HISTONE H2A.Z IS ESSENTIAL FOR CARDIAC MYOCYTE HYPERTROPHY BUT OPPOSED BY SILENT INFORMATION REGULATOR 2ALPHA

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Running title: Sir2α antagonizes H2A.z

In this study we show that the histone variant, H2A.z, is upregulated during cardiac hypertrophy. Upon knockdown with RNAi, hypertrophy and the underlying increase in growth-related genes, protein synthesis, and cell size, were downregulated. During attempts to understand the mode of regulation of H2A.z, we found that over-expression of silent information regulator 2alpha (Sir2α) specifically induced down-regulation of H2A.z via NAD-dependent activity. This effect was reversed by the proteasome inhibitor epoxomicin, suggesting a Sir2α-mediated ubiquitin/proteasome-dependent mechanism for degradation of H2A.z. An increase in Sir2α also resulted in a dose-dependent reduction of the response to hypertrophic stimuli, while its inhibition resulted in enhanced hypertrophy and apoptosis. We show that Sir2α directly deacetylates H2A.z. Mutagenesis proved that lysines 4, 7, 11, and 13 do not play a role in H2A.z’s stability, whereas, K15 was dispensable. Meanwhile, K115 and, conserved, ubiquitinatable K121, are critical for Sir2α-mediated degradation. Fusion of the C-terminus of H2A.z (a.a. 115-127) to H2A.x or green fluorescence protein (GFP) conferred Sir2α-inducible degradation to the former protein only. Since H2A.x and H2A.z have conserved N-tails, this implied that both the C- and N-termini are critical for mediating the effect of Sir2α. In short, the results suggest that H2A.z is required for cardiac hypertrophy, where its stability and the extent of cell growth and apoptosis are moderated by Sir2α. We also propose that Sir2α is involved in deacetylation of H2A.z, which results in ubiquitination of K115 and K121 and its degradation via a ubiquitin/proteasome-dependent pathway.

H2A.z is an essential histone variant. Disruption of H2A.z in mice is embryonic lethal at a very early stage (E 4.5) (1). Likewise, H2A.z is vital for the development of Drosophila melanogaster (2), and is indispensable in Tetrahymena thermophila (3). In yeast, disruption of H2A.z results in slow growth and formamide sensitivity and is not rescued by the major H2A, but is rescued by Tetrahymena thermophila H2A.z variant (4). These reports provide evidence that H2A.z has a vital and non-redundant role and that it is conserved among species.

One of the mechanisms for transcriptional regulation involves the replacement of core histones with more specialized variants. A report by Abbott et al shows that nucleosomes harboring H2A.z are destabilized, being less compact than nucleosomes harboring the core histone H2A (5). This is also supported by the crystal structure of a nucleosome core containing H2A.z, which exhibits subtle differences compared to the H2A nucleosomes, which account for instability between the H2A.z-H2B dimer and the H3-H4 tetramer (6). This is consistent with a role for H2A.z in nucleosomal remodeling to reduce its structural compactness, thereby, facilitating the binding of transcription factors. But it should be noted, though, that this might not be the sole function of H2A.z in the cell. It has been shown that during development H2A.z is first turned on at the onset of differentiation and appears to localize to the pericentric heterochromatin (7), where it has been suggested to play a role in chromosome segregation (8).

In yeast, there is abundance of evidence that suggests that H2A.z is a transcriptional regulator,
for which multiple mechanisms have been described. One mechanism proposes that H2A.z recruits RNA polymerase II (9). The study showed that depletion of H2A.z abrogates GAL1-10 expression, which was rescued by H2A.z replacement but not by a mutant with a C-terminal domain of H2A. This domain of H2A.z interacted with RNA polymerase II and recruited it to the GAL1-10 promoters. Another mechanism for H2A.z function that has proven operational in yeast is reminiscent of nucleosomal remodeling, which is a prerequisite for recruitment of transcriptional factors. Nucleosomal remodeling is a function of the SNF/SWI complex, which can be, to a great extent, substituted for by H2A.z in a SNF deletion mutant (10). A third mechanism proposes that H2A.z prevents the spread of silent heterochromatin into euchromatin regions. Microarray studies showed that H2A.z is bound to genes near the telomeres (11). Deletion of H2A.z silenced those genes, but interestingly a second deletion of the telomere-silencing gene, Sir2, rescued the phenotype. This suggested that H2A.z functions by antagonizing the spread of Sir2-dependent gene silencing.

Sir2 is a class III histone deacetylase (12), also known to deacetylate p53 (13). In mammalian cells, it has been proposed that SirT1 (the human homologue of Sir2) functions by deacetylating H3-K16 and H3-K9, recruiting H1, thereby, promoting formation of heterochromatin (14). Similar to yeast, it has been shown that calorie restriction in mammalian cells activates SirT1, which reduces stress-induced apoptosis, thus, increasing the life span of the cell (15). Likewise, in cardiac myocytes Sir2α (the mouse homologue of Sir2) is anti-apoptotic (16). There are 7 isoforms of mammalian Sir2 that exhibit differential cellular and tissue distribution, where SirT1 appears to have the highest homology to yeast Sir2. Noteworthy, is that disruption of its gene in a mouse model resulted in embryonic lethality due to cardiac defects (17).

In this study we show that H2A.z and Sir2α oppose one another to regulate cardiac myocyte hypertrophy. Both are found upregulated in the hypertrophic heart, where H2A.z is essential for the expression of some of the growth related genes, such as Cdk7 and ribosomal S6. On the other hand, Sir2α induces H2A.z degradation and reduces hypertrophy. The effect of Sir2α is NAD-dependent and mediated through the N- and C-tails of H2A.z. The results suggest that Sir2α deacetylates K15, resulting in ubiquitination of K115 and K121 and degradation of H2A.z.

**Experimental Procedures**

**Culturing cardiac myocyte and adenovirus infection** - Cardiac myocytes were prepared as previously described (18). Briefly, hearts were isolated from 1-2 day old Sprague-Dawley rats. After dissociation the cells were subjected to Percoll gradient centrifugation followed by differential pre-plating to enrich for cardiac myocytes and deplete non-myocytes. Cells were then plated in DMEM/Ham F12 with 10% fetal calf serum, at a density of 0.5-1 x 10^5 cells / cm^2. Twenty-four hr after plating, serum was removed and the cells were infected with recombinant adenoviruses at a multiplicity of infection (moi) of 10-20 particles / cell. These cells were analyzed 20-24 hrs after infection, unless otherwise indicated. For cells infected with shRNA expressing virus, the cells were harvested after 96 hr to allow enough time for depletion of endogenous proteins.

**Mutagenesis** - H2A.z and H2A.x were cloned by PCR from a mouse heart cDNA library. The H2A.z mutants were generated by PCR, where the mutations were built into the primers. All construct were subsequently validated by sequencing then cloned into adenoviruses. The H2A.x/H2A.z and GFP/H2A.z were likewise constructed by a PCR reaction in which the 3’ primer included the H2A.z tail (bp 440-482). The H2A.x/H2A.z fusion excludes the H2A.x c-tail.

**Construction of adenoviruses** - Recombinant adenoviruses were constructed, propagated and tittered as previously described by Dr. Frank Graham (19). Briefly, pBH Gloā13Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC shuttle vector containing the gene of interest, into 293 cells using Lipofectamine (Invitrogen). Through homologous recombination, the test genes integrate into the ΔE1-deleted adenoviral genome. The viruses were propagated on 293 cells and purified using CsCl2 banding followed by dialysis against 20 mM Tris buffered saline with 2% glycerol. Tittering was performed on 293 cells overlaid with Dulbecco’s...
Modified Eagle’s Medium (DMEM) plus 5% equine serum and 0.5% agarose. All viruses listed in the manuscript were constructed in the lab, except for the Sir2α virus, which was kindly provided by Dr. Sadoshima.

**Construction of short hairpin RNA (shRNA) adenoviral expression vectors** – pSilencer 1.0-U6 expression vector was purchased from Ambion. The U6 RNA polymerase III-dependent promoter and the polycloning region were subcloned into the adenoviral shuttle vector pDC311 (Microbix). The hairpin forming oligo corresponding to bases 460-480 of mouse H2A.z, accession # NM_016750, and its antisense with Apal and Hind III compatible overhangs, were synthesized, annealed, and subcloned distal to the U6 promoter (20). A recombinant adenovirus was generated using homologous recombination in 293 as described above.

**Cell fractionation and Western blotting** – Cells were fractionated into cytosol (cyto), membranes (mem), nuclei (nuc), and cytoskeleton (cytosk), using Subcellular ProteoExtract kit from Calbiochem, according to the manufacturer’s directions. Ten µg of protein was analyzed on a 4-20% gradient SDS-PAGE (Criterion gels, Biorad).

The antibodies used include: anti-Sir2, anti-H2A, anti-H2A.x, anti-acetyl-H3, anti-acetyllysine, anti-SirT2, anti-H2B, anti-phosphoH2B (Upstate Biotechnologies); anti-Cdk7 (Santa Cruz); anti-ribosomal S6 (Cell Signaling).

**Development of H2A.z monoclonal antibody** – A 19-mer peptide was synthesized by Rockland Immunochemicals, Inc. (VIPHIHKSLLIGKGGQKTVC-KLH). The conjugate, mixed with the adjuvant (Titermax Gold for 1st injection and Freund's incomplete adjuvant for subsequent boost doses), was used to immunize Balb/C mice. The mouse with highest antibody titer was used for fusion of the spleen cells with Mouse Myeloma cells, Sp2/0 using standard hybridoma technique. The CELLine device CL-1000 (BD Biosciences) was used for producing high titer monoclonal antibody, which was purified using Immunopure IgG purification kit (Pierce Biotechnolgy). The specificity of the antibody was verified using recombinant H2A and H2A.z protein (Upstate).

**Immunocytochemistry** – The cells were fixed in 3% paraformaldehyde plus 0.3% Triton x-100 in PBS at 25°C for 5 min followed by 3% paraformaldehyde in PBS at 25°C for 20 min. The cells were then incubated with anti-myosin heavy chain (MF-20) at 1:100 in Tris-buffered saline with 1% BSA. After an over night incubation the cells were washed and the secondary antibody-Alexa-598 plus phalloidin-Alex488 (Molecular Probes) were added to the cells. After washing, the slides were mounted using Prolong Gold anti-fade with DAPI (Molecular Probes).

**Cell Stretching** – Cells were cyclically stretched by 15 % for 24 h, using the Flexercell Tension Plus, FX-4000T, which is a computer-controlled apparatus that mimics strain conditions using vacuum suction to deform cells cultured on special flexible, matrix-bonded, growth surfaces.

**Monitoring proteins synthesis** – Protein was monitored by the incorporation of [3H]-leucine into total cellular protein and normalized to the DNA content, as previously described (21).

**Transverse aortic constriction** – Mice were anesthetized (i.p.) with a mixture of ketamine (0.066 mg/g), xylazine (0.013 mg/g), and acepromazine (0.002 mg/g). The animals were then ventilated via tracheal intubation connected to a rodent ventilator (Harvard Apparatus) with a tidal volume of 0.2 mL and a respiratory rate of 110 breaths per minute. The left chest was opened at the second intercostal space and the thymus glands were superiorly reflected. The transverse thoracic aorta between the innominate artery and left common carotid artery was dissected and a 7-0 nylon suture tied around the aorta. A control group underwent thoracotomy and aortic dissection without constricting the aorta.

**Deacetylation reaction** – Cardiac myocytes were infected with adenovirus over-expressing H2A.z or a K4, 7, 11,13R mutant. Cells were lysed and H2A.z was immunoprecipitated. The complex was washed with HDAC Assay Buffer (10mM Tris-HCl, pH 8.0, 150mM NaCl, 10% Glycerol). The precipitate was resuspended in HDAC Assay Buffer plus 4 mM NAD cofactor in the absence or presence of 1 µg active SirT1. The suspension was incubated at 30°C for 2 h, shaking. The
reaction was terminated by adding loading dye and analyzed by Western blotting.

RESULTS

Using subtractive hybridization, we have previously found that histone H2A.z mRNA was differentially upregulated during pressure overload cardiac hypertrophy in the mouse (22). In figure 1a we show that this is paralleled by an increase in nuclear H2A.z protein, but not the core histone H2A. Sir2α, previously reported to antagonize H2A.z in yeast (11), was also upregulated in the cytosol, membranes, and nucleus. Interestingly, H2A.z is uniformly expressed in all neonatal rat organs tested, but is reduced in the adult rat heart and skeletal muscle, and to a lesser extent in the brain (Fig. 1b). Thus, the expression pattern of H2A.z positively correlates with growth and proliferation.

To address the significance of H2A.z in cardiac growth, we knocked down the protein using short hairpin H2A.z (H2A.z-shRNA) delivered to the myocytes via recombinant adenovirus. After 4 days the cells were subjected to mechanical stretch for an addition 24 h. Stretch is used to mimic mechanical load as a stimulus for hypertrophic growth. The results show that stretch-induced upregulation of growth-related genes, ribosomal S6 and Cdk7, was abrogated in the absence of H2A.z, but acetylation of H3 was unchanged (Fig. 2a). Interestingly, membrane associated and nuclear Sir2α were consistently upregulated in the absence of H2A.z.

Similarly, endothelin (ET-1)-induced myocytes spreading and sarcomere organization that characterize cellular hypertrophy, were also inhibited by pretreatment with H2A.z-shRNA (Fig. 2b). In addition, protein synthesis, as monitored by [3H]-Leucine incorporation, was significantly inhibited after 24 h, but not 9 h, of hypertrophic stimulation by endothelin or stretch. This may suggest that H2A.z is not directly involved in protein synthesis, but that H2A.z-dependent de novo gene expression is required for sustaining growth. In contrast, over-expression of H2A.z was insufficient for induction of protein synthesis or cell spreading (data not shown). Thus, we propose that H2A.z is required, but not sufficient, for hypertrophic growth, through regulating the expression of growth-related genes.

In yeast, it has been shown that H2A.z antagonizes heterochromatin formation by Sir2α (11). Therefore, to investigate the relationship between H2A.z and Sir2α in cardiac cells, we over-expressed the latter in a time-dependent fashion. In addition, the cells were supplemented with adenoviral-delivered H2A.z to augment the signal/noise ratio. We had expected possible inhibition of H2A.z’s effect on gene expression. What we found was a dose dependent reduction of total H2A.z accompanied by inhibition of its induction of cyclin-dependent kinase 7 (Cdk7) expression (Fig. 3a), a previously reported marker of cellular hypertrophy (21). In contrast, Sir2α did not affect endogenous H2A.x. Also, this effect was not reproduced by over-expressing the close family member SirT2 (Fig. 3b). To address the possibility of Sir2α inhibiting the CMV promoter that drives the expression of the virally delivered H2A.z, the experiment was repeated with over-expression of CMV-driven H2A.x, the outcome of which eliminated that possibility (Fig. 3c).

Sir2 is a NAD-dependent deacetylase (12). To determine whether the observed effect required this activity, the myocytes were treated with Si2α in the absence or presence of high concentrations of the inhibitor nicotinamide (NAM), which is a product of the deacetylase reaction. The results demonstrate that Sir2α-induced H2A.z degradation was inhibited dose-dependently by NAM. In corroboration, H2A.z-induced Cdk7 expression was also rescued by NAM. Interestingly, there was more H2A.z accumulated in the presence of NAM plus Sir2α than in control or NAM only treated cells. It is plausible that excess inactive Sir2α stabilizes H2A.z. Thus, the results suggest that Sir2α specifically regulates the translation and/or degradation of H2A.z and/mRNA stability through a NAD-dependent deacetylase activity.

To address whether a proteasome/ubiquitin pathway is involved in the down-regulation of H2A.z, we inhibited proteasomal activity using epoxomicin. This approach resulted in a dramatic increase in H2A.z protein abundance and the inhibition of its Sir2α-dependent degradation (Fig. 4). It should be noted that the levels of H2A.z in the nucleus appear to be saturable and any excess
accumulates extra-nuclear. The results indicate that Sir2α induces H2A.z degradation via a proteasome/ubiquitin-dependent mechanism.

To investigate the significance of the upregulation of both H2A.z and Sir2α during hypertrophy, we determined the effect of the hypertrophic growth factor endothelin (ET-1) on cells over-expressing or inhibited in, Sir2α. Western blot analysis revealed that ET-1 treatment in the presence of the Sir2α inhibitor, NAM, resulted in increased H2A.z, as well as, the hypertrophic marker, Cdk7. But it was previously reported that Sir2α has an anti-apoptotic effect in cardiac myocytes (16). In agreement, our results show that 2, 5 or 10 mM NAM treatment induced the apoptotic marker phospho-H2B 1.45, 2.6, 3.9 folds, respectively, after normalization to total H2B (Fig. 5a). This is in contrast to the effects seen with over-expression of Sir2α that resulted in reduced H2A.z and Cdk7, but had no effect on phospho-H2B. It should by noted, that chronic hypertrophy is associated with apoptosis, which would not be detected in our relatively short-term (24 h) experimental hypertrophy. Morphological analysis also confirmed normal or slightly exaggerated ET-1-induced cell spreading and organization of sarcomeres in the presence of 10mM NAM, which was minimized in the presence of Sir2α, although alone, Sir2α appeared to slightly enhance cell size (Fig. 5b). To confirm these results we monitored protein synthesis by [3H]-leucine incorporation in the absence or presence of these molecules. In corroboration, NAM dose-dependently enhanced protein synthesis, while Sir2α inhibited it. The inhibition seen by Sir2α reached a plateau at ~50% of control value (Fig. 5c). Thus, we propose that Sir2α curbs both hypertrophy and apoptosis. But it remains unclear whether the inhibition of apoptosis is primary or secondary, or both, to inhibition of hypertrophy.

Sir2α is a histone deacetylase known to deacetylate H3 (acetyl-K9 and -K14) and H4 (acetyl-K16). In addition, we have shown above that its effect on H2A.z requires its NAD-dependent deacetylase activity. Thus, a plausible mechanism for its effect is the deacetylation of a critical lysine. To identify any potential lysine targets, we individually mutated the 5 lysines (K4, K7, K11, K13, K15), in the N-tail of H2A.z, into arginine (R). The results show that the R4, R7, R11, R13 mutants were stably expressed in the myocytes and were down-regulated upon over-expression of Sir2α, similar to the wild-type protein (Fig. 6a-c). It should be noted, though, that the R7 mutant did not integrate into the nucleus but remained sensitive to Sir2α (Fig. 5b). In contrast, we detected the mRNA but not the protein of the R15 point mutant or an encompassing deletion mutant (not shown), which leads us to predict that K15 is critical for H2A.z protein stability. Therefore, neither K4, 7, 11, or 13, play a role in Sir2α’s effect on H2A.z, while K15 is a potential candidate, equivalent to H4-K16.

To confirm direct deacetylation of H2A.z by Sir2α, we used an in vitro deacetylase assay. First, we immunoprecipitated H2A.z or an R(4,7,11,13) mutant from myocytes over-expressing these proteins. The immunoprecipitate was then incubated with recombinant GST-Sirt1 lacking the N-terminus, in the presence of NAD cofactor. The resultant reaction was analyzed by Western blotting. The data show that H2A.z and the R(4,7,11,13) mutant, were deacetylated to the same extent (~50%) in the presence of recombinant SirT1 and NAD (Fig. 6d), suggesting that H2A.z is indeed a direct target of Sir2α.

Although H2A and its variants, H2A.z and H2A.x, possess unique c-tails, there are 2 lysine residues that are conserved amongst them (K118-119 in H2A and H2A.x, corresponding to K120-121 in H2A.z) (Fig. 7a, boxed residues), where K119 is well known to be ubiquitinated in H2A, affecting its function but not stability (23). To assess the role of the lysines in the c-tail of H2A.z, we individually mutated K115, K120, K121, and K125 into R residues (Fig. 7a, bold residues). The results demonstrate that R115 and R121 mutants were resistant to Sir2α-induced degradation, via over-expressing Sir2α or activating it by resveratrol (Fig. 7b-c). While R125 mutant retained the wild type characteristic (Fig. 7d), and R120 resulted in no detectable protein (not shown). In agreement, H2A.z(R115 or R121)-induced Cdk7 and ribosomal S6 expression was unaffected by Sir2α. Interestingly, though, resveratrol independently down-regulated Cdk7,
and S6. Thus, K115 and K121 are both required for Sir2α-induced H2A.z degradation. It should be noted, that K115 does not have a match in H2A or H2A.x, which may account for the difference in response to Sir2α and the molecules’ stability.

From the above results we know that both K115 and K121 in the c-tail are necessary for Sir2α-induced degradation. But since the R15 mutant protein is unstable, at best, we can conclude its necessity for H2A.z stability, but we are unable to conclusively prove the requirement of the n-tail for the sensitivity towards Sir2α. To partially address this issue, we made a fusion of GFP or H2A.x with a.a 115-127 of the c-tail of H2A.z (GFP.z and H2A.xz, respectively). H2A.x is homologous to H2A.z, with the most divergence in the c-tail sequence, while its n-terminus retains conserved lysines corresponding to K7, K11, and K15 of H2A.z (Fig. 8a). The assumption was that if the n-terminus, in addition to the c-terminus, of H2A.z is indispensable for responsiveness to Sir2α then H2A.xz, but not GFP.z, will be destined for degradation in the presence of Sir2α; but if only the c-terminus is required, then both would be degraded. The results show that H2A.xz, but not GFP.z, was down-regulated by over-expressing Sir2α in cardiac cells. Thus, both the N- and C-tails of H2A.z are necessary for conferring Sir2α-dependent degradation to a protein.

DISCUSSION

Although we know that H2A.z is essential for development in mammals, we do not know its mechanism of function or mode of regulation. Here we show that it is differentially regulated during development, where its levels drop significantly in the adult heart, unless induced to undergo hypertrophic growth. While in yeast it has been mainly reported to differentially regulate transcription, in mammalian cells it has been shown to play a role in chromosomal segregation. Now in addition, our results show that knockdown of H2A.z inhibited the expression of hypertrophy-induced genes, including Cdk7 and ribosomal S6, and consequently hypertrophic growth. Although over-expression of H2A.z induced the expression of these genes, it was incapable of induction of fulminate hypertrophy. This suggests that H2A.z is necessary but not sufficient for induction of hypertrophy, and that its effect on Cdk7 and ribosomal S6 is possibly direct. The full spectrum of genes that are regulated by H2A.z in the heart is still under investigation. Therefore, the results are consistent with its role as a transcriptional regulator. This also agrees with its expression pattern, wherein the neonatal, but not the adult heart, is undergoing hypertrophic growth.

In an attempt to understand the mechanism of H2A.z regulation, we turned to previous studies in yeast cells for clues. There it was shown that H2A.z antagonized Sir2 and the spread of heterochromatin. This prompted us to test the relationship between Sir2α and H2A.z in cardiac myocytes. Our results demonstrate that over-expression of Sir2α resulted in an ubiquitin/proteasome-dependent degradation of H2A.z and, vice versa, knockdown of H2A.z resulted in an increase in Sir2α protein seen in the membrane and nuclear fractions. Thus, it appears that H2A.z and Sir2α negatively regulate each other. In this study we have determined that Sir2α regulated H2A.z’s protein stability, but we have not determined yet the mode of action of H2A.z on Sir2α. In accordance with its role as a transcriptional regulator, we think that it negatively regulates Sir2α at the transcriptional level. Interestingly, inhibition of Sir2α by NAM not only inhibited its effect on H2A.z, but also reversed it. So excess, NAM-inhibited, Sir2α stabilizes H2A.z. Therefore, we predict that there are other deacetylases for H2A.z, which are competitively displaced by excess inactive Sir2α. We have also knocked down Sir2α using shRNA, which resulted in a dramatic increase in apoptosis (data not shown). In this experiment, though, we did not observe an increase in H2A.z. This further supports the idea that there are other H2A.z deacetylases.

H2A.z is an H2A variant, unique mainly in the N- and C-termini. In Tetrahymena it has been shown that if all 6 lysines in the N-terminus are mutated to arginine, it results in a lethal phenotype (24). Whereas replacing any one of these with an acidic amino acid or retaining at least one lysine restores viability, although growth of the organism becomes very slow. Similarly, deletion of the whole domain restores growth. These results imply that the N-terminal lysine residues serve as
a positive charge patch that requires acetylation to neutralize it. On the other hand, in *Drosophila*, deletion of the C-terminus tail of its H2A.z proved it to be an essential domain that confers the H2A.z-specific functionality (25). Our results establish the importance of both the N- and C-tails of mouse H2A.z. Unlike in *Tetrahymena*, deletion of the N-terminus resulted in an unstable protein. Upon individually mutating the lysines in the N-terminus, we found the K15 was critical for protein stability, while K7 was important for nuclear localization. On the other hand, the C-terminal K115 and K121 were necessary for H2A.z degradation via the ubiquitin/proteasome pathway. It is important to note that mutations of these lysines into arginine did not affect H2A.z-induced Cdk7 or ribosomal S6 expression. It is known that the conserved K121 in H2A is ubiquitinated (23). But in that case ubiquitination is associated with Polycomb silencing. Similarly, H2B is ubiquitinated at K123, which regulates histone H3 methylation (26). The difference in the effect of ubiquitination on H2A vs. H2A.z might be attributed to K115, present in the latter protein only, wherein its mutation completely inhibited H2A.z degradation by Sir2α. In short, both the N- and C-tails of H2A.z function to regulate the protein’s activity, localization, and stability.

While the role of histones has not been studied in cardiac growth before, circumstantial evidence is provided from studies involving the histone deacetylases (HDAC) and histone acetyltransferases (HAT). Zhang et al found that during hypertrophy a novel kinase phosphorylates and deactivates class II HDACs (27). Overexpression of an HDAC mutant resistant to deactivation in cultured myocytes inhibited cardiac hypertrophy and the associated gene expression. This was associated with reduction of acetyl-H3 associated with the promoters of the hypertrophic markers, ANF and β-MHC. In vivo studies of an HDAC9 knockout mouse that developed a late onset cardiac hypertrophy and exhibited early hypersensitivity to hypertrophic stimuli, confirmed that HDAC is an inhibitor of cardiac hypertrophy. On the other hand, over-expression of the transcriptional co-activators p300 and CBP induced cardiac hypertrophy, dependant on their HAT activity (28).

We found that during hypertrophy both H2A.z and Sir2α are upregulated. Sir2α is anti-apoptotic in cardiac myocytes, at least partly through deacetylation of p53 (16). In addition, we show that Sir2α induces degradation of H2A.z, which is required for hypertrophic growth, through upregulating Cdk7 and ribosomal S6. In accordance, over-expression of Sir2α partially reduced hypertrophy. In contrast, inhibiting Sir2α with NAM resulted in exaggerated hypertrophy associated with increased apoptosis. While initially hypertrophy is compensatory, its progression is associated with increasing apoptosis, which culminates into cardiac failure. Thus, it is likely that Sir2α protects the cells by moderating the extent of hypertrophy and, thereby, secondary apoptosis. We also show that resveratrol, a polyphenol compound found mainly in the skin of red grapes and a known activator of Sir2, induces H2A.z degradation. Therefore, resveratrol may be beneficial for patients with cardiac hypertrophy and failure.

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**FOOTNOTES**

We thank Dr. S. Vatner, Chairman of the Department of Cell Biology and Molecular Medicine, for his advice and support. This study is supported by the American Heart Association, Grant-in-Aid # 0555773T, and the National Institute of Health Grant 2RO1 HL057970-06.

The abbreviations used are: Sir2α, silent information regulator 2alpha; TAC, transverse aortic constriction; shRNA, short hairpin RNA; SirT1, silent information regulator T1; Cdk7, cyclin-dependent kinase 7

**FIGURE LEGENDS**

Fig 1. H2A.z is upregulated during pressure overload and neonatal hypertrophic growth. a. Hearts were isolated from mice, 3 weeks after undergoing transverse aortic constriction (TAC). After lysis and
fractionation into cytosol (cyto), membrane (mem), nuclei (nuc), and cytoskeleton (cytosk), 10 µg of protein was analyzed by Western blotting, using antibodies against the proteins indicated on the left. b. Spleen (sp), liver (lr), heart (ht), stomach (st), brain (bn), kidney (kd) and skeletal muscle (sk) from 1 day neonatal and 8 week old adult rats were lysed and the nuclear fractions were analyzed by Western blotting, using antibodies against the proteins indicated on the left.

**Fig. 2.** Knockdown of H2A.z inhibits hypertrophy. Myocytes were treated with adenoviruses expressing either H2A.z-shRNA or Lacz-shRNA (control) for 96 h under serum-free conditions. Following that: a. Cells were cyclically stretched by 15% for 24 h. They were then lysed, fractionated, and 10 µg of protein was analyzed by Western blotting, using antibodies against the proteins indicated on the left. b. Cells plated on uncoated glass slides, expressing H2A.z-shRNA (right panels) or Lacz-shRNA (left panels), were stimulated with endothelin (ET-1, lower panels). They were then fixed and stained with anti-myosin heavy chain (red) and phalloidin (green). The slides were imaged using an inverted microscope with a 60x lens. c. Myocyte were stretched or treated with ET-1 for 9 or 24 h, as indicated, in the presence of [3H]-leucine. The cells were lysed and total protein and DNA were extracted. The results are reported as counts per minute (cpm) of [3H]-leucine / µg DNA. The error bars represent standard error of the mean of samples (from 2 experiments). *P <0.05 ET-1 or stretch versus control, calculated by unpaired Student’s t test. #P <0.01 ET-1 or stretch in H2A.z knockdown versus non-knockdown cells, calculated by unpaired Student’s t test.

**Fig. 3.** Sir2α induces degradation of H2A.z. a. Myocytes were infected with adenovirus (Ad) expressing Sir2α for 6, 12, 24 h before lysis. In addition, they were infected with Ad-H2A.z for 24 h, under serum-free conditions, as indicated by a + sign. Cells were then lysed, fractionated, and 10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left. b-c. Cells were infected with Ad-H2A.z, -SirT2, -Sir2α, or -H2A.x for 24 h, under serum-free conditions, as indicated with a + sign. Cells were then lysed and fractionated and 5-10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left. d. Cells were infected with Ad-Sir2α and simultaneously treated with 10 or 20 mM nicotinamide (NAM) for 24 h, under serum-free conditions, as indicated with a + sign. Cells were then lysed, fractionated, and 5-10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left. e. The intensities of the H2A.z bands were measured and normalized to H2A.x using “un-scan-it” software. The results are reported as total H2A.z abundance relative to basal levels (no additives) adjusted to 1, averaged from 3 different experiments. The error bars represent standard deviation. *P <0.05 with respect to basal.

**Fig. 4.** Sir2α induces H2A.z degradation through a ubiquitin/proteasome pathway. a. Cells were infected with Ad-H2A.z or –Sir2α, in the presence or absence of the proteasome inhibitor, epoxomicin (0.1 µM) for 24 h, under serum-free conditions, as indicated by the + signs. Cells were then lysed, fractionated, and 5-10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left. b. The relative intensities of the H2A.z bands were measured and normalized to H2A.x using “un-scan-it” software. The results are reported as total H2A.z abundance relative to basal levels (no additives) adjusted to 1, averaged from 3 different experiments. The error bars represent standard deviation. *P <0.01 with respect to basal. #P <0.01 with respect to H2A.z.

**Fig. 5.** Sir2α inhibits hypertrophy and apoptosis. a. Cells were treated with ET-1 in the absence or presence of nicotinamide (NAM, 2, 5, 10 mM) or Sir2α (6, 12, 24h), as indicated by the slope of the triangle. Cells were then lysed, fractionated, and 10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left. b. Cells plated on uncoated glass slides and treated with 10 mM NAM (middle panels) or Sir2α (right panels), were stimulated with endothelin (ET-1, lower panels) for 24 h. They were then fixed and stained with anti-myosin heavy chain (red) and phalloidin (green). The slides were imaged using an inverted microscope with a 40x lens. c. Cells were treated with ET-1 in the absence or presence of nicotinamide (NAM, 2, 5, 10 mM) or Sir2α (6, 12, 24h), in the presence of [$^3$H]-leucine. The cells were lysed and total protein and DNA were extracted. The results
are reported as counts per minute (cpm) of [3H]-leucine / µg DNA. The error bars represent standard error of the mean of 6 samples (from 2 experiments). *P <0.05 ET-1 + NAM or Sir2α versus ET-1, calculated by unpaired Student’s t test.

Fig. 6. Lysines 4, 7, 11, 13 are dispensable for Sir2α-mediated degradation of H2A.z. a-c. Cells were infected with Ad-H2A.z, -H2A.z(R4), -H2A.z(R7), -H2A.z(R11), or -H2A.z(R13) in the absence or presence of Ad.Sir2α for 24 h, under serum-free conditions, as indicated with a + sign. Cells were then lysed, fractionated, and 5-10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left. d. Myocytes were infected with Ad.H2A.z or H2A.z(R4,7,11,13) for 24 h, under serum-free conditions, as indicated by the + sign. Histones were acid extracted and mouse IgG (lane 1) or anti-H2A.z (lanes 2-5) were added to 100 µg of the extract. The precipitate was then subjected to deacetylation by recombinant SirT1 and the product analyzed by Western blotting, using the antibodies against the protein listed on the left. e. The relative intensities of the acetyl-lysine (Ac-lys) bands in (d.) were measured and normalized to H2A.z using “un-scan-it” software. The results are reported as acetyl-lysine content relative to acetyl-H2A.z adjusted to 1, averaged from 3 different experiments. The error bars represent standard deviation. *P <0.01 with respect to acetyl-H2A.z. #P <0.01 with respect to acetyl-H2A.zR4,7,11,13.

Fig. 7. Lysines 115 and 121 are indispensable for Sir2α-mediated degradation of H2A.z. a. Sequence alignment of H2A.z (a.a 109-127), H2A.x (a.a. 107-142), and H2A (a.a. 107-129). The bolded K115, K120, K121, K125 in H2A.z are the sites mutated into arginine (R), below. The boxed amino acids are conserved K120-121 of H2A.z found in all 3 isoforms. b-d. Cells were infected with Ad-H2A.z, -H2A.z(R115), -H2A.z(R121), or -H2A.z(R125), in the absence or presence of Ad.Sir2α or 40 µM resveratrol for 24 h, under serum-free conditions, as indicated with a + sign. Cells were then lysed, fractionated, and 5-10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left.

Fig. 8. The c-terminus of H2A.z confers Sir2α-mediated degradation to H2A.x but not GFP. a. Sequence alignment of H2A.z (a.a 1-15), H2A.x (a.a. 1-13). Boxed amino acids are conserved lysines 7, 11, 15 in H2A.z, corresponding to K5, K9, K13 of H2A.x. b. Cell were infected with the hybrid protein H2A.x/H2A.z (H2A.xz) or GFP/H2A.z (GFP.z) in the absence or presence of Sir2α for 24 h under serum-free conditions. Cells were then lysed, fractionated, and 5-10 µg were analyzed by Western blotting, using antibodies for the proteins indicated on the left.
Figure 1

(a) 

| TAC | cyto | mem | nuc | cytosk |
|-----|------|-----|-----|--------|
| H2A.z | +   | +   | +   | +      |
| H2A   |     |     |     |        |
| Sir2α |     |     |     |        |

(b) 

|       | sp | ir | nt | s1 | bn | kd | g1 |
|-------|----|----|----|----|----|----|----|
| H2A.z |    |    |    |    |    |    |    |
| H2A   |    |    |    |    |    |    |    |
Figure 2b-c

b. Lacz-shRNA  H2A.z-shRNA

vehicle  

ET-1  

c. [3H]Leucine/µg DNA (cpm)

|                | Lacz  | H2Az |
|----------------|-------|------|
| 9 h shRNA      |       |      |
| 24 h shRNA     |       |      |

*p < 0.05
#p < 0.01

Duration of ET-1 or stretch stimulation
Figure 3a-c
Figure 4

|                | cyto | mem | nuc | cytosk |
|----------------|------|-----|-----|--------|
| Sir2α          | -    | -   | -   | -      |
| Epox           | -    | +   | +   | +      |
| H2A.z          | +    | +   | +   | +      |

**H2A.z**

**H2A.x**

**Ubq**

**Sir2α**

**b.**

|                | Relative H2A.z abundance |
|----------------|---------------------------|
| Sir2α          | -                         |
| Epox           | -                         |
| H2A.z          | -                         |

* * *

# #

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by guest on March 25, 2020
Figure 5

a. 

b. 

Cdk7 - 

p-H2B - 

H2B - 

C.

Figure 5

b.

C.

Cdk7 - 

p-H2B - 

H2B - 

C.

Figure 5

b.

C.

Cdk7 - 

p-H2B - 

H2B - 

C.

Figure 5

b.

C.

Cdk7 - 

p-H2B - 

H2B - 

C.

Figure 5

b.

C.

Cdk7 - 

p-H2B - 

H2B - 

C.

Figure 5

b.

C.

Cdk7 - 

p-H2B - 

H2B - 

C.

Figure 5

b.

C.

Cdk7 - 

p-H2B - 

H2B - 

C.

Figure 5

b.

C.

Cdk7 - 

p-H2B - 

H2B - 

C.
Figure 6

a.  

|          | cyto | mem | nuc | cytosk |
|----------|------|-----|-----|--------|
| H2A.z(R4) | -    | -   | +   | +      |
| H2A.z    | -    | -   | +   | +      |
| Sir2α    | -    | +   | -   | -      |

b.  

|          | cyto | mem | nuc | cytosk |
|----------|------|-----|-----|--------|
| Sir2α    | -    | -   | +   | -      |
| H2A.z(R7) | -    | -   | +   | +      |
| H2A.z    | +    | +   | -   | +      |


c.  

|          | cyto | mem | nuc | cytosk |
|----------|------|-----|-----|--------|
| Sir2α    | +    | +   | -   | -      |
| H2A.z(R13)| -   | -   | +   | +      |
| H2A.z(R11)| +   | +   | -   | +      |


d.  

|          | cyto | mem | nuc | cytosk |
|----------|------|-----|-----|--------|
| SirT1    | -    | -   | +   | +      |
| H2A.z    | +    | +   | -   | -      |
| H2A.z^R4,7,11,13 | -    | -   | +   | +      |


e.  

| Relative acetylation content | SirT1 | H2A.z | H2A.z^R4,7,11,13 |
|-------------------------------|-------|-------|------------------|
|                               |       |       |                  |

* Significant difference compared to control
# Significant difference compared to H2A.z^R4,7,11,13
Figure 7

|   | cyto | mem | nuc | cytosk |
|---|------|-----|-----|--------|
| a. |       |     |     |        |
| H2A.z(109) | VIPHIHSLIGKKQOKTV |     |     |        |
| H2A.x(107) | VLPNIQAVLLPKKSSATVGPKAPAVGKKA SQASQ |     |     |        |
| H2A(107)   | VLPNIQAVLLPKRTEHHKA KGK |     |     |        |
| b. |       |     |     |        |
| Sir2α      | -    | -+  | -+  | -+     |
| RSV        | -    | +   | +   | +      |
| H2A.z(R115)| -    | +   | +   | +      |
| H2A.z      | -    | +   | +   | +      |
| c. |       |     |     |        |
| Sir2α      | -    | -+  | -+  | -+     |
| RSV        | -    | +   | +   | +      |
| H2A.z(R121)| +    | +   | +   | +      |
| H2A.z      | -    | +   | +   | +      |
| d. |       |     |     |        |
| Sir2α      | -    | -+  | -+  | -+     |
| RSV        | +    | -   | -   | -      |
| H2A.z(R125)| +    | +   | +   | +      |
| H2A.z      | -    | +   | +   | +      |
### Figure 8

#### a.

|       | H2A.Z(1) | AGGKAGGDSCKAKTK |
|-------|----------|-----------------|
| H2A.X(1) | SG.R.GRTGKRARAK |

#### b.

|       | cyto | mem | nuc | cytoSk |
|-------|------|-----|-----|--------|
| Sir2α | +    | -   | +   | -      |
| H2A.xz | +    | +   | +   | +      |
| GFP.z  | +    | +   | +   | +      |

GFP.z

H2A.xz

H2A.z

Sir2α
Histone H2A.Z is essential for cardiac myocyte hypertrophy but opposed by silent information regulator 2 alpha
Ieng-Yi Chen, Jacqueline Lypowy, Jayashree Pain, Danish Sayed, Stan Grinberg, Ralph R. Alcendor, Junichi Sadoshima and Maha Abdellatif

J. Biol. Chem. published online May 10, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M601443200

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