

Crosstalk between glucocorticoid and thyroid hormone signaling at post-embryonic transition.

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Abstract

Endocrine signaling mediated by nuclear receptors form a set of highly conserved pathway among chordates. Thyroid hormones signaling has a wide range of biological effects involved in the control of homeostasis and development. TH also control the onset of amphibian metamorphosis, which resemble the mammalian perinatal period. Indeed, *Xenopus* metamorphosis has been an instrumental model in building our current understanding of TH signaling during vertebrates development. Glucocorticoids (GC) mediate stress response in a physiological or developmental context. They also act as a relay that channel environmental inputs and modulate the corresponding biological processes. Although deeply adaptative in nature, GC mediated responses may also promote more detrimental long term effects. Similarly, the clinical use of GC is limited by long term adverse effects. Surprisingly, although functional interactions between TH and GC signaling are known, there are supported by little molecular data.

Here, we used extensive analysis of RNA-Seq data to collect the molecular details of the crosstalk between TH and GC signaling during limb development, *in vivo*. We show that the transcriptional response to TH and GC is highly archetypal and that crosstalk affects expression of a restricted number of genes (~ 300), mostly involved in bone formation and resorption or linked to human skeletal pathologies. We also show that GC treated metamorphic tadpoles have a strong phenotype with early ossification of long bones. Thus, our work provide a first molecular landscape of TH and GC crosstalk with potential applications for human health.

Introduction

Adversity early in life is thought to elicit developmental adaptations that serve to improve perinatal survival and prepare the organism for a range of postnatal environments ([1]; [2];[3]). These processes, although adaptive in their nature, may later prove to be inadequate or disadvantageous ([1]). Early life conditions are now known to contribute to several pathologies such as mental and cardiovascular diseases, obesity and cancer, occurring much later in life ([4]). Furthermore, as dysfunctions could involve epigenetic modifications set early in life, they have the potential, in certain cases, to be transferred to subsequent generations. The perinatal period is marked by endocrine signaling pathways that control a number of morphological, developmental and physiological processes. Two of these endocrine signals are thyroid hormones (TH) and glucocorticoids (GC).

TH have prominent effects on growth, development, and metabolism of virtually every cell and organ ([5]). Thyroid diseases are among the most common endocrine disorders worldwide ([5]). On the one hand, TH deficiency has a large spectrum of effects, ranging from lower strength and mild impairment of cognitive development to severe brain and neurological damage. On the other hand, excess TH leads to thyrotoxicosis and death. Both hypothyroid and hyperthyroidism have been linked to increased risk of cardiovascular disease and osteoporosis. This also have well characterized effects on mitogenesis and apoptosis, probably explaining the fact that some thyroid diseases may be linked to increased risk of malignant disorder ([6]). Moreover, while thyroid dysfunction can have strong adverse effect on quality of life, symptoms are very general and frequently misleading. As a result, thyroid dysfunction is difficult to diagnose and often fail to be appropriately treated. Importantly, TH signaling is critical at the perinatal period in mammals, since deficiency results in abnormal development with severe mental retardation and growth defects. This syndrome, dubbed cretinism, is prevented by a single pulse of exogenous TH at birth ([7]).

GC also mediate various physiologic processes including metabolism, immune responses, stress responses and electrolyte homeostasis ([8]). Synthetic GCs are a large class of potent drugs used since five decades for treatment of inflammation, autoimmune disorders, brain

oedema, organ transplant rejection and cancer, to name a few. However, despite clinically relevant effects, GC also promote a wide range of adverse outcomes. For example, prenatal exposure to elevated GC levels, either clinically or through maternal stress or malnutrition, can re-program gene expression and increased blood pressure (a risk factor of cardiovascular-related diseases), retardation of childhood cognition development, alterations of long-term behavior, increased insulin resistance, impaired peripheral glucose intake and increased adipose tissue lypolysis ([9]). This is a cause of concern, since synthetic GCs are also used in perinatal medicine to induce lung maturation in case of threatened or preterm birth [10]. Importantly, a few molecular details of crosstalk between GC and TH signaling have been addressed, mainly through the regulation of TH metabolizing enzymes (Dio2, Dio3, [11;12]). It is noteworthy that antenatal GC treatment increases early total TH levels in premature infants, and that GC regulate peripheral metabolism of TH. In addition, human populations are exposed to increasing amounts of TH and/or GC-disruptors, which are found in biological fluids in adults, children, pregnant women and even amniotic fluid. It should be emphasized that endocrine disruptors can perturb not only the TH and GC signaling *per se*, but also their functional interactions. However, although their medical, environmental and societal cost are increasingly recognized and are believed to be high, this point is difficult to address fully because the molecular basis of many of these effects are not known. In this context, we set out to address the molecular and phenotypic crosstalk between TH and GC, *in vivo*, in a model of perinatal development.

Our working model is amphibian metamorphosis, the well known post-embryonic developmental transformation of an aquatic tadpole into an air breathing frog. This complex process implies the growth *de novo* of new body parts (limbs) and the remodeling of (almost all) organs. Interestingly, metamorphosis parallels many aspects of the perinatal period of mammals (transition aquatic vs air breathing life style, conserved gene regulatory networks, tissue resorption... [13]), including their strict control on regulation by TH. In fact, it has been proposed that TH driven metamorphosis would be an ancestral feature of vertebrates and that evolutionary and ecological inputs would provide the basis of “variations on a theme”, where subtle variations in a core signaling pathway in a range of species translates into diverse post-embryonic developmental scenarios. During natural metamorphosis, plasma GC levels rise markedly at metamorphic climax and follow that of circulating TH. Interestingly, the timing of metamorphosis is in part modulated by environmental inputs through GC signaling, which

thus acts as a physiological interface that integrates environmental cues into regulatory networks. The genomic effects of TH and GC signaling are mediated by their cognate receptors, which are ligand-dependant transcription factors belonging to the super family of nuclear receptors.

In this paper, we probe the crosstalk between TH and GC at the onset of *Xenopus tropicalis* metamorphosis. To this end, we measured transcripts levels by RNA sequencing of developing limbs and we provide evidence of a crosstalk between TH and GC signaling. This crosstalk is quite general and is not limited to the regulation by GC of the TH metabolizing factors Dio2 and Dio3, as previously acknowledged. Strikingly, GC/TH crosstalk also impact the expression of many genes involved in skeletal development, which translate in early ossification of long bones. Given that some of these genes (REL, LEPR and Dio2) are known biomarkers of human bone pathologies, our work provide the scientific community with a set of novel candidates genes which might be of interest for the development of new molecular markers of bone pathologies.

Material and Methods

Animal care

X. tropicalis adult frogs were obtained from NASCO (Fort Atkinson, WI) and maintained at 24°C in aquatic housing system (MPAquarien, Rockenhausen, Germany). Mating was induced by injection of 200 U of human chorionic gonadotropine for females and 100 U for males (Chorulon; Intervet, Beaucouze, France). Tadpoles were raised at 26°C. Animal care was in accordance with institutional and national guidelines (ref: 68008, delivered by the Cuvier Ethic Committee).

Hormonal treatments

T₃ (T2752, SIGMA) was dissolved in 0.1N NaOH and added to the culture medium to a final concentration of 10 nM. CORT (C2505, SIGMA) was dissolved in 100% DMSO (D8418, SIGMA), and added to the culture medium or the tank to a final concentration of 100 nM. All treatments received an equivalent amount of DMSO vehicle (0.001%).

Hindlimb culture

Hindlimb explant cultures were carried out in order to investigate the peripheral effects of TH and GC on a developing tissue. We dissected Nieuwkoop-Faber stage 52-54 *X. tropicalis* tadpoles anesthetized on ice. The hind limb buds were amputated just above the bud and separated from the tail by resection. Special care was taken not to separate the two buds or damage the tissue between them. The pairs of hind limb buds were then dipped in 100% ethanol and washed with 65% L15 (11415-049, GIBCO) + antibiotic/antimycotic (15240-96, GIBCO). Each pair of hindlimbs were then cultured in 24 wells culture plates (FALCON), with 1 ml 65% L15 + antibiotic/antimycotic and T₃ and/or CORT at 24°C, protected from light. For each treatment, 5 hind limb buds were used. After 24 hours, the hind limb buds were dissected from the mass of tissue, snap frozen in liquid nitrogen, and stored at -80°C. Three independent biological replicates were used for the RNA-Seq and three independent replicates were used for RT-qPCR validations.

RNA isolation and gene expression assessment

The tissues from either cultured hindlimb buds or whole tadpoles were processed as described in [14]. The endogenous control *rpl8* was selected based on NormFinder [15] analysis of a panel of candidates genes. Raw results were processed using the $-2^{\Delta\Delta Ct}$

method: Data were normalized on the endogenous control *rp18* (ΔCt). For each treatment (T_3 , CORT, T_3 +CORT), ΔCt were normalized on the non-treated control. Resulting values corresponds to the expression fold-change compared to the non-treated control. Statistical significance was addressed with a two-tailed student *t*-test.

RNA-Seq data processing

Sequencing runs quality was assessed with the FASTQC toolkit

(<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Redundant reads were filtered by keeping the read with the best quality score. We used *fastx* toolkit (v 0.0.13) to clip the 3' end of reads when score dropped below 30 on the Sanger scale (Phred+33). Preprocessed reads were mapped on the version 4.1 of the *X. tropicalis* genome ([16]) using bowtie 0.12.3 ([17]) with the following parameters: “-5 10 -m1 -n2 -l28”. Gene expression call is based on models aggregated from Ensembl (version 61) ([18]) and Xenbase [19]. Consistency between each replicate was assessed by Principal Component Analysis (PCA): raw read counts were subjected to a variance-stabilization transformation as described in ([20]), and PCA was applied on the top 1000 most variable genes. The eigen vector of the component corresponding to batch effects (consistent variability between replicates instead of treatments) was set to 0 and the dataset reconstructed, resulting in a denoised version of the data. Differential expression between treatments was performed with DESeq ([20]) version 1.12 with the following parameters: *method*=“pooled”; *sharing-mode*=“maximum”; *fit-type*=“parametric”. Genes with low expression value were discarded as described in the DESeq, with $\theta=0.4$. Genes statistically differentially expressed were called at an FDR of 5%. Genes were grouped in 81 clusters according to their expression profiles. We used fuzzy c-mean clustering, a method akin to k-mean clustering but less sensitive to noise, using the mfuzz package ([21]). We kept clusters corresponding to interactions between T_3 and CORT, and discarded the others. “Antagonistic” genes and “potentiated” genes were distinguished in two lists, and subjected to a final round of clustering to cancel out over-clustering. Culture effects were removed by filtering out genes with similar expression profiles in hindlimb buds and whole tadpoles. Functional annotation and biological “contexts” (tissues, pathologies, biological processes) are based on manual curation of NCBI gene database (<http://www.ncbi.nlm.nih.gov/gene>), the HuGe Navigator database ([22]), and literature. Functional interactions between genes were collected from BioGrid ([23]). Automated functional annotation was done using the g:Profiler suite of tools ([24]).

Skeletal preparations on tadpoles

Tadpoles stage NF54 were treated as previously described and sacrificed 24 hours after the end of the treatment. After eyes and viscera removal, tadpoles were washed for 15 min in ethanol baths of increasing concentration (25%, 50%, 75%, 100%) and stored at -20 C. Cartilage staining was carried out by incubation with Alcian Blue (A3157, SIGMA) 0.05% in a 4:1 EtOH 100% / glacial acetic acid solution overnight at 4°C and light agitation. After 2 EtOH 70% washes (2h each), progressive rehydration and tissue maceration in 0.5% KOH / 3% H₂O₂, bones were stained in 0.05% Alizarin Red (A5533, SIGMA) in KOH 1% for 3 hours. After thorough washing, stained tadpoles are stored in glycerol 100% at 4°C. Pictures were taken on a MZ16F (LEICA), and acquired thanks to a Retiga SRV (QIMAGING) coupled to an RGB-HM-S-IR filter (QIMAGING).

Results

Characterization of hindlimb bud transcriptome by RNA-Seq

In order to address the interactions between TH and GC signaling pathways at the transcript level without the confounding central effects, we cultured pre-metamorphic *X. tropicalis* hindlimb buds treated or not with T_3 and / or CORT. Total RNA were extracted after 24h and subjected to quality controls (Fig S1, “Biological sample collection and processing”). Proper biological response to hormonal treatments was assessed prior to sequencing by measuring transcript levels of *klf9*, a well known CORT and T_3 target (Fig S2). Transcript abundance was strongly induced following T_3 and CORT treatment. The simultaneous treatment with both hormones resulted in higher transcript level compared to each treatment alone. These results agree well with previous reports ([25]), and further confirm the combined effects of both T_3 and CORT in our experiment.

After deep sequencing, raw data were subject to standard RNA-Seq processing procedures (“low level processing” Fig S1) with quality controls. Mapping statistics are shown in Table S1. Importantly, we confirmed that sequencing depth was high enough and resulted in about 73% of gene detection (Fig S3). One library (CORT treatment, replicate 3, Table S1) was sequenced at a much higher depth, which marginally affected the detection of expressed genes (bottom panel Fig S3). Also, we corrected for batch effects after principal component analysis (PCA) by removing the corresponding principal component (Fig S4A before batch effect removal, Fig S4B after batch effect removal).

Overall, we found 3,793, 29 and 4,367 genes differentially expressed in T_3 , CORT and T_3 +CORT treatments respectively (Fig 1, Table S2), for a total of 4,741 genes differentially expressed in at least one treatment compared to the control. Induction folds (in log2) varied over a large dynamic range: between -6.33 and 7.52 for T_3 treatment, -2.48 and 3.74 for CORT, and -4.53 and 8.19 for T_3 +CORT.

The technical validation of RNA-Seq data was carried out by RT-qPCR on a number of genes selected over a wide range of expression levels and induction folds (Fig S5A). We found a good correlation ($r = 0.847$) between RT-qPCR and RNA-Seq measures of gene expression (Fig S5B).

Glucocorticoids interfere with thyroid hormone signaling

Interactions between TH and GC signaling can result in two different expression profiles: “antagonistic” (where the effect of one hormone decreased the effect of the other) and “potentiated” (where the effect of one hormone increased the effect of the other). To isolate “potentiated” and “antagonistic” genes, we carried out expression profile-based clustering on genes that were differentially expressed in at least one treatment (Fig S1, “clustering”). This has the benefit of better controlling the level of false negatives relative to approaches purely based on *p*-values. This may have important biological consequences since many genes may be assigned to incorrect categories. We could classify genes into the two categories of expression profiles: 203 “antagonistic” genes and 440 “potentiated” genes. We further filtered these gene lists to remove culture effects. To this end, we kept genes for which the expression profile was consistent in both cultured hindlimb buds and hindlimb buds isolated from whole treated tadpoles (Fig S1). This resulted in a final dataset of 11 clusters composed of 112 “antagonistic” (Fig 2A,B) and 206 “potentiated” genes (Fig 2C,D), Table S3. For most clusters, the T_3 effect is altered in T_3 and CORT co-treatment (Fig 2A,C). A modulation of the CORT effect by T_3 is found in only two clusters (Fig 2B,D).

The number of genes per cluster ranged from 3 (cluster 3) to 70 (cluster 11). For antagonistic profiles, 30% of the genes (34/112) correspond to a decrease of the T_3 -mediated induction by CORT, while of 70% of the genes (78/112) correspond to a decrease of the T_3 -mediated repression. For “potentiated” genes, this situation is less contrasted, with 42% of the genes corresponding to a “potentiated” T_3 effect are upregulated and 58% downregulated.

Interestingly, in co-treatment, antagonism of T_3 effects (Fig 2A) arises irrespectively of the CORT effects alone. Indeed, clusters 1, 2 and 4 show little or no CORT effect alone, while the T_3 effect is strongly affected in co-treatment. A similar conclusion can be drawn for cluster 3 and clusters 5, with a small to strong induction or repression in CORT treatment alone. In cluster 6, the strong CORT effect is weakened by T_3 , although T_3 alone has no effect (Fig 2B). “Potentiated” effects (i.e. CORT driven increase of T_3 effect, Fig 2C) occur only when the CORT and T_3 effects both correspond to an induction or a repression of gene expression. This is also true for cluster 11, where T_3 and CORT co-treatment increases the CORT effect, although T_3 alone has no effect (Fig 2D).

Glucocorticoid affect TH driven expression of genes involved in skeletal establishment and maintenance

Gene ontology analysis on the final datasets revealed enrichment in terms typically

associated with response to TH and GC: inflammation, immune system, metabolism and cell cycle. Comparison with KEGG pathways and the REACTOME database lead to a similar conclusion. These categories are consistent with known biology of TH and GC at a physiological, cellular and molecular level. We then set out to further characterize the function of the genes composing our final dataset, and completed these analyses with a systematic and manual review of the biological functions and pathologies associated to these genes (bibliography and NCBI gene pages). We then derived a map connecting each gene to biological functions / pathologies (Fig S6, black links). We also included the functional interaction map from the BioGrid database ([23]) (Fig S6, magenta links). Strikingly, we found strong connections with skeletal development, maintenance and the associated pathologies (Fig 3A). A summary of these interactions is shown Fig 3B, where the dataset is restricted to the categories related to skeletal structure, development and maintenance (“matrix”, “bone”, “cartilage”) and pathological contexts (“rheumatoid arthritis”, “osteoarthritis”, “osteoporosis”, and “spondylitis ankylosis”). We could confirm RNA-Seq results by RT-qPCR (Fig S7).

Almost half of the genes (24/53) are involved in limb development and skeletal structure establishment, while 35/53 are linked to pathologies. We found a limited number of functional interactions between genes. In fact, with the exception of *capn1-sh3bgr*, all interactions connect together *kif22*, *tat*, *il8*, *txnip*, *txn*, *cd40*, *tank*, *traf3m*, *pik3r1*, *dlx2*, *sco1*, most of which are connected to various skeletal pathologies (7/11). Only two genes (*tat* and *sco1*) are not connected either to structural or to pathological components. Genes are mostly connected to osteoarthritis and rheumatoid arthritis (53%, 28/53). Interestingly, 31% (12/39) of genes connected to pathologies are known to be involved in the genesis of skeletal alterations and disorders.

Crosstalk between GC and TH affects skeletal development during metamorphosis

We next addressed whether the interaction between TH and GC at the transcriptomic level translates into morphological changes during hindlimb development. To this end, we treated premetamorphic NF54 tadpoles with a 24h pulse of 10 nM T₃ and/or 100 nM CORT, and carried out bone and cartilage staining after 48h (no difference was observed just after the treatment). For the control experiment as well as the T₃ and CORT treatment, the developing hindlimb showed strong and uniform blue staining corresponding to cartilage. Strikingly, after co-treatment with T₃ and CORT, limb showed a strong and localized red staining at the center of the long bones, indicative of early ossification (Fig 4A).

Discussion

In this paper, we show that GC and TH pathways interact at the post-embryonic transition. The importance of TH and GC signaling as integral components of the functional relays coordinating developmental processes is well described ([26]). In addition to their role in development and the control of homeostasis, perturbations of these two pathways are also linked to a number of human diseases and pathologies. The GC pathway is of special interest because of the popular use of GC-based anti-inflammatory therapies, for example in provoking lung maturation in newborns. Little is known about the crosstalks between TH and GC pathways at this post-embryonic transition and their potential effects later in life.

What is the transcriptional landscape of the GC - TH crosstalk?

After TH and GC co-treatment, the vast majority of genes follow a very stereotyped response corresponding either to a pure TH or GC effect. Surprisingly, only a moderate number of genes (~ 300) display altered expression profile and reflect interactions between TH and GC signaling ('potentiated' and 'antagonistic' genes). Given the large number of TH-responsive genes, this effect is qualitatively modest. Strikingly, we found a very limited number of GC regulated genes, suggesting that in absence of TH, limb growth is poorly responsive to GC or that GC effects are efficiently buffered by non genomic cascades. Therefore, relative to GC signaling, the crosstalk effect is massive and results in ~ 10 times more genes differentially expressed. This may have strong functional implications.

At first, it may be tempting to summarize the GC – TH crosstalk as a direct consequence of the modulation of transcripts abundance of *dio2* and *dio3*. If true, one would expect an enhanced TH response corresponding only to a 'potentiating' effect. This is clearly not the case because the number of 'potentiated' genes is rather modest, relative to the TH effect. In addition, this view fails to account for 'antagonistic' expression profiles. Alternatively, one might argue that the expression of a large number of genes already reached a maximum, thus masking the potentiating effect of the crosstalk. By inducing general metabolism, TH treatments may result in an increase of the total mRNA content per cell. This is unlikely, however, because in our datasets the total read count is not dominated by a limited subset of highly TH responsive genes. Therefore, the crosstalk between GC and TH signaling may not

be limited to the consequences of the *dio2* and *dio3* deregulation, and involves additional molecular mechanisms. In support of this, Kulkarni and Buchholz ([27]) reached similar conclusions and proposed a number of possible regulatory scenarios.

The fact that an interaction exists between these two signaling pathways is not surprising and is already known, and crosstalks take place at different levels in signaling pathways. For example, GC induce the expression of the TH activating enzyme *dio2* and reduce expression of the inactivating enzyme *dio3*. Thus the levels of active TH in target tissues increase and fuel an enhanced TH response ([28]). Also, TSH and ACTH levels are both controlled by CRH (Denver, 1997, Denver, R.J., 1999. Evolution of the corticotropin-releasing hormone signaling system and its role in stress-induced phenotypic plasticity. *Ann. N.Y. Acad. Sci.* 897, 46–53.), which plays a key role in controlling TH dependant post embryonic development. GC is also known to increase the binding capacity of THR to T3 ([29]). At the transcriptional level, GC enhance TRb transcripts level ([25]), and GC and TH synergistically enhance KLF9 expression ([30]). In addition, transcripts levels of GR are modulated by TH in a tissue specific manner ([31]). A number of additional cis-regulatory mechanisms of interactions between GR and other nuclear receptors have also been described ([32]; [33]).

Kulkarni and Buchholz reported recently a micro-array analysis of the GC – TH crosstalk at the post-embryonic transition, in *Xenopus tropicalis* tail. The most striking difference between their dataset and ours is that they found a strong GC effect (~1900 vs ~30 genes) and a large number of genes responding to the crosstalk (~1200 vs ~300 genes). We note, however, that the comparison is somewhat limited because of tissue specific differences, distinct technological platforms (micro-array vs RNA-Seq) and analysis pipelines (Venn diagrams vs clustering), as discussed by Grimaldi et al. ([34]). Nonetheless, we describe here important datasets providing novel insights on the phenotypic output of the GC-TH crosstalk, at the end of post-embryonic development and their potential long terms effects.

What is the functional output of the TH – GC crosstalk?

Strikingly, the crosstalk affects various mediators of the formation and turnover/homeostasis of bone and cartilage, with clear links to pathologies of skeletal structures (Fig 3). This is mediated mostly by alteration of extracellular matrix metabolism and cellular communications.

The corresponding pathways are affected (e.g. TOLL, TNF), although for each of them, the expression of only a few components is affected in the crosstalk. For example, in the TOLL pathway, the expression profile of the pro-inflammatory cytokine IL8 [35] and CD40 ([36]) are consistent with an anti-inflammatory action, following the potentiated repression of TRAF3. The synthesis of extracellular matrix (ECM) components are also affected (ADAMTSL5, EMILIN2, MMP13, MMP13L). They are consistent with ECM remodeling and a rapid turnover of cartilage and bone cell populations.

Our datasets provide novel connections between known pathways or factors and skeletal pathologies. For example, QSOX1 expression follows the clinical grade of breast tumor, but was not previously linked to skeletal pathologies [37]. Of note, breast cancer cells often metastasize to bone structures. The product of the SLC35A3 gene, of unknown function, was thought to be connected to vertebral malformations in human and cattle, although no clear evidence could be reported. TAT expression is related to the Richner-Hanhart syndrome but no link to skeletal pathologies has been reported [38].

The links we provide here illustrate the potential of our datasets as a source of factors potentially involved in the development of human pathologies. Obviously, developmental alterations of skeletal anatomy at the perinatal period (*i.e.* the end of the post-embryonic transition) will most certainly have strong effects later in life and could be detected early. More subtle phenotypes, such as alteration of bone internal fine structure or of signaling pathways, would be more difficult to detect and may lead to delayed diagnosis, treatment and increased societal and economic cost. By providing a number of potential candidates interfering with proper TH and GC at the post-embryonic transition, we hope to contribute to the development of novel markers of perinatal pathologies.

Importantly, we would like to highlight the fact that although we used GC as a direct mediator of stress, the source of crosstalk may have multiple origins. In addition to the clinical use of GC, exposure to endocrine disruptors may affect the balance of circulating levels of GC and TH, thus potentially increasing exposure to crosstalk. Currently, the extent of the crosstalk in other tissues is poorly described and would need further investigation. In a broader perspective, integrative mechanisms, and especially the TH and GC signaling, are essential for adaptation to ongoing global change because they modulate life-history trade-offs. Our results show that crosstalk may counter-balance these adaptive responses and, thus, may affect the ability to cope with a changing world([1]).

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Figure legends

Fig 1: **TH and GC affect hindlimb transcriptome.** Relationship between the mean expression level and the difference of expression measured by RNA-Seq (MA plot). Genes reported as differentially expressed by DESeq (FDR=0.05) relative to the control are colored in blue.

Fig 2: **Interactions between TH and GC signaling results in a limited number expression profiles.** Expression profiles are categorized in 11 clusters. Within each cluster, expression values are summarized by a box plot. Upper and lower "hinges" correspond to the first and third quartiles. The upper and lower whiskers extend from the hinge to respectively the highest or lowest value that is within 1.5 times the inter-quartile range of the hinge. Data beyond the end of the whiskers are outliers and plotted as points. A) "Antagonistic" profiles: GC decrease either TH induced repression (clusters 1-3) or induction (clusters 4 and 5). B) Cluster 6 contains genes for which the GC induced up-regulation is decreased by TH. C) "Potentiating" clusters: GC enhances either TH driven induction (clusters 7 and 8) or repression (cluster 9 and 10). D) Cluster 11 contains genes for which the GC induced up-regulation is increased by TH. For each gene, the expression level was adjusted to a variance of 1, and normalized by the mean expression level accross all four conditions. Ø=control; T=T3; C=corticosterone; T+C=T3+corticosterone.

Fig 3: **Genes co-regulated by TH and GC are functionally enriched in terms related to skeletal structures.** A) Biological terms or pathologies associated to "antagonistic" or "potentiated" genes. Light blue terms are associated to the skeleton structure. B) Interaction map between the genes linked to skeletal components (corresponding to light blue terms in A) and human pathologies. Magenta edges between genes correspond to interactions described in the BioGrid database.

Fig 4: **Brief glucocorticoid treatment affects skeletal development during natural metamorphosis.** Morphology of hindlimb buds 24 hours after a 24 hour treatment (upper

panel). Skeletal preparation (Alcian/Alizarin) of the same samples (bottom panel). Cartilage is stained with alcian blue and bone with alizarin red.

Supplementary Figures (available from the website)

Fig S1: **General workflow of data production, processing and analysis.**

Fig S2: **Measure of KLF9 gene expression by rt-qPCR.** Letters a and b indicate significant differences between the group means based on a two tailed student t-test.

Fig S3: **Saturation analysis of RNA-seq data.** The number of expressed gene (y axis) is plotted as a function and increasing number of reads (x axis).

Fig S4: **Principal component analysis of RNA-Seq data.** The analysis was carried out on raw data (A) or after reduction of batch effects (B).

Fig S5: **RT-qPCR validations of gene expression levels measured by RNA-Seq.** Thirteen genes were chosen from RNA-Seq results over wide dynamic range of expression, as illustrated on the MA plots (A). Expression levels were measured by RT-qPCR and normalized using rpl8 as internal control, with three biological replicated. The relationship between fold changes (in log2 scale) measured by RT-qPCR and by RNA-Seq is shown in panel B.

Fig S6: **Interaction network between genes differentially expressed and biological functions and pathologies.** Yellow hexagons identify biological functions and pathologies, red vertices identifies genes with antagonistic expression profiles, green vertices identifies genes with potentiated expression profiles. Black links correspond to connections between genes and biological processes or pathologies, red dotted links represent connections between pathologies and blue links interactions between gene products.

Functional interaction data between genes were collected from the BioGrid database (Stark et al., 2011).

Fig S7: Interaction network between genes differentially expressed and biological functions and pathologies.

Supplementary Tables (available from the website)

Table S1: **Mapping statistics.**

Table S2: **Expression level of differentially expressed genes.**

Table S3: **Expression level of genes affected by TH and GC crosstalk.**