Klf9 is a key feedforward regulator of the transcriptomic response to glucocorticoid receptor activity

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Abstract

The zebrafish has recently emerged as a model system for investigating the developmental roles of glucocorticoid signaling and the mechanisms underlying glucocorticoid-induced developmental programming. To assess the role of the Glucocorticoid Receptor (GR) in such programming, we used CRISPR-Cas9 to produce a new frameshift mutation, GR^{369-}, which unlike previously-reported frameshift mutations eliminates all potential in-frame initiation codons upstream of the DNA binding domain. Using RNA-seq to ask how this mutation affects the larval transcriptome under both normal conditions and with chronic cortisol treatment, we find that GR mediates most of the effects of the treatment, and paradoxically, that the transcriptome of cortisol-treated larvae is more like that of larvae lacking a GR than that of larvae with a GR, suggesting that the cortisol-treated larvae develop GR resistance. The one transcriptional regulator that was both underexpressed in GR^{369-} larvae and consistently upregulated in cortisol-treated larvae was *klf9*. We therefore used CRISPR-Cas9-mediated mutation of *klf9* and RNA-seq to assess Klf9-dependent gene expression in both normal and cortisol-treated larvae. Our results indicate that Klf9 contributes significantly to the transcriptomic response to chronic cortisol exposure, mediating the upregulation of proinflammatory genes that we reported previously.

Introduction

The vertebrate hypothalamus-pituitary-adrenal (HPA) axis orchestrates physiological, behavioral, and metabolic adjustments required for homeostasis, by dynamically regulating production and secretion of adrenal steroids known as glucocorticoids. In humans the primary glucocorticoid is cortisol, the biological activity of which is mediated by two regulatory proteins in the nuclear receptor family, the ubiquitous glucocorticoid receptor (GR) and the more tissue-restricted mineralocorticoid receptor (MR). The GR binds cortisol less avidly than the MR and is thus more dynamically regulated over the normal physiological range of cortisol fluctuations[1,2]. The GR and MR function both as transcription factors and as non-nuclear signaling proteins, including in the central nervous system where both proteins are highly expressed[1-5]. Given that the GR is more widely expressed and more dynamically regulated by cortisol, it is generally thought to be the principal mediator of cortisol-induced genomic responses to circadian rhythms and acute stress[5].

A GR target gene that was recently shown to mediate circadian regulation of cell proliferation in human epidermis is *KLF9*[6], which encodes a ubiquitously expressed[7] member of the krüppel-like family of zinc finger transcription factors. Klf9 is a repressor important for neurogenesis and control of neural plasticity[8-11], and is a central player in the GR-responsive gene regulatory network in macrophages[12], where it functions to repress another GR target gene, *klf2*. In this context the GR, *klf9*, and *klf2* constitute a regulatory circuit known as an 'incoherent type-1 feed-forward loop' (i1-FFL)[12], a network motif commonly used to generate pulsatile dynamics and accelerate responses[13-15]. GR signaling often exhibits incoherent feed-forward regulatory logic[16-18]. In epidermis many of the genes repressed by Klf9 are also activated by the GR[6]. It was recently shown that *klf9* is upregulated in mouse hippocampus in response to the onset of chronic stress, and blocking that upregulation prevents chronic stress-induced pathologies[19]. In the same study it was also shown that hippocampuses of women with major depression were found to overexpress *KLF9*[19]. Finally, it was recently discovered that Dexamethasone-induced upregulation of Klf9 promotes hepatic gluconeogenesis and hyperglycemia in mice[20]. Thus, *klf9* is a GR target gene that dynamically controls GC signaling, and its overexpression is implicated in stress-induced pathology.

The zebrafish has recently emerged as a model system well-suited to investigating the developmental functions of glucocorticoid signaling and mechanisms underlying developmental programming in response to chronic glucocorticoid exposure such as occurs with chronic early life stress[21-25]. As in humans, the endogenous glucocorticoid in zebrafish is cortisol, which is produced by the interrenal gland, the functional equivalent of the mammalian adrenal. Thus, the zebrafish homolog of the HPA axis is the Hypothalamus-Pituitary-Interrenal (HPI) axis. Eight different zebrafish GR mutants have been described in the literature to date[26-29]. The first of these, *gr*^{s357}, is a missense mutation that substitutes a cysteine for an arginine in the DNA binding domain, disrupting DNA binding activity[26]. The other mutations, all produced by targeted mutagenesis using CRISPR-Cas9 or TALEN technology, consist of frameshift indels in exons 2 and 5[27-29], which respectively encode domains N- and C-terminal to the DNA binding domain (see Fig. 1). All of the zebrafish GR mutants manifest behavioral defects but are viable as homozygotes.

We showed previously that zebrafish larvae treated chronically with 1 μM cortisol upregulate proinflammatory genes and give rise to adults that maintain chronically elevated cortisol and display aberrant regulation of those same genes[23]. Thus in zebrafish, chronic cortisol exposure during early development results in persistent, long-term dysregulation of the HPI axis and gene expression downstream thereof, a 'developmental programming' effect similar to what has been observed with early life stress in mammals[23,25]. To ask whether and to what extent that developmental programming depends on the GR, we used CRISPR-Cas9 technology to

mutate the GR-encoding gene *nr3c1*. Here we report a new loss-of-function mutation, consisting of a deletion in *nr3c1* exon 3 that results in a frameshift and premature stop codon immediately upstream of the DNA binding domain which eliminates GR activity as a transcriptional activator. Using this mutant line, we performed an RNA-seq experiment to identify genes regulated by the GR and to parse GR-dependent and GR-independent effects of chronic cortisol treatment on larval gene expression. We found that most of the effects of the chronic cortisol treatment are mediated by the GR and identified *klf9* as the one transcriptional regulatory gene consistently upregulated by the GR under both normal conditions and in response to chronic cortisol treatment. We therefore used CRISPR-Cas9 and RNA-seq to interrogate the role of *klf9* with respect to the transcriptomic response to chronic cortisol treatment. Our results indicate that *klf9* mediates the cortisol-induced upregulation of pro-inflammatory genes that we previously reported[23].

Results

A frameshift deletion introduced into exon 3 of zebrafish *nr3c1* eliminates GR transcriptional function. To mutate the GR, we employed CRISPR-Cas9 using a guide RNA (gRNA) that targets *nr3c1* exon 3 (the second coding exon; Fig. 1A). This resulted in a 20 base deletion and frameshift that introduces sixteen new amino acids after E369, followed by a premature translational stop immediately upstream of the DNA binding domain (Fig. 1B, C), a mutation hereafter referred to as GR^{369-}. F0 males with GR^{369-} in the germline were identified and outbred to wild-type (AB) females, and siblings of that cross were mated to generate F2s, from which homozygous males and females were identified. Homozygous GR^{369-} males crossed with wild-type females produced viable embryos, whereas most homozygous GR^{369-} females crossed with either mutant or wild-type males produced embryos that all died within 24 hours of fertilization. However, one produced 10 viable offspring that were raised to adulthood, indicating that maternal GR is not essential for early development. When inbred these F3 fish produced no viable offspring. However, females homozygous for GR^{369-} following another generation of outcrossing produced variable numbers of viable offspring. These results suggest that loss of GR function may compromise egg quality, albeit in a context-dependent way.

The zebrafish *nr3c1* transcript has multiple AUG codons that could potentially initiate translation to allow production of alternative N-terminal isoforms from a single mRNA (Fig. 1C, E; Fig. S1), as occurs with human *nr3c1*[30] and some other transcription factors (e.g.[31]). One potential in-frame initiation codon upstream of the DNA binding domain lies downstream of all previously reported frameshift mutations in exon 2[27-29] (Fig. 1 C,E), suggesting that *nr3c1* mRNA transcribed in those mutants might still allow translation of an N-terminally truncated GR isoform with an intact DNA binding domain. To assess the transcriptional activity of different N-terminal isoforms we injected homozygous GR^{369-} zygotes with mRNA encoding full-length GR (FL) or GRs lacking the first 310 or 369 amino acids of the full-length GR (N310 and N369, Fig. 1D). The N310 mRNA corresponds to the shortest translation initiation variant with a DNA binding domain potentially available to the previously described exon 2 mutants[27-29], whereas the N369 corresponds to what is available in GR^{369-} mutants. The embryos were collected at 6 hours post fertilization (hpf) and quantitative reverse transcription and polymerase chain reaction (qRT-PCR) was used to measure the expression of the GR-target gene *fkbp5*. Unlike uninjected embryos or embryos injected with N369 or a *Xenopus* control mRNA, embryos injected with N310 mRNA upregulated *fkbp5*, indicating that N310 retains activity as a transcription factor (Fig. 1F). Zygotes injected with mRNA encoding full length GR showed ~50-fold stronger upregulation of *fkbp5* than those injected with N310 (Fig. 1F). We conclude that the previously described *nr3c1* exon 2 frameshift mutations[27-29] strongly abrogate but do not abolish GR transcriptional function, whereas GR^{369-} eliminates potential for translating a transcriptionally active GR.

RNA-seq shows that the transcriptomic response to chronic cortisol treatment is largely GR-dependent but converges with that caused by loss of GR function. To identify GR-dependent zygotic gene expression and assess the extent to which the GR contributes to the transcriptional effects of chronic cortisol exposure[23], we used a visual background adaptation (VBA) assay to identify homozygous GR^{369-} larvae from a heterozygous cross. This assay makes use of the fact that larval melanophores mount a camouflaging response to changes in background, dispersing when larvae are transferred from the dark to a light background[26]. This response is mediated by the GR, so larvae lacking a functional GR can be identified as those in which melanophores fail to respond, remaining clustered in a dark patch after transfer to a light background. At four days post fertilization (dpf) we used the VBA screen to separate homozygous GR^{369-} (VBA-) larvae from their heterozygous and wild type (VBA+) siblings developed under normal (vehicle-treated) conditions or in medium supplemented with 1 μM cortisol (Fig. 2A). In support of the screen's validity, the number of VBA- larvae was ~1/4 of the total number of larvae, the expected Mendelian ratio for homozygous mutants. The larvae developed for another day under the same conditions (vehicle- or cortisol-treated), at which time four biological replicates of each sample were collected for RNA-seq (Fig. 2A).

A principal component (PC) analysis of the RNA-seq data indicated that 60% of the variance in gene expression among samples is captured in the first two PCs, which respectively correlate with the two treatments (chronic cortisol and absence of a GR, respectively accounting for 46% and 14% of the variance; Fig. 2B). The eight VBA+ (i.e. mixed wild type and heterozygous mutant) samples cluster according to whether they were treated with cortisol, with the two clusters (cortisol-treated and vehicle-treated controls) segregating toward opposite poles of PC1. This is not the case in the VBA- (i.e. homozygous GR^{369-}) fish, the four cortisol-treated replicates of which are widely dispersed along PC1. This indicates that the global effect of the cortisol treatment captured in PC1 is GR-dependent and suggests as well that gene expression is less constrained overall in larvae lacking a GR. PC2 correlates with the presence and absence of a GR (VBA+ vs. VBA- respectively). Interestingly, along PC2 chronic cortisol treatment displaces the VBA+ transcriptome toward the VBA- pole, suggesting that the cortisol-treated fish adapt to the exposure by developing GR resistance.

The regulatory roles of the GR were further assessed by analyzing differential gene expression (DGE) between pairs of treatments, using an adjusted *p*-value (false-discovery) threshold of 0.05 as the criterion for differential expression. Comparison of VBA+ and VBA- larvae identified 405 genes affected by loss of the GR in 5-day larvae, 103 of which are underexpressed (Fig. 2C) and 302 of which are overexpressed in VBA- larvae compared to their VBA+ counterparts (Fig. S2A). Gene ontology (GO) analysis shows that the underexpressed genes (i.e. genes normally upregulated by the GR) are involved in sugar metabolism and response to heat, whereas the overexpressed genes (i.e. genes that are normally downregulated via the GR) are involved in basement membrane organization, epidermis development, cell adhesion, locomotion, and growth (Figs. S3 and S4; Table S1). A DGE analysis comparing cortisol-treated VBA+ fish to their vehicle-treated VBA+ counterparts showed that in cortisol-treated larvae with a functional GR, 4,298 genes were differentially expressed (Fig. S2B), 2,177 of which were upregulated (Fig. 2C) and 2,121 of which were downregulated. GO enrichment analysis of the upregulated genes identified biological processes related to nervous system development and function as well as cell adhesion, locomotion, and growth, whereas the downregulated genes were largely involved in protein synthesis and metabolism (Figs. S5, S6; Table S2). Interestingly, the transcriptome of cortisol-treated VBA+ larvae overlapped more with that of vehicle-treated VBA- larvae than with that of vehicle treated VBA+ larvae (Fig. S2C), and accordingly, many of the biological processes affected by the absence of GR function in VBA- larvae were similarly affected by the chronic cortisol-treatment in VBA+ larvae (Fig. S7). This again suggests that the latter larvae develop a GR resistant phenotype.

We reasoned that some of the effects of chronic cortisol exposure might stem from GR-induced upregulation of a GR target gene that functions as a feedforward transcriptional regulator of GR signaling. Such a gene should be both underexpressed in VBA- larvae and upregulated in VBA+ larvae in response to chronic cortisol (i.e., opposite of the predominant trend noted above). Of the 2,177 genes upregulated in cortisol-treated VBA+ larvae only four were basally underexpressed in VBA- larvae (Fig. 2C), two of which encode transcription factors: *klf9* and *per1a*. Of these, only *klf9* was also found to be significantly upregulated in our previous RNA-seq analysis of the effects of chronic cortisol treatment in wild-type fish (Fig. S2C), being one of the most highly upregulated transcription factors[23]. A plot comparing *klf9* expression in each of the conditions reveals that the GR contributes to both its normal developmental expression and its upregulation in response to chronic cortisol treatment (Fig. 2D). However, the plot also suggests that cortisol affects *klf9* in a GR-independent fashion, albeit more variably, as indicated by the range of expression levels in the cortisol-treated VBA- samples shown in Fig. 2D, which correlate with the spread of the cortisol-treated VBA- samples along PC1 shown in Fig. 2B.

In contrast to the situation in VBA+ larvae, only 8 genes were differentially expressed in cortisol-treated VBA- larvae compared to their vehicle-treated VBA- siblings, all of them upregulated (Fig. 2C, Fig. S2D). The genes included the immediate early genes (IEGs) *npas4a*, *egr1*, *egr4*, *fosab*, and *ier2b* (Fig. 2C, Fig. S8). The GR-independence of their cortisol-induced upregulation is clearly seen in a plot of the expression levels of the most highly upregulated gene of this set, *npas4a* (Fig. 2D), a neuronal IEG that along with the other IEGs was also found to be upregulated in our previous RNA-seq analysis of cortisol-treated larvae (Fig. S2D)[23]. This indicates that the GR mediates nearly all the transcriptomic effects of chronically elevated cortisol, except for a small subset that appears to relate to neuronal activity.

A frameshift deletion introduced into exon 1 of zebrafish *klf9* eliminates the DNA binding domain and significantly reduces expression of the mature transcript. The fact that *klf9* was the transcriptional regulatory gene most consistently found to be upregulated by chronic cortisol exposure in a GR-dependent way suggested that it may contribute to the transcriptomic effects of the exposure. To test this, we mutated *klf9* using CRISPR-Cas9 with a gRNA that targets exon 1 (Fig. 3A, B). This resulted in a frameshift mutation upstream of the DNA binding domain (Fig 3A, B), producing a defective transcript and truncated protein predicted to lack function as a transcription factor. *Klf9* loss-of-function mutations are viable in mice[32] and similarly, the *klf9* mutant fish were viable and fertile when bred to homozygosity, although mutant embryos survive at a lower rate than wild type. To ask how the mutation affects *klf9* expression we used quantitative reverse transcription and polymerase chain reaction (qRT-PCR) to compare *klf9* transcript levels in wild type and *klf9* homozygous mutant (hereafter referred to as *klf9*$^{-/-}$) larvae under both normal conditions and in response to chronic cortisol treatment. This provided further confirmation that chronic cortisol treatment leads to upregulation of *klf9* and revealed that mature *klf9* mRNA levels are significantly reduced in the *klf9*$^{-/-}$ larvae, probably due to nonsense-mediated decay triggered by the premature stop codon (Fig. 3C). In contrast and as expected, there was no effect on *klf9* pre-mRNA levels, measured by qRT-PCR of the intron (Fig. 3C). We conclude from these experiments that the frameshift mutation introduced into *klf9* exon 1 abrogates Klf9 function.

RNA-seq shows that *klf9* functions as a feedforward regulator of glucocorticoid receptor activity. To identify Klf9 target genes and ask whether Klf9 contributes to the transcriptomic response to chronic cortisol treatment we used RNA-seq to query gene expression in 5-day old wild type and *klf9*$^{-/-}$ mutant larvae from sibling parents, developed both normally and in the presence of 1 μM cortisol. Samples were collected at the same time on day 5 post-fertilization as in the previous GR knockout experiment and processed similarly.

However, PC analysis revealed the largest source of variance in this experiment was not due to genotype or treatment, but rather the order in which the samples were collected and processed (Fig. S9A). GO analysis of genes correlated with the first principal component showed that the later samples (replicates 3 and 4) had increased synaptic signaling and decreased translation (Table S3), suggestive of a physiological response to the stress of capture. However, a further confound is that PC1 also correlates with preparation of the RNA in two batches on separate days, suggesting that the variance that it captures may also be technical (see Materials and Methods). Reassuringly, after normalizing for this variation the samples segregate along two principal components representing genotype and treatment (Fig. S9B). Further analyses of the unnormalized data included the first PC as a covariate to control for the artifact introduced by the collection/preparation order. We were thus able to detect variance in the effect of cortisol treatment specifically attributable to *klf9* function, as hierarchical clustering identified 228 genes that were upregulated in cortisol-treated wild-type larvae but not cortisol-treated *klf9*$^{-/-}$ larvae (Fig. 4A and Fig. S10). About a fifth (43) of those genes were also identified in our previous study of the effects of chronic cortisol exposure[23] (Fig. 4B, C). Gene ontology term enrichment analysis of the subset of genes upregulated by chronic cortisol treatment in wild-type but not *klf9*$^{-/-}$ embryos revealed that they are involved in defense and immunity (Fig. 4D, Table S4), the same biological processes that we previously found to be the most strongly affected by the chronic cortisol treatment[23]. Similar results were obtained when qRT-PCR was used to measure expression of immune genes of interest in the same RNA samples (Fig. S11), indicating that the result is not an artifact of how the RNA-seq data were processed and analyzed. These results indicate that Klf9 is a GR-activated feedforward regulator that contributes significantly to the pro-inflammatory gene expression induced by of chronic cortisol exposure.

Discussion

The zebrafish has recently emerged as a model system for investigating the developmental functions of the GR and its role in glucocorticoid-induced developmental programming. Several previously published studies have characterized loss-of-function mutations of the zebrafish GR, including four frameshifting indels that introduce a premature stop codon in exon 2[27-29] (Fig. 1C). Unlike those mutations, our frameshift deletion (GR^{369-}) lies within exon 3 (Fig. 1A, B), which eliminates all possible in-frame initiation codons upstream of the DNA binding domain (Fig. 1C). The human GR exists as multiple translation initiation variants, all of which are transcriptionally active, including the shortest, which lack over 300 amino acids of N-terminal sequence contained in the full length GR[30]. One or more potential start codons remain available as in-frame initiation sites in all the previously reported exon 2 mutations of zebrafish *nr3c1* (Fig. 1), and thus those mutants produce mRNA that could potentially be translated into an N-terminally truncated GR. That such an isoform might retain activity as a transcription factor is shown by our observation that homozygous GR^{369-} zygotes injected with mRNA encoding a GR that lacks the first 310 amino acids of the full-length protein activate expression of the GR target *fkbp5*, albeit much less strongly than zygotes injected with mRNA encoding a full-length GR (Fig. 1F). In contrast, zygotes injected with mRNA encoding a GR lacking the first 369 amino acids and all start codons upstream of the DNA binding domain do not activate *fkbp5* (Fig. 1F). We conclude that GR^{369-} affords a complete loss of transcriptional function.

We used RNA-seq to determine how loss of GR function affects the larval transcriptome, both in larvae developed under normal conditions and in larvae treated chronically with cortisol. To overcome the low survival of embryos from homozygous mutant females and avoid nonspecific maternal effects that might be associated with poor egg quality, we took advantage of a visual background adaptation (VBA) screen to identify homozygous GR^{369-} progeny of a heterozygous cross, as those larvae lack a VBA response (VBA- larvae).

Based on the observation that VBA- larvae comprised ~¼ of the population, as expected for a recessive Mendelian trait, larvae that successfully mount a VBA response (VBA+ larvae) are predicted to consist of a 1:2 mixture of homozygous wild-type and heterozygous GR^{369-} mutants, and hence to contain at least one intact *nr3c1* allele encoding a functional GR. A principal component analysis of the RNA-seq data revealed that complete absence of the GR has a profound effect on gene expression in both normal and cortisol-treated larvae, such that transcriptomes from larvae lacking a GR are clearly distinguished from those that have one, and that cortisol treatment produces a coherent effect only in larvae with a functional GR (Fig. 2A). A pair-wise comparison of gene expression between VBA+ and VBA- larvae (accounting for PC2) identified with statistical significance about four hundred genes that are regulated by the GR in 5-day larvae at the time they were collected (midmorning). GO term enrichment analysis indicated that genes upregulated by the GR are involved in metabolism and stress response, as would be expected, while genes downregulated by the GR are involved in epidermis development, cell adhesion, growth, and basement membrane formation, suggesting that the GR may function as a switch to downregulate those morphogenetic processes in late development, or to temporally segregate them to a certain time of day given the circadian dynamic of glucocorticoid signaling. Interestingly, numerous biological processes and individual genes affected by loss of GR function were similarly affected by chronic cortisol treatment in larvae with a GR. This suggests that one effect of the chronic treatment is to promote development of GR resistance, and moreover, that it does so via the GR. Such resistance might be construed as an adaptive response to the chronic exposure.

Comparing gene expression in VBA+ larvae treated with cortisol versus vehicle (accounting for PC1) identified over four thousand genes that are differentially expressed in response to chronic cortisol treatment. This latter number is substantially larger than the 555 differentially expressed genes identified in our previous analysis[23] of the effects of chronic cortisol treatment and yielded a somewhat different result when subjected to GO term enrichment analysis. One major difference between the present analysis and that reported previously[23] is that in the present analysis the embryos and larvae were cultured in a light-dark cycle, whereas previously they were cultured in the dark. In zebrafish larvae the circadian clock is not synchronized until the fish are exposed to a light-dark cycle[33,34], so our previous results may have had circadian asynchrony as a confounding variable. However, arguing against this is that the RNA-seq results reported here comparing wild-type and *klf9*$^{-/-}$ larvae produced similar results to those of our earlier study and were from embryos developed with light-dark cycles. Another difference between the earlier study and that depicted in Figure 2 was that the latter measured transcriptomic effects of chronic cortisol treatment in a 1:2 mixture wild-type and heterozygous mutant larvae, whereas the previous was carried out in wild-type; thus the results in VBA+ larvae would be expected to be less sensitive to any effects of the chronic exposure for which the GR is haplo-insufficient.

Despite these differences, numerous genes identified as differentially expressed in the previous study were also identified in both RNA-seq experiments presented in the present study. These included *klf9*, one of only four GR-dependent genes in 5-day larvae that were upregulated in response to chronic cortisol, and only one of two that encode transcription factors, the other being *per1a* (Fig. 2B). Interestingly both *klf9* and *per1a* are involved in circadian regulation, and both have been shown to be GR targets in other vertebrate models[6-8,35]. Of these, *klf9* was one of the most highly upregulated transcriptional regulatory genes in our previous RNA-seq analysis, and was found to be the most commonly upregulated transcription factor in a recent meta-analysis of glucocorticoid-induced gene expression in the brain[36]. We have found by ATAC-seq that the promoter region of *klf9* is one of the most differentially open regions of chromatin in blood cells of adults derived from cortisol-treated embryos, possibly reflecting the chronically elevated cortisol levels maintained by those adults (Hartig et al., submitted). In mice *klf9* was recently shown to mediate glucocorticoid-induced metabolic dysregulation

in liver[20]. Our RNA-seq results from *klf9*$^{-/-}$ larvae show that Klf9 mediates the pro-inflammatory gene expression induced by chronic cortisol exposure that we reported previously[23]. Among other things Klf9 functions as a transcriptional repressor[10,11], and in mouse macrophages as an incoherent feedforward regulator of the GR target *klf2*[12], which functions to control inflammation[37]. Further work is needed to determine how *klf9* regulates pro-inflammatory gene expression, which could either be directly as a feedforward activator, or indirectly as a feedforward repressor of an anti-inflammatory regulator like Klf2.

Perhaps unsurprisingly, our results indicate that the GR mediates nearly all the transcriptomic effects of chronically elevated cortisol. However, eight genes upregulated by the treatment were found in the RNA-seq analysis to be upregulated in both VBA+ and VBA- larvae (Fig. 2B), indicating that their upregulation is not mediated by the GR. Interestingly most of these genes are known IEGs, and include the neuronal IEG *npas4a*, the mammalian homologue of which is directly repressed by the GR[38]. One possible explanation is that the genes are upregulated by increased MR activity, which was recently shown to contribute to stress axis regulation in zebrafish larvae[28]. Further work in MR mutant fish[28] will be needed to test this.

Gene ontology analysis of genes upregulated by chronic cortisol treatment indicated a strong effect on biological processes associated with nervous system development and function. This is consistent with a recent report that injection of cortisol into eggs leads to increased neurogenesis in the larval brain[24]. In this regard it is interesting that *klf9* is a stress-responsive gene that regulates neural differentiation and plasticity[8,19]. The long-term dysregulation of the HPA/I axis caused by early life exposure to chronic stress and/or chronically elevated cortisol suggests that the exposure perturbs brain development and activity. Given its role in regulating plasticity in brain regions relevant to neuroendocrine function, it will be interesting to determine whether *klf9* contributes to those effects.

Materials and Methods

Zebrafish strains, husbandry, and embryo treatments. The AB wild-type strain was used for all genetic modifications. Husbandry and procedures were as described previously[23]. All animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the MDI Biological Laboratory. Embryo culture and cortisol treatments were performed as previously described[23], with one difference: embryos were cultured in a diurnal light-dark cycle (14 hours light – 10 hours dark). Briefly, fertilized eggs were collected in the morning, disinfected and at ~4 hours post fertilization placed in dishes with either 1uM cortisol or vehicle (DMSO) added to embryo media. Embryos developed in a 28.5° C incubator with a 14/10 light/dark cycle synchronized with the core fish room. Media was changed daily.

Construction of *nr3c1* and *klf9* mutant lines. To mutate *nr3c1* and *klf9* we used CRISPR-Cas9[39], injecting zygotes with multiple guide RNAs for each gene and mRNA encoding Cas9. Guide RNAs were designed using the CHOP-CHOP algorithm[40,41].

To generate the GR^{369-} mutant line fertilized wild-type AB embryos were injected at the 1 cell stage with 1-2 nL of a gRNA cocktail targeting *nr3c1* exons 2 and 3 (final concentration 40 ng/µL for each gRNA, 230 ng/µL Cas9 mRNA, 0.1 M KCl, and 0.01% phenol red indicator dye). Individual whole injected larvae were screened for mutations of the targeted regions by high resolution melt analysis (HRMA) of PCR amplicons containing those regions[42]. Detected mutations were then verified by Sanger sequencing of a PCR amplicon containing the targeted region. F0 adults bearing mutations were identified by HRMA of DNA extracted from tailfin clips, and germline mutations were then identified by PCR and HRMA/sequencing of sperm. F0 males with germline mutations were outcrossed to AB females, and heterozygous progeny were screened via sequencing from

tailfin clips. The GR^{369-} mutation was identified and selected for by breeding over 2 additional generations to yield F3 homozygous progeny. Subsequent generations were maintained as heterozygotes for health and breeding purposes.

To generate the *klf9*$^{-/-}$ mutant line fertilized wild-type AB embryos were injected at the 1-cell stage with <20% cell volume of injection mix consisting of 200ng/ul Cas9 mRNA, 100ng/ul guide RNA, 0.05% phenol red dye, and 0.2M KCl. Individual F0 larvae were screened with HRMA to confirm CRISPR efficacy. Larvae were placed into system and raised. Young adult fish were genotyped via HRMA using DNA extracted from fin clips, and mutations were confirmed by Sanger sequencing. F0 fish positive for mutation were outcrossed to wild-type (AB) fish. F1 offspring of this cross were screened by HRMA to confirm germline transmission, and Sanger sequencing identified the 2bp frame-shift mutation in one female founder. This female was out-crossed with WT AB males. The resulting F2 fish were screened as young adults via fin clip and HRMA, and males positive for mutation were back-crossed to the F1 founder female. Resulting F3 generation fish were screened via fin clip HRMA and sequenced to identify homozygous mutants as well as homozygous wild-type siblings. F3 generation was Mendelian 1:2:1 wild-type:heterozygote:homozygous-mutant ratio.

Visual Background Adaptation (VBA) screen. To identify larvae lacking a functional GR a VBA screen was performed on 4-day old larvae as described[26]. Briefly, larvae were incubated for 20 minutes in a dark incubator then transferred to a white background and immediately examined under a stereomicroscope with brightfield optics. Larvae that failed to mount a VBA response were identified by the failure of melanophores to disperse, remaining clustered in a dark patch on the dorsal surface[26]. Larvae were segregated as VBA+ and VBA- cohorts and returned to culture for an additional day before they were collected for RNA-seq.

RNA-seq and Data Analysis. At 3 hours zeitgeber time (post lights-on) on day 5 post-fertilization four biological replicates of cortisol-treated and control embryos were collected as follows. For the first RNA-seq experiment, one by one, a dish of larvae corresponding to a single condition was removed from the incubator, and 8 larvae per replicate (4 replicates) were collected in a 1.5 mL tube with minimal water and immediately snap frozen in liquid nitrogen. All replicates from one condition were collected sequentially before moving on to the next condition. Total RNA was extracted using the Qiagen RNA-Easy Plus mini kit (Qiagen). For the second RNA-seq experiment, four replicates of n=10 larvae were collected from a single dish for each condition and immediately snap frozen in liquid nitrogen. Collection of 16 samples occurred over 22 minutes. RNA was prepared as described above on two different days. On the first day (experimental replicates 1 and 2) the lysis buffer was added to all 8 frozen samples before homogenization, while on the second day the lysis buffer was added to each sample which was then immediately homogenized. This difference in sample preparation likely accounts for the large variance between samples prepared on day 1 and day 2. RNA was sent to the Oklahoma State Genomics Facility for Illumina library preparation and single-end sequencing.

Fastq formatted read files were preprocessed with Trimmomatic[43] version 0.38 with default options, and then aligned to the Zebrafish genome version 11 as presented in Ensembl[44] version 93, using the STAR aligner[45] version 2.6.1b. The Ensembl transcriptome was preprocessed with a splice junction overhang of 100 nt. Following alignment, the resulting BAM files were processed with RSEM[46] version 1.3.0 for isoform and gene-level expression estimates. The resulting gene-level expression values were merged into a single expression matrix with an in-house python script, and differential expression analysis was carried out in R version 3.6.1 with the DESeq2[47] version 1.24.0. DESeq2 was also used to generate a rlog-matrix which was subsequently Z-transformed to normalize each gene across all samples. The Z-transformed rlog matrix was then loaded into JMP version 13 and used for PCA after thresholding to an average rlog expression value of 7.5.

DESeq2 output tables were filtered with the following restrictions for the identification of statistically significant differentially expressed genes: BaseMean >= 75 (average of 5 counts per sample), padj <= 0.05, |log2foldchange| >= 0.5, estimated standard error of the log2foldchange < 1.0.

Quantitative Reverse Transcription and Polymerase Chain Reaction (qRT-PCR). Total RNA purified from snap-frozen larvae using the Trizol method and used as template to synthesize random-primed cDNA using the Primescript cDNA synthesis kit (TaKaRa). Relative gene expression levels were measured by qRT-PCR, using the delta-delta Ct method as described previously, and *eif5a* as a reference gene. Examination of the results of multiple RNA-seq data sets indicated that *eif5a* activity was highly stable across treatments and genotypes. In many experiments beta-actin was also used as a reference gene, and this did not substantially change the results.

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Author Contributions

IG and EIH respectively performed most of the experiments and data analysis and did the matings, microinjections and husbandry to establish the *klf9* and *nr3c1* mutant zebrafish strains; SZ designed the gRNAs and performed the initial PCR and sequencing analyses to identify fish containing the desired *nr3c1* mutations; IG performed the mRNA injection experiments and qRT-PCR data analysis; ART contributed to the RNA-seq experimental design and data analysis; LH wrote the code used in the RNA-seq data analysis; NM performed the initial analysis of the RNA-seq data; JHG directed the computational analyses and performed the PC and DEG analyses; JAC conceived the study, contributed to analysis of the data and drafted the manuscript.

Figure Legends

Figure 1. A new CRISPR-Cas9-induced mutation of the zebrafish GR. (A) Schematic of the *nr3c1* gene and encoded GR showing location of the targeted sequence and resulting premature stop codon with respect to the protein domains: N-terminal domain (NTD), DNA binding domain (DBD), and ligand binding domain (LBD). (B) Nucleotide sequence of nr3c1 exon 3, showing sequence targeted by the gRNA (underlined), the 20 base deletion resulting from injection of that gRNA and Cas9 mRNA (gray), and the resulting premature stop codon (bold italic). (C) Predicted amino acid sequence of the full length zebrafish GR, plus the extra amino acids introduced by the frameshift shown in (B) (red font). The sequence eliminated by the premature stop is shown in gray, with the DBD underlined. Methionines corresponding to potential alternative initiation sites are shown in green. The positions of all reported frameshift mutations (including that reported here, E369) are boxed. The arginine that is changed to a cysteine in the gr^{s357} mutation is shown in orange. (D) Schematic of the GR showing locations of the two previously reported exon 2 frameshift mutations resulting in truncations at amino acids 178 and 310, and the one in exon 3 reported here truncating at amino acid 369, and residues included in N310 and N369 mRNA constructs produced for microinjection. (E) Relative expression of *fkbp5* in 6 hour homozyogous GR^{369-} embryos, either uninjected or injected with mRNA encoding full-length GR (FL), N-terminally truncated isoforms N310 and N369, or a nonspecific control mRNA encoding *Xenopus* elongation factor 1α (Xeno).

Figure 2. RNA-seq identifies GR-dependent and GR-independent genes and effects of chronic cortisol treatment. (A) Diagram of experimental design using Visual Background Adaptation (VBA) selection. (B) Principal component plot of the RNA-seq data, showing the location of each replicate sample with respect to the first two principal components. Cortisol-treated samples are shown in red, vehicle-treated controls in black. VBA+ samples (containing at least one functional GR allele) are filled shapes, VBA- samples are empty shapes. (C) Venn diagram showing numbers of genes upregulated in each of the indicated comparisons (keyed as in (A)): VBA+ vs. VBA- (vehicle-treated controls); cortisol-treated vs. vehicle-treated VBA+; and cortisol-treated vs. vehicle treated VBA-. Upregulated genes in common respectively between the first two and the last two comparisons are listed on the right. (D) Box plots of Z-transformed expression levels of *klf9* and *npas4a* obtained from the RNA-seq data.

Figure 3. CRISPR-Cas9-mediated disruption of zebrafish *klf9*. (A) Schematic of the *klf9* locus and encoded protein showing location of the gRNA target in exon 1, which introduces a premature stop codon upstream of the DNA binding domain. (B) Sequence of *klf9* exon 1, with 5' untranslated region in small case and coding sequence in upper case. The location of the 2 base pair deletion generated by CRISPR is shown in gray font; the gRNA target sequence is underlined; and the premature stop codon introduced by frameshift is noted with an asterisk. (C) Effect of chronic cortisol treatment and the frameshift mutation on relative levels of *klf9* mRNA and pre-mRNA, measured by qRT-PCR. The results show the averages and SEM of three biological replicates; significance values were obtained by ANOVA.

Figure 4. RNA-seq analysis of the effect of loss of *klf9* function on the transcriptomes of normal and cortisol-treated larvae. (A) Box plots of Z-transformed expression levels of genes upregulated by chronic cortisol treatment in wild type larvae but not in *klf9*$^{-/-}$ larvae. (B) Venn diagram showing number of genes from (A) and those upregulated by chronic cortisol in our previous experiment and intersection between the two sets. (C) Examples of two of the genes upregulated by chronic cortisol in a klf9-dependent fashion that were also identified in our previous analysis. (D) Revigo treemap of gene ontology biological process terms found by GOrilla to be enriched in the set of genes upregulated by chronic cortisol in a *klf9*-dependent way.

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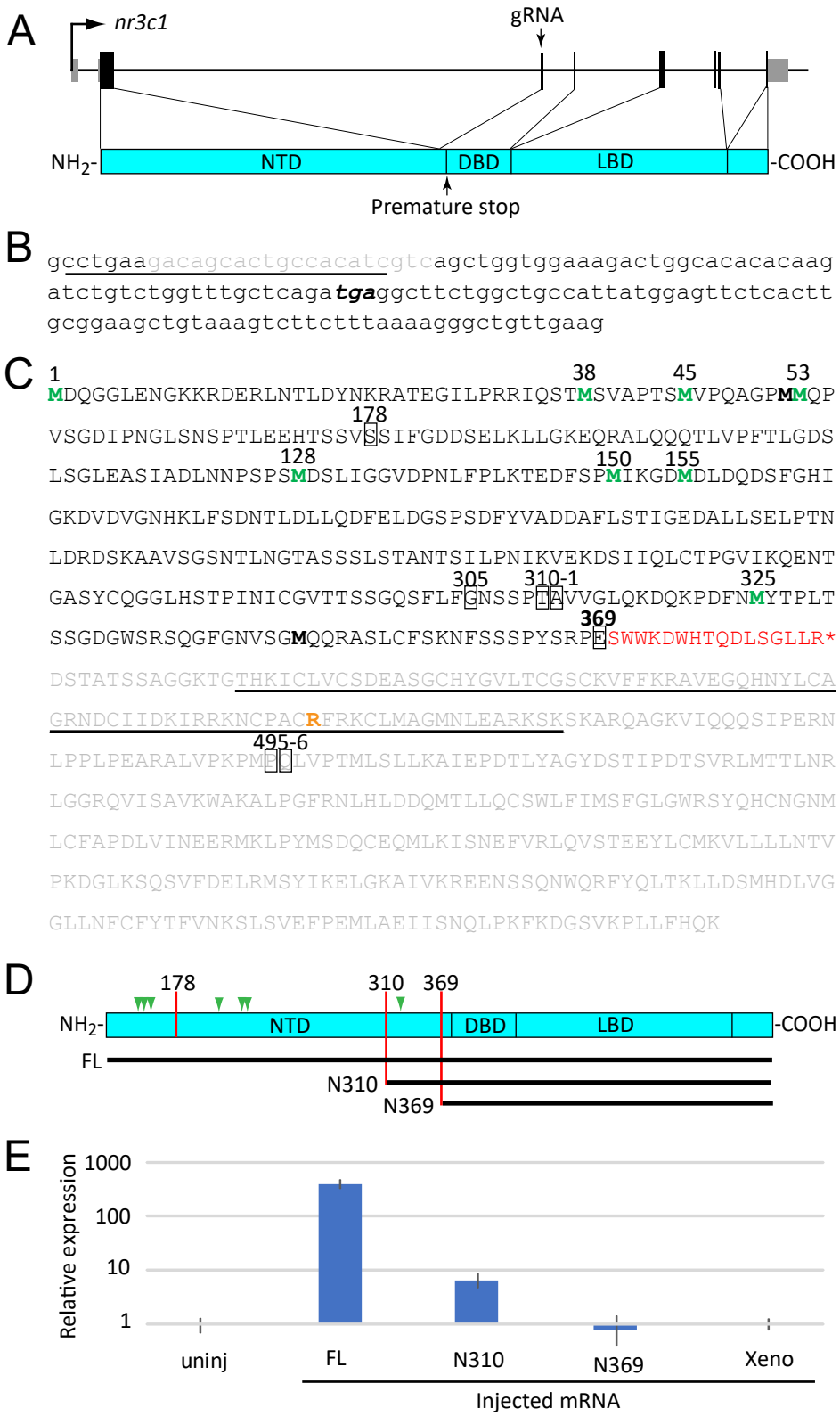
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D
178
310
369
NH2-
NTD
DBD
LBD
-COOH
FL
N310
N369
E
Relative expression
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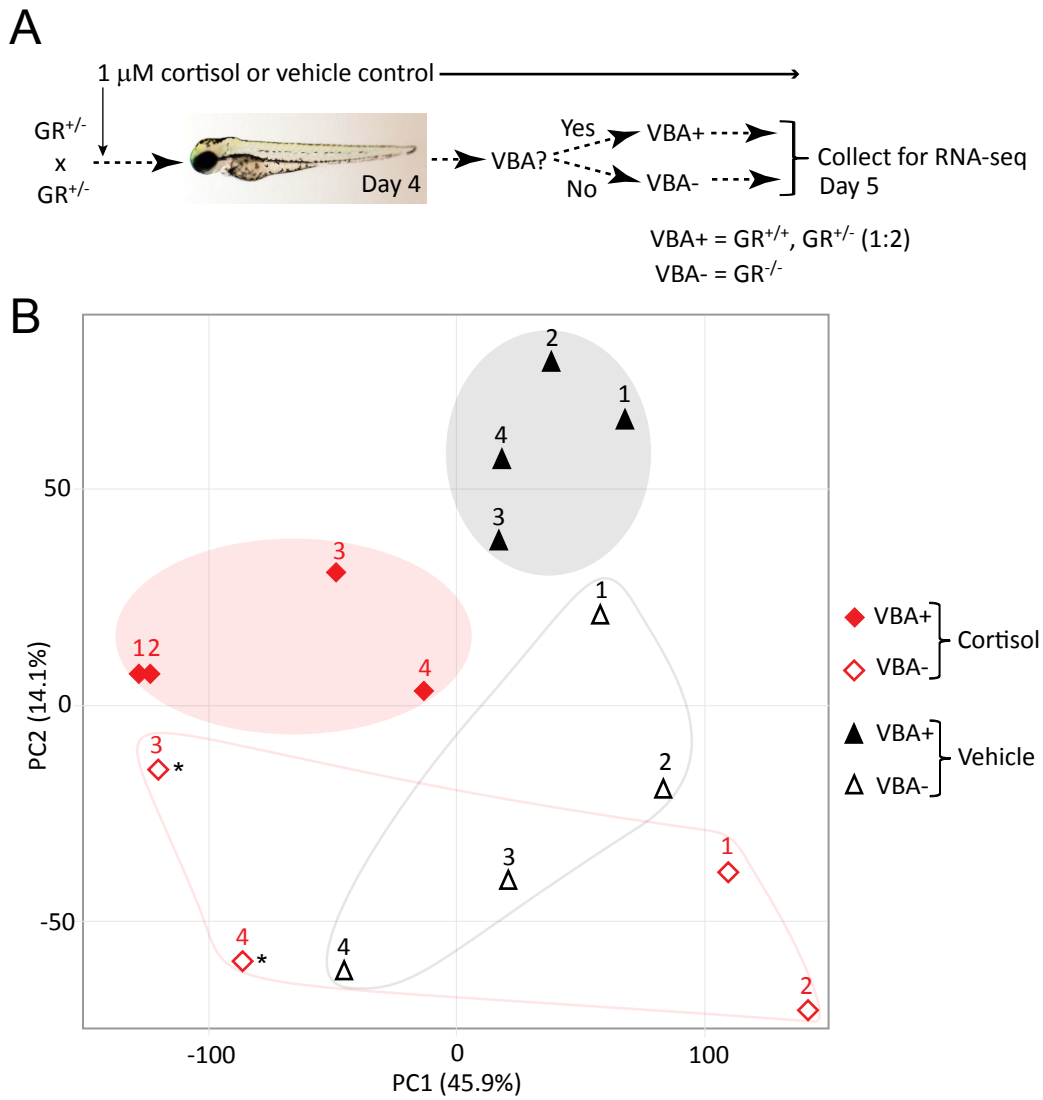
100
10
1
uninj
FL
N310
N369
Xeno
Injected mRNA

Figure 1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GR+/-
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VBA?
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No
VBA+
VBA-
Collect for RNA-seq
Day 5
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VBA- = GR-/-
B
PC2 (14.1%)
PC1 (45.9%)
50
0
-50
-100
0
100
VBA+
VBA-
Cortisol
VBA+
VBA-
Vehicle
C
99
4
2165
8
klf9
per1a
psme4a
hsp90aa1.2
npas4a
ier2b
egr4
egr1
gpr186
sik1
fosab
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VBA+
VBA-

Figure 2

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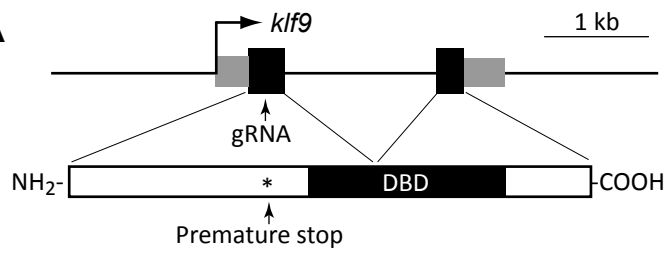

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C

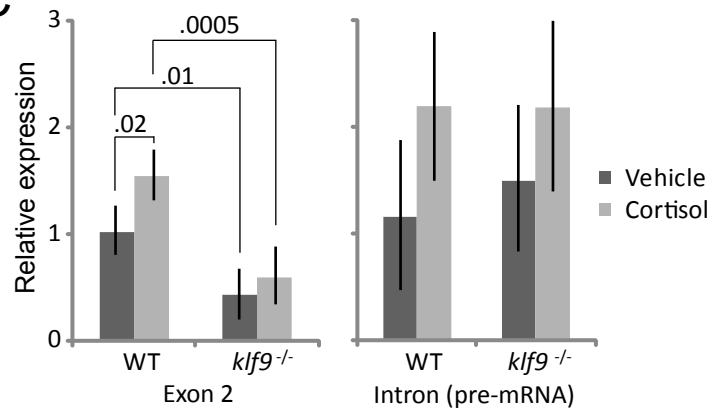
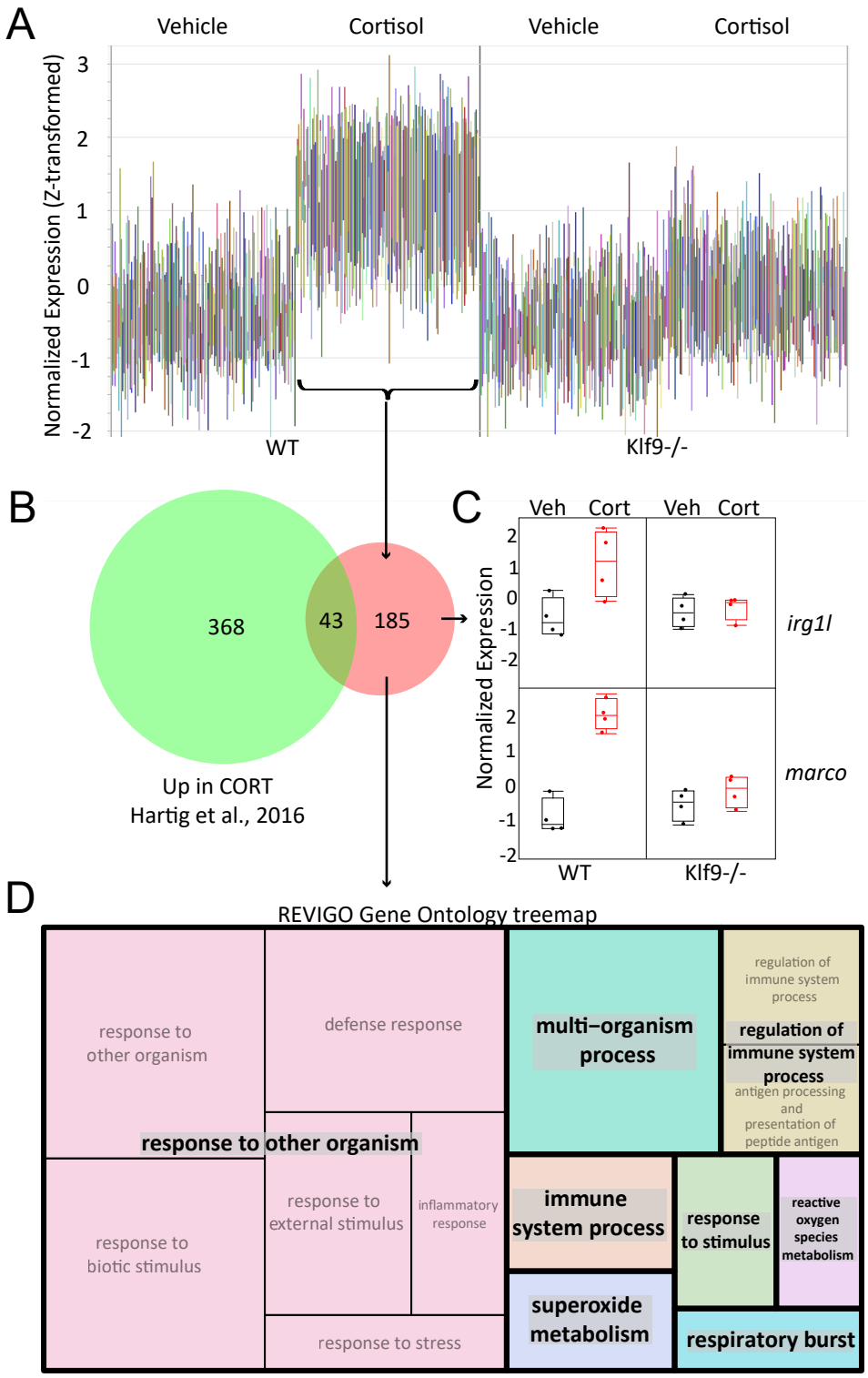


Figure 3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Vehicle
Cortisol
Vehicle
Cortisol
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-1
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Klf9-/-
B
368
43
185
Up in CORT
Hartig et al., 2016
C
Veh
Cort
Veh
Cort
Normalized Expression
irg1l
marco
WT
Klf9-/-
D
REVIGO Gene Ontology treemap
response to other organism
defense response
multi–organism process
regulation of immune system process
regulation of immune system process
antigen processing and presentation of peptide antigen
response to other organism
response to biotic stimulus
response to external stimulus
inflammatory response
immune system process
response to stimulus
reactive oxygen species metabolism
superoxide metabolism
respiratory burst
response to stress

Figure 4