A ChIP-cloning approach linking SIRT1 to transcriptional modification of DNA targets

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*The mammalian protein deacetylase SIRT1 (sirtuin1) is widely recognized for its link to calorie restriction and longevity. SIRT1 not only modulates the function of protein targets such as p53 or NFkappaB, but it also affects gene transcription by causing hypoacetylation of associated nucleosomal histones. However, the identification of SIRT1-specific DNA targets that confer chromosomal stability and cell longevity have remained elusive. Here, we report the usefulness of a ChIP-cloning approach for the identification of an endogenous DNA target intimately linked with SIRT1 activity. Using the aforementioned technique, we identified a gene encoding the neuro-oncological ventral antigen2 (*nova2*) as a SIRT1 target.* Nova2 *regulates the alternative splicing of* scn1a*, which encodes the α-subunit of a neuronal sodium channel targeted by antiepileptic drugs. This finding demonstrates that ChIP-cloning is an innovative approach for the identification of SIRT1-specific DNA targets.*

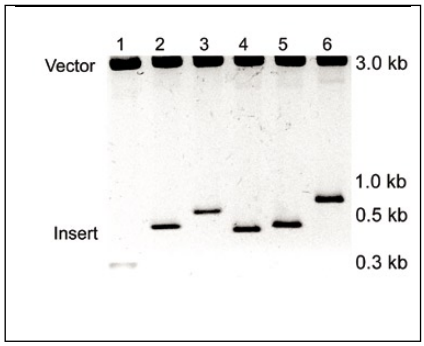

Figure 1. Ethidium bromide–stained agarose gel showing restriction analysis of endogenous DNA targets of SIRT1 derived from ChIP-cloning. Plasmid DNA (1 µg) from each of six randomly chosen bacterial transformants (1–6) carrying SIRT1-specific DNA targets (insert) in pGEM-T Easy (Vector) was subjected to *Eco*RI restriction for 4 h at 37°C. Agarose gel–electrophoresis (1.5%) revealed the linearized vector at 3.0 kilobase pairs (kb) and SIRT1-specific targets ranging from approximately 0.9 kb to 0.3 kb. All six DNA clones were subsequently subjected to DNA sequencing analysis.

Limiting food intake increases life span (1). For instance, calorie restriction (CR) extends mean and maximum life span in a variety of organisms, including yeast (2), fruit flies (3), nematodes (4), and rodents (5). Food restriction also appears to promote longevity in nonhuman primates (6), suggesting therefore that CR may be an untapped potential procedure for modifying life span in humans. The mammalian SIR2 ortholog, SIRT1 (Sirtuin1), mediates in part the physiological effects produced by CR (7). SIRT1 is nuclear and functions as a protein deacetylase, capable of modulating the actions of other proteins in a (NAD+)-dependent manner. SIRT1 targets a wide range of proteins, including p53 (8), NFkappaB (9), MyoD (10), and histone proteins that form the nucleosome. Much of the work on SIRT1 has focused on linking Sirt1-induced protein modification with age-onset diseases. In contrast, the search for specific target genes directly affected by SIRT1 have been largely neglected, particularly those genes associated with epigenetic changes of the histone code. In this regard, once tethered to DNA, SIRT1 influences chromatin structure by deacetylating specific lysine residues of histones H1, H3, and H4 (11,12). Histone deacetylation in eukaryotic cells is generally associated with heterochromatin-formation and transcriptional repression.

Two major obstacles for the analysis of previously unidentified DNA targets are the lack of detailed knowledge of SIRT1/DNA linkage and the generally poor intergenic resolution of genome scanning techniques such as those found in microarrays procedures. To circumvent these two limitations, we decided on a technique that combines both chromatin immunoprecipitation (ChIP) with cloning of the generated DNA piece, followed then by sequence analysis (13). In this context, all ChIP techniques (14) take advantage of the fact that proteins that link with chromatin can be crosslinked to the site of their association in cell lineages. Subsequent fragmentation of the endogenous DNA, followed by immunoprecipitation using an antibody specific for the protein in question, allows for the selection of a specific protein/DNA complex. The purified DNA fraction is most often screened for explicit targets by PCR using sequence-specific DNA primers. To find previously unknown DNA targets, the precipitated DNA can be used in a genome-spanning DNA microarray (ChIP-chip), which is often not only prohibitively expensive but also limited by the degree of intergenic coverage. This latter issue is especially problematic when working with proteins binding to the large extent of regulatory versus coding DNA. We therefore chose to pursue a ChIP-cloning approach followed by DNA sequence identification of the precipitated DNA. The application of this combinatorial approach provides an innovative novelty to the field of SIRT1 biology, allowing the identification of endogenous DNA targets to be viewed in a true genome-wide search format.

For the following experiment, adherent human embryonic kidney cells (HEK-293) were grown to 80%–90% confluence on 10 cm cell culture dishes under standard culture conditions (Dulbecco's Modified Eagle Medium, 10% fetal bovine serum, at 37°C with 5% CO_2). Ten 10 cm dishes (approximately 1–2 × 10^8 cells) went into one single experiment. ChIP and subsequent DNA purification was accomplished by using the EZ ChIP kit (Upstate, Lake Placid, NY, USA) according to the manufacturer's specifications. The crosslinked DNA was sheared into approximately 200 to 1000 bp fragments by sonication of the cell lysate with five sets of 10 s pulses on wet ice using a Cell Disruptor (Heat-Systems Ultrasonics, Farmingdale, NY, USA) with a 2 mm tip and set to 30% of maximum power. SIRT1/DNA complexes were immunoprecipitated by either using 10 µg of a monoclonal (Clone 2G1/F7, Upstate) or a polyclonal anti-SIRT1 antibody (Bethyl Laboratories, Montgomery, TX, USA). Following the manufacturer's specifications, 5 µl of the purified DNA solution was ligated to an *Eco*RI adaptor and excess adaptor subsequently removed using spin columns prepared with Sephacryl S-400 (Universal Riboclone cDNA Synthesis System, Promega, Madison, WI, USA). Purified DNA was amplified by PCR using primers matching the sequence of the *Eco*RI adaptor (forward and reverse primer: AATTCCGTTGCTGTCG). The

amplified DNA was briefly separated on a 1.5% agarose gel and fragments larger than 200 bp were extracted (Qiagen, Valencia, MD, USA). Purified DNA was ligated into the pGEM-T Easy Vector System and transformed into the *E. coli* strain JM109 (Promega). Plasmid DNA from positive transformants (blue-white screen) was subjected to restriction with the endonuclease *Eco*RI, and analyzed on a 1.5% agarose gel (Figure 1). Inserts larger than 200 bp were DNA-sequenced and identified using the "Basic Local Alignment Search Tool" (BLAST; www.ncbi.nlm.nih.gov/blast). A selection of identified DNA targets was verified by PCR using sequence-specific DNA primers (Integrated DNA Technologies, Coralville, IA, USA) on unamplified ChIP DNA (Figure 2). One of the identified target sequences corresponds to an intronic sequence of the gene encoding the neuro-oncological ventral antigen2 (*nova2*). The type of association of SIRT1 with *nova2* is not yet clear. Because SIRT1 has not yet been demonstrated to bind DNA by itself, the concomitant presence of other protein factors linking SIRT1 to the *nova2* gene locus seems likely to occur. Regardless of this circumstance, it is conceivable that SIRT1 might facilitate local histone deacetylation followed by the formation of heterochromatin and transcriptional repression of *nova2*. Interestingly, NOVA2 is known to regulate the alternative splicing of *scn1a*, which encodes the α-subunit of a neuronal sodium channel associated with the pharmacological effects of antiepileptic drugs (e.g., carbamazepine and phenytorin; 15). These drugs are thought to control seizures in people by acting on certain neuronal channels of the neuromuscular system. Of further interest, antiseizure drugs markedly extend mean and maximum life span of the nematode *Caenorhabditis elegans* (16). Our results open up the possibility that there may be new SIRT1 targets not yet explored that affect life span and neuromuscular function.

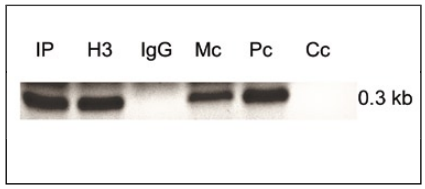

Figure 2. Ethidium bromide–stained agarose gel showing the PCR identification of *nova2* using unamplified ChIP DNA. The product size is approximately 0.3 kb (forward primer: TAGGTCT TCGAGGCTGTCCTTCAT; reverse primer: TGT GCTGAGTGCTTTGTGAGCTT). Standard Taq-PCR was performed on the following six samples: IP, Input; H3, Acetyl-histone H3 immunoprecipitation; IgG, Immunoglobulin G immunoprecipitation; Mc, Monoclonal SIRT1 immunoprecipitation; PC, Polyclonal SIRT1 immunoprecipitation; Cc, Contamination control for PCR.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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