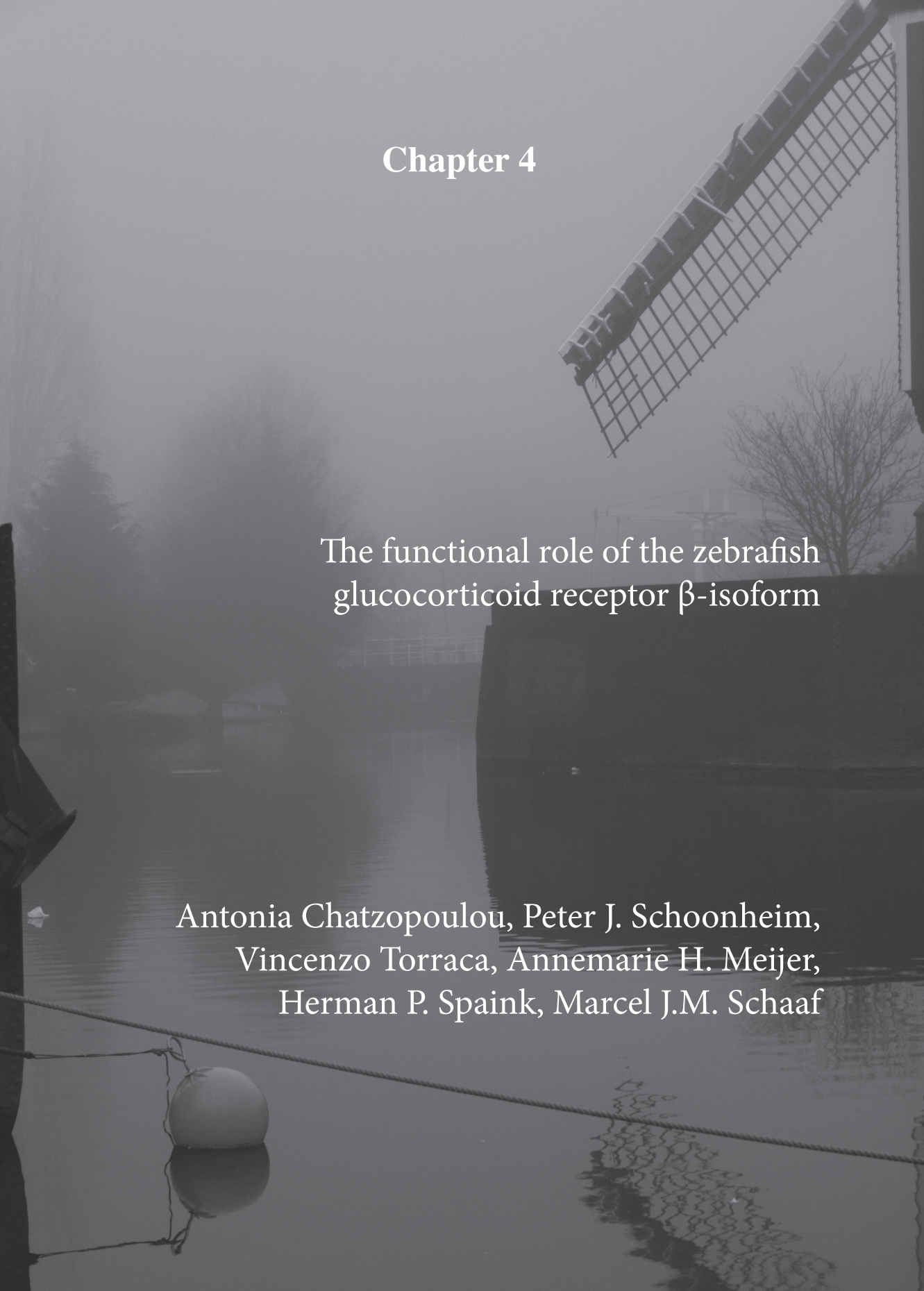


Chapter 4

The functional role of the zebrafish glucocorticoid receptor β-isoform

Antonia Chatzopoulou, Peter J. Schoonheim, Vincenzo Torraca, Annemarie H. Meijer, Herman P. Spaink, Marcel J.M. Schaaf

Abstract

In humans, two splice variants of the glucocorticoid receptor (GR) exist: the canonical human GR α-isoform (hGRα), acting as a ligand-activated transcription factor, and the human GR β-isoform (hGRβ), which has been shown to have a dominant-negative effect on hGRα. Several studies have shown that elevated hGRβ levels are associated with resistance to anti-inflammatory glucocorticoid treatment. In the present study we have used the zebrafish model system in order to elucidate the functional role of the GR β-isoform *in vivo*. Luciferase reporter assays in transiently transfected COS-1 cells demonstrated a dominant-negative effect of the zebrafish GR β splice form (zGRβ) on the transactivation properties of the GRα isoform (zGRα). However, no such effect was observed in zebrafish PAC2 cells using induction of the *fkbp5* gene as readout. In addition, upon zGRβ mRNA injections in 1-2 cell stage zebrafish embryos, no effect was observed on the zGRα-induced transactivation of the *fkbp5*, *pepck* and *nfkbiaα* genes. Subsequently, we generated a transgenic fish line with inducible zGRβ overexpression. Transcriptome analysis by microarray and subsequent qPCR validation using this line did not reveal any regulatory effect of zGRβ (either as a dominant-negative inhibitor of zGRα or as a transcription factor independent of zGRα). Based on these results, we suggest that the zebrafish GR β-isoform does not have a regulatory role in transcription and that splicing of the GR pre-mRNA into a messenger encoding an alternative splice variant could instead represent a physiological mechanism to downregulate the levels of the canonical receptor variant

Introduction

The glucocorticoid receptor (GR) is expressed almost ubiquitously in the human body and regulates a wide range of biological processes such as our metabolism, growth, reproduction, vascular tone, bone formation, immune response and brain function [1-6]. The GR is activated by binding of its cognate ligands (glucocorticoids (GCs)), and upon activation it acts as a transcription factor. In its inactive state, the GR mainly resides within the cytoplasm in a multiprotein complex containing chaperones and immunophilins. Upon glucocorticoid binding, the GR is released from its cytoplasmic complex and translocates to the nucleus, where it orchestrates gene expression via DNA-binding dependent and independent mechanisms. DNA-binding dependent mechanisms involve GR binding to glucocorticoid response elements (GREs), where it recruits transcriptional coregulators and thus, directs (positively or negatively) the transcription rate of target genes. The mode of GR action by which DNA binding leads to upregulation of gene expression is known as *transactivation*. Independent of DNA-binding, the GR can also physically interact with other transcription factors (e.g. NF-κB and AP-1), via which it can either synergistically induce gene expression or repress activation of genes. The latter mode of action is called *transrepression* and comprises one of the classical ways by which GCs exert their anti-inflammatory effects [1, 2, 7-11].

Cloning of the human GR gene revealed the occurrence of two splice variants, named as hGRα and hGRβ, which derive from alternative usage of an acceptor splice site within the last coding exon (exon 9) [12, 13]. The two isoforms are identical between amino acids 1 and 727, thus sharing the same N-terminal domain and DNA binding domain (DBD), and only differ in the C-terminal part of their ligand binding domain

(LBD). The GR α-isoform (777 amino acids) is able to interact with GCs, and represents the canonical receptor. The hGR β-isoform (742 amino acids) has a shorter LBD with a unique C-terminal 15 amino acid sequence, which renders it unable to respond to GCs [12-14].

Almost ten years after the discovery of hGRβ, it was shown (using *in vitro* reporter assays) that this isoform had a pronounced dominant-negative inhibitory effect on hGRα's transactivational properties [15-17]. Moreover, hGRα-mediated transrepression of *in vitro* reporter assays was also reported to be inhibited by hGRβ [17]. These findings were soon coupled to clinical data demonstrating a positive correlation between high expression levels of the β-isoform and GC resistance of patients suffering from immune-related disorders, like asthma [18-23], ulcerative colitis [24-26], leukemia [27-29] and rheumatoid arthritis [30, 31]. Further cell-based research supports an inhibitory role for hGRβ on both hGRα-induced transactivation and transrepression of endogenous genes (MKP-1, myocilin, fibronectin, TNFα and IL6 [23, 32, 33]), as well as on hGRα-mediated regulation of cell death, proliferation and phagocytosis [34-36]. Moreover, recent data support the notion that hGRβ can have its own intrinsic transcriptional activity. Overexpression of zGRβ has been demonstrated to attenuate NF-κB and AP-1 induction of luciferase reporter constructs [37], as well as GATA3-mediated activation of IL5 and IL13 promoters of luciferase genes [38]. Additionally, transcriptome analyses of cultured cells showed that hGRβ can direct gene transcription independently of hGRα activation [39, 40].

Despite several studies indicating a dominant negative function or intrinsic transcriptional activity of the GR β-isoform, its physiological relevance has often been questioned. First, in many studies the dominant-negative role of hGRβ on hGRα's transcriptional properties could not be demonstrated in *in vitro* reporter assays [37, 38, 41-46]. Second, despite the fact that hGRβ is expressed in almost all human tissues, its expression levels are significantly lower compared to hGRα [15, 16, 34, 47-49]. This raises doubts about its *in vitro* dominant negative effect, since in most studies this required transfection of a 10-molar excess of GRβ expression vector compared to hGRα [15-17].

Until recently, functional studies of GRβ were complicated by the fact that expression of this isoform was only found in humans, and that rodents contained a mutation in the splice site required for GRβ expression [50]. It was therefore remarkable that we discovered the occurrence of a GR β-isoform in zebrafish [51]. Like humans, zebrafish contain a single GR gene that contains 9 exons and gives rise to two splice variants, the GR α- and β-isoform. Like their human equivalents, both zebrafish GR α- and β-isoforms share the same N-terminal domain and DBD and only differ at the C-terminus of their LBD. In contrast, the gene organization and splicing events leading to the expression of GRβ are different between humans and zebrafish. Whereas in humans the β-specific sequence is found in exon 9, in the zebrafish it is located in exon 8. Usage of the most 5' splice donor site in this exon results in a shorter version of exon 8 and an mRNA encoding zGRα. Usage of the most 3' splice donor site leads to an extended version of exon 8 that introduces a stop codon within that exon, resulting in an mRNA encoding zGRβ [51]. Thus, the human and zebrafish β-isoforms derive via different splicing mechanisms and there is low homology between their β-specific sequences, but they share the same point of divergence at the protein level [51, 52]. Interestingly, an alternative splice event in exon 8 similar to that leading to the expression of a GR β-isoform in zebrafish was recently discovered in mice [53].

Both the human and zebrafish GR β-isoforms exhibit the same predominantly nuclear localization, and zGRβ also acts as a dominant-negative inhibitor of zGRα-

mediated transactivation in *in vitro* reporter assays [51]. Like its human equivalent, zGRβ is ubiquitously expressed, and at significantly lower levels compared to zGRα [51]. All these data point to convergent evolution of hGRβ and zGRβ, which most likely emerged due to a common biological need for regulation of specific signaling pathways [51].

In the present study we have used the zebrafish as an *in vivo* model to elucidate the biological significance of GRβ. Over the last decade, the zebrafish has emerged as a major contributor to biomedical research for an array of human diseases [54, 55]. It is easily handled and maintained and produces big clutches of embryos, which are fertilized externally and develop rapidly [56]. Moreover, its genome has been sequenced and embryos can easily be subjected to genetic manipulations [56, 57].

The scope of this study was to investigate the role of zGRβ in gene transcription either as a dominant negative inhibitor of zGRα or as a distinct transcription factor independent of zGRα. We have utilized both *in vitro* and *in vivo* approaches. For the *in vitro* studies, we performed luciferase assays in COS-1 cells transiently transfected with zGRβ. Additionally, we used the zebrafish PAC2 cell line, in which we have stably overexpressed zGRβ and studied the transcription rate of several endogenous target genes. For *in vivo* experiments, we have overexpressed zGRβ by means of mRNA injections in embryos, and have generated a transgenic zebrafish line with an inducible zGRβ expression construct. While a clear dominant-negative effect of zGRβ was observed on luciferase assays in COS-1 cells, our data do not reveal any regulatory effect of zGRβ on the expression of endogenous genes. Therefore, our study does not support the proposed functions of the GR β-isoform as a dominant negative inhibitor of zGRα or as a transcription factor independent of zGRα, but rather suggest an alternative function of alternative splicing, such as downregulation of the GRα expression level.

Materials & Methods

Cell cultures

COS-1 cells (African green monkey kidney cells were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). Cells were grown at 37°C and 5% CO_2. PAC2 zebrafish cells were cultured in Leibovitz's medium (Invitrogen), supplemented with 15% fetal bovine serum (Invitrogen) and 1% penicillin and streptomycin (Invitrogen). Cells were grown at 28°C.

Luciferase reporter assays in COS-1 cells

COS-1 cells were seeded into 24-well plates ($3x10^4$ cells/well). Transfection was performed 24h later using the *Trans*IT®-COS Transfection Kit (Mirus Bio) according to the manufacturer's instructions. For the experiments in which the transactivation activity of zGRα was assayed, 200ng MMTV-luciferase reporter construct was transfected, with a range of PCS+zGRα plasmid concentrations (1-300ng) and/or 100ng pCS2+zGRβ expression vector (previously described in [51]), together with 2ng CMV-renilla (Promega). For the experiments in which the transrepression activity was assayed, cells were transfected with 50ng of a κB-luciferase reporter construct (containing 5 κB response elements (Stratagene)) and 50ng of a human p65 expression vector (pCMV4-p65, described in [58]), together with a range of pCS2+zGRα concentrations (0-1000ng) in the presence and absence of 100ng pCS2+zGRβ. The total amount of transfected DNA was

always kept equal among groups by transfecting empty pCS2+ vector. Twenty-four hours after transfection, cells were treated with 100nM dexamethasone (Sigma) and 24h later, they were assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega). Bioluminescence was detected using a Wallac 1450 MicroBeta Luminometer. For each sample, the luciferase activity was normalized to the renilla activity. Per sample, measurements were performed in duplicate, and data shown are averages ± s.e.m. of three individual experiments.

Generation of the PAC2-zGRβ cell line

For the generation of a stable cell line overexpressing zGRβ, the pCS2+zGRβ plasmid [51] and a neomycine resistance plasmid were transfected into PAC2 zebrafish cells using the Amaxa® Cell line Nucleofector kit V and the Nucleofector™ II device set at T27 programme (Lonza). Four days after transfection, cells were subjected to selection for antibiotic resistance by supplementing their culture medium with 500µg/ml of geneticin (G418, Invitrogen). Resistant cells were propagated to establish the PAC2-zGRβ cell line.

Glucocorticoid treatment of PAC2 cells for gene expression analysis

PAC2 wild type and PAC2-zGRβ cells were seeded in 6 well plates ($2x10^5$ cells/well) the day before treatment with either 10µM betamethasone 17-valerate (Sigma) or vehicle (<2% DMSO) in complete culture medium for 3h. Samples were collected in TRIzol® reagent (Invitrogen) and total RNA was isolated following the supplier's manual (Invitrogen).

RNA isolation & cDNA synthesis

Total RNA was extracted using the TRIzol® reagent (Invitrogen) according to the manufacturer's instructions (Invitrogen). RNA was dissolved in water and denatured for 5 min at 60°C. Samples were treated with DNAase using the DNA-*free*™ kit (Ambion). For microarray analysis, RNA was further purified using the RNeasy MinElute™ Cleanup kit from Qiagen and its integrity was checked with a lab-on-chip analysis using the 2100 Bioanalyzer (Agilent Technologies). For subsequent cDNA synthesis, at least 200ng of total RNA was added as a template for reverse transcription using the iSCRIPT™ cDNA Synthesis Kit (Biorad). Non reverse-transcribed samples were also generated to be used as negative controls during the quantitative polymerase chain reaction step.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR analysis was performed using the MyiQ Single-Color Real-Time PCR Detection System (Biorad). PCR reactions were performed in a total volume of 25µl containing 2µl cDNA, 1µl forward and reverse primer (10µM) and 12.5µl of 2x iQ™ SYBR® Green Supermix (Biorad). Cycling conditions were 95°C for 3 min, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. Ct values (cycle number at which a threshold value of the fluorescence intensity was reached) were determined for each sample. A dissociation protocol was added, determining dissociation of the PCR products from 65°C to 95°C, allowing discrimination of specific products. In order to determine the efficiency of the amplification reaction, serial dilutions of cDNA were used and standard curves were generated plotting the Ct values against the relative cDNA concentration. The linear slope of the standard curve for each primer pair was determined and the efficiency was calculated based on the following formula: $Efficieny = 10^{-(\frac{1}{slope})}$. This was done for the target gene and a reference gene. Finally, the ΔCt value was determined by subtracting the Ct value of the experimental sample from the Ct value of a control sample, and the fold

change of gene expression was calculated using this formula: $Foldchange = \frac{\left(Efficiency_{t\arg et}\right)^{\Delta Ct_{t\arg et}}}{\left(Efficiency_{reference}\right)^{\Delta Ct_{reference}}}$. In all qPCR experiments, a non-reverse transcribed sample and a water-control were included. All cDNA samples were assayed in duplicate. Values shown are means ± s.e.m of three individual experiments. Sequences of all primers used for qPCR analysis are included in Suppl. Table 4.

Zebrafish strains, husbandry & egg collection

Wild type adult ABxTL zebrafish and the transgenic lines *Tg*(*hsp70*:*Gal4)* (provided by Dr. H. Baier, University of California, San Francisco, USA) and *Tg(UAS:GFP-zGRβ)* (see 'Generation of the *Tg(UAS:GFP-zGRβ)* transgenic line' later in this section) were used in this study. Livestock was maintained and handled according to the guidelines from http://zfin.org. Fertilization was performed by natural spawning (single crossings) at the beginning of the light period and eggs were raised at 28°C in E3 egg water medium containing 60µg/ml Instant Ocean sea salts supplemented with 0.0025% methylene blue (GURR). All experimental procedures were conducted in compliance with the directives of the animal welfare committee of Leiden University.

zGRβ mRNA synthesis & injections

pCS2+zGRβ plasmid was linearized with NsiI (New England Biolabs) and used as a template for the synthesis of zGRβ mRNA using the mMESSAGE mMACHINE SP6 kit from Ambion. Newly synthesized mRNA was purified using the RNeasy MinElute™ Cleanup kit (Qiagen). RNA was denatured at 60°C for 5min and its integrity was checked on an agarose gel. Prior to injections, zGRβ mRNA was denatured at 60°C for 5min and diluted at a final concentration of 5ng/µl in 1x Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES at pH 7.6) containing 0.05% phenol red (Sigma). Approximately 1nl of zGRβ mRNA solution was injected into the yolk of 1-2 cell stage fertilized eggs.

Glucocorticoid treatment of zGRβ mRNA injected embryos

Twenty-eight hours post fertilization embryos injected with zGRβ mRNA were dechorionated and placed in 2% agarose-coated Petri dishes, where they were incubated with 100µM dexamethasone (Sigma) in E3 medium for 6h at 28°C. Samples were collected in TRIzol® reagent (Invitrogen) and total RNA was isolated according to the manufacturer's instructions (Invitrogen).

Generation of the UAS:GFP-zGRβ expression vector

The zGRβ coding sequence was amplified out of the pCS2+zGRβ plasmid [51] using the CCCTCGAGCACCATGTACCCATACGATGTTCCAGATTATGCTATGGATCAAGG-AGGCTGGA forward and CCGGATCCTCAGTTTGAAAACTGCCTTTGAA reverse primer. This primer pair also introduced the Xho and BamH1 restriction sites flanking the 5' and 3' zGRβ ORF, respectively, together with an HA tag upstream of zGRβ. The resulting amplicon was, then cloned into a pME-MCS vector ([59], provided by Dr. K. Kawakami (National Institute of Genetics, Japan). The HA-zGRβ-ME-MCS was further used as template for PCR amplification using the TCAGAATTCACCATGCTATGGATC-AAGGAGGACTGG forward and CTAAAGGGAACAAAAGCTGGAGCTC reverse primer. The forward primer omitted the HA tag, while introducing an EcoRI site at the 5' end of the zGRβ sequence, whereas the reverse primer was designed downstream of a

unique NotI restriction site at the 3' end of the zGRβ sequence. The resulting amplicon was cloned into the 14xUAS E1b Tol2 transposon-based vector (provided by Dr. H. Baier, University of California, San Francisco, USA). Furthermore, the destabilized green fluorescent protein (d1GFP) sequence was amplified from pd1EGFP-N1 vector (purchased from Clontech) using the TCAGAATTCATGGTGAGCAAGGGCGAGG forward and TCAGAATTCCGTTGTACAGCTCGTCCATGCC reverse primer, introducing EcoRI restriction sites at both ends of the ORF of d1GFP as well as a deletion of the stop codon at the 3' end. The resulting amplicon was cloned into the 14xUAS:zGRβ construct, upstream of and in frame with the zGRβ coding sequence.

Generation of the Tg(UAS:GFP-zGRβ) transgenic line

The plasmid pCS2FA-transposase (provided by Dr. K. Kawakami) was linearized with NotI (New England Biolabs) and further used as a template for the mRNA synthesis using the mMESSAGE mMACHINE SP6 kit from Ambion [60]. Newly synthesized mRNA was purified using the RNeasy MinElute^{TM} Cleanup kit from Qiagen and its integrity was checked on a 1.5% agaroze electrophoresis gel.

The UAS:GFP-zGRβ construct and the Tol2 transposase mRNA were diluted at a final concentration of 30 and 50ng/μl respectively, in 1x Danieu's buffer (58mM NaCl, 0.7mM KCl, 0.4mM $MgSO_4$, 0.6mM $Ca(NO_3)_2$, 5mM HEPES at pH 7.6) containing 0.05% phenol red [60]. 1nl of mixed UAS:GFP-zGRβ DNA and transposase mRNA solution was microinjected at the single-cell stage eggs. Positive F0 founders were outcrossed again with wild type fish and their offspring were raised to adulthood. F1 adult fish were genotyped and positive F1 fish were subsequently used in crossings with the *Tg*(*hsp70*:*Gal4)* line [61].

Heat shock and glucocorticoid treatment of transgenic embryos

The *Tg*(*hsp70*:*Gal4)* and *Tg(UAS:GFP-zGRβ)* lines were crossed and their offspring were heat-shocked at 1dpf in Petri dishes filled with pre-warmed (37°C) E3 egg water medium in a 37°C incubator for 2.5h. The next day, embryos were screened for GFP expression and wild types as well as their GFP expressing siblings were subjected again to the heat shock protocol. Three day old hatched wild types and their GFP expressing siblings were checked again for GFP signal and treated with either vehicle (<2% DMSO) or 25μM beclomethasone for 6h. Samples were collected in TRIzol® reagent (Invitrogen) for subsequent RNA isolation.

Microarray design

A 4x180k microarray chip platform (customized by Agilent Technologies, (Design ID:028233)) was used in this study. This array consists of all probes already present in an earlier 45.219 custom-made array [62], and another 126.632 newly designed zebrafish probes had been added as described in [63]. A total of 16 samples (4 experimental groups from 4 replicate experiments) were processed for transcriptome analysis. On each 4x180k slide, 4 samples from the different experimental groups within the same replicate were hybridized against a common reference.

Microarray amplification & labeling

Amplification and labeling of RNA was performed at the MicroArray Department (MAD) of the University of Amsterdam (Amsterdam, The Netherlands). Per sample, 0.5μg total RNA was amplified and combined with Spike A according to the Agilent Two-Color Microarray-Based Gene Expression Analysis kit (Agilent technologies). As a common

reference sample an equimolar pool of all test samples was made and 0.5µg samples were amplified similarly as the test samples with the exception that Spike B was used. Amino-allyl modified nucleotides were incorporated during the aRNA synthesis (2.5mM of each GTP, ATP, UTP (GE Healthcare), 0.75mM CTP (GE Healthcare), 0.3mM AA-CTP (TriLink Biotechnologies)). Synthesized aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit (Omega Bio-Tek). The quality was inspected on the BioAnalyzer (Agilent Technologies) with the Agilent RNA 6000 kit (Agilent Technologies). Test samples were labeled with Cy3 and the reference sample was labeled with Cy5. Five µg of aRNA was dried out and dissolved in 50mM carbonate buffer pH 8.5. Individual vials of Cy3/Cy5 from the mono-reactive dye packs (GE Healthcare) were dissolved in 200µl DMSO. To each sample, 10µl of the appropriate CyDye dissolved in DMSO was added and the mixture was incubated for 1h. Reactions were quenched with the addition of 5µl 4M hydroxylamine (Sigma-Aldrich). The labeled aRNA was purified with the E.Z.N.A. MicroElute RNA Clean Up Kit. Yields of aRNA and CyDye incorporation were measured on the NanoDrop ND-1000.

Microarray hybridization, scanning & data processing

Each hybridization mixture was made up from 825ng Test (Cy3-labeled) and 825ng Reference (Cy5-labeled) material. Hybridization mixtures were using the Agilent Two-Color Microarray-Based Gene Expression Analysis kit according to the manufacturer's instructions (Agilent technologies). The samples were loaded onto the microarray chips and hybridized for 17h at 65°C. Afterwards the slides were washed and scanned (20 bit, 3µm resolution) in an ozone-free room with the Agilent G2505C scanner. Data was extracted with Feature Extraction (v10.7.3.1, Agilent Technologies) with the GE2_107_Sep09 protocol for two-color Agilent microarrays. The Agilent output from the 16 hybridizations was then imported into the Rosetta Resolver 7.2 software (Rosetta Biosoftware, Seattle, Washington) and subjected to a factorial design with a re-ratio with common reference application. Data analysis was performed setting cutoff for the p-value of $<10^{-5}$ and for fold change of either >2 or <-2.

Statistical analysis

Statistical analyses (t-tests and two-way ANOVAs with Bonferroni post-hoc tests) were performed using the GraphPad Prism version 4.00 (GraphPad Software, La Jolla, USA).

Results

Dominant-negative activity of zGRβ in luciferase assays in COS-1 cells

Previously, we have demonstrated that zGRβ has a dominant-negative effect on zGRα-mediated transactivation of an MMTV-luciferase construct in COS-1 cells [51]. In the present study, we aimed at studying this effect on a broader range of zGRα/zGRβ ratios and exploring whether zGRβ has a dominant-negative effect on zGRα-mediated transrepression as well. For the studies on transactivation, COS-1 cells were transiently transfected with an MMTV-luciferase reporter construct and a range of zGRα expression vector amounts in the presence and absence of 100ng zGRβ plasmid. Upon transfection, cells were incubated with 100nM dexamethasone (dex) for 24h and assayed for luciferase activity. The results show that, as expected, the luciferase signal was increased upon increasing concentrations of zGRα (Fig. 1A). In the presence of zGRβ, the luciferase activity was strongly reduced, even when equal amounts of the zGRα and zGRβ

expression vector were transfected. These data demonstrate that zGRβ exhibits a dominant-negative effect on the transactivation properties of zGRα in these reporter assays.

In order to study the effect of zGRβ on the transrepression activity of zGRα, COS-1 cells were transiently transfected with a κB-luciferase construct, an expression vector for the human p65 subunit of the NF-κB transcription factor complex and a range of zGRα expression vector amounts in the presence and absence of 100ng zGRβ plasmid. Upon transfection, cells were incubated with 100nM dex for 24h and assayed for luciferase activity. The results, shown in Fig. 1B, reveal that with increasing concentrations of zGRα, NF-κB-mediated luciferase activation was dramatically repressed, reflecting the transrepression activity of zGRα. In these experiments, the presence of zGRβ did not affect the observed luciferase activity levels, indicating that zGRβ does not have a dominant-negative inhibitory role on the transrepression activity of zGRα.

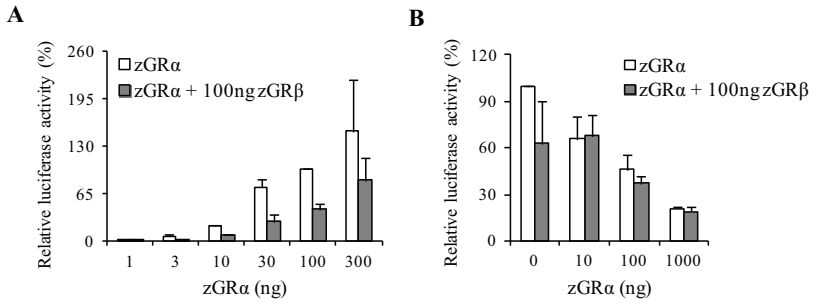


Figure 1. A. Dominant-negative activity of zGRβ on transactivation analyzed by luciferase assays. COS-1 cells were transiently transfected with an MMTV-luciferase reporter construct and a range of zGRα expression vector amounts in absence (white bars) and presence (grey bars) of 100ng zGRβ, and incubated with 100nM dex for 24h. The relative luciferase activity values (setting the 100ng zGRα transfected group value as 100%) shown are the means ± s.e.m. of three independent experiments. Statistical analysis by ANOVA revealed an effect of zGRβ expression ($p=0.001$). **B.** Lack of dominant-negative activity of zGRβ on transrepression analyzed by luciferase assays. COS-1 cells were transiently transfected with a κB-luciferase construct, an expression vector for the human p65 subunit of the NF-κB transcription factor complex and a range of zGRα expression vector amounts in the absence (white bars) and presence (grey bars) of 100ng zGRβ plasmid, and incubated with 100nM dex for 24h. The relative luciferase activity values (setting the 0ng zGRα transfected group value as 100%) shown are the means ± s.e.m. of three independent experiments. Statistical analysis by ANOVA demonstrated no significant effect of zGRβ expression.

Dominant-negative activity of zGRβ in PAC2 cells

Transiently transfected reporter constructs contain simple promoters and are not embedded into chromatin structure. In order to study the effect of zGRβ on endogenous promoters *in vitro*, we generated a PAC2 cell line that stably overexpresses the zebrafish GR β-isoform (PAC2-zGRβ). In PAC2-zGRβ cells, the zGRβ mRNA expression level was 260 fold higher than in wild type PAC2 cells (PAC2-wt), whereas the expression of zGRα was not different between these two cell lines (Fig. 2A and B). That renders zGRβ as the predominant GR splice variant in the PAC2-zGRβ line, with a 10-fold higher absolute

mRNA abundance than that of zGRα (Suppl. Fig. 1). Treatment with GCs did not significantly alter the expression of either splice variant (Fig. 2A and B).

The zGRα signaling in those two cell lines was evaluated upon incubation with 10μM betamethasone-17-valerate (BV) for 3h. Hence, we investigated the expression profile of a well-known endogenous GR-target gene, *fkbp5* [64], by means of qPCR. Upon BV treatment, a more than 10-fold induction of *fkbp5* was observed in both PAC2-wt and PAC2-zGRβ cells, and that was not statistically different between the two lines examined (Fig. 2C). Thus, zGRβ does not exhibit any dominant-negative activity on zGRα-induced transactivation of the *fkbp5* gene in PAC2 cells. Several other known GR target genes (*gilz, slc5α1, agtxb, hsd11b2, pepck, nfkbiαα*) were tested for induction upon BV treatment in PAC2 cells. None of these genes showed increased mRNA expression upon treatment, leaving *fkbp5* as the only gene that could be used to study the effect of zGRβ on the transactivation properties of zGRα in these cells.

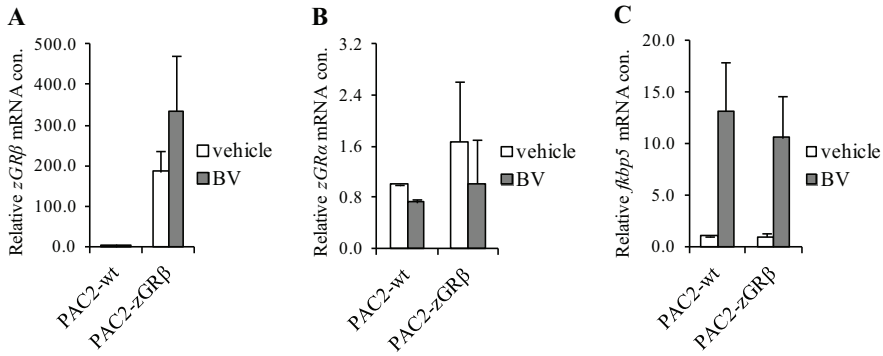


Figure 2. A. Stable overexpression of the zebrafish GR β-isoform in PAC2 cells. Cells were stably transfected with a zGRβ expression vector for the generation of the PAC2-zGRβ line. The zGRβ expression level of this cell line was determined by qPCR. The data show that the PAC2-zGRβ cell line highly overexpresses (> 250 fold) the zGR β-isoform compared to its parental wild type line. Stimulation of both lines with 10μM BV for 3h did not significantly affect zGRβ mRNA expression. **B.** Expression levels of zGRα mRNA in PAC2-wt and PAC2-zGRβ cells treated with either 10μM BV or vehicle for 3h. Statistical analysis by ANOVA demonstrated that there is no significant effect on the expression of the zGR α-isoform due to either BV treatment or overexpression of the zGR β-isoform. **C.** Expression levels of *fkbp5* mRNA in PAC2-wt and PAC2-zGRβ cells, determined by qPCR. Cells from both lines were compared for their ability to upregulate the expression of *fkbp5* upon treatment with either 10μM BV or vehicle for 3h. Statistical analysis revealed that *fkbp5* induction due to BV treatment does not differ between the two lines examined. In all figures mRNA expression measurements were normalized to those of *bactin1* and the relative mRNA concentration values are the means ± s.e.m. of three independent experiments.

Injections of zGRβ mRNA in zebrafish embryos

In order to assess the zGRβ effect on transactivation *in vivo*, we injected 1-2 cell stage zebrafish embryos with 5pg zGRβ mRNA. The next day we treated them with 100μM dex for 6h, and total RNA was isolated from the embryos and processed for qPCR analysis of three GRα target genes: *fkbp5*, *pepck* and *nfkbiaa*. Dex treatment of control embryos resulted in approximately 100 (± 10.6 s.e.m.), 3.5 (± 0.9 s.e.m.) and 1.6 (± 0.25 s.e.m.) fold induction of *fkbp5*, *pepck* and *nfkbiaa* mRNA, respectively (Fig. 3A, B and C). zGRβ-injected embryos treated with dex showed similar induction levels of these genes (Fig. 3A, B and C). These results confirm *in vivo* the lack of a dominant-negative zGRβ effect on

fkbp5 as already observed in PAC2 cells, and additionally demonstrate no dominant-negative effect of zGRβ on the induction of *pepck* and *nfkbiaa* gene expression by zGRα.

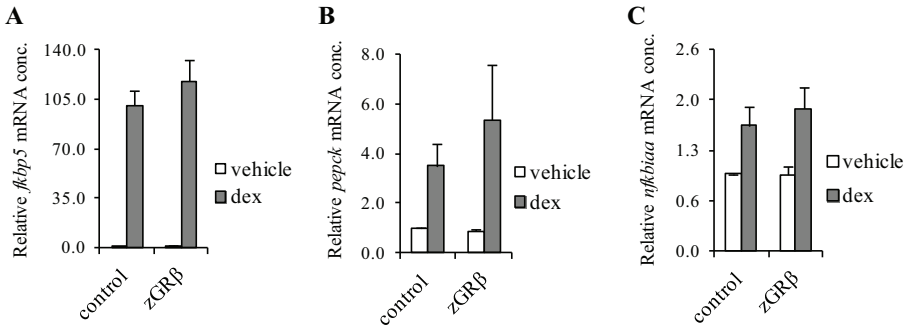


Figure 3. Expression levels of the GR target genes *fkbp5* (A)*, pepck* (B)*,* and *nfkbiaa* (C) in zGRβ mRNA injected embryos, determined by qPCR. Embryos were injected at the 1-2 cell stage with 5pg zGRβ mRNA. At 28 hours post fertilization, they were treated with 100μM dex for 6h. Expression levels were normalized to those of *bactin1* and the relative mRNA concentration values shown are the means ± s.e.m. of three independent experiments. Statistical analysis by ANOVA demonstrated that *fkbp5, pepck,* and *nfkbiaa* induction upon dex treatment were not affected by zGRβ mRNA injection. These data show that zGRβ does not exhibit any dominant-negative effect on the zGRα-induced transactivation of any of the genes tested in 1 day post fertilization zebrafish embryos.

Characterization of a Tg(UAS:GFP-zGRβ) transgenic line

Subsequently a fish line was generated which stably overexpressed zGRβ, using the Gal4/UAS expression system. We generated a *Tg(UAS:GFP-zGRβ)* line, in which the GR β-isoform is fused to a green fluorescent protein (GFP) at its N-terminus and is downstream of a 14xUAS promoter that responds to the Gal4 transcription factor. This line was crossed with the *Tg*(*hsp70*:*Gal4)* line (in which Gal4 expression is controlled by a heat shock-inducible promoter), yielding embryos in which the zGRβ expression was induced upon a heat shock. Embryos at 1 and 2 day post fertilization (dpf) were heat shocked and the GFP signal was readily detectable within a few hours, reflecting expression of the GFP-tagged zGR β-isoform. Expression was clearly present in the muscles, yolk and eyes of 3dpf larvae (Fig. 4B). Further magnification suggested that GFP-zGRβ is localized in the nuclei of the cells (Fig. 4C), which was confirmed by co-staining with Sytox Orange (data not shown). This observation is in line with previous data showing that a YFP-tagged zGRβ expressed in COS-1 cells resulted in a more nuclear localization compared to YFP-zGRα in the same cell type [51].

In order to confirm that the fusion protein was properly expressed, we performed a western blot analysis on samples from fluorescent and non-fluorescent embryos using an anti-GFP antibody. We could identify a specific band at the expected size (approximately 108kDa) which was lacking from control embryos (data not shown). In addition, using an expression vector for YFP-zGRβ we showed that the YFP tag does not affect zGRβ's dominant-negative activity in COS-1 cells, indicating that this type of tag does not interfere with the dominant-negative activity of the zGR β-isoform (Suppl. Fig. 2).

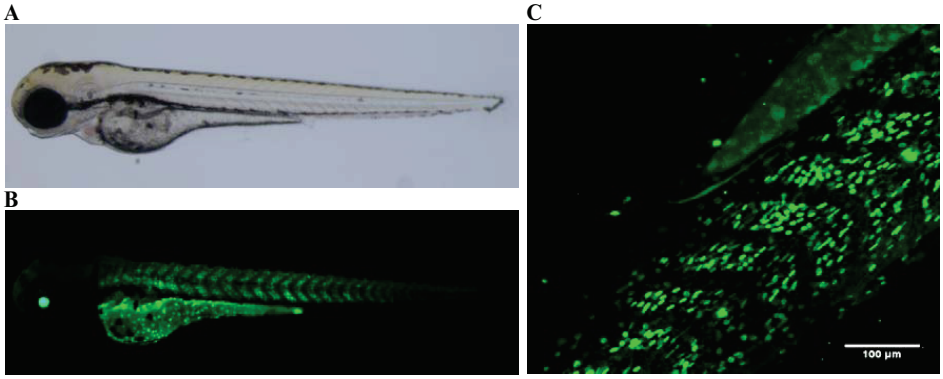


Figure 4. Images of a 3dpf zebrafish embryo generated by crossing the *Tg*(*hsp70*:*Gal4)* and *Tg(UAS:GFP-zGRβ)* transgenic lines. Embryos were heat shocked at 37°C for 2.5h at 1 and 2dpf. **A.** Transmitted light microscopy image showing representative embryo, indicating that transgenesis and heat shock treatment do not alter the morphology of zebrafish embryos. **B.** Fluorescence microscopy image showing expression of the GFP-zGRβ fusion protein. The green fluorescent signal is clearly present in the muscles, on the yolk sac and in the eyes. **C.** Larger magnification fluorescence microscopy image showing expression of GFP-zGRβ in muscle tissue. The image shows that GFP-zGRβ is localized in the nuclear cell compartment.

Microarray analysis of zGRβ transcriptional activity

Next it was explored at the whole transcriptome level whether zGRβ plays a role either as a dominant-negative inhibitor of the zGR α-isoform or as an intrinsic transcription factor (i.e. independent of zGRα), using a custom-designed Agilent 180k zebrafish microarray platform [63]. The *Tg*(*hsp70*:*Gal4)* line was crossed with the *Tg(UAS:GFP-zGRβ)* line. The resulting embryos were identified, upon heat shock, based on the observed fluorescence as either wild type (WT, lacking fluorescence) or zGRβ-overexpressing (zGRβ, showing green fluorescence). Both populations were treated with either 25µM beclomethasone (beclo) or vehicle for 6h at 3dpf. Thus, 4 groups were generated: WT/vehicle, WT/beclo, zGRβ/vehicle, zGRβ/beclo. Total RNA samples from 4 biological replicates (16 samples in total) were processed for the microarray study using a common reference design. Data were analyzed using Rosetta Resolver 7.2 software, setting signatures for significantly regulated probes at a p-value cutoff of $p<10^{-5}$ and fold changes either >2 or <-2. Figure 5 gives a schematic overview of different microarray group comparisons that were performed to analyze beclo- and zGRβ-mediated effects on transcription.

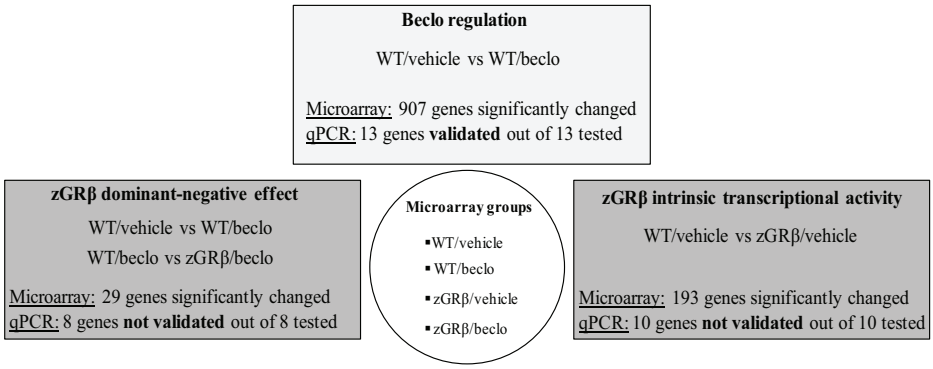


Figure 5. Schematic overview of microarray analysis and subsequent qPCR validation of beclo and zGRβ effects using a 180k zebrafish microarray platform. The *Tg*(*hsp70*:*Gal4)* line was crossed with the *Tg(UAS:GFP-zGRβ)* line and the resulting embryos were heat shocked at 37°C for 2.5h at 1 and 2dpf. At 3dpf, embryos were sorted based on their GFP signal and treated with 25µM beclo for 6h. Thus, 4 groups were generated: WT/vehicle, WT/beclo, zGRβ/vehicle, zGRβ/beclo. Total RNA samples were processed for transcriptome analysis by microarray. Results for beclo- as well as zGRβ-mediated effects on transcription were analyzed using Rosetta Resolver 7.2 software. Numbers of genes refer to annotated genes found to be significantly regulated at a p-value cutoff of $p<10^{-5}$ and fold changes either >2 or <-2. Beclo regulation of genes is defined as significant up- or down-regulation based on the WT/beclo versus WT/vehicle group comparison. The dominant-negative activity of zGRβ is defined as a significant up- (or down-) regulation by beclo, and a decrease (or increase) in the GRβ/beclo group as compared to the WT/beclo group (see also Fig. 7). The intrinsic transcriptional activity of zGRβ is defined as significant up- or down-regulation based on the WT/vehicle versus zGRβ/vehicle group comparison. Annotation and probe assignment was based on the Ensemble Gene ID codes (ENSDARG).

As a first step, we studied genes regulated upon beclo treatment in the wild type fish (WT/vehicle vs WT/beclo comparison). We identified 1805 probes corresponding to genes significantly upregulated by beclo treatment and 703 probes corresponding to downregulated ones. Of these 2508 total probes, 1592 could be attributed to an annotated gene (in total, 907 beclo-regulated genes). For 13 randomly chosen genes (10 up-regulated, 3 down-regulated, Suppl. Table 1) which were represented in this cluster by at least two probes (enabling less false-positive discoveries), validation by qPCR was performed, and a significant effect of beclo treatment was confirmed for all genes tested (Fig. 6).

We, then, took the latter subset of probes representing beclo-regulated genes and compared the expression of these genes between the WT/beclo and the zGRβ/beclo group, in order to examine a dominant-negative effect of zGRβ. For 30 of the 1805 probes corresponding to beclo-upregulated genes, a downregulation was observed upon overexpression of zGRβ (in the presence of beclo), indicating a dominant-negative effect of zGRβ. In addition, for 35 of the 703 probes corresponding to genes downregulated by beclo treatment, an upregulation was observed upon overexpression of zGRβ (in the presence of beclo), also indicating a dominant-negative effect of zGRβ (Fig. 7). Thus, in our microarray experiment, zGRβ appeared to exhibit a significant inhibitory role in zGRα signaling for 65 probes, which accounts for only 2.6% of beclo-regulated ones.

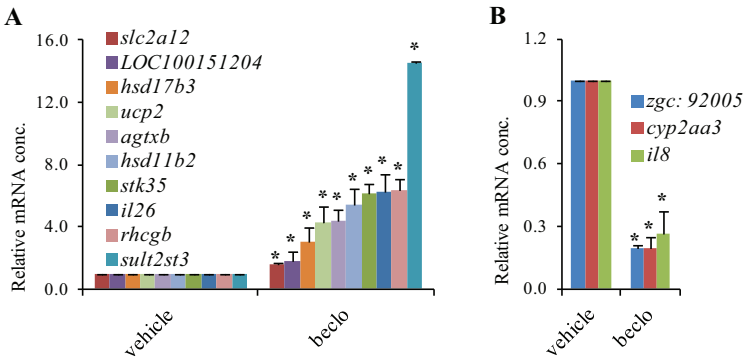


Figure 6. Validation by qPCR of the effect of beclo treatment on the expression of several genes identified as beclo-responsive in the microarray analysis. Expression rates of all genes were normalized to those of *bactin1* and relative mRNA concentration values are the means ± s.e.m. of three independent experiments. **A.** Validation for genes identified as up-regulated by beclo treatment in the microarray study. Statistical analysis demonstrated that for all genes the mRNA expression was significantly higher in the beclo-treated group compared to the vehicle-treated one. **B.** Validation for genes identified as down-regulated by beclo treatment in the microarray study. Statistical analysis demonstrated that for all genes the mRNA expression was significantly lower in the beclo-treated group compared to the vehicle-treated one. Asterisks (*) correspond to a statistically significant difference between the beclo- and vehicle-treated group ($p<0.05$). These data confirm the effects of beclo treatment observed in the microarray analysis.

Within the cluster of 65 probes that indicated a dominant-negative activity of zGRβ, 34 probes could not be attributed to an annotated gene. The other 31 probes could be attributed to 29 genes, since 27 genes were represented by one probe and only two genes (*LOC100151204* and *crybb2*) by two probes (Table 1). This is not a result of these genes being represented by a low number of probes on the array, since only one of these 27 annotated genes is represented by one probe on the 180k array whereas expression of the rest can be detected by multiple probes. *LOC100151204* together with 7 randomly chosen genes from the pool of 29 annotated genes with putative dominant-negative regulation were used for validation of the microarray results by qPCR. In this validation, we could not verify any significant zGRβ dominant-negative effect on any of the genes tested (Suppl. Table 2).

Subsequently, we investigated whether zGRβ has intrinsic transcriptional activity, able to regulate the expression of target genes, independent of zGRα activity. For that reason we compared the WT/vehicle and zGRβ/vehicle groups and we identified 258 probes corresponding to up-regulated transcripts due to the zGRβ overexpression and 223 probes corresponding to down-regulated ones.

Of these 481 regulated probes, 271 could not be attributed to an annotated gene. The other 210 probes corresponded to 193 genes. As expected, the GR gene itself was present among the genes up-regulated in the zGRβ transgenic line, and this up-regulation was detected with 12 probes. In addition, the *hsp47* gene (5 probes) and the *LOC100151204* and *zgc: 111983* genes (2 probes) were represented by more than 1 probe (Table 1). The *hsp47* and *zgc: 111983* genes and eight randomly selected genes were used for validation. Again, qPCR analysis failed to verify any significant zGRβ effect on all genes tested, (Suppl. Table 3).

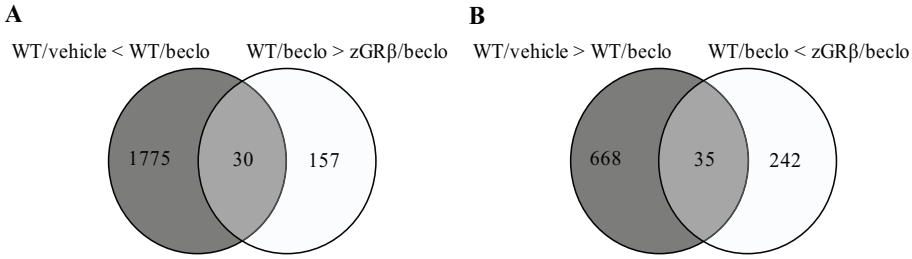


Figure 7. Analysis of the dominant-negative activity of zGRβ observed in the microarray. **A.** Venn diagram depicting the pool of probes corresponding to transcripts upregulated in the WT/beclo group compared to the WT/vehicle group (grey circle, 1805 probes), and the pool of probes corresponding to transcripts downregulated in the zGRβ/beclo compared to the WT/beclo group (white circle, 187 probes). The overlap between these pools (30 probes) represents transcripts which were significantly upregulated upon beclo treatment, reflecting a possible dominant-negative effect of zGRβ on transactivation properties of zGRα. **B.** Venn diagram depicting the pool of probes corresponding to transcripts downregulated in the WT/beclo group compared to the WT/vehicle group (grey circle, 703 probes), and the pool of probes corresponding to transcripts downregulated in the zGRβ/beclo compared to the WT/beclo group (white circle, 277 probes). The overlap between these pools (35 probes) represents transcripts which were significantly downregulated upon beclo treatment, reflecting a possible dominant-negative effect of zGRβ on the transrepression properties of zGRα.

Differences between the clusters of beclo- and zGRβ-regulated genes

As presented previously, we were not able to verify by qPCR the regulation of genes due to zGRβ overexpression (dominant-negative or intrinsic transcriptional activity), whereas gene regulation due to beclo treatment was confirmed in all cases studied. This prompted us to further investigate differences between the clusters of beclo- and zGRβ-regulated genes.

In our custom-made 180k microarray platform, the annotated (provided by ENSEMBL, www.ensembl.org) genes are represented by either one or multiple probes, with the largest number of them being represented by two probes (Table 1). Our analysis revealed that approximately 33% of the beclo-regulated genes were represented within the cluster of beclo-regulated genes by more than one probe (Table 1), which is a dramatically higher percentage than that found in the subset of genes regulated by GRβ in a dominant-negative way (7%), or as an intrinsic transcription factor (2% , Table 1). Limiting the analysis to genes that show regulation for at least two probes would have left very few zGRβ-regulated genes (6 genes in total, 2 for zGRβ dominant-negative activity and 4 for zGRβ intrinsic transcriptional activity), whereas still a significant number of beclo-regulated genes (302 genes) would have been found in the array. The disadvantage of this analysis is that effects on genes that are represented by only one probe on the array (19% of genes represented on the 180k chip) are overlooked. Second, the p-values corresponding to the (significant) regulation of the individual probes were studied, and for each subset of regulated probes (beclo-regulation, zGRβ dominant-negative effect, zGRβ intrinsic transcriptional activity) the distribution of these p-values was plotted (Fig. 8A). Interestingly, zGRβ-regulated probes (both zGRβ intrinsic transcriptional and dominant-negative activity), mainly show higher p-values, whereas beclo-regulated probes show a more even distribution over a wider range of p-values (Fig. 8A). Obviously, using a lower p-value as significance threshold would decrease the number of false positive results. For example, using a significance threshold of $p=10^{-10}$, zGRβ would show a dominant-negative

effect on only 11 probes (previously, that number was 65) and an intrinsic transcriptional activity on only 105 probes (previously, it was 481). In contrast, beclo treatment would significantly still affect 1809 probes (previously they were up to 2508). Lowering p-values shows that the cluster of beclo-regulated probes is altered much less that those of zGRβ-regulated ones, since 72% of the beclo-affected probes previously regulated at a cutoff of $p=10^{-5}$ are still regulated at a cutoff of $p=10^{-10}$. As for zGRβ, the equivalent numbers correspond to less than 17% for its dominant-negative effect and almost 22% for its intrinsic transcriptional activity. However, clusters deriving from a lower p-value could still contain a large number of false positives, since from the pool corresponding to zGRβ dominant-negative effect already two genes have been shown to be false positive hits by qPCR (Suppl. Table 2), and from the pool corresponding to zGRβ intrinsic transcriptional activity four genes have already been shown to be false positives by qPCR (Suppl. Table 3).

	180k array	**Regulation by beclo**	**Regulation by zGRβ**	
			Dominant-negative effect	**Intrinsic transcriptional activity**
Total no. probes	**175975**	**2508**	**65**	**481**
probes without annotation	81534	916	34	271
probes with gene annotation	94441	1592	31	210
Total (annotated) genes	**23837**	**907**	**29**	**193**
genes with 1 probe	4539 (19%)	605 (66.7%)	27 (93%)	189 (98%)
genes with 2 probes	4578 (19.2%)	127 (14%)	2 (7%)	2 (1%)
genes with 3 probes	3920 (16.4%)	78 (8.6%)		
genes with 4 probes	3008 (12.6%)	47 (5.2%)		
genes with 5 probes	2334 (9.8%)	21 (2.3%)		1 (0.5%)
genes with 6 probes	1728 (7.2%)	14 (1.5%)		
genes with 7 probes	1238 (5.2%)	8 (0.9%)		
genes with 8 probes	852 (3.6%)	1 (0.1)		
genes with 9 probes	528 (2.2%)	2 (0.2%)		
genes with 10 probes	330 (1.4%)	4 (0.4%)		
genes with 11 probes	222 (0.9%)			
genes with 12 probes	126 (0.5%)			1 (0.5%)
genes with >12 probes	434 (1.8%)			

Table 1: Overview of microarray results. Shown are the numbers of probes and annotated genes represented on the microarray and the numbers of probes and annotated genes showing significant regulation (p-value cutoff of 10^{-5} and fold changes either >2 or <-2). Annotation of probes was performed based on the Ensembl Gene ID codes (ENSDARG). Percentages correspond to the number of genes represented by a certain number of probes relative to the total number of annotated genes. The analysis shows that within the cluster of (beclo- and zGRβ-) regulated probes the majority of corresponding annotated genes is represented by one probe, despite the fact that most of these can be detected by more than one probe on the array. Furthermore, approximately 33% of beclo-regulated genes are represented by more than one probe, which is dramatically more than that observed for zGRβ-regulated genes (2%).

In conclusion, a more stringent analysis of the microarray data (increasing significance threshold and excluding genes showing regulation for only one probe) dramatically decreases the size of the clusters of zGRβ-regulated genes, whereas it has a much smaller impact on the cluster of beclo-regulated ones. This suggests that the relative number of false-positives among the zGRβ-regulated genes is considerably larger than among the beclo-regulated genes, which was confirmed by our qPCR validation that verified the up- or downregulation of several beclo-regulated genes, but failed to validate any zGRβ effect on several genes observed in the microarray analysis.

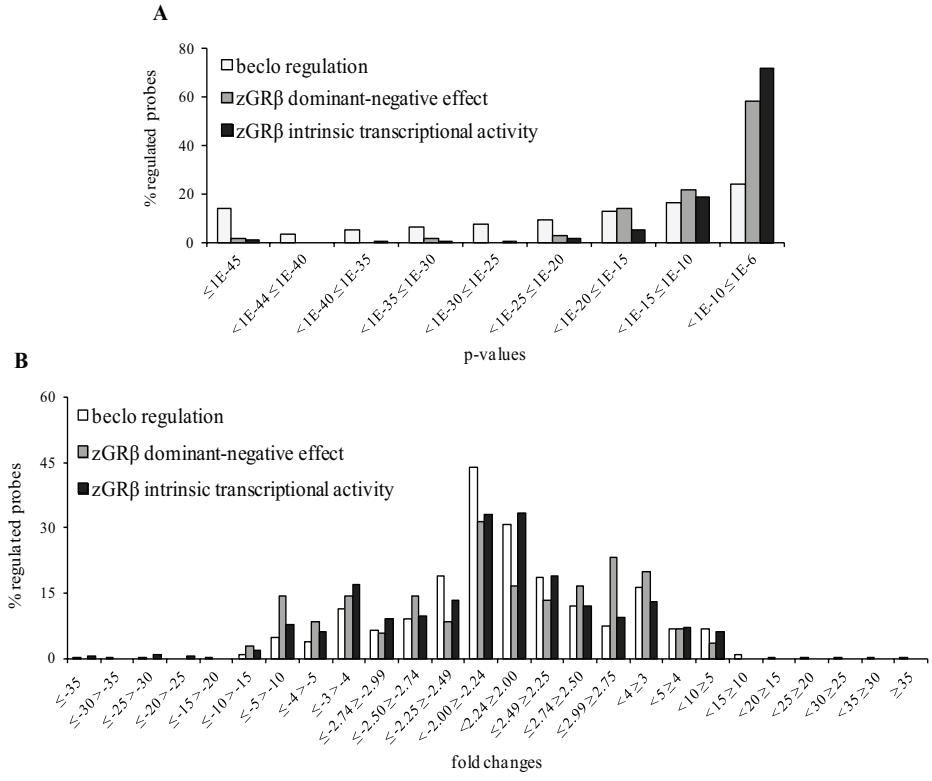


Figure 8. Distribution of significantly regulated probes (cutoff set at p-value $<10^{-5}$ and fold change of either >2 or <-2) from our microarray expression data analysis for beclo as well as zGRβ-mediated effects on transcription. **A.** Percentage (of the total number of regulated probes) of signature probes which fall into a certain range of p-values for beclo regulation (probes corresponding to transcripts up- or down-regulated in the WT/beclo group compared to the WT/vehicle group), zGRβ dominant-negative effect (subset of beclo-regulated probes corresponding to transcripts up- or down-regulated in the zGRβ/beclo group compared to the WT beclo group (see Fig. 6)) and zGRβ intrinsic transcriptional activity (probes corresponding to transcripts up and down-regulated in the WT/vehicle group compared to the zGRβ/vehicle group). For beclo regulation (white bars), there is a substantial probe presence over a wide range of p-values even lower than 10^{-20}. In contrast, for zGRβ-mediated effects (grey and black bars), their significantly regulated probes are mainly in p-values higher that 10^{-20}. **B.** Percentage (based on the total number of up- or down-regulated probes) of signature probes which fall into a certain range of fold changes. The data show that for both beclo and zGRβ-regulated probes, fold changes mainly fall into a 3/-3 range.

Discussion

In the present study we have used the zebrafish as a model organism to investigate the regulatory role of the zebrafish GR β-isoform on gene transcription. We first looked at its dominant-negative effect on both transactivation and transrepression properties of the GR α-isoform. By means of luciferase reporter assays using transient transfections in COS-1 cells we demonstrated that zGRβ strongly inhibits zGRα's transactivation properties even when we transfected equal amounts of zGRα and zGRβ expression vectors. This dominant-negative activity had already been observed in our laboratory using a 1:10 zGRα/zGRβ ratio [51], and recapitulates data obtained in similar reporter assays in which the human [15, 17, 52, 65] and mouse [53] GRβ showed a dominant-negative effect at 1:10 and 1:2 α/β transfection ratio, respectively. In contrast, we did not observe an effect of zGRβ on the transrepression activity of zGRα. These data are in line with most of the literature on the human GR β-isoform, since in most experimental approaches employing reporter assays and transient transfections in various cell lines (e.g. COS-1, A549, Jurkat T-cells) the human GRβ was also reported not to exhibit any dominant-negative activity on hGRα transrepression properties [37, 38, 44, 46], even at a 1:20 α/β transfection ratio in COS-7 cells [43]. In contrast, one study has demonstrated a specific dominant-negative effect on transrepression rather than transactivation using reporter assays [45] and a dominant-negative effect on the dex-induced repression of TNFα and IL6 was demonstrated by siRNA knockdown of hGRβ expression in human monocytes [33].

The reporter constructs used in our luciferase assays have simple promoters and are not embedded into chromatin like the promoters of endogenous gene. Therefore, we next addressed the question whether zGRβ also exhibits dominant-negative properties on the zGRα-induced transactivation of endogenous genes. From studies in human and mouse cell cultures there is a solid body of evidence that GRβ inhibits GRα-induced transactivation of a number of endogenous genes, like MKP-1 in human monocytes [33] and BAL macrophages [23], myocilin and fibronectin in human trabecular meshwork eyes cells [32], CANT1, NEK2, TWSGI1, and LATS2 in Hela cells [40], and GILZ, PDK4, and G6Pase in mouse MEF cells [53].

In the present study, we started our investigation on endogenous genes by first establishing a cell-based system that subsequently could allow us to further characterize the molecular properties of zGRβ. We chose the PAC2 zebrafish cell type which expresses both zebrafish GR isoforms and is easily subjected to transfection protocols. Thus, we generated a PAC2 cell line that stably overexpressed zGRβ (on average ~260 fold higher at the mRNA level compared to wild type). We determined that zGRβ is expressed more than 10 times higher in these cells than zGRα, rendering the zGR β-isoform as the predominant GR receptor at the mRNA level in these cells. The GRβ protein has been shown to be more stable than GRα in humans [66] and zebrafish [51], so it can be assumed that the higher zGRβ expression level compared to zGRα observed in our cells at the mRNA level is translated to the protein level as well.

In this zGRβ-overexpressing cell line we investigated whether zGRβ had a dominant-negative activity on the zGRα-induced transactivation of *fkbp5* (a well-known GRα target gene [67]). Surprisingly, we did not observe any effect of zGRβ overexpression on the induction of *fkbp5* by zGRα. Thus, it appears that zGRβ does not exhibit a general dominant-negative activity on zGRα-induced transactivation. Interestingly, recent studies in mice reported a lack of GRβ overexpression effect on GRα-induced *fkbp5* transcription, whereas a dominant-negative activity on other, mostly metabolism-related genes, was observed [53]. Unfortunately, due to a limited

responsiveness of our PAC2 line to GC treatment, we were not able to assess the zGRβ counteractive effect on other GRα-target genes.

Previously, we had observed that 1dpf zebrafish embryos are responsive to GC treatment, as shown by an upregulation of the expression of the GR target genes *pepck*, *nfkbiaa*, and *fkbp5* [64]. Therefore we further investigated a possible dominant-negative effect of zGRβ *in vivo* by injecting mRNA encoding the zGR β-isoform into 1-2 cell stage embryos. Like in the PAC2 cells, overexpression of the zGR β-isoform did not suppress zGRα-mediated gene induction, as shown on all three genes tested (Fig. 3).

Subsequently, in order to get a genome-wide and unbiased view of the zGRβ transcriptional properties, we performed a transcriptome analysis upon zGRβ overexpression *in vivo*. We generated the *Tg(UAS:GFP-zGRβ)* transgenic line that we crossed with the *Tg*(*hsp70*:*Gal4)* one. To our knowledge, this is the first attempt to identify novel pathways of zGRβ influence within the physiological content of a living organism. Therefore, using our *in vivo* transgenic embryo model combined with a microarray approach, we expected to elucidate the biological significance of the zGR β-isoform. Our results showed an effect of zGRβ overexpression on 481 probes (independent of zGRα activity), and 65 probes displayed a dominant-negative effect of zGRβ, out of a total of 180,000 probes present on the array. However, after careful analysis of the microarray data we concluded that at least the vast majority of these data represent false positive results. The distributions of p-values and number of probes per gene showing regulation within the subsets of zGRβ-regulated genes (either independent of zGRα or dominant-negative) was remarkably different from those found for the cluster of probes showing regulation by beclo treatment (Fig. 8A and Table 1), raising questions about the confidence level of the results on zGRβ regulation. Indeed, using qPCR of a randomly selected set of genes showing regulation by zGRβ in the microarray we could not verify the effect of zGRβ on any of the selected genes. Using qPCR on genes showing regulation by beclo could verify the results from the microarray on all selected genes, supporting the validity of our study. These results led us to suggest that the effects of zGRβ on transcription in our transgenic embryo model are absent. Combined with the negative results obtained in PAC2 cells and upon zGRβ mRNA injection in embryos, these data suggest that, if existing, any effect of zGRβ on transcription is probably limited to a very small subset of genes or highly specific for a certain tissue or condition, and is obscured in our experimental systems.

The lack of an effect of the GR β-isoform in zebrafish presented in this study follows the trend of controversy over the significance of the GR β-isoform in humans. After more than 15 years of research, a consistent view on a possible dominant-negative activity on either transactivation or transrepression is still lacking. The observations on the intrinsic transcription factor activity of hGRβ [39, 40] have recently added more inconsistent data, since a large set of genes appeared to be oppositely regulated by hGRβ in different studies [14]. Our work on the functional role of zGRβ does not support a physiologically significant role for the GR β-isoform, and we conclude that the effect of this type of GR splice variant on transcription is anything but universal or generic. Nevertheless, gene-, cell type-, condition-, or species-specific effects can obviously not be ruled out, and these effects may explain the large inconsistencies in the literature on the GR β-isoform.

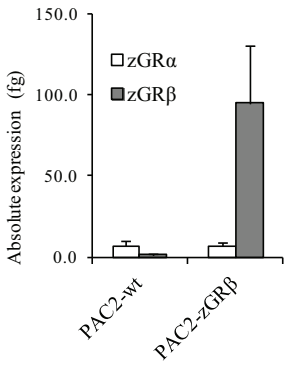
Finally, C-terminal splice variants of nuclear receptors are widespread [68], and most of these splice variants have been shown to display dominant-negative activity on the transactivational properties of their canonical equivalent in reporter assays. However, the presented studies on the zGR β-isoform demonstrate that dominant-negative activity observed in reporter assays is not necessarily followed by a similar effect *in vivo*. We

suggest that splicing of a pre-mRNA into a messenger encoding an alternative splice variant could instead be a physiological way to downregulate the levels of the canonical receptor variant. In such a case, the GR β-isoform does not have an active inhibitory role but its expression would result in less GRα available to mediate glucocorticoid signaling.

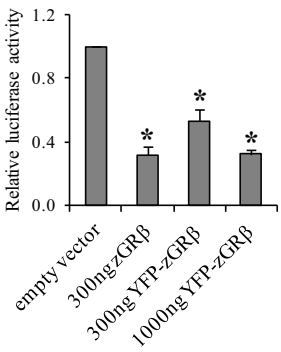
Acknowledgements

The authors would like to thank Dr. H. Baier for kindly providing the transgenic line *Tg*(*hsp70*:*Gal4)* and the 14xUAS E1b Tol2 transposon-based vector, as well as Dr. K. Kawakami for kindly providing the pME-MCS vector and pCS2FA-transposase plasmid. Additionally, the authors would like to gratefully acknowledge Huma Safdar, Enice Bagci and Özge Zelal Aydin for their technical assistance during the experiments, as well as Dr. Oliver W. Stockhammer for his assistance with THE microarray analysis. This work was financially supported by the SmartMix program of The Netherlands Ministry of Economic Affairs and the Ministry of Education, Culture and Science.

Supplemental data



Supplemental Figure 1. In order to measure the absolute mRNA concentration for zGRα and zGRβ, 500ng of total RNA samples from both PAC2-wt and PAC2-zGRβ cells were analyzed by qPCR for zGRα and zGRβ mRNA expression. Absolute amounts (± s.e.m.) of both transcripts were calculated based on standard curves. Analysis showed that in PAC2-wt cells, the average amount of zGRα is 7.08 ± 2.55fg per 500ng total RNA and 6.95 ± 2.63fg in PAC2-zGRβ. As for zGRβ, its mRNA concentration in PAC2-wt cells is 1.35 ± 0.34fg, per 500ng total RNA whereas in PAC2-zGRβ, it is dramatically higher: 95.05 ± 35.85fg. These results clearly demonstrate that in PAC2-wt cells, there is a more than 5-fold excess of zGRα mRNA as compared to zGRβ mRNA. In stark contrast, in PAC2-zGRβ cells, zGRβ mRNA becomes the dominant receptor isoform transcript, since it is expressed almost 14 times higher than zGRα mRNA.



Supplemental Figure 2. Dominant-negative activity of tagged and non-tagged zGRβ on transactivation analyzed by luciferase assays. COS-1 cells were transiently transfected with MMTV-luciferase reporter gene and zGRα plasmid together with either empty vector, 300ng zGRβ, 300ng or 1000ng YFP-zGRβ expression vectors and incubated with 100nM dex for 24h. Relative luciferase activity values are the means ± s.e.m. of three independent experiments. The analysis shows that in the presence of both tagged and non-tagged zGRβ, zGRα-mediated activation of luciferase gene is significantly suppressed. These data demonstrate that the YFP tag does not impair the dominant-negative activity of zGRβ on zGRα-induced transactivation in *in vitro* luciferase reporter assays. Asterisks (*) correspond to a statistical significance of $p < 0.001$ as calculated by a Bonferroni's multiple comparison test between empty vector group and each other one present in the upper graph.

Gene name	Gene symbol	Gene ID	Probes regulated by beclo	Probes/gene on the array	Fold change (microarray)	P-value (microarray)	Fold change (qPCR validation)	P-value (qPCR validation)
Genes upregulated by beclo								
sulfotransferase family 2 cytosolic sulfotransferase 3	*sult2st3*	777792	5	7	16.27 15.68 15.33 13.52 11.62	<1E-45 <1E-45 <1E-45 <1E-45 1.17E-9	14.53	<0.05
LOC100151204 cytochrome P450 2F2-like	*LOC100151204*	*100151204*	2	3	2.90 2.71	9.22E-19 <1E-45	1.80	<0.05
interleukin 26	*il26*	553971	4	10	8.49 7.67 5.92 4.07	7.6E-27 9.48E-22 6.72E-11 1.15E-6	6.24	<0.05
serine/threonine kinase 35	*stk35*	568080	5	6	5.40 5.31 4.95 4.91 4.76	1.9E-20 5.19E-38 4.96E-32 1.63E-16 <1E-45	6.17	<0.05
uncoupling protein 2	*ucp2*	555812	10	12	4.20 3.95 3.90 3.82 3.81 3.66 3.63 3.59 3.52 2.60	2.56E-33 8.36E-25 2.2E-31 1.51E-30 2.39E-26 4.67E-25 2.17E-27 2.72E-19 <1E-45 2.44E-7	4.29	<0.05
hydroxysteroid 11-beta dehydrogenase 2	*hsd11b2*	334098	6	6	3.83 3.80 5.72 4.99	<1E-45 4.23E-11 <1E-45 <1E-45	5.37	<0.05

					4.96	<1E-45		
					4.95	<1E-45		
rhesus blood group C glycoprotein b	*rhcgb*	792274	5	8	3.96	3.6E-37	6.30	<0.05
					3.8	3.54E-31		
					3.36	<1E-45		
					2.32	1.55E-16		
					4.18	<1E-45		
solute carrier family 2 (facilitated glucose transporter) member 12	*slc2a12*	393510	4	6	3.3	6.12E-21	1.63	<0.05
					2.47	<1E-45		
					2.3	<1E-45		
					2.15	1.22E-15		
hydroxysteroid (17-beta) dehydrogenase 3	*hsd17b3*	393335	5	7	2.81	<1E-45	3.06	<0.05
					2.74	1.71E-36		
					2.66	3.64E-44		
					2.43	1.03E-36		
					2.38	<1E-45		
alanine-glyoxylate aminotransferase b	*agxtb*	79378	5	6	5.64	<1E-45	4.42	<0.05
					5.51	<1E-45		
					5.42	<1E-45		
					4.21	4.12E-36		
					6.50	<1E-45		
Genes downregulated by beclo								
zgc:92005	*zgc:92005*	445477	3	6	-5.47	<1E-45	-5.26	<0.05
					-5.83	<1E-45		
					-11.79	<1E-45		
cytochrome P450 family 2 subfamily AA polypeptide 3	*cyp2aa3*	386638	2	3	-2.94	1.14E-29	-5.26	<0.05
					-3.06	<1E-45		
interleukin 8	*il8*	100002946	6	6	-2.93	2.81E-14	-3.70	<0.05
					-2.94	2.07E-25		
					-3.19	3.28E-23		
					-4.47	9.69E-24		
					-2.06	6.39E-29		
					-2.71	5.86E-10		

Supplemental Table 1: Details of 13 genes found by microarray analysis to be significantly (p-value $<10^{-5}$ and a fold change of either > 2 or <-2) regulated by beclo treatment and subsequently subjected to qPCR validation (Fig. 5). Fold changes refer to beclo-mediated fold induction or suppression per probe based on WT/vehicle vs WT/beclo comparison for both microarray and qPCR analysis.

Gene name	Gene symbol	Gene ID	Probes regulated by beclo	Probes/gene on the array	Fold change (microarray)	P-value (microarray)	Fold change (qPCR validation)	P-value (qPCR validation)
Genes upregulated by beclo								
LOC100151204 cytochrome P450 2F2-like	*LOC100151204*	100151204	2	4	**2.90** / *-3.97* **2.71** / *-3.56*	**9.22E-19** / *1.4E-12* **<1E-45** / *4.6E-14*	**1.8** / *2.9*	**<0.05** / *>0.05*
FGFR1 oncogene partner 2	*fgfr1op2*	335511	1	5	**3.86** / *-5.56*	**3.93E-8** / *3.69E-12*	**-1.15** / *-1.11*	**>0.05** / *>0.05*
novel immune-type receptor 2e	*Nitr2e*	368526	1	3	**3.33** / *-2.52*	**3.35E-8** / *4.43E-6*	**-1.05** / *-1.25*	**>0.05** / *>0.05*
zinc finger, CCHC domain containing 9	*zcchc9*	386639	1	3	**3.11** / *-3.59*	**9.27E-6** / *1.92E-6*	**-1.01** / *1.05*	**>0.05** / *>0.05*
si:dkey-14d8.21	*si:dkey-14d8.21*	558317	6	14	**3.15** / *-2.03*	**1.37E-21** / *7.99E-7*	**1.62** / *1.56*	**>0.05** / *>0.05*
zgc:165670	*zgc:165670*	100073345	2	3	**2.98** / *-2.03*	**1.6E-31** / *2.48E-13*	**1.82** / *2.56*	**<0.05** / *>0.05*
Genes downregulated by beclo								
chemokine (C-C motif) receptor 12.3	*ccr12.3*	563803	1	1	**-6.45** / *8.69*	**6.8E-10** / *1.9E-12*	**-1.35** / *-1.11*	**>0.05** / *>0.05*
protocadherin 2 gamma 5	*pcdh2g5*	554053	1	3	**-2.55** / *4.45*	**4.7E-6** / *1.3E-10*	**1.10** / *-1.05*	**>0.05** / *>0.05*

Supplemental Table 2: Details of 8 genes found by microarray analysis to be significantly (p-value $<10^{-5}$ and a fold change of either >2 or <-2) regulated by a dominant negative zGRβ effect and subsequently subjected to qPCR validation. Numbers in **bold** refer to beclo-mediated fold change per probe based on WT/vehicle vs WT/beclo comparison for both microarray and qPCR. Numbers in *italics* refer to zGRβ-mediated fold change per probe based on WT/beclo vs zGRβ/beclo comparison for both microarray and qPCR analysis.

Gene name	Gene symbol	Gene ID	Probes regulated by zGRβ	probes/gene on the array	Fold change (microarray)	P-value (microarray)	Fold change (qPCR validation)	P-value (qPCR validation)
acyl-CoA thioesterase 11a	*Acot11a*	554842	1	7	9.02	3.6E-8	1.12	>0.05
G protein-activated inward rectifier potassium channel 1-like	*LOC557095*	557095	1	5	5.52	1E-13	1.12	>0.05
Zinc finger, AN1-type domain 1	*zfand1*	445257	1	3	-4.95	3.7E-6	-1.01	>0.05
Cytochrome P450, family 17, subfamily A, polypeptide 1	*cyp17a1*	399692	1	6	-29.94	<1E-45	-1.19	>0.05
melatonin receptor 1C	*mtnr1c*	30661	1	3	-7.23	5.4E-9	-1.04	>0.05
serine/threonine kinase 33	*stk33*	565828	1	6	-4.15	5.1E-6	-1.18	>0.05
trh1 protein	*trh1*	259250	1	3	-3.26	1.3E-17	-1.01	>0.05
heat shock protein 47	*hsp47*	30449	5	9	-2.75 -2.89 -3.04 -2.96 -2.84	2.2E-6 7.1E-6 7E-6 1.4E-6 3.3E-6	-1.59	>0.05
zgc:111983	*zgc:111983*	550501	2	11	-2.31 -25.43	2.1E-7 <1E-45	-1.2	>0.05
synaptotagmin VIII	*Syt8*	570237	1	4	-3.29	1.1E-7	-1.19	>0.05

Supplemental Table 3: Details of 10 genes found by microarray analysis to be significantly (p-value $<10^{-5}$ and a fold change of either >2 or <-2) regulated by zGRβ and subsequently subjected to qPCR verification. Fold changes refer to zGRβ-mediated fold induction or suppression per probe based on WT/vehicle vs zGRβ/vehicle comparison for both microarray and qPCR analysis.

.

Gene name	Gene symbol	Forward primer sequence	Reverse primer sequence
Zinc finger, AN1-type domain 1	*zfand1*	TGGCTGAACTAGACATTGGAAAGC	AACAGCTGCTACACACGAATGGT
acyl-CoA thioesterase 11a	*Acot11a*	GGCTTCCGTGAACACCGTCAAT	CGCAAACTTGCCTTAGGAGTCTG
G protein-activated inward rectifier potassium channel 1-like	*LOC557095*	GCCCCCGAGAAGAAAAGGAA	CGGCCGTTCTTGTCCACAAA
Cytochrome P450, family 17, subfamily A, polypeptide 1	*cyp17a1*	GTTCCACATGGAAGTTTCACCG	GAACCTTCTCCAAACATGCAAAGA
melatonin receptor 1C	*mtnr1c*	GATCTTCACCACCGTGGCTG	TGCATTCCTCAGTTTCTTGTTCC
serine/threonine kinase 33	*stk33*	TAGAGATGATGCGTCAGTTCCG	GTGCAGTCCCTCGCTCGTCT
trh1 protein	*trh1*	CGATGAAAGCGAGCCTGATG	TGCGCTCTTCTTCTTCTCcG
heat shock protein 47	*hsp47*	GGAGTGGGACACAGAGGGAAAC	TCCTCATCTTCTCACTGCcG
zgc:111983	*zgc:111983*	AGCCACAACAGCTTCAACCG	TTGTGGCTCCAGATGCAGTG
synaptotagmin VIII	*Syt8*	TTTCTCTCCGCTATGTCCCG	CTGAAGAGCCTACCTGGTCCATC
Cytochrome P450 2F2-like	*LOC100151204*	GTTGACTGCACTGGTTTTGTTGTG	CGCCGAATTCTCACTTGCAAG
FGFR1 oncogene partner 2	*fgfr1op2*	CCCGTTGCTGCTGATAAGGA	TGGCATCAGCCAGAACCTTCTC
novel immune-type receptor 2e	*Nitr2e*	TCACATTTGGAAACAGCACAGACC	CAGACTCGATGTTCACTCCACCAG
zinc finger, CCHC domain containing 9	*zcchc9*	TAATTCCATCACTCTGGGCCG	CACATGCACGTCCTCATGGTC
si:dkey-14d8.21	*si:dkey-14d8.21*	CTGCAAAGCTCACCACCAGAC	GATATACTGTTGTGCAGTGCcG
zgc:165670	*zgc:165670*	CTGAACGAAGCCTTCCGTGTT	TCCCAATGAGAACAGCACcG
chemokine (C-C motif) receptor 12.3	*ccr12.3*	TGAAGGCAGCATCGTAACCG	TGAATCGAATCACATCGGCTTT
protocadherin 2 gamma 5	*pcdh2g5*	CCTCAGATATTATACCCCTCTCCG	CAGCTTTGGGCACCATCTCG
Bactin1	*Bactin1*	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
FK506 binding protein 5	*fkbp5*	TCTGCCAGCACAAGATTCGTGAGC	GACCCTGCTTATTCTGATCGGAAA
phosphoenolpyruvate carboxykinase 1	*pepck*	CAGGGCGATCTGGCGTCTCT	CTGCTGTCGATGAACTCCcG
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha ALPHA	*nfkbiaa*	CTTGGGCTAAAGTAGTCACcG	GATGGCAAGGTGCAGATACGTG
nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*zGRbeta*	GATGAACTACGAATGTCTTA	GCAACAGACAGCCAGACAGCTCACT
nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)	*zGRa*	AACTGGCAACGGTTCTATCAGCTCA	TTCTGGTGAAAGAGCAGCGG
zgc:92005	*zgc:92005*	AGGAGGGATACGGGAGACcG	CGTCTATCTCACGGTGGATGTG
cytochrome P450, family 2, subfamily AA, polypeptide 3	*cyp2aa3*	CCGTGTGTTAGAGGAAAGCcG	CACTGGCTTGCCTTCATCTTTG
interleukin 8	*il8*	TGTGTTATTGTTTTCCTGGCATTTC	GCGACAGCGTGGATCTACAG
sulfotransferase family 2, cytosolic sulfotransferase 3	*sult2st3*	CTTCGTGGAGTCTTGTGCcG	TACTGACGACACGATCCAAAGC
interleukin 26	*il26*	TAAACCCTTCCATCGGATATTGG	TCATCATAGATTTCCAGGACAcG
serine/threonine kinase 35	*stk35*	CCACAGGGACCTCAAACCAGA	CCTTGATGACAGGATTGCcG
uncoupling protein 2	*ucp2*	CGTTCCTCCTACTGCCACcG	GGTGTCCAGTGGAAAGGTGAAG

hydroxysteroid 11-beta dehydrogenase 2	*hsd11b2*	AACCCCAGGTGCGATACTAcG	AACCTGTCGCTGATGCTGAGTG
rhesus blood group, C glycoprotein b	*rhcgb*	GTGCTTCGTCTGGCAGGTGT	GTGTCTGCCTCCTCGTTATACcG
solute carrier family 2 (facilitated glucose transporter), member 12	*Slc2a12*	ACTATGCAGAGCCGCACACAC	CGTGAAGTTTGCTGTATTACcG
hydroxysteroid (17-beta) dehydrogenase 3	*hsd17b3*	CGCAATGACAGGACATCAGAAG	CTTCTCACAAACTCCTCAGCcG
alanine-glyoxylate aminotransferase b	*agxtb*	TGAAGCATCACCAGATCGAGTTT	GTATCCCATGAGCCCAATCcG

Supplemental Table 4: Sequences of all primers used for qPCR analysis.

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