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**Poly(ADP-ribose) polymerase-1 (PARP-1) transcriptionally regulates
angiotensin AT2 receptor (AT2R) and AT2R binding protein (ATBP) genes**

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ABSTRACT

The renin-angiotensin system (RAS) plays a crucial role in cardiovascular and neuronal (patho-)physiology. The angiotensin AT2 receptor (AT2R) seems to counteract the proinflammatory, prohypertrophic and profibrotic actions of the AT1 receptor. Recently, we identified a novel protein, termed "AT2R binding protein" (ATBP/ ATIP) which seems essential for AT2R mediated growth inhibition. Poly(ADP-ribose) polymerase-1 (PARP-1) can act as a nuclear integrator of angiotensin II-mediated cell signalling, and has been implicated in the pathogenesis of cardiovascular and neuronal disease.

In this study, promoters of human AT2R and ATIP1 were cloned and two transcriptional start sites in the ATIP1 promoter were identified whereas only one was detected in the AT2R promoter. Promoter assays indicated that the exon 1-intron 1 region of AT2R is necessary and sufficient for AT2R promoter activity. Inverse cloning experiments indicated that this regulatory region is a promoter but not an enhancer element implicating (a) further start site(s) in this region. Consistently, the exon 1-intron 1 region of AT2R was shown to tether the basal transcriptional machinery. Overexpression, pharmacological inhibition and ablation of PARP demonstrated that PARP-1 activates the ATIP1 gene but represses the AT2R on promoter and mRNA levels *in vitro*, and in brain tissue *in vivo*. Additional experiments indicated that AT2R activation does not modulate PARP-1 transcript levels but increases AT2R promoter activity, thereby creating a positive feedback mechanism.

Our results demonstrate that PARP-1 acts as novel node within the RAS network based on its ability to regulate downstream targets such as AT2R and its adapter protein ATBP.

Keywords:

renin-angiotensin system (RAS)

promoter

PARP-1

angiotensin AT2 receptor

ATBP genes

cardiovascular pathophysiology

Abbreviations: RAS, renin-angiotensin system; AT1R, angiotensin AT1 receptor; AT2R, angiotensin AT2 receptor; PARP-1, poly(ADP-ribose) polymerase-1; SHP-1, SH2 domain-containing phosphatase 1; PLZF, promyelocytic zinc finger protein; ATBP, AT2R binding protein; ATIP, AT2 receptor interacting protein; MTSG, mitochondrial tumor suppressor gene 1; PAR, poly(ADP-ribose); MEFs, mouse embryonic fibroblasts; RLA, relative luciferase activity; ChIP, chromatin-immunoprecipitation; TBP, TATA box-binding protein

1. Introduction

The renin-angiotensin system (RAS) constitutes one of the best characterized systems within mammalian physiology and pathophysiology. It is causally involved in a broad spectrum of diseases ranging from arterial hypertension, cardiac hypertrophy, diabetic nephropathy and stroke to cancer and inflammation [1,2,3,4]. The effects of the RAS are mainly mediated by two receptors, the angiotensin AT1 receptor (AT1R) and the angiotensin AT2 receptor (AT2R) [1]. Several groups have demonstrated that AT2R activation can counteract the vasoconstrictory, pro-hypertrophic and pro-proliferative effects of AT1R activation [1,5,6], in addition to its ability to mediate neuroprotective effects [7,8]. Nevertheless, the (patho)physiological role of AT2R is still controversial [9,10]. We recently postulated that the type of adapter protein recruited to the AT2R determines its cellular effects [11]. The AT2R can couple to certain G proteins [12,13] and the SH2 domain-containing phosphatase 1 (SHP-1) [14]. The physical interaction with the latter is probably related to the antiproliferative effects of the AT2R [9,15]. In contrast, recruitment of the promyelocytic zinc finger protein (PLZF) to the AT2R was shown to be associated with an increase in protein synthesis of cardiomyocytes and cardiac hypertrophy [16].

Our group was recently able to identify ATBP (AT2R binding protein) as a novel protein with the ability to bind to the cytoplasmic C-terminus of the AT2R [17]. ATBP is expressed in at least three different isoforms termed ATBP50, ATBP60 and ATBP135 [17]. ATBP50 plays a crucial role concerning the transport of the AT2R from the Golgi compartment to the cell membrane, and mediates the inhibitory effects on MAP kinases as well as the antiproliferative effects of the AT2R in neuroblastoma cells [17]. The ATBP gene was simultaneously cloned by

two other groups who termed this gene ATIP (AT2 receptor interacting protein) [18,19] and MTSG (mitochondrial tumor suppressor gene 1) [20], respectively, and who also demonstrated the inhibitory effects of this protein on cell proliferation and MAP kinases.

ATIP1 (= ATBP50) is the major isoform since it was initially identified by the yeast two-hybrid screenings [17,18], is expressed ubiquitously [17,19] and constitutes the most abundant isoform e.g. in brain, heart, breast and ovary [19]. ATBP can also function as a tumor suppressor gene [20] and very recently it was shown that a genetic copy number variant (CNV) affecting the ATBP gene locus as a deletion polymorphism is significantly associated with a decreased risk of breast cancer [21].

PARP-1 is an ubiquitous nuclear protein catalysing the attachment of poly(ADP-ribose) (PAR) polymers to several different acceptor proteins such as histones and RNA polymerase II [22,23]. PARP-1 is involved in pathophysiologies such as hypertension, stroke, neuronal injury, diabetes and inflammation [24] thereby contributing to similar disease states as the RAS. Consistently, animal experiments indicated that cardiac hypertrophy and also endothelial dysfunction induced by angiotensin II are absent in homozygous PARP-1 deficient mice [25,26]. Nevertheless, the regulatory mechanisms of this RAS-PARP interplay are largely unknown. Therefore, we analysed basal and PARP-mediated regulation of the AT2R and its interaction partner ATBP/ATIP on chromatin, promoter and mRNA levels.

2. Materials and methods

2.1 Cell culture

Human endothelial EA.hy926 cells were kindly provided by C.-J. Edgell (Chapel Hill, NC, USA) and cultured in high glucose DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% FBS, 100 U/ml penicillin and 100 microg/ml streptomycin, 1 mM sodium pyruvate, 1% non-essential amino acids and 2% HAT (all from Invitrogen).

The human neuroblastoma cell line KELLY was a kind gift of the Physiology Department (Campus Mitte) of the Charité, Berlin, Germany. KELLY cells were cultured in RPMI (Invitrogen) supplemented with 10% FCS, 100 U/ml penicillin and 100 microg/ml streptomycin. For transfection experiments KELLY cells were cultured on plates coated with poly-L-lysine (0.005 mg/ml; Biochrom, Bremen, Germany). The human breast adenocarcinoma cell line MCF-7, a kind gift of G. Schönfelder (Toxicology Department, Charité), was cultured in high glucose DMEM supplemented with 10% FCS and 1% penicillin and streptomycin (100 IU/ml). Mouse embryonic fibroblasts (MEFs) were obtained from Z.-Q. Wang (Leibniz Institute for Age Research, Jena, Germany) and were grown in high glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 microg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.2% beta-mercaptoethanol (Invitrogen).

All cell lines were grown in a humidified atmosphere of 5% CO₂ in air and at 37°C.

Cellular total RNA was isolated using the RNA Isolation kit from Macherey-Nagel (Düren, Germany).

For stimulation experiments the following small molecules were used: the non-selective PARP inhibitor 3-aminobenzamide (3-AB; Sigma, Hamburg, Germany; 10 mM (EA.hy926) or 30 mM (KELLY)), the AT2R agonist Compound 21 (1 µM in

water or PBS; a kind gift of Vicore Pharma, Gothenburg, Sweden), the AT1R blocker irbesartan (10 μ M in DMSO/PBS; applied 30 min before angiotensin II treatment; Sigma) and angiotensin II (0.1 μ M in water; Sigma). Stimulation was performed 48 hours after transfection and 24 hours after starving. Harvest for RNA extraction or luciferase assay was performed 7 hours after stimulation.

2.2 Tissue samples

Tissue samples from homozygous PARP-1 knockout mice (PARP-1 $-/-$) derived from the knockout line which was described previously [27]. Total RNA of these samples was isolated with Trizol (Invitrogen, Karlsruhe, Germany) method. mRNA quantification by real-time PCR was performed based on 6 wild type and 6 knockout animals, each in technical triplicate. In brain tissue of these 6 + 6 mice, mRNA expression was evaluated based on three independent reverse transcriptions due to larger standard deviations.

The AT2R knockout mice were a kind gift of Michael Bader (Max Delbrück Center for Molecular Medicine (MDC), Berlin, Germany); real-time PCR data represent 2 animals in each group and technical triplicates.

2.3 Constructs

Serial deletion mutants of the human AT2R promoter were subcloned into the luciferase reporter vector pGL3-basic (Promega, Mannheim, Germany) using a common antisense primer located directly upstream of exon 2 (5'-gtaagagaaacagcagctaaataat) and following sense primers: 5'-atagcttgtaatagcacctaccata-3' [-1316-construct], 5'-gagctgggttgtaaagccaggtag-3' [-1116-construct], 5'-tggtgtgtagttattgccccac-3' [-916-construct], 5'-agccaacaaaaactgcgcaaagcaa-3' [-716-construct], 5'-gaaaaaaggaagagaaaattctgta-

3' [-516-construct] and 5'-ctggcaagggttcataagtcagccc-3' [-316-construct]. The "-1100"-construct of human AT2R promoter was subcloned using an antisense primer located directly upstream of exon 1 (5'-aatagcaccagatctctggttct-3') and the sense primer used for the "-1316"-construct. The "-216"-construct and "-148"-construct based on the antisense primer located directly upstream of exon 2 (see above) and the sense primers 5'-acgtcccagcgtctgagagaacgag-3' and 5'-taagtatgaacaatttatataat-3', respectively. The "-216-inverse"-construct was subcloned as the "-216"-construct but exchanging the restriction sites within the anchor primers.

Serial deletion mutants of the human ATIP1 promoter were subcloned into the luciferase reporter vector pGL3-basic (Promega) using a common antisense primer located directly upstream of the translational start site (5'-gtcttcggagcaggtggcgagattt-3') and following sense primers: 5'-tattcggtggttaatggaattagct-3' [-1100-construct], 5'-gaaagaccactataagagaccac-3' [-900-construct], 5'-cttttgacaatctgtcattaatatatt-3' [-700-construct], 5'-acacagtttactttcaggaaatcc-3' [-500-construct], 5'-agcatgcacagtgtgatgtggt-3' [-300-construct] and 5'-gtatttcttggccttgaagagtac-3' [-100-construct].

A PCR product (5'-gggaggatggcggagtcttcggata-3' (sense primer) and 5'-ttaccacaggagggtcttaaaattg-3' (antisense primer)) comprising the complete open reading frame of human PARP-1 was subcloned into the expression vector pcDNA3.1-V5-His-TOPO (Invitrogen, Karlsruhe, Germany).

All constructs were confirmed by sequencing.

2.4 Quantitative RT-PCR

First-strand cDNA was synthesized by MLV-RT (Promega) using random hexamer primers (Promega). cDNA was quantified by real-time PCR using Power Mix

(Applied Biosystems, Darmstadt, Germany). Data analysis was performed according to the $\Delta\Delta C_T$ method. Following primer pairs were used for real-time PCR:

AT2R (human, 107 bp): 5'-aacattaccagcggtcttcac-3' (sense),

5'-ggaattgcatctaaatgcttatctgatgg-3' (antisense);

AT2R (rat, 80 bp): 5'-aatccctggcaagcatcttatgt-3' (sense),

5'-cggaaataaaatgttggaatg-3' (antisense);

AT2R (mouse, 100 bp): 5'-aggagagctcggaactgaaag-3' (sense),

5'-ttgccagtgttaagcagttatc-3' (antisense);

PARP-1 (mouse, 151 bp): 5'-gcagcgagagtattccaag-3' (sense),

5'-aagccatccacctaacaac-3' (antisense);

ATIP1 (human, 86 bp): 5'-tgcttcgaaaccttcgactt-3' (sense),

5'-gaggattctttgcctgctc-3' (antisense);

ATBP50 (rat, 115 bp): 5'-tgggctcaggaaaaacactg-3' (sense),

5'-cctttcggtggacagcacat-3' (antisense);

ATBP50 (mouse, 107 bp): 5'-ggctcaggaaaaacactgtca-3' (sense),

5'-ggaggacagcacatctggag-3' (antisense);

18s rRNA (human, rat, mouse, 108 bp): 5'-ccgcagctaggaataatggaata-3' (sense),

5'-tctagcggcgcaatacgaat-3' (antisense);

beta-actin (mouse, 144 bp): 5'-gacaggatgcagaaggagattactg-3' (sense),

5'-gctgatccacatctgctggaa-3' (antisense).

The identities of the AT2R-specific transcripts were confirmed by sequencing in KELLY cells, MEFs of PARP-1 knockout mice as well as cardiac tissue of PARP-1 +/+ and PARP-1 -/- animals.

2.5 Small interfering RNA (siRNA)

siRNA directed against human PARP-1 (5'-gauuucaucuggugugauuu-3' (sense), 5'- uaucacaccagaugaaaucuu-3' (antisense); Dharmacon/ Thermo Fisher Scientific, Lafayette, Colorado, USA) and a siRNA negative control (sc-37007, Santa Cruz Biotechnology, Heidelberg, Germany) were transfected using HiPerFect (Qiagen, Hilden, Germany). Efficiency of transcript suppression (to 1.5% of control) was evaluated by real-time PCR.

2.6 RNA ligase-mediated (RLM)- 5'-RACE

Transcriptional start sites of human AT2R and ATIP1 genes were determined using the GeneRacer kit (Invitrogen, Karlsruhe, Germany) which only amplifies capped mRNA. Cardiac mRNA (left ventricle) was a generous gift from H. Witt (CCR, Berlin). Reverse transcriptions were performed with random hexamer primers followed by nested PCRs using indicated primers: 5'-cacagcgagggtgaagatgt-3' (AT2R; antisense; first round PCR), 5'-gtgaagaccgctggtaatgt-3' (AT2R; antisense; second round PCR), 5'-gagctgcaggataaatccac-3' (ATIP1; antisense; first round PCR) and 5'-acagtgtctctcctaaaccc-3' (ATIP1; antisense; second round PCR). Reaction products were subcloned and sequenced. To determine transcriptional start sites on the pGL3-basic reporter vector we used following primers: 5'-ctcgatatgtgcatctgtaaaagca-3' and 5'-cttcatagccttatgcagttgctc-3' (nested).

2.7 Promoter luciferase assays

Promoter reporter assays were performed as described previously [28,29] using the Dual-Luciferase Reporter Assay System (Promega). Cotransfection of the phRL-null plasmid (encoding humanized renilla luciferase; Promega) served for standardisation. Relative luciferase activity (RLA) is defined as the mean value of

the firefly-luciferase/ renilla-luciferase ratios of each construct related to the insertless reporter plasmid pGL3-basic [28]. RLA data represent the mean \pm standard deviation of at least four single, parallel transfection experiments.

2.8 Chromatin-immunoprecipitation (ChIP)

EA.hy 926 cells were treated with 10 mM 3-AB and respective control for 24 hours without starving. Fixation at a confluence of about 90% was performed using 1% formaldehyde (Merck, Darmstadt, Germany) in 1x PBS (Invitrogen) for 7 min at 37°C. Cells were then rinsed twice with ice-cold 1x PBS, scraped of in 1x PBS and centrifuged for 5 min at 440 x g. Each pellet was resuspended in 2.5 ml lysis buffer (1% SDS, 50 mM Tris-HCl, 1x Complete protease inhibitor cocktail (Roche, Mannheim, Germany), 5 mM EDTA (final concentration); pH 8.1), followed by a 20 min incubation on ice. Sonification, immunoprecipitation and reversal of crosslink were performed according to Bryant and Ptashne [30] using 3 μ g antibody directed against the following antigens: TBP (sc-204, Santa Cruz Biotechnology) and RNA polymerase II (sc-5943, Santa Cruz Biotechnology).

Sonification itself was performed using the Sonoplus HD 2070/ UW 2070 sonifier with the tip MS 72 (Bandelin Electronic, Berlin, Germany; output control (power %)= 100; time= 20 sec (once); constant duty cycle).

Quantitative PCR was performed as described above applying the following primers:

Human ATIP1 promoter around the transcriptional start site: 5'-aggatctgtgacattattggtt-3' (sense), 5'-aaaatgatgctcttctctgaa-3' (antisense) and human beta-actin promoter: 5'-aatgctgcactgtgcggcga-3' (sense), 5'-ggcggatcggcaaaggcga-3' (antisense).

Input (total) and genomic DNA served as positive controls.

Calculation of transcription factor (TF) recruitment X (e.g. to human ATIP promoter) standardised to the recruitment of TBP to the TATA box region of the human beta-actin promoter was calculated according to

$$X = \frac{2^{-[C_T[IP(TF), PCR(ATIP)] - C_T[input, PCR(ATIP)]]}{2^{-[C_T[IP(TBP), PCR(\beta\text{-actin})] - C_T[input, PCR(\beta\text{-actin})]}}$$

X was calculated separately for the incubation condition (i.e., 3-AB) [X(i)] and vehicle control [X(c)]. X(i) divided by X(c) served as a measure for relative PARP-1 recruitment. Indicated standard deviation is based on three on three real-time PCR measurements on each template.

For combined ChIP and siRNA experiments, KELLY cells were transfected with siRNA against PARP-1 (#J-006656-05, Dharmacon, Chicago, USA) or a negative control siRNA (sc-37007, Santa Cruz) using Dharmafect (Dharmacon). 27 hours after transfection cells were fixed using 1% formaldehyde for 3 min at 37°C. Sonification was performed in five steps [1x 20 sec, output control= 100, duty cycle= 5; 3x 20 sec, output control= 30, duty cycle= constant; 1x 10 sec, output control= 100, duty cycle= 5] using the Sonoplus HD 2070/ UW 2070 sonifier with the MS 72 tip. After sonification, samples were diluted 1: 50 in dilution buffer (0.01% SDS, 4 mM EDTA, 50 mM Tris-HCl, pH= 8.1; 1x Complete protease inhibitor cocktail (Roche)). Protein A-coated paramagnetic beads (Diagenode, Liege, Belgium) and following antibodies were used for immunoprecipitation (4°C, overnight): Normal rabbit IgG (3 µg; #2729S, Cell Signaling, Danvers, USA), PARP-1 (3 µg; sc-8007, Santa Cruz Biotechnology) and RNA polymerase II (3 µg; sc-899, Santa Cruz Biotechnology). Real-time PCR measurements were performed in technical duplicate using following primers: Human AT2R promoter around the transcriptional start site: 5'-agctggcaagggttcataag (sense), 5'-

caggctgcagaatgctttga-3' (antisense); human AT2R upstream promoter region: 5'-ctaggcatgatttaggcact-3' (sense), 5'-caaaccagctctaccactt-3' (antisense); beta-actin (see above). Relative recruitment indicates the standardisation to the recruitment of RNA polymerase II to the TATA box region of the human beta-actin promoter.

2.9 Immunofluorescence staining

KELLY and MEF cells were cultured in 24-well plates on coverslips to a confluence of 70%. Afterwards, KELLY cells were stimulated with the PARP inhibitor 3-AB (30mM) or the vehicle DMSO for 10 hours. KELLY and MEF cells were washed with PBS and fixed on the coverslips with ice-cold methanol (-20°C, 10 min). Coverslips were blocked with donkey serum (10% in PBS, 1 hour, room temperature; PAN Biotech GmbH, Aidenbach, Germany). Blocking buffer was removed and coverslips were incubated with rabbit polyclonal AT2R antibody (1:100 in 5% donkey serum in PBS, 4°C, overnight; sc-9040, Santa Cruz Biotechnology). After incubation, primary antibody was rinsed from the coverslips three times with PBS. Cy3-conjugated anti-rabbit IgG (1:150 in 5% donkey serum in PBS; Chemicon, Schwalbach, Germany) was incubated for 1 hour at room temperature. The secondary antibody was rinsed three times with PBS and the coverslips were treated with DAPI nucleic acid stain (50 ng/ml, 15 min, room temperature; Molecular probes, Karlsruhe, Germany). After washing for three times with PBS, coverslips were mounted on slides with Mounting medium (Dako Cytomation, Hamburg, Germany) and air dried. A Leica DM IRE2 microscope (Leica Microsystems, Wetzlar, Germany) was used to generate the images.

2.10 Statistical analysis

A two-tailed t-test was applied and statistical significance was assumed at $p < 0.05$. Vertical lines in the histograms indicate standard deviations.

2.11 Gene nomenclature

Since no official consensus publication regarding the terminology exists, the term ATBP is used for our experiments performed in murine cells and tissues whereas the term ATIP refers to the identical gene in the human species.

3. Results

3.1 Basal characterisation of human AT2R and ATIP promoters

To analyse the mechanisms of AT2R and ATIP gene expression we initially determined the respective transcriptional start sites by a modified 5'-RACE protocol which only amplifies capped mRNA. Two transcriptional start sites were identified in the human ATIP1 promoter whereas only one single start site - in appropriate distance to a TATA box - was detected in the human AT2R promoter (Fig. 1A&B). To gain insight into activating and repressing promoter regions we subcloned serial deletion mutants of human AT2R and human ATIP1 promoters into a reporter vector. Transient transfections indicated that the 216 bp region consisting of exon 1 and intron 1, termed [-216], of human AT2R is necessary and sufficient for AT2R promoter activity since the construct [-1100] without this region did not exhibit significant luciferase activity whereas the 216 bp region itself displayed promoter activity (Fig. 2A). A similar effect was observed in human endothelial EA.hy926 cells (not shown). Furthermore, upstream promoter elements (> 316 bp relative to exon 2) do not contribute to human AT2R promoter activity to a major degree (Fig. 2A). To examine whether the 216 bp exon 1-intron 1 region constitutes an independent promoter or an enhancer element - with the latter defined by an orientation independence (31) - we subcloned this region in an inverse orientation into the reporter vector *quasi* abolishing promoter activity (Fig. 2B). This promoter property of the exon 1-intron 1 region of the AT2R gene implies an additional transcriptional start site. We therefore performed a further 5'-RACE experiment after transfection of the [-216] promoter construct using antisense primers complementary to the luciferase gene of the reporter plasmid in order to

increase the sensitivity. Sequencing of the RACE PCR product revealed the expected splicing of intron 1 (Fig. 1C). In addition, we observed a transcriptional start site about 60 bp upstream of the 5' end of exon 1 (Fig. 1C). This indicates that the 216 bp region consisting of exon 1 and intron 1 of the AT2R gene is capable of tethering the basal transcriptional machinery.

Concerning the serial deletion mutants of the human ATIP1 promoter, the construct comprising 300 bp directly upstream of the translational start site (i.e., the [-300] construct) showed significant promoter activity (Fig. 2C). Extension to 500 bp further increased luciferase activity (Fig. 2C). These observations are consistent with the location of the two transcriptional start sites that reside in the [-300] and [-500] construct, respectively. Maximum promoter activity was measured using the [-700] construct of human ATIP1 promoter in epithelial MCF7 (Fig. 2C) as well as in endothelial EA.hy926 cells (Fig. 4).

3.2 PARP-1 represses the AT2R gene

To examine whether PARP-1 regulates the AT2R gene as a downstream target, human neuronal KELLY cells were incubated with the PARP inhibitor 3-AB. These cells were chosen because of their basal AT2R mRNA expression in contrast to 9 different human endothelial (EA.hy926), epithelial (HeLa-S3, HEK293, MCF7), glial (T98G, U-87MG, U-373MG) and neuronal (SK-N-AS, SH-SY5Y) cell lines (not shown). Pharmacological inhibition of PARP caused an increase in AT2R mRNA to over 150% compared to the vehicle control (Fig. 3A). To validate these findings, AT2R mRNA expression was quantified in embryonic fibroblasts derived from homozygous PARP-1 knockout mice. In wild type control cells AT2R mRNA was

almost undetectable (as assayed by a high C_T value and faint bands in the corresponding agarose gel (not shown)). In contrast, AT2R expression was increased about 3-fold in PARP-1 deficient cells (Fig. 3B).

To analyse corresponding regulatory mechanisms on promoter level, serial deletion mutants of the human AT2R promoter were transfected in the presence or absence of the PARP inhibitor 3-AB. PARP inhibition caused a significant derepression of all constructs containing intron 1 but not of the [-1100] construct without this region (Fig. 3C). Consistently, an increase in luciferase reporter activity of all promoter constructs containing the intron 1 region was also observed in PARP-1 knockout cells (Fig. 3D).

Finally, the complete coding sequence of human PARP-1 was subcloned into an expression vector and cotransfected into neuronal KELLY cells (Fig. 3E). PARP-1 overexpression decreased the promoter activities of three different constructs of the AT2R regulatory region containing intron 1 but not of the [-1100] reporter plasmid without this sequence. The 216 bp exon 1-intron 1 region alone was repressed by PARP-1 cotransfection to a similar degree (i.e., to about 45%) compared to the full length construct (Fig. 3E). The exon 1-intron 1 region cloned in an inverse orientation was not modulated by PARP-1 cotransfection (data not shown).

To analyse the regulation of AT2R by PARP on the protein level, KELLY cells were incubated with 3-AB and AT2R expression was visualised by immunocytochemistry (Fig. 3F). PARP inhibition caused a clear increase of the AT2R in the plasma membrane region. Consistently, PARP-1 ablation caused a general upregulation of the AT2R protein expression in MEF cells (Fig. 3F).

The direct involvement of PARP-1, i.e., the binding of PARP-1 to the upstream and downstream promoter region of the AT2R genes, was demonstrated in neuronal

cells by chromatin-immunoprecipitation (ChIP) (Fig. 3G). The specificity of these findings was confirmed by siRNA directed against PARP-1 and by a negative antibody control (Fig. 3G).

3.3 PARP-1 activates the ATIP1/ ATBP50 gene

To study putative effects of PARP-1 on ATIP1 gene expression, endothelial cells were incubated with the PARP inhibitor 3-AB causing a downregulation of ATIP1 mRNA to 24.3% compared to vehicle control (Fig. 4A). This finding was further confirmed in cells derived from PARP-1 knockout mice (Fig. 4B). PARP deficiency decreased ATBP50 mRNA to about 73.6% compared to wild type control cells. Additionally, we quantified ATIP1 mRNA expression after PARP-1 knockdown by siRNA in human endothelial cells and observed a strongly reduced ATIP1 expression (about 20% compared to the control transfection) (Fig. 4F).

To further analyse whether this regulation occurs on the transcriptional level, the serial deletion mutants of human ATIP1 promoter were transfected into EA.hy926 cells in the presence or absence of the PARP inhibitor 3-AB. Consistently with the real-time PCR data, PARP inhibition caused a repression of all promoter luciferase reporter constructs tested (Fig. 4C). Moreover, these serial deletion mutants were transfected in cells derived from PARP-1 knockout mice and respective controls and, similar to 3-AB, a suppression of ATIP1 promoter activities by PARP-1 ablation could be observed (Fig. 4D). *Vice versa*, recombinant overexpression of human PARP-1 caused an increase in promoter activities of these deletion mutants (Fig. 4E).

Finally, we performed a quantitative ChIP to analyse the promoter regulation within the endogenous chromatin context. Consistent with the data on mRNA level,

pharmacological PARP inhibition decreased the recruitment of TBP and especially of RNA polymerase II (Fig. 4G).

3.4 Downstream effects of an AT2 receptor activation

To address whether AT2 receptor activation itself affects PARP-1 expression, i.e. whether there is a reciprocal interaction between AT2R and PARP-1, we performed stimulation experiments using angiotensin II and the novel AT2R agonist compound 21 (32). Real-time PCR analyses indicated that PARP-1 mRNA remains unchanged after AT2R activation (Fig. 5A). Consistently, quantification of PARP-1 mRNA in cardiac tissue and brain of AT2R knockout mice and respective controls by real-time PCR yielded similar expression levels, indicating that PARP-1 itself is not a downstream target of the AT2R (Fig. 5B).

In the context of downstream effects of the AT2R it was also analysed whether there is a feedback of the AT2 receptor on its own promoter. Since AT2R mRNA was almost undetectable in MEF cells derived from wild type mice (not shown and Fig. 3B) the stimulation experiments were only evaluated in MEF cells of homozygous PARP-1 knockout mice. AT2R activation caused a modest, but significant increase of both AT2R mRNA expression (Fig. 5C) and promoter activity of the 216 bp regulatory exon 1-intron 1 region (Fig. 5D).

ATBP50 mRNA was not modulated by AT2 receptor activation in PARP $-/-$ and PARP $+/+$ MEF cells (data not shown).

3.5 The regulation of AT2R and ATBP50 genes by PARP-1 in vivo

In order to analyse the downstream regulatory effects of PARP-1 on AT2R and ATBP50 gene *in vivo*, we measured transcript levels of these genes in homozygous PARP-1 knockout mice and respective wild type controls. PARP-1 deficiency decreased AT2R mRNA to 34.1% and 41.7% in cardiac and renal tissues, respectively (Fig. 6A, B). In contrast, in the brain, PARP-1 ablation caused a derepression of AT2R mRNA (Fig. 6C) consistent with the regulation in neuronal cells *in vitro* (Fig. 3). Basal PARP-1 mRNA expression was demonstrated by real-time PCR in heart, kidney and brain of wild type, but - as expected - not of PARP-1 $-/-$ mice assuring that PARP ablation is responsible for the observed expression differences of the AT2R (data not shown) .

Regarding ATBP50 expression we did not observe expression differences between PARP-1 knockout and wild type mice in heart, kidney and brain by real-time PCR (data not shown).

4. Discussion

The AT₂ receptor and PARP are major players within human cardiovascular pathophysiology. In this work we deciphered a novel regulatory network in which PARP-1 constitutes a nodal point based on the findings that this nuclear factor acts as an upstream regulator of AT₂R and ATIP1 genes (Fig. 7). Furthermore, AT₂R activation causes a PARP-independent induction of AT₂R promoter activity thereby creating a positive feedback loop (Fig. 7, left dotted line), in line with the literature [6].

Additionally, a regulatory interplay between the transcriptional and posttranslational levels is present since PARP-1 regulates the transcription of the AT₂R and also the level of ATIP/ ATBP, the direct protein-protein interaction partner of the AT₂R [17,18] (Fig. 7, right dotted line).

The reciprocal regulation of AT₂R and ATIP1 genes by PARP-1 *in vitro* - i.e., activation in the case of ATIP1 and repression in the case of AT₂R - was shown for the promoter and mRNA levels as well as for the chromatin context regarding the ATIP gene. In this context it is important to note that the repressive effects of PARP-1 on the AT₂R gene observed in neuronal cells *in vitro* are consistent with the regulation in brain tissue *in vivo*. On the protein level, an upregulation of the AT₂R by pharmacological PARP inhibition or genetic PARP-1 ablation was observed by immunocytology (Fig. 3F). Nevertheless, tissue-specific mechanisms and/ or differences between the *in vitro* and *in vivo* gene regulation appear likely because PARP-1 ablation causes a decreased AT₂R mRNA expression in heart and renal tissue *in vivo*.

Our experiments, consistent with a previous publication [33], also demonstrated the important regulatory function of the exon 1-intron 1 region of the AT₂R gene

consisting of 216 bp. In human MCF7 and KELLY cells, this exon1-intron 1 region is necessary and sufficient for AT2R promoter activity, whereas upstream promoter elements (> 316 bp relative to exon 2) do not contribute to promoter activity to a major degree (Fig. 2&3). Inverse cloning experiments (Fig. 2) revealed that this 216 bp region of the AT2R is a promoter but not an enhancer element implicating (a) further start site(s) in this region. This hypothesis was further confirmed by 5'-RACE (Fig. 1) which indicated that the exon 1-intron 1 region is capable of tethering the basal transcriptional machinery. The binding of PARP-1 to the AT2R promoter was demonstrated by ChIP (Fig. 3G), indicating that PARP-1 is directly involved in the regulation of the AT2R gene.

The AT2R is technically "hazardous" because of its very low expression levels in adult tissues [1,34]. This observation is also reflected on the promoter level based on low relative luciferase activities compared to other genes [29,35] and also extends to the analysis of transcriptional start sites since a substantial number of 5'-RACE clones had to be sequenced (data not shown). In this context, our result of a single transcriptional start site within the TATA box positive AT2R promoter is consistent with the promoter architecture of a tissue-specific gene [36] in contrast to strong housekeeping genes associated with multiple start sites and the absence of a TATA motif [29,35]. It is noteworthy that two transcriptional start sites *upstream* the TATA box were identified in human uterus RNA by primer extension analysis [33] (Fig. 1A) which might reflect tissue specific differences and could affect mRNA stability. Furthermore, processed, expressed pseudogenes of the AT2R seem to exist competing with the canonical transcript for detection (unpublished data). Consistently, it is important to note that publications analysing the AT2R may be biased by overexpression experiments to circumvent the low expression [37]. Since the AT2R expression is high in fetal tissues [38], a

repressor of the AT2R gene has to be postulated. Thus, our present finding that the AT2R can be strongly repressed by PARP-1 is of physiological and putative therapeutic relevance. As discussed above, the role of the AT2R still remains controversial, but assuming that AT2R activation is beneficial to the nervous [7,8,39] and cardiovascular [40] system, an activation of this receptor could be a worthwhile therapeutic task. This can be achieved by direct receptor stimulation with small molecule agonists such as the novel compound 21 on the protein level [32]. Nevertheless, our data indicate that indirect activation of the AT2R protein by intervention on the transcriptional level using PARP inhibitors is a further exciting approach, at least in the nervous system, whereas the qualitative and quantitative contribution of AT2Rs to the beneficial cardiac effects of PARP inhibitors remains elusive. The constitutive receptor activation of the AT2R [41] also favours the latter. In this context it is of interest that pharmacological PARP inhibitors have successfully passed preclinical development regarding e.g. stroke, neuronal injury, myocardial infarction and heart failure, and have already entered human clinical trials for the indications myocardial infarction and different neoplasms [24,42].

Two parameters are crucial to qualitatively and quantitatively assess the contribution of AT2Rs to the effects of PARP inhibitors. First, it must be known - as discussed above - if the AT2R *per se* exerts beneficial or detrimental functions in the respective biological context. Second, the number of target genes downstream of PARP-1 and their respective functions are important. PARP-1 affects transcription in the absence of DNA damage due to several different mechanisms ranging from direct sequence-specific DNA binding, via recruitment by transcription factors, to poly(ADP-ribosyl)ation of other transcription factors and histones [22,23]. Besides AT2R and ATBP50 identified in this study, it was shown that the gene encoding alpha-synuclein can be directly regulated by PARP-1 [44].

A microarray analysis indicated the direct or indirect effect of PARP-1 on the expression of about 90 genes in fibroblasts [43]. PARP's enzymatic activity is required for its transcriptional regulatory functions in some contexts but not in others [22,44,45]. Our data illustrate that inhibition of the enzymatic activity of PARP using 3-AB and PARP-1 ablation/ siRNA exert similar effects, indicating that PARP's enzymatic activity is involved in the regulation of AT2R and ATBP50 genes.

In conclusion, our data reveal that PARP-1 is an upstream transcriptional regulator of the AT2 receptor *in vitro* and *in vivo*. Pharmacological interference within this AT2R-PARP-AT2R network by approved (e.g. angiotensin AT1 receptor blockers), experimental (e.g. PARP inhibitors and AT2R agonists) or future (e.g. decoy oligonucleotides) drugs - and their respective combinations - represents a current or putative therapeutic approach in cardiovascular and neuronal disease.

Acknowledgements

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Figure legends

Fig. 1. Analysis of the transcriptional start sites of human AT2R and human ATIP1 genes. A RNA ligase-mediated 5'-RACE was performed on human adult heart mRNA using primers specific for AT2R (A) and on mRNA from SH-SY5Y cells in the case of human ATIP1 (B), respectively. Resulting PCR products were subcloned for sequencing. (A) Yellow: exon 1 of human AT2R; blue: exon 2; green: transcriptional start site; bold and underlined: TATA box. The sequence shown corresponds to GenBank accession no. GI:51477555. The two start sites identified by Warnecke, C. et al. (1999) are indicated by grey rectangles. (B) Yellow: first ATIP1-specific exon; green: transcriptional start sites; bold, underlined

and capital: translational start site. The sequence shown corresponds to GenBank accession no. GI:51467159.

(C) A 5'-RACE was performed after transfection of a luciferase plasmid containing the [-216] AT2R regulatory region into EA.hy926 cells. The resulting PCR product was subcloned and sequenced. Sequencing results are depicted. Anchor: sequence of the anchor primer ligated to the mRNA; horizontal grey arrow: nested RACE antisense primer; X: splicing event; vertical black arrow: transcriptional start site; black lines: sequence of the luciferase reporter vector.

Fig. 2. Promoter activities of human AT2R and human ATIP1 genes under basal conditions. Serial deletion mutants of human AT2R (A, B) and human ATIP1 promoters (C) were subcloned into the pGL3-basic firefly luciferase vector and transiently transfected into the human epithelial cell line MCF7. Numbers indicate the length of respective constructs in base pairs. Relative luciferase activity (RLA) indicates promoter activity relative to an insertless pGL3-basic vector and standardised to a cotransfected phRL-null renilla luciferase vector.

Fig. 3. PARP-1 represses the AT2R gene. (A) Serum-cultured neuronal KELLY cells were incubated with the PARP inhibitor 3-AB (30 mM; grey columns) or vehicle (white columns) for 8 hours followed by analysis of AT2R mRNA by real-time PCR.

(B) RNA was extracted from MEF cells derived from wild type and homozygous PARP-1 knockout mice. AT2R mRNA was quantified by real-time PCR. Additionally, the identity of the AT2R PCR products was confirmed by sequencing.

(C) Serial deletion mutants of human AT2R promoter were transfected into KELLY cells cultured in 3-AB (30mM; grey columns) or vehicle (white columns) for 8 hours. Promoter activities of these luciferase reporter constructs are expressed as RLA as described above.

(D) Serial deletion mutants of human AT2R promoter were transfected into MEF cells derived from homozygous PARP-1 knockout mice (grey columns) and wild type MEF control cells (white columns).

(E) KELLY cells were cotransfected with luciferase reporter constructs comprising serial deletion mutants of human AT2R promoter and a PARP-1 expression vector (dark grey columns) or an insertless control vector (white columns), respectively.

(F) KELLY cells were incubated with 3-AB or vehicle control for 10 hours; MEF cells derived from PARP-1 knockout mice and wild type controls were cultured without 3-AB. AT2R expression was analysed by immunofluorescence staining. The "overlay" panel represents the combination of the AT2R staining ("anti-AT2R") and the nuclear staining with DAPI. Incubation without primary antibody ("secondary antibody only") served as negative control. Exposition was identical between intervention (i.e., 3-AB or PARP -/-) and respective control. Numbers ("20x", "63x") indicate the magnification.

(G) KELLY cells were transfected with siRNA against PARP-1 (grey columns) or a negative control siRNA (white columns). ChIP was performed with antibodies against PARP-1. Immunoprecipitation using IgG antibodies served as negative control. Transcription factor recruitment was quantified by real-time PCR using two different primer pairs located around the transcriptional start site ("TSS") or in the upstream region ("upstream") of the human AT2R promoter. The ordinate

represents the recruitment of PARP-1 to the AT2R promoter standardised to the recruitment of RNA polymerase II to the housekeeping promoter beta-actin.

Fig. 4. PARP-1 activates the ATIP1 gene. (A) Serum-cultured endothelial EA.hy926 cells were incubated with the PARP inhibitor 3-AB (10 mM; grey columns) or vehicle (white columns) for 5 days followed by analysis of ATIP1 mRNA by real-time PCR. mRNA expression of the vehicle control was set to 100%.

(B) RNA was extracted from MEF cells derived from wild type (white columns) and homozygous PARP-1 knockout mice (grey columns). ATBP50 mRNA was quantified by real-time PCR. mRNA expression of wild type cells was set to 100%.

(C) Serial deletion mutants of human ATIP1 promoter were transfected into EA.hy926 cells cultured in 3-AB for 24 hours (grey columns) or vehicle (white columns). Harvest was performed 48 h after transfection. Promoter activities of these luciferase reporter constructs are expressed as RLA as described above.

(D) Serial deletion mutants of the human ATIP1 promoter were transfected into MEF cells derived from wild type (white columns) and homozygous PARP-1 knockout mice (grey columns).

(E) EA.hy926 cells were cotransfected with luciferase reporter constructs comprising serial deletion mutants of the human ATIP1 promoter and a PARP-1 expression vector (dark grey columns) or an insertless control vector (white columns), respectively. Harvest was performed 24 h after cotransfection.

(F) EA.hy926 cells were treated with siRNA against human PARP-1, followed by RNA isolation after 24 hours. ATIP1 mRNA was quantified by real-time PCR.

(G) EA.hy926 cells were incubated with 3-AB (grey columns) or vehicle control (white columns) followed by ChIP using antibodies against TATA box-binding protein (TBP) or RNA polymerase II (pol II). Recruitment of these factors - standardised to the recruitment of TBP to the beta-actin promoter - was quantified by genomic real-time PCR using primers located in the core promoter region of human ATIP1.

Fig. 5. Downstream effects of an AT2 receptor activation. (A) MEF cells derived from wild type mice were incubated with the AT2R agonist compound 21 (C21) or angiotensin II (ang) (in the presence or absence of the angiotensin AT1 receptor blocker irbesartan (irb)) and the respective vehicle controls (V). mRNA of PARP-1 was quantified by real-time PCR. The results of 4 independent real-time PCRs, each in technical triplate, are shown; vehicle was set to 1.

(B) PARP-1-specific mRNA was quantified in heart and brain of AT2R knockout mice (grey columns) and respective wild type controls (w.t., white columns) by real-time PCR. Expression in w.t. animals was set to 100%.

(C) MEF cells derived from homozygous PARP-1 knockout mice were incubated with the AT2R agonist compound 21 (C21) or angiotensin II (ang) (in the presence or absence of the angiotensin AT1 receptor blocker irbesartan (irb)) and the respective vehicle controls (V). mRNA of AT2R was quantified by real-time PCR. N: condition without stimulation or vehicle.

(D) MEF cells derived from homozygous PARP-1 knockout were transfected with the indicated constructs of the human AT2R promoter, followed by an incubation with angiotensin II (ang) or compound 21 (C21) and the respective vehicle controls (V). Promoter activities are expressed as relative luciferase activity (RLA).

Fig. 6. Regulation of the AT2R gene in vivo. AT2R-specific mRNA from heart (A), kidney (B) and brain (C) of homozygous PARP-1 knockout mice (PARP-1 ^{-/-}, grey columns) and of respective wild type controls (w.t., white columns) was quantified by real-time PCR. Data represent 6 wild type and 6 knockout animals, each quantified by 3 (heart and kidney) or 9 (brain) C_T values. Expression in w.t. animals was set to 100%.

Fig. 7. Transcriptional regulation of AT2R and ATIP1 genes. PARP-1 acts as a transcriptional regulator of AT2R and ATIP1 gene promoters but is not modulated itself downstream of an AT2R activation. In addition, the AT2R stimulation can induce AT2R promoter activity via a PARP-independent mechanism. The dotted lines indicate that promoter activities are reflected in the respective protein levels and that ATBP is a direct protein-protein interaction partner of the AT2R.

A

ttcataagtcagccctagaattatgtaggttg
aaggtctccagtggaacgaccaaaca**tataag**
aaggaaaccagagatctggtgctatt**acgtcc**
cagcgtctgagagaaacgagtaagcacagaatt
caaagcattctgcagcctgaattttgaaggta
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aagtagaacatacattaaatgaaaatattttt
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agctgctgtttctcttac**aggagtgtgttag**
gcactaagcaagctgatttatgataactgctt
taaacttcaacaacca

AT2R

B

gagaccaccactttctcctccctttaccat
ctcgatgaaaaccataacaactc**cacaagca**
tgc**acagtgtgatgtggttcaga****ggcagcttc**
tagacctgcaggaggagattgtattcagagg
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ATIP1

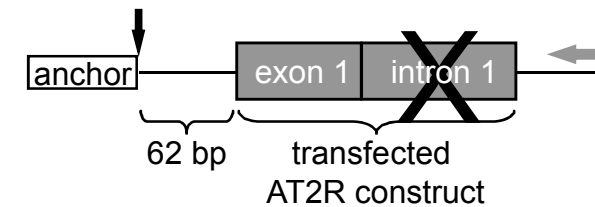
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figure 1

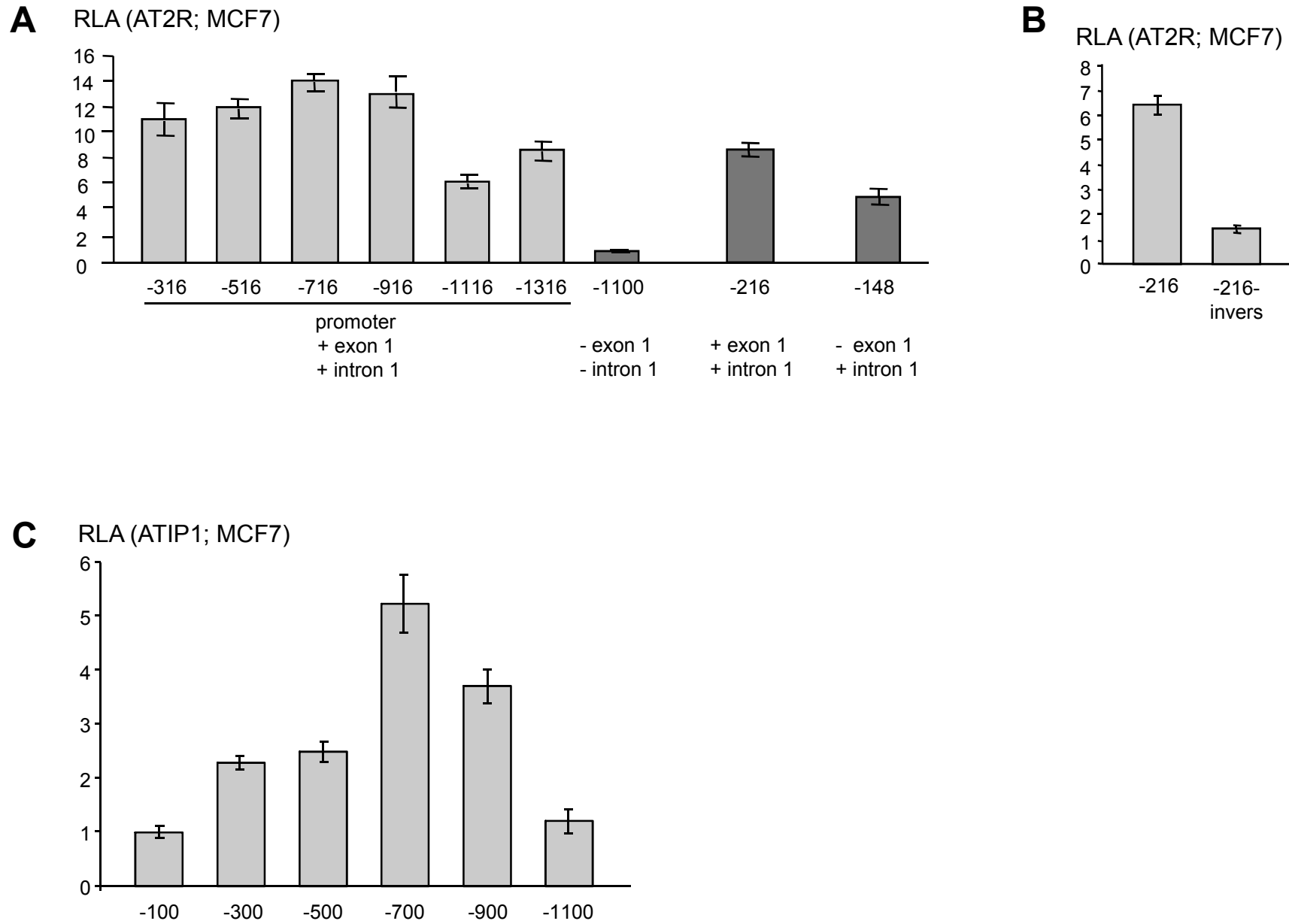


figure 2

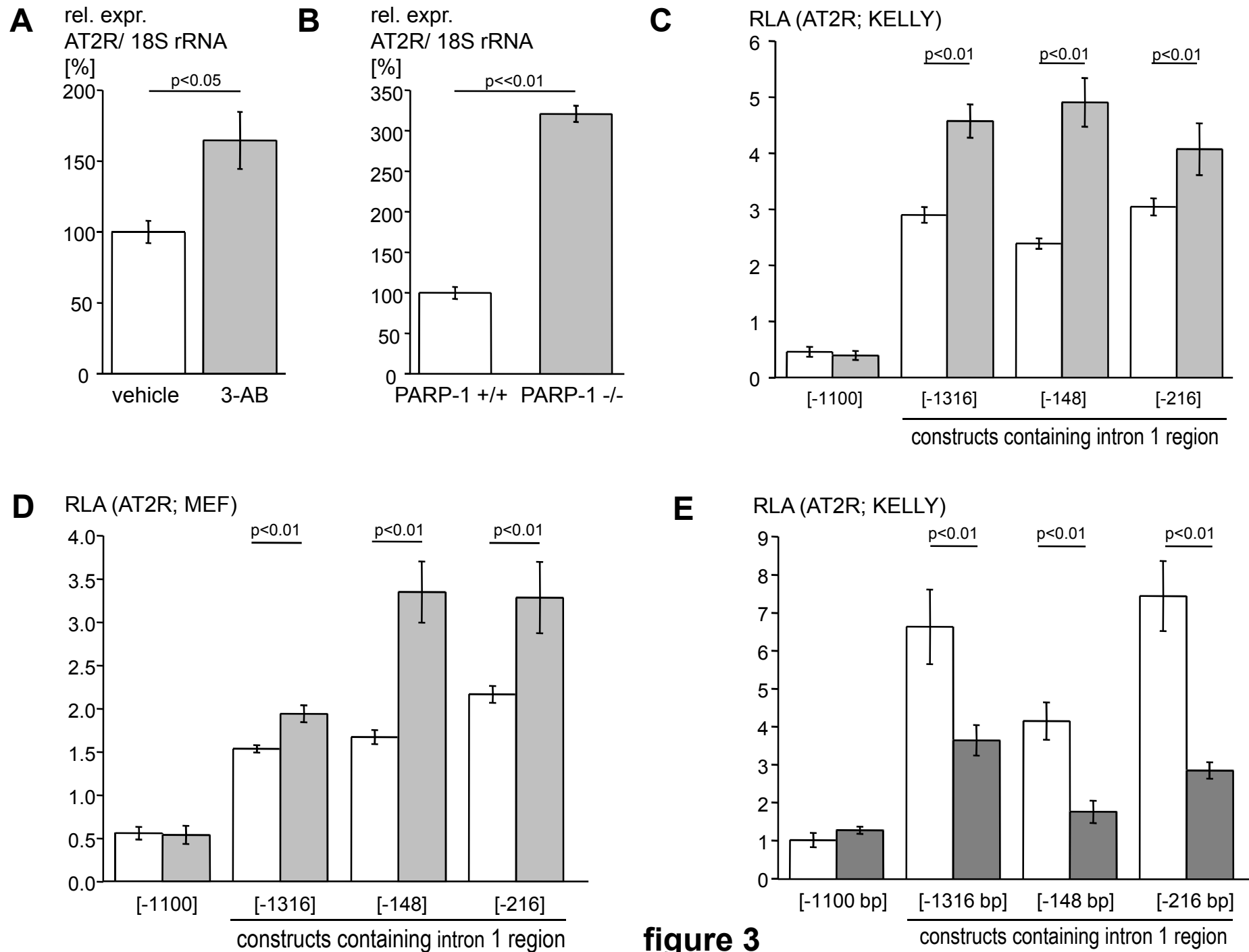
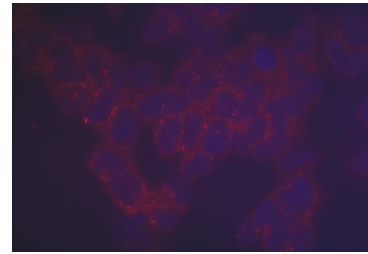
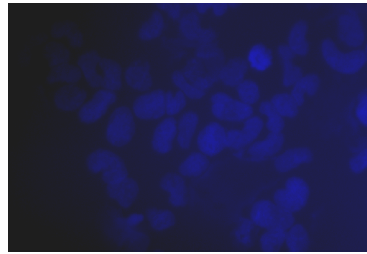
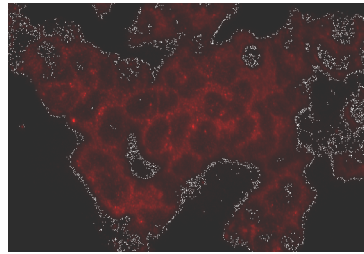


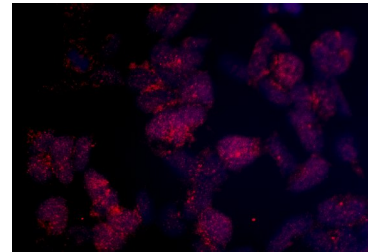
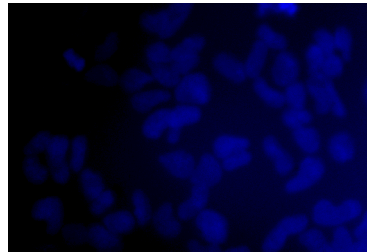
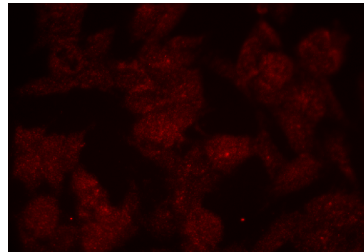
figure 3

F

KELLY (3-AB)



KELLY (vehicle)



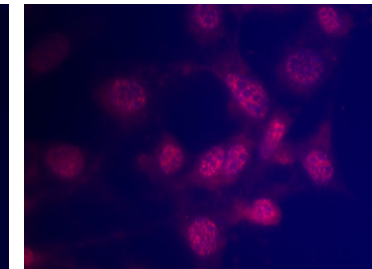
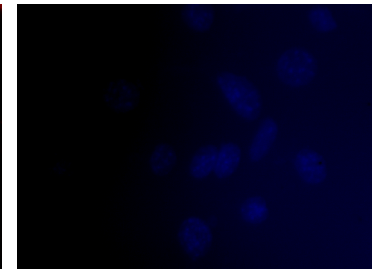
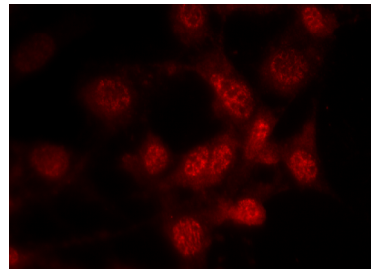
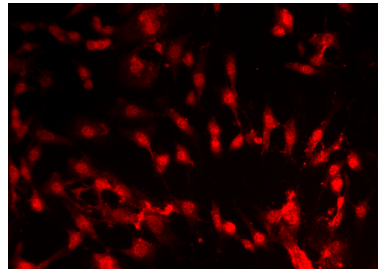
anti-AT2R (63x)

DAPI (63x)

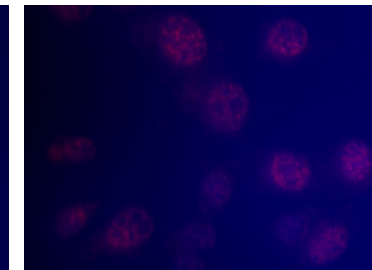
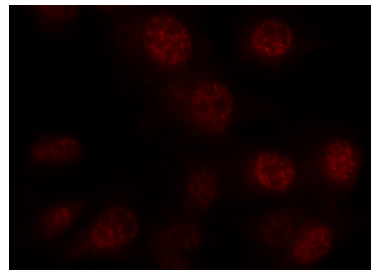
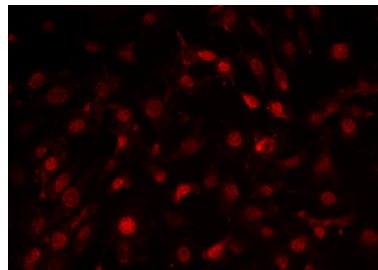
overlay (63x)

secondary antibody only

MEF (PARP-1 -/-)



MEF (PARP-1 +/+)



anti-AT2R (20x)

anti-AT2R (63x)

DAPI (63x)

overlay (63x)

figure 3

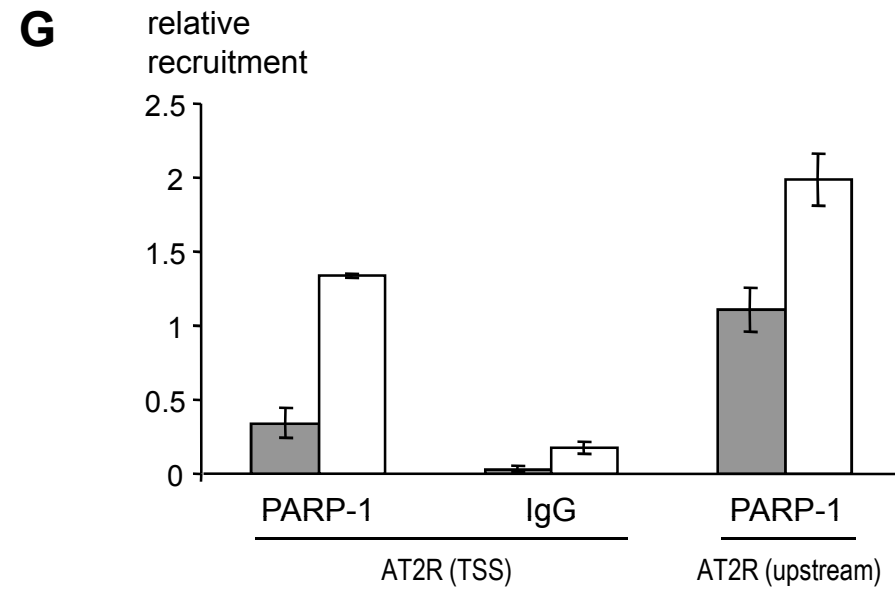


figure 3

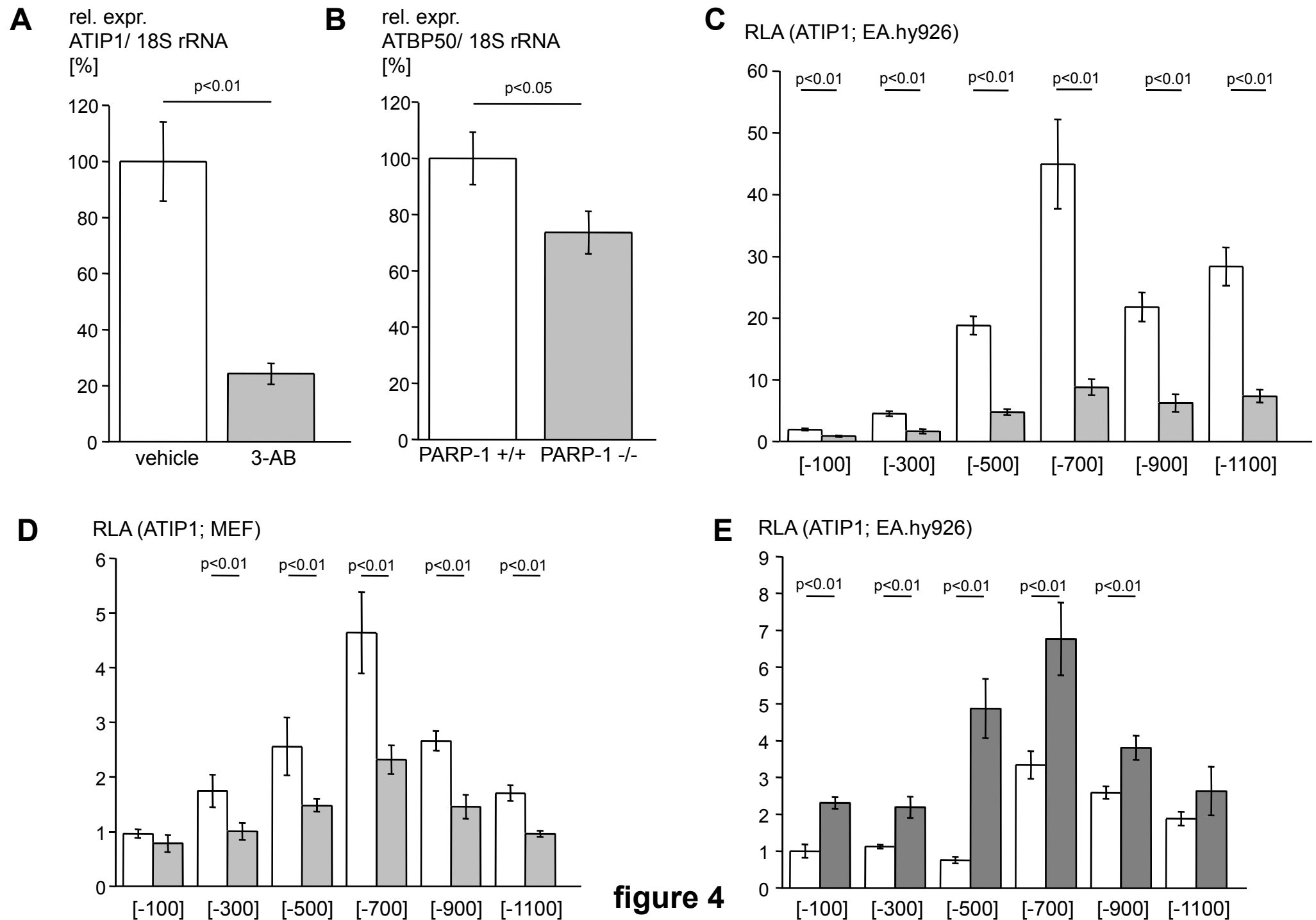
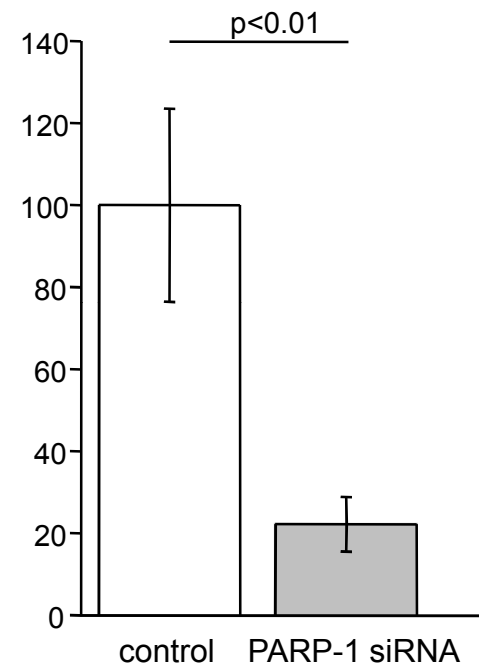


figure 4

F

rel. expr.
ATIP1/ 18S rRNA
[%]



G

relative
recruitment

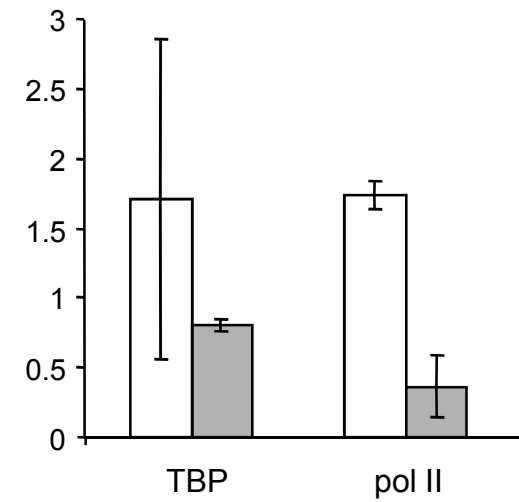
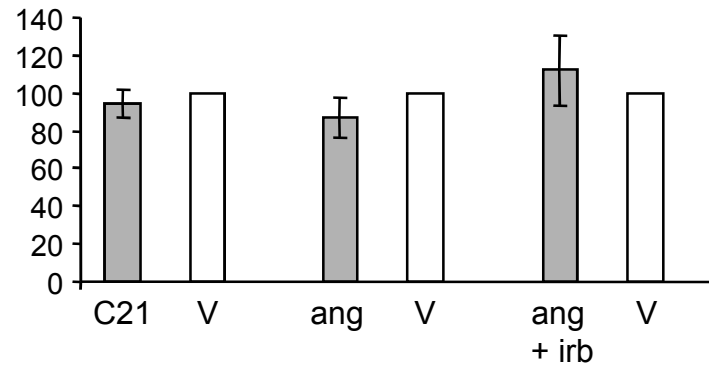


figure 4

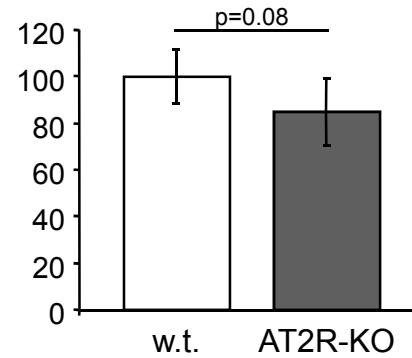
A

rel. expr.
PARP-1/ 18S rRNA
[%]

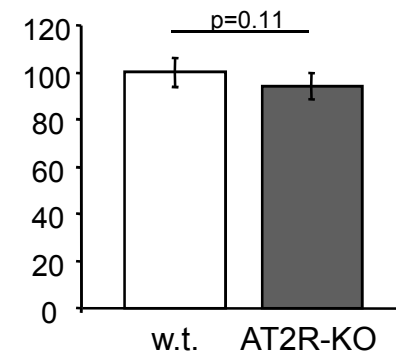


B

rel. expr.
PARP-1/ β -actin rRNA
[%] - heart

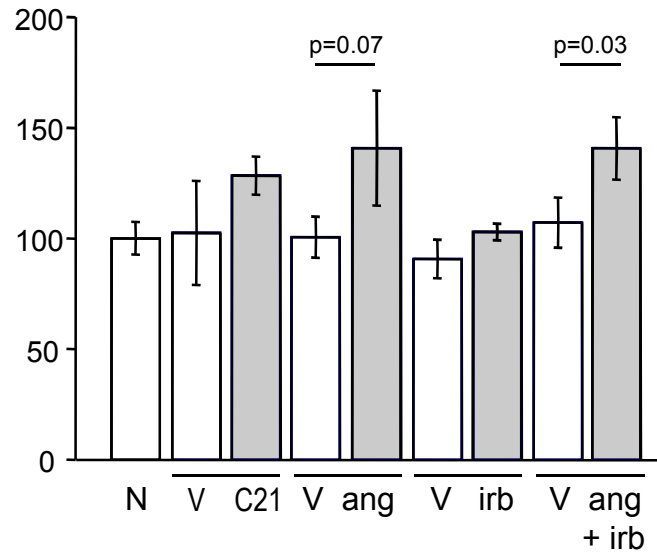


rel. expr.
PARP-1/ β -actin rRNA
[%] - brain



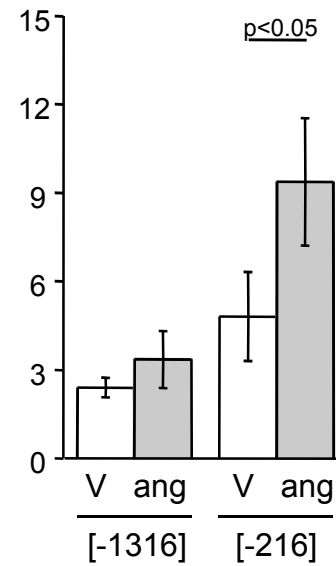
C

rel. expr.
AT2R/ 18S rRNA
[%]



D

RLA (AT2R; PARP -/-)



RLA (AT2R; PARP -/-)

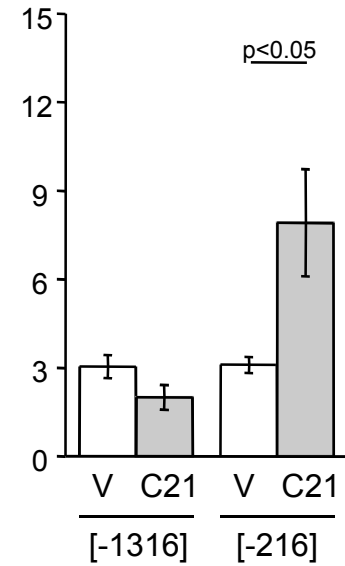


figure 5

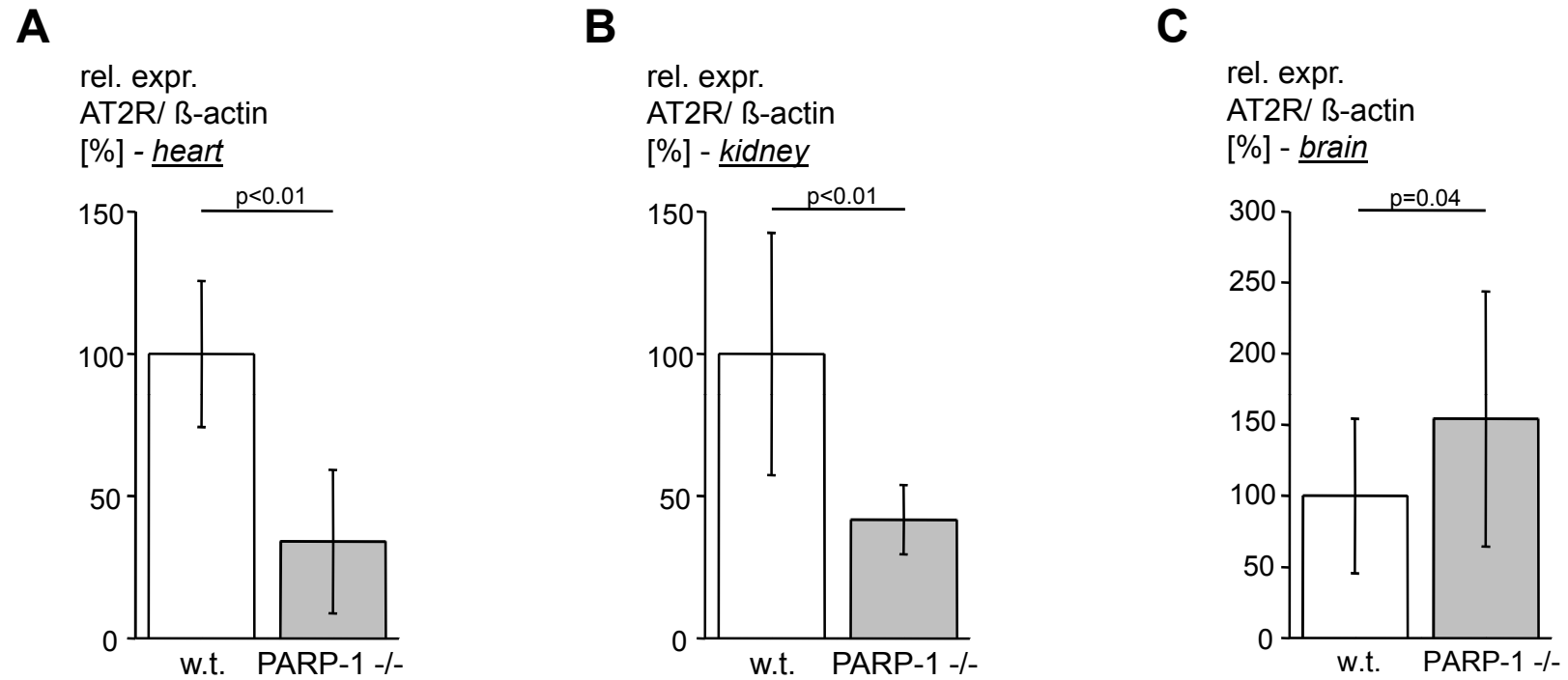


figure 6

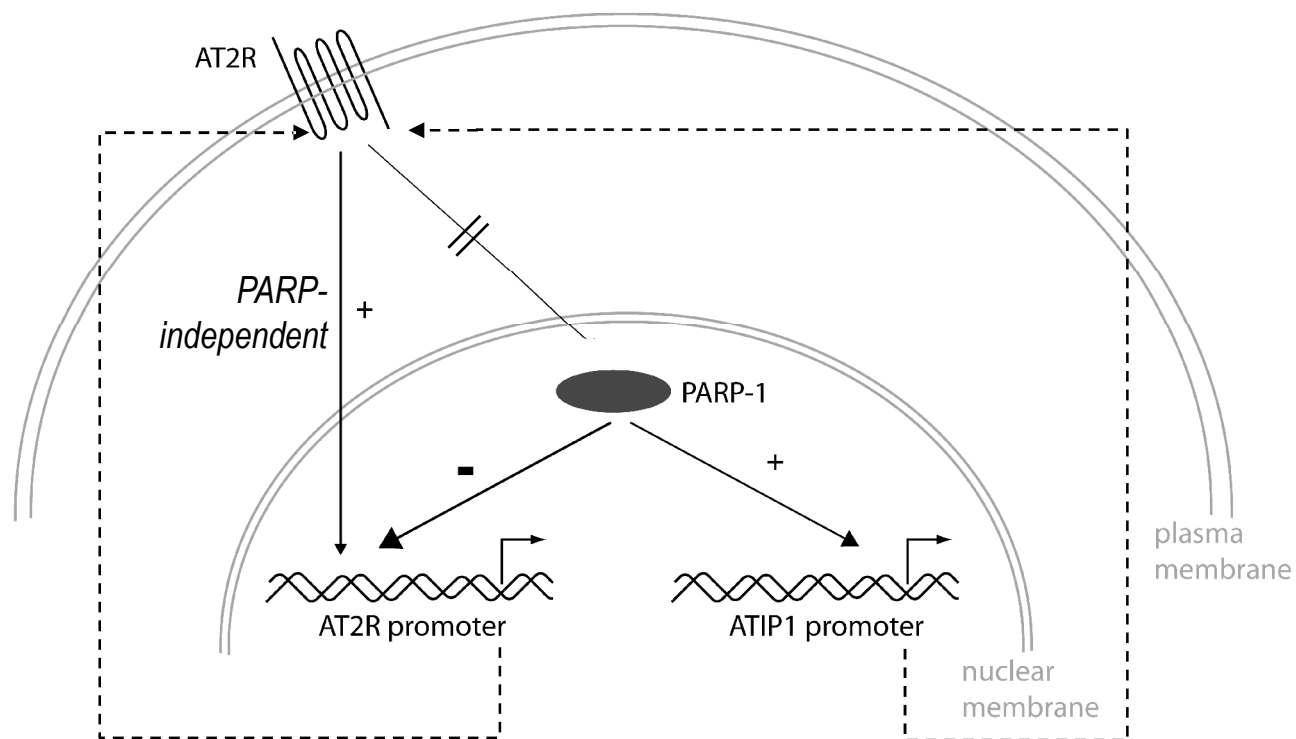


figure 7