a, but a much more significant impact on its level of expression.

To rule out the possibility that the changes in NOXA and miR-23a expression observed in the induced PErTA70 cells were mediated by doxycycline and not HSP70, we treated the parental cell line PErTA with doxycycline for 24 h and measured NOXA mRNA and miR-23a transcript levels by RT-qPCR in control and heat-shocked cells (Figure 1f and Supplementary Figure 3). Heat shock treatment increased NOXA mRNA and decreased miR-23a levels in a manner similar to what was observed in the non-induced PErTA70 cells. This pattern was not altered by doxycycline treatment indicating that the effects shown for the induced PErTA70 cells are attributable to HSP70.

We next used semi-quantitative RT-PCR to determine whether a similar pattern of miR-23a and NOXA mRNA expression occurred in other cells exposed to hyperthermia (Figure 2a). Although basal and heat-induced levels of expression varied for each of the human cancer cell lines, in every case, heat shock treatment resulted in an increase in NOXA mRNA and a corresponding decrease in miR-23a levels. The increased abundance of NOXA mRNA was accompanied by an increase in NOXA protein levels in each of the heat-shocked cells with the exception of U937. We also examined the temporal changes in NOXA mRNA and miR-23a levels in heat-stressed HeLa cells (Figure 2b). Again, an inverse correlation between levels of NOXA mRNA and miR-23a was observed.

To directly determine whether miR-23a regulated NOXA mRNA levels, we transiently transfected HeLa cells with a miR-23a expression vector or a non-targeting (C-miR) vector and performed RT-PCR (Figure 2c). Overexpression of miR-23a substantially reduced the abundance of NOXA mRNA and protein but not Puma mRNA, which does not contain a miR-23a-binding site within its 3′ UTR. Additionally, we transiently transfected HeLa cells with a plasmid encoding an shRNA targeting the miR-23a sequence and found that this resulted in an increase in NOXA mRNA and protein abundance (Figure 2d). Transfection with a mutant version of this plasmid in which five nucleotides in the miR-23a targeting sequence were altered had no effect on the levels of miR-23a or NOXA mRNA and protein. Finally, we cloned the NOXA 3′ UTR sequence from human genomic DNA and inserted it into a luciferase reporter plasmid for transient co-transfection assays with the miR-23a expression plasmid or C-miR. We also created a mutated version of the NOXA 3′UTR reporter plasmid in which the miR-23a-binding site was altered by site-directed mutagenesis. In transiently transfected HeLa cells, we observed a 65% reduction in luciferase activity from the NOXA 3′UTR reporter plasmid when miR-23a was over-expressed (Figure 2e). Also, deletion of the miR-23a-binding site from the NOXA 3′UTR resulted in a fourfold increase in luciferase activity that was unaffected by miR-23a expression. Together, these data demonstrate that miR-23a is capable of binding the NOXA mRNA 3′UTR resulting in a reduction in NOXA mRNA levels.

In order to assess the consequences of miR-23a expression on resistance to heat-induced apoptosis, we created stably transfected cell lines overexpressing miR-23a using the same parental cell line (PEER) that was used to create the PErTA70 cell line. Analysis of miR-23a levels by RT-qPCR showed a 100 to over 1000-fold elevation in miR-23a levels in the three overexpressing clones (Figure 3a). These cells had correspondingly decreased levels of NOXA mRNA. Exposure to hyperthermia reduced miR-23a levels in all clones; however, the overexpressing clones still maintained levels that were higher than the basal level in the parental cell line. As a result, while NOXA mRNA levels increased nearly 1000-fold in the parental and control miRNA-expressing cell line, NOXA mRNA levels in the miR-23a-expressing clones remained repressed. As a consequence, basal and heat-induced levels of NOXA protein were also repressed in these cells and heat shock treatment failed to result in caspase-3 processing (Figure 3b). The extent of apoptosis was quantitated in these cells by measuring Annexin-V staining (Figure 3c) and caspase-3 activity (Figure 3d). While heat shock treatment resulted in a significant increase in both indicators of apoptosis in the parental and control miRNA lines, the miR-23a-expressing clones were essentially unaffected by the heat treatment. Therefore, forced overexpression of miR-23a reduces NOXA mRNA and protein levels and protects cells from heat-induced apoptosis.
resistant to hyperthermia than either the non-induced PEER cell line (Supplementary Figure 4). miR-23a and NOXA mRNA levels were examined by RT-qPCR in the parental U937 cell line, in two clones expressing the miR-23a targeting shRNA (23a-shRNA) as well as two clones (C-shRNA) expressing a non-targeting shRNA (Figure 4a). Levels of miR-23a were reduced about 40-fold in the parental and non-targeting shRNA clones following exposure to hyperthermia. However, miR-23a levels were 1000-fold less in the miR-23a shRNA-expressing clones relative to the parental line, and exposure to hyperthermia further reduced these levels by an additional 500-fold. NOXA mRNA levels in the miR-23a shRNA cells were elevated by approximately 200-fold in non-stressed cells relative to the parental cells and exposure to hyperthermia resulted in a further 200-fold increase relative to their own basal level. Western blot analysis revealed that unlike the PEER cell line, exposure of U937 cells to 43 °C hyperthermia did not significantly affect NOXA protein levels or result in caspase-3 processing (Figure 4b). However, NOXA protein levels and caspase-3 processing were substantially enhanced when the miR-23a shRNA-expressing cells were exposed to hyperthermia. Furthermore, these cells were highly sensitized to heat-induced apoptosis as shown by an increased percentage of Annexin-V staining (Figure 4c) and DEVDase activity (Figure 4d). Therefore, knockdown of miR-23a increases NOXA mRNA levels and sensitizes cells to heat-induced apoptosis.

Having established that miR-23a regulates NOXA expression and that this is a critical factor regulating heat resistance, we next sought to determine the mechanism responsible for the elevated levels of miR-23a in cells overexpressing HSP70.

Figure 2  NOXA mRNA abundance is regulated by miR-23a binding to the NOXA mRNA 3′ UTR. (a) Semi-quantitative RT-PCR analysis of pri/pre-miR-23a and NOXA mRNA transcript levels and western blot (WB) analysis of NOXA protein levels in response to hyperthermic exposure in a panel of human cancer cell lines: PEER (acute lymphoblastic leukemia), U937 (histiocytic lymphoma), A498 (kidney carcinoma) and HCT116 (colon carcinoma). Cells were exposed to 43 °C for 1 h and then incubated at 37 °C for 6 h. (b) RT-PCR analysis of pri/pre-miR-23a and NOXA transcript levels in HeLa cells exposed to 43 °C for 1 h and then incubated at 37 °C for 0 to 9 h. Levels of NOXA protein are also shown. (c) RT-PCR analysis of HeLa cells transiently transfected with either a control miRNA or miR-23a expression plasmid. (M = mock-transfected cells) (d) RT-PCR analysis of HeLa cells transiently transfected with a control shRNA or an shRNA targeting miR-23a. (e) Luciferase activity assay of HeLa cells transiently transfected with either a control luciferase expression plasmid (psiCHECK-2) containing a minimal 3’ UTR with an SV40 polyA sequence, or a plasmid in which this sequence is replaced with the NOXA mRNA 3′ UTR or the NOXA 3′ UTR containing a mutation in the miR-23a binding site. Cells were co-transfected with the reporter plasmids plus either a control miRNA or an miR-23a expression plasmid (mean ± S.E.M., n = 3)
We considered the possibility that HSP70 could affect miR-23a transcription, nuclear export or turnover. miR-23a is transcribed by RNA polymerase II as part of a pri-miRNA cluster that includes miR-27a and miR24-2. We measured the levels of this pri-miRNA by semi-quantitative RT-PCR in the PErTA70 cell line (Figure 5a). Expression of the pri-miRNA transcript was 2.7-fold higher in the HSP70-expressing cells compared with the non-induced cells indicating either elevated rates of transcription or enhanced stability (Figures 5a and b). Levels of the pri-miRNA were reduced following exposure to hyperthermia in both the non-induced and the HSP70-expressing cells, although the cells with HSP70 still contained twofold higher levels of the pri-miRNA after heat shock than did the non-induced cells that were not exposed to hyperthermia (Figures 5a and b). Levels of the pri-miRNA were reduced by only 26% in the HSP70-expressing cells but by over 72% in the non-induced cells.

To address differences in pre-miRNA nuclear export, we measured pri/pre-miR-23a levels by RT-qPCR in isolated nuclear and cytosolic fractions (Figure 5c). Fold changes in levels are expressed relative to the cytosolic value of the control non-induced cells. Cytosolic levels of pre-miR-23a decreased in both the non-induced and HSP70-expressing cells; however, the HSP70-expressing cells maintained higher levels of cytosolic pre-miR-23a as a result of its higher total abundance. Calculation of the percentage of total pre-miR-23a that was present in the cytosolic fraction shows that HSP70 had a beneficial effect on the nuclear export of pre-miR-23a (Figure 5d). Cytosolic levels of pre-miR-23a dropped 15-fold in the non-induced cells but only 3.6-fold in the HSP70-expressing cells.

To determine whether the elevated levels of miR-23a in the HSP70-expressing cells is due to a reduced rate of turnover, we incubated cells with actinomycin D and measured the
remaining amount of miR-23a at various times during incubation at 37 °C (Figure 6a). In the non-induced cells, miR-23a levels were reduced 10-fold within 90 min, whereas in the HSP70-expressing cells this required nearly 4 h representing a 2.7-fold slower turnover rate. Consequently, NOXA mRNA levels decayed more rapidly in the HSP70-expressing cells compared with the non-induced cells (Figure 6a). We also examined the effect of exposure to hyperthermia on miR23a stability. For this, we exposed cells to 43 °C for 60 min and then incubated them at 37 °C in the presence of actinomycin D for 30–120 min (Figure 6b). Heat shock treatment resulted in a more rapid turnover of both miR-23a and NOXA mRNA. In the non-induced cells, miR-23a levels decreased by more than 10-fold within 30 min. However, in the presence of HSP70 a 10-fold reduction did not occur until about 90 min. Therefore, the stability of miR-23a is enhanced by threefold in HSP70-expressing cells exposed to hyperthermia compared with the non-induced cells. The enhanced stability of miR-23a in the HSP70-expressing cells was again correlated with a more rapid turnover of NOXA mRNA following heat exposure.

In summary, miR-23a levels are reduced in cells exposed to hyperthermia most likely due to the combined effects of the inhibitory effects of high temperature on RNA polymerase II-directed transcription, an impairment of pre-miR-23a nuclear export and an elevated rate of turnover. HSP70-expressing cells have higher levels of miR-23a, which is primarily due to a reduced rate of turnover, and maintain high levels of miR-23a following exposure to hyperthermia in part due to the protective effects of HSP70 on pri-miRNA transcription, pre-miRNA nuclear export and maturation. However, the increased stability of miR-23a provided by HSP70 expression is most likely the major factor contributing to its preservation in heat-shocked cells leading to a more rapid loss of NOXA mRNA.

**Figure 4** Overexpression of an shRNA targeting miR-23a in stably transfected U937 cells results in increased NOXA mRNA and protein levels and increased sensitivity to heat-induced apoptosis. (a) RT-qPCR analysis of miR-23a and NOXA mRNA levels in the parental U937 cell line, two stable clones expressing a control shRNA and two stable clones expressing a miR-23a targeting shRNA (mean ± S.E.M., n = 3). miR-23a transcript levels represent both pri-miRNA and pre-miRNA. (b) Immunoblot analysis of NOXA and cleaved caspase-3 levels in heat-shocked cells. (c) Quantification of apoptosis by Annexin-V staining and flow cytometry (mean ± S.E.M., n = 3). (d) Caspase-3 activity assay (mean ± S.E.M., n = 3). Heat-shocked cells were exposed to 43 °C for 1 h and then incubated at 37 °C for 6 h (HS)
All living organisms must employ strategies to cope with environmental stress, particularly proteotoxic stress which can impair cell structure and function, and if uncontrolled, will lead to cell death. Although removal of irreparably damaged cells by apoptosis is beneficial to the organism, adaptive measures must act to prevent inappropriate cell elimination when faced with fluctuating environmental conditions. The heat shock response is an evolutionarily conserved response that allows cells to increase their level of resistance to proteotoxic stress by stress-dependent synthesis of heat shock proteins. The primary function of this family of molecular chaperones is the maintenance of proteostasis by assisting in the proper folding of cellular proteins. This ability provides a powerful anti-apoptotic function not only by preventing protein misfolding in general but also by intervening at key steps in apoptotic pathways. For example, exposure of cells to hyperthermia triggers BAX oligomerization, cytochrome c release and caspase activation all of which are inhibited in cells overexpressing the molecular chaperone HSP70. As NOXA protein levels increase following heat shock treatment, we explored the possibility that this was due to the loss of a microRNA targeting the NOXA mRNA. We have now demonstrated that NOXA expression is regulated by miR-23a, that synthesis of this microRNA is repressed in cells exposed to hyperthermia and that cells expressing HSP70 resist this repression. Consequently, HSP70 can protect cells from...
heat-induced apoptosis by maintaining miR-23a levels and thereby prevent the accumulation of NOXA mRNA and protein. BH3-only proteins act at the critical tipping point in the cell death decision-making process by overcoming the inhibitory effect that the anti-apoptotic BCL2 proteins have on BAX/BAK activation. NOXA performs this task by selectively binding to assist in the loading of miRNA duplexes into the RISC complex by an ATP-dependent conformational change of the Ago proteins. Our results point to a role for HSP70 in regulating the cellular abundance of microRNAs. Heat shock is a well-documented inhibitor of transcription and translation following heat stress would help to buffer these changes and could significantly influence cell fate. Hyperthermia is also known to disrupt mRNA precursor processing and prior heat shock protein synthesis protects intron splicing in heat-stressed cells. We speculate that pri-microRNA processing is another activity that could be disrupted in heat-stressed cells and which could be protected by HSP70.

Tumor cells often have elevated levels of heat shock proteins including HSP70, which provide a selective pro-survival advantage that contributes to the process of tumorigenesis. HSP70 has been implicated in the suppression of multiple apoptotic regulators from stress kinase signaling to BCL2 and caspase family regulation. Our results demonstrate that HSP70 can also intervene in pathways leading to stress-induced cell death by altering the microRNA profile of cells. The elevated levels of miR-23a in HSP70-expressing cells could result from inhibition of microRNA degradation perhaps by preventing its deadenylation in the nucleus and/or by stabilizing interactions with Drosha/DGCR8 or Exportin-5. Alternatively, HSP70 could affect the abundance or activity of transcription factors that are responsible for the regulation of miR-23a expression. We suspect that alteration of microRNA expression could be a general mechanism by which HSP70 contributes to tumorigenesis.

Materials and Methods

Cell lines and treatments. The PErT A70 cells with tetracycline-regulated expression of HSP70 (HSPA1) were described previously and are derived from the acute lymphoblastic T-cell line PEER that was engineered to express the reverse tetracycline-controlled transactivator protein rtTA (PErTA). A control miR expression plasmid and an miR-23a overexpression plasmid containing the pre-miR-23a sequence in the 3' UTR of a GFP reporter plasmid (pEZx-MR04) were obtained from GeneCopoeia (Rockville, MD, USA). PEER cells stably overexpressing the control miR or miR-23a were generated by electroporation and selection with puromycin at 0.8 μg/ml (HyClone Thermo Scientific, Markam, ON, Canada). Drug-resistant clones were screened by flow cytometry using a Beckman Coulter FC500 (Beckman Coulter Canada, Mississauga, ON, Canada). An shRNA targeting miR-23a (5′-ggaaacgccgactgaggt-3′) was constructed using psSUPER-puro (Oligoengine, Seattle, WA, USA). A control shRNA plasmid was created by altering five nucleotides in the miR-23a targeting sequence (5′-gatccgatggtagctgag-3′). U937 cells stably expressing these plasmids were generated by electroporation and selection with puromycin at 0.025 μg/ml. Drug-resistant clones were screened by PCR analysis of genomic DNA. Transient transfection of HeLa cells was performed using calcium phosphate precipitation.

For heat shock treatments, suspension cells (PEER, U937) were pelleted and resuspended in fresh media (RPMI with 10% FBS and 10 mM HEPES pH 7.2; all media supplies were obtained from HyClone) at a concentration of 5 × 10^6 cells/ml in a 15 ml centrifuge tube and submerged in a circulating water bath at 43 °C for 1 h. The cells were either collected immediately after the heat shock treatment or transferred to cell culture flasks with fresh media to dilute the cells to 1 × 10^6/ml and incubated at 37 °C for up to 9 h. Adherent cells (HeLa, A498, HCT116) were grown in DMEM with 10% FBS and heated while attached to 10 cm culture dishes (with the addition of 10 mM HEPES pH 7.2) that were wrapped in parafilm and floated on top of the water surface of a circulating water bath.

Nuclear export of miR-23a was examined by RT-qPCR in detergent lysed cells. Following treatment, cells were collected by centrifugation, washed twice with cold PBS and resuspended in 500 μl of a hypotonic lysis buffer (20 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM MgCl2). After incubation on ice for 15 min, 25 μl 10% NP40 was added and the samples were vortexed at high speed for 10 s. The lysates were separated into a nuclear pellet fraction and a cytosolic supernatant fraction by centrifugation at 3000 rpm for 10 min at 4°C. The pellet fraction was washed by resuspension in 500 μl hypotonic lysis buffer and centrifuged again. RNA was then isolated from each fraction.

To measure RNA stability, cells were pelleted and resuspended in fresh media (RPMI with 10% FBS and 10 mM HEPES pH 7.2) at a concentration of 1 × 10^6/ml.
Renilla values were standardized to the firefly activity to account for transfection and firefly luciferase activity was measured with the dual-luciferase reporter assay.

μ

site-directed mutagenesis replacing the sequence 5′-tcctggttctgctgaacatgactggaggttttg-3′. For the detection of

μ

5′-tggcagagccagctgactggaggttttg-3′. The design of

ΔΔ

Ct after correcting the Ct values for expression of RPL4. Primers

RPL4-fwd: 5′-gctggaagtcgagtgtgcta-3′; NOXA-rev: 5′-cgcccggtgcgggctggggt-3′; miR-23a-rev: 5′-ccgccgggaagcttcagagctcagggtcggttgg-3′; GeneCopoeia

PUMA-fwd: 5′-ggagtctgaagggaggggatg-3′; NOXA-rev: 5′-agcacactcgacttccagc-3′; miR-23a-rev: 5′-ccgccgggaagcttcagagctcagggtcggttgg-3′. Each 25 μl reaction contained 10 μM primers and 1 μl cDNA in 1× GoTaq Flexi buffer. All PCR reactions were 30 cycles except for miR-23a, which was 35 cycles. PCR products were mixed with RedSafe dye (FroggBio, Toronto, ON, Canada) analyzed by agarose gel electrophoresis and imaged using a Bio-Rad GelDoc XR® imaging system (Bio-Rad, Laboratories, Mississauga, ON, Canada).

RT-qPCR

Burlington, ON, Canada). RNA was quantified by Nanodrop and cDNA was

PBS, and RNA was isolated using TRIzol Reagent (Invitrogen-Life Technologies, Burlington, ON, Canada) analyzed by agarose gel electrophoresis and imaged using a Bio-Rad GelDoc XR® imaging system (Bio-Rad, Laboratories, Mississauga, ON, Canada). For RT-qPCR, cDNA was synthesized from 0.017 μg of either the GeneCopoeia

3′-gactagctcgagtgactgcatcaaaaacttgcatgagg-3′, followed by PCR using primers: pri-fwd: 5′-cccttgctgccatagacagccagtgcacctgac-3′, pri-rev: 5′-ggccggg cccctcctcgactggtcgg-3′. PCR was carried out using GOTOaq Flexi DNA Polymerase (Promega, Madison, WI, USA) with the following gene-specific primers:

μ

5′-aatgtgaa-3′ of either the GeneCopoeia

miR-23a promotes cell survival by inhibiting NOXA

Roufayel

moiety plays an essential role in the MCL-1 survival function.

miR-23a family: implications for physiology and therapy.

Trends Biochem Sci 2014; 39: 101–111.

Chen L, Willis SN, Wei A, Smith BJ, Fletcher JI, Hinds MG et al. Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. Mol Cell 2005; 17: 393–403.

Ploner C, Koffler R, Villunger A: Noxa: at the tip of the balance between life and death.

Cell Death & Differ 2014; 51: e1052.

Wills SN, Adams JM. Life in the balance: how Bcl-3 only proteins induce apoptosis. Curr Opin Cell Biol 2005; 17: 617–623.

Stankiewicz AR, Lachappe G, Foo CP, Radicson SM, Mosser DD. Hsp70 inhibits heat-induced apoptosis upstream of mitochondria by preventing Bax translocation. J Biol Chem 2005; 280: 38729–38739.

Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

Percivalle RM, Opferman JT. Delving deeper: MCL-1's contributions to normal and cancer biology. Trends Cell Biol 2013; 23: 22–29.

18. Craxton A, Butterworth M, Harper N, Fairall L, Schwabe J, Ciechanover A et al. NOXA, a sensor of proteasome integrity, is degraded by 26 S proteasomes by an ubiquitin-independent pathway that is blocked by MCL-1. Cell Death Differ 2012; 19: 1434–1434.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.

Stankiewicz AR, Livingstone AM, Mohseni N, Mosser DD. Regulation of heat-induced cell death pathways. Cell Biol Int 2005; 19: 277–281.

11. Pagliari LJ, Kuwana T, Bonzon C, Newmeyer DD, Tzu S, Beere HM et al. The multidomain proapoptotic proteins Bax and Bak are directly activated by heat. Proc Natl Acad Sci U S A 2005; 102: 17975–17980.
23. Leung AK, Sharp PA. MicroRNA functions in stress responses. Mol Cell 2010; 40: 205–215.
24. DiLeva G, Garfolo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol 2014; 9: 287–314.
25. Lima RT, Busacca S, Almeida GM, Gaudino G, Fennell DA, Vasconcelos MH. MicroRNA regulation of core apoptosis pathways in cancer. Eur J Cancer 2011; 47: 163–174.
26. Jahid S, Sun J, Edwards RA, Dixon D, Panarelli NC, Milsom JW et al. miR-23a promotes the transition from indolent to invasive colorectal cancer. Cancer Discov 2012; 2: 540–553.
27. Chen Q, Xu J, Li L, Li H, Mao S, Zhang F et al. MicroRNA-23a/b and microRNA-27a/b suppress Apaf-1 protein and alleviate hypoxia-induced neuronal apoptosis. Cell Death Dis 2014; 5: e1132.
28. Mao J, Li Z, Zhuang Y. MicroRNA-23a is involved in tumor necrosis factor-alpha induced apoptosis in mesenchymal stem cells and myocardial infarction. Exp Mol Pathol 2013; 97: 23–30.
29. Place RF, Noonan EJ. Non-coding RNAs turn up the heat: an emerging layer of novel regulators in the mammalian heat shock response. Cell Stress Chaperones 2014; 19: 159–172.
30. Oshlag JZ, Devasthanam AS, Tomasi TB. Mild hyperthermia enhances the expression and induces oscillations in the Dicer protein. Int J Hyperthermia 2013; 29: 51–61.
31. Wilming GJ, Roth CL, Ibev BL, Ketchum N, Bernhard J, Cerna CZ et al. Identification of microRNAs associated with hyperthermia-induced cellular stress response. Cell Stress Chaperones 2010; 15: 1027–1038.
32. Yin C, Wang X, Kukreja RC. Endogenous microRNAs induced by heat-shock reduce myocardial infarction following ischemia-reperfusion in mice. FEBS Lett 2008; 582: 4137–4142.
33. Yu J, Liu F, Yin P, Zhu X, Cheng G, Wang N et al. Integrating miRNA and mRNA expression profiles in response to heat stress-induced injury in rat small intestine. Funct Integr Genomics 2011; 11: 203–213.
34. Iwasaki S, Kobayashi M, Yoda M, Sakaguchi Y, Katsumi S, Suzuki T et al. Hsc70/Hsp90 chaperone machinery mediates ATP-dependent RISC loading of small RNA duplexes. Mol Cell 39: 292–299.
35. Liu RY, Li X, Li L, Li GC. Expression of human hsp70 in rat fibroblasts enhances cell survival and facilitates recovery from translational and transcriptional inhibition following heat shock. Cancer Res 1992; 52: 3667–3673.
36. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J 2004; 23: 4051–4060.
37. Yost HJ, Lindquist S. RNA splicing is interrupted by heat shock and is rescued by heat shock protein synthesis. Cell 1986; 45: 185–193.
38. Kramer MF. Stem-loop RT-qPCR for miRNAs. Curr Protoc Mol Biol 2011; Chapter 15: Unit 15. 10.