nature climate change

LETTERS

PUBLISHED ONLINE: 1 AUGUST 2016 | DOI: 10.1038/NCLIMATE3087

Molecular signatures of transgenerational response to ocean acidification in a species of reef fish

Celia Schunter[1], Megan J. Welch[2], Taewoo Ryu[1,3], Huoming Zhang[4], Michael L. Berumen[5], Göran E. Nilsson[6], Philip L. Munday[7]* and Timothy Ravasi[1]*

The impact of ocean acidification on marine ecosystems will depend on species capacity to adapt[1,2]. Recent studies show that the behaviour of reef fishes is impaired at projected CO_2 levels[3,4]; however, individual variation exists that might promote adaptation. Here, we show a clear signature of parental sensitivity to high CO_2 in the brain molecular phenotype of juvenile spiny damselfish, *Acanthochromis polyacanthus*, primarily driven by circadian rhythm genes. Offspring of CO_2-tolerant and CO_2-sensitive parents were reared at near-future CO_2 (754 μatm) or present-day control levels (414 μatm). By integrating 33 brain transcriptomes and proteomes with a *de novo* assembled genome we investigate the molecular responses of the fish brain to increased CO_2 and the expression of parental tolerance to high CO_2 in the offspring molecular phenotype. Exposure to high CO_2 resulted in differential regulation of 173 and 62 genes and 109 and 68 proteins in the tolerant and sensitive groups, respectively. Importantly, the majority of differences between offspring of tolerant and sensitive parents occurred in high CO_2 conditions. This transgenerational molecular signature suggests that individual variation in CO_2 sensitivity could facilitate adaptation of fish populations to ocean acidification.

The uptake of additional carbon dioxide from the atmosphere is changing ocean chemistry, with potentially far-reaching impacts on marine life[5]. Recent studies show that the behaviour of marine fishes and some invertebrates can be impaired by projected near-future CO_2 levels, with implications for key ecological processes such as navigation, habitat selection, recruitment, competition and predator–prey interactions[6,7]. Impaired behaviour at high CO_2 levels have been found in a variety of fish taxa, including sharks[8], stickleback[9] and salmon[10]. Fish use chemical cues from injured conspecifics (chemical alarm cues (CAC)) to detect the threat of predation and respond by moving away from CAC and decreasing activity[11]. However, in high CO_2 conditions juvenile fish exhibit a decreased avoidance of CAC and do not learn to associate an increased risk of predation with the presence of CAC[12–14]. The failure to react to predation threat can have immediate consequences for individual survival and may affect population replenishment[4,13,14]. Furthermore, there appears to be limited capacity for within- or between-generation acclimation of impaired behavioural responses to high CO_2 (refs 3,12). Consequently, species will need to adapt to avoid adverse effects of ocean acidification on behaviours that are critical to individual performance and population success.

The adaptive potential of a population depends on the presence of genetic variation upon which selection can act[1]. Previous studies have observed variable levels of behavioural impairment among individuals exposed to near-future CO_2 levels[4,13,15]. However, whether this variation could be transmitted between generations is unknown. One previous study has shown that the average response of juvenile fish to CAC in high CO_2 does not change with parental exposure to high CO_2 (ref. 12), but no studies have yet explored the relationship between individual variation in response to high CO_2 between parents and their offspring. We used a transgenerational rearing experiment to investigate the potential heritability of variation in behavioural sensitivity to ocean acidification in the spiny damselfish, *Acanthochromis polyacanthus*. The underlying cause of behavioural changes in reef fish exposed to high CO_2 appears to be an effect of ionic change from acid–base regulation on the function of the $GABA_A$ receptor, the major inhibitory neurotransmitter receptor in the vertebrate brain[14,16,17]. Therefore, we focused on transgenerational molecular signatures of CO_2 tolerance and sensitivity in the fish brain. We first tested the behavioural sensitivity of adult fish to elevated CO_2 (a projected near-future level of 754 μatm). Fish that retained an innate avoidance of CAC in high-CO_2 water were considered 'tolerant' to high CO_2, whereas fish that became attracted by CAC in high CO_2 were termed 'sensitive'. Adult males and females of similar tolerance or sensitivity to high CO_2 were paired for breeding, with half of the pairs breeding in control conditions and half of the pairs breeding in high CO_2 conditions. Offspring of these parental pairs were then reared in the same CO_2 conditions as their parents. Full brain transcriptomes were sequenced and proteomes were obtained for a total of 33 offspring from the four parental-sensitivity by CO_2 rearing conditions: nine offspring from tolerant parents reared in control conditions; six offspring from tolerant parents reared in high CO_2 conditions; nine offspring from sensitive parents

[1]KAUST Environmental Epigenetic Program (KEEP), Division of Biological and Environmental Sciences & Engineering and Division of Applied Mathematics and Computer Sciences, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia. [2]ARC Centre of Excellence for Coral Reef Studies and College of Marine and Environmental Sciences, James Cook University, Townsville, Queensland 4811, Australia. [3]APEC Climate Center, Busan 48058, South Korea. [4]Proteomics and FACS facility, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia. [5]Red Sea Research Center, Division of Biological and Environmental Sciences & Engineering, King Abdullah University of Science and Technology, Thuwal 23955-6900, Kingdom of Saudi Arabia. [6]Section for Physiology and Cell Biology, Department of Biosciences, University of Oslo, Oslo NO-0316, Norway. [7]ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland 4811, Australia.
*e-mail: philip.munday@jcu.edu.au; timothy.ravasi@kaust.edu.sa

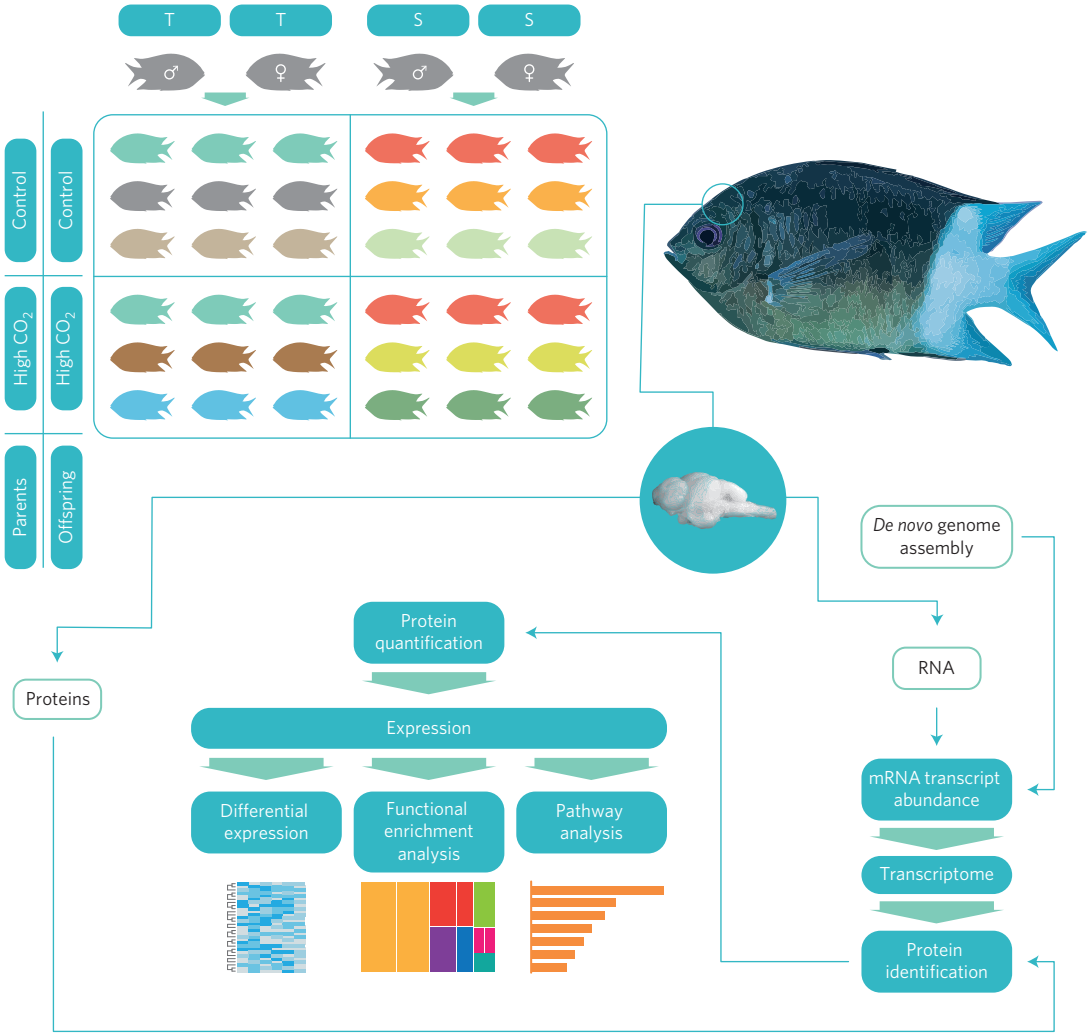

Figure 1 | Sampling design of juvenile fish for molecular analysis of brain transcriptomes and proteomes. Offspring were sampled from breeding pairs of spiny damselfish characterized as tolerant or sensitive to the behavioural effects of elevated CO_2 (see grid). T are 'tolerant' parents, whose behaviour is not impeded, and S are 'sensitive' parents, whose behaviour changed when exposed to high CO_2. Three offspring (biological replicates) from different parental pairs each were sampled from four parental-sensitivity by CO_2 rearing conditions. The colour of the fish indicates different family lines. Fish brains were dissected and processed for transcript and protein differential expression analysis by using a *de novo* assembled genome as the reference.

reared in control conditions; and nine offspring from sensitive parents in high CO_2 conditions (Fig. 1). The *de novo* genome of *A. polyacanthus* was sequenced and assembled to facilitate the assembly and annotation of the transcriptome and proteome data sets (Supplementary Methods).

The expression of brain messenger ribonucleic acid (mRNA) and proteins differed markedly between offspring reared in elevated CO_2 compared with control conditions, as well as between offspring of tolerant and sensitive parents (Fig. 2a). We identified 173 and 62 mRNA transcripts (Fig. 2b) and 109 and 68 proteins (Fig. 2c) showing differential expression between control and CO_2 conditions for offspring of tolerant and sensitive parents, respectively. Only seven transcripts and eighteen proteins were commonly differentially expressed in response to high CO_2 in both parental groups (Fig. 2b,c), revealing a distinct parental influence in responses to CO_2 exposure. Importantly, the majority of differences between these two groups of offspring occurred in fish reared at high CO_2, with 152 transcripts and 99 proteins differentially expressed, compared with 14 transcripts and 46 proteins in control conditions (Fig. 2 and Supplementary Fig. 1).

The general response to high CO_2, irrespective of parental sensitivity, involved genes and proteins associated with the brain's glucose, serine and glycine metabolism. *pck1*, cytosolic phosphoenolpyruvate, is the main control gene for gluconeogenesis and the most upregulated gene of the seven found differentially expressed between high CO_2 and control conditions regardless of parental phenotype (Supplementary Table 1). Thirty-three per cent of these differentially expressed genes matched to differentially expressed proteins directly, but multiple glycolytic proteins involved in similar pathways, such as fructose-bisphosphate aldolase or glyceraldehyde-3-phosphate dehydrogenase, also showed increased expression at the high CO_2 level (Supplementary Table 2). Several other fish species exhibit increased blood glucose levels when exposed to stress and environmental perturbations such as pH changes[18]. Furthermore, upregulation in *pck1* has also been shown to promote a glucose side-branch metabolism: the serine and glycerol-3-phosphate pathways[19]. *Phgdh*, phosphoglycerate dehydrogenase, catalyses the early step of the L-serine synthesis from 3-P-glycerate and is upregulated at the transcript and protein level here. *Shmt2* (serine hydroxymethyltransferase 2) then converts serine into glycine. Serine and glycine are involved in a wide range of processes, such as the biosynthesis of lipids and proteins, and are necessary for cell proliferation. Upregulation of these metabolic pathways in the brain has also been seen

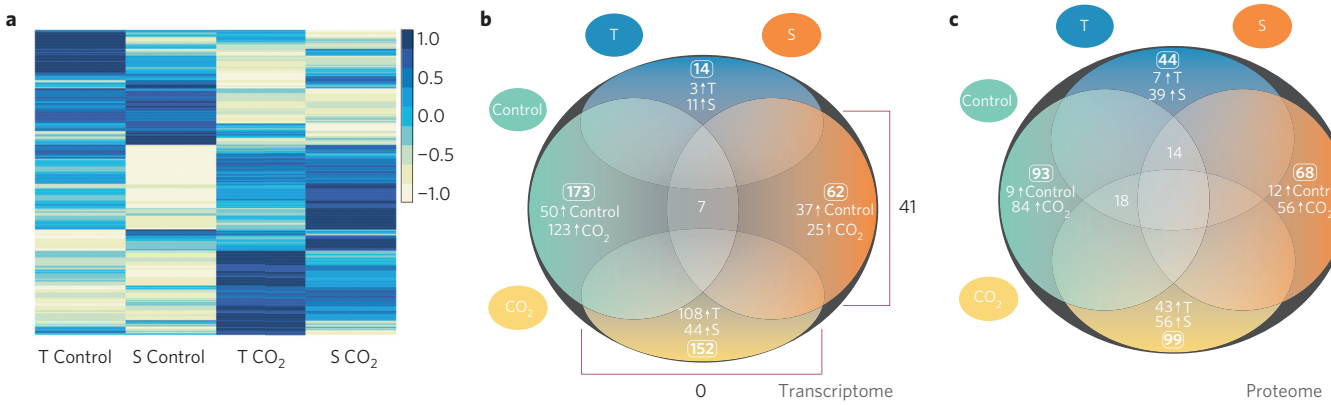

Figure 2 | Differential expression of transcripts and proteins for the four different comparisons of parental-sensitivity by CO_2 rearing conditions. **a**, Heatmap of all differentially expressed transcripts with hierarchical gene clustering. Expression level is indicated by the *z*-score. **b**,**c**, Venn diagram of differentially expressed transcripts (**b**) and differentially expressed proteins (**c**). Square brackets in **b** represent the overall factorial comparisons, for example, 41 transcripts are differentially expressed for control versus CO_2, regardless of parental phenotype. Values enclosed in rectangles show the total amount of differential expression. Upward arrows represent the number of transcripts/proteins that are upregulated in the respective condition. T, tolerant parents; S, sensitive parents; Control, control condition; CO_2, high CO_2 condition.

in zebrafish after the exposure to chemicals[20]. All high-CO_2 individuals, regardless of parental phenotype, exhibited an increased expression of genes related to serine biosynthesis (Supplementary Table 3), revealing a common cost in the stress response to high CO_2 exposure.

The molecular phenotype of offspring from CO_2-sensitive parents was substantially different to that of offspring from CO_2-tolerant parents (Fig. 2). At the protein level, offspring of sensitive parents exhibited a ninefold overexpression of histone 1 (H1), possibly compacting the chromatin and regulating gene expression by inaccessibility to transcription factors[21]. On the transcript level, gene ontology analysis showed that genes involved in transfer ribonucleic acid (tRNA) aminoacylation were uniquely enriched in the sensitive-parents group (Supplementary Table 3). These included several tRNA synthetases, such as *aars*, *dars* and *kars*. tRNA synthetases are necessary for the translation from mRNA into proteins, as they bind the proper amino acid to tRNA. Until recently, tRNA synthetases were thought to be housekeepers, but new evidence links differential expression and mutation in these synthetase genes to human diseases[22], stress response and rapid adaptation to environmental stressors in yeast and *Escherichia coli*[23]. tRNA synthetases are also responsible for adaptive translation[23] and, although rarely studied in fish, seem to be involved in temperature acclimation[24]. Thus, the elevated expression of tRNA synthetases in offspring of sensitive parents may be triggered by an unsuccessful attempt to acclimate. It is possible that this may even become maladaptive in offspring of sensitive parents.

Not only were tRNA synthetases more highly expressed in the transcriptomes of offspring from CO_2-sensitive parents, but there were also differences between the sensitive and tolerant offspring group in the genomic sequence of two tRNA-related genes. We measured the fixation index (F_{ST}) of all single nucleotide polymorphisms (SNPs) found across the transcriptomes of all tolerant-parent offspring against all sensitive-parent offspring to evaluate a potential difference due to a fixed genetic variation. Four outliers were found with different genotypes for offspring of tolerant and sensitive parents within the sequenced coding regions (Supplementary Fig. 2). One SNP was found in the *coro1a* gene involved in immune deficiency and another just upstream of the *igdcc3* (immunoglobulin superfamily) coding region. For both SNPs, the sensitive-parents offspring revealed homozygosity (both copies of the same allele), possibly indicating less adaptive potential for these genes. The other two outlier SNPs were located in *trnt1* and *iars*, tRNA-synthetase-related genes of which *iars* was also differentially expressed for sensitive-parent offspring at high CO_2. This is consistent with a role of tRNA synthetase in the cellular response to environmental stressors[23]. In this study we focused on coding regions of the genome, but additional important genetic variants might be located in upstream regulatory regions of the differentially expressed genes.

The main inhibitory neurotransmitter receptor in vertebrate brains, the gamma-aminobutyric acid receptor A ($GABA_A$), is an ion channel with conductance for Cl^- and HCO_3^-, and its function has been shown to be affected by the exposure to near-future CO_2 levels[16,17]. Fish with impaired behaviour regain normal behaviour after treatment with gabazine, a $GABA_A$ receptor antagonist, and the underlying mechanism is thought to be related to pH regulatory processes altering the neuronal gradients for Cl^- and HCO_3^- (refs 9,14,17). The GABA receptor genes were highly expressed in the transcriptomes of all our tested fish, but at the same level across treatments. However, on the protein level we found aldehyde dehydrogenase 9 member A1 (Al9a1), a protein involved in the dehydrogenation of gamma-aminobutyraldehyde to GABA, to be 1.7-fold overexpressed at high CO_2 in offspring of tolerant parents. Altered GABA receptor function with high CO_2 exposure could be expected to affect the expression of transporter genes and proteins such as the solute carrier family (slc). However, only one non-GABA-related neurotransmitter transporter gene (*slc6a15*) and the glycine neurotransmitter transporter protein (Sc6a5) were differentially expressed, but again were upregulated in offspring of CO_2-tolerant parents in high CO_2 conditions (Supplementary Table 4). This upregulation might help fish deal with the interference of high CO_2 with the function of the $GABA_A$ receptor and at least partly explain individual variation in CO_2 tolerance.

Fish have acid–base and osmo-regulatory mechanisms allowing them to avoid tissue acidosis when exposed to high CO_2, which is one of the predicted physiological costs of acidified oceans[25]. The importance of this is demonstrated by the overexpression of the arginine vasotocin protein in our fish at high CO_2 level, which is a key component in the coordination of osmotic challenges[26]. Most fish closely regulate their acid–base relevant ions (primarily Cl^-, HCO_3^- and H^+) in response to environmental fluctuations of CO_2 (ref. 16). Many processes involving osmoregulation are under circadian regulation, such as acid–base regulation when exposed to different levels of pH (refs 27,28). In this study we find the molecular signature of CO_2 tolerance to be defined by

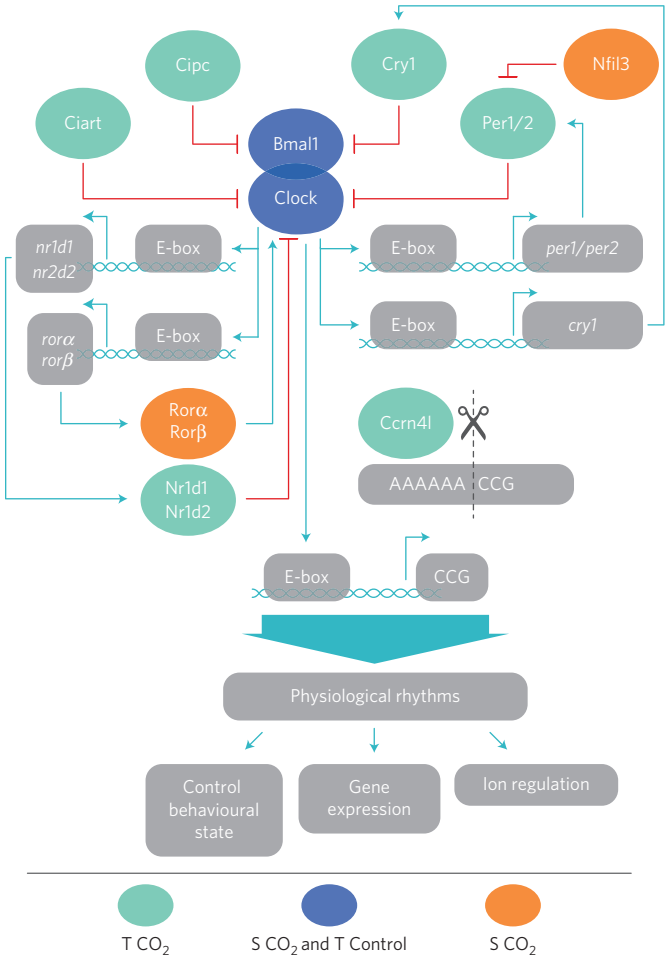

Figure 3 | Differential regulation of circadian rhythm genes for offspring of tolerant parents at high CO_2 condition. All pathway genes are differentially expressed and mRNA upregulation is represented by different colours and refers to different offspring/treatment groups. Pathways of activation (green arrows) and repression (red lines) of different genes[29]. Importantly, repressors are upregulated for offspring of tolerant parents reared in high CO_2 conditions, whereas activators are found downregulated for this group. The scissors represent the posttranscriptional activity of *ccrn4l* by degrading the poly-A tails.

the differential regulation of nearly all components of the circadian rhythm system (Fig. 3). Differential expression of most circadian genes and several proteins are found in offspring of tolerant parents in high CO_2, in comparison to offspring of the same parents at control CO_2 and offspring of CO_2-sensitive parents at high CO_2 levels. Circadian rhythm and rhythmic process are also enriched biological functions (Supplementary Table 3). The main circadian rhythm activator genes such as *bmal1* (also known as ARNTL in mammals) or *clock* were downregulated, whereas circadian rhythm repressors such as *per1, nr1d1* or the Paraspeckle component protein 1 were upregulated in offspring of tolerant parents in high CO_2 conditions. Altered levels of circadian rhythm genes evoke a phase shift in the circadian clock[29] and such phase shifts can provide an adaptive advantage when faced with environmental change[30]. Opposing this downregulation in circadian rhythm for offspring of tolerant parents, we find *asmt* (acetylserotonin O-methyltransferase), the enzyme that catalyses the final reaction in the synthesis of melatonin, a key regulator of the circadian rhythm, upregulated in offspring of sensitive parents. Ion-regulatory adjustments in fish are managed by melatonin, concurrent with the circadian rythm[31]. It is possible, therefore, that offspring of sensitive parents display more pronounced ion-regulatory adjustments in response to elevated CO_2, which in turn leads to more profoundly altered Cl^- and HCO_3^- gradients that interfere with the $GABA_A$ receptor function. We hypothesize that offspring of tolerant parents inherit the 'flexibility' in ion-regulatory control, and therefore the ability to phase shift the circadian clock and avoid a maladaptive reaction to elevated levels of CO_2. This transgenerational signal suggests the adaptive potential of impaired behaviours from high CO_2 due to existing natural variation.

Received 21 March 2016; accepted 21 June 2016; published online 1 August 2016

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Acknowledgements

This study was supported by the Australian Research Council (ARC) and the ARC Centre of Excellence for Coral Reef Studies (P.L.M.), the Office of Competitive Research Funds OCRF-2014-CRG3-62140408 from the King Abdullah University of Science and Technology (T.R., M.L.B., P.L.M., T.Ryu, C.S.), and the University of Oslo (G.E.N.). This project was completed under James Cook University (JCU) ethics permit A1828. We thank the Marine and Aquaculture Research Facilities Unit (JCU), Integrative Systems Biology Laboratory (KAUST), and Biosciences Core Laboratory (KAUST) for support and assistance.

Author contributions

M.J.W. and P.L.M. designed and managed the fish rearing experiments. M.J.W. performed the adult fish behavioural phenotyping. C.S. prepared the samples for RNA sequencing, and together with H.Z. protein samples for mass spectrometry. T.Ryu performed the genome assembly and gene annotation and wrote the corresponding part. C.S. analysed transcriptome expression data, and performed quantitative real-time PCR expression validation and variant analysis. C.S. analysed mass spectrometry data and integrated the data sets. G.E.N. assisted in interpreting the expression data. C.S., P.L.M., T.Ravasi and G.E.N. wrote the paper and all authors read and approved the final manuscript.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. RNA-seq transcriptome sequences have been deposited in GenBank under BioProject ID PRJNA311159. Correspondence and requests for materials should be addressed to P.L.M. or T.Ravasi.

Competing financial interests

The authors declare no competing financial interests.