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Secondary Analysis of ChIP-SEQ Dataset and Genomic Interactions of ICER

Karem Rivera

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Abstract

The Inducible cyclic AMP (cAMP) Early Repressor (ICER) is an endogenous transcriptional repressor of the cAMP-mediated signaling gene transcription pathway and belongs to the CRE-binding protein (CREB)/CRE modulator (CREM) gene family. ICER is associated to many functions and biological processes in the immune system, brain, regulation of spermatogenesis, circadian control of transcription, apoptosis and metabolic functions, and of particular interest, has been shown to act like a tumor suppressor. Recent findings from our laboratory suggest that ICER might have a role in DNA repair, but further experiments need to be performed. In this research, we aimed to elucidate the function of ICER and its role in DNA repair, by first re-analyzing ICER's DNA binding sites and genome interaction from a recently published Chromatin Immunoprecipitation - Seq dataset by Seidl et al, 2020. Then, using PCR and EMSA we empirically showed that ICER binds to some important genes thought to be involved in variety of DNA Repair pathways such as PARP1, RAD51C, XRCC6 and XRCC2, suggesting that ICER might play a role in DNA repair mechanisms. These results are important and will inform future experiments to understand ICER's function in DNA repair.

MONTCLAIR STATE UNIVERSITY

SECONDARY ANALYSIS OF CHIP-SEQ DATASET AND GENOMIC INTERACTIONS OF ICER

by

Karem Rivera

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Thesis Committee

[redacted]

Dr. Carlos A. Molina

Thesis Sponsor

[redacted]

Dr. Charles Du

Committee Member

[redacted]

Dr. John Gaynor

Committee Member

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Table of Contents

List of Figures

List of Tables

Introduction

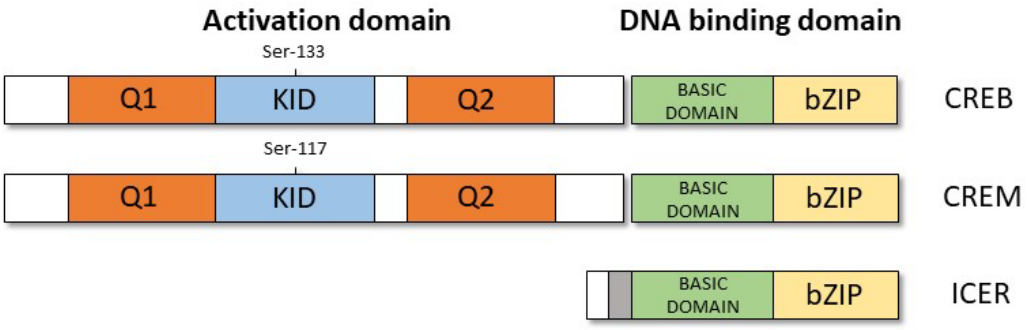
Transcription is a highly regulated process controlled not only by an array of regulatory proteins but also regulatory sequences. Proteins that regulate transcription respond to various signaling pathways, such as the cAMP (cyclic Adenosine Monophosphate) dependent system (Lee et al., 1995). The intracellular concentration of the secondary messenger cAMP in different cell types is essential for the activation or inhibition of transcription of many genes, and in doing so is involved in regulating many physiological and pathological processes (Zhang et al., 2020). Two protein members of this pathway that are important for gene regulation are CREM (cAMP Response Element Modulator) and CREB (cAMP Response Element Binding protein). Both of these proteins, together with AFT1 (Activator Transcription Factor 1), belong to a class of nuclear transcription factors called the CREB family and respond to a specific palindromic regulatory sequence composed of eight nucleotides: TGACGTCA, denominated CRE (cAMP Response Element). Although variations on this motif exist, almost every CRE contains at least a conserved half sequence (TGACG/CGTCA) (Lonze & Ginty, 2002).

cAMP responsive proteins are structurally similar; they are characterized by the presence of a highly conserved basic leucine zipper (bZIP) domain: an α-helical coiled-coil structure with an adjacent basic domain located at the C-terminus, that facilitates DNA binding and dimerization with other bZIP family members (Servillo et al., 2002). They also have a Kinase Inducible Domain (KID) which contains a Serine within the Protein Kinase A (PKA) phosphorylation site (RRP**S**Y) (Zhang et al., 2020), located between two glutamine rich domains (Q1 and Q2) that constitute the transcriptional activation domain

(Xiao et al., 2010). The functional domains of CREB, CREM and ICER are shown on Figure 1.

Figure 1

Structure of CREB and CREM showing the Activation and DNA binding domains and the Serine residues phosphorylated by activating protein kinases. ICER structure is also illustrated.



Note: Image adapted from Servillo et al, 2002

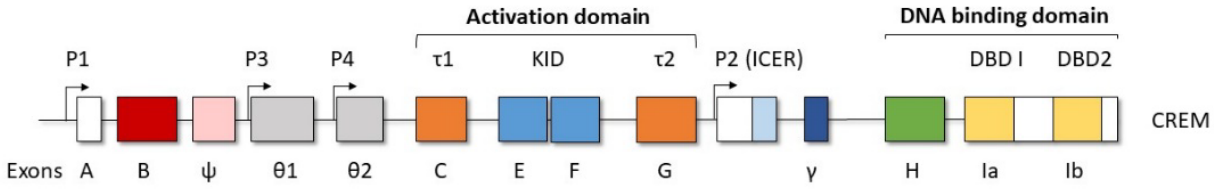
The *CREB* and *CREM* genes are complex and encode extensive homologous proteins with activating or suppressing functions as a result of alternative splicing (Molina et al., 1993) (Ortega-Martínez, 2015). The *CREB* gene produces only activator isoforms, whereas *CREM* can produce isoforms that are either activators or repressors, depending on the exons transcribed (Sassone-Corsi, 1995)

The *CREM* gene encodes more than 20 isoforms due to alternative promoter usage and alternative splicing (29 isoforms reported on Uniprot ("UniProt: the universal protein knowledgebase in 2021," 2021)) (Rauen et al., 2013) (Figure 2). Promoter 1 (P1) is located in the upstream region of the *CREM* gene and is constitutively active. The KID, located in the central region, is encoded by exons E and F. This domain interacts with coactivators

such as the histone acetyltransferases CREB Binding Protein (CBP) and P300 and mediates cAMP inducibility (Daniel et al., 2000). Exon C and the 3′-region of exon B encodes the upstream glutamine-rich *trans*-activation domain Q1 (also named τ1) and exon G encodes the downstream domain Q2 (τ2). Additionally, the *CREM* gene contains two DNA-binding or dimerization domains (bZIP structure) encoded by exons H and I. Interestingly, exon I, which has two alternative splice sites including the 3′-untranslated region (UTR) gives rise to two DNA-binding domains (DBD1 and DBD2) but only one is included in the final protein (Daniel et al., 2000) (Borlikova & Endo, 2009).

Figure 2

Schematic representation of the human CREM gene structure depicting exons and promoters.



Note: Image adapted from Don et al, 2002

CREMα, CREMβ and CREMγ spliced isoforms originate from P1 and act as antagonists (suppressors) of cAMP induced transcription (Foulkes et al., 1991), these isoforms lack both glutamine rich (Q1 and Q2) domains important for the transactivation function (Don & Stelzer, 2002). CREMτ, CREMτ1, CREMτ2 isoforms originate from the same promoter (P1) as the suppressor isoforms, but in contrast to them, these isoforms act as transcriptional activators and include the glutamine rich-domains in their sequence (Don & Stelzer, 2002) (Foulkes et al., 1992). Two additional isoforms termed CREMθ1 and

CREMθ2 are product of two exons (θ1 and θ2) containing two CREM alternative promoters (P3 and P4 respectively). These two promoters are also cAMP responsive, and can regulate the transcription of trans-activator forms of CREM when activated by a cAMP-dependent protein kinase signaling (Daniel et al., 2000).

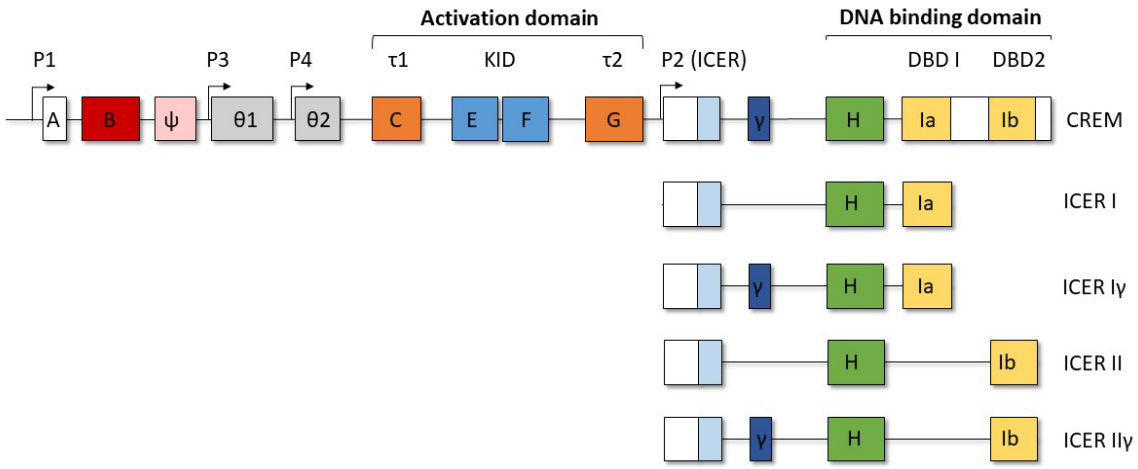
ICER (Inducible cAMP Early Repressor), a particular isoform of the *CREM* gene, is generated by an alternative promoter, P2, located within an intron near the 3' end of the *CREM* gene. ICER is actually defined as a family of small 12-13 kDa proteins that lack the transactivation and KID domain but contain the DBD (bZIP structure) that allows for DNA binding and dimerization with CREM and CREB proteins (Molina et al., 1993). ICER acts as a powerful transcriptional repressor and is induced by cAMP on its own promoter. ICER expression is activated by transcriptional activators (e,g CREB/CREM) that bind to P2, which contains four closely spaced CREs termed CAREs (cAMP Autoregulatory Elements). This activation increases ICER's intracellular concentration and leads to a strong repression of cAMP-induced transcription of CRE-containing genes, and negatively regulates ICER's own expression by binding to its own promoter (Molina et al., 1993) (Yehia, Schlotter, et al., 2001).

Due to alternative splicing, ICER transcript generates four isoforms called ICER I, ICER Iγ, ICER II, and ICER IIγ (Figure 3). DBD1 and DBD2 are present on ICER I and ICER II respectively and exon γ is absent on the γ isoforms (ICER Iγ and ICER IIγ) (Sassone-Corsi, 1995). Another inducible isoform called small ICER (smICER) was recently described in 2014 by Seidl et al. (Seidl et al., 2014). This isoform is generated by a CRE-dependent internal promoter (P6) and is structurally similar to ICER but lacks the ICER-specific exon and contains the γ domain (exon x) and one of the DBD. Its function

is not well defined but it was shown to be induced after β-adrenergic stimulation in the heart and researchers suggest that it could be responsible for the biological functions attributed to ICER alone and is possibly present beyond the cardiac tissue (Seidl et al., 2020).

Figure 3

Organization of ICER gene and structure of ICER isoforms.



Note: Image adapted from Borlikova et al, 2009.

ICER is associated with many functions and biological processes in the immune system (Bodor et al., 2007) (Vaeth et al., 2011), brain (Borlikova & Endo, 2009) (Porter et al., 2008), regulation of spermatogenesis (Don & Stelzer, 2002), circadian control of transcription (Stehle et al., 1993), apoptosis (Tomita et al., 2003), metabolic functions (Favre et al., 2011), and of particular interest, its role as a tumor suppressor gene product. Studies have shown that ICER repressed the expression of growth controlling genes such as *c-fos* and cyclin A, and inhibited the DNA synthesis and growth of human

choriocarcinoma and pituitary tumor cells, by mediating cAMP antiproliferative activity (Razavi et al., 1998). ICER forced expression inhibited cell growth of prostate cancer cell lines LNCaP, reversed their transformed phenotype and arrested cells at the G1 phase of the cell cycle (Mémin et al., 2002) (Yehia, Razavi, et al., 2001). Additionally, exogenous ICER expression in leukemia cell line HL60, potentially repressed leukemia in vitro and controlled tumor progression (Pigazzi et al., 2008).

Although these studies highlight the importance of ICER expression in the regulation of cell growth, they have also revealed that significantly lower levels of ICER protein, are not only present in some primary prostate tumors cells (Yehia, Razavi, et al., 2001), but are also linked with melanomagenesis (Healey et al., 2013) and acute leukemia (Pigazzi et al., 2008). Researchers demonstrated that in cancer cells, ICER mRNA levels remained relatively unaltered when compared to ICER protein levels, thus suggesting that this down-regulation was in fact the result of post-translational modifications targeting ICER for proteasomal degradation (Folco & Koren, 1997) (Yehia, Schlotter, et al., 2001) (Mémin et al., 2011) (Healey et al., 2013) (Cirinelli et al., 2022).

Selective targeting of the ubiquitin – proteasome pathway to destroy ICER at a high rate, negatively affects its stability and transcriptional repressor functions. In cancer cells, it has been shown that the level of ICER protein concentration is regulated by two mechanisms: phosphorylation and/or ubiquitination. Yehia et al, (Yehia, Schlotter, et al., 2001) revealed that the activation of the Mitogen Activated Protein Kinase (MAPK) pathway, increased ICER phosphorylation at Serine 41 and targeted ICER for ubiquitination; Mémin et al, (Mémin et al., 2011) presented evidence that ICER is phosphorylated by the Cdk1/cyclinB mitotic kinase complex and then gets ubiquitinated

during the cell cycle, and most recently, Cirinelli et al. (2022), determined that ICER gets ubiquitinated in response to the Ac/N-end rule pathway by the E3 ubiquitin protein ligase UBR4. This protein recognizes the acetylated N-terminus sequence of ICER for polyubiquitination and mediates ICER proteasomal degradation in human melanoma cells. Contrary to this, when the MAPK pathway was inhibited by cAMP in choriocarcinoma and pituitary tumor cells, it stabilized ICER protein and prevented its phosphorylation and degradation (Yehia, Schlotter, et al., 2001).

The aforementioned MAPK cascade plays a key role in cell proliferation, differentiation, survival and death (Zhang & Liu, 2002). Cross-communication of this pathway with other signaling cascades in the cells, such as cAMP, is crucial for regulation of cell proliferation and help us understand its association with cancer and other diseases (Dhillon et al., 2007). Mutations in this cascade are frequent and they are linked to cancer development and progression, especially those affecting the RAS-RAF-MEK-ERK pathway, a very important component of the MAPK signaling pathway controlling survival and development of tumor cells (Guo et al., 2020). One link between ICER and the RAS-RAF-MEK-ERK pathway has been previously demonstrated by Healey et al, (Healey et al., 2013) who determined that ICER gets targeted for proteosomal degradation in human tumors where RAS is mutated, leading to melanoma tumorigenesis, whereas inhibition of RAS activity or the proteasome, rescued ICER from being degraded. Taken together, more studies are needed for in-depth understanding of the impact that post translational modifications and subsequent degradation of ICER have on these diseased states.

It has been hypothesized that ICER might be involved in DNA repair mechanisms and that its expression is up-regulated in response to DNA damage caused by UV light;

experiments performed at Dr. Molina's laboratory showed that upon UV exposure and subsequent DNA damage, ICER gets accumulated and also gets phosphorylated by the DNA-dependent Protein Kinase (DNA-PK), an important protein in the DNA damage response, DNA repair machinery and genome integrity maintenance (Goodwin & Knudsen, 2014), however more evidence is needed to support this hypothesis. DNA mutations and damage occur often from all the endogenous and exogenous agents that not only cause genomic instability, but also interfere with important biologicals processes such as DNA replication and transcription. To compensate for the damage, cells have developed mechanisms that senses the damage and mediates its repair called the DNA Damage Response (Dexheimer, 2013). If this response is perturbed, the DNA repair capacity will be reduced and the accumulated mutations will lead to tumor development (Helleday et al., 2008). There are five main DNA repair pathways: base excision repair (BER), mismatch repair (MMR), nucleotide excision repair (NER), and double-strand break repair, including both homologous recombination (HR) and non-homologous end joining (NHEJ) (Dexheimer, 2013). Each mechanism will correct specific lesions and will be performed by specific genes associated to it.

To better understand ICER's biological function, this research focused on ICER's genomic DNA binding to help elucidate its role in the DNA damage response processes. The research recently published by Seidl et al., (2020), provided a list of the DNA binding sites of ICER and smICER obtained through ChIP-SEQ using ICER-HA and smICER-HA constructs over-expressed in HEK-293 cells. HA-tags (Human Influenza Hemagglutinin tags) are immunoreactive polypeptides attached to a protein of interest for its separation, isolation, purification and detection (Zhao et al., 2013). Since ICER is a very small protein,

the use of affinity tags such as HA-tags is very common due to the difficulties of producing antibodies targeting ICER specifically. HA- tags are small in size and unlikely to interfere with the functions or reactivity of ICER as demonstrated by previous experiments. (Cirinelli et al., 2022).

A broad analysis of ICER and smICER DNA binding locations was performed and after a comprehensive examination of the supporting dataset, it was possible to determine that ICER interacts with essential DNA repair associated genes. Next, we took the information from the in-silico analysis and verified these interactions in a skin cancer cell line, via Polymerase Chain Reaction (PCR) and Electrophoretic Mobility Shift Assay (EMSA). Confirmation of interactions in Melanoma cell line was important because secondary analysis of genes originating from the previously published ChIP-SEQ data used polyclonal antibodies for the pull-down possibly resulting in non-specific binding. They also used Human Embryonic Kidney (HEK293) cells; a type of cell lines derived from a human fetus that are widely used for the expression of proteins. These types of cells are not likely to contain somatic mutations and were possibly different from the skin cancer line we were interested in.

This study provides tremendous value to better understand ICER's biological function and minimize the lack of information regarding ICER's role in the regulation of important genes associated to DNA repair pathways, because a similar process can be replicated for other biological functions of interest.

Materials and methods

Identification of ICER/SmICER interacting genes and function

Seidl et al., (2020) provided a supplementary Excel spreadsheet containing ChIP-SEQ data of validated ICER genome-wide binding locations from the ICER-HA and smICER-HA constructs overexpressed in HEK293 cells. The information from the spreadsheet contained the Gene ID, Gene Symbol, genomic coordinates, and relationship to the expected transcriptional start site (TSS). This information was categorized based on Gene Ontology (GO) annotations by using a batch query via UniProt Knowledgebase. (Dimmer et al., 2012). This provided relevant information such as related biological functions for all the genes in this dataset.

Genes located upstream of the TSS region at which ICER, smICER and both were binding were grouped according to related biological processes as defined by their Gene Ontology terms. However, some genes have implications in many biological processes. To facilitate the sorting and grouping based on Biological Function, a Macro program was created in Microsoft Excel to automatically search and display all the genes that match a given search term entered via a drop down. In this program 47,200 Biological Processes terms can be queried against the gene list from the Seidl et al., 2020 paper. Matching terms were exported to a new sheet which reduced search time and also allowed for easy comparison against similar functions. The list was filtered for different key functions such as Apoptosis, DNA damage and DNA repair and tables containing the genes that have reported those biological functions were obtained for analysis. Genes that matched “DNA Repair” were selected for investigational purposes. The coordinates of candidate genes involved in DNA Repair that were shown to bind upstream of the TSS were converted to

an interval file through Galaxy (Afgan et al., 2018), based on hg19. Sequences were then manually inspected using the UCSC genome browser, to confirm the presence of CRE's in the selected genomic coordinates.

While it is likely that smICER also inhibits transcription by binding to CRE's, there were some smICER specific gene targets pulled down by ChIP-SEQ. These discrepancies were not previously addressed by the group that published the initial ChIP-SEQ dataset. To investigate if there were any other potentially relevant regulatory regions or sites in smICER, sequences from this secondary analysis were examined using MEME-ChIP motif analysis (Bailey et al., 2015). Shared genes by both ICER and smICER, known to contain full canonical CRE's half CRE's or no CRE's were also analyzed using MEME ChIP and Galaxy as a control.

Polymerase Chain Reaction

PCR was carried out using Chromatin immunoprecipitated (ChIP) DNA after transfection of Human melanoma cells SK-MEL-24 (ATCC® HTB-71™) with HA-tagged ICER. Cells were obtained from ATCC and cultured in Eagle's Minimum Essential Medium (EMEM) with 15% Heat inactivated FBS) and 5mL of Penicillin/Streptomycin. pcDNA3.1™(+) plasmids were used for ICER-Iγ expression with HA-tag on N or C-terminus. Chromatin Immunoprecipitation was performed according to manufacturer protocol using Simple ChIP ® Enzymatic Chromatin IP Kit containing Magnetic beads. ChIP grade polyclonal Anti-HA tag antibodies were obtained from ABCAM and used as recommended by manufacturer.

Primers were designed to be centered around the previously identified CRE(s), and had a predicted amplicon between 150 and 200bp and GC content ~50% (Table 1) ChIP DNA was sonicated to a fragment size ~150-900bp and so a smaller amplicon was preferred to avoid missing the target loci. ChIP DNA was quantified using dsDNA HS qubit assay kit (High-Sensivity double stranded DNA). Briefly, mix was prepared by combining 199 µL of buffer with 1 µL of dye. 199 µL of buffer+dye was then combined with 1 µL of ChIP-DNA. Measurements taken in triplicate and concentration for normalization was based on average of the triplicate concentrations in ng/µL. ChIP DNA was normalized to a concentration 0.5 ng/µL in nuclease free Molecular Biology Grade water. Human male genomic DNA (Promega cat # G1471) and EGFP transfection controls prior to pull down served as positive controls, and Nuclease-Free water was the No Template Control (NTC).

Table 1

Primers designed for PCR

Name	Sequence	Amplicon size
INTS3_Forward	5’ GAGACGGGGTTTCACCACGT 3’	193bp
INTS3_Reverse	5’GAGTGCATCACACAGGCACG 3’	193bp
PAGR1_Forward	5’ CAGGAGTTCAAGACCAGCCTCG 3’	176bp
PAGR1_Reverse	5’CAGTGGCATGATCTTGGCTCAC 3’	176bp
PARP1 Forward	5’ GTCGCCCATGTTTGATGGAAAAG 3’	185bp
PARP1_Reverse	5’GCCCCAGTATGTACACACCTGTC3’	185bp
RAD51C_Forward	5’GACTGCGCAAAGCTGCAAG 3’	174bp
RAD51C_Reverse	5’CATTTCAAAGCGGAACGTCTTCC 3’	174bp
XRCC2_Forward	5’ GACAAGAAAAGCACCAGCTGTC 3’	144bp
XRCC2_Reverse	5’GGAGCATGCGCAATAGGGTG 3’	144bp
JMY_Forward	5’TCAGCATCGCCACAATGTTGC 3’	152bp
JMY_Reverse	5’TCAGGGGACGCCGAGAGGA 3’	152bp
LIG3_Forward	5’GTGGCTAATTATGGGGCCTAACT 3’	203bp
LIG3_Reverse	5’GCCCAGTGACTTCAGCATTCTC 3’	203bp

USP1_Forward	5'GTTCTCTCTGCCCGCACCA 3'	185bp
USP1_Reverse	5'CGAGGGATGTAAAGGCATTCCC 3'	185bp
POLB_Forward	5'CTGGGCTGTCATTCTGAGGTGC 3'	197bp
POLB_Reverse	5'GACCGGCGGAACAATGGTT 3'	197bp

PCR primers were synthesized by Eurofins and reconstituted to 100µM in TE buffer (10mM Tris-HCl, 1mM EDTA; pH6.8) and PCR was performed according to KAPA HiFi HotStart Ready Mix PCR Kit (KAPA Biosystems) protocol, containing KAPA HiFi HotStart DNA Polymerase as follow: 5µl of ChIP DNA (as described above) was amplified in total of 25 µL PCR reaction containing 1.5 µL of forward and reverse primers at 10µM and 12.5 µL of 2x Kapa HiFi HotStart ReadyMix using the following program: 95 °C for 3 minutes; 35 cycles of 98 °C for 20 seconds, 63 °C for 15 seconds; and 72 °C for 30 seconds; and a final elongation at 72 °C for 1 minute. PCR products were visualized on a 2% (w/v) agarose gel. Confirmation of amplification of two genes with particular relevance were visualized on an Agilent TapeStation 2200 using high sensitivity DNA tape.

Electrophoretic Mobility Shift Assay

For the supershift assay, IRD®700 end- labeled DNA oligonucleotides (Integrated DNA Technologies, IA) containing the canonical CRE sequence and complementary strand of each oligonucleotides without end-labeled IRDye (Integrated DNA technologies, IA) were designed as shown in Table 2.

Table 2

Primers designed for EMSA

Name	Sequence	Scale	Purification
PARP1_Forward	/5IRD700/CCGGCACCC**TGACGT**TGAGGTGGAT	100nm	HPLC
PARP1_Reverse	/5IRD700/ATCCACCTCA**ACGTCA**GGGTGCCGG	100nm	HPLC

RAD51C_Forward	/5IRD700/CTTTACGTC**TGACGTCA**CGCCGCAC	100nm	HPLC
RAD51C_Reverse	/5IRD700/GTGCGGCG**TGACGTCA**GACGTAAAG	100nm	HPLC
XRCC6_Forward	/5IRD700/ACATGCGTGA**TGACGT**AGAGGGCGT	100nm	HPLC
XRCC6_Reverse	/5IRD700/ACGCCCTCT**ACGTCA**TCACGCATGT	100nm	HPLC
XRCC2_Forward	/5IRD700/GTTCCTG**TGACGTCA**GACTTCCGCG	100nm	HPLC
XRCC2_Reverse	/5IRD700/CGCGGAAGTC**TGACGTCA**CAGGAAC	100nm	HPLC

Oligonucleotides were reconstituted to 100µM in TE buffer (10mM Tris-HCl, 1mM EDTA; pH6.8) and annealed. Annealed oligonucleotides were diluted to 200nM and Mobility shift assays were performed according to Odyssey infrared EMSA reagent Kit (Li-COR Biosciences). 1µL of the purified protein was incubated with 2 µL of 10X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5),1µL of poly dl.dC (1 µg/µl in 10 mM Tris, 1 mM EDTA; pH 7.5), 1µL of 100nM MgCl2, 1µL of double strand oligonucleotides and 1 µL of anti HA-ICER for supershift determination for 20 min at room temperature avoiding light in a 20 µL final volume reaction. After incubation, 2µL 10X orange Loading Dye was added prior to electrophoresis. 22µL of binding reaction was loaded onto a 5% polyacrylamide TBE gel at 100V for 1hr. The signal was detected using the Li-Cor Odyssey Imaging system (LI-COR. Inc., USA).

Results

Secondary analysis of ICER binding sites data set

ChIP-Sequencing supplementary data obtained from Seidl et al. (2020), revealed 2095 smICER and 1606 ICER binding sites, from which 1592 were found to be identical between ICER and smICER, and the vast majority (32-35%) were located upstream of the TSS (Seidl et al., 2020). A list with these genes was derived using UniProt Knowledgebase and filtered to obtain the Gene Ontology (GO) biological functions. More than 1500 GO terms were obtained representing the main processes and or related functions among the genes and the number of appearances for each term. Using the GO term, it was possible to link these terms to their more related term or "parent term" and group the most common functions associated within them. Interestingly, DNA repair was one of the processes that appeared into the first 30 GO terms, together with other well established ICERs functions such as: transcription regulation, cell division, spermatogenesis and apoptosis. Table 3 displays the first 30 most common GO terms ("child" terms) associated with the genes and their immediate GO Process ancestor ("parent" term).

Table 3

ICER and smICER upstream genes GO terms processes

Region	#counts of GO terms	GO term	GO accession ID number	GO process ancestor
Upstream	34	positive regulation of transcription by RNA polymerase II	GO0045944	Biological regulation
Upstream	25	regulation of transcription by RNA polymerase II	GO0006357	Biological regulation
Upstream	19	positive regulation of transcription DNA-templated	GO0045893	Biological regulation
Upstream	18	cell division	GO0051301	Cellular Process
Upstream	14	regulation of transcription DNA-templated	GO0006355	Metabolic process

Upstream	14	protein phosphorylation	GO0006468	Metabolic process
Upstream	13	post-translational protein modification	GO0043687	Metabolic process
Upstream	12	regulation of transcription by RNA polymerase II	GO0006357	Biological regulation
Upstream	12	spermatogenesis	GO0007283	Reproductive process
Upstream	12	apoptotic process	GO0006915	Cellular Process
Upstream	11	transcription by RNA polymerase II	GO0006366	Metabolic process
Upstream	11	signal transduction	GO0007165	response to stimulus
Upstream	11	regulation of signal transduction by p53 class mediator	GO1901796	response to stimulus
Upstream	10	RNA splicing	GO0008380	Metabolic process
Upstream	10	protein ubiquitination	GO0016567	Metabolic process
Upstream	10	protein polyubiquitination	GO0000209	Metabolic process
Upstream	9	transcription initiation from RNA polymerase II promoter	GO0006367	Metabolic process
Upstream	9	protein transport	GO0015031	Localization
Upstream	9	protein stabilization	GO0050821	Biological regulation
Upstream	9	protein deubiquitination	GO0016579	Metabolic process
Upstream	9	protein autophosphorylation	GO0046777	Metabolic process
Upstream	8	negative regulation of transcription by RNA polymerase II	GO0000122	Biological regulation
Upstream	8	retrograde vesicle-mediated transport Golgi to endoplasmic reticulum	GO0006890	Localization
Upstream	8	response to oxidative stress	GO0006979	Response to stimulus
Upstream	8	regulation of cell cycle	GO0051726	Biological regulation
Upstream	8	protein folding	GO0006457	Cellular Process
Upstream	8	MAPK cascade	GO0000165	Biological regulation
Upstream	8	intracellular signal transduction	GO0035556	Biological regulation
Upstream	8	DNA repair	GO0006281	Cellular Process

Re-analysis of the list showed that the main functions to all those genes can be linked to two main sources: *Cell* and *Metabolic Processes*; these terms are a broad reference to all chemical reactions and pathways where compounds are formed as part of the normal metabolic (anabolic and catabolic) processes carried out at the cellular level.

It is clear that ICER, a transcription regulator and relevant member of the CREM family, is interacting with genes taking part in critical processes such as: organic substance metabolic processes (57.5%), primary metabolic processes (56.3%), cellular metabolic processes (55.6%), nitrogen compound metabolic processes (42.7%) and biosynthetic processes (37.5%). Table 4. shows the distribution of the functions discovered for the genes at which both ICER and smICER bind.

MEME ChIP Motif analysis of the regulatory regions of ICER, smICER and shared genes demonstrated that within a span of +/- 100bp from the central region of the coordinates an a partial CRE motif (TGA**T**GTCA) was present in the few genes at which ICER alone was binding (Figure 4A). Interestingly, when the coordinates were uploaded to MEME ChIP, no canonical CRE motif (full or half) was identified for the genes that were specific to smICER DNA interactions (Figure 4B). To ensure we did not miss regions that should contain a CRE, we expanded the span of the coordinates until we reached +/- 500bp from each side of the center peak, repeated the analysis and no CRE motif was found. Finally, we uploaded the coordinates from the shared regions and a full canonical CRE motif (TGACGTCA) was identified as the top-ranked motif among all the genes (Figure 4C).

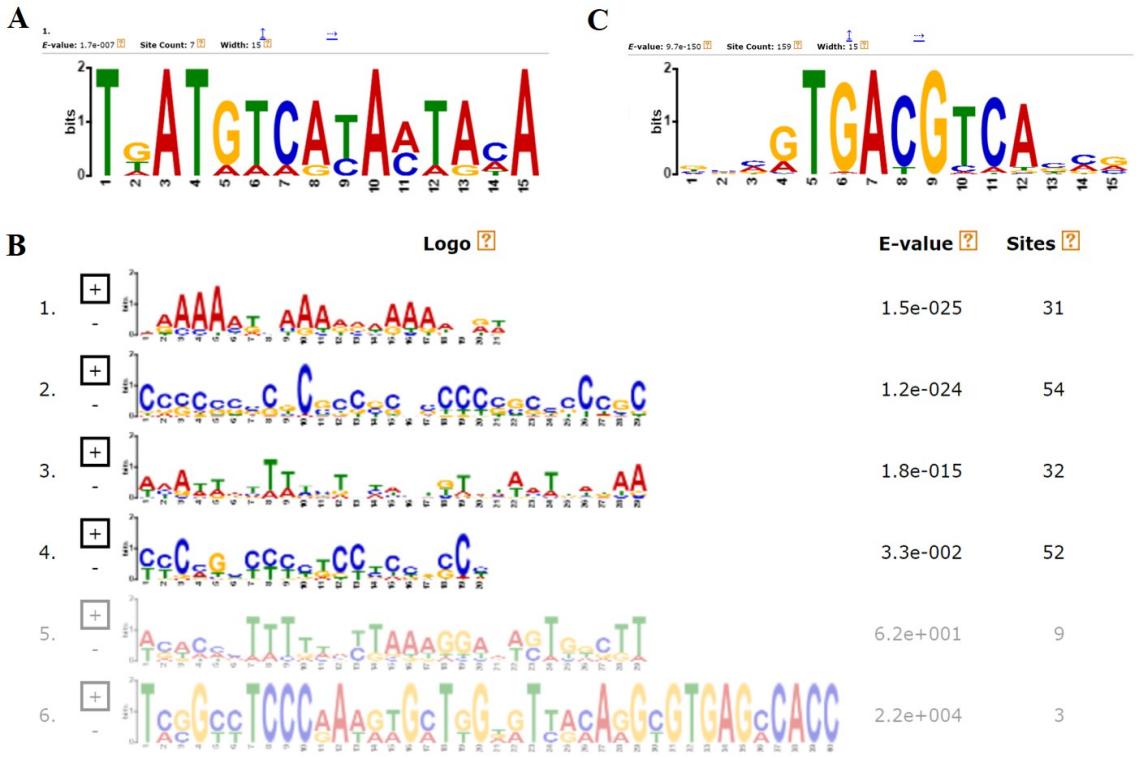
Table 4

Upstream ICER and smICER GO terms and/or functions distribution.

GO TERM	GENES	COUNT	%	P-VALUE	BENJAMINI
Organic substance metabolic process		275	57.5	1.50E-04	3.80E-03
Primary metabolic process		269	56.3	2.60E-05	1.40E-03
Cellular metabolic process		266	55.6	7.00E-05	2.70E-03
Nitrogen compound metabolic process		204	42.7	6.10E-07	9.30E-05
Biosynthetic process		178	37.2	1.30E-04	3.80E-03
Regulation of metabolic process		171	35.8	5.10E-04	1.10E-02
Cellular component organization		166	34.7	1.80E-03	3.50E-02
Cellular component biogenesis		88	18.4	2.60E-03	4.40E-02
Macromolecule localization		84	17.6	4.80E-03	7.30E-02
Cellular localization		75	15.7	1.00E-02	1.20E-01
Catabolic process		59	12.3	2.80E-02	2.30E-01
Cell cycle process		55	11.5	2.70E-05	1.40E-03
Interspecies interaction between organisms		36	7.5	6.20E-03	8.60E-02
Multi-organism cellular process		35	7.3	7.40E-03	9.40E-02
Response to abiotic stimulus		31	6.5	8.80E-02	6.40E-01
Methylation		13	2.7	7.60E-02	5.70E-01
Antigen processing and presentation		12	2.5	1.40E-02	1.40E-01
Multi-organism metabolic process		11	2.3	2.30E-02	2.20E-01
Protein folding		11	2.3	3.50E-02	2.80E-01
Positive regulation of multi-organism process		9	1.9	2.70E-02	2.30E-01
Modification of morphology or physiology of other organism		8	1.7	1.30E-02	1.40E-01

Figure 4

Discovered Motifs found by MEME using a +/- 100bp span from the middle of the peak on the coordinates



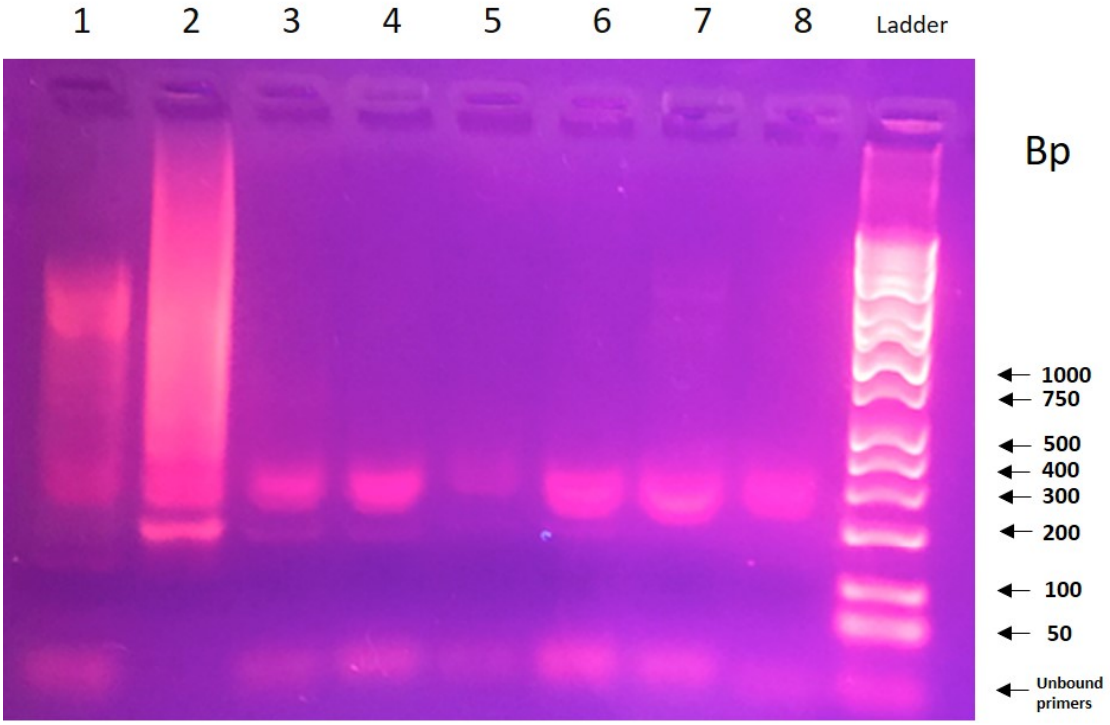
Note: A) ICER, motif showing a partial CRE on. B). SmICER, NO canonical CRE discovered, C). Shared genes, full canonical CRE found.

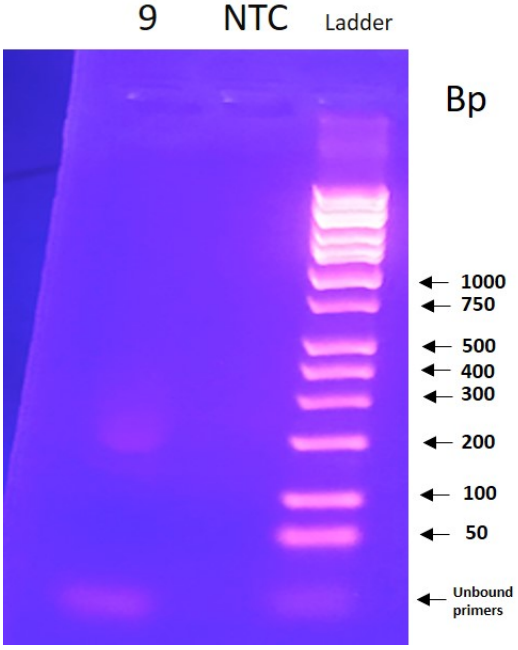
PCR demonstrated ICER interactions with genes related to repair pathways

We took the information from the in-silico analysis and focused on genes that matched the gene search for DNA repair and downsized the list to a group of genes involved in DNA repair pathways. Using ChIP DNA, we aimed to test a set of different primers designed to amplify the putative CRE binding motif of $_{N}$HA-ICER in the following genes: INTS3, PAGR1, USP1, PARP1, POLB, LIG3, JMY, XRCC2 and RAD51C. Gel electrophoresis results from the PCR assay revealed that most DNA fragments were appropriately amplified for the genes of interest (Figure 5).

Figure 5

2% agarose gel Electrophoresis results from the PCR assay.





Number	Gene Name	Predicted Amplicon Size
1	INTS3	193
2	PAGR1	176
3	USP1	185
4	PARP1	185
5	POLB	197
6	LIG3	203
7	JMY	152
8	XRCC2	144
9	RAD51C	174
10	NTC	0

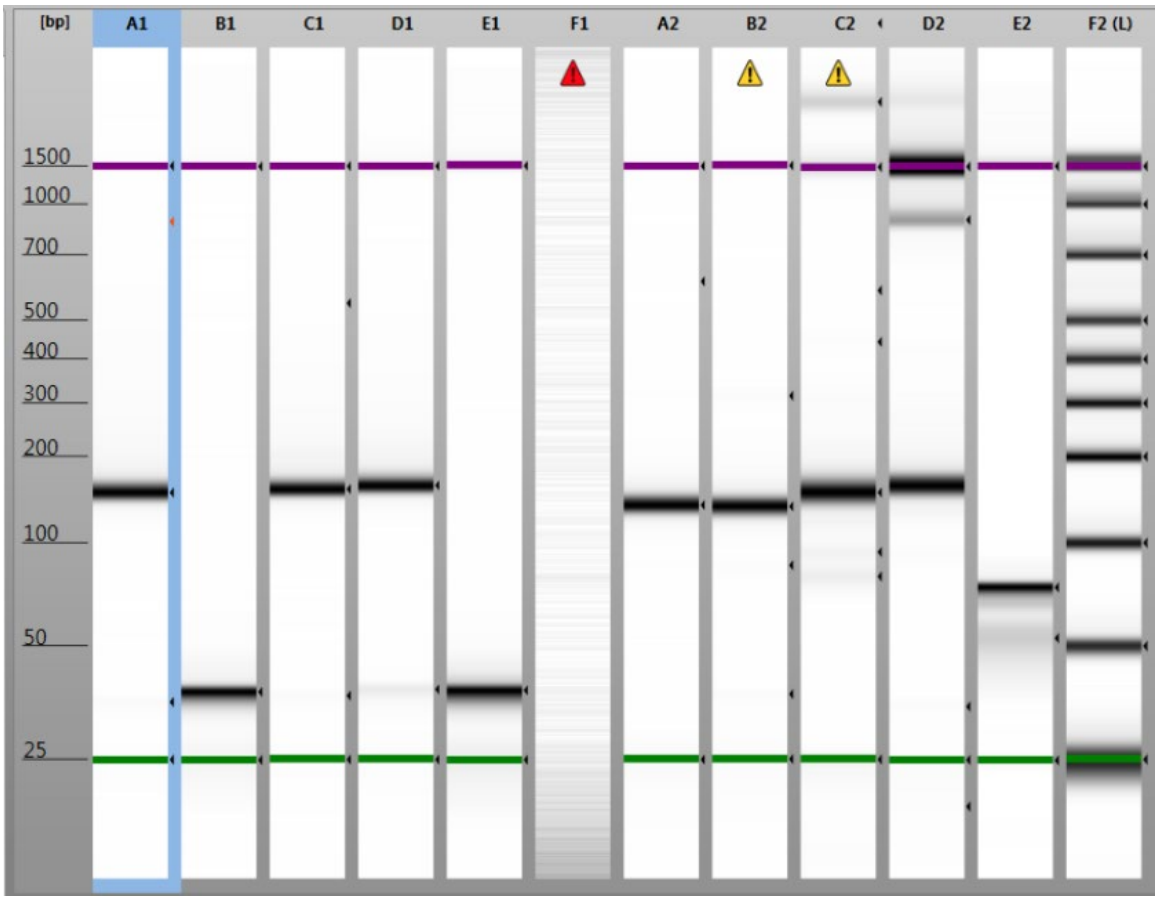
Note: Bands represent the amplification of genes of interest on ChIP DNA from Human melanoma cells SK-MEL-24 transfected with HA-tagged ICER. Ladders are located on the right side of both gels. Lanes are described in the table below image.

Bands 3 to 9 had positive results, suggesting that the genes USP1, PARP1, POLB, LIG3, JMY, XRCC2 and RAD51 were pulled down via ChIP in SK-MEL-24 cells following transient transfection with HA-ICER. Most bands were clear and run near the 200 bp in accordance with the predicted amplicon sizes (Figure 5). Bands 1 and 2 representing INTS3 and PAGR1 genes appeared smeared. This inadequate amplification of the DNA fragments was probably due to primer design, sequence not present on the cell type used or quality of sample and loading. No contamination or non-specific amplifications were detected as shown in the No template Control (NTC).

We then prioritized those genes whose functions are of particular interest for future studies and selected PARP1 and RAD51C to verify the data and confirm the proper amplification of their products with a second PCR. TapeStation gel image (Figure 6) shows the size and quality of the amplified DNA fragments. Lanes A1 to E1 correspond to PARP1 assays and lanes A2 to E2 to RAD51C assays. Lanes A1 and A2 show ChIP DNA fragments that were bound to $_{N}$HA-ICER with a size of 170-180bp as expected. Lane B2 represents ChIP DNA fragments bound to ICER-$_{C}$HA having a similar size as $_{N}$HA-ICER + RAD51C. Due to low yield, not enough ChIP DNA of ICER-$_{C}$HA was obtained to test it with PARP1 primers, and the assay had to be run with nuclease free water as seen in lane B1. To assess for specificity, positive and negative controls were amplified. Lanes C1, C2, D1 and D2 represent the positive controls. EGFP was effectively fragmented cell genomic DNA prior to the immunoprecipitation (IP) step and was used as positive control together with intact genomic DNA from Promega. Control fragments (170-190bp) are visible in both lanes. Lanes E1 and E2 represent negative controls using nuclease free water.

Figure 6

TapeStation High Sensitivity DNA electrophoresis results.



Position	Description
A1	$_{N}$HA-ICER + PARP1
B1	NTC + PARP1
C1	EGFP + PARP1
D1	Promega Genomic DNA + PARP1
E1	NTC + PARP1
F1	Blank
A2	$_{N}$HA-ICER + RAD51C
B2	ICER-$_{C}$HA + RAD51C
C2	EGFP + RAD51C
D2	Promega Genomic DNA + RAD51C
E2	NTC + RAD51C
F2	MWM

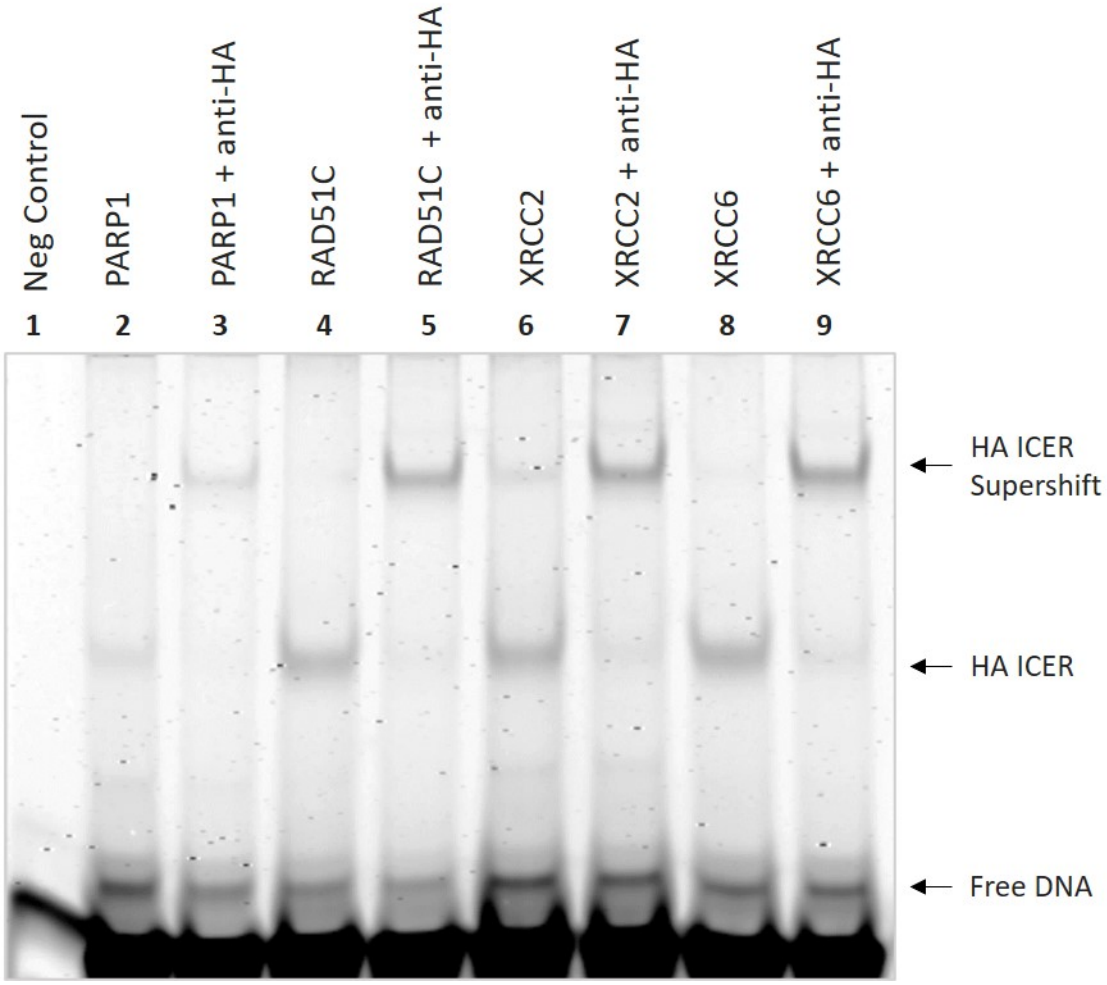
Note: A1, A2, B2: ChIP DNA amplification of genes PARP1 and RAD51C; C1, C2, D1, D2: positive controls; E1, E2: negative controls; F2 lane: high Sensitivity ladder.

ICER binds to genes involved in DNA repair pathways

To confirm that ICER's interaction with the genome from the previously published ChIP-SEQ data was specific, EMSA was carried out as described in materials and methods. Seidl et al., (2020) used polyclonal antibodies in their procedures, and despite these being used as the standard reagent for ChIP-Seq, they can lead to higher nonspecific background and complex formations with nonspecific proteins (Busby et al., 2016). The use of monoclonal antibodies, in contrast, offers the advantage of highly specific antibody – antigen interactions and is highly recommended (DeCaprio & Kohl, 2017). For the immune supershift assay, oligonucleotides were incubated with anti-HA-ICER, a specific monoclonal antibody that recognizes the HA tag in the protein. Figure 7 displays the DNA binding activity of ICER to four of the selected genes associated with DNA repair. Results revealed that ICER could bind to the canonical CRE sequence present in PARP1, RAD51C, XRCC2 and XRCC6 oligonucleotides as seen on lines 1,3,6 and 7, and this is confirmed by the addition of anti-HA ICER that formed a complex and generated a distinct supershift that appears clear in lines 2, 4, 6 and 8.

Figure 7

EMSA analysis of ICER's DNA binding activity with four genes related to DNA repair.



Note: The oligonucleotides contain the canonical CRE binding site. Lanes 1,3,5,7 correspond to the oligonucleotides; lanes 2,4,6,8 correspond to the oligonucleotides + anti-HA ICER.

Since ICER is part of the CRE binding proteins that mediates gene expression, these specific interactions between ICER and DNA repair associated genes are suggestive that ICER could not only bind to all those genes with great affinity but most likely influence their expression and/or activities within the cell.

Discussion

Numerous studies have established a connection between ICER and cell development, apoptosis, transcription regulation, antitumor properties and other functions within the immune, nervous and neuroendrocrine system. Since ICER is an endogenous transcriptional repressor that belongs to the CREB/CREM gene family, most of its effects are mainly due to ICER's expression in response to cAMP signaling cascade stimuli. While the impact of ICER in acting as a tumor suppressor has been documented, more research is needed to learn how ICER may contribute to reducing tumorigenesis in certain cancers. In an attempt to better understand ICER's function, a series of computational and experimental approaches were carried out in this study to elucidate the impact of ICER in DNA Repair.

ChIP-SEQ results from experiments performed by Seidl et al. (2020), demonstrated that ICER and smICER were binding to a wide range of specific sites within the genome. After a secondary analysis of the supplementary dataset, consisting of an extensive examination and organization of the information provided, it was possible to retrieve a list of interacting genes located upstream to the TSS and the functions associated to them using the GO resource. The fact that processes such as, transcription regulation, cell division, apoptosis and spermatogenesis appeared in the top 10 activities associated with ICER, was no surprise, as those roles have been demonstrated in the past by several studies. Gene ontology terms also revealed that *Cell* and *Metabolic Processes* are the molecular-level activities at which ICER is mostly performing, this is in agreement with all the evidence highlighting the various important functions that ICER has as a small nuclear transcriptional repressor.

ChIP-SEQ data revealed the presence of ICER and smICER shared binding sites as well as some ICER specific targets. Interestingly, gene targets that where specific only for smICER were also pulled down from the assay. We investigated these discrepancies further to identify any other potentially regulatory regions or sites that were relevant for smICER since this was not addressed by the authors. Using MEME-ChIP motif analysis, results showed that within a span of +/- 100bp from the peak region of the coordinates for ICER specific target genes, an almost full canonical CRE motif was found in the database as expected (TGA**T**GTCA). Similarly, a full putative CRE (TGACGTCA) was also found for the shared genes as depicted in Figure 4, but no canonical full or half CRE was found in the sequences at which smICER was specifically binding, even when the sequence length was increased by hundreds of base pairs and the analysis was repeated.

smICER is a new isoform identified not long ago that gets regulated by a novel intronic promoter (P6) within the CREM gene and differs from ICER in its structure (it lacks exon X and the canonical N-terminus) (Seidl et al., 2014) but its biological function is not yet well-defined. MEME-ChIP findings raised the question where is smICER binding if is not binding to any full or half CRE? Despite that smICER is a slightly different protein, it still contains the DNA binding domain needed to bind the CRE, hence it should be behaving similarly to ICER protein and bind to the canonical motif instead of being precipitated binding to a great number of genes that do not contain a full or even half CRE motif. On the other hand, binding of smICER to a CRE located in another region and not upstream of the gene, is less likely since these regulatory regions are typically located within the promoter or enhancer regions.

Contamination of DNA during the ChIP procedure or non-optimal conditions are always contributors to the variability in the results and could be some of the reasons for the inconsistencies. In addition to this, researchers ideally should have used specific monoclonal antibodies instead of polyclonal antibodies to enrich the specific targeted protein (and specific DNA region bound to it) during the immunoprecipitation. The use of polyclonal antibodies probably yielded the very high background noise that resulted in a great number of non-specific interactions between smICER and the genes pulled down through ChIP. In future studies, it would be useful to experiment with monoclonal antibodies to improve the quality, specificity and reproducibility of the results.

Interactions between ICER and the genome were found to be commonly occurring among genes associated with apoptosis and DNA damage and repair processes. Since recent evidence from the laboratory suggested that DNA damage could somehow be up-regulating ICERs expression, it was of particular interest to find the “DNA repair” term within the top 30 results on the obtained list. This was an important starting point to find which specific genes ICER could be interacting with and continue exploring its possible roles in this pathway. This also has considerable implications considering that in certain tumors, ICER is targeted for degradation via ubiquitination. If ICER is degraded, then the lack of transcriptional repression of these target genes are of great importance as future drug targets in diseased states.

Because it has been previously publicized that ICER induces apoptosis (Ding et al., 2005) (Cirinelli et al., 2022) we focused on studying ICER’s possible critical involvement in DNA repair as a novel function. DNA repair is a very important process that mitigates the effects of any mutagenic agent either inside or outside the cells that can lead to tumor

development. Identification of the target genes of ICER as a transcription factor is relevant for the understanding of its role in DNA damage and repair regulation. Genes such as INTS3, PAGR1, USP1, PARP1, POLB, LIG3, JMY, XRCC2 and RAD51C play all important roles in DNA repair pathways, either by forming protein complexes that promote DNA repair or by conducting the repair itself. PCR results showed that amplification of the CRE containing sequences of the targeted genes was successful for most of them, (Figure 5) Although not all genes in this study could be verified to amplify correctly either due to primer design or sequence not being present in the DNA extracted from human melanoma cells, most PCR products had the expected size and demonstrated that designed primers were suitable for future experiments as detected in the gel electrophoresis. Using the TapeStation system, DNA fragments from PARP1 and RAD51C genes were amplified and at the expected size. PARP1 (Poly (ADP-ribose) polymerase 1) is an ADP-ribosylating enzyme important for mediating DNA repair response and is activated in response to DNA base damage, DNA single-strand and double-strand breaks as well as in DNA maintenance of genomic integrity (Kamaletdinova et al., 2019). RAD51C (RecA-like DNA recombinase 51-C) is a paralog of RAD51 gene and plays an important role in DNA double-strand break repair via homologous recombination (HR) (Li et al., 2019).

In this context, the confirmation of these genomic interactions suggested that ICER is somehow regulating the expression of these genes or the function of their protein products. Additionally, these results are in agreement with the EMSA analysis, which revealed that HA-ICER could bind the canonical CRE sequence present not only in PARP1 and RAD51C but other important genes associated with DNA repair such as, XRCC2 and XRCC6. XRCC2 (X-ray Cross Complementing 2) is also a paralog of RAD51 and it is

associated in the HR pathway by initiating recruitment of RAD51 proteins to the DNA damage sites (Zhao et al., 2021). XRCC6 gene is an essential component of the NHEJ pathway and it is involved in maintenance of genome integrity, hence important for genome stability and cell survival (Bau et al., 2011) (Jia et al., 2015).

The mechanism by which ICER participates in the regulation of DNA repair genes is an important topic for future research. PARP1 for example, one of the repair proteins of interest, is a major target protein in breast and ovarian cancer treatments. Its downregulation by PARP inhibitors (PARPi) results in a lack of DNA repair and therefore cytotoxicity in tumor cells with mutated BRCA1/2 genes (Mateo et al., 2019). BRCA1 and BRCA2 proteins play a critical role in the pathway of genome protection, and mutations in these genes increase breast and ovarian cancer susceptibility (Patel et al., 2021). If there is a defect in one DNA repair pathway, it can be compensated for by other pathway, for instance, PARPi have been proposed as an effective treatment for this type of cancers since they work under the concept of "synthetic lethality", meaning that the loss of cell viability is produced by the concomitant inactivation of two different genes: PARP1 and the already dysfunctional BRCA1/2. Experiments focusing on understanding the mechanisms by which ICER could be interacting with PARP1 and/ or regulating its mechanisms of action on transfected ovarian cancer cells with and without BRCA1/2 mutations will provide tremendous value to better understanding ICER's biological function, because a similar process can be replicated for other biological function of interest.

This study showed a novel correlation between ICER and DNA repair and provided evidence that will serve as a basis for future research. Therefore, I strongly suggest continuing experiments on different cell types and even using whole organisms to identify

the target genes of ICER to help understand transcriptional networks and contribute to elucidate ICER's function and up-regulation in DNA damage response.

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