# HIC2 activates transcription of *SIRT1* gene

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**Directed by Professor Man-Wook Hur**

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**Ji-Yang Song**

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# This certifies that the Doctoral Dissertation of Ji-Yang Song is approved.

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**May. 16. 2019**

**Ji-Yang Song**

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## ABSTRACT

### HIC2 activates transcription of *SIRT1* gene

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The histone deacetylase SIRT1 is crucial to numerous physiological processes, including aging, metabolism, and autoimmunity. SIRT1 is repressed by various transcription factors. HIC1 is one of the well-known transcription factors that repress *SIRT1*. I found that HIC2, a highly homologous protein with HIC1, interacts with HIC1 through their zinc-finger domains and activates *SIRT1* transcription. The structural differences between the HIC1 and HIC2 intermediate domains confer opposing transcriptional regulation of *SIRT1*.

Mammalian two-hybrid assays showed that p300 domains interacted with HIC2 middle domain 2, but not with the HIC1 middle domain 2, despite its conserved p300 acetylation lysine residues at *a.a.* 313-317 (MKHEP). The difference in protein interaction with p300 may explain differences in transcriptional regulatory properties of HIC1 and HIC2.

This relationship between HIC2 and SIRT1 could be important for cardiac development, which associates with both proteins. In this study, I investigated that ectopic HIC2 increases the expression of SIRT1, which results in less apoptosis in ischemia/reperfusion injury in primary cardiomyocyte and H9c2 cells. Considering the fact that treatment of HIC2 adenovirus with SIRT1 knockdown did not reduce apoptosis caused by HIC2 overexpression, HIC2 has cardioprotection function only by increasing the SIRT1 expression. I found that unlike its structural homolog HIC1, HIC2, is a pivotal transcriptional activator of SIRT1, and consequently, may protect the heart from I/R injury.

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Key words: HIC2, SIRT1, HIC1, transcription, heart, ischemia/reperfusion injury

# HIC2 activates transcription of SIRT1 gene

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## I. INTRODUCTION

SIRT1 (a member of the sirtuin family) is a nicotinamide adenosine dinucleotide ($NAD^+$)-dependent deacetylase that removes acetyl groups from a variety of proteins.[1] SIRT1 is involved in many biological processes, including energy metabolism, metabolic diseases, cancer, aging, cellular senescence, neurodegeneration, inflammation, and development.[2-4]

In many of these processes, hypermethylated in cancer-1 (HIC1), and the ubiquitous tumor suppressor p53, repress *SIRT1* transcription; conversely, SIRT1 deacetylates and inactivates p53.[5,6] In addition, p53 binds to and activates the *HIC1* gene promoter.[7] In response to DNA damage, HIC1, SIRT1,

and p53 regulate each other to form a regulatory loop, and abnormalities in this loop may lead to cancer, as now demonstrated in cell, animal models, and human cancer patients.[6,8] HIC1 is also a transcriptional repressor of *SIRT1* and a tumor suppressor whose promoter is epigenetically hypermethylated in various carcinomas.[9] HIC1 is a POK family member containing a BTB/POZ domain critical for interacting with other proteins at its N-terminus, in addition to a zinc-finger domain that recognizes and binds a specific DNA sequence (GGCA) at its C-terminus.[10,11] The middle domain of HIC1 has two functional motifs: a GLDLSKK motif that binds to the co-repressor C-terminal-binding protein (CtBP), and a MKHEP motif that undergoes an acetylation/SUMOylation switch.[12-15] Depending on the situation, HIC1 binds to CtBP or Metastasis Associated-1 (MTA1), to form different repressive complexes that bind to the promoters of target genes and repress their expression.[16]

HIC2 (hypermethylated in cancer 2) is a POK family protein, and the amino acid sequence of its BTB/POZ and zinc-finger domains are similar to those of HIC1.[17] Specifically, the amino acid sequences of the POZ domains of HIC2 and HIC1 are 88% identical, while their zinc-fingers are more than 80% identical.[13] However, other parts of HIC1 and HIC2, including their middle domains, show little homology.

While the structure and functions of HIC1 are relatively well studied, much

less is known about HIC2. In 2014, HIC2 was reported to be essential for a crucial stage of heart development, and homozygosity for a *Hic2* mutant allele is embryonic lethal, while *Hic2* hemizygosity causes cardiac malformation, including ventricular septal defects.[18] Subsequent studies have shown that when *Hic2* is deleted, expression of fetal stage genes, in the heart and blood, is increased during mouse development.[19] These results suggest that HIC2 plays a role in suppressing fetal genes for normal development of the heart and circulatory system, in embryogenesis.[18,19] Despite this knowledge, the structural and biochemical characteristics and functions of HIC2, as a transcription factor, remain unknown.

Interestingly, *Sirt1* KO mice and embryos also show cardiac defects, in addition to other deleterious phenotypes such as body size, malformation of the skull, and reduced sperm numbers.[20] Moreover, *SIRT1* is a direct target gene of HIC1 in normal cells under stress or metabolic challenges.[6] Specifically, HIC2 has a DNA-binding domain nearly identical to that of HIC1, and the overlapping abnormal heart phenotypes of *Hic2* and *Sirt1* KO mice suggest HIC2 might be a critical transcriptional regulator of *SIRT1*. Ventricular septal defects, which are common phenotypes in both *Hic2* and *Sirt1* KO mice, are caused by excessive myocardial apoptosis during development.[21] Apoptosis is an important process in development, causing deformities if not properly

controlled.[22]

Transition from embryonic to fetal development requires additional oxygen due to increased workload, including vastly increased heart rate.[23] Dykes *et al.* suggested that HIC2 may also respond to hypoxia.[19] Also, SIRT1 is known to protect the heart by inhibiting pro-apoptotic molecules during ischemia/reperfusion injury,[24] and HIC2 may also play a role in such protection.

Considering the various physiological functions of SIRT1, it is important to identify the mechanism(s) of how its expression is regulated. Here, we investigated and found that HIC2 is a newly discovered transcription activator of *SIRT1*.

## II. MATERIALS AND METHODS

### 1. Animals

To investigate whether ectopic HIC2 could increase SIRT1 expression, we isolated primary cardiomyocytes and heart from the mice as follow. Primary cardiomyocytes were isolated from the young mice (2-3 days postnatal) using Pierce Primary Cardiomyocyte Isolation Kits (Thermo Scientific) were used. To isolate adult male mice hearts, control or HIC2 adenovirus was directly injected to the hearts of 10-week old adult male mouse (C57BL/6), following

anesthetization with a cocktail of ketamine and xylazine, intubation, and mechanical ventilation. Adenovirus ($7x10^{10}$ viral particles), in a volume of 100 μl, was injected into the apex of the left ventricle with a 30-gauge needle.

**2. Plasmids and recombinant proteins**

The HIC2 ORF was amplified from a human cDNA library by PCR, and cloned into pcDNA3.1, which has a Myc epitope and 6 copies of His epitopes. pGL2-SIRT1-Luc, pcDNA3.1-LacZ, pcDNA3.0-FLAG-HIC1, pcDNA3.1-CtBP, and pcDNA3.1-p300 were used for transcriptional analysis. For mammalian two-hybrid assays, cDNA fragments encoding the HIC1-POZ (*a.a.* 1-126), HIC1-middle 1 (*a.a.* 127-301), HIC1-middle 2 (*a.a.* 302-411), HIC1-zinc fingers (*a.a.* 412-615), HIC1-C terminus (*a.a.* 616-714), HIC2-POZ (*a.a.* 1-140), HIC2-middle 1 (*a.a.* 141-295), HIC2-middle 2 (*a.a.* 296-430) or HIC2-zinc fingers (*a.a.* 431-615), were cloned into GAL4 DNA binding domain-expressing pBIND vector, and cDNA fragments encoding the p300 domains (*a.a.* 1-648, 1051-1158, 1196-1280, 1281-1519, 1662-1808, 1809-2414) were cloned into a VP16-expressing pACT vector. To prepare recombinant GST proteins, cDNA fragments encoding the HIC1-POZ (*a.a.* 1–126), HIC1-middle 1 (*a.a.* 127-301), HIC1-middle 2 (*a.a.* 302-411), HIC1-zinc fingers (*a.a.* 412-615), HIC1-C terminus (*a.a.* 616-714), HIC1- zinc

fingers/C terminus (*a.a.* 412-714), HIC2-POZ (*a.a.* 1-140), HIC2-middle 1 (*a.a.* 141-295), HIC2-middle 2 (*a.a.* 296-430) or HIC2-zinc fingers (*a.a.* 431-615), were cloned into pGEX4T1. The recombinant GST-fusion proteins were expressed in *E. coli* DH5α cells. All plasmid constructs were verified by DNA sequencing. IPTG was added to a final concentration of 1 mM for protein induction.

**3. Cell culture**

HEK293A cells and H9c2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin. The cells were maintained in a 5% $CO_2$ incubator at 37℃.

**4. Transient transcription analysis**

Various combinations of pGL2-SIRT1-Luc, pcDNA3.0-HIC1, pcDNA3.1-HIC2, pcDNA3.1-CtBP, pcDNA3.1-p300, or control vector were transiently transfected into HEK293A cells using Lipofectamine Plus reagent (Invitrogen). Transient transcription analysis was performed as previously reported.[25]

**5. Reverse transcription and quantitative real-time PCR (RT-qPCR)**

Total RNA was isolated from cells using TRIzol reagent (Invitrogen). cDNAs were synthesized using 2 μg of total RNA, random hexamers (10 pmol), and M-MLV Reverse Transcriptase (200 units/μl), in a total volume of 20 μl, using a reverse transcription kit (Promega). RT-qPCR was performed using SYBR Green Master Mix (Applied Biosystems). Oligonucleotide primers sets used for RT-qPCR are listed in Table 1. All reactions were performed in triplicate.

**6. Western blot and antibodies**

Western blotting was performed as previously described.[25] The following antibodies were used: FLAG tag (SIGMA F3165), His tag (R&D system MAB050), Myc tag (Cell Signaling #2278), HIC1 (Santa Cruz sc-271499), HIC2 (Thermo Fisher Scientific PA5-37293), SIRT1 (Millipore 07-131), p300 (Millipore 05-257), acetylated-lysine (Cell Signaling #9441), and GAPDH (Santa Cruz sc-32233). Primary antibody incubation was followed by washing and incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Thermo Scientific #31430 and #31460, respectively). Protein bands were visualized by enhanced chemiluminescence solution (Thermo Scientific #32106).

**7. Co-immunoprecipitation**

HEK293A cells were harvested 48 h after transfection with HIC1 and/or HIC2 expression vectors and lysed in RIPA buffer. Cell lysates were incubated with anti-FLAG or anti-His antibodies, on a rotating platform, at 4℃ for 16 hr. The mixtures were then incubated with protein A-sepharose fast flow beads for 3 hr at 4℃, and the beads collected by centrifugation.

**8. GST-fusion protein purification, in vitro transcription and translation, and GST-fusion protein pull-down assays**

Recombinant GST fusion proteins were prepared in E. coli BL21 (DE3) cells grown for 4 hr at 37℃ in LB broth containing 1 mM IPTG, lysed, and proteins purified using glutathione-agarose, 4-bead affinity chromatography (Peptron, Taejeon, Korea). HIC1 and HIC2 polypeptides were prepared by incubating 2 μg of either pcDNA3.0-FLAG-HIC1 or pcDNA3.1-his-HIC2 with $T_NT$ Quick-Coupled Transcription/Translation Extract (Promega) containing 40 μl of $T_NT$ Quick Master Mix and 2 μl of [$^{35}$S]-methionine (1175.0 Ci/mol, PerkinElmer Life Sciences) at 30℃ for 90 min. We then performed GST-fusion protein pull-down assays, as previously described.[25]

**9. Oligonucleotide pull-down assays**

The following double-stranded oligonucleotides (HRE1, HRE2, and HRE3)

were used (only sense strands are shown and 5' biotin-labeled).

HRE1, 5'-TGCTCCAGGCAGATGCCATAACAAACACTG-3';

HRE2, 5'-GAGTCACAGTGTGCCAGAATTTCAGGGAGA-3';

HRE3, 5'-CGGAGCCGCGGGGGCGCCAGTGCCGCGCGTCGA-3'.

Oligonucleotide pull-down assays were performed as previously described.[26]

**10. ChIP-qPCR**

Chromatin immunoprecipitation (ChIP) assays were performed as we have previously reported.[27] PCR reactions were conducted using the following oligonucleotide primer sets:

SIRT1 promoter HRE1 (forward, 5'-TGGCCAGAACCCATACTAGG-3';

reverse, 5'-CCATGGTAGATCTAGAGCCAGTG-3'),

HRE2 (forward, 5'-CCATGGGTTATATGGGTCCTT-3';

reverse, 5'-TTCAAAAGGCTTAGTGGAAAGC-3'),

HRE3 (forward, 5'-ACTACACGCTCGCCACAAAG-3';

reverse, 5'-GGGCCAGACCACAACACTAC-3').

**11. Simulated ischemia/reperfusion**

Culture medium of H9c2 cells was removed, replaced with ischemia buffur (4 mM HEPES, 117 mM NaCl, 12 mM KCl, 0.9 mM $CaCl_2$, 0.49 mM $MgCl_2$, 20

mM sodium lactate, 5.6 mM 2-deoxyglucose, pH 6.2),[28] cells placed in a hypoxia incubator with 94% $N_2$, and 5% $CO_2$ for 2 hr, and transferred back to a standard $CO_2$ incubator with normal culture medium, for 1 hr.

**12. Adenovirus production**

The adenoviruses used in the experiments were purchased from Goma Biotech.

**13. *In vitro* acetylation assays**

Recombinant His-tagged full-length HIC1 (*a.a.* 1-714), His-tagged full-length HIC2 (*a.a.* 1-615), and His-tagged full-length HIC2 K341R mutant (*a.a.* 1-615) proteins were expressed in *E. coli* DH5α cells, and purified by Ni-NTA affinity chromatography. Full-length p300 was purchased from Protein One. Each recombinant POK protein (6 μg) and acetyl-coenzyme A (0.83 mM) were incubated with p300 (500 ng) in 1X HAT assay buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol), at 37℃ for 2 hr. Reaction mixtures were resolved by SDS-PAGE, and analyzed by western blot, using an anti-acetyl lysine antibody to evaluate POK acetylation.

**14. Statistical Analysis**

Student's *t* test was used for the statistical analysis. *p* values <0.05 were

considered statistically significant.

**Table 1. Oligonucleotide primers sets used for RT-qPCR**

| Species | Oligonucleotides | | Sequence |
|---|---|---|---|
| Human | *HIC2* | Sense | 5’-AGACTCACACGGAGGAAGAGCT-3’ |
| | | Antisense | 5’-GTCTTCTCGCAGACCGAACACT-3’ |
| | *HIC1* | Sense | 5’-GGCTTCTTGTGCGACGTGATCA-3’ |
| | | Antisense | 5’-TGTCATGGTCCAGGTTGAGCAG-3 |
| | *SIRT1* | Sense | 5’-TCAGTGGCTGGAACAGTGAG-3’ |
| | | Antisense | 5’-AGCGCCATGGAAAATGTAAC-3 |
| | *GAPDH* | Sense | 5’-ACCACAGTCCATGCCATCAC-3’ |
| | | Antisense | 5’-TCCACCACCCTGTTGCTGTA-3’ |

## III. RESULTS

### 1. HIC2 activates transcription of SIRT1

We first investigated whether HIC2, a potential transcription factor with high amino acid sequence homology to HIC1, regulates transcription of SIRT1. Transient transcription assays in HEK293A cells showed that HIC1 repressed transcription of the SIRT1 promoter fused to a reporter gene (pGL2-SIRT1-Luc-1.26 kb), as reported previously.[6] Interestingly, HIC2 activated the SIRT1 promoter by 3.5-fold (Fig. 1A). By contrast, ectopic HIC1 repressed endogenous SIRT1 and HIC2 activated transcription of SIRT1. (Fig. 1, B and C). These data suggest that HIC2, despite high amino acid sequence homology to HIC1 in key functional domains, activates (not suppresses) SIRT1 transcription.

Next, we tried to validate HIC2's function in transcriptionally activating SIRT1, by RNA interference or knockdown using siRNA. However, we were unable to obtain satisfactory data, due to very low levels of HIC2 expression, under such test conditions.

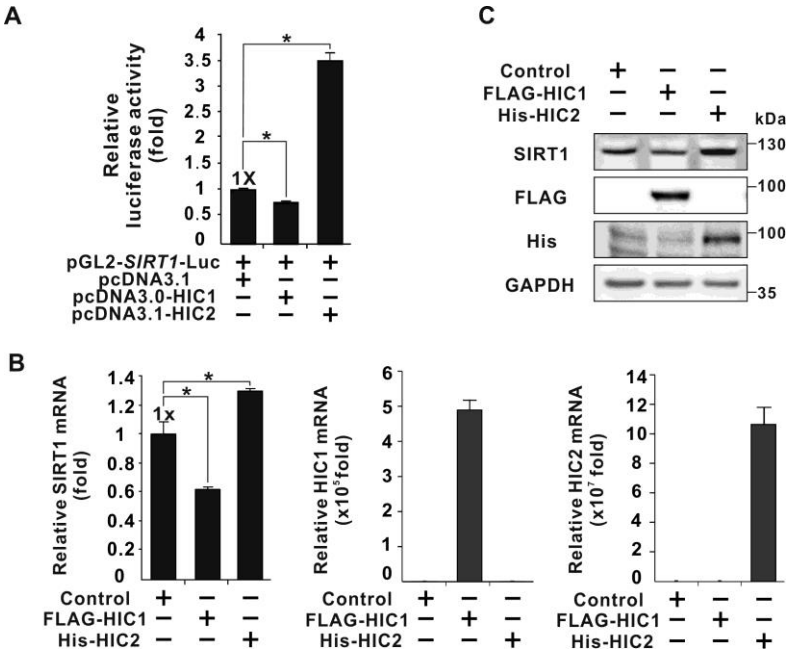


**Figure 1. HIC2 activates endogenous *SIRT1*, which is repressed by HIC1 in HEK293 cells.** (A) Transient transcription assay. HEK293A cells were transfected with various combinations of pGL2-*SIRT1*-Luc, pcDNA3.1, pcDNA3.0-*HIC1*, or pcDNA3.1-*HIC2*. Luciferase activity was measured 48 h after transfection, and normalized to cotransfected β-galactosidase activity. The results are average of three independent assays. **P* < 0.05. (B) RT-qPCR analysis of endogenous *SIRT1* mRNA expression, following transfection with *HIC1* and/or *HIC2* expression vectors. RT-qPCR results are average of three independent assays. GAPDH mRNA, normalization control. **P* < 0.05. (C)

Western blot analysis of endogenous SIRT1 expression in HEK293A cells transfected with pcDNA3.1, pcDNA3.0-*HIC1*, or pcDNA3.1-*HIC2* expression vectors. Whole cell extracts were separated by SDS-PAGE and analyzed using the antibodies indicated. GAPDH was used as a control.

**2. HIC2 interacts with HIC1**

As HIC1 and HIC2 have been previously reported to interact each other,[17] we conducted co-immunoprecipitation and GST-fusion protein pull-down assays to confirm and analyze this interaction. Co-immunoprecipitation and western blot assays of the cell lysates, prepared from HEK293A cells, transiently transfected with FLAG-HIC1 and/or His-HIC2 expression vector, showed that HIC2 interacts with HIC1 (Fig. 2A).

To "narrow down" the interacting domains of HIC1 and HIC2, we prepared [$^{35}$S]-methionine-labeled synthetic HIC2 or HIC1 protein by *in vitro* transcription and translation, and recombinant GST fusion protein fragments of HIC1 and HIC2 (Fig. 2, B-E). GST fusion protein pull-down assays of the reaction mixture of synthetic [$^{35}$S]-labeled HIC2 or HIC1 protein, and various recombinant GST fusion protein fragments, showed that HIC1 interacts with HIC2's POZ-domain and zinc-finger DNA-binding domains. Analogously, these assays also showed that HIC2 interacts with HIC1's POZ-domain, middle domain 1, and zinc finger DNA-binding domains. Interestingly, we found interactions between full-length HIC1 and the HIC2ZF, and also between full-length HIC2 and the HIC1ZF. It remains to be seen whether the interaction between full-length HIC1 and HIC2, via HIC1ZF, is also robust (Fig. 2, B and D).

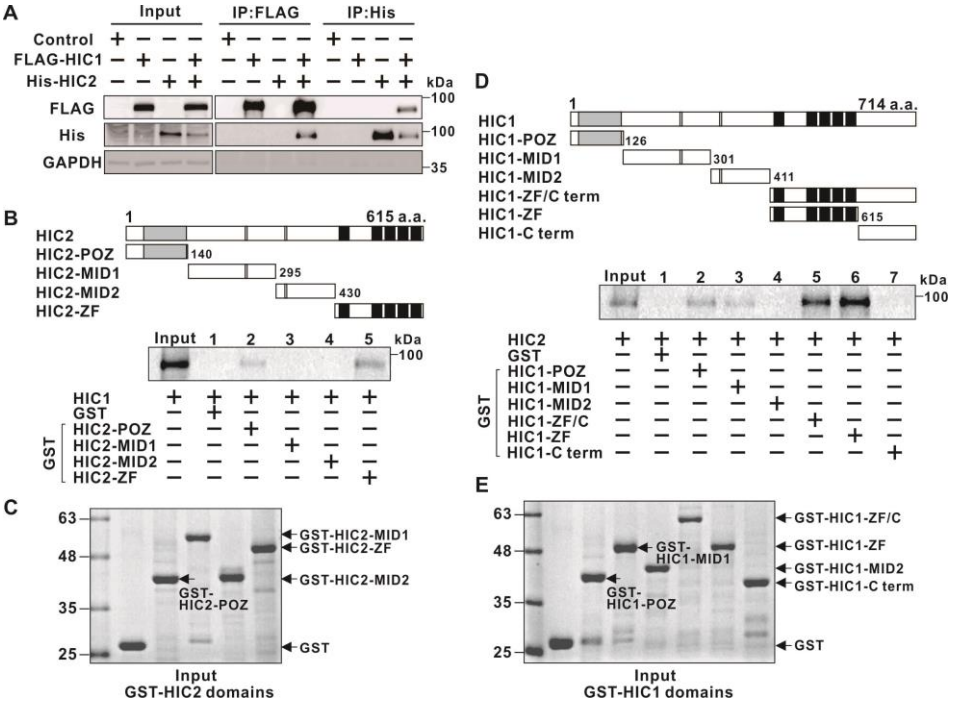


**Figure 2. HIC2 interacts directly with HIC1.** (A) Co-immunoprecipitation of HIC1 and HIC2. Cell lysates prepared from HEK293A cells, transfected with pcDNA3.0-FLAG-HIC1 or pcDNA3.1-His-HIC2 expression vectors, were immunoprecipitated using anti-FLAG and/or anti-His antibodies, and analyzed by western blot using the antibodies indicated. (B-E), GST-fusion protein pull-down assays. Schematic diagram of the domains of HIC1 and HIC2 tested. Recombinant GST protein and GST-fusion proteins were incubated with *in vitro* synthesized [$^{35}$S]-methionine-labeled HIC1 or HIC2, precipitated, resolved by 10% SDS-PAGE, and analyzed by autography.

**3. HIC2 activates transcriptional activation of SIRT1 via HIC1-binding elements**

Previously, Van Rechem *et al*. showed that HIC1 repressed SIRT1 by binding to two distal, and one proximal, elements within the SIRT1 promoter.[12] Since the DNA-binding domain of HIC2 is highly similar to that of HIC1, HIC2 might regulate SIRT1 by binding to its HIC1 binding elements. Transient transfection and transcription assays of various SIRT1 promoter reporter-gene fusion constructs, differing in lengths of their 5' upstream regulatory sequences in HEK293A cells (Fig. 3A), showed that HIC2 activates reporter gene expression. When the distal promoter region (bp, -1260 ~ -911), with two HIC1-binding elements deleted, SIRT1 transcriptional activation by HIC2 decreased by ~50%. While deletion of a further downstream promoter region (bp, -910 ~ -311) did not affect transcriptional activation by HIC2, deletion of a near proximal region (bp, -310 ~ -1), containing one HIC1-binding element, virtually eliminated transcriptional activation by HIC2 (Fig. 3B). These data show that HIC2 might regulate SIRT1 by acting on the same DNA elements as HIC1. Oligonucleotide pull-down assays of whole cell lysates transfected with empty pcDNA3.1 or pcDNA3.1-Myc-HIC2 showed that HIC2 could bind to all three HIC1-binding elements (Fig. 3C). When the distal region (bp, -1260 ~ -911) was deleted, *SIRT1* transcriptional activation by HIC2 decreased by ~50%,

and deletion of a near proximal region (bp, -310 ~ -1) decreased HIC2 transactivational activity by ~50%. One may argue that the binding patterns and transcriptional regulation do not match, as these particular experiments were carried out using deletion constructs of the *SIRT1* promoter and 5'upstream sequences. To more extensively assess the function of each HRE in transcriptional activation by HIC2, we introduced mutations by site-directed mutagenesis, while maintaining the length of 5'upstream sequence and promoter, and then performed transient transfection and transcription assays. Mutation of HREs -1 and -2 decreased reporter expression by 23% and 18%, respectively, mutation of HRE3 decreased reporter expression by 35%, and mutations of all three elements resulted in a 44% decrease (Fig. 3D). These data were consistent with *in vitro* binding patterns of HIC2, the potential presence of HIC1-binding elements, and HIC2 not binding to the mutated HREs.

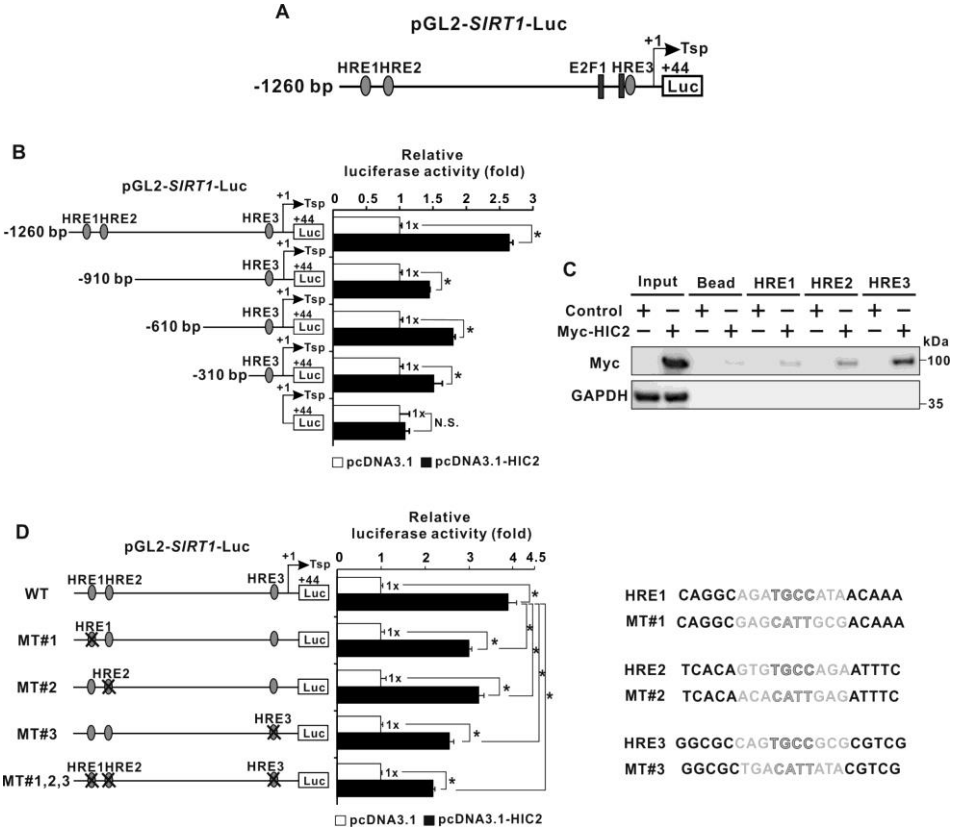


**Figure 3. HIC2 activates transcription of *SIRT1* via distal and proximal promoter HIC1-binding elements.** (A) Diagram of the *SIRT1* promoter luciferase gene fusion reporter construct. Key regulatory elements such as HIC1-binding elements (HRE1, HRE2, HRE3), and E2F1-binding elements, are indicated. The transcription start point (Tsp, +1) is marked by an arrow; filled oval circles, HIC1 or HIC2 binding elements; filled rectangular boxes, E2F1 binding elements. (B) Transient transcription assays. *HIC2* expression plasmid and reporter plasmids were transiently co-transfected into HEK293A cells. The

structures of various pGL2-*SIRT1*-Luc constructs are shown on the left. Data presented is the average of three independent assays. Luciferase activity was measured 48 hr after transfection and normalized by co-transfected β-galactosidase activity. Bars represent standard deviations. *$P < 0.05$. (C) Oligonucleotide pull-down assays of HIC2. Structure of *SIRT1* promoter with HIC1-binding elements are indicated in (A). HEK293A cells were transfected with pcDNA3.1 or pcDNA3.1-HIC2 plasmids, and harvested after 48 h. Biotinylated oligonucleotide probes, including HRE1, HRE2 or HRE3, linked to streptavidin agarose beads, were incubated with the cell lysates. The precipitates were then analyzed by western blotting using the antibodies indicated. (D) Transient transcription assays and structures of four additional *SIRT1* promoter and luciferase gene fusion constructs designed to confirm whether HIC2 binds to the HREs. HRE, HIC1-response element; GAPDH was used as a control.

**4. HIC2 decreased HIC1 binding to HIC1-binding elements**

Considering the molecular interactions between HIC1 and HIC2, HIC2 binding to HREs, and the negative effects of HRE mutation on transcriptional activation by HIC2, HIC1 and HIC2 may interfere with each other to affect HRE binding. To assess this scenario, we performed oligonucleotide pull-down assays of whole cell lysates prepared from HEK293A cells transfected independently with HIC1 or HIC2 expression vectors, showing that HIC1 and HIC2 interfere with each other for binding HREs (Fig. 4, A and B). Overall HIC2 binding was relatively weak, compared to HIC1 binding. Unlike cell lysates prepared from cells co-transfected with HIC1 and HIC2 expression vectors, HIC2 protein levels were not changed in the reaction mixture containing cell lysates of ectopic HIC1 or HIC2 expression.

Overall, oligo pull-down assays showed that HIC1 and HIC2 bind HREs-1, -2, and more strongly to HRE3, consistent with the data in Fig. 3C. HIC2 binding was strongly affected by HIC1. Interestingly, both HIC1 and HIC2 decreased HIC2 and HIC1 binding to the HREs strongly, indicating molecular interaction between HIC1 and HIC2 (Fig. 4B). Furthermore, chromatin immunoprecipitation (ChIP) assays likewise showed that HIC1 binds to HREs -1 and -2, and quite strongly to HRE3 (Fig. 4C). In contrast, HIC2 binding to HRE1 was relatively weak, and its binding to HREs -2 and -3 was higher than

to HRE1. These *in vivo* binding patterns, revealed by ChIP assays, were consistent with the *in vitro* binding patterns shown in Figs. 3C and 4B. The expression pattern of co-transfected HIC1 and HIC2, and that of HIC1 and HIC2, independently expressed (used as a mixture in oligo pull-down assays), may suggest that HIC1 and HIC2 can regulate transcription of *SIRT1* either by direct binding competition or by an indirect "titration'" effect by "neutralizing" each other's DNA-binding domain, through direct interaction of the two proteins.

Accordingly, we investigated whether transcriptional regulation of the *SIRT1* promoter reporter fusion construct could recapitulate the binding patterns of HIC1 and HIC2 to the promoter HREs, as revealed by *in vitro* oligo pull-down and *in vivo* ChIP assays. Indeed, transcriptional activation by HIC2 was drastically repressed by progressively increasing HIC1 expression (Figs. 4E and G). However, transcriptional repression of *SIRT1* by HIC1 was only marginally affected by progressively increasing HIC2 (Fig. 4F and G). Ectopic expression of *HIC2* was affected by the presence of ectopic *HIC1*, while ectopic HIC1 expression remained relatively constant (Figs. 2A and 4G). These assays well represented the interference of HIC2 expression by HIC1, and interference of HIC1 and HIC2 binding, by the molecular interaction between the two proteins.

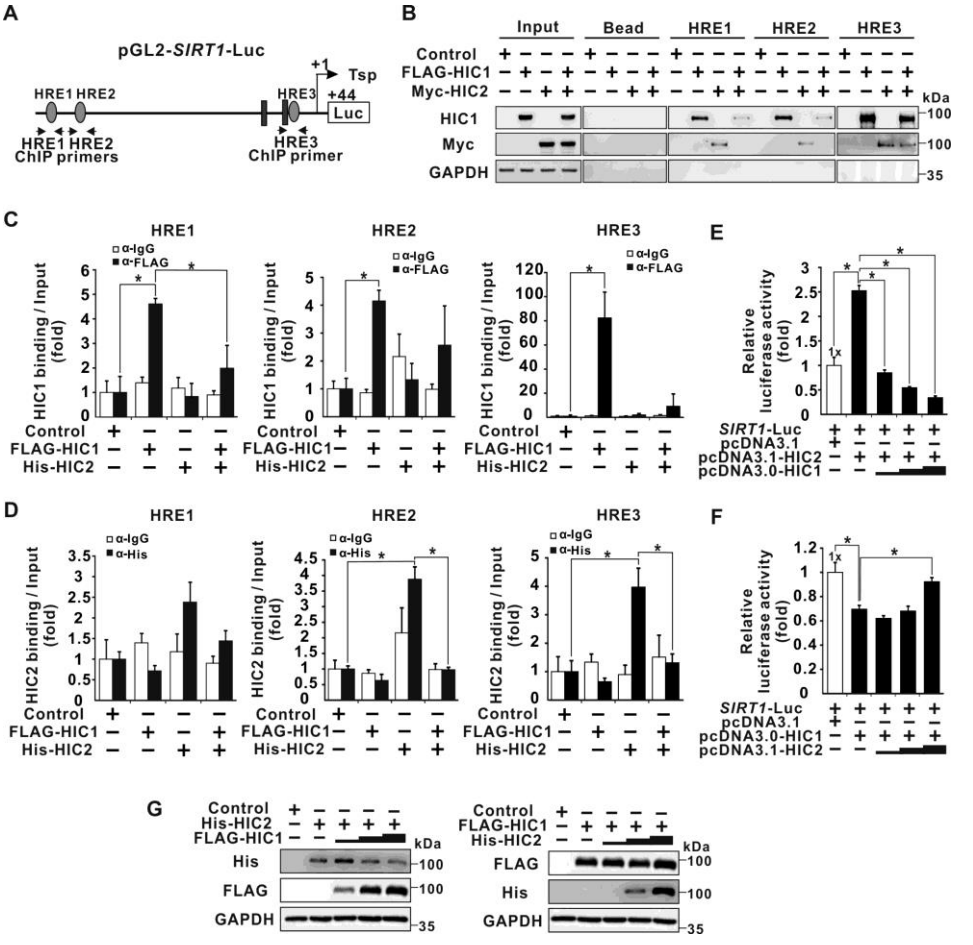


**Figure 4. HIC2 interferes with HIC1 binding to HREs in the *SIRT1* promoter.** (A) Structure of the *SIRT1* gene promoter. Arrows at the promoter regions indicate the locations of ChIP PCR primer-binding sites. (B) Oligonucleotide pull-down assays. Whole cell lysates of HEK293A cells transfected with pcDNA3.1, pcDNA3.0-HIC1, or pcDNA3.1-HIC2 plasmids were incubated with oligonucleotide probes conjugated to streptavidin agarose

beads. Proteins bound to the probes were precipitated by centrifugation and analyzed by western blotting. (C, D) qChIP assays. HEK293A cells were transfected with FLAG-HIC1 and/or His-HIC2 expression vector, as above. Chromatin was immunoprecipitated with the antibodies indicated and analyzed by qRT-PCR. The results are averages of three independent assays. IgG, negative control. *$P$ < 0.05. (E, F) Transient transfection and transcription assays. HEK293A cells were co-transfected with pGL2-*SIRT1*-Luc reporter constructs, a combination of FLAG-HIC1 and increasing amounts of His-HIC2 expression vector, or a combination of His-HIC2 and increasing amounts of FLAG-HIC1 expression vector. Luciferase activity was measured 48 hr after transfection and normalized to co-transfected β-galactosidase activity. The results are averages of three independent assays. Bars represent standard deviations. *$P$ < 0.05. (G) Western blots analyses of the lysates of the cells co-transfected with FLAG-HIC1 and increasing amounts of His-HIC2 expression vector, or vice versa.

**5. The middle domain of HIC2 activates transcription of the pG5-Luc reporter construct and interacts with p300**

Although HIC1 and HIC2 show high amino acid sequence homology (>85%) in their respective POZ and ZF domains, the two proteins differ in their middle domains 1 and 2. Because HIC1 represses, and HIC2 activates SIRT1 gene transcription, we suspected that these middle domains may contribute to the two proteins' differential effects on SIRT1. Therefore, we investigated which domains of HIC1 and HIC2 are responsible for transcriptional activation or repression of SIRT1, using the pG5-UAS-Luc system with yeast Gal4 DNA-binding elements at the proximal promoter. HEK293A cells were cotransfected with pG5-UAS-Luc, and the expression vectors of the domains of HIC1 or HIC2 fused with Gal4-DBD. In the case of HIC1, the middle domain 1 of HIC1 gave statistically significant repression, while POZ domain, middle domain 2, ZF domain, and C-terminus domain showed relatively weak effects in transcription. In the contrast, HIC2 middle 1, -2 domains and ZF domain showed transcriptional activation. The middle domain 2 showed more strong transcription activation of reporter than other domains (Fig. 5, A-D).

Because the middle domains of HIC1 and HIC2 showed opposite effects on the pG5-UAS-Luc reporter expression, the domains may confer transcriptional activation or repression potential of HIC1 and HIC2 on the SIRT1 promoter.

HIC1 contains a conserved GLDLSKK peptide motif that recruits the corepressor C-terminal-binding protein in the middle domain 1.[13] Overexpression of the histone acetyltransferase p300 acetylated HIC1 residues, including lysine 314, in the conserved ΨK314XEP, an acetylation/ SUMOylation switch motif in the middle domain 2.[15] Although the middle domains between HIC1 (a.a. 127-411) and HIC2 (a.a. 141-430) showed little amino acid sequence homologue, the GLDLSKK motif in the middle 1 domain, and ΨKXEP motif in the middle 2 domain, were preserved in HIC2 (Figs. 5 A and B). We postulated that differences in interactions between the middle domains of HICs and cofactors, corepressor CtBP or coactivator p300, may determine transcriptional activation or repression potential of HIC1 and HIC2 on the SIRT1 promoter.

Transient transcription assays in HEK293A cells showed that HIC1 repressed and HIC2 activated pGL2-*SIRT1*-Luc. Coexpression of HIC1 and CtBP repressed transcription further. Transcriptional activation of SIRT1 by HIC2 was significantly attenuated by coexpressed CtBP. These data suggested that the interaction between HIC1 and CtBP is important transcriptional repression of SIRT1 by HIC1 as reported previously by others (Fig. 5E).[15]

HIC1 was shown to interact with p300, which acetylated MKHEP motif (called acetylation/SUMOylation switch) in the middle domain 2.[15] Transient

transcriptional assays showed that HIC1 repressed transcription as above and coexpression of p300 showed little effects in transcriptional repression of SIRT1. However, coexpressed p300 enhanced transcriptional activation by HIC2 (Fig. 5F). The differences in molecular interactions between the middle domains of HICs and p300, may determine transcriptional activation or repression potential of HICs on the SIRT1 promoter.

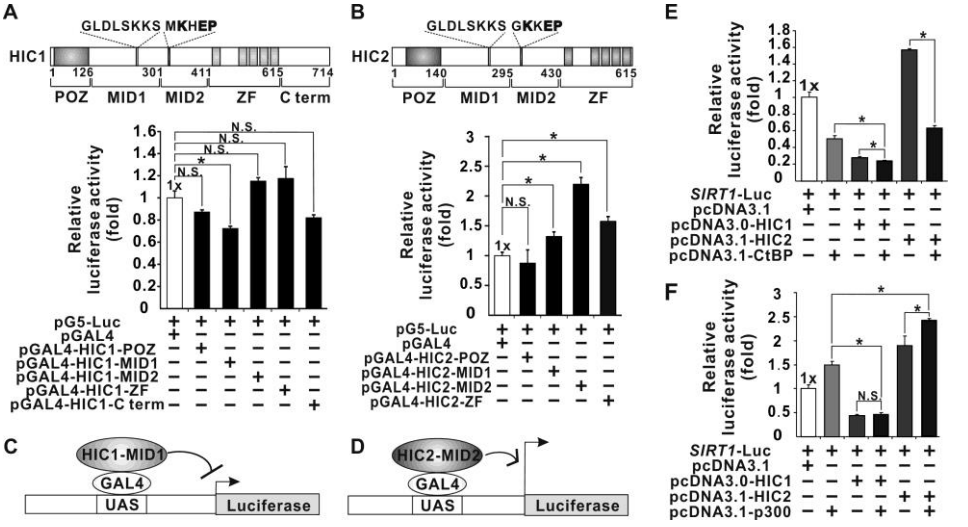


**Figure 5. The middle domains of HIC1 and HIC2 show opposite transcriptional regulatory activity.** (A-D) Transient transfection and transcription assays. HEK293A cells were co-transfected with pG5-UAS-Luc reporter construct, and expression vectors of the domains of *HIC1* or *HIC2* fused with Gal4-DBD. The results are average of three independent assays. (E, F) Transient transfection and transcription assays. HEK293A cells were co-transfected with the pGL2-*SIRT1*-Luc reporter constructs and a combination of HIC1 and/or HIC2 expression vectors, with CtBP or p300 expression vector. Luciferase activity was measured 48 hr after transfection, and normalized to co-expressed β-galactosidase activity. The results are average of three independent assays. **P* < 0.05.

**6. Middle domain 2 of HIC2 interacts with p300 to activate transcription of target genes**

Transcriptional activators often interact with coactivators, such as p300/CBP, PCAF, and Tip60.[29] Because above data suggested that HIC2 may interact with p300 and has a conserved p300 acetylation MKHEP motif in the middle domain 2, we suspected that the middle domain 2 might interact with p300. We prepared pGAL4 and pVP16 fusion protein expression vectors which expressed HIC2-MID2 fused with GAL4-DBD and p300 domains fused with the VP16 transcription domain (Fig. 6A). Mammalian two-hybrid assays in HEK293A cells, using pG5-Luc, pVP16-p300 domains and pGAL4-HIC1-POZ, pGAL4-HIC2-POZ, pGAL4-HIC1-MID2 or pGAL4-HIC2-MID2 fusion protein expression vectors, showed that only middle 2 domain of HIC2 interacted with p300 domains (Fig. 6B-E). The POZ domains of HIC1 and HIC2, which are known to form homo- and heterodimers and also with other regulatory proteins, did not bind to the p300 domains. Except p300 domain 1, p300 domains interacted with HIC2 middle domain 2, but not with the HIC1 middle domain 2, despite its conserved p300 acetylation lysine residues at a.a. 313-317 (MKHEP). The difference in protein interaction with p300 may explain differences in transcriptional regulatory properties by HIC1 and HIC2.

Although interaction of HIC1 and p300 was not certain, acetylation of HIC1 by p300 on lysine 314 was reported to disrupt its interaction with MTA1, and curtail HIC1's transcriptional repression potential.[15] Consequently, we investigated the molecular interaction between HIC2 and p300 by co-IP and western blot assays, showing that HIC2 interacts with p300 (Fig. 6F), and is acetylated at lysine 341 by p300. As expected, when HIC2 lysine 341 was mutated with arginine, the acetylation of HIC2 was significantly reduced (Fig. 6G).

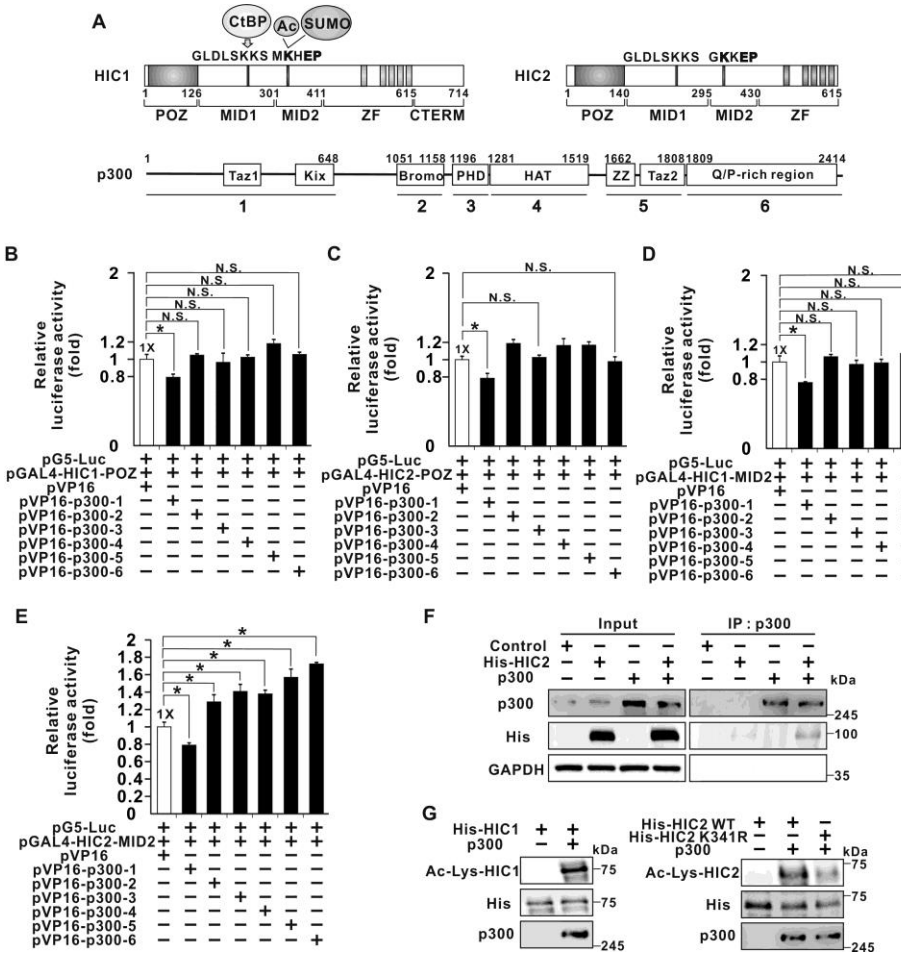


**Figure 6. HIC2 interacts with p300 to activate transcription of pG5-Luc.** (A) Structures of HIC1, HIC2, and p300. Key regulatory motifs are indicated in the sequences. (B) Mammalian two-hybrid assays. HEK293A cells were transfected with pG5-Luc, pVP16-p300 domains and pGAL4-HIC1-POZ, pGAL4-HIC2-POZ, pGAL4-HIC1-MID2, or pGAL4-HIC2-MID2. Luciferase

activity was measured 48 h after transfection and normalized to co-expressed β-galactosidase activity. The results are average of three independent assays. *$P < 0.05$. (F) Co-IP and western blot analysis of the molecular interaction between HIC2 and p300. HEK293A cells were transfected with pcDNA3.1-His-BCL6 and/or pcDNA3.1-p300, lysed, and precipitated with anti-His or anti-p300 antibody. The precipitates were then analyzed by western blotting. GAPDH, control. (G) *In vitro* acetylation assays of HIC1, HIC2 WT, and a HIC2 K341R mutant. Recombinant POK proteins (HIC1, HIC2 WT, and HIC2 K341R) were incubated with p300. 10% of the reaction mixture was separated by 6% SDS-PAGE. Acetylated POK proteins were detected by western blot analysis, using an antibody against acetyl-lysine.

**7. HIC2 increases Sirt1 expression in the mouse heart and cardiomyocytes cell line**

Considering the fact that *Sirt1* and *Hic2* KO mice have the same phenotype in the heart, we supposed SIRT1 upregulation by HIC2 could occur in the heart. To study such in vivo effects, we injected adult mice hearts with $7x10^{10}$ HIC2 viral particles or controls. One week later, SIRT1 protein levels were significantly increased in the hearts of mice overexpressing HIC2 (Fig. 7A). Similarly, infection with HIC2 adenovirus increased Sirt1 expression in mouse primary cardiomyocytes (Fig. 7B). Moreover, SIRT1 is well known to protect the heart from myocardial ischemia/reperfusion injury and HIC2 may also respond to hypoxia.[19,24] In H9c2 cardiomyocyte cells, SIRT1 reduced by I/R injury was rescued by overexpression of HIC2, and the amount of FOXO3, a target of SIRT1-induced apoptosis, decreased (Fig. 7C). Apoptosis assays in primary mouse cardiomyocytes showed that ectopic *Hic2* decreased apoptosis by I/R injury (Fig. 7D). To confirm that HIC2 protects cardiomyocytes by increasing SIRT1, we induced I/R injury after knockdown of *Sirt1* in H9c2 cardiomyocyte cells. Western blots further showed that when *SIRT1* was knocked down, FOXO3 did not decrease, even when HIC2 was overexpressed. These data suggest that the cardioprotective effect of overexpressed HIC2 is SIRT1-dependent (Fig. 7E), potentially by inhibiting apoptosis following I/R

injury, through increased expression of SIRT1.

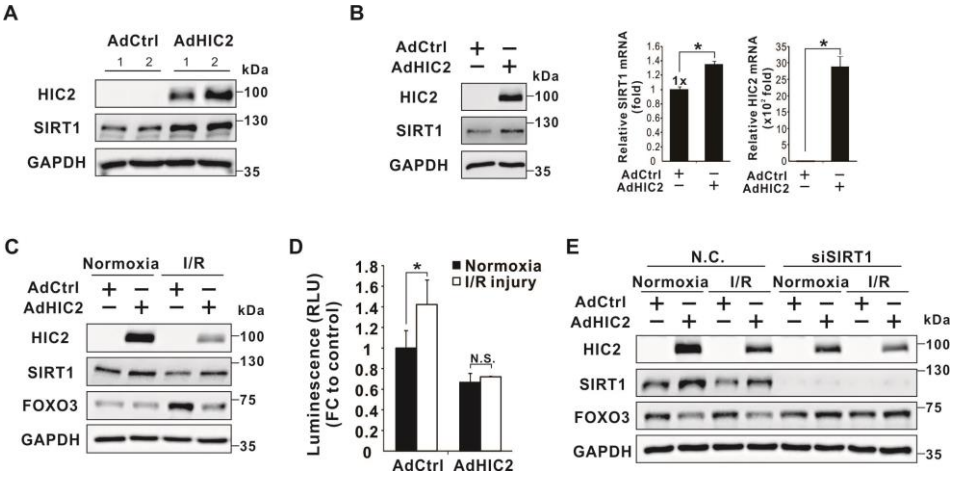


**Figure 7. HIC2 decreases apoptosis by increasing SIRT1 in I/R injury.** (A) Western blot analysis of endogenous SIRT1 expression in the heart of adult mice infected with HIC2 adenovirus. (B) Western blot analysis of endogenous SIRT1 expression in mouse neonatal primary cardiomyocytes. Cells were infected with $1x10^8$ control or HIC2 viral particles per well, in six-well plates, for 48 hr. (C) Western blot analysis of endogenous SIRT1 and FOXO3 expression in H9c2 cells. Cells were infected with $1x10^9$ control or *HIC2* viral particles, in 60mm dishes, for 48 hr. Cells were then subjected to I/R injury. GAPDH was used as a control. (D) Apoptosis assays. Luminescence data shows the degree of phosphatidylserine exposure during apoptosis. $*P < 0.05$. (E) Western blot analysis of endogenous SIRT1 and FOXO3 expression in H9c2 cells. Cells were transfected with the indicated siRNAs, and infected with $1x10^9$

control or HIC2 viral particles for 48 h, prior to I/R injury. GAPDH, control.

## IV. DISCUSSION

In this study, we demonstrated that HIC2 is a transcriptional activator of SIRT1. Since SIRT1 plays an important role in a variety of biological processes,[2-4] it is meaningful to understand its transcriptional regulation. After SIRT1 was shown to inhibit aging and improve various diseases, SIRT1 activators have been developed.[30,31] Previously, HIC2 was considered another transcriptional repressor like HIC1, since the POZ and zinc-finger domains of HIC2 are similar to those of HIC1,[17] and HIC2 co-localized with CTBP1, a corepressor of HIC1.[18] Contrary to that expectation, however, we show here that HIC2 is actually an activator of SIRT1.

Although HIC2 has high amino acid similarity to HIC1, particularly in its POZ and zinc-finger domains, the rest of the amino acid sequences are quite different. Interestingly, two functional motifs in the HIC1 middle domain, which forms repression complexes with corepressors, are also conserved in HIC2. In present study, we showed that the middle domain 2 of HIC2, including the ΨKXEP functional motif, increases SIRT1 transcription (Fig. 5). Previous studies showed that when SIRT1 deacetylates the K314 lysine residue of the MKHEP motif of HIC1, this residue is SUMOylated by HDAC4, resulting in an acetylation/SUMOylation switch.[15] SUMOylated HIC1 binds to MTA1, a

subunit of the NuRD (nucleosome remodeling and histone deacetylase) complex, to form a repressive complex.[12] Considering that several domains of p300 interact with the middle domain 2 of HIC2, HIC2 may form an activation complex with other cofactors, maintaining the acetylation form of this motif, rather than a repression complex, by an acetylation/SUMOylation switch. Acetylation by p300 in the ΨKXEP motif is important for HIC1 and HIC2 to act either as a transcriptional repressor or activator. Further studies are necessary to investigate detailed mechanisms of how cofactors, including p300, interact with HIC2 through the ΨKXEP motif of HIC2, to facilitate transcriptional activation.

Both Hic2 and Sirt1 KO mouse embryos showed similar ventricular septal defects,[18,20] possibly due to excessive apoptosis triggered by oxidative stress.[21] Previous studies have shown that SIRT1 protects the heart during oxidative stress, including I/R injury.[24,32-34] In particular, a previous study using cardiac-specific overexpressing Sirt1 mice have reported that moderate expression of Sirt1 can mitigate oxidative stress and apoptosis.[32] Also, HIC2 expression has been reported to be regulated by hypoxia signals.[19] Therefore, we suspected that SIRT1 expression may well be regulated by HIC2, in the heart. Our data showed that Hic2 overexpression in mouse heart, neonatal primary cardiomyocytes, and H9c2 cardiomyocyte cell lines, increasd Sirt1 expression. Accordingly, HIC2 may be upregulate SIRT1 in the heart, and

potentially, many other SIRT-regulated physiological processes.

SIRT1 protects cells from apoptosis by deacetylating and inhibiting the expression of the pro-apoptotic Forkhead box O (FOXO) family of transcription factors, decreasing their activities.[35,36] In previous studies, FOXO3 has been reported to be activated in myocardial ischemia/reperfusion (I/R) injury.[37,38] Considering that FOXO3, increased by I/R, is reduced to normoxic levels by HIC2 overexpression, HIC2 may play a role in protecting cells from apoptosis by increasing SIRT1. Further investigation is required to identify changes in other SIRT1 target genes, followed by increasing of SIRT1 expression by HIC2 in I/R.

In conclusion, we were able to not only discover HIC2 as a transcriptional activator of SIRT1, but also HIC2's molecular mechanism of how it transcriptionally activates SIRT1. Here, despite having high sequence homology to HIC1, HIC2 acts not as a transcription repressor, like HIC1. Considering the importance of SIRT1 in various biological processes, and in addition to heart injury, these findings have significant implications for numerous SIRT1-mediated processes, including aging, metabolism, and autoimmunity.

## V. CONCLUSION

Our group found that HIC2, highly homologous to HIC1, is a transcriptional activator of SIRT1, due to opposite activity of the intermediate domains of the two homologs. This relationship between HIC2 and SIRT1 could be important for cardiac development, which associates with both proteins. Here, we assessed whether ectopic expression of HIC2, and subsequent upregulation of SIRT1, might decrease apoptosis in H9c2 cardiomyocytes under simulated ischemia/repurfusion injury conditions. Our results demonstrate that unlike its structual homolog HIC1, HIC2, is a pivotal transcriptional activator of SIRT1, and consequently, may protect the heart from I/R injury.

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**ABSTRACT (in Korean)**

## HIC2는 SIRT1 유전자의 전사를 활성화 시킨다.

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송 지 양

히스톤 탈 아세틸화 효소인 SIRT1은 노화, 대사, 자가면역 등의 다양한 생리학적 과정에서 중요한 작용을 하며 여러가지 전사인자들에 의해서 발현이 억제된다. HIC1은 SIRT1을 억제하는 것으로 잘 알려져 있는 전사인자이다. 우리 연구팀에서는 HIC1과 높은 구조적 상동성을 지닌 HIC2가 HIC1과 zinc-finger domain을 통해 상호작용한다는 사실 및 HIC2가 SIRT1의 전사 활성자라는 사실을 밝혔다. 중간 도메인의 구조적 차이로 인해 두 단백질은 SIRT1의

전사를 상반되게 조절한다. 우리는 mammalian two-hybrid assay 실험을 통해서 p300 도메인들이 HIC2의 두번째 중간 도메인과 결합한다는 사실을 확인했다. 하지만 p300에 의해서 아세틸레이션 되는 것으로 보고 된 HIC1은 p300 도메인들과 결합하지 않았다. p300과의 결합 여부가 전사 조절에서 HIC1과 HIC2의 차이를 유발할 가능성이 있다.

HIC2와 SIRT1은 각각 심장 발달에서 중요한 역할을 하기 때문에 두 단백질 사이의 관계는 심장 발달과 관련이 있을 수 있다. 본 연구에서 우리는 과발현된 HIC2가 허혈재관류 손상 (ischemia/ repurfusion injury) 시, 세포사멸로부터 H9c2 심근세포를 보호할 수 있다는 사실을 확인했다. SIRT1의 발현을 낮춘 상태에서는 HIC2를 과발현 시켜도 허혈재관류 손상시 세포사멸을 유발하는 FOXO3가 감소하지 않았다. 따라서 HIC2는 SIRT1을 조절함으로써 세포사멸로부터 심근세포를 보호한다는 사실을 알 수 있었다. 본 연구 결과는 HIC1과 구조적 유사성이 높은 HIC2가 SIRT1의 전사 활성자로서 허혈재관류 손상으로부터 심장을 보호할 수 있다는 가능성을 제시하였다.

**핵심되는 말: HIC2, SIRT1, HIC1, 전사, 심장, 허혈재관류 손상**

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3. Choi WI, Yoon JH, Song JY, Jeon BN, Park JM, Koh DI, et al. Zbtb7c is a critical gluconeogenic transcription factor that induces glucose-6-phosphatase and phosphoenylpyruvate carboxykinase 1 genes expression during mice fasting. Biochim Biophys Acta Gene Regul Mech 2019;1862;643-656.

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5. Jeon BN*, Song JY*, Huh JW, Yang WI, Hur MW. Derepression of

matrix metalloproteinase gene transcription and an emphysema-like phenotype in transcription factor Zbtb7c knockout mouse lungs. FEBS Lett 2019; doi:10.1002/1873-3468.13501. (* Co-First)