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Zebrafish Mutant Lines Reveal the Interplay between nr3c1 and nr3c2 in the GC-Dependent Regulation of Gene Transcription

Alberto Dinarello 1, Annachiara Tesoriere 1, Paolo Martini 2, Camilla Maria Fontana 1, Davide Volpato 1, Lorenzo Badenetti 1, Francesca Terrin 1, Nicola Facchinello 1, Chiara Romualdi 1, Oliana Carnevali 3, Davide Volpato 1, Luisa Dalla Valle 1,*,†,‡ and Francesco Argenton 1,†

1 Department of Biology, University of Padova, 35121 Padova, Italy; alberto.dinarello@phd.unipd.it (A.D.); annachiara.tesoriere@phd.unipd.it (A.T.); camillamaria.fontana@phd.unipd.it (C.M.F.); davide.volpato.4@studenti.unipd.it (D.V.); lorenzo.badenetti@phd.unipd.it (L.B.); francesca.terrin@phd.unipd.it (F.T.); nicola.facchinello@unipd.it (N.F.); chiara.romualdi@unipd.it (C.R.); francesco.argenton@unipd.it (F.A.)
2 Department of Molecular and Translational Medicine, University of Brescia, 25121 Brescia, Italy; paolo.martini@unibs.it
3 Department of Life and Environmental Sciences, Università Politecnica delle Marche, 60131 Ancona, Italy; o.carnevali@univpm.it
* Correspondence: luisa.dallavalle@unipd.it
† These authors contributed equally to this work.
‡ These authors contributed equally to this work.

Abstract: Glucocorticoids mainly exert their biological functions through their cognate receptor, encoded by the nr3c1 gene. Here, we analysed the glucocorticoids mechanism of action taking advantage of the availability of different zebrafish mutant lines for their receptor. The differences in gene expression patterns between the zebrafish gr knock-out and the grs357 mutant line, in which a point mutation prevents binding of the receptor to the hormone-responsive elements, reveal an intricate network of GC-dependent transcription. Particularly, we show that Stat3 transcriptional activity mainly relies on glucocorticoid receptor GR tethering activity: several Stat3 target genes are induced upon glucocorticoid GC exposure both in wild type and in grs357 larvae, but not in gr knock-out zebrafish. To understand the interplay between GC, their receptor, and the mineralocorticoid receptor, which is evolutionarily and structurally related to the GR, we generated an mr knock-out line and observed that several GC-target genes also need a functional mineralocorticoid receptor MR to be correctly transcribed. All in all, zebrafish mutants and transgenic models allow in vivo analysis of GR transcriptional activities and interactions with other transcription factors such as MR and Stat3 in an in-depth and rapid way.

Keywords: glucocorticoid receptor; mineralocorticoid receptor; zebrafish; CRISPR/Cas9

1. Introduction

The glucocorticoid receptor (GR) is encoded by the NR3C1 gene and normally localized in the cytoplasm in a multimeric complex composed of heat shock protein (HSP) 70 and 90 and immunophilins. When glucocorticoids (GCs) bind to GR, the FK506-binding protein 51 (FKBP51) is substituted by FKBP52 and dynein is recruited to allow the migration of GC/GR in the nucleus [1]. Once in the nucleus, GR detaches from HSP90 [2] and regulates the transcription of target genes directly interacting with DNA at the level of hormone response elements (HRE). HREs are cis elements shared with other steroid receptors such as the mineralocorticoid receptor (MR), the androgen receptor (AR) or the progesterone receptor (PR) [3]. Additionally, GR can affect the activity of other transcription factors through tethering or complex protein–protein–DNA interactions described in Ratman et al. [4]. The lack of GR in mice is lethal: Nr3c1 knock-out (KO) animals die after birth for severe delay of lung development [5]. On the other hand, Galdimon mice, characterized by the A458T
mutation in the second zinc finger that abrogates GR dimerization and the subsequent HRE-dependent transactivation, are vital and reach adulthood, hence underlining alternative GR-dependent mechanisms of transcription activation [6]. Although GR monomers expressed by Gr<sup>dim/dim</sup> mice mutants have been proven to bind DNA efficiently [7,8], recent data obtained by Johnson and collaborators demonstrated that GR monomers poorly bind to chromatin and induce the transcription of a restricted number of GC-related genes [9].

However, with reduced transcriptional activity, GR monomers can indeed bind several transcription factors such as nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), signal transducer and activator of transcription 3 (STAT3) and activator protein-1 (AP-1) [10,11] and thus the Gr<sup>dim/dim</sup> model can allow discriminating between GR activity as dimer or monomer.

As recently reviewed by Dinarello et al. [12], in the last 20 years, zebrafish has gained importance for the study of GC/GR activities and several mutants have been generated for the genes encoding GR or the enzymes involved in the synthesis of GCs. In zebrafish, GR is encoded by a single gene called nr3c1, which shares a high similarity with the human orthologue [13].

In this work, taking advantage of two zebrafish mutant lines, the nr3c1<sup>ia30</sup> [14] and the nr3c1<sup>s357</sup> [15,16], we decided to better investigate in vivo the different mechanisms used by GR in the regulation of gene transcription. The nr3c1<sup>ia30</sup> (herein called gr<sup>ia30/ia30</sup>) line has been generated with CRISPR/Cas9 technology and is characterized by an insertion of 5 nucleotides in the second exon of the nr3c1 gene. This mutation leads to a frameshift and a premature stop codon, and the mutation affects the response to stress stimuli, the feeding entrainment of zebrafish circadian clock and reproduction [14,17,18]. On the other hand, the nr3c1<sup>s357</sup> mutant line, generated with N-ethyl-N-nitrosourea (ENU) mutagenesis, has a point mutation that prevents GR interaction with the DNA [19]. However, nr3c1<sup>s357/s357</sup> mutants (herein called gr<sup>s357/s357</sup>) can still synthesize an entire GR protein (replacing an arginine 443 with a cysteine in the second zinc finger) available for interaction with other possible proteins and, hence, retaining all its HRE independent transcriptional activities. Starting from the experimental evidence shown by Facchinello et al. [14] and Vettori et al. [20], we wanted to analyse in more detail the differences between these two gr mutant lines, aiming to show how their use can help to uncover the functions that GR accomplishes by directly interacting with the DNA and the processes affected through the regulation of other transcription factors.

A complete evaluation of GR activity as a transcription factor cannot disregard the role of MR, that, interacting with GR in heterodimeric or heterotetrameric complexes, seems to regulate GR-dependent activities [21,22], affecting the response to GCs at the transcriptional level. In detail, GR and MR can interact with each other [23,24] and the heterodimer can affect gene transcription, thus increasing the potential roles of GC in tissues such as the brain, that expresses both receptors [22,24,25]. Consequently, the MR:GR ratio is critical for the maintenance of neuronal functions [26] and its imbalance was connected to behavioural dysfunctions, cognitive disorders [27] and depression in humans [28–30]. The higher expression of MR in the teleost brain also suggests that, in zebrafish, MR has a major role in regulating stress axis behaviour [31,32].

The GR and MR collaboration in the regulation of target genes transcription is particularly interesting in teleosts. GR and MR are structurally, evolutionarily, and functionally tightly connected [33] but, while in mammals MR also binds a specific ligand, aldosterone, and regulates ion balance and homeostasis [21,34], it has been demonstrated that in zebrafish GR accomplishes these functions [35–40]. Hence, in zebrafish, MR exerts only cortisol-related activities and Danio rerio can be an excellent model to study all the MR/cortisol-dependent processes that are also conserved in mammals, such as obesity, heart failure and depression [21]. For these reasons and to better elucidate the interplay between GR and MR, we decided to generate a new mr (nr3c2) KO zebrafish line with CRISPR/Cas9 technology, to investigate the role of MR in the regulation of GC/GR-dependent transcription.
2. Results
2.1. The Expression of GC-Dependent Genes klf9, epas1a and ucp2 Confirms Differences between gr\textsuperscript{ia30/ia30} and gr\textsuperscript{s357/s357} Mutants’ GCs Response

Prior to the analysis of the differences between the two gr mutant lines at the transcriptional level, we confirmed the downregulation of \textit{nr3c1} mRNA expression only in gr\textsuperscript{ia30/ia30} compared to gr\textsuperscript{+/+} siblings (named gr\textsuperscript{+/+}, to distinguish them from gr\textsuperscript{+/+}, siblings of gr\textsuperscript{s357/s357} larvae) (Figure 1A,B). Therefore, only in the gr\textsuperscript{ia30/ia30} mutants the protein is absent or, when present, it is truncated and completely non-functional.

Figure 1. GR direct target genes show slight differences of expression between gr\textsuperscript{ia30} and gr\textsuperscript{s357} mutant lines. (A) RT-qPCR analysis of \textit{nr3c1} in 6 dpf gr\textsuperscript{+/+} and gr\textsuperscript{ia30/ia30} larvae. (B) RT-qPCR analysis of \textit{nr3c1} in 6 dpf gr\textsuperscript{+/+} and gr\textsuperscript{s357/s357} larvae. (C) RT-qPCR analysis of klf9 in 6 dpf gr\textsuperscript{+/+} and gr\textsuperscript{ia30/ia30} (C) and in gr\textsuperscript{+/+} and gr\textsuperscript{s357/s357} (C') larvae with or without Dex treatment. (D) RT-qPCR analysis of epas1a in 6 dpf gr\textsuperscript{+/+} and gr\textsuperscript{ia30/ia30} (D) and in gr\textsuperscript{+/+} and gr\textsuperscript{s357/s357} (D') larvae with or without Dex treatment. (E) RT-qPCR analysis of ucp2 in 6 dpf gr\textsuperscript{+/+} and gr\textsuperscript{ia30/ia30} (E) and in gr\textsuperscript{+/+} and gr\textsuperscript{s357/s357} (E') larvae with or without Dex treatment. Statistical analyses were performed with Student’s \textit{t} test. Mean ± SEM. * \textit{p} < 0.05; ** \textit{p} < 0.01; *** \textit{p} < 0.001.

Mainly, our experimental approach was based on RT-qPCR analysis of the expression of GC-dependent genes in basal conditions or after 6 h of treatment with the synthetic GC Dexamethasone (Dex).

Since both lines, gr\textsuperscript{ia30/ia30} and gr\textsuperscript{s357/s357}, lack a GR that can bind to HREs (respectively due to gr KO and to the point mutation in GR DNA-binding domain), we found the expression of GR direct target genes significantly dampened in both lines. As already reported by Facchinello and collaborators [14], two important GC/GR target genes like \textit{fk506-binding protein 5} (\textit{fkbp5}), which belongs to the negative feedback loop induced by GC/GR for self-regulation, and \textit{forkhead box protein O 3b} (\textit{foxo3b}), which is upregulated by GCs [41], are significantly downregulated in both mutant lines compared to wild type (WT) and, in both gr\textsuperscript{ia30/ia30} and gr\textsuperscript{s357/s357} homozygous mutants, Dex treatment does not induce the expression of these two genes.

Here, as reported in Figure 1C,D, we confirmed the previous results by analysing the expression of other two GC/GR-dependent genes: \textit{krueppel-like factor 9} (\textit{klf9}), whose GC-dependent induction has a pro-inflammatory effect upon prolonged cortisol exposure [42], and \textit{endothelial PAS domain protein 1a} (\textit{epas1a}), a GC-regulated gene that encodes for hypoxia-
inducible factor 2α (HIF2α) [43,44]. These results demonstrate that both mutant lines cannot induce the expression of klf9 and epas1a when exposed to exogenous GCs, confirming that HRE-related gene transcription cannot be properly induced in both mutant lines. Of note, in gr^ia30/ia30 line, at the basal level the epas1a expression is significantly upregulated, suggesting that the high levels of cortisol in gr^ia30/ia30 larvae [14] can determine the upregulation of GC-target genes probably acting via other steroid hormone receptors, such as the MR that has a high affinity for cortisol [26].

Furthermore, we measured the level of expression of uncoupling protein 2 (ucp2) that encodes for a proton transporter [45] and allows the GCs regulation of mitochondrial biogenesis in muscle [46]. Interestingly, it is severely downregulated in gr^ia30/ia30 compared to gr^+/-1. On the other hand, no significant differences were observed between gr^+/-2 and gr^s357/s357, thus revealing a basal differential expression of ucp2 in gr^ia30/ia30 and gr^s357/s357. While a 6 h long treatment with Dex in gr^+/-1 determines an upregulation of this gene, both mutant lines appeared to be insensitive to Dex treatment since we could not detect significant differences in ucp2 expression levels between treated and untreated mutants, suggesting that this gene is properly activated via the DNA-binding domain (Figure 1E).

These results confirmed that direct DNA-binding-dependent GC-induced transcription is significantly dampened in these two lines, even if some small differences are evident.

2.2. Analysis of GC-Dependent Transcription in mr Mutant Zebrafish Larvae

As mentioned above, MR is an important modulator of GR activity for its capability to regulate GC-dependent transcription generating MR/GR heterodimers and heterotetramers and to bind HRE promoter regions on DNA. MR is encoded by the nr3c2 gene and in zebrafish has a high affinity for cortisol and for 11-deoxycorticosterone (DOC) [31]. To test whether MR has a function in the different expression patterns of gr^ia30 and gr^s357 zebrafish lines and to evaluate the possible roles of MR/GR interaction in the expression of GC-target genes, we decided to generate a new nr3c2 zebrafish mutant line with CRISPR/Cas9 approach. The nr3c2^ia32/ia32 (herein called mr^ia32/ia32) zebrafish mutant line is characterized by a deletion of 11 nucleotides in the third exon of the nr3c2 gene. This mutation determines a frameshift with the subsequent generation of a premature stop codon (Figure S1A–D). Homozygous mutants are predicted to encode an aberrant, truncated protein of 628 amino acids, 17 of which are determined by the mutation. The mutated MR lacks the DBD, the LBD and the activation domain in the C-terminus (Figure S1A).

Moreover, homozygous mutants are also characterized by a significant reduction of nr3c2 mRNA expression compared with mr^+/- siblings (Figure 2A), possibly due to the nonsense-mediated mRNA decay (NMD) commonly activated in KO zebrafish lines [47]. mr^+/-, mr^ia32 and mr^ia32/ia32 animals can be easily distinguished by PCR with the primers listed in Table 1 (Figure S1E). Homozygous mutants are viable and reach adulthood, even if the mutation has some effects on survival: as reported in Figure S1F, the percentage of mutants observed at one-month post-fertilization is significantly lower (17%) than the expected one (25%). Interestingly, nr3c1 mRNA expression is more upregulated in mr^ia32/ia32 larvae than mr^+/- siblings (Figure 2B), suggesting that compensation mechanisms are triggered in this mutant line. Of note, nr3c2 mRNA levels are significantly higher in gr homozygous mutants compared to WT siblings, while this transcript is significantly downregulated in gr^s357/s357 mutants compared to WT (Figure 2C,D), showing another substantial difference between these two gr mutant lines.
Figure 2. Analysis of GC-dependent genes in mr mutant zebrafish larvae. (A) RT-qPCR analysis of nr3c2 in 6 dpf mr+/+ and mria32/ia32. (B) RT-qPCR analysis of nr3c1 in 6 dpf mr+/+ and mria32/ia32. (C) RT-qPCR analysis of nr3c2 in 6 dpf gr+/+ and gria30/ia30. (D) RT-qPCR analysis of nr3c2 in 6 dpf gr+/+ and gria30/ia30. (E) RT-qPCR analysis of klf9 in 6 dpf mr+/+ and mia32/aia32 larvae with or without Dex treatment. (F) RT-qPCR analysis of epas1a in 6 dpf mr+/+ and mia32/aia32 larvae with or without Dex treatment. (G) RT-qPCR analysis of ucp2 in 6 dpf mr+/+ and mia32/aia32 larvae with or without Dex treatment. Statistical analyses were performed with Student’s t test. Mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

Table 1. List of primers (5′–3′ sequences) used for genotyping and for RT-qPCR.

| Gene | Forward Sequence (5′–3′) | Reverse Sequence (5′–3′) | Use |
|------|--------------------------|--------------------------|-----|
| nr3c1 | ACCAATCTCAACGGCAGCAAG | CGGCCTTCTGTATCCTGCC | Genotyping |
| nr3c2 | GACGACCAAGTGTGTTGCTG | TGGACCGCATTCTGACCTG | Genotyping |
| stat3 | GGCCCTCTCTGATAGTGACCG | GAGAAACCATATGTTGGAC | RT-qPCR |
| ube2a | CATCATGGTCTGGAACGCTG | GAGGAACCGTCAAGGCACTG | RT-qPCR |
| nr3c1 | CAACACAATTACCTGTGTGCTG | CTTGACGTGCCTTTGACTTG | RT-qPCR |
| nr3c2 | CGAATGAGCAACGTGTGCC | CAGAAGAGAGTGGTGCTG | RT-qPCR |
| klf9 | GGGGAGGAGGAGGAGGAGG | TTTGGGAGGAGGAGGAGG | RT-qPCR |
| epas1a | CTTGCGGACATGGCAGCGA | CGTACAAAGACCCCGATG | RT-qPCR |
| ucp2 | CTGCGGAAAACATTCCTCA | CTCAGCCACATCTTACCT | RT-qPCR |
| sla2s5a | CATGAGACAGGAGGAGGAGG | TGAATCTCAGATACCATG | RT-qPCR |
| soc3a | GAAACCTCAGACACGGCAGAC | GAAAGACGGGAGGAGGAGG | RT-qPCR |
| ilk2 | GAAACAGACCTCAGCTCTC | TCTTCTGAGGAGGAGGAGG | RT-qPCR |
| hif1al | ATGGTGCAGCTATGCTG | AGAACAGACTTCATATG | RT-qPCR |
| pmpla3 | CCTCTGAGCGACGCT | GGAAGAGAGGAGGGAATT | RT-qPCR |
| ddit4 | GAACCTGACCCGCAACCAC | TTACAAAGGCGCTTCTCGT | RT-qPCR |
| pomca | TCTGCGACACCCGACGAC | TCTTCTGAGGAGGAGGAGG | RT-qPCR |
| fdp5 | GTGTGTCCGACACTACAC | TCTTCTGAGGAGGAGGAGG | RT-qPCR |

The upregulation of nr3c1 transcript and the possible compensatory effect that characterizes mia32/ia32 mutants is further supported by the higher fluorescence intensity of mia32/ia32 and mr+/ia32 larvae in Tg(9xGCRE-HsflUI23:EGFP)ia20 background (the zebrafish reporter line of GC-related transcription described by Benato et al. [48]) when compared to mr+/+ siblings (Figure S2A). This phenomenon probably derives from the increased expression of nr3c1 mRNA in mutants compared to WT since, as reported by Faught and Vijayan [32] with their mria402 zebrafish KO line, the lack of Mr does not lead to hypercortisolism, a feature that is further confirmed in the mia32 line, where the levels of expression of pomca are not affected in mr mutants compared to wild type siblings (Figure S2B).
Then, we tested the expression levels of the genes previously analysed in gr mutants, comparing mr+/+ and mrα32/α32 larvae and testing their responsiveness to Dex. Interestingly, mr KO determines a strong insensitivity to exogenous GCs since the expression levels of klf9, epas1a and ucp2 are significantly different in treated mrα32/α32 compared to treated mr+/+ (Figure 2E–G). Additionally, it is worth noting that the lack of a functional MR significantly affects the basal expression of epas1a and ucp2. These results suggest that MR is essential for the correct GC-dependent transcriptional response and that it exerts many functions in the regulation of hypoxia response and mitochondrial homeostasis.

To better investigate this last aspect, we tested the expression of other mitochondrial proteins in the mutant lines of interest. We analysed the level of expression of ucp3 and solute carrier family 25 member 25 (SLC25A25). As shown in Figure 3, these transcripts are upregulated by Dex in gr+/+ and mr+/+ larvae. However, while grα30/α30 show a basal lower level of expression of these transcripts compared to gr+/+ siblings and appear to be totally insensitive to exogenous GCs (Figure 3A,C), grα357/α357 larvae do not show significant differences compared to gr+/+ and Dex upregulates the expression of these genes either in gr+/+ and in grα357/α357 (Figure 3A,C). It is worth mentioning that all these transcripts are significantly downregulated in grα30/α30 compared to gr+/+ in basal conditions and we could not see any statistically significant difference between gr+/+ and grα357/α357, suggesting that the two mutant lines have different transcriptional profiles in basal conditions. Considering also the expression profiles of the same genes in the mrα32/α32 larvae, only ucp3 showed a reduction in basal expression in mrα32/α32 larvae if compared to mr+/+ siblings (Figure 3B,D). Interestingly, these genes do not show a response to Dex stimulation in mrα32/α32 larvae, as observed in grα30/α30 (Figure 3B,D). These results suggest that possibly GR regulates the expression of these genes in a DNA-binding independent way and the expression of the identified mitochondrial proteins is also connected to MR function.

![Figure 3](image-url)

**Figure 3.** GC-dependent genes reveal different expression levels in the two nr3c1 zebrafish mutant lines. (A) RT-qPCR analysis of ucp3 in 6 dpf gr+/+ and grα30/α30 (A) and in gr+/+ and grα357/α357 (A')
larvae with or without Dex treatment. (B) RT-qPCR analysis of ucp3 in 6 dpf mr+/+ and mria32/ia32 larvae with or without Dex treatment. (C) RT-qPCR analysis of slc25a25 in 6 dpf gr+/+ and gria30/ia30 (C) and in gr+/+ and gria357/s357 (C') larvae with or without Dex treatment. (D) RT-qPCR analysis of slc25a25 in 6 dpf mr+/+ and mria32/ia32 larvae with or without Dex treatment. Statistical analyses were performed with Student’s t test. Mean ± SEM. * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ns = not significant.

2.3. gria30/ia30 and gria357/s357 Zebrafish Lines Reveal DNA-Binding Independent Mechanisms of Regulation of Stat3

The tight connection between GR and other transcription factors has already been studied [49]. Specifically, the crosstalk of GR with STAT3 was widely described by Langlais and collaborators [50]. Here, for the first time with in vivo models, we decided to better elucidate how the GR–Stat3 crosstalk works and identify some Stat3-dependent transcripts which need GR to be properly activated. The analysis of the crosstalk between GR and Stat3 allowed us to highlight again the differences between gria30/ia30 and gria357/s357 zebrafish mutant lines. Firstly, taking advantage of the Tg(7xStat3-Hso.Ul23:EGFP)ia28 zebrafish reporter line that expresses EGFP in Stat3-positive cells [51], we tested whether Stat3-dependent transcription in intestinal stem cells is altered in gria30/ia30 and gria357/s357 mutant lines. Interestingly, as reported in Figure 4A, gria30/ia30;Tg(7xStat3-Hso.Ul23:EGFP)ia28 6 dpf larvae are characterized by a significantly lower intestinal fluorescence compared to gr+/+;Tg(7xStat3-Hso.Ul23:EGFP)ia28 siblings. On the other hand, we could detect a slight but not significant difference in EGFP fluorescence between gria357/s357;Tg(7xStat3-Hso.Ul23:EGFP)ia28 compared to gr+/+;Tg(7xStat3-Hso.Ul23:EGFP)ia28 larvae (Figure 4B). This result suggests that Stat3 transcriptional activation is different in the two gr zebrafish mutant lines analysed and that GR-dependent regulation of Stat3 activity relies mainly on DNA-binding independent mechanisms of GR. Interestingly, 24 h long treatment with 10 µM Dex did not affect the fluorescence of Stat3 reporter in the intestine of WT larvae (Figure S3A). Furthermore, we also decided to see whether Stat3 signalling affects GR transcriptional activity. To do so, we analysed the levels of fluorescence of the GC zebrafish reporter line Tg(9xGCRE-Hso.Ul23:EGFP)ia20 [48] after chemical inhibition of the Stat3 pathway with AG490 (Figure 4C). No significant differences in reporter fluorescence were detected, meaning that the inhibition of Stat3 does not affect GC/GR-dependent transcription. To confirm this result, we also measured the fluorescence of Tg(9xGCRE-Hso.Ul23:EGFP)ia20 in stat3 mutant background, taking advantage of a stat3ia23 zebrafish mutant line [51,52]. No significant differences between stat3+/+, stat3+/ia23 and stat3ia23/ia23 6 dpf larvae were observed (Figure S3B). Additionally, Dex treatment determines a significant increase of fluorescence in all the three genotypes analysed compared to untreated controls (Figure S3B). These results demonstrated that the lack of Stat3 does not significantly interfere with the nuclear activities of the GC/GR pathway. Moreover, a further confirmation of this result came from RT-qPCR analysis of fbkp5 performed on stat3+/+, stat3+/ia23 and stat3ia23/ia23 after Dex treatment: we could not detect significant differences in fbkp5 expression between stat3+/+, stat3+/ia23 and stat3ia23/ia23 and Dex treatment significantly induced the expression of this gene in all the genotypes under investigation (Figure S3C). However, it is worth noting that 3-day long treatment of Tg(9xGCRE-Hso.Ul23:EGFP)ia20 with leukaemia inhibitory factor (LIF), an activator of the Jak/Stat3 pathway that upregulates the intestinal fluorescence of Stat3 reporter line (Figure S3D), determines a significant upregulation of GC reporter fluorescence (Figure 4C,C'), demonstrating that the overstimulation of the Jak/Stat3 pathway determines the activation of GR transcriptional activity.
Figure 4. Analysis of crosstalk between GR and Stat3 with zebrafish mutant and transgenic lines. (A) Representative pictures (A) and fluorescence quantification (A’) of Tg(7xStat3-Hsv.Ul23:EGFP)ia20 6 dpf larvae at the level of the intestine in gr+/a1, gr+/a30 and gra30/a30 genetic background. Scale bar= 100 μm. (B) Representative pictures (B) and fluorescence quantification (B’) of Tg(7xStat3-Hsv.Ul23:EGFP)ia28 6 dpf larvae at the level of the intestine in gr+/a2, gr+/a357 and gra357/a357 genetic background. Scale bar = 100 μm. (C) Representative pictures (C) and fluorescence quantification (C’) of Tg(9xGCRE-Hsv.Ul23:EGFP)ia28 incubated from 3 to 6 dpf with DMSO, 50 μM AG490 and 20 μM LIF. Scale bar = 500 μm. (D) Representative pictures of 6 dpf larvae generated by the breeding between gra30/a30/stat3+/a23 zebrafish. Scale bar = 500 μm. (E) Table of observed (OV) and expected (EV) values of animals belonging to the 9 different genotypes obtained from breedings between gra30/a30/stat3+/a23 zebrafish: χ² test shows not significant differences between OV and EV in genotype distribution until 72 hp (p-value = 0.7196); significant differences between OV and EV were detected at 4 dpf (** p-value = 0.0058) and 3 mpf (**** p-value = 4.30949 × 10⁻²²). Mean ± SEM. Statistical analyses were performed with Student’s t test (A,B,C) and χ² test (E). * p < 0.05; ** p < 0.01; *** p < 0.001; ns = not significant.

Further data showing the different impacts of gr/a30 and gr/a357 mutations on Stat3 are shown in Figure 4D. We bred gr/a30 mutants with stat3+/a23 larvae and subsequently, we performed gr/a30/stat3+/a23 double heterozygotes crosses to obtain the 9 possible genotypes. Interestingly, gr/a30/stat3+/a23 double homozygous mutants are characterized by severe developmental defects like large cardiac oedema, lack of yolk absorption, small head size and start dying at 4 dpf (Figure 4D,E). The severe phenotype of these double mutants was expected, since the roles of Stat3 and GR in development are well-known [53,54]. Interestingly, the defects described above were also detected in gr/a30/stat3+/a23, gr/a30/stat3+/a23.
and in some gr\textsuperscript{ia30}/stat3\textsuperscript{ia23} larvae. Notably, gr\textsuperscript{ia30/ia30}/stat3\textsuperscript{ia23} and gr\textsuperscript{ia30}/stat3\textsuperscript{ia23} cannot reach adulthood (Figure 4D,E). Finally, in five breedings between double heterozygous, only three adult gr\textsuperscript{ia30/ia30}/stat3\textsuperscript{ia23} have been observed reaching the humane endpoint at 3 months of age. Conversely, the offspring generated by the breeding between gr\textsuperscript{fa30}/stat3\textsuperscript{fa23} double heterozygotes adult zebrafish did not show any morphological defects and adult gr\textsuperscript{fa30/faf357}/stat3\textsuperscript{fa23} have been detected with the expected frequency (12.5%). These results confirmed that GR and Stat3 activities are tightly connected and that GR DNA-binding properties are not required for Stat3 activation and the proper induction of Stat3 transcription.

2.4. GR Regulates Stat3-Transcriptional Activities in DNA-Binding Independent Mechanisms with a Contribution of MR

Further investigations of GR-dependent control of Stat3 transcriptional activity were performed with RT-qPCR analysis of Stat3 target genes in both gr\textsuperscript{ia30/ia30} and gr\textsuperscript{fa30/faf357} 6 dpf zebrafish larvae. We analysed the levels of expression of suppressor of cytokine signalling-3a (socs3a), hypoxia-inducible factor 1x like (hif1xal), unc-51 like autophagy activating kinase 2 (ulk2), patatin-like phospholipase 3 (pnpla3) and DNA damage-inducible transcript 4 (ddit4). socs3a is a Stat3 target gene that represses the Jak/Stat3 pathway and binds Janus Kinases (Jaks), inhibiting their function as an activator of Stat3 [55]. The hif1xal gene encodes for hypoxia-inducible factor 3x (Hif3x), a factor involved in hypoxia response—a process in which both GR and Stat3 are well-established players [20,56–60]. In addition, its promoter is predicted to harbour Stat3 binding elements (https://maayanlab.cloud/Harmonizome/ accessed on 12 September 2021). Ulk2 is an autophagy inducing kinase and its transcription is positively modulated by Stat3 [61]. Pnpla3 encodes for an enzyme that has hydrolase activity on retinyl esters and triglycerides [62] whereas ddit4 is upregulated in response to different stressors and its transcription is induced by GCs [63] and phosphorylated Stat3 [64].

socs3a and hif1xal expression are not different between gr\textsuperscript{fa30/ia30} and gr\textsuperscript{fa30/faf357} mutants and their respective WT siblings, meaning that, in basal conditions, they are not differentially expressed in the two genetic backgrounds. Additionally, they are significantly induced in gr\textsuperscript{fa30} upon Dex treatment, confirming their GC-based transcriptional regulation. However, while gr\textsuperscript{fa30/ia30} appeared to be completely insensitive to exogenous GCs (Figure 5A,B), we could observe a not significant increase in socs3a and hif1xal expression in gr\textsuperscript{fa30/faf357} larvae upon Dex treatment (Figure 5A′,B′). Evidence about the differential control of Stat3 target gene expression in the two gr mutant lines came from the analysis of ulk2, pnpla3 and ddit4. Dex significantly induces the expression of these three genes in gr\textsuperscript{fa30}, gr\textsuperscript{fa30/faf357} and gr\textsuperscript{fa30/faf357}, when compared to untreated siblings, but had no effect on gr\textsuperscript{fa30/ia30} larvae (Figure 5A–E,C′–E′). These results demonstrate that GCs regulate the expression of some Stat3-related genes mainly in a GR–DNA binding independent mechanism.

Moreover, we sought to assess whether the differential control of Stat3 target genes observed in gr\textsuperscript{fa30/ia30} and gr\textsuperscript{fa30/faf357} can be due to compensating effects by MR. A first analysis of the mr mutant in Tg(7xStat3-Hsa:U123:EGFP)mr\textsuperscript{ia28} Stat3 reporter line background demonstrated that, at least in the intestine, the lack of a functional MR does not affect Stat3-dependent transcription (Figure S2C). However, RT-qPCR analysis of the genes already reported in Figure 4 demonstrated that the absence of a functional MR partially affects the GC-mediated control of Stat3-related genes. As reported in Figure 5, the responsiveness of mr\textsuperscript{ia32/ia32} for Dex is not significantly affected as regards the expression levels of socs3a, hif1xal and ddit4, since we could not see significant differences in their expression in treated mr\textsuperscript{ia32/ia32} compared to treated mr\textsuperscript{ia32/ia32}. However, ulk2 and pnpla3 mRNA expression is not upregulated in mutants, revealing that mr\textsuperscript{ia32/ia32} show defects in the correct expression of Stat3-target genes and demonstrating that MR plays a role in this process together with GR. These transcriptional patterns show that some of these Stat3-dependent genes are controlled by GR alone (like socs3a, hif1xal and ddit4), but other genes, like pnpla3 and ulk2, need both GR and MR to be properly induced. Of note, the basal upregulation of ddit4...
in $mr^{ia32/ia32}$ compared to $mr^{+/+}$ can be due to the significant upregulation of $nr3c1$ that characterizes $mr$ KO animals (Figure 2B).

**Figure 5.** Stat3-dependent genes are differentially expressed in $gr^{ia30}$ and $gr^{s357}$ mutant lines. (A) RT-qPCR analysis of $socs3a$ in 6 dpf $gr^{+/+}$ and $gr^{ia30/ia30}$ (A) and in $gr^{+/+}$ and $gr^{s357/s357}$ (A') larvae with or without Dex treatment. (B) RT-qPCR analysis of $hif1al$ in 6 dpf $gr^{+/+}$ and $gr^{ia30/ia30}$ (B) and in $gr^{+/+}$ and $gr^{s357/s357}$ (B') larvae with or without Dex treatment. (C) RT-qPCR analysis of $ulk2$ in 6 dpf $gr^{+/+}$ and $gr^{ia30/ia30}$ (C) and in $gr^{+/+}$ and $gr^{s357/s357}$ (C') larvae with or without Dex treatment. (D) RT-qPCR analysis of $pnpla3$ in 6 dpf $gr^{+/+}$ and $gr^{ia30/ia30}$ (D) and in $gr^{+/+}$ and $gr^{s357/s357}$ (D') larvae with or without Dex treatment. (E) RT-qPCR analysis of $ddit4$ in 6 dpf $gr^{+/+}$ and $gr^{ia30/ia30}$ (E) and in $gr^{+/+}$ and $gr^{s357/s357}$ (E') larvae with or without Dex treatment. (F) RT-qPCR analysis of $socs3a$ in 6 dpf $mr^{+/+}$ and $mr^{ia32/ia32}$ larvae with or without Dex treatment. (G) RT-qPCR analysis of $hif1al$ in 6 dpf $mr^{+/+}$ and $mr^{ia32/ia32}$ larvae with or without Dex treatment. (H) RT-qPCR analysis of $ddit4$ in 6 dpf $mr^{+/+}$ and $mr^{ia32/ia32}$ larvae with or without Dex treatment. (I) RT-qPCR analysis of $ulk2$ in 6 dpf $mr^{+/+}$ and $mr^{ia32/ia32}$ larvae with or without Dex treatment. (J) RT-qPCR analysis of $pnpla3$ in 6 dpf $mr^{+/+}$ and $mr^{ia32/ia32}$ larvae with or without Dex treatment. Statistical analyses were performed with Student’s $t$ test. Mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; ns = not significant.

3. Discussion

In this work, we decided to investigate the possibility of in vivo distinction between the different GCs-GR mechanisms of transcription regulation. Although the $gr^{s357/s357}$ zebrafish mutant line has been used as a KO model by several research groups [16,20,65–70], our previous works have already suggested that DNA-binding independent mechanisms are still present in the $gr^{s357/s357}$ [14,20]. GR transcriptional activity mechanisms have been debated for a long time. The protein works as a transcription factor in a homodimeric complex binding DNA in several different conformations [71], but some research has also shown that GR monomers can interact with DNA activating the transcription of a small set of target genes [7–9]. Notably, GR activity relies on several phosphorylations that regulate
was further confirmed by both the severe phenotype of gr (Figure 1) suggesting that its basal expression might be regulated by GR in a DNA-binding dependent way. This GR protein cannot bind to DNA, but still maintains the possibility of regulating the activity of other related receptors that have a different affinity for corticosteroids. The higher affinity of MR allows the receptor to be occupied in basal conditions while the lower affinity of GR facilitates the regulation of different target genes without the interaction of GR with HREs [50]. This hypothesis has been confirmed by the Dex-related upregulation of these genes was detected in treated gr+/+ and gr<sup>s357/s357</sup> but not in gr<sup>ia30/ia30</sup>, thus confirming a substantial difference between gr<sup>ia30/ia30</sup> and gr<sup>s357/s357</sup>. We suppose that the GR regulation of Stat3 activity is due to nuclear interactions between these two transcription factors, a tethering mechanism that can control the expression of different target genes without the interaction of GR with HREs [50]. This hypothesis was further confirmed by both the severe phenotype of gr<sup>ia30/ia30</sup>/stat3<sup>ia23/ia23</sup> larvae, not detected in gr<sup>s357/s357</sup>/stat3<sup>ia23/ia23</sup> mutants, as well as the inability of the gr<sup>ia30/ia30</sup>/stat3<sup>+/+1</sup> and gr<sup>+/ia30</sup>/stat3<sup>3n23/23a23</sup> to reach adulthood.

Among the GC-responsive genes we analysed, ucp2, a member of the mitochondrial solute carrier 25 family largely involved in mitochondrial homeostasis, also controlled by Stat3 [76,77], is clearly downregulated in gr<sup>ia30/ia30</sup> compared to gr<sup>+/+</sup> siblings. Of note, we could not observe significant differences in ucp2 expression between gr<sup>+/+</sup> and gr<sup>s357/s357</sup> (Figure 1) suggesting that its basal expression might be regulated by GR in a DNA-binding dependent way. However, we did not observe a significant increase of ucp2 expression after Dex treatment of gr<sup>s357/a357</sup>, implying that ucp2 regulation by GC depends on the binding of GR to DNA. This trend represents an intermediate expression pattern between these two transcription factors, a tethering mechanism that can control the expression of different target genes through the interaction of GR with HREs [50].

Moreover, we need to keep in mind that GCs can bind GR and MR. Two different but related receptors that have a different affinity for corticosteroids. The higher affinity of MR allows the receptor to be occupied in basal conditions while the lower affinity of GR determines its activation in response to GCs-increase following the stress or in phase with the GCs-circadian increase [82].
Both receptors are members of the steroid receptor family and show a high level of similarity: they compete for the same ligands and share the same hormone-responsive elements (HREs) on DNA [21]. Hence, to better understand the mechanisms underlying the differential expression of the genes analysed in basal and GC-stimulated conditions in the \( \text{gr} \) mutant lines, we took into consideration MR and generated a new \( \text{mr}^{\text{ia32/ia32}} \) zebrafish line. The homology between GR and MR and the similar mechanisms of action justify the compensation mechanism detected in both mutant lines: \( \text{gr}^{\text{ia30/ia30}} \) and \( \text{mr}^{\text{ia32/ia32}} \) showed an upregulated expression of respectively \( \text{mr}3c2 \) and \( \text{mr}3c1 \) transcripts when compared to WT.

Of note, GR and MR can form heterodimers with each other, regulating the expression of specific target genes probably with a different efficacy: more specifically, MR homodimers are the main complex working at low levels of GCs, while MR/GR heterodimers and GR homodimers formations are predominant in stress conditions [21]. This fact can be an interesting starting point for the interpretation of our data. In the \( \text{mr}^{\text{ia32/ia32}} \) line, the expression of canonical GC-dependent genes \( \text{klf9} \) and \( \text{epas1a} \) is downregulated in basal conditions; on the contrary, in \( \text{gr}^{\text{ia30/ia30}} \) \( \text{klf9} \) expression does not show significant differences when compared to \( \text{gr}^{+/+} \) and \( \text{epas1a} \) seems to be even more expressed in \( \text{gr}^{\text{ia30/ia30}} \) rather than \( \text{gr}^{+/+} \). Interestingly, regarding these two genes, the transcriptional response to Dex is completely erased in both \( \text{gr} \) and \( \text{mr} \) KO lines. Hence, considering the model of Gomez-Sanchez and collaborators [16], we can hypothesize that in basal conditions the expression of \( \text{klf9} \) and \( \text{epas1a} \) is regulated by MR homodimers. For this reason, in \( \text{gr}^{\text{ia30/ia30}} \) line the expression of \( \text{klf9} \) and \( \text{epas1a} \) is not impaired and the upregulation of \( \text{mr}3c2 \) in \( \text{gr}^{\text{ia30/ia30}} \) explains the higher levels of expression of \( \text{epas1a} \) in \( \text{gr} \) KO. Upon Dex treatments, the response is possibly driven by GR/MR heterodimers as the sensitivity of these two mutant lines to GCs is severely dampened. Additionally, \( \text{pnpla3} \) also showed expression profiles in mutant lines that are compatible with the mechanism of regulation described by Gomez-Sanchez et al. [21]: GR seems to be not involved in the regulation of their expression in basal conditions, while \( \text{mr}^{\text{ia32/ia32}} \) line showed a downregulated expression of \( \text{pnpla3} \) in basal conditions suggesting that MR homodimers control their transcription. Furthermore, \( \text{ULK2} \) seems to be sensible to Dex stimulation in an MR and GR-dependent way. On the contrary, \( \text{hifl1l} \), \( \text{ddit4} \) and \( \text{soc53a} \) do not seem to be regulated by MR: \( \text{soc53a} \) and \( \text{hifl1l} \) levels of expression in basal conditions are not impaired in \( \text{mr}^{\text{ia32/ia32}} \) line, in both basal and stimulated conditions; in the same way, \( \text{ddit4} \) does not show a difference between \( \text{mr}^{+/+} \) and \( \text{mr}^{\text{ia32/ia32}} \) larvae. Moreover, the significant upregulation in \( \text{mr}^{\text{ia32/ia32}} \) compared to WT suggests the dependency of \( \text{ddit4} \) to GR, since \( \text{mr} \) mutants are characterized by high levels of expression of \( \text{gr} \). We can conclude that MR probably cooperates with GR in the regulation of some Stat3 targets, regulating both their basal expression (\( \text{pnpla3} \)) and Dex induced response (\( \text{pnpla3} \) and \( \text{ulk2} \)).

Starting from the interesting expression profile of \( \text{ucp2} \) in \( \text{gr}^{\text{ia30/ia30}} \) and \( \text{gr}^{\text{ia357/ia357}} \) zebrafish lines, we have investigated the expression pattern of other solute carrier family 25 members (\( \text{ucp3} \) and \( \text{slc25a25} \)) to clarify how both MR and GR could be involved in the regulation of mitochondrial homeostasis. These two targets are upregulated by exogenous GCs and their expression relies on GR: in basal conditions the expression of these transcripts is significantly downregulated in \( \text{gr} \) KO compared to WT, but not in \( \text{gr}^{\text{ia357/ia357}} \) compared to \( \text{gr}^{+/+} \), suggesting a basal control of their expression by DNA-binding independent mechanism. After Dex exposure, the scenario slightly changes, and expression profiles demonstrate a more intricate mechanism of regulation. While \( \text{ucp2} \) appears to be regulated by GR-DNA-binding dependent mechanisms, the response to Dex of \( \text{ucp3} \) and \( \text{slc25a25} \) in \( \text{gr}^{\text{ia357/ia357}} \) is independent from GR-DNA-binding. Interestingly, \( \text{ucp2} \) and \( \text{ucp3} \) genes showed similar expression patterns in \( \text{gr}^{\text{ia30/ia30}} \) and \( \text{mr}^{\text{ia32/ia32}} \), with a downregulation of transcripts in both basal and stimulated conditions compared to \( \text{gr}^{+/+} \) and \( \text{mr}^{+/+} \) respectively, suggesting that both receptors can be involved in the regulation of mitochondrial functions. On the contrary, the basal expression of \( \text{slc25a25} \) in \( \text{mr}^{\text{ia32/ia32}} \) is not impaired, while in \( \text{gr}^{\text{ia30/ia30}} \) it is downregulated. Finally, transcriptional data suggest that both GR
and MR are involved in Dex stimulation of *upc3* and *slc25a25* genes. The level of expression of the analysed genes is summarized in Table 2.

| Gene          | gr*ia30/ia30 vs. gr*+/+ | gr*ia357/ia357 vs. gr*+/+ | mr*ia32/ia32 vs. mr*+/+ |
|---------------|-------------------------|---------------------------|-------------------------|
| *klf9*        | =                       | =                         | =                       |
| *epas1a*      | ⇑                       | ⇓                         | ⇓                       |
| *ucp2*        | ⇓                       | =                         | ⇓                       |
| *ucp3*        | ⇓                       | =                         | ⇓                       |
| *scl25a25a*   | ⇓                       | =                         | ⇓                       |
| *socs3a*      | ⇓                       | =                         | ⇓                       |
| *hif1a1*      | =                       | =                         | =                       |
| *ulk2*        | =                       | =                         | =                       |
| *pnpla3*      | =                       | =                         | =                       |
| *ddit4*       | =                       | =                         | =                       |

In conclusion, we demonstrated in vivo that GC-dependent genes are regulated through several mechanisms that rely on both GR and MR. As already known, GR exerts its transcriptional activities by both binding directly to HRE, but also by tethering with other transcription factors. Our models allowed us to verify in vivo the two different mechanisms and to demonstrate that Stat3 is one of the most important transcriptional partners of GR. Notably, GR regulates Stat3 transcriptional activity mostly in a DNA-binding independent way, even if we cannot exclude a marginal role of canonical GR transcriptional activities in the modulation of the Jak/Stat3 pathway. Finally, it is worth mentioning that mitochondrial homeostasis is heavily regulated by GR, confirming the strong involvement of this protein in mitochondria activities [83–85]. Notably, the GR effect on the mitochondrial genes here analysed seems to be GR–DNA-binding independent and needs MR to be properly determined. All in all, the results obtained highlight the central role of MR as a prominent regulator and enhancer of GR transcriptional activity and underline the potential of the *gr* KO and *gr*ia357/ia357 mutants for deepening knowledge on GC-dependent transcriptional mechanisms.

4. Materials and Methods

4.1. Animal Husbandry and Zebrafish Lines

Animals were staged and fed as described by Kimmel et al. [86] and maintained in a large-scale aquaria system. Embryos were obtained after natural mating, raised in Petri dishes containing fish water (50×: 5 g NaHCO₃, 39.25 g CaSO₄, 25 g Instant Ocean for 1:l) and kept in a 12:12 light/dark cycle at 28 °C. All experimental procedures complied with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU).

*gr*ia30 [14], *mr*ia32 and *stat3ia23* [51] mutant lines are genotyped by PCR amplification and 3% agarose gel migration. *gr*ia357 animals are genotyped as described in Facchinello et al. [9]. The Tg(7xStat3-Hsv.Ul23:EGFP)ia28 line and the Tg(9xGCRE-Hsv.Ul23:EGFP)ia20 line have been respectively characterized by Peron et al. [51] and by Benato et al. [48].

4.2. Generation of mr Zebrafish Mutant Line

The generation of the *mr* mutant zebrafish line was performed using the CRISPR/Cas9-mediated genome editing. Briefly, the CHOPCHOP (https://chopchop.rc.fas.harvard.edu)
accessed on 26 January 2022) and E-CRISP (available at http://www.e-crisp.org/E-CRISP/ accessed on 26 January 2022) software were used to design the gene-specific guide RNA (sgRNA) (GAGGCGTCAGGATGCCACTACGG) to target \textit{nr3c2} gene on exon 2 following the protocol described in Gagnon et al. [87]. The guide was subsequently produced using the protocol described in Gagnon et al. [87]. Fertilized eggs were injected with 1 nL of a solution containing 280 ng/µL of Cas9 (M0386T, New England Biolabs) and 3 pmol/µL of \textit{nr3c2}-targeting sgRNA. Genomic DNA was extracted from 4 dpf injected larvae to test the presence of mutations and to confirm the activity of the Cas9 enzyme. Injected embryos were raised to adulthood and screened, by F1 genotyping, for germline transmission of the mutation. An F1 mutant carrier harbouring a deletion of 11 nucleotides was selected and bred with WT to obtain the F2 generation. The resulting heterozygous F2 individuals were incrossed, to obtain F3 generation that includes homozygous mutants.

4.3. Imaging

For in vivo imaging, transgenic larvae were anaesthetized with 0.04% tricaine, embedded in 1% low-melting agarose and mounted on a depression slide. Nikon C2 confocal system was used to acquire images from \textit{Tg(7xStat3-Hsv.Ul23:EGFP)ia28} transgenic larvae. \textit{Tg(9xGCRE-Hsv.Ul23:EGFP)ia20} transgenic larvae were mounted in 1% low-melting agarose and observed with a Leica M165 FC microscope equipped with a Nikon DS-Fi2 digital camera. All images were analysed with Fiji (ImageJ2, Madison, WI, USA) software and fluorescence integrated density was calculated setting a standard threshold on non-fluorescent samples as described in Facchinello et al. [88].

4.4. Animal Treatments

We exposed 6 dpf \textit{gr}^{+/+}, \textit{gr}^{ia30/ia30}, \textit{gr}^{s357/s357}, \textit{mr}^{+/+}, and \textit{mr}^{ia32/ia32} larvae to 10 µM Dex for 6 h and the treatments started at 3 p.m. At the end of the treatment, larvae were anaesthetized and sacrificed for RNA extraction. \textit{Tg(9xGCRE-Hsv.Ul23:EGFP)ia20} reporter larvae were treated with 50 µM AG490, 10 µM Dex and 20 µM LIF from 3 to 6 dpf.

4.5. mRNA Isolation and Quantitative Real-Time Reverse Transcription PCR (RT-qPCR)

Total RNAs were extracted from pools of 20 larvae at 6 dpf with TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA, 15596018) and incubated at 37 °C for 30 min with RQ1 RNase-Free DNase (Promega, M6101, Madison, WI, USA). cDNA synthesis was performed using random primers (Promega, C1181) and M-MLV Reverse Transcriptase RNase H (Solis BioDyne, 06-21-010000) according to the manufacturer’s protocol. qPCRs were performed in triplicate with SybrGreen method by means of CFX384 Touch-Real Time PCR Detection System and the 5x HOT FIREPol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia, 08-36-00001) and \textit{ube2a} and \textit{actb} were used as internal standards in each sample. The amplification protocol consists of 95 °C for 14 min followed by 45 cycles at 95 °C for 20 s, 60 °C for 20 s and 72 °C for 25 s. Threshold cycles (Ct) and melting curves were generated automatically by CFX384 Touch-Real Time PCR Detection System (Biorad, Hercules, CA, USA) and results were obtained with the method described in Livak and Schmittgen [89]. Sequences of genes of interest primers are listed in Table 1.

4.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Different experimental groups for RT-qPCR and fluorescence quantifications were compared using Student’s \textit{t} test. Observed genotype distributions (OV) were compared to the expected values (EV) (+/+ = 25%; +/− = 50%; −/− = 25%) using χ² test. * \textit{p} < 0.05; ** \textit{p} < 0.01; *** \textit{p} < 0.001, **** \textit{p} < 0.0001; ns = not significant.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/ijms23052678/s1.
Author Contributions: Conceptualization, A.D., L.D.V. and F.A.; methodology, A.D., A.T., C.M.F., D.V., L.B., F.T. and N.F.; validation, A.D., A.T. and C.M.F.; formal analysis, A.D., A.T., P.M., C.R., O.C. and L.D.V.; investigation, A.D., A.T., D.V., L.B., F.T. and N.F.; resources, F.A., O.C. and L.D.V.; writing—original draft preparation, A.D., A.T. and L.D.V.; writing—review and editing, F.A. and L.D.V. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Progetti di Ricerca di Ateneo (CPDA134095), University of Padova (to L.D.V.); Associazione Italiana per la Ricerca sul Cancro grant IG 2017 19928 (to F.A.); Fondo di Ateneo 2019 (to O.C.).

Institutional Review Board Statement: All animal experiments were performed under the permission of the ethical committee of the University of Padova and the Italian Ministero della Salute (23/2015-PR and 112/2015-PR).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Acknowledgments: We are thankful to the personnel at the Zebrafish Centre of the University of Padova (Luigi Pivotti, Shkendy Iljazi, Martina Milanetto and Natascia Tiso). We thank Herwig Baier (Max Planck Institute of Neurobiology, Germany) for sharing the grs*357A357 zebrafish mutant line.

Conflicts of Interest: The authors declare no conflict of interest.

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