INTRODUCTION

Regulation of CRE (cAMP-responsive element)-mediated gene transcription by members of the CREB1/CREM (CRE-binding protein/CRE-modulator)/ATF-1 (activating transcription factor-1) protein family affects a multitude of cellular processes. However, the underlying genes can encode several different isoforms with different functions. In the Crem gene, six internal promoters (P1 to P6) have been identified. Consequently, together with alternative splicing, a multitude of CREM repressor isoforms exist in the heart, namely for ICER and smICER (small ICER), which are induced in response to β-adrenergic stimulation in a transient- and saturation-like manner, respectively. This time-shifted induction pattern, driven by two internal promoters in the Crem gene, leads to the predominant transcription of smICER after prolonged β-adrenergic stimulation. Using an ICER knockout mouse model with preserved smICER induction, we show that the transient-like induction of Icer itself has minor effects on gene regulation, cardiac hypertrophy or contractile function in the heart. We conclude that the functions previously linked to ICER may be rather attributed to smICER, also beyond the cardiac background.

KEYWORDS
cyclic AMP (cAMP), gene regulation, heart, transcription factors

Abstract

ICER (inducible cAMP early repressor) isoforms are transcriptional repressors encoded by the Crem (cAMP responsive element modulator) gene. They were linked to the regulation of a multitude of cellular processes and pathophysiological mechanisms. Here, we show for the first time that two independent induction patterns for CREM repressor isoforms exist in the heart, namely for ICER and smICER (small ICER), which are induced in response to β-adrenergic stimulation in a transient- and saturation-like manner, respectively. This time-shifted induction pattern, driven by two internal promoters in the Crem gene, leads to the predominant transcription of smICER after prolonged β-adrenergic stimulation. Using an ICER knockout mouse model with preserved smICER induction, we show that the transient-like induction of Icer itself has minor effects on gene regulation, cardiac hypertrophy or contractile function in the heart. We conclude that the functions previously linked to ICER may be rather attributed to smICER, also beyond the cardiac background.

Abbreviations: BCL2, B-cell lymphoma 2; Ca++, intracellular Ca++; ChIP, Chromatin immunoprecipitation; CRE, cAMP-responsive elements; CREM, cAMP-responsive element modulator; CREB, cAMP-responsive element binding protein; DBD, DNA binding domains; HA, hemagglutinin tag (HA); ICER, inducible cAMP early repressor; IKO, ICER knockout mice; ISO, isoproterenol; KID, kinase-inducible domain; NGS, next generation sequencing; PDE3A, phosphodiesterase 3a; PKA, protein kinase A; qRT-PCR, quantitative realtime polymerase chain reaction; RACE, Rapid Amplification of cDNA Ends; RT, room temperature; shRNA, short RNA; SL, sarcomere length; smICER, small ICER; TUNEL, terminal uridine deoxynucleotidyl transferase dUTP nick end labeling.
and alternative translation initiation the expression of a multitude of transcriptional activators or repressors is possible.\textsuperscript{3-8}

Among these, the strong transcription inhibitor ICER (inducible cAMP early repressor) is involved in many functions, for example, in the regulation of spermatogenesis,\textsuperscript{9,10} functions of the immune system\textsuperscript{11,12} and the brain,\textsuperscript{13-15} and metabolic functions.\textsuperscript{16,17} ICER is a collective name for four different isoforms of ICER produced from the \textit{Crem} gene by use of the ICER-specific P2 promoter. ICER proteins contain a short and conserved N-terminal sequence, encoded by exon \textit{X}, followed by a γ-domain encoded by the exon γ (absent in γ-isoyforms ICER γI and ICER γII) and one of the DNA binding domains (DBD) encoded by exon \textit{H} and exon \textit{Ia} (ICER I) or \textit{Ib} (ICER-II) in the \textit{Crem} gene.\textsuperscript{18} ICER is rapidly induced by several external stimuli. The cAMP-dependent protein kinase A (PKA)/CREB1-ICER signaling cascade is the most prominent signal transduction pathway regulating \textit{icerc} transcription. When phosphorylated by PKA at Ser133 within its kinase inducible domain (KID), CREB1 induces the transcription of \textit{icerc} via four autoregulatory CRE elements in the ICER-specific P2 promoter. Additionally, by binding to these elements ICER is able to repress the activity of its own promoter, resulting in a negative feedback loop.\textsuperscript{18} Previously, we identified another internal promoter (P6) within the \textit{Crem} gene.\textsuperscript{6} This promoter encodes short transcriptional inhibitory isoforms called small smICER (smICER).

ICER and smICER are structurally related. Due to the location of the P6 promoter, downstream of the exon \textit{X} smICER isoforms lacking the exon \textit{X} sequence, and also contain the γ-domain (absent in some smICER variants) and one of the DBDs. ICER and smICER do not contain the KID and transactivation domains and inhibit the transcription of CRE dependent promoters including their own P6 and P2 promoters in a mutual manner.\textsuperscript{6,18} It should be noted that several publications, which aimed to specifically investigate the function of ICER, might actually have targeted smICER, too. Bieganska et al already identified the problem of inactivation of ICER by unspecific siRNA approaches also targeting other CREM splice variants, and developed a specific method to silence ICER selectively via shRNA\textsuperscript{19}. Therefore, functions previously attributed solely to ICER might have to be reconsidered, taking the existence of smICER beside ICER into account.

In the heart, stimulation of β-adrenoceptors induces \textit{icerc} mRNA within 3 to 12 hours. ICER is regarded as a negative regulator of cardiac hypertrophy and a positive mediator of apoptosis, in part due to the downregulation of BCL2 (B-cell lymphoma 2).\textsuperscript{20} It was suggested that the induction of ICER perpetuates the elevation of cAMP by inhibition of the phosphodiesterase 3a (PDE3A), which would impair the degradation of cAMP, resulting in a sustained induction of ICER. This so called PDE3A-ICER feedback loop was proposed to support the progression of heart failure in human patients by promoting cardiomyocyte apoptosis.\textsuperscript{21-24} Moreover, inhibition of micro-RNAs by ICER was linked to cardiac electrical remodeling after postmyocardial infarction, for example, by downregulation of the potassium current \textit{I}_{\text{Ko}} associated channel subunit KV4.2/KCNN2.\textsuperscript{25} While knowledge about the specific functional role of smICER isoforms in the heart and other tissues is still rather scarce, more is known about the functions of \textit{CREM-\textit{IbΔC-X}}, a short splice variant isolated from human heart tissue.\textsuperscript{26} Proteins translated from \textit{CREM-\textit{IbΔC-X}} were identical to proteins encoded by some smICER splice variants.\textsuperscript{6} Transgenic mice over-expressing CREM-\textit{IbΔC-X} in the heart show a complex cardiac phenotype including atrial remodeling, which results in spontaneous atrial fibrillation.\textsuperscript{27,28} Moreover, arrhythmogenic alterations in ventricular cardiomyocytes are present.\textsuperscript{29} Similar to ICER, smICER reportedly exhibits pro-apoptotic effects in human tumor cells.\textsuperscript{6}

Until now, the profile of CREM isoforms and their complex transcriptional regulation in the heart is not understood in detail. Using an ICER knockout mouse model, we assessed the role of ICER in the heart at functional and transcriptional level in response to chronic β-adrenergic stimulation. A detailed analysis of \textit{Crem} splice variants transcribed in murine cardiomyocytes and quantified by next generation sequencing (NGS) revealed that \textit{smicerc} is the main isoform induced after prolonged β-adrenergic stimulation in the heart, while \textit{icerc} is only transiently induced under chronic β-adrenergic stress. These results provide novel insights into the complex temporal controlled network of CREM isoforms. Moreover, we conclude that long-term induction of smICER rather than transient induction of ICER might represent an important mechanism to reprogram cardiac myocytes to a remodeling phenotype.

2    MATERIALS AND METHODS

2.1    Experimental animals and ethics statement

ICER knockout mice (IKO) generated by exon \textit{X} deletion in the \textit{Crem} gene\textsuperscript{14} were bred on a C57BL/JRccHsd (Envigo, Huntingdon, UK) background. For our experiments, both male and female mice were used at the age of 16-20 weeks. Wild-type littermates (WT) served as controls. For quantification of mRNA levels in whole hearts, we used also FVBN mice. All experimental animal procedures were in accordance with local animal welfare authorities, conform to the Directive 2010/63/EU of the European Parliament and were approved by the LANUV—Regional Authority for Nature, Environment and Consumer protection in North Rhine-Westphalia, Germany (Permit numbers: 84-02.04.2011.A179 and 84-02.04.2016.A104).
2.2 Treatment of mice with isoproterenol

Osmotic mini-pumps (model 2001, Alzet, Cupertino, CA, USA) were implanted into IKO and WT mice for continuous application of 10 mg/kg body weight/day (10 mg kg⁻¹ d⁻¹) of isoproterenol (ISO) (DL-isoproterenol hydrochloride, Merck, Darmstadt, Germany) dissolved in 0.9% of NaCl/2 mM of HCl solution for 6, 24 hours, and 7 days according to the manufacturer's instructions.

2.3 Hemodynamic experiments

Left ventricular function was assessed in anesthetized mice via left-ventricular catheterization by the use of a diagnostic pressure/volume catheter (SPR-839; Millar Instruments, Houston, TX, USA) as described previously. Increasing doses of isoproterenol were administered into the cannulated left external jugular vein using an automated syringe pump. Data were recorded using Chart 5 software (ADInstruments, Bella Vista, NSW, Australia) and analyzed using a photomultiplier-based system (Myocyte Calcium System, Ionoptix, Westwood, MA USA). Ca²⁺ transients and sarcomere shortening were recorded as described previously. 29 In brief, isolated cardiomyocytes were loaded with Indo-1/AM (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA) and intracellular Ca²⁺ (Ca²⁺⁺) transients and sarcomere shortening were recorded using a photomultiplier-based system (Myocyte Calcium and Contractility System, Ionoptix, Westwood, MA USA).

2.4 Cardiomyocyte isolation and calcium imaging

Primary adult ventricular cardiomyocytes were isolated by collagenase/protease digestion in a Langendorff apparatus and intracellular Ca²⁺ (Ca²⁺⁺) transients and sarcomere shortening were recorded as described previously. 29 In brief, isolated cardiomyocytes were loaded with Indo-1/AM (Molecular Probes, Thermo Fisher Scientific, Waltham, MA, USA). Ca²⁺⁺ transients and sarcomere shortening were recorded simultaneously (0.5 Hz stimulation, RT) under basal conditions and during acute perfusion with 1 μM of ISO using a photomultiplier-based system (Myocyte Calcium and Contractility System, Ionoptix, Westwood, MA USA).

2.5 Isolation of mRNA and identification of Crem splice variants

Total RNA was isolated from ventricular cardiomyocytes or complete hearts by the use of TRIzol (Thermo Fisher Scientific) and 1 μg was randomly reversely transcribed to cDNA using transcriptor first strand cDNA synthesis kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. Transcripts of the Crem gene were amplified by polymerase chain reaction (PCR) (annealing temperature 51-55°C) using forward primers for exon B or X 5'-ATGGGCTGTAACCTGGAGATGAAAC-3' (ExB_for), 5'-ATGGCTGTAACTGGAGATGAAAC-3' (ExX_for) and reverse primers for exon Ia or Ib 5'-GTATTTCTCACACGGCCACA-3' (ExIa_rev) and 5'-GAACCTTCCACGTCGACT-3' (ExIb_rev). The PCR mix contained 10 μL of 5xPhusion HF buffer, 1 μL of dNTP mix (10 mmol each), 0.5 μL of Phusion DNA polymerase, (New England Biolabs, Frankfurt, Germany), 2.5 μL sense and antisense primers (10 pmol), and 10 μL cDNA in a final volume of 50 μL. The thermal cycling conditions were: initial denaturation for 30 minute at 98°C, followed by 35 cycles for 10 seconds at 98°C, 30 seconds at 51-55°C, 30 seconds at 72°C, and 1 cycle for 5 minutes at 72°C. PCR products were separated on a 1.5% of agarose gel, purified with the Zymoclean Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA) and cloned into a pJET 1.2 vector (CloneJET PCR Cloning Kit, Thermo Fisher Scientific) according to the manufacturer's instructions. Sequences of clones were verified by sequencing (Eurofins Scientific, Luxemburg, Luxemburg). For 5′-Rapid Amplification of cDNA Ends (5′RACE)-PCR, mRNA was isolated from FVBN mice treated with isoproterenol (3 mg kg⁻¹ d⁻¹) for 10 hours via osmotic-mini-pumps. For amplification, a 5′RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Thermo Fisher Scientific) was used according to the manufacturer's instructions. In brief, purified mRNA was reverse transcribed with a primer for exon Ia or Ib 5′-TTATGGGACATAAAGGT-3′ (Race_Ia) and 5′-CCTCTATAAGCTTCTT-3′ (Race_Ib), mRNA was degraded and cDNA was purified. Terminal deoxynucleotidyl transferase was used to add homopolymeric tails to the 3′ ends of the cDNA, which was then amplified with a nested exon Ia or Ib primer 5′-CTTCTGACACTCAAGACACTCC-3′ (nRace_Ia) or 5′-ACTCGACTCTCTGAAAAGGT-3′ (nRace_Ib) and an anchor primer. After a second nested PCR using an Abridged Universal Amplification Primer and two further nested specific primers for exon Ia or Ib 5′-TTCTTCTCTTCTCGACACTCCG-3′ (2nRace_Ia) or 5′-GCTGACATTCTTACGACTTCGACT-3′ (2nRace_Ib), mRNA products were separated on a 1.5% of agarose gel and sequences were determined via cloning as described above. Identified transcript variants were aligned to the murine Crem gene (MGI:88 495; Genbank: NC_000084.6 complement sequence from 3 266 354 to 3 337 589 bp) and exon usage and in frame start sites were investigated by sequence analysis with the Software Geneious (Version 7, Biomatters Ltd, Auckland, New Zealand).

2.6 Plasmid construction of ICER-HA and smICER-HA and Ctr-HA (truncated Ia-HA)

For the generation of ICER-HA, murine cDNA (Xγ-H-Ib) was amplified using ExX_for and an exon Ib 5′-CTAAATGGGAGAGATGAAAC-3′ (ExIbrev) primer and
were inserted into the pJET1.2 vector as described before. After purification of the plasmids, an EcoRI, a hemagglutinin tag (HA) with a glycine linker and a Not-I restriction site were introduced by PCR using 5’-AAAAAAGAATTCAGGATGGCTGTAACTGGAGATGGAACTTG-3’ and 5’-ATTATTATTGCGGCCGCTATGGGAATCTGCTATTGCTATGGGTAACCCCAACGCCCACCCACCTTCACCTGTTTGGGAGAGCA-3’ (HAlong_rev) as primer. After gel extraction, the sample was amplified again using 5’-AAAAAAGAATTCAGGATGGCTG-3´ and 5´-ATTATTATTGCGGCCGCCTATGGGAATCTGCTATTGCTATGGGTAACCCCAACGCCCACCCACCTTCACCTGTTTGGGAGAGCA-3’ (HA_rev) as primer pair, cutted by EcoRI, and NotI and ligated to the mammalian expression vector pcDNA3.1+ (Thermo Fisher Scientific). The smICER-HA construct was generated from the ICER-HA vector by the use of 5´-AAAAAGAATTCACTGATGGAGGAGACTGACCT-3’ and the HA-rev primer. The truncated Ib-HA construct was generated by PCR using 5´-AAAAAAGAATTCAGGATGTGCTCTCCAAAAGATGGAA-3´ and HAlong_rev as primer pair. Both were digested and ligated into pcDNA3.1 as described for ICER-HA. Construct inserts were verified by sequencing (Eurofins Scientific).

2.7 Cell culture, transfection and immunoblotting

HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C in an atmosphere of 5% of CO₂. Media was supplemented with 10% of fetal calf serum, 100 U penicillin and 0.1 mg of streptomycin per ml, and 7 mM of glutamine. Cells were maintained in 75 cm² culture flasks to 80% confluency and transfected with 4 μg of ICER-HA, smICER-HA or Ib-HA DNA and 12 μL of X-tremeGENE in vitro Transfection Reagent (Roche) according to manufacturer’s protocol. 24 hours after transfection HEK293 cells were washed with PBS, harvested by a cell scraper, pelleted in PBS (4°C) by centrifugation (900 × g) and lysed in 475 μL of NaHCO₃ (10 mM) supplemented with protease inhibitors (Pierce Protease Inhibitor Tablets, Thermo Fisher Scientific). Lysates were prepared by subjecting the cells to a freeze-thaw cycle followed by sonification (HTU Soni130, G. Heinemann, Schwäbisch Gmünd, Germany; 3 × 6 seconds, amplitude 60%) to a total of three times. Then, 125 μL of SDS (20%) was added, followed by sonification and centrifugation at 13 000 × g for 20 minutes. Protein content of the supernatant was determined according to Bradford with bovine serum albumin as standard. Each sample was mixed 1:1 (20 μL + 20 μL) with 2× Laemmli buffer, boiled at 95°C for 10 minutes, 15-50 μg/lane were loaded onto 12% of SDS-polyacrylamide gels and run at 60 mA. The gel was electroblotted onto nitrocellulose membranes (GE Healthcare, Chicago, IL, USA) in 50 mmol/L sodium phosphate buffer (pH 7.4) for 180 minutes at 1.5 A at 4°C. Membranes were blocked with 5% of milk powder in TBS-T for 1 hour at RT. Immunological detection with an anti-HA-Tag antibody (Abcam, Cambridge, UK; ab9110; 1:1000 in blocking solution) was performed overnight at 4°C, followed by 3× 10 minutes washing with TBS-T. The secondary antibody (ECL anti-rabbit IgG, HRP-linked whole Ab; 1:10 000, donkey, GE Healthcare) was incubated for 2 hours at RT. After 3× 10 minutes washing in TBST signals were visualized via horseradish peroxidase activated chemiluminescence (SERVA Light Helios CL HRP WB Substrat-Kit, SERVA Electrophoresis GmbH, Heidelberg, Germany) detected on ChemiDoc MP Imaging System with Image Lab Software (BioRad, Hercules, CA, USA).

2.8 Quantitative reverse transcription PCR (qRT-PCR)

The qRT-PCR was conducted with a mix of 2 μL isolated RNA, 2 μL of each primer (10 PM, each), 10 μL of Quantitect SYBR Green PCR Master Mix (Roche), and 4 μL of H₂O. Reactions were incubated at 95°C for 5 minutes, followed by 45 cycles at 95°C for 10 seconds, 60°C for 15 seconds, and 72°C for 20 seconds, followed by a melting curve on a LightCycler 480 system (Roche) with LightCycler 480 Software release 1.5.1.62. The following specific murine primer pairs were used: 5´-CCTGCTAGCTGCCAACTTGA-3´ (Pde3a_for), 5´-AGCCCACTCTCGGAATCACT-3´ (Pde3a_rev), 5´-GAACTGGGGGAGGATTGTGG-3′ (Bcl2_for), 5′-GGGGTGA CATCTCCCTGTG-3’ (Bcl2_rev) 5′-ATGAGCAGCAGAGTGA TTAGAATCTGCTATTGCTATGGGTAACCCCAACGCCCACCCACCTTCACCTGTTTGGGAGAGCA-3’ (HAlong_rev) as primer pair. Both were digested and ligated into pcDNA3.1 as described for ICER-HA. Construct inserts were verified by sequencing (Eurofins Scientific).

2.9 Detection of DNA fragmentation as a marker for apoptosis in heart tissue

Excised hearts were fixed overnight in neutral-buffered formaldehyde (4%) and embedded in paraffin. A 5 μm thick sections were cut from each heart and placed on glass slides. Heart sections were dewaxed in xylene and rehydrated in graded alcohols. DNA fragmentation was detected by terminal uridine deoxynucleotidyl transferase nickend labeling (Click-iT TUNEL Alexa Fluor 488 Imaging Assay, Thermo Fisher Scientific), according to the manufacturer’s instructions. Nuclei were stained
by 4',6-diamidino-2-phenylindole (DAPI, 10 pg/mL in distilled water) and mounted (Fluorescence mounting medium, Dako Deutschland GmbH, Hamburg, Germany). The slides were digitized using a camera (DS-Ri2) attached to a fluorescent microscope (Ti-E Eclipse, Nikon GmbH, Düsseldorf, Germany). Quantitative analysis under standardized calibrated magnification was performed manually using a computer-based software system (NIS-Elements AR 5.11, Nikon GmbH). For quantification, apoptotic green cells were counted in the central heart tissue omitting atria, connective, aortic tissue, and the outer surface of the heart. Green sparks, exhibiting no blue signal in the DAPI staining, were also omitted from the quantification.

2.10 RNA sequencing

Isolated total RNA samples from cardiomyocytes were prepared for sequencing using the NEBNextPoly(A)mRNA Magnetic Isolation Module (New England BioLabs) to isolate poly(A)+ RNA from total RNA. The eluted RNA was transcribed in cDNA using the mRNA Library Prep Kit for Illumina (New England BioLabs) and purified by magnetic beads (Agencourt AMPure XP, Beckman Coulter GmbH, Krefeld, Germany). End repair and dA tailing of cDNA library was performed, immediately followed by adaptor ligination by Multiplex Oligos for Illumina Kit (New England BioLabs) according to the manufacturer's instructions. Quality control was performed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Libraries were sequenced on a HiScanSQ sequencing system using a TruSeq SR Cluster v3 Kit and a TruSeq SBS v3 Kit (both Illumina, San Diego, CA, USA) in single-end mode (50 cycles). The fastq data are quality controlled with FastQC. The software Trimmomatic was used for adapter trimming and read filtering.32 The resulting reads were aligned to the mouse reference genome (GRCm38.p6) using HISAT2 (ver. 2.05).33 The aligned reads were sorted using samtools (ver. 1.2.1).34 The aligned reads were sorted using samtools (ver. 1.2.1).34 The sorted and aligned reads were counted per genes using bedtools software (ver. 2.28.0).38

2.11 Chromatin immunoprecipitation (ChIP) and ChIP DNA sequencing (ChIP-Seq)

HEK293 cells transfected with ICER-HA, smICER-HA or Ib-HA were harvested 24 hours after transfection with a cell scraper and suspended in 1 mL PBS. The following incubation steps were done by the use of an overhead shaker. After crosslinking with 1% of formaldehyde (Thermo Fisher Scientific, *28 906, methanol-free) for 10 minutes at room temperature (RT), the reaction was stopped by the addition of glycine to a final concentration of 0.125 M. Until now all steps were done on ice or at 4°C. The cells were collected by centrifugation (3 minutes at 2500 g), rinsed in PBS, and again centrifugated for 10 minutes (13 000 g). The cell pellet was lysed in cell lysis buffer (1% of SDS, 10 mM of EDTA, 50 mM Tris-HCl pH 8, supplemented with protease inhibitor cocktail tablets). Samples were sonicated for 10 seconds with 60% amplitude (HTU Soni130, G. Heinemann), incubated for 120 minutes, and then, frozen at −80°C. After thawing, samples were sonicated for 10 seconds with 60% amplitude in total five times. After 10 minutes centrifugation (13 000 g) chromatin samples were diluted with 700 μL of ChIP dilution buffer (0.01% of SDS, 1.1% of Triton-X-100, 1.2 mM of EDTA, 16.7 mM of Tris-HCl pH 8.1, and 167 mM of NaCl supplemented with protease inhibitor cocktail tablets) and precleared with 80 μL of protein A/G (50/50%)-agarose beads for 1 hour. Before use, the agarose beads were blocked with 0.024% of glycogen and 0.5% of bovine serum albumin overnight at 4°C. Afterward, chromatin was incubated with rabbit polyclonal HA tag antibody (5 μg, ab9110, Abcam) over-night, precipitated with 80 μL blocked protein A/G beads for 2 hours, and centrifugated at 150 g for 2 minutes. Subsequently the beads were washed with 500 μL low-salt buffer (0.1% of SDS, 1% of Triton-X-100, 2 mM of EDTA, 20 mM of Tris-HCl pH 8.0, and 150 mM of NaCl); high-salt buffer (0.1% of SDS, 1% of Triton-X-100, 2 mM of EDTA, 20 mM of Tris-HCl pH 8.0, and 500 mM of NaCl); LiCl buffer (250 mM of LiCl, 1% of Nonident 40, 1% of deoxycholic acid (sodium salt), 1 mM of EDTA pH 8.0, and 10 mM of Tris-HCl pH 8.0); and two times with TE buffer (1 mM of EDTA and 20 mM of Tris-HCl pH 8.0). After each washing step, the slurry was centrifugated at 150 g for 1 minutes. Immune complexes were eluted by a 15 minutes incubation of the agarose beads with 150 μL of fresh elution buffer (1% of SDS and 0.1 M of NaHCO3) at 37°C, centrifugation at 150 g, and a second elution with 100 μL of elution buffer. The collected supernatants were combined and crosslinks were reversed by the addition of 20 μL of 5 M NaCl per sample and a subsequent incubation at 67°C over night. The eluate was resuspended in 0.08 mg/mL proteinase K and 0.1 mg/mL RNase A for 1.5 hours at 55°C. DNA was purified with Mini PCR Purification Kit (Qiagen GmbH, Hilden, Germany) and amplified with the GenomePlex Complete Whole Genome Amplification (WGA2) Kit (Merck) following manufacturer's instructions. For next generation sequencing, the quality of enriched fragments was checked and the amount of DNA was quantified by the use of a Qubit dsDNA HS Assay.
Kit (Q32851, Thermo Fisher Scientific) and a fluorimeter (Qubit 3.0, Thermo Fisher Scientific). For library preparation, the NEBNext Ultra II DNA Library Prep Kit for Illumina was used and adapters were be used by the use of the NEBNext Multiplex Oligos for Illumina Primer Sets 1 und 2 (both New England BioLabs). Quality control was performed using a 2100 Bioanalyzer (Agilent Technologies). For sequencing, a NextSeq 500/550 v2 kit and a High output flow cell v2 (Illumina) were used. Validated sequences were mapped with the software PAVIS39 to the human genome (Hg38). Analysis was conducted with Model-based analysis of ChIP-seq (MACS2 Vers. 2.1.0.20150731),40 and bound regions of ICER and smICER were compared. From these, regions of the Ib-group also found in the range of 1000 base pairs were manually subtracted and results were presented as a proportional Venn-diagram.41

2.12 Statistical analysis

If not indicated otherwise, data are presented as mean ± SD. The number of experiments is reported as n = number of independent experiments. A Shapiro-Wilk-Test or Spearman’s rank correlation test was used to test normal distribution and heteroscedasticity, respectively. One-way ANOVA, ANOVA on ranks, two-way ANOVA, and two-way repeated-measures ANOVA were chosen to compare several experimental groups as indicated in the figure legends. To keep the statistical analysis for the Ca2+ transient and exons coverage straight forward, we used a rank sum test to compare the genotypes at a specific condition. Statistical analysis of qRT-PCR data in Figure 8C,D and S6 was performed using the relative expression software tool (REST 2009 Version 2.013; Qiagen) Random statistical analysis was performed with 10 000 iterations.

3 RESULTS

ICER is supposed to play a pivotal role in pathophysiological conditions of enhanced β-adrenergic stimulation of the heart like cardiac hypertrophy.20 Hence, we investigated the relevance of ICER induction for morphometric and functional effects in the heart using global knockout mice with a specific deletion of the exon X (IKO) vs wild-type (WT) mice. Mice were treated with isoproterenol (ISO) 10 mg kg−1 d−1 via osmotic mini-pumps for 6, 24 hours, or 7 days termed in the manuscript as WT6h, IKO6h, WT24h, IKO24h, WT7d, and IKO7d. Control mice termed WTCtr and IKO Ctr were left untreated, to assess basal levels of ICER transcription, without potential ICER induction caused by stress, for example, anesthesia and surgery procedures, which may as well lead to additional endogenous catecholamine release.

3.1 Increased cardiomyocyte cell length in IKO mice after β-adrenergic stimulation

Morphometric analysis revealed an increased heart weight to body weight ratio by 15 and 13% after 7 days of ISO treatment in both WT7d and IKO7d compared to untreated mice, but no differences between the genotypes were detectable. A similar pattern was found in the heart weight to tibia length ratio (Figure 1A,B).

In untreated animals, no differences in cardiomyocytes length (Figure 1; WTCtr: 132.7 ± 11.6 μm, n = 7; IKO Ctr: 133.0 ± 10 μm, n = 9) or width (Figure 1D; WTCtr: 27.5 ± 1.7 μm, n = 7; IKO Ctr: 27.8 ± 1.5 μm, n = 9) were detected. After 7 days of ISO treatment IKO7d cardiomyocytes cell length slightly increased by 11% to 147.6 ± 11.0 μm (n = 8), while WT7d cardiomyocytes (137.8 ± 15.2 μm) showed no alteration in comparison to WTCtr myocytes (Figure 1C, n = 8). In tendency, cell width showed a similar pattern without any significant changes among groups (Figure 1D).

3.2 Transient decrease of heart performance in IKO mice after 7 days of isoproterenol treatment

Untreated animals (IKO Ctr vs WTCtr) showed a similar heart performance in all parameters. Investigation of heart performance by catheter measurements after 7 days of ISO treatment revealed a decrease of cardiac output by 35%, of maximal contraction velocity by 16%, and of stroke volume by 32% in KO7d vs WT7d mice under highest levels of acute ISO stimulation (Figure 1E,F and S1A). No differences in heart rate, maximum relaxation velocity and ejection fraction were detected between genotypes at this time point (Figures 1G,H and S1B). To examine whether cardiac function is still diminished after longer β-adrenergic stimulation, mice were treated with ISO for 14 days. At this time point, both genotypes showed similar heart performance in all hemodynamic parameters measured under basal conditions and acute ISO treatment (Figure S2).

3.3 Differences in calcium transients and contractility of IKO cardiomyocytes after 24 hours of isoproterenol treatment

To analyze changes in cardiac function at cellular level, intracellular Ca2+ (Ca2+ i) cycling and sarcomere shortening were investigated in isolated ventricular cardiomyocytes. Cardiomyocytes of WT Ctr and IKO Ctr mice showed no differences in diastolic Ca2+ i levels, Ca2+ i transient amplitude, Ca2+ i release (time to peak) or time to 50% Ca2+ i decay under basal conditions (Figure 2A-D) and during acute isoproterenol stimulation (10−6 M, Figure S3A-D). In accordance
FIGURE 1  Determination of morphometric and functional parameters of WT and IKO hearts after β-adrenergic stimulation. Heart weight to body weight-ratio (A), heart weight to tibia length (B), cardiomyocyte cell length (C) and width (D) of cardiomyocytes are shown for untreated WT_Ctr (open circle, grey) and IKO_Ctr (open rectangle, light red) control (Ctr) animals as well as 7 days ISO treated WT_7d (circle, black) and IKO_7d (rectangle, red) mice. Notably, cell length of IKO_7d was increased compared to IKO_Ctr animals. Hemodynamic parameters such as cardiac output (E), maximal contraction velocity (F), heart rate (G), and maximum relaxation velocity (H) were measured in WTCtr, IKOCtr, WT7d, and IKO7d animals under basal (B) and increasing concentrations of ISO. Raw data in A-H are displayed as scattered plots and the horizontal bars with errors represent the mean values ± SD; A and B n = 14-17, *P < .05 vs Ctr, ANOVA on ranks Dunn post hoc test; C and D n = 7-9, based on 60 cells each, two-way ANOVA *P < .05 vs Ctr, Holm-Sidak post hoc test; E-H n = 5-6, *P < .05, main effect in genotype, *P < .05 vs IKO, two-way repeated-measures ANOVA calculated for WT_7d and IKO_7d. Sidak post hoc test.
FIGURE 2  Determination of calcium transient and contractility parameters of WT and IKO cardiomyocytes under basal conditions. Diastolic calcium level (A) and sarcomere length (SL) (E), calcium amplitude (B) and SL shortening (F), as well as time to maximum calcium release (C) or to maximum SL contraction (G), and time to 50% calcium decay (D) or 50% SL relaxation (H) were measured in WT_ctr (n = 8; grey, open circles), WT_6h (n = 8), WT_24h (n = 9), WT_7d (n = 8; black, circles) and IKO_ctr (n = 7; faint red, open rectangles), IKO_6h (n = 8), IKO_24h, (n = 8), and IKO_7d (n = 8; red rectangles) cardiomyocytes. Each parameter is illustrated by the small insets. Notably, while no differences in the parameters were observed in Ctr and in 6 hours and 7 days ISO treated animals, differences were observable at 24 hours—for time to maximum calcium release (C) and SL contraction (G) were faster in IKO_24h vs WT_24h. An outlier (E) omitted from the analysis in the IKO_24h group identified by Grubs test (α < .01) is marked by an open red triangle. Data are shown as mean values ± SD; *P < .05 vs WT, Mann-Whitney rank sum test.
with these results no differences in diastolic sarcomere length (SL), SL shortening during contraction, time to maximum SL contraction or time to 50% SL relaxation were found under basal conditions (Figure 2E-H). During acute ISO stimulation diastolic SL was increased in IKO_Ctr myocytes (Figure S3E), while the other parameters of sarcomere shortening (Figure S3F-H) were not different between genotypes. After chronic ISO stimulation parameters started to change in cardiomyocytes isolated from treated mice, for example, time to 50% ISO stimulation parameters started to change in cardiomyocytes of both genotypes respectively. Unexpectedly, only two genes: Xist (Xist) upregulated in IKO 24h vs WT 24h specifically transcript (Xist) were upregulated in IKO Ctr mice. After chronic ISO stimulation, cardiomyocytes of both genotypes exhibited comparable functional parameters again.

### 3.4 Deletion of exon X in IKO mice leads to minor changes in gene transcription after β-adrenergic stimulation

We further investigated the impact of the deletion of ICER on gene transcription by determination of mRNA levels in cardiomyocytes via next generation sequencing (NGS). Analysis revealed one outlier in the IKO_24h and the WT_7d group, which were omitted from gene transcription analysis (n = 6 per group in all other groups). After pairwise calculation of altered genes between the experimental groups, genes exhibiting less than 25 fragments in mean (base mean) over all samples were excluded, leading to 12 377 genes in each group used for further analysis (Table S1). An overview of regulated genes is presented in Figure 3A. Transcriptional alterations were only detectable when comparing each treatment group (6, 24 hours, and 7 days ISO) to the respective untreated Ctr group in both genotypes. After 6 hours of treatment, 5737 and 5151 genes were changed in WT_6h and IKO_6h mice compared to the WT_Ctr and IKO_Ctr cardiomyocytes, respectively. After 7 days, still 2687 and 1228 genes were changed in WT_7d vs WT_Ctr and IKO_7d vs IKO_Ctr cardiomyocytes, respectively. Unexpectedly, only two genes: Crem downregulated in IKO_6h vs WT_6h and X inactive specific transcript (Xist) upregulated in IKO_24h vs WT_24h animals were significantly altered between the genotypes. Distribution plots of all genes also revealed only a slight difference between WT and IKO mice during ISO stimulation (Figure 3B-D). After 24 hours moderately regulated genes (log2-fold change −0.5 to 0.5) in IKO_24h compared to IKO_Ctr seemed to be shifted toward higher transcription levels than genes in WT_24h compared to WT_Ctr mice. After 7 days of treatment WT_7d exhibited a higher number of downregulated and stronger upregulated regulated genes in comparison to WT_Ctr, while IKO_7d exhibited more nonregulated and slightly upregulated genes in comparison to IKO_Ctr mice (Figure 3D).

### 3.5 Identification of Crem splice variants in murine hearts

The lack of ICER in IKO might be compensated by other splice variants transcribed from the Crem gene, which could explain the minor effects on gene transcription, morphometry, and cell physiology observed in IKO cardiomyocytes. Thus, we assessed alternative splicing within the Crem gene in an exploratory approach using polymerase chain reaction (PCR), subsequent cloning and sequencing. We were able to detect 16 different splice variants in untreated murine cardiomyocytes or ventricular tissue (see Figure 4). For PCR, the primer pairs B-Ia, B-Ib, X-Ia, and X-Ib were used. We identified 4 long exon B comprising splice variants (B-E-F-γ-H-Ia, B-E-F-γ-H-Ib, B-E-F-H-Ia, and B-E-F-H-Ib) containing the exons E and F as a part of the kinase inducible domain, the alternatively spliced exon γ and one of the exons Ia or Ib coding for the DNA binding domain. Furthermore, we found 4 short exon B comprising variants B-γ-H-Ia, B-γ-H-Ib, B-H-Ia, and B-H-Ib. No splice variants with the transactivation domains τ1 or τ2, encoded by exon C and G, were found. 9Icer splice variants including the exon X were X-γ-H-Ib, X-H-Ia, and X-H-Ib, known as Icer II, Icer γI, and Icer γII. Further splice variants were detected by 5′RACE-PCR, using specific primers for exon Ia or Ib in ventricular tissue of mice treated for 10 hours with ISO (10 mg kg⁻¹ d⁻¹). Using this approach, we identified that ISO induced only the Icer variant X-γ-H-Ia (Icer I) and the smIcer variants S-γ-H-Ia, S-γ-H-Ib, S-H-Ia, and S-H-Ib (see Figure 5). Notably, no other Crem splice variants could be detected by RACE-PCR. Overall, we were not able to detect splice variants having both, one of the transactivation motifs (exon C or G) and the kinase inducible domain (exon E and F).

### 3.6 Exon S encoded the 3’UTR for smIcer splice variants

Interestingly, 5′-RACE-PCR identified exon S as a new exon between exon X and exon γ transcribed by the P6 promoter. Sequence comparison with public available databases revealed identity with the recently updated transcript variants ENSMUST00000124747.7 or NM_001311066. This exon exhibits no translational start site and forms a part of the UTR in all smIcer transcripts transcribed by the P6 promoter5 (see Figure S4A).
Hence, the presence of exon $X$ is specific for $Icer$ and the presence of exon $S$ is specific for $smIcer$ splice variants. Analysis of the coding sequences of $Icer$ and $smIcer$ revealed several possible translational start sites in the exon $X$, $\gamma$ and $H$. Overexpression of hemagglutinin-tagged $Icer$ ($X$-$\gamma$-$H$-$Ib$-$HA$) and $smIcer$ ($\gamma$-$H$-$Ib$-$HA$) constructs in Hek293 cells...
and consecutive immunoblotting revealed the functionality of these translational start sites embedded in the exons. The protein X-γ-H-Ib was the most abundant protein translated from the Icer construct (X-γ-H-Ib-HA) in HEK293 cells using the start site in exon \(X\). Furthermore, two smaller proteins H I-Ib and H II-Ib were translated from this construct using two alternative start sites in the exon \(H\). Overexpression of smICER resulted in the translation of γ-H-Ib and, to a higher extent HI-Ib and H II-Ib. This demonstrates that the expression of the proteins HI-Ib and H II-Ib, with identical sequences, can be regulated by at least two different promoters, namely P2 (Icer) and P6 (smIcer; Figure S4A-C).

### 3.7 Icer and smIcer are the predominant Crem splice variants after chronic β-adrenergic stimulation in mouse hearts

To quantify the transcription of Crem splice variants induced by β-adrenergic stimulation in the heart in more detail, we studied mRNA levels using the data of the NGS-approach, by summarizing exon spanning reads (splice junctions) between exons in each group (\(n = 6\); Figures 5 and S5). Overall analysis of splice junctions confirmed those splice variants previously identified by PCR and presented in Figure 4. Some individual splice junctions were also found for exon C and G, for example, 4 reads in 7 days treated WT animals (C-E-F), suggesting the presence of splice variants with one or both transactivation domains together with the KID (C-E or F-G), while junctions without transactivation domains B-E, E-F, and F-H or F-γ were found to be 3 to 4 times more abundant.

Most of the exon B containing variants were regulated by the P1 promoter starting with the exon A0 upstream of the exon B, while only a few splice variants originated from the P5 promoter. No splice variants were transcribed from the P3 or P4 promoter (Figure S5). After β-adrenergic stimulation Icer and smIcer regulated by the P2 or P6 promoter were the most abundant splice variants. Since all splice variants contain exon H, coverage of this exon could be used as a direct measure of Crem mRNA levels, which is more or less the

![FIGURE 4 Qualitative identification of Crem splice variants in murine hearts. Top: schematic genomic structure of the murine Crem gene (adapted from Gellerson et al5) Exons are indicated as grey boxes, promoters P1 to P6 are marked by white flags. Black arrows indicate primer used for PCR to identify isoforms. Exon \(ψ\) described for the human Crem gene was omitted.\(^5\) Functional domains are the two transactivation domains \(τ1\) and \(τ2\) the kinase inducible domain (KID) and the two DNA binding domains (DBDI and DBDII). Splice variants were identified by PCR, subsequent cloning and sequencing in mouse ventricular tissue (\(P\)_V), cardiomyocytes (\(P\)_C) or by 5′RACE-PCR in ventricular tissue from ISO treated mice (\(R\)_V). The recently described exon S was identified as unique for smIcer splice variants by RACE-PCR (also see Figure S4). Notably, no splice variants with both, a complete transactivation and a kinase inducible domain (C-E-F-G) were found (also see Figure S5).}
FIGURE 5  Analysis of Icer (exon X) and smIcer (exon S) exon coverage and exon spanning reads after β-adrenergic stimulation. Sashimi plot of the data obtained by NGS in cardiomyocytes RNA-Seq of ISO stimulated WT6h-7d and IKO6h-7d mice (n = 6), or untreated WTCtr and IKOCtr mice. Exons are colored in grey, exon spanning reads are depicted as arcs and numbers indicate abundance of exon spanning reads. Bars indicate the number of reads per nucleotide in the exons (for quantification see Figures 6 and 7). Reads originate from the exon X (red) increased in WT6h animals, but already declined after 24 hours of ISO treatment indicating a rapid but transient induction of ICER isoforms. In IKO, almost no reads in the exon X were found. Reads originate from the exon S (blue) increased in both genotypes after ISO treatment and exhibit high levels also after 7 days. For quantification, see Figures 6 and 7.
sum of Icer (exon X) and smIcer (exon S) which were the predominant Crem splice variants after beta adrenergic stimulation. While in untreated WT animals 4 splice junctions for exon X-H were detectable, 26 S-H and 12 S-γ junctions were found. After 6 hours of ISO stimulation 143 X-γ and 144 X-H junctions as well as 79 S-γ and 101 S-H junctions were detectable (Figure 5).

The quantitative analysis of exon X coverage revealed an increase from 0.9 ± 1.4 reads per nucleotide (n = 6, Figure 6A) in WT_Ctr to 69.3 ± 40.4 in WT_iso animals, which then declined to 30.3 ± 10.5 and 4.9 ± 2.4 after 24 hours and 7 days of ISO stimulation, respectively. In contrast to exon X, exon S coverage started already at a higher level of 8.0 ± 4.6 reads per nucleotide and increased in a saturation-like manner to 21.7 ± 13.0 reads at 6 hours, 31.2 ± 8.9 after 24 hours and 24.8 ± 7.3 reads after 7 days of β-adrenergic stimulation (Figure 6A). Moreover, after 7 days of ISO stimulation smIcer splice variants (identified by exon S) were the most abundant of all Crem variants, superseding the mRNA levels of Icer variants (also compare Figure 5 and Figure S5).

Comparison of exon usage after 7 days of ISO treatment, in line with a transient induction of Icer levels of exon usage after 7 days of ISO treatment, in line with a transient induction of Icer.

3.8 Deletion of exon X did not affect the composition of other splice variants of the Crem gene

In IKO cardiomyocytes analysis of exon coverage and splice junctions confirmed the absence of mRNA originating from exon X, while no differences in splice variant combination for the exons A0, B, C, E, F, G, and S were observed between the genotypes (Figure 7). Coverage of the exons γ, H, Ia, and Ib was reduced, due to the loss of Icer splice variants, proving the successful deletion of Icer splice variants transcribed by the P2 promoter. Since the difference between IKO and WT animals in the transcription of all splice variants (mainly transcriptional repressors) can be assessed by the abundance of exon H (grey area exon H, Figure 7). Exon H coverage indicated that Crem transcription in IKO and WT cardiomyocytes only differed at 6 and 24 hours and exhibited identical levels of exon usage after 7 days of ISO treatment, in line with a transient induction of Icer.

3.9 No evidence for the existence of the Icer-Pde3a-loop when comparing IKO and WT mice after prolonged ISO treatment

Published data suggested that ICER perpetuates its own expression by binding to the Pde3a promoter inhibiting the production of the gene's alternative DNA binding sites (Figure 6C).
expression of this phosphodiesterase. This would elevate cAMP levels and consequently maintain ICER expression and, as one possible consequence might lead to enhanced apoptosis by downregulation of BCL2. Quantification of mRNA levels of Pde3a and Bcl2 in cardiomyocytes of untreated and ISO treated mice, based on the NGS results and qRT-PCR (Figure 8A-D) revealed no long-term changes in Pde3a or Bcl2 mRNA levels after 7 days of ISO treatment. Pde3a was upregulated by 50% in IKO6h vs WT6h animals, which was found by qRT-PCR but not by NGS. However, Bcl2 mRNA levels were found downregulated in WT24h (NGS) and WT6h (qRT-PCR) animals by 50% in comparison
to WT<sub>Ctrl</sub> (Figure 8B,D). Furthermore, qRT-PCR results from complete hearts taken from wild-type FVBN mice treated with ISO (3 mg kg<sup>−1</sup> d<sup>−1</sup>) for 10, 24 hours, and 7 days did not show any differences in mRNA levels of Pde3a. However, Bcl2 levels were reduced at 10 and 24 hours by about 30% (Figure S6). Quantification of apoptotic cells in heart tissue of WT<sub>Ctrl</sub>, IKO<sub>Ctrl</sub>, WT<sub>7d</sub>, and IKO<sub>7d</sub> animals revealed a significant increase of apoptosis due to ISO treatment, but no differences between genotypes (Figure 8E,F and Figure S7).

### 3.10 ICER binding sites in the genome are also bound by smICER

The minor alterations of gene transcription in IKO mice suggested that the increase of smICER might compensate the absence of the ICER induction after β-adrenergic stimulation, by binding to DNA target sequences also occupied by ICER. To identify DNA binding sites, ICER-HA, smICER-HA (see Figure S4C) and a truncated I<sub>b</sub>-HA control construct were over-expressed in HEK293 cells. ChIP and subsequent NGS-based analysis (data from n = 8 independent experiments) revealed in summary 2095 smICER and 1606 ICER binding sites. 1592 of these sites were identical between ICER and smICER (Figure 9A; for details see Table S2). Annotation of binding sites showed that 33%-35% were located upstream of the transcriptional start sites of known genes, for example, polymerase beta (Polb) or early growth response 1 (Egr1) (Figure 9B). Notably, a binding of ICER or smICER to the Pde3a or Bcl2 promoters was not found.

### 4 DISCUSSION

As main results of this study we show (i) that there is a complex and time-controlled regulation of the transcription of multiple CREM repressor isoforms from the <i>Crem</i> gene in response to β-adrenergic stimulation, (ii) that a short-term transient-like induction of I<sub>cer</sub> is followed by a long-lasting saturation-like induction of smI<sub>cer</sub>, (iii) that transient induction of I<sub>cer</sub> plays a minor role for the regulation of gene transcription and cardiac remodeling and (iv) that smICER may redundantly take over the function of ICER. Overall our results suggest that a saturation-like induction of smI<sub>cer</sub>, instead of I<sub>cer</sub>, induces a long-lasting alteration in the CRE-mediated gene expression along with detrimental cardiac effects of prolonged β-adrenoceptor stimulation.

CRE-mediated transcription is controlled by the CREB1/CREM/ATF-1 protein family. In this context, the expression of CREM isoforms comprising transcriptional activators and repressors was shown to play a critical role in spermatogenesis, the immune system, and for endocrine or neuronal functions. Our analysis of NGS data allowed for the first time a detailed analysis of the complex regulation of CREM isoforms during β-adrenergic stimulation. Our results show that transcriptional activator splice variants such as CREM-τ isoforms, containing the kinase inducible and at least one of the τ<sub>1</sub> or τ<sub>2</sub> domains (exon C or G), were nearly absent in our experimental conditions. Other variants transcribed by the P1 promoter like CREM α or β exhibit the kinase inducible domain (exon E and F), but lack the transactivation domain. These and short variants like B-H-I<sub>b</sub> that completely miss both the kinase inducible and transactivation domains are described as transcriptional repressors. Along with the repressors I<sub>cer</sub> and smI<sub>cer</sub>, only inhibitory splice variants were transcribed in cardiomyocytes. In this context, the usage of the exon <i>la</i> was higher than of the alternative spliced <i>lb</i>, both forming alternative DNA binding domains, which is in line with a higher stability of the <i>la</i> containing ICER isoform X<sub>γ-H-Ia</sub> compared to X<sub>γ-H-Ib</sub>. Chronic β-adrenergic stimulation increased I<sub>cer</sub> splice variants rapidly and transiently, probably because of the auto-regulatory inhibition of its own promoter. In contrast, smI<sub>cer</sub> was increasingly induced in a long-term saturating manner becoming the predominant splice variant after 7 days of β-adrenergic stimulation. Other splice variants transcribed by the P1 promoter were induced on an overall low level (compare Figure 7 exon A<sub>γrG</sub>). It is not clear so far how the sustained elevation of smI<sub>cer</sub> is achieved. We can speculate that smI<sub>cer</sub> mRNA exhibits a prolonged lifetime or that the P6 promoter is modulated by other factors. Interestingly, proteins H<sub>1</sub>-I<sub>b</sub> and H<sub>1r</sub>-I<sub>b</sub> translated from smI<sub>cer</sub> splice variants exhibit the same protein sequence as proteins translated from the P1 or P5 promoter driven CREM-I<sub>b</sub>ΔC-X (B-H-I<sub>b</sub>) splice variant, identified in the human heart termed H-B-1 and H-B-1-II.26 Overexpression of CREM-I<sub>b</sub>ΔC-X in mice led to atrial dilatation, atrial fibrillation, and arrhythmogenic alterations in ventricular cardiomyocytes. These data suggest that the long-term induction of smI<sub>cer</sub> contributes to the pathophysiological remodeling after β-adrenergic stimulation, and may explain in some aspects why a prolonged stimulation of the β-adrenoceptor, for example, in the context of heart-failure, leads to cardiac electrical remodeling, whereas a short and single stimulation is innoxious. However, the exact role of smICER in vivo has to be addressed in a specific model of smICER deletion in future studies.

In the human failing heart ICER was found upregulated along with a downregulation of PDE3a. This led to the hypothesis of the existence of an ICER-PDE3A feedback loop: induction of ICER perpetuates the elevation of cAMP by repressing the cAMP degrading PDE3A, resulting in turn in a sustained induction of ICER. Our experiments did not reveal this sustained induction of ICER. A transient induction of ICER in response to isoproterenol stimulation was regularly observed on RNA level in our mice and was also shown...
by Tomita et al. on mRNA and protein levels in rat cardiomyocytes. The constant upregulation of ICER protein expression was reported by Ding et al. primarily after angiotensin stimulation. In line with a transient, ICER-dependent regulation, we did not observe a sustained downregulation of PDE3a following ISO infusion, neither in WT or IKO mice.
We only noticed a trend of PDE3a downregulation at 6 hours when PDE3a expression between WT and IKO was significantly different (Figure 8C). The finding that PDE3a was not downregulated at mRNA level is in line with the absence of ICER or smICER binding to \( Pde3a \) promoter in our study. As a limitation, our study investigated the binding of ICER and smICER to the \( Pde3a \) promoter in ChIP experiments in HEK293 cells using an over-expression approach. However, the inhibition of the \( Pde3a \) promoter by ICER was also shown in HEK293 cells by Ding et al.\(^{21}\) Since HEK293 cells may be artificial compared to the endogenous situation in cardiomyocytes, the exact impact of ICER on the \( Pde3a \) promoter in cardiomyocytes is still not clear. Future experiments may clarify the exact conditions required for the activation of the ICER/PDE3a loop and the involvement of ICER and smICER in PDE3a downregulation.

Tomita et al showed that adenoviral over-expression of ICER prevented hypertrophy and increased apoptosis in neonatal cardiomyocytes, while antisense inhibition of ICER significantly enhanced \( \beta \)-adrenergic dependent hypertrophy and inhibited myocyte apoptosis.\(^{20}\) Even though the mean cardiomyocyte length was only elevated by 7% in IKO vs WT mice after 7 days of ISO treatment, our results support the observed anti-hypertrophic effect of ICER in vivo. It should be noted again that ICER is induced within 24 hours only transiently and that the long-term induction of inhibitory smICER splice variants, still present in IKO mice, might replace ICER inhibition of CRE-mediated transcription. Our data show that smICER is able to bind to 99% of the DNA-binding sites also targeted by ICER. Furthermore, it cannot be excluded that the antisense construct (\( X-H-Ib \)) used by Tomita et al repressed smICER isoforms as well, so that their study unintentionally might have addressed the combined function of smICER and ICER.

As mentioned before, overexpression of ICER was linked with an induction of apoptosis by downregulation of the antiapoptotic protein BCL2 after \( \beta \)-adrenergic stimulation. The P1 promoter of \( Bcl2 \) possesses a functional CRE-element, shown to be bound by transcription factors CREB1 and NF-\( \kappa \)B.\(^{51}\) We observed a downregulation of \( Bcl2 \) mRNA concurrently with \( Icer \) mRNA upregulation, but \( Bcl2 \) mRNA levels were not further reduced after 7 days of ISO stimulation in line with the diminished \( Icer \) but contrary to the increased \( smIcer \) levels, since both are acting as repressors of CRE-mediated transcription. Moreover, this downregulation may be only indirectly dependent on CREM isoforms, since no binding of ICER or smICER to the \( Bcl2 \) promoter was found in our ChIP experiments. In line with this argument, a recent publication showed that ICER binds to the p65 subunit of NF-\( \kappa \)B and inhibits directly the transcriptional activity of NF-\( \kappa \)B.\(^{52}\) Moreover, it was shown that NF-\( \kappa \)B factors bind to the CRE element in the \( Bcl2 \) promoter (see above) and that NF-\( \kappa \)B is able to induce \( Bcl2 \) transcription.\(^{52}\) Hence, ICER might indirectly inhibit BCL2 expression without targeting CRE elements in the promoter of \( Bcl2 \), implicating that the CRE-mediated transcriptional regulation of \( Bcl2 \) is of minor relevance in this context and that BCL2 expression might be inhibited by ICER but not smICER. However, the number of apoptotic cells was increased in the heart tissue after 7 days of ISO treatment. Hence, apoptosis induced by chronic \( \beta \)-adrenergic stress seems to be independent of ICER and BCL2, and one may speculate that rather smICER, which is also showing proapoptotic properties,\(^{6}\) is involved in this progress.

In the genome, multiple CRE elements exist. In testis, Martianov et al identified about 6800 binding sites occupied by CREM isoforms and in 83% of these full-site CRE elements or variants of it occurred. Interestingly, in spite of over 5400 binding sites occupied by CREM isoforms in the promoter region of genes, only 58 genes were affected by a loss of CREM.\(^{53}\) In good agreement to this, the absence of the transient induction of ICER in our study had minor impact on the
gene regulation in cardiomyocytes, despite the fact that over 1600 possible binding sites were identified and about 30% were localized upstream of transcriptional start sites. The distribution of regulated genes at 24 hours suggested that ICER acts as a general inhibitory modulator of gene transcription induced after β-adrenergic stress, damping short-term transcriptional response, while smICER controls CRE-mediated transcription in a long-term steady-state manner.

The relevance of ICER for short-term adaption to β-adrenergic stress is reflected by functional alterations in cardiomyocytes and the whole heart. In this context, the absence of ICER seems to accelerate physiological alterations on the cellular level. For example, Ca2+ i release and time to 50% decay were different at 24 hours and reached the same levels after 7 days of ISO stimulation in IKO, in comparison to WT24h and WT7d mice. Also in IKO7d mice, we detected cell hypertrophy and a transient decrease of cardiac performance in comparison to WT7d mice. Hence, we conclude that the transient induction of Icer, plays only a minor role for long-term cardiac remodeling. Instead, a saturation-like induction of smIcer causes a long-lasting alteration in the CRE-mediated gene expression along with detrimental cardiac effects of chronic β-adrenoceptor stimulation. In this context, the duration, intensity, and frequency of stress stimuli as well as the pathophysiological context may determine the intensity of ICER and smICER modulation and their physiological relevance. The fine-tuned regulation of ICER and smICER isoforms as repressors of the CRE-mediated transcription might have been unrecognized in experimental approaches so far and could have an important impact on functions so far attributed to ICER alone, also beyond the cardiac context.

ACKNOWLEDGMENTS
We thank Nina Goda, Maria Schulik, Stefanie Triebel, and Melanie Voß for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (DFG Mu1376/11-1 and Mu1376/11-3).

CONFLICT OF INTEREST
All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Conception and design: M.D. Seidl, J.S. Schulte, and F.U. Müller; performed experiments: M.D. Seidl, B. Fels, D. Kranick, A. Sternberg, K. Grimm, and F.T. Stümpel; analysis and interpretation: M.D. Seidl, B. Fels, D. Kranick, A. Sternberg, K. Grimm, A. Huge, F. Pluteanu, J.S. Schulte, F.U. Müller; for important intellectual content: F. Pluteanu, J.S. Schulte, A. Heinick, N. Koijma, S. Endo, M. Stoll; F.U. Müller supervised the project and received the funding; M.D. Seidl wrote the initial manuscript, which was revised and approved by all the authors.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

**How to cite this article:** Seidl MD, Fels B, Kranick D, et al. Induction of ICER is superseded by smICER, challenging the impact of ICER under chronic beta-adrenergic stimulation. *The FASEB Journal*. 2020;34:11272–11291. [https://doi.org/10.1096/fj.201902301RR](https://doi.org/10.1096/fj.201902301RR)