# BMS397
# LABORATORY RESEARCH PROJECT

# SEMESTER 1, 2020-21

## Laboratory Report

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## Abstract

Glucocorticoids play a vital role in mediating the stress response in mammals. They serve a range of functions such as the production of glucose to provide cells with more ATP and the inhibition of inflammation to prevent its damage to the organism. These effects are mediated through the alteration of gene transcription in target cells, where certain genes are up or downregulated in order to produce a cellular response to stress. It is crucial that an understanding of this effect on transcription is gained, as a lack of these processes can have severe clinical outcomes, such as those seen in congenital adrenal hyperplasia (CAH). This study uses a zebrafish model of CAH in order to assess the effects of glucocorticoid inactivity on the genome of zebrafish larvae that lack cyp21a2, a gene crucial in the production of cortisol, the primary glucocorticoid in both zebrafish and mammals. This study also assessed the effects of hydrocortisone treatment on the animals, to view whether the mutated phenotype could be recovered and to assess the effects of elevated glucocorticoid signalling on the larvae. The results identified a range of genes mediated by glucocorticoids which could be linked to the symptoms of CAH, such as trim63a, a protein involved in muscle atrophy. Treatment with hydrocortisone appeared to have similar effects on both the mutant and wildtype samples, with significant effects on immune regulation. Questions where therefor raised about the efficacy of this treatment and whether it provides results more extreme than endogenous glucocorticoid signalling.

## Lay abstract

When our body experiences a stressful situation its needs change. For example, in order to escape danger our muscles require more energy, or in order to survive infection inflammation must be controlled. Cortisol is a hormone that is released in these situations and has a range of effects in different locations in the body. These effects can include breaking down muscle and fat for energy or stopping prolonged inflammation from harming our bodies. When this system goes wrong, it can have debilitating effects on the individual. A key example of this is in congenital adrenal hyperplasia (CAH), where individuals are born with lower levels of cortisol which can impair a range of bodily functions. In order to further understand disorders like this we can recreate them in animals to see their effects, and then apply this to our knowledge of humans. In this study zebrafish were used to assess the effects of an absence of cortisol similar to that in CAH. The effects of treatment with hydrocortisone where also assessed, as this is a common drug used as a replacement for cortisol in CAH patients. The hydrocortisone was shown to be effective in reversing the effects of cortisol deficiency, however it also produced a range of unexpected effects on the immune system in the zebrafish. More studies may need to be done in order to understand the effects of hydrocortisone on the immune response.

# 1.0: Introduction

Glucocorticoids are a class of corticosteroid hormone that acts as a ligand to a group of glucocorticoid receptors. Upon binding, the ligand receptor complex enters the nucleus and regulates the transcription of an array of genes involved in metabolism, development and the immune and inflammatory responses. The primary glucocorticoid in both humans and zebrafish is cortisol.

## 1.1 Cortisol Production

The process of cortisol steroidogenesis in humans is well documented. Cortisol production occurs primarily in the adrenal cortex in mammals and in the interrenal gland in zebrafish (Clark, et al., 2011). Like all other steroid hormones, cortisol is produced from cholesterol through a stepwise series of biochemical reactions involving multiple precursors. These reactions are mediated by a collection of oxidative enzymes within the cytochrome P450 family (Miller & Auchus, 2011). This is illustrated by fig. 1 below:

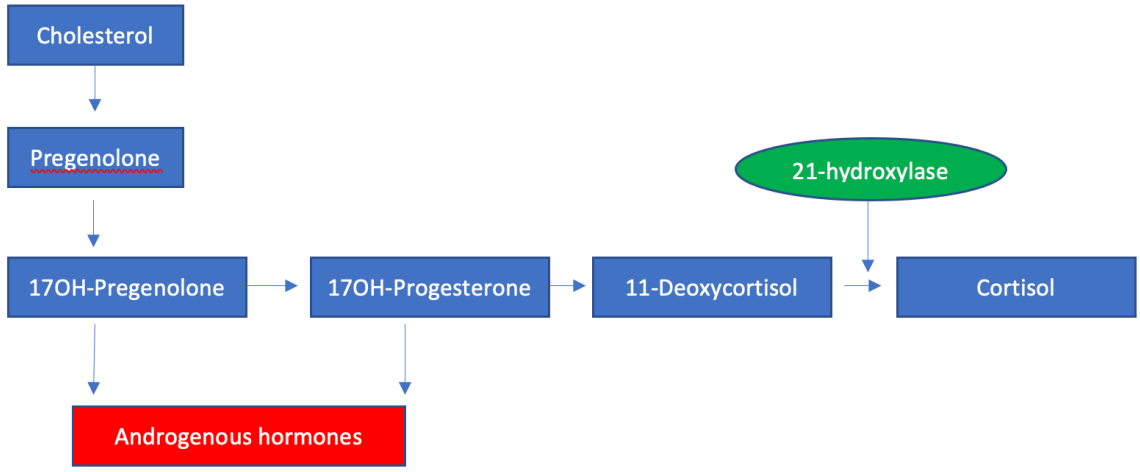


*Figure 1: Steroidogenesis pathway of Cortisol from its precursors.*

The two precursors 17OH-Pregenolone and 17OH-Progesterone are also precursors for androgens, therefore they are not exclusively fated to synthesise cortisol. It is the conversion of 17OH-Progesterone (hydroxyprogesterone) to 11-Deoxycortisol and the subsequent step that specifically determines cortisol production.

The conversion of hydroxyprogesterone to 11-Deoxycortisol is catalysed by the cytochrome P450 enzyme 21-hydroxylase. 21-hydroxlase is a type 2 cytochrome P450 enzyme coded for by a gene named CYP21A2, making it a key gene in cortisol synthesis.

## 1.2 Cortisol function and transcriptomic impact

Cortisol is involved primarily in the regulation of the body's response to stress, it's release is mediated by the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis consists of the hypothalamus, the anterior lobe of the pituitary gland and the adrenal gland. The activity of these structures results in a negative feedback loop mediated by cortisol, seen in fig. 2 (Gonzales-Alvarez, et al., 2012).

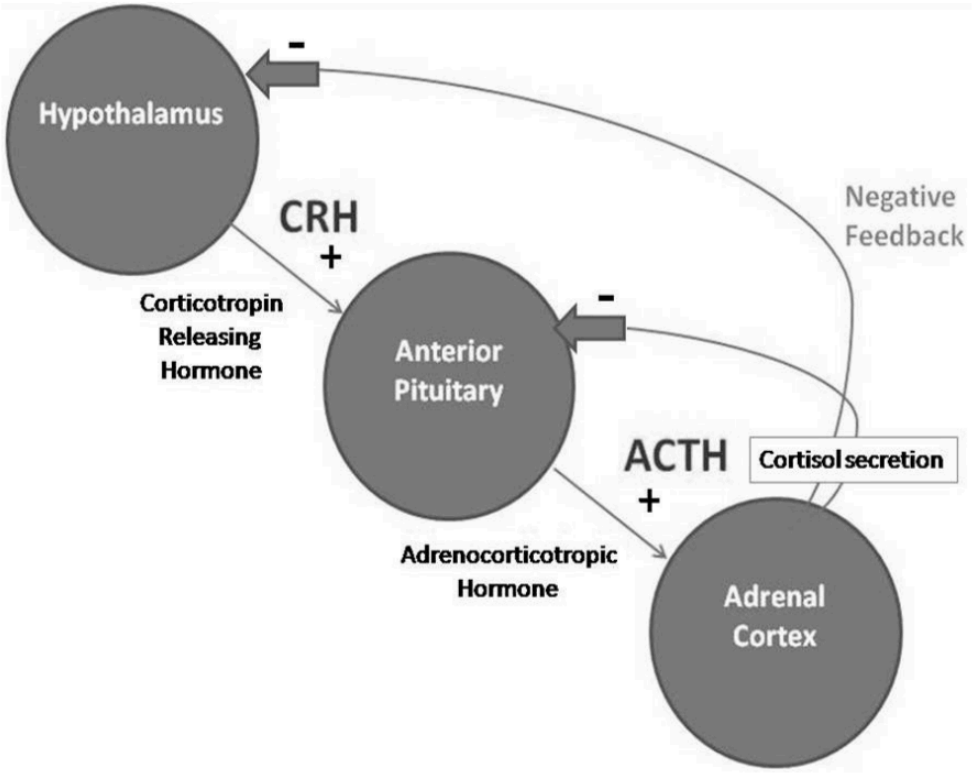


*Figure 2: A diagram showing action of the HPA axis and the role of cortisol in its regulation through negative feedback. When a stressful stimulus is encountered the Hypothalamus releases corticotropin releasing hormone. This goes on to stimulate Adrenocorticotropic hormone from the pituitary which in turn stimulates cortisol release from the adrenal cortex. Cortisol then goes on to repress the action of the Hypothalamus and Anterior pituitary, resulting in a negative feedback loop that inhibits cortisol secretion. Adapted from (Gonzales-Alvarez, et al., 2012)*

Upon its release Cortisol is transported through the blood where it can diffuse into target cells. Upon entering the cell, it binds to the cytosolic glucocorticoid receptor, resulting in a conformational change that switches it to an active state. The ligand-receptor complex is then translocated to the nucleus where it alters the expression of target genes. Its primary cellular effect is to induce gluconeogenesis; the breakdown of proteins and fats to form

glucose, therefor providing cells with a larger source of energy during times of metabolic stress. Cortisol has a range of other functions, such as inhibiting the inflammatory responses.

The current understanding of the effect of cortisol signalling on the transcriptome comes from animal model studies. Cortisol is the primary stress hormone in zebrafish and is released by the interrenal gland, which is the zebrafish equivalent of the adrenal gland in humans. Zebrafish therefore have a hypothalamic-pituitary-interrenal (HPI) axis that forms the stress response in the same way that the HPA axis does in humans.
A range of target genes have already been identified within animal models. Recent knockout studies show that glucocorticoid receptor action increases expression of genes related to stress response and metabolism and downregulation of genes involved in cell adhesion and growth (Gans, et al., 2020). This shows one of the primary impacts of Cortisol is to maximise cellular resources in order to respond to the allostatic load caused by stress. Although some genes have already been implicated in glucocorticoid signalling, there remains a distinct set of questions to be asked in terms of how glucocorticoid levels can impact the transcription of these genes. This research is of high clinical significance, as dysfunctions in cortisol signalling have been implicated in a range of disorders.

## 1.3 Congenital adrenal hyperplasia (CAH)

CAH is one of the most prominent inherited metabolic disorders and is caused by mutations in genes responsible for the steroidogenic enzymes (Krone & Arlt, 2009). One of the most common causes is a loss of function mutation in CYP21A2, the gene that encodes the enzyme 21-hydroxylase (White & Speiser, 2000). The loss of 21 hydroxylase results in lack of cortisol production. As a result, there is no negative feedback within the HPA axis leading to increased ACTH production, which in turn leads to increased production of precursor molecules to cortisol. These molecules are converted to sex hormones, resulting in patients exhibiting high levels of androgens. As a result, female patients may form ambiguous genetalia that represent male organs rather than female ones. Furthermore, both sexes suffer from poor control of blood pressure, stress and glucose metabolism.

The main method of treating CAH is through the use of hydrocortisone, a replacement glucocorticoid. This can prevent the serious outcomes of the disorder, however side effects such as weight gain and fatigue can still lead to a lower quality of life (Wiebke Arlt, 2010). More research is required in order to fully understand both the effects of CAH and artificial glucocorticoids on the transcriptome.

The first step in this research is to successfully model the disease. A recent study illustrated a zebrafish model for interrenal hyperplasia, a homologue for human adrenal hyperplasia. This involved the successful knockout of CYP21A2 to create a phenotype in the zebrafish larvae that accurately modelled adrenal hyperplasia, causing increased activity in the API axis and reduced expression of glucocorticoid mediated target genes (Eachus, et al., 2017). This study will build off their research, using a similar animal model to identify target genes affected by a lack of glucocorticoid signalling in CYP21A2 mutant zebrafish. The effect of hydrocortisone on this transcription will also be assessed. The hypothesis for this study is therefore that the hydrocortisone treated CYP21a2 mutants should show a reversal of gene expression when treated with hydrocortisone.

# 2.0 Methods

## 2.1 Quality analysis of the read data

Data was received in the form of multiple csv files, containing the read data for each sample. These were imported into R studio using the readr package (Wickham & Hester, 2020). A deseq data set was created using deseq2 (Love, et al., 2014), combining all results into one object. A log2 transformation was applied to the read data, which was then visualised using a boxplot, principal component analysis and cluster heatmap. The heatmap was generated using the pheatmap package (Kolde, 2019) whilst the other two used deseq2.

## 2.2 Differential expression analysis

The deseq dataset was subset into 4 different parts containing data for each comparison. 4 contrasts of gene expression were performed as follows: mutant (MUT) untreated vs wildtype (WT) untreated, MUT treated vs MUT untreated, MUT treated vs WT untreated and WT treated vs WT untreated.

Fold changes in expression and adjusted p values where generated using the deseq2 library and this data was then visualised. MA plots where generated using deseq2, after the data was subset to only include genes where $p < 0.05$. Volcano plots where generated using EnhancedVolcano (Blighe, et al., 2020). Pheatmap was used to generate heatmaps of the read data, that again had a log2 transformation applied.

## 2.3 Gene set enrichment analysis

Differentially expressed genes were then ranked according to their adjusted P value, from most to least significant. The resulting list was inserted into the Gorilla algorithm (Eden, et al., 2009), which assigned gene ontology terms to the list and the p value was set at 10-5. The resulting list of GO terms was input into revigo, a web server that visualises lists of ontology terms into treemaps (Supek, et al., 2011).

# 3.0 Results

## 3.1 Quality analysis of the read data

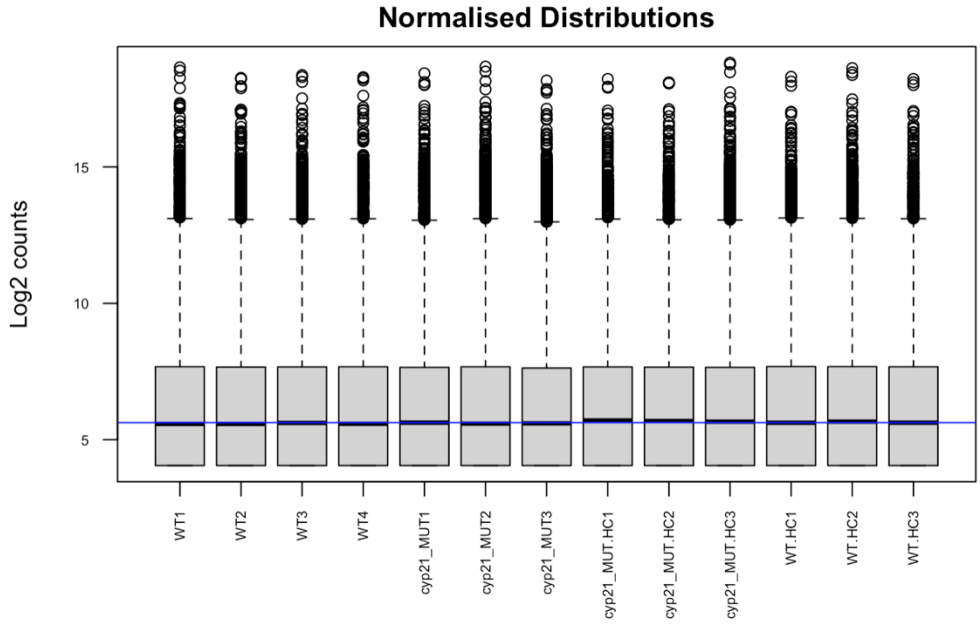


*Figure 3: A boxplot of the normalised count data. X axis indicates sample id and y axis shows reads after a log2 transformation was applied. The blue line indicates where the median values line up.*

The read data was normalised using a log 2 transformation. In order to assess the distribution of the data a boxplot was produced.

The boxplot indicates that a lot of read values lie outside of the mean. However, whilst the data is spread out, the medians for each sample still line up closely, with a small amount of variation between samples. This indicates that the data has been successfully normalised.

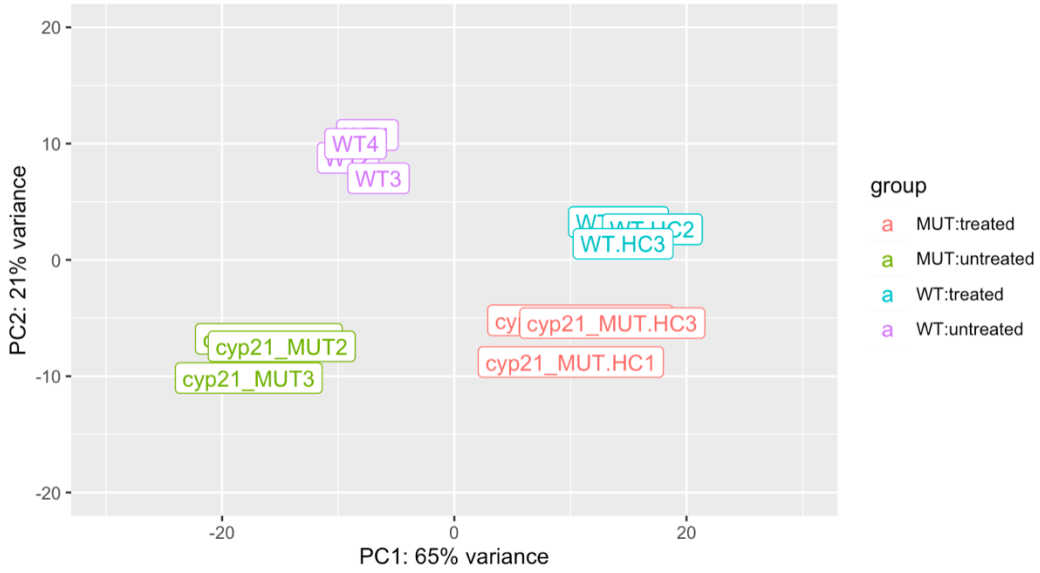


*Figure 4:A PCA plot of the normalised count data. Each data point is labelled with the sample name and colour coded according to its treatment group and genotype. Samples are compared to each other and plotted based on how their two principal components vary from one another. Samples are therefore grouped together where they show more similarity.*

A principal component analysis (PCA) was performed to visualise differences between samples. It should be expected that samples of the same treatment and genotype should show significant overlap and occupy distinct quadrants on the graph, as shown in Figure 4. There is some erraticism within groups which indicates some extraneous biological variability between samples. The fact that each group differs is good as it indicates that gene expression is different between each experimental condition.

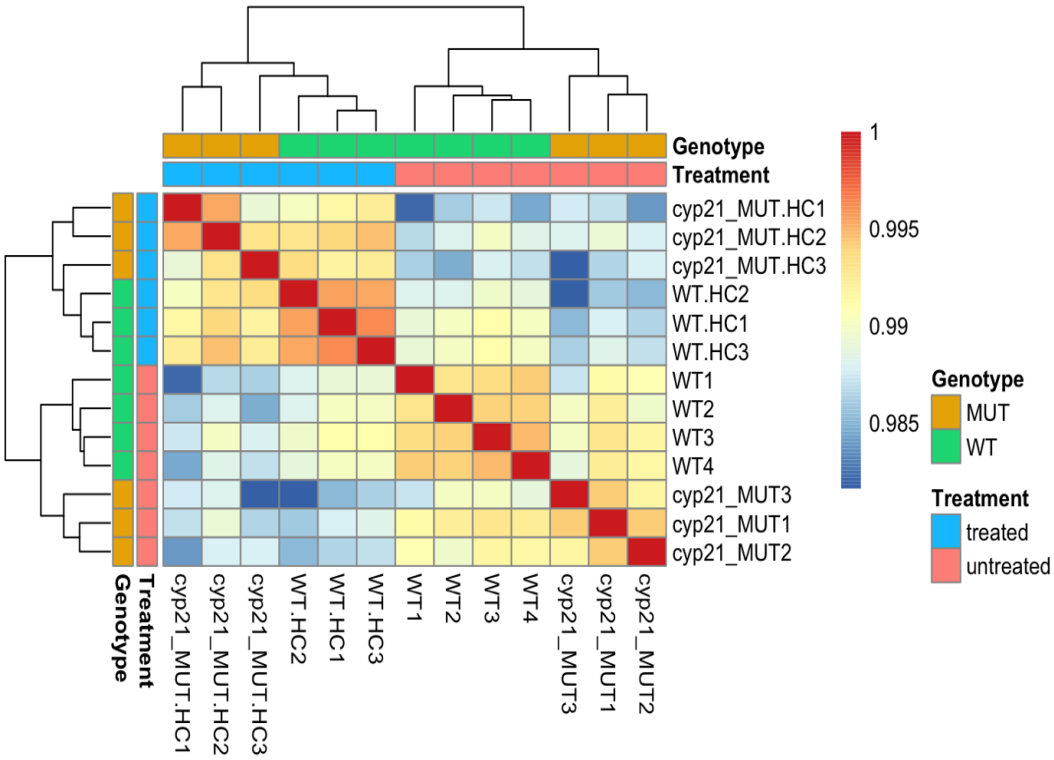


*Figure 5: A cluster heatmap of the normalised count data. The rows and columns show the sample names, with each individual rectangle showing degree of similarity between samples. Deep red indicates high similarity and deep blue indicates low similarity. Rows and columns are colour coded to show treatment and genotype of each group.*

To further assess sample similarity a cluster heatmap was produced, showing distinct clusters of red/orange where samples of the same group are compared. Differences can be seen within the mutant groups, indicating that there is a high-level of biological variability between the mutants. Interestingly it also appears that the mutant untreated samples are more similar to the wildtype untreated samples than they are the mutant treated samples, which gives some indication as to what the differential expression analysis will show.

## 3.2 Differential expression between the mutant and wild type samples

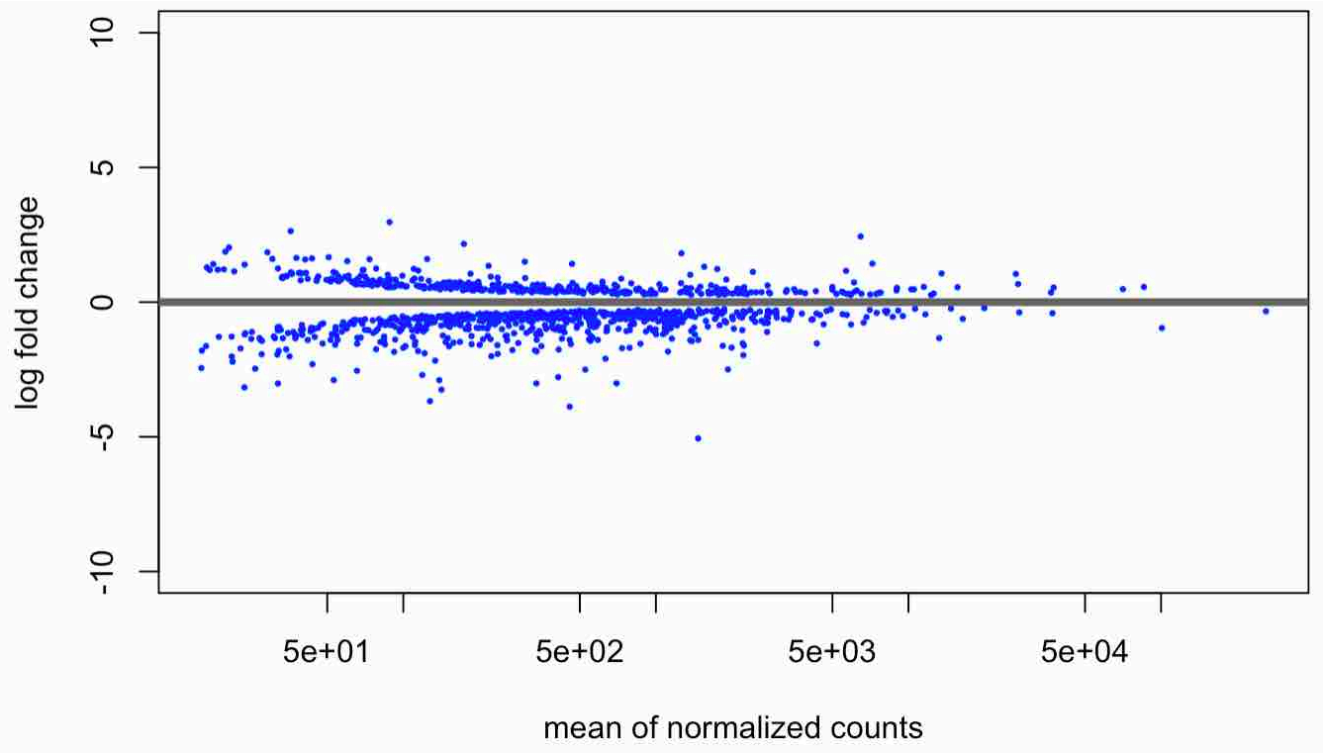


*Figure 6: ma plot of the mutant vs wildtype data. Data was subset to only include results where p < 0.05. X axis shows the mean normalised count data for each gene and the y axis shows log fold change. A negative fold change indicates decreased expression in the mutant and a positive fold change indicates increased expression.*

To identify the effects of the mutation on glucocorticoid signalling the first comparison was done on the mutant vs wildtype samples. Fig. 6 above shows an MA plot from these results. The plot indicates a higher level of downregulation in the significantly differentially expressed genes than upregulation, with one gene in particular having a very negative fold change less than minus five.

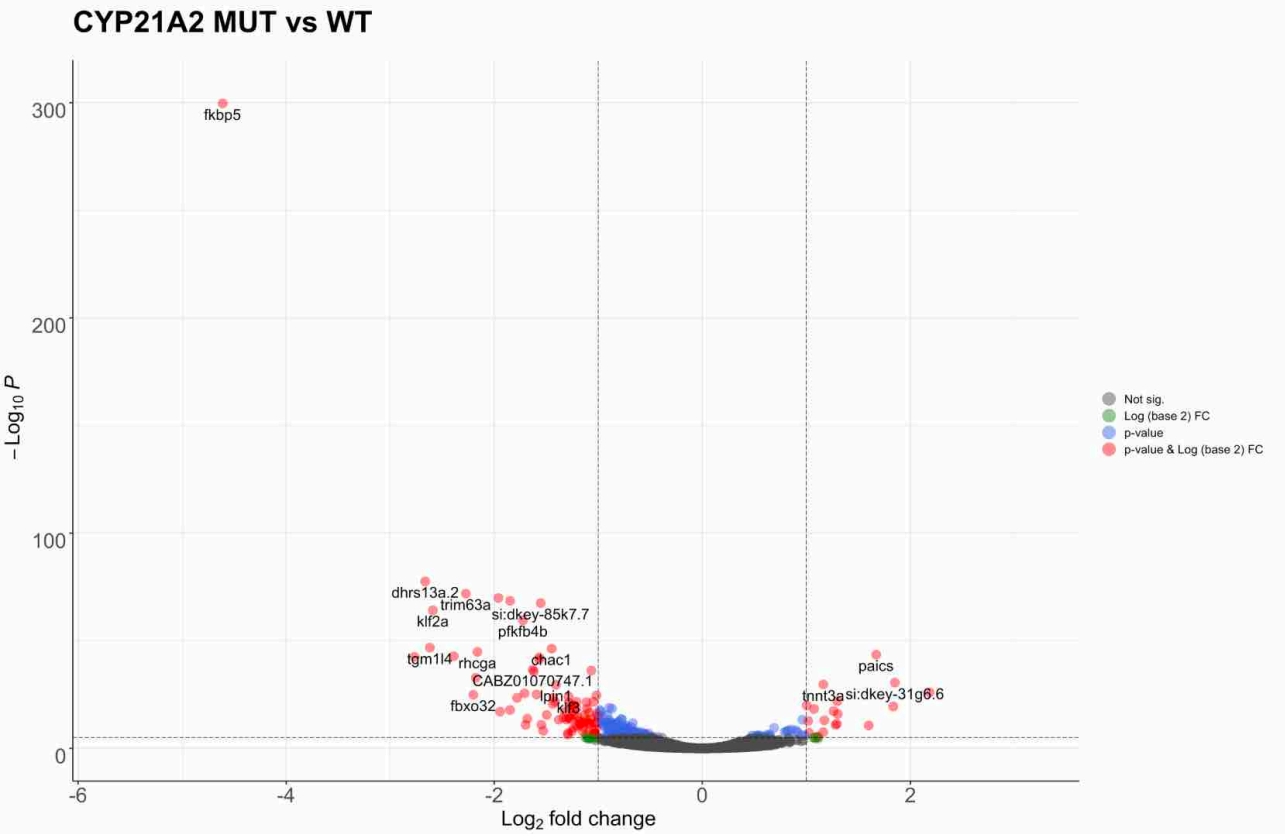


*Figure 7:A volcano plot of CYP21A2 mutant Vs wildtype samples. X axis shows the log2 fold change in gene expression for each gene, whilst Y shows the -log10 pvalue. Genes with a higher p value show a more statistically significant change. Genes with a p value <0.05 and a foldchange > 1 are highlighted in red. Labels were added to the top 30 most significant results where they do not overlap.*

In order to identify the differentially expressed genes a volcano plot was produced to examine log fold change, which confirms what figure 6 showed. The most significantly downregulated gene was fkbp5, with paics being the most statistically significant upregulated gene.

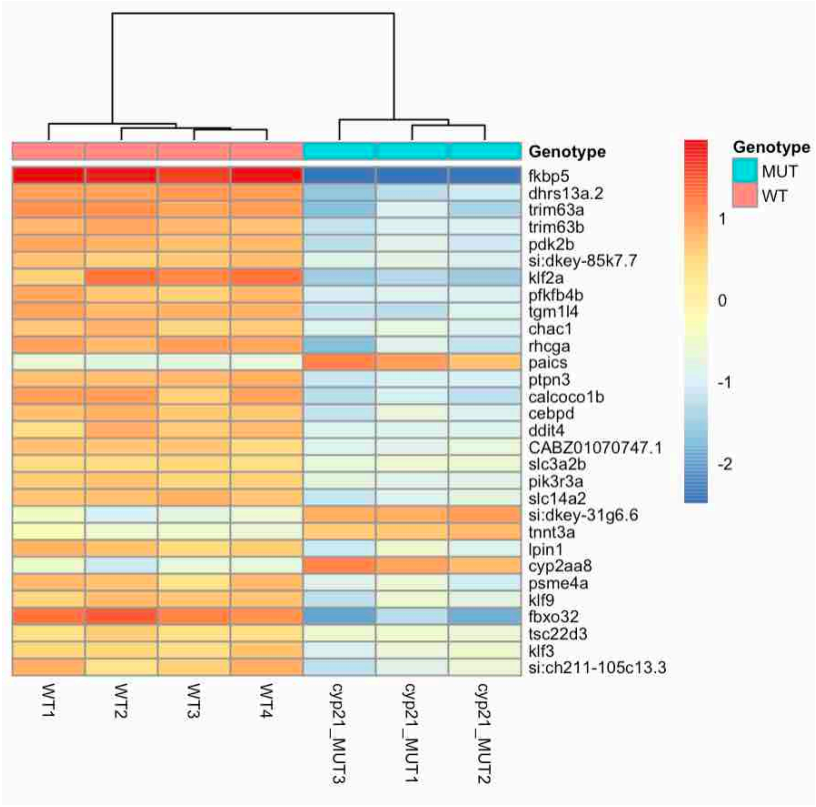


*Figure 8:A heatmap showing counts for each gene per sample group. The degree of redness in each cell indicates the level of expression of each gene, with a deep red showing high expression and a deep blue indicating low expression. Sample groups are annotated based on their genotype. Each gene is labelled according to its external gene name.*

A heatmap was used in order to visualise the expression of individual genes in each sample. The most significant difference in expression was fkbp5, with an extremely high expression in the WT samples compared to the mutants. It appears that of the top 30 most significant changes in expression, only 4 genes where upregulated in the mutants compared to the wildtype. These include paics and cyp2aa8. These differences are not as pronounced as those seen in most of the downregulated genes.

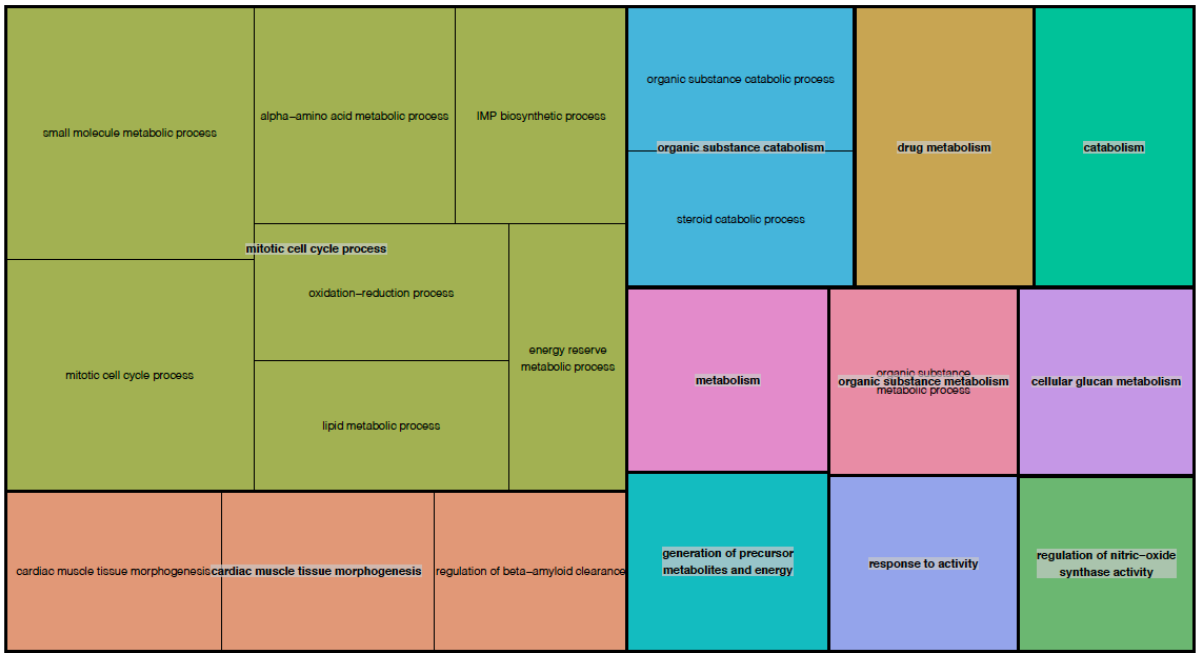


*Figure 9:A revigo ontology treemap showing gene ontology terms linked with the most significantly differentially expressed genes ($p < 0.04$). linked processes are colour coded, with the size of each box indicating significance of the term.*

In order to assess the biological significance of these differentially expressed genes, a gene set enrichment analysis was performed.

Figure 9 reveals that a wide range of processes are implicated in a lack of glucocorticoid signalling. It appears that metabolic and catabolic processes appear most frequently, followed by processes related to the control of the cell cycle and cell growth. Interestingly processes related to stress responses did not appear, despite that being one of cortisol's primary functions.

## 3.3 Differential expression between the mutant treated and untreated samples

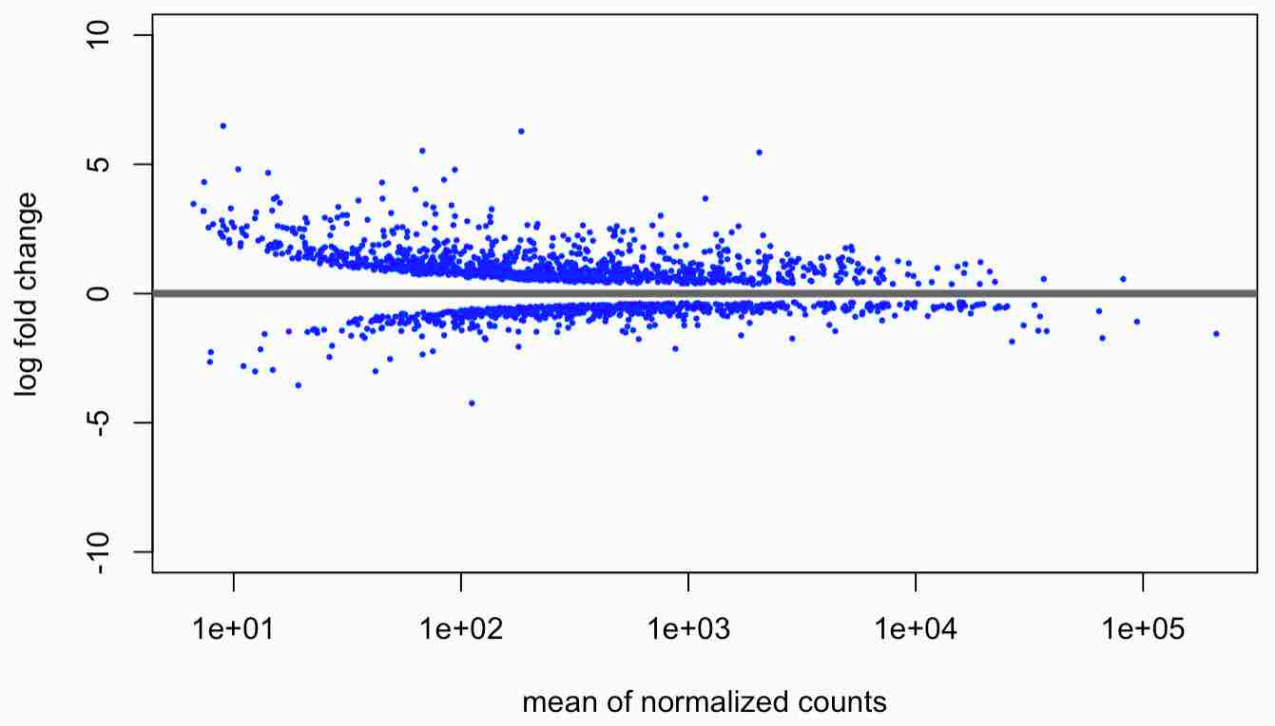


*Figure 10: ma plot of the mutants treated with hydrocortisone vs untreated mutant's data. Data was subset to only include results where p < 0.05. X axis shows the mean normalised count data for each gene and the y axis shows log fold change. A negative fold change indicates decreased expression in the mutants and a positive fold change indicates increased expression*

Comparisons of the gene expression were then made between the mutants treated and untreated to assess the effect on the mutant phenotype. An MA plot was produced to assess overall trends in gene expression.

Figure 10 shows a significant upregulation of genes in the treated mutants. In addition, there are more significantly differentially expressed genes than those that where differentially expressed between the mutants and the wild type samples. Most of the results in figure 10 also show greater foldchanges in expression. Overall, this indicates that the treatment may have a greater effect on gene expression than the mutation on its own.

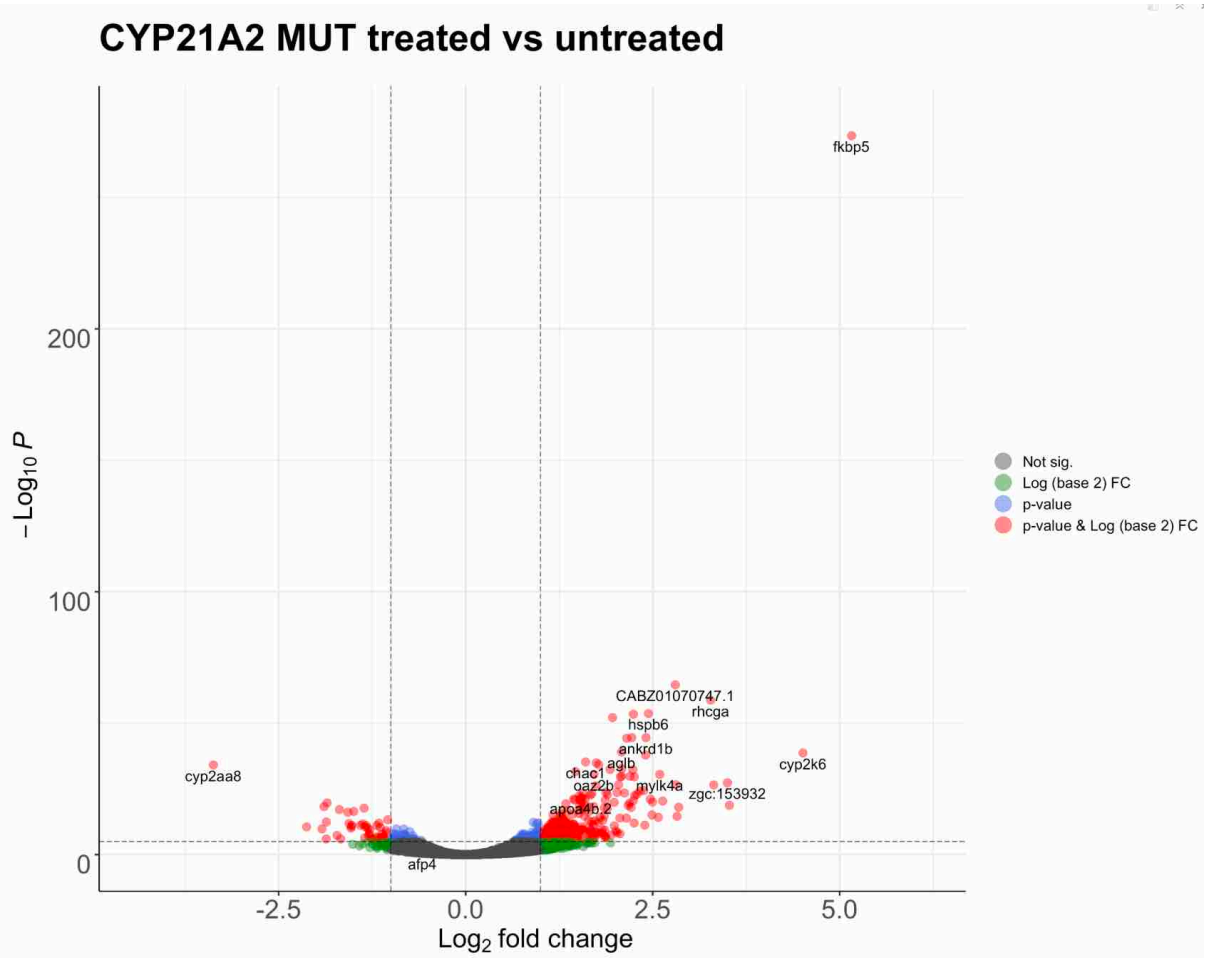


*Figure 11:A volcano plot showing the mutant treated vs mutant untreated comparison. X axis shows log2 fold change and y shows -log10p value. Genes highlighted in red have a foldchange greater than one and p < 0.05. The top 30 most significant changes where labelled where they do not overlap.*

In order to better identify differentially expressed genes a volcano plot was produced. In this case it is clear that there is more upregulation of genes in the treated mutants. Interestingly fig. 11 appears to almost mirror fig. 7, with fkbp5 being significantly upregulated in the treated group. Furthermore, cyp2aa8 is now downregulated here. This may indicate a reversal of some of the transcriptomic effects seen in the mutation.

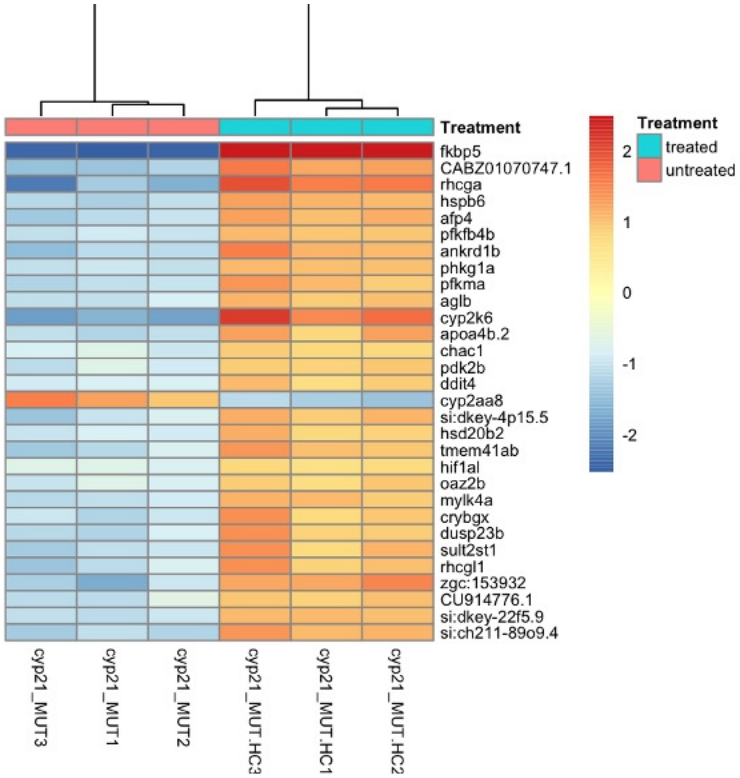


*Figure 12:A heatmap showing gene expression in the mutant treated and untreated groups. The colour of each cell indicates level of gene expression, with deep red indicating high expression and deep blue indicating low. Each column is annotated with the sample name and coloured blocks show the treatment group of each sample.*

To further asses the differences in expression, a heatmap was created from the normalised read data of the top 30 most significant genes.

There is significant upregulation shown in the top 30 genes. It appears that fkbp5 is significantly upregulated, implying that the treatment had a reversal effect on its transcription in the mutants. Other genes also show reversed effects such as cyp2aa8 which was upregulated in the mutants (fig. 8) and is now downregulated when treated with hydrocortisone. Finally, pfkfb4b also shows the same reversal in transcription.

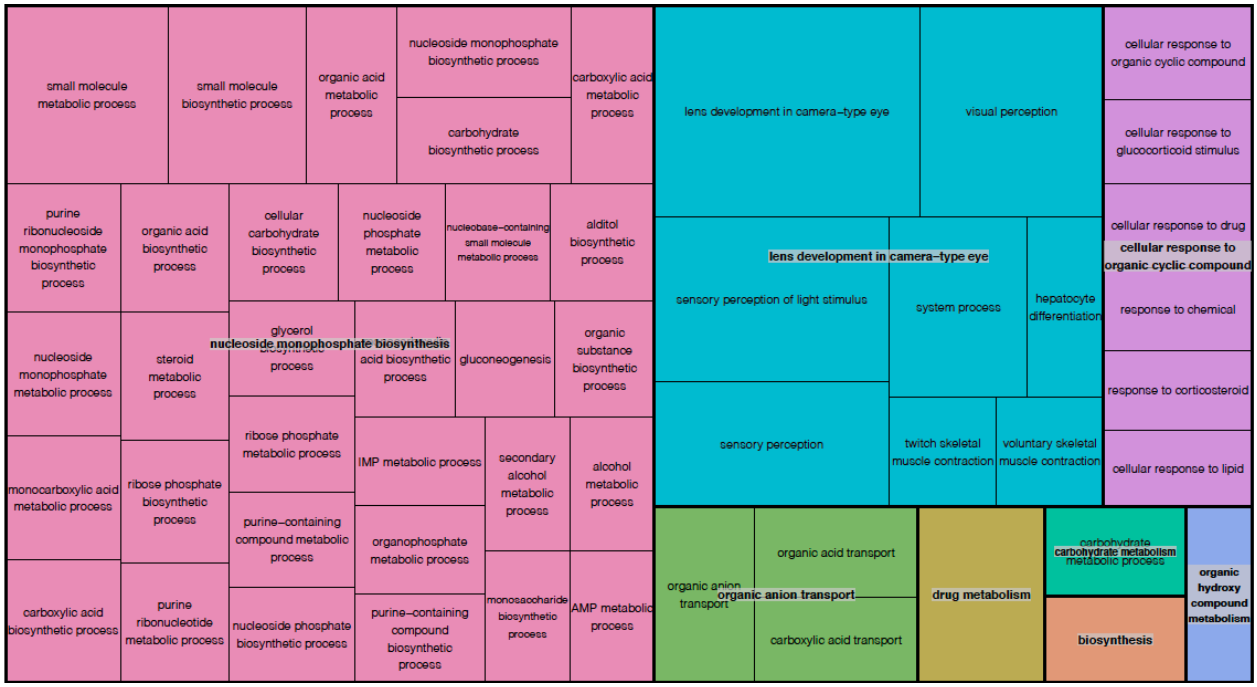


*Figure 13:A revigo gene ontology treemap showing enriched GO terms generated from the list of differentially expressed genes between mutant treated and untreated samples. Colors indicate linked processes and the size of each box indicates significance. Analysis was done where $p < 0.04$.*

A gene enrichment analysis was performed as before in order to assess the implicated biological processes

Fig. 13 shows a significantly higher list of terms when compared to fig. 9, which is due to the fact that there were more significantly differentially expressed genes. Terms of special note include response to glucocorticoid stimulus on the right as well as a range of steroid metabolic processes. This indicates that the treatment had a significant effect on glucocorticoid signalling in the mutant zebrafish.

## 3.4 Differential expression between the treated mutants and untreated wild type samples

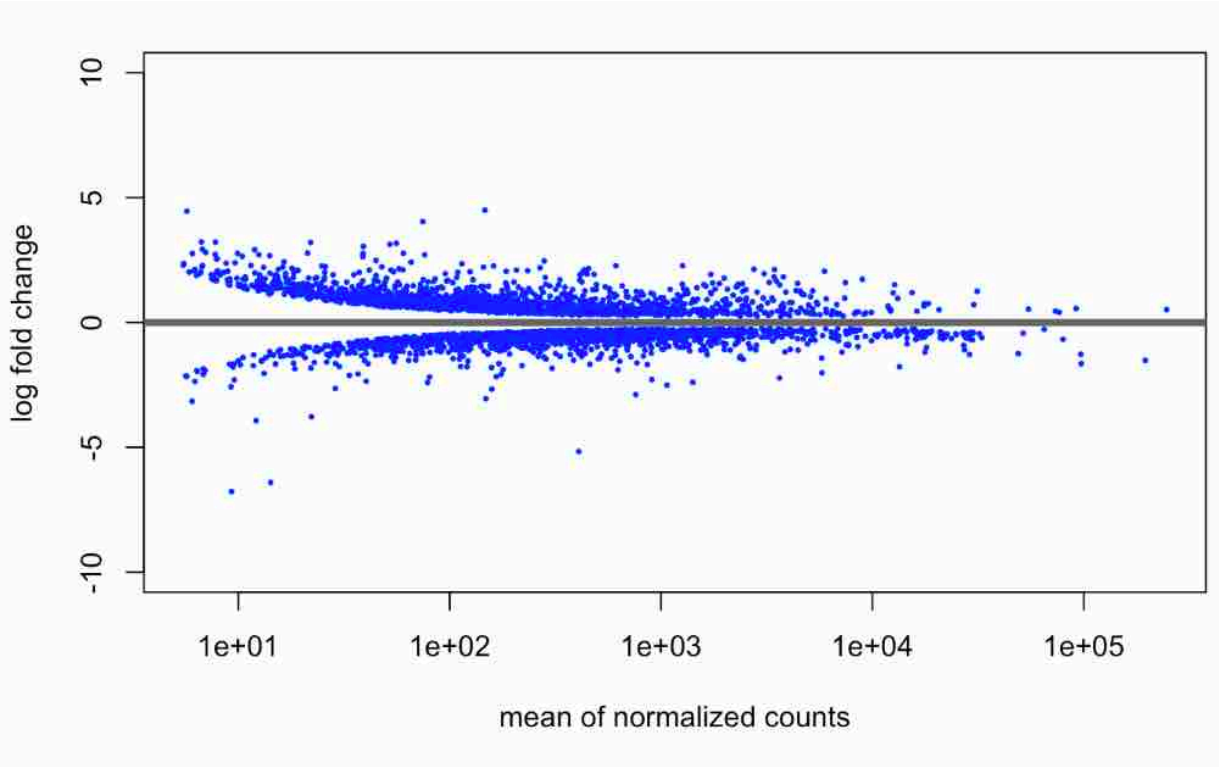


*Figure 14: ma plot of the treated mutant vs wildtype untreated data. Data was subset to only include results where p < 0.05. X axis shows the mean normalised count data for each gene and the y axis shows log fold change. A negative fold change indicates decreased expression in the mutant and a positive fold change indicates increased expression*

In order to assess how the mutants treated with hydrocortisone differ from the wild type animals the differential expression was analysed between the mutant treated and wild type untreated samples.

Once again, an MA plot was used to gain an overview of the differential expression.

This analysis showed far more differentially expressed genes than the previous two analyses produced. There appears to be little favour for either upregulation or downregulation, with a large number of genes with both a positive and negative fold change. This already suggests that the mutants treated with the hydrocortisone still differ significantly from the wild type, despite the fact that one might assume they would be more similar.

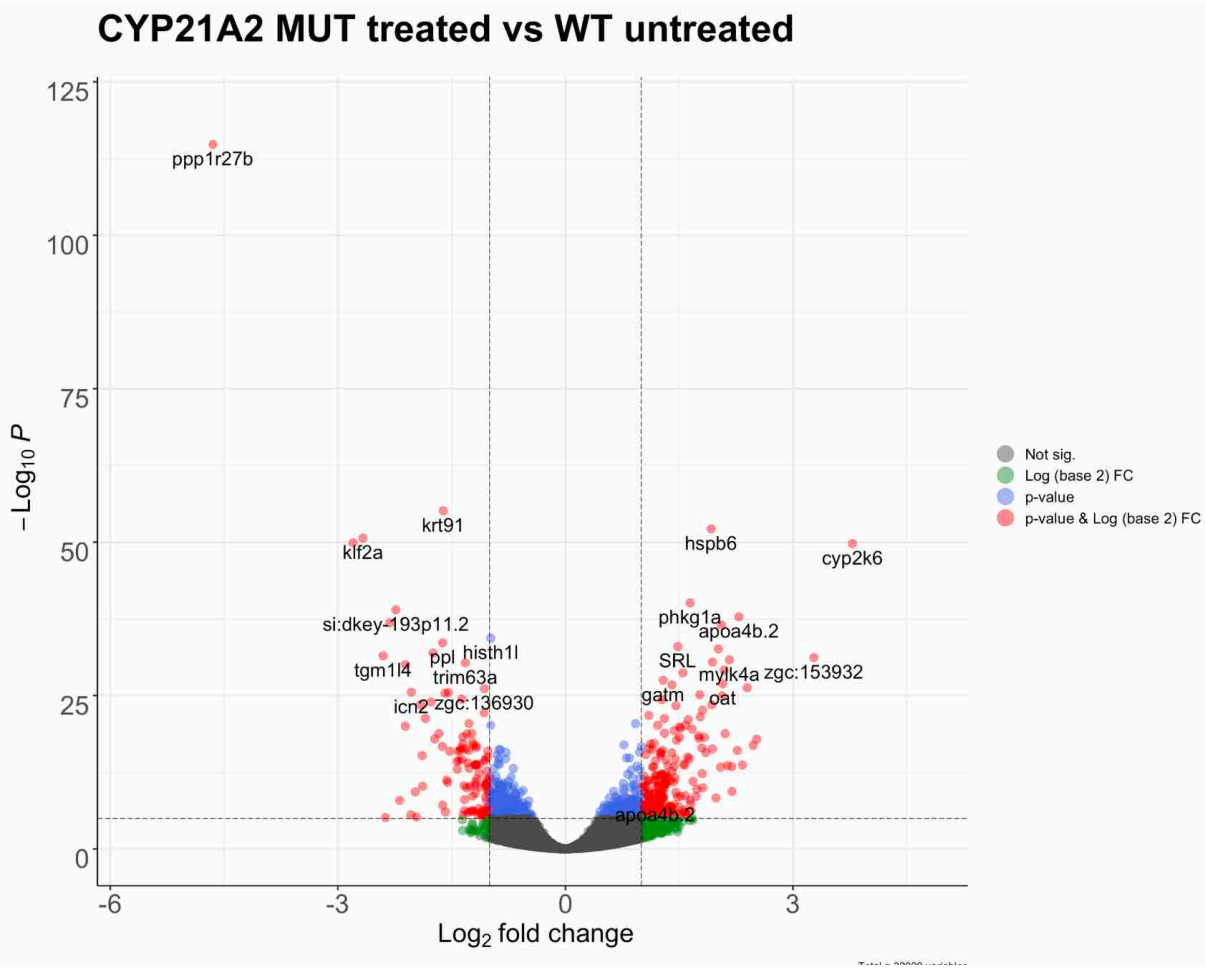


*Figure 15: A volcano plot of the mutant treated vs wildtype comparison. Genes in red where both statistically significant ($p < 0.05$) and had a fold change > 1. X axis shows log 2-fold change and y shows a log10 adjusted p value. The top 30 most significant genes where labelled where they do not overlap.*

To further assess these results a volcano plot was generated.

The volcano plot shows an almost equal amount of upregulation compared to downregulation. The one gene that stands out however is ppp1r27b, which showed a highly significant downregulation in the mutants treated with hydrocortisone. Other downregulated genes of significance include anxa1c and klf2a. Some genes where also significantly upregulated, including hspb6 and cyp2k6.

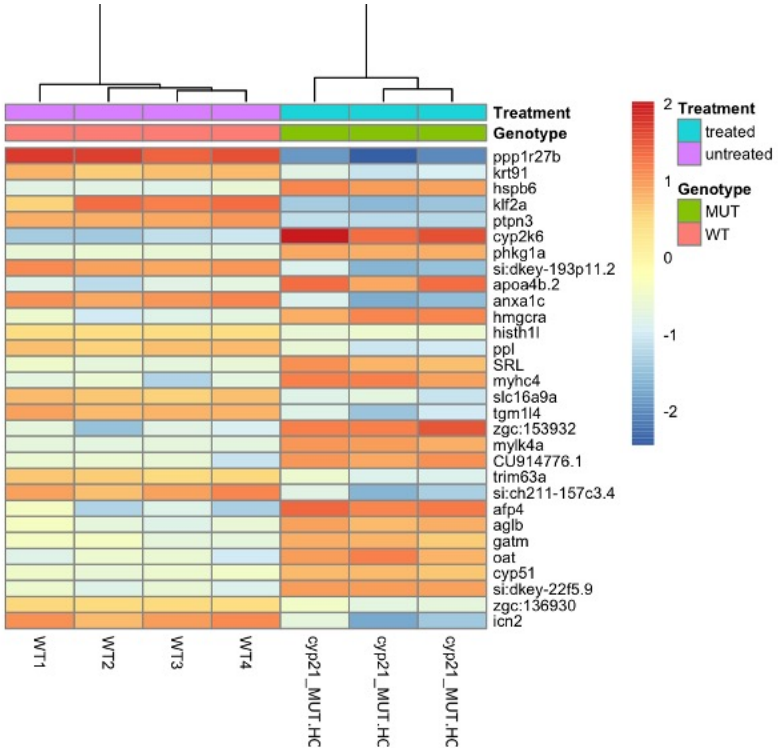


*Figure 16:A heatmap showing expression of genes in the mutants treated with hydrocortisone and the wild type untreated samples. Columns are annotated with colours depicting the genotype and the treatment of each sample. Each square indicates degree of gene expression, with deep red indicating high expression and deep blue indicating low expression. Genes are annotated with their gene symbol.*

A heat map was produced from the top 30 most differentially expressed genes.

Fig. 16 differs wildly from the previous heatmaps, with a lot more variability seen in the expression of these genes. The most pronounced difference was the downregulation of ppp1r27b in the mutants treated with hydrocortisone. When compared to the other heatmaps it appears that the differences in the reads between each sample are less extreme. This raises the question as to what the reason for these differences are, as it could be inferred that biological variability between the animals themselves could be playing a role.

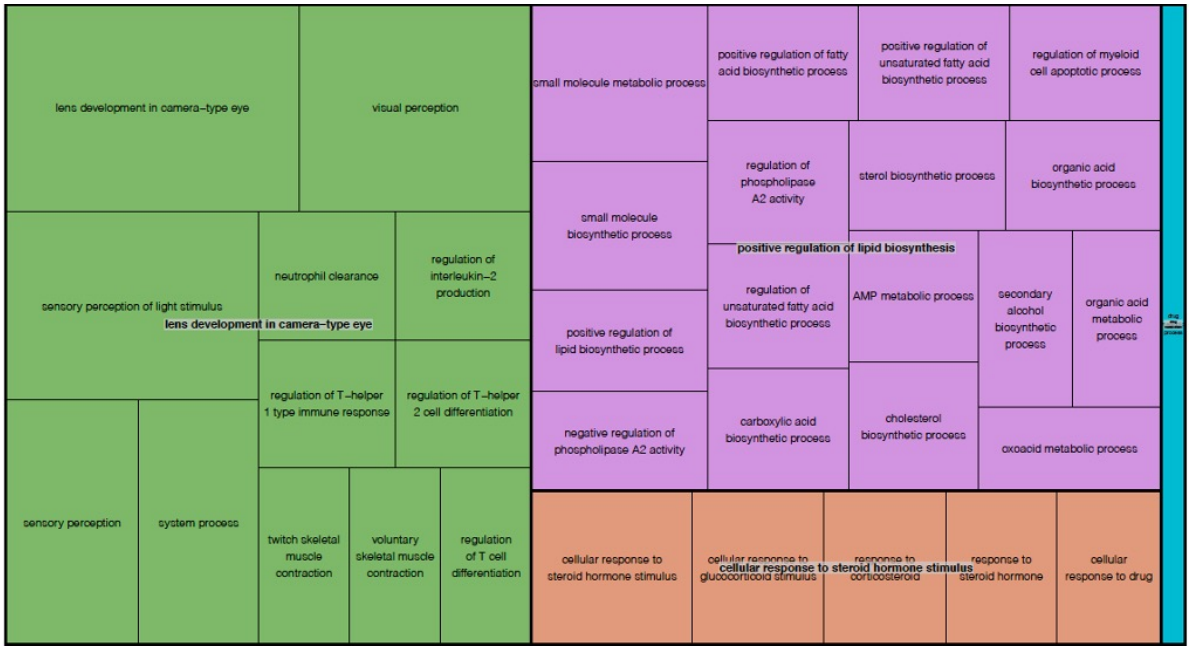


*Figure 17:A revigo gene ontology treemap generated from the list of differentially expressed genes between mutant treated and WT untreated samples. Processes that relate to one another are color coded. The size of each box indicates significance.*

In order to infer what processes the differentially expressed genes are involved in, a revigo gene ontology analysis was conducted.

A range of processes relating to immune cell function have been implicated. Furthermore, once again cellular response to hormones and glucocorticoids was also significantly impacted, indicating that the mutants and WT still have significant differences in glucocorticoid related processes, despite the treatment with hydrocortisone.

Very few of these terms overlap with those seen in figure 9, which seems to imply that these differences are more likely caused by the treatment as opposed to the mutation itself.

## 3.5 Differential expression between the WT treated and untreated samples

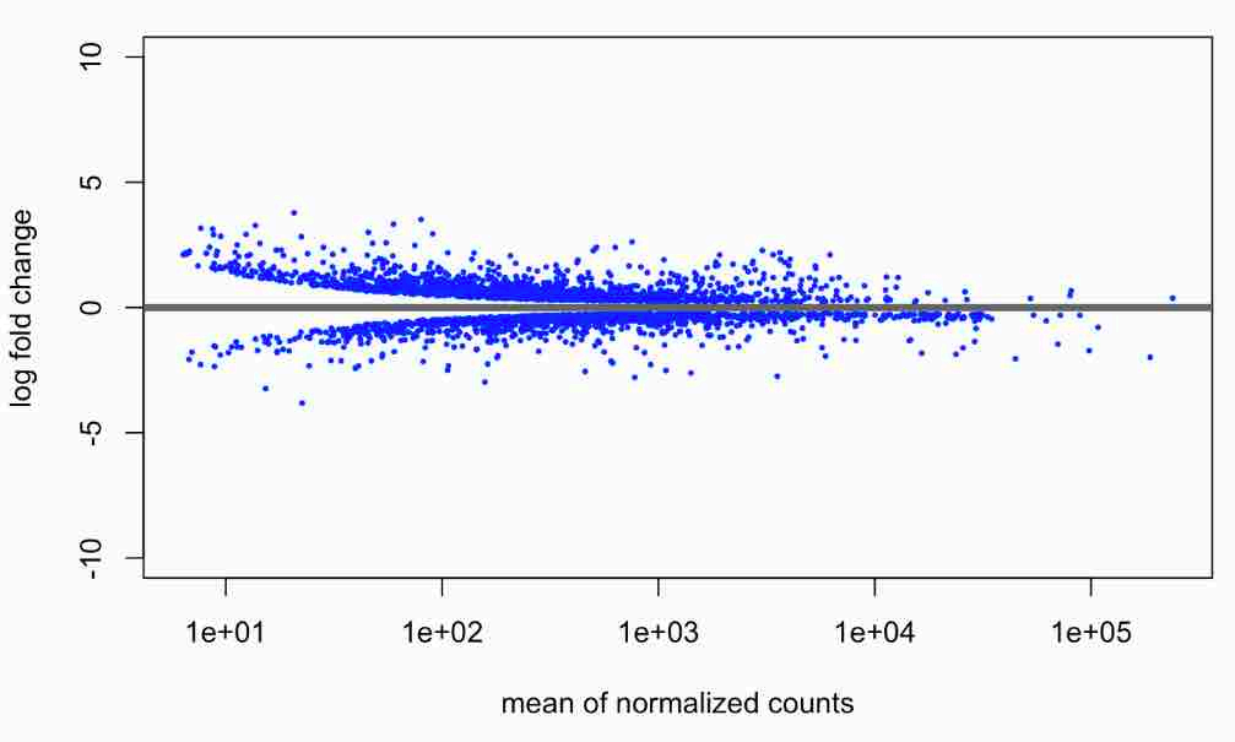


*Figure 18:An MA plot generated from the WT treated vs WT untreated comparison. X shows the mean of the normalised counts for each gene and Y shows the log fold change. Negative fold change indicates decreased expression In the treated group, and a positive fold change indicates an increase. Results where subset to only include those that where statistically significant ($p < 0.05$).*

Finally, in order to assess the effects of elevated glucocorticoid signalling and compare them with the results seen in section 3.4 the expression between the WT untreated and treated samples was assessed.

Fig. 18 shows similar results to fig. 14, however it appears that there were slightly more significantly differentially expressed genes in this comparison. This is interesting as it suggests that increased glucocorticoid activity has a more significant effect on gene transcription than the mutation itself.

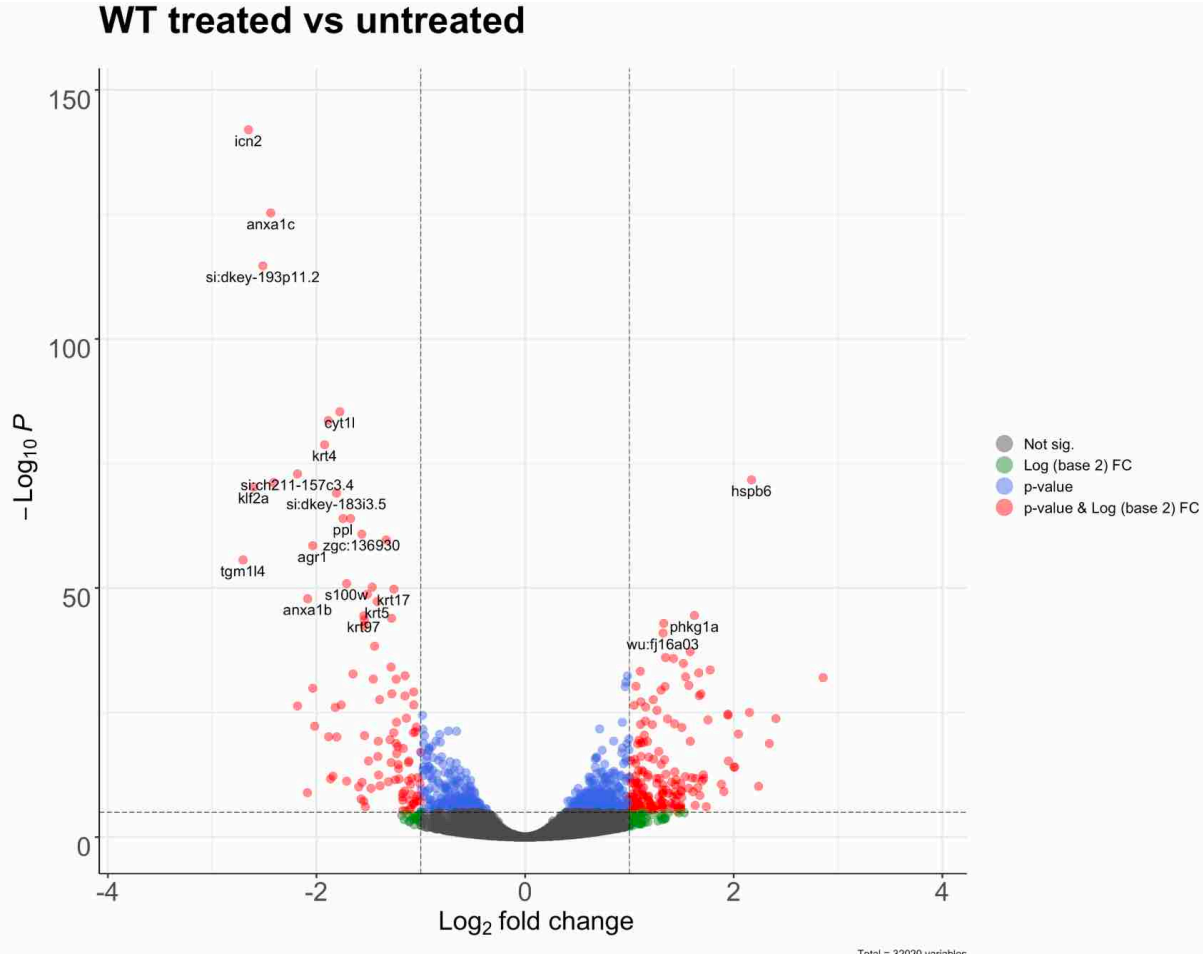


*Figure 19: A volcano plot of the WT treated vs wildtype untreated comparison. Genes in red where both statistically significant ($p < 0.05$) and had a fold change > 1. X axis shows log 2 fold change and y shows a log10 adjusted p value. The top 30 most significant genes where labelled where they do not overlap.*

To further assess these findings a volcano plot was produced.

A similar number of genes where downregulated and upregulated, however it seems that those that were downregulated show greater statistical significance. What's more interesting is that a sizeable number of downregulated genes in this comparison were also seen to be downregulated in figure 15, including anxa1b and ppp1r27b. hsbp6 was once again upregulated in the treated group, which also shows a similar effect to that seen in figure 15.

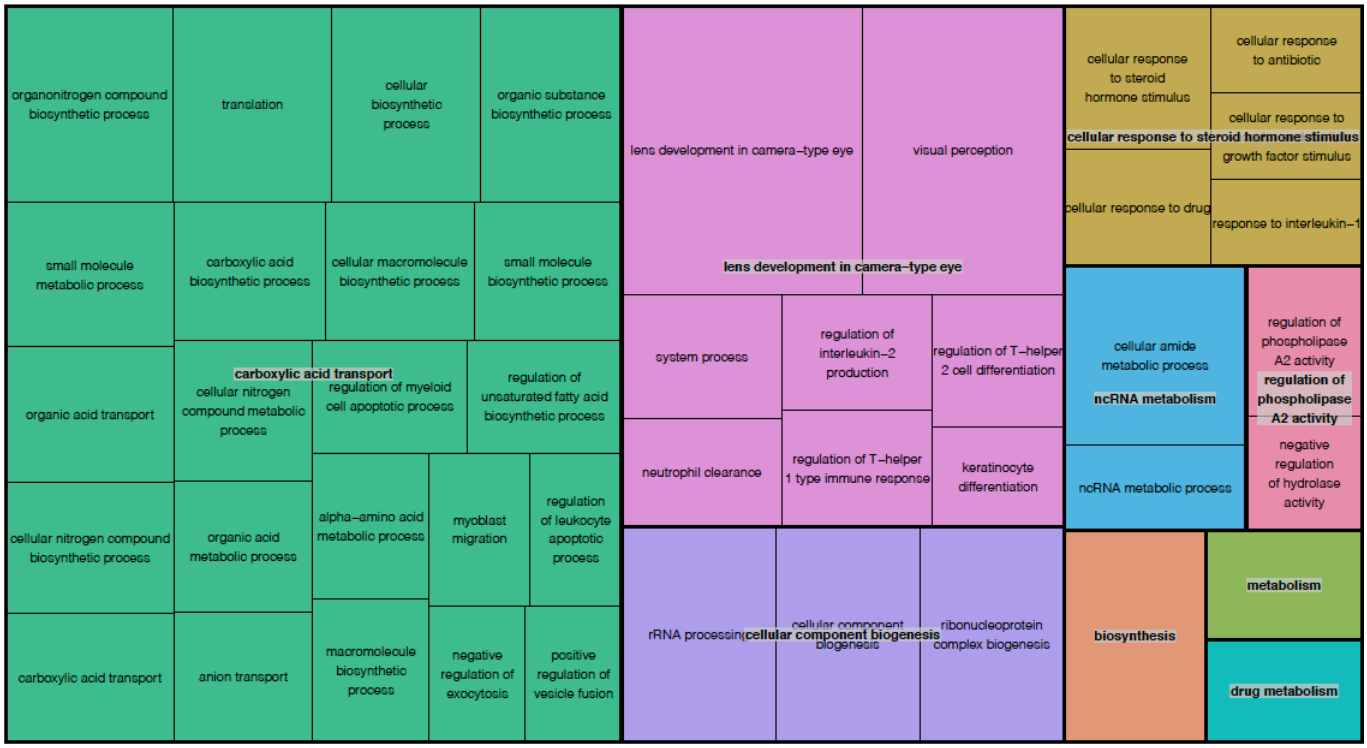


*Figure 20: A revigo gene ontology treemap generated from the list of differentially expressed genes between WT treated and WT untreated samples. Processes that relate to one another are color coded. The size of each box indicates significance.*

A revigo gene ontology analysis was once again conducted

A significantly large number of terms where produced due to the increased size of the DE gene list. Terms in this comparison appear to be focused on processes related to immune response and metabolism, as well as response to steroid hormones. This is very similar with those seen in fig. 17, which implies that elevated glucocorticoid signalling may produce similar effects to the hydrocortisone on the mutants. This raises the question as to why these effects occur.

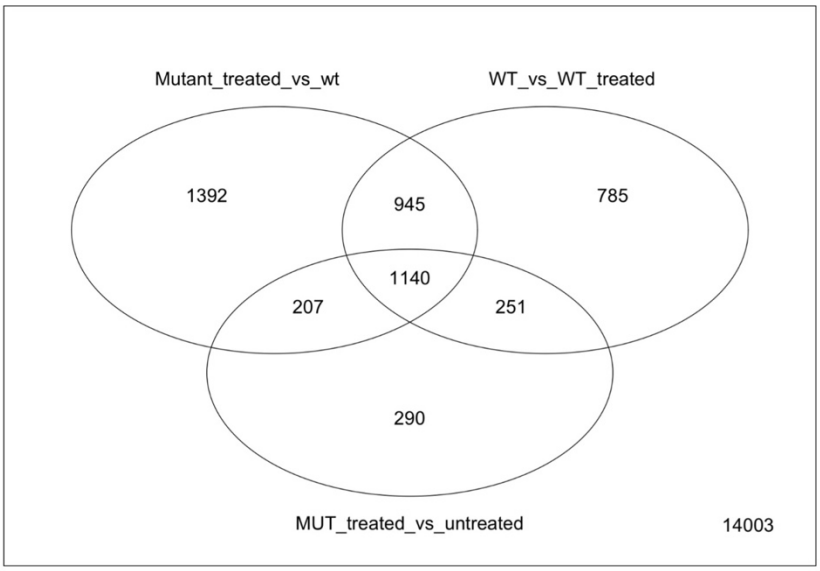


*Figure 21: A venn diagram comparing 3 de gene lists: mutant treated vs wildtype, WT vs WT treated and MUT treated vs MUT untreated. Data was subset to only include statistically significant results ($p < 0.05$).*

In order to view the differences and similarities between the de gene lists a Venn diagram was created of the significantly differentially expressed genes in each comparison.
Fig. 21 shows a large amount of similarity between the different gene lists, with 1140 genes in common between the three. It seems that there was slightly more similarity between the mutant treated vs untreated and WT treated vs untreated lists, which shows that some of the effects of elevated glucocorticoid signalling are likely present in the mutants that where treated, despite their initial lack of glucocorticoid signalling.

# 4.0 Discussion

## 4.1 CYP21a2 mutants show reduced glucocorticoid signalling

In order to assess the impact of the cyp21 knockout it must first be determined whether the mutation did in fact cause glucocorticoid deficiency. To do so the results of this study were compared with prior work to assess the function of previously identified glucocorticoid regulated genes. One such gene is fkbp5, which encodes a cochaperone protein directly involved in the trafficking of the activated glucocorticoid receptor to the nucleus, where it enacts its effects (Grad & Picard, 2007). Since this gene is directly involved in the function of the activated GR, it is a good candidate to indicate levels of glucocorticoid signalling. Prior studies on 21-hydroxylase deficiency have used fkbp5 as an indicator. A previous study using a cyp21a2 zebrafish knockout showed reduced fkbp5 expression in mutants, compared to wild type samples (Eachus, et al., 2017). The results seen in fig.7 and 8 mirror this. The lack of fkbp5 expression indicates that fkbp5 is not being recruited to translocate the GR to the nucleus, and therefore a reduced amount of glucocorticoid signalling is implied.

For further confirmation of this point, the levels of klf9 transcription were also studied. Prior studies have shown that klf9 is key gene regulated by glucocorticoid signalling, as it functions to mediate the cortisol controlled immune responses (Gans, et al., 2020). Fig. 8 shows that klf9 was significantly downregulated in the mutants.

There is strong indication that genes known to be mediated by glucocorticoid activity have been downregulated and therefore glucocorticoid signalling has been impaired.

## 4.2 The transcriptomic effects of glucocorticoid insufficiency link to CAH symptoms.

A range of genes and relating ontology terms were identified in the analysis of the differential expression between the mutants and wildtype animals. Ontology terms fit with some of the general known functions of cortisol, including a range of metabolic processes. These terms can be linked to specific genes identified in the differential expression analysis. Trim63a is one such gene. Classical studies have shown that the trim63 protein, sometimes called murf1, is an e3 ubiquitin ligase. Its primary function is the atrophy of both skeletal and cardiac muscle tissue (Bodine, et al., 2001). Trim63a was shown to be downregulated in the CYP21a2 mutants (fig. 7). This shows that the lack glucocorticoid signalling is likely to impact muscle atrophy. This can be linked to some of the ontology terms seen in fig. 8, such

as “generation of metabolic precursors for energy” and “cardiac muscle morphogenesis”. This is likely to have a significant effect on the whole organism, as these processes are critical for normal heart muscle development. Previous studies have identified Trim63 deficiency in a range of pathogenies involving cardiomyopathy. A genetic study performed on 302 hypertrophic cardiac myopathy patients implicated mutations in the trim63 protein as being a possible cause, implying that the lack of protein degradation in the muscle of the heart can cause excess muscle and therefore hypertrophy (Chen, et al., 2012). Congenital adrenal hyperplasia patients are also known to be at risk of cardiac myopathies. A study in 2015 showed that children with CAH showed a statistically significant increase in left ventricle mass index, indicating ventricular hypertrophy (Nengom, et al., 2017). It can therefore be theorised that a lack of trim63 protein in CAH patients may be one of the potential causes of their heart abnormalities. However, it should be noted that these studies were performed on human CAH patients, as opposed to the zebrafish model in this study, so further research may need to be done in order to solidify this hypothesis.

Only a small number of genes appeared to be upregulated in the mutants, one of these being another cytochrome p450 encoding gene: cyp2aa8. Cyp2aa8 belongs to the zebrafish specific cyp2aa family of genes. Little is known as to the specific functions of cyp2aa8; however, it has been implicated in steroidogenesis (Ruzicka, et al., 2019). Its upregulation could therefor imply that its function is in the production of androgens, which in excess are the cause of a range of symptoms in CAH. One of the gene ontology terms identified in fig. 9 was steroid catabolism, which may link to this gene. However, the previous CYP21a2 knockout study in zebrafish did not find any hyperandrogenism in the samples studied, implying that an excess of male steroid hormones may not be a symptom of this specific model of adrenal hyperplasia (Eachus, et al., 2017). This study did not monitor physical characteristics of the larvae and it cannot be determined whether these zebrafish exhibited androgenous traits. As a result, more research is required as to the function of cyp2aa8 in zebrafish and its relation to cortisol and steroid hormone synthesis.

Paics was another gene that showed significant upregulation in the mutants (fig. 8). Paics is an enzyme that catalyses the conversion of precursors in the steps required to produce purine nucleotides. Its upregulation in the mutants would therefor indicate an increase in purine nucleotide biosynthesis. Studies have shown that human CAH patients exhibit

increased purine synthesis. For example, (Alwashih, et al., 2017) showed that patients who were treated with a low level of hydrocortisone showed higher levels of inosine (a purine). This shows that the upregulation of Paics may be the cause of this effect.

### Treatment with hydrocortisone reverses some effects of mutation

Both fig. 11 and 12 show some reversal of the effects seen in the mutation. FKBP5 was shown to be significantly upregulated in the mutants treated with hydrocortisone, and since this is a marker of glucocorticoid activity it can therefore be assumed that glucocorticoid signalling was at least somewhat restored in the mutant zebrafish. Fig 13 certainly reflects this, with a range of glucocorticoid relating GO terms being identified, such as “cellular response to glucocorticoid stimulus” and “response to corticosteroid”. The literature is sparse in terms of specific functions of many of the upregulated genes and how these may relate to glucocorticoid signalling. However, the most significantly downregulated gene was shown to be cyp2aa8, which as mentioned earlier may be an indicator of androgen production. The fact that this gene was downregulated upon recovery of some glucocorticoid function suggests that this gene is at least very closely linked to the activity of glucocorticoids. More research may need to be carried out to determine its link to steroid hormone synthesis.

### Treatment with hydrocortisone in mutants produced comparable results to elevated glucocorticoid activity in the wildtype.

The most interesting result of this study comes from the comparison of the mutant samples with the wild type. It seems that whilst hydrocortisone treatment recovered some glucocorticoid signalling, it also produced a range of effects not seen in the wild type animals. A range of GO terms come up in Fig. 17, with some relating to immune cell regulation and phospholipase-a production. Fig. 15 and Fig. 16 give indication as to the cause of this, with genes such as anxa1 being downregulated in the treated mutants. Anxa1 encodes annexin a1, which is an anti-inflammatory protein that acts in the immune system to inhibit inflammation. Annexin a1 is induced by glucocorticoid activity to prevent inflammatory responses from becoming too prolonged, hence causing damage to the organism (Vago, et al., 2012). The downregulation of anxa1 in the treated mutants is

therefore confusing, as one might assume that hydrocortisone treatment would result in anxa1 upregulation due to increased glucocorticoid activity. Classical studies have identified annexins as a marker for glucocorticoid activity, showing that serum glucocorticoid levels directly correlate with annexin 1 levels (Mulla, et al., 2005).

Interestingly similar effects were seen in the WT animals treated with hydrocortisone. They too showed downregulation of anxa1c, and also anxa1b with GO terms also relating to immune response. These effects further contradict those seen in the literature, as these animals had fully functioning cortisol secretion with the addition of hydrocortisone, which should therefor increase annexin levels. These effects can perhaps be explained by the developmental stage of the animals. Since these zebrafish where in the larval stage it could be possible that the hydrocortisone disrupts normal development of the glucocorticoid system. It is known that exposure to stress in early life, even prenatally can result in a range of later life symptoms in mammals, including chronic inflammation (Harris & Seckl, 2011). A study from 2016 showed that treating early-stage zebrafish embryos with glucocorticoids resulted in aberrant expression of immune response related genes (Hartig, et al., 2016). Whilst annexin wasn't specifically mentioned in this study, this may suggest a reason as to the downregulation of the anxa1c and anxa1b genes in both mutant and wildtype zebrafish treated with hydrocortisone.

Fig. 21 implicates that many of the same genes were affected by the hydrocortisone treatment in both genotypes, so the adverse transcriptional affects are likely to be universal. This may imply that hydrocortisone is somewhat of an overkill treatment, as it seems to produce the effects of elevated glucocorticoid signalling in the mutants. This raises questions as to how the treatment may be affecting humans, and whether some of the common side effects may be attributed to this exaggerated effect on gene transcription.

## Conclusion

A distinct set of glucocorticoid regulated genes were identified in the mutants, which were linked to the symptoms of CAH, further cementing the idea that interrenal hyperplasia in zebrafish can lead to a further understanding of the disease. The hydrocortisone treatment managed to reverse some of the transcriptional effects of the mutation, however it also appeared to produce similar effects when it was both given to mutant and wildtype animals.

This research therefor opens up many questions as to the effects of hydrocortisone concentration on both gene transcription and steroid hormone synthesis

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