An osmolality/salinity-responsive enhancer 1 (OSRE1) in intron 1 promotes salinity induction of tilapia glutamine synthetase

Chanhee Kim & Dietmar Kültz

Euryhaline tilapia (Oreochromis mossambicus) are fish that tolerate a wide salinity range from fresh water to > 3× seawater. Even though the physiological effector mechanisms of osmoregulation that maintain plasma homeostasis in fresh water and seawater fish are well known, the corresponding molecular mechanisms that control switching between hyper- (fresh water) and hypo-osmoregulation (seawater) remain mostly elusive. In this study we show that hyperosmotic induction of glutamine synthetase represents a prominent part of this switch. Proteomics analysis of the O. mossambicus OmB cell line revealed that glutamine synthetase is transcriptionally regulated by hyperosmolality. Therefore, the 5′ regulatory sequence of O. mossambicus glutamine synthetase was investigated. Using an enhancer trapping assay, we discovered a novel osmosensitive mechanism by which intron 1 positively mediates glutamine synthetase transcription. Intron 1 includes a single, functional copy of an osmoresponsive element, osmolality/salinity-responsive enhancer 1 (OSRE1). Unlike for conventional enhancers, the hyperosmotic induction of glutamine synthetase by intron 1 is position dependent. But irrespective of intron 1 position, OSRE1 deletion from intron 1 abolishes hyperosmotic enhancer activity. These findings indicate that proper intron 1 positioning and the presence of an OSRE1 in intron 1 are required for precise enhancement of hyperosmotic glutamine synthetase expression.

Euryhaline fish have evolved the capacity to utilize a suite of osmoresponsive genes for rapidly switching between hypo- and hyper-osmoregulation in response to salinity stress to maintain plasma ionic and osmotic homeostasis. Mozambique tilapia (O. mossambicus) are representative euryhaline fish belonging to the family of cichlidae, which consists of many species that are uniquely adapted to specific environments. A remarkable adaptive trait of O. mossambicus is its ability to tolerate large and rapid salinity fluctuations, ranging from 0 to 120 g/kg even though their osmoregulatory balance starts being compromised beyond 60–65 ppt. The corresponding changes in plasma osmolality are normally low and within the range of 305–330 mOsmol/kg. However, when salinity increases chronically to values greater than 65 g/kg or acutely by more than 30 g/kg then plasma osmolality increases between 450 and 550 mOsmol/kg have been reported. Even more moderate but acute salinity stress occurring during transfer of tilapia from freshwater to 25 g/kg results in plasma osmolality increasing up to 460 mOsmol/kg at 15 h. This species has evolved molecular mechanisms for rapidly turning on and off genes that encode enzymes and transporters involved in hypo- and hyper-osmoregulation. Many of the genes and proteins involved in transepithelial ion transport and osmoregulation of euryhaline fish have been identified using candidate gene approaches such as qPCR and Western blotting or large-scale discovery approaches such as transcriptomics and proteomics. However, the regulatory mechanisms deciphering how abundances of the corresponding mRNAs and proteins are regulated are still largely elusive. For example, for many of the regulated genes it is not known whether their abundance change is due to transcriptional regulation or posttranscriptional RNA processing and whether cis- and trans-elements that regulate gene expression are involved. This lack of knowledge contrasts with the evolutionary diversity of fish, which have radiated into virtually any aquatic ecological niche. Previous studies investigating which parts of the genome have a functional
role in the evolution of organisms have stressed cis-regulatory elements (CREs) as major targets of evolutionary adaptation. Therefore, alterations of CREs are considered potent drivers of evolutionary adaptation.

CREs typically contain binding sites for transcriptional regulators that orchestrate gene expression in response to altered environmental and developmental contexts. Many studies have focused on characterizing enhancers, the most studied type of CREs, involved in diseases, development, and cell- and tissue-type specificity, especially in mammalian models. For example, in human renal cells the hyperosmotic induction of the sodium/myo-inositol cotransporter (SMIT) is mediated via several enhancers found in its 5′-untranslated region (UTR). In contrast to these findings in mammalian models, a comprehensive understanding of enhancer functions in fish exposed to salinity stress is still very limited. We have recently identified several copies of a CRE, the osmolality/salinity-responsive enhancer 1 (OSRE1) in the inositol monophosphatase (IMPA1.1) and myo-inositol phosphate synthase (MIPS) genes of O. mossambicus. Enhancers such as OSRE1 are generally considered to function independent of whether they occur in the 5′ or 3′ regulatory regions or in introns. Although most enhancers, including OSRE1 in O. mossambicus IMPA1.1 and MIPS genes, are found in the 5′ regulatory region, intronic enhancers have been previously reported. For example, using human cell lines, Harris et al. have identified a tissue-specific enhancer in intron 1 of the cystic fibrosis transmembrane conductance regulator gene (CFTR). Another study has reported that enhancers located in intron 4 are responsible for differential expression of the Bone Morphogenetic Protein 6 gene (Bmp6), which underlies phenotypic differences between fresh water and seawater populations of threespine sticklebacks (Gasterosteus aculeatus).

The glutamine synthetase gene (GS) encodes an evolutionarily highly conserved enzyme that catalyzes the conversion of ammonia to glutamine. It is thought to be crucial for detoxification of ammonia as a part of the role in the evolution of organisms have stressed cis-regulatory elements (CREs) as major targets of evolutionary adaptation. Therefore, alterations of CREs are considered potent drivers of evolutionary adaptation.

Hyperosmotic induction of glutamine synthetase is transcriptional and mediated by intron 1. Actinomycin D applied to OmB cells during exposure to hyperosmotic stress prevented glutamine synthetase production, which confirms that glutamine synthetase upregulation is mediated by transcriptional induction (Fig. 1). Quantitation of glutamine synthetase abundance revealed a 4.65 ± 0.18-fold increase during hyperosmotic stress (mean ± s.e.m, p < 0.0015, Fig. 1). This increase in glutamine synthetase abundance was completely abolished by including 10 µM actinomycin D in the media to yield a slight 0.85 ± 0.09-fold reduction during hyperosmotic stress (mean ± s.e.m, p = 0.2726, Fig. 1).

The 3.4-kb 5′ regulatory sequence (RS), including the 5′-UTR, of the O. mossambicus GS was cloned, sequenced, and submitted to GenBank (GenBank Accession Number: MN631059). The start codon (SC) for translation was located in exon 2 (Fig. 2a). The first region tested for hyperosmotic enhancer activity was very long and spanned base pairs − 2,825 to the SC (+ 499). The corresponding plasmid construct with the first region inserted for a luciferase reporter assay is shown in Supplementary Fig. S2. This region conferred a 4.65 ± 0.18-fold increase during hyperosmotic stress (mean ± s.e.m, p < 0.0015, Fig. 1). This increase in glutamine synthetase abundance is controlled by intron 1 for the hyperosmotic induction of the sodium/myo-inositol cotransporter (SMIT) is mediated via several enhancers found in its 5′-untranslated region (UTR). In contrast to these findings in mammalian models, a comprehensive understanding of enhancer functions in fish exposed to salinity stress is still very limited. We have recently identified several copies of a CRE, the osmolality/salinity-responsive enhancer 1 (OSRE1) in the inositol monophosphatase (IMPA1.1) and myo-inositol phosphate synthase (MIPS) genes of O. mossambicus. Enhancers such as OSRE1 are generally considered to function independent of whether they occur in the 5′ or 3′ regulatory regions or in introns. Although most enhancers, including OSRE1 in O. mossambicus IMPA1.1 and MIPS genes, are found in the 5′ regulatory region, intronic enhancers have been previously reported. For example, using human cell lines, Harris et al. have identified a tissue-specific enhancer in intron 1 of the cystic fibrosis transmembrane conductance regulator gene (CFTR). Another study has reported that enhancers located in intron 4 are responsible for differential expression of the Bone Morphogenetic Protein 6 gene (Bmp6), which underlies phenotypic differences between fresh water and seawater populations of threespine sticklebacks (Gasterosteus aculeatus).

The glutamine synthetase gene (GS) encodes an evolutionarily highly conserved enzyme that catalyzes the conversion of ammonia to glutamine. It is thought to be crucial for detoxification of ammonia as a part of the role in the evolution of organisms have stressed cis-regulatory elements (CREs) as major targets of evolutionary adaptation. Therefore, alterations of CREs are considered potent drivers of evolutionary adaptation.

The glutamine synthetase gene (GS) encodes an evolutionarily highly conserved enzyme that catalyzes the conversion of ammonia to glutamine. It is thought to be crucial for detoxification of ammonia as a part of...
all four shortened constructs to yield constructs that contain fragments whose 3′ end coincided with the end of exon 1 (+131 bp downstream of the transcription start site, TSS) (Fig. 3a). Removal of intron 1 completely abolished the hyperosmotic induction of reporter activity for all four of these constructs (−718 to +131, −257 to +131, −108 to +131, and −60 to +131) (Fig. 3b). This result demonstrates that intron 1 of GS is required for its hyperosmotic transcriptional induction. To test the hyperosmotic induction of intron 1 in a more physiological context using the endogenous rather than a heterologous core promoter we isolated the GS core promoter (GS-CP). For this purpose, a reporter plasmid containing the GS-CP (−257 to +131) was constructed (Fig. 3a). The functional GS-CP region (−257 to +131) was selected from four putative GS-CP regions because previous studies have shown that for many genes the functional promoter spans from approximately 250 bp upstream of the TSS to 100 bp downstream35. Deleting the region spanning −257 to −108 bp from the GS-CP abolishes GS-CP activity. In addition, we have identified three downstream promoter elements (DPEs) in the GS-CP by motif searching for the ‘RGWYVT’ consensus motif (Fig. 3a).

Intron 1 contains a single, functional copy of OSRE1. Systematic bioinformatics searches of the entire intron 1 sequence for the occurrence of a previously identified OSRE1 was performed by utilizing the OSRE1-consensus (DDKGGA AWW DWWYDNRB) and several specific OSRE1 sequences (incl. O. mossambicus IMPA1.1-OSRE1: AGTGGAAAAATCTAAG) that yielded high hyperosmotic induction of reporter activity in a previous study18. This approach enabled us to identify a single copy of OSRE1-like sequence (AGTGGAAAAATACAAC) in intron 1 of GS. This GS-OSRE1 was 16 bp long and almost identical (88%) to IMPA1.1-OSRE1, harboring only one gap and a single mismatch. GS-OSRE1 was localized on the reverse strand (Fig. 4a).

To verify whether GS-OSRE1 has functional activity as an enhancer element during salinity stress, a series of luciferase reporter plasmids driven by the endogenous GS-CP were constructed. Synthetic oligonucleotides harboring different numbers of copies of GS-OSRE1 were used to validate its function as an osmoreponsive enhancer. Constructs containing either a single copy or up to five copies of GS-OSRE1 were tested using dual luciferase reporter assays (Fig. 4b). Each of these constructs conferred hyperosmotic induction of reporter activity. Moreover, the extent of induction was proportional to the number of GS-OSRE1 copies. However, a single copy yielded only a very small albeit significant degree of hyperosmotic induction 1.2 ± 0.11(s.e.m)-fold (p < 0.01). In contrast, two copies (2.2 ± 0.23(s.e.m)-fold, p < 0.01), three copies (4.6 ± 0.86(s.e.m)-fold, p < 0.01),
four copies (6.6 ± 0.55(s.e.m)-fold, \(p < 0.001\)), and five copies (7.6 ± 0.40(s.e.m)-fold, \(p < 0.001\)) of GS-OSRE1 yielded much greater hyperosmotic induction (Fig. 4c). These data demonstrate that GS-OSRE1 functions as an osmoreponsive CRE during hyperosmotic stress. However, they also show that a single copy of GS-OSRE1 is insufficient to explain the 3.4 to 3.7-fold hyperosmotic GS induction mediated by intron 1 (Fig. 2c).

After confirming the enhancer function of GS-OSRE1 we refined the consensus sequence for OSRE1 by including the GS-OSRE1 sequence in the consensus. This inclusion resulted in a change of the overall OSRE1 motif from DDKGGA AWY DWWY DNRB to DDKGGA AWY DWWY NNRB (Fig. 5).

**Hyperosmotic induction of GS depends on the location of intron 1 and requires OSRE1.** The dependence of hyperosmotic induction of GS on the location of intron 1 was investigated to address whether OSRE1-containing intron 1 behaves as a conventional position-independent enhancer. Unexpectedly, when intron 1 was positioned downstream of the GS-CP (which represents its native genomic context) the hyperosmotic induction of reporter activity was much lower than when it was trans-positioned upstream of the GS-CP (3.4-fold vs. 9.9-fold, Fig. 6a,b). This result shows that intron 1-mediated transcriptional regulation of GS during salinity stress depends on the location of intron 1, which is atypical for conventional enhancers\(^6\). This atypical but pronounced position-dependency of intron 1 mediated enhancement represents a potential mechanism for evolutionary tuning of enhancer responsiveness via trans-positioning regulatory elements.

In addition to establishing the position-dependency of intron 1 enhancement (Fig. 6a, b) and functionally validating OSRE1 (Fig. 4), we investigated whether GS-OSRE1 is necessary for the enhancer function of intron 1. To test whether the presence of GS-OSRE1 is essential for intron 1-mediated hyperosmotic transcriptional induction of GS the 16 bp OSRE1 sequence was deleted from intron 1. The rationale for this experiment was that, although a single copy of GS-OSRE1 was insufficient to account for the hyperosmotic induction of the GS gene (Fig. 4c), it may still be required as an essential component triggering the formation of an inducible transcription factor complex. Two luciferase reporter plasmids with deletions of GS-OSRE1 were constructed and tested for luciferase activity in OmB cells exposed to iso- and hyperosmotic media (Supplementary Fig. S3). One of these constructs harbored intron 1 downstream of the TSS in its native context and the other contained intron 1 trans-positioned upstream of the TSS (Fig. 6c). Selectively deleting GS-OSRE1 (16 bp) from intron 1 completely
abolished the hyperosmotic transcriptional induction conferred by intron 1, independent of the location of intron 1 (Fig. 6d). This result demonstrates that GS-OSRE1 is necessary for the enhancer activity of GS intron 1.

Discussion
In this study, we discovered a novel molecular mechanism where intron 1 harboring an osmoreponsive CRE (GS-OSRE1) positively regulates transcription of the tilapia GS under hyperosmotic stress. Furthermore, we identified that intron 1-mediated hyperosmotic GS induction requires the OSRE1 element and that its enhancer activity depends on the location of intron 1. The molecular mechanism of intron 1 enhancement of GS transcription during hyperosmotic stress differs from that of conventional context-inducible transcriptional enhancers, many of which have been previously shown to function independent of orientation or distance (relative position) to the TSS.37,38

Euryhaline fish embrace a tolerance stage before osmoregulatory mechanisms have fully adjusted to altered salinity, which makes them temporarily vulnerable to dysregulation of osmotic homeostasis before adaptive mechanisms have been remodeled and can take effect.39 Our group and others have documented that tilapia induce organic osmolyte synthesis in multiple tissues during salinity stress, which implies that plasma osmolality increases significantly under those conditions.40-42 OmB cells were exposed to 650 mOsmol/kg, which exceeds the plasma osmolality increase documented in intact tilapia (450–550 mOsmol/kg, see introduction section). However, 650 mOsmol/kg is still below the maximal osmotic tolerance of this cell line (735 mOsmol/kg) and mechanistic dissection of physiological mechanisms is best performed when the corresponding mechanisms are robustly induced, even under slightly exaggerated conditions.43 Moreover, cells and organisms have evolved a safety margin of physiological capacity in response to demand that exceeds physiological conditions actually experienced by as much as ten-fold.44

Previously, we analyzed the 5′ RS of O. mossambicus IMPA1.1 and MIPS genes and identified six osmotically responsive CREs with a common 17 bp consensus motif, which we named OSRE1.18 All of these OSRE1 elements were located between –232 and +56 bp relative to the TSS in both genes. The first osmotically responsive enhancers were identified in mammalian cell lines and named Tonicity responsive element (TonE) and, alternatively, osmotic response element (ORE).45 Bai et al. have also characterized a distinct osmotic-responsive element (OsmoE) in a mouse kidney cell line and revealed its genomic locus to be further upstream (–808 to

---

**Figure 3.** Identification of intron 1 as the genomic region necessary for hyperosmotic GS induction. (a) The genomic sequences used for reporter assays are shown. The Ex1_3′ primer contains a NcoI restriction site at the 3′ end of exon 1. The grey bars indicate constructs that exclude intron 1 from the original constructs tested in Fig. 2 (black bars). The light green bar presents the functional GS core promoter that is used for constructing the backbone reporter vector GS-CP + 4.23. Three purple bars in the light green bar indicate downstream promoter elements (DPEs) that match to the ‘RGWYVT’ motif. (b) Hyperosmotic induction of reporter activity is completely abolished when intron 1 is excluded from the luciferase constructs. The normalized F/R ratio expresses inducible Firefly luciferase activity versus constitutive Renilla luciferase activity. This ratio was measured for both isosmotic (315 mOsmol/kg) and hyperosmotic (650 mOsmol/kg) conditions and normalized by setting isosmotic controls to one. One-way ANOVA was performed to assess statistical significance of the data and calculate p values using the R Stats package software (https://www.R-project.org/). The number of asterisks indicates the statistical significance of the hyperosmotic induction (**p < 0.001; *p < 0.01, *p < 0.05, ns not significantly different). Figure layout and abbreviations are as outlined in Fig. 2.
−791 bp relative to the TSS) in the NHE-2 gene encoding the Na⁺/H⁺ exchanger-2⁴⁶. In addition to the discovery of OsmoE, this study also identified a TonE-like element far upstream (−1,201 to −1,189 bp) in the same gene⁴⁶. Therefore, our initial attempt to identify an osmotically responsive CRE in the GS gene focused on the 3.4-kb region of 5′ RS spanning from −2,825 to +526 bp. Our tilapia study and these previous observations in mammalian model systems suggest (with rare exceptions) that osmoreponsive CREs are located preferentially very close (within a 50-bp range).

Figure 4. Identification of an osmoreponsive element (OSRE1) in intron 1 of GS. (a) Pairwise alignment of the GS intron 1 sequence against the 17 bp IMPA1.1-OSRE1 sequence yielded a match with 15 identical bases and 88.2% of pairwise identity (one gap, one mismatch), which is referred to as GS-OSRE1. The image was generated with Geneious 11.0 (Biomatters, https://www.geneious.com). (b) Reporter constructs containing different copy number of GS-OSRE1 are depicted. (c) GS-OSRE1 represents an inducible enhancer whose transcriptional potency is proportional to copy number. (cp = copy, cps = copies). The normalized F/R ratio expresses inducible Firefly luciferase activity versus constitutive Renilla luciferase activity. This ratio was measured for both isosmotic (315 mOsmol/kg) and hyperosmotic (650 mOsmol/kg) conditions and normalized by setting isosmotic controls to one. One-way ANOVA was performed to assess statistical significance of the data and calculate p values using the R Stats package software (https://www.R-project.org/). The number of asterisks indicates the statistical significance of the hyperosmotic induction (**p < 0.01, *p < 0.05, ns not significantly different).

Figure 5. Refinement of osmolality/salinity-responsive enhancer 1 (OSRE1) consensus sequence. A multiple sequence alignment of GS-OSRE1 with previously identified OSRE1 motifs in IMPA1.1 and MIPS genes⁴⁸ is shown. This alignment yields the refined consensus sequence DDKGGAAWWDYWNNRB. The image was generated with Geneious 11.0 (Biomatters, https://www.geneious.com).
few hundred bp) to the TSS. This knowledge informs comparative studies and future searches for osmoreponsive CREs in other genes and/or species.

With the discovery of functional OSRE1 in GS, IMPA1.1 and MIPS (see above), further genes are deduced to be regulated via OSRE1. The production of compatible osmolytes represents a common functional role of all three genes (GS, IMPA1.1, MIPS). Therefore, it is possible that other genes with the same function are also regulated via OSRE1. One possible candidate is the aldose reductase gene (AR) which produces the organic osmolyte sorbitol and was shown to harbor a TonE/ORE less than 1 kb upstream of the transcription start site in a mammalian model. Furthermore, the sodium- and chloride-dependent taurine transporter gene may be controlled by OSRE1 as its mRNA increases with salinity in tilapia. Another OSRE1 candidate gene is glycine synthase, which increases during hyperosmotic stress and promotes the production of glycine (a neutral amino acid) that can also function as a compatible osmolyte. However, in all of these cases it is not sufficient to find OSRE1 consensus sequences near the promoter without experimentally validating them.

In addition to the role of hyperosmotically induced genes and cellular mechanisms of osmoregulation, a variety of other endpoints have been documented at higher levels of biological organization for euryhaline fish undergoing salinity adaptation. For example, physiological differences in organ function and phenotypic differences of tissues have been detected in osmoregulatory organs such as the gill, kidney, and intestine. Drinking rates and intestinal water absorption are increased in parallel to salinity and the number and size of ionocytes in gill epithelium of euryhaline fish increase during hyperosmotic stress. Salinity adaptation at the whole organism level also includes significant integrative effects of hormones such as growth hormone (GH), insulin-like growth factor 1 (IGF-1), and cortisol to facilitate systemic integration of salinity adaptation to hyperosmolality.

We elucidated that intron 1 in combination with the endogenous GS-CP mediates transcriptional induction of the GS under salinity stress. Intron 1 has been shown to boost gene expression in numerous ways including by providing binding sites for transcription factors, regulating the rate of transcription, promoting nuclear export, and stabilizing transcripts. Several studies of plant species have identified a positive effect of introns on transcription or mRNA accumulation in a constitutive rather than context-dependent manner. Moreover, most reported cases of intron-mediated transcriptional enhancement are stimulus-independent. Only a small number of studies has thus far investigated the stimulus-responsiveness of introns. However, some previous studies using different cell lines have shown that stimulus-dependent transcriptional regulation of a variety...
of genes is mediated by intron 1. For example, in a human breast cancer cell model, intron 1 of the ERBB2 proto-oncogene (ERBB2) contains a 409 bp sequence that mediates ERBB2 transcriptional changes in response to estrogens. These previous studies reporting stimulus-dependent intron 1 mediated enhancement are consistent with our finding that intron 1 enhances GS transcription during hyperosmotic stress. Therefore, our study provides evidence that introns, which have often been regarded as "junk DNA" that is spliced out during mRNA processing, represent functional genomic targets for evolutionary adaptation to environmental changes.

Our observation that the degree of hyperosmotic transcriptional GS induction mediated by intron 1 is position-dependent suggests that the corresponding mechanism is distinct from typical CRE-mediated enhancement. A position-dependent effect of an intronic enhancer was also reported for intron 2 of the human beta-globin gene demonstrating that changes in the location of intron 2 relative to the promoter alters transcriptional activity three-fold. This result is very similar to the three-fold change in transcriptional activity observed in our study when the location of GS intron 1 was altered. Moreover, the position dependence of intron-mediated enhancement (IME) has been well documented in plants.

In other cases reported for IME, however, intron 1 has been shown to act independent of its location. It is likely that sequence rearrangements around the TSS result in conformational changes of the transcriptional machinery, which then affects transactivation efficiency. Therefore, it is possible that intron 1 trans-positioning changes the structural conformation of the transcriptional machinery in a way that increases transactivation. The location of OSRE1 in intron 1 in a position that does not maximally enhance transactivation suggests that evolution has favored moderate over strong transcriptional induction of GS during hyperosmolality. Otherwise, transposition of the CRE elements included in intron 1 upstream of the TSS would have been evolutionarily favored. Possible reasons for limiting the extent to which GS is induced during hyperosmotic stress are as follows: Glutamine synthetase abundance during hyperosmotic stress may represent a compromise between its ability to produce a compatible osmolyte (glutamine) on the one hand and its consumption of energy (ATP) on the other hand. In most organisms including fish, glutamine synthetase is an essential enzyme that mediates bidirectional biochemical reactions, ammonia assimilation and glutamine biosynthesis. Thus, a moderate increase of glutamine synthetase abundance during hyperosmotic stress may be evolutionarily favored as the most cost-effective strategy during salinity stress.

This study demonstrates that the GS-OSRE1 element in intron 1 is essential for transcriptional induction during hyperosmotic stress. The prime candidate transcription factor for activating OSRE1 during hyperosmotic stress is nuclear factor of activated T cells (NFAT5). Mammalian NFAT5 is a fundamental regulator of the cellular response to osmotic stress in mammals. It binds to the Ton/E/ORE enhancer. Since Ton/E/ORE and OSRE1 share a common core motif (TGAGAAA), tilapia NFAT5 has high potential for binding to OSRE1 and controlling its enhancer activity during hyperosmolality. NFAT5 also contributes to osmosensory or osmoregulatory mechanism in fish but its precise role and whether it binds to OSRE1 is still unclear. Another candidate of an OSRE1 binding protein is the tilapia homolog of transcription factor II B (TFIIB). Tiliapia TFIIB mRNA is induced rapidly and transiently within a short period of exposure of fish to salinity stress (fourfold within 2 h) whereas other stressors (oxidative stress and heat stress) did not trigger its induction. Mammalian TFIIB is known to bind to a specific DNA sequence (B recognition element, BRE: SSRCGCC) to promote transcription of a gene by stabilizing the general transcriptional machinery. Thus, TFIIB is less likely to interact with OSRE1 directly but rather might be involved in stabilization of a multi-protein enhancer complex. However, no sequence that resembles the mammalian BRE element is present in the proximal promoter region of GS suggesting that the homologous tilapia sequence diverges significantly from that of mammals, occurs in a region that is more distant from the GS core promoter, or is not involved in the osmotic regulation of the GS gene. Our results suggest that a combination of inducible transcription factors is necessary for promoting transcriptional enhancement since a single copy of GS-OSRE1 outside its native intron 1 sequence context was inefficient for enhancing transactivation. We conclude that other, yet to be identified CREs, are present in intron 1 that interact with OSRE1 to result in transcriptional enhancement. Such combinatorial interactions between different CREs and corresponding transcription factors are common. One important focus of future research will be to characterize such complexes and their interactions.

In conclusion, GS intron 1 was revealed to contain a single OSRE1 (GS-OSRE1) and to enhance transcriptional induction of GS in a tilapia (O. mossambicus) cell line exposed to hyperosmolality. The mechanism for this transcriptional enhancement of GS expression during hyperosmolality has two characteristics: 1. Its extent is dependent on the location of CREs in intron 1 relative to the TSS, 2. It requires GS-OSRE1 for intron 1 enhancer function. Furthermore, our data strongly suggest that the previously identified osmoreponsive CRE OSRE1 consensus sequence can be used for bioinformatics screening approaches that identify candidate OSRE1 sequences on a genome-wide basis. Identification of the transcription factor(s) that bind to GS-OSRE1 and potential other osmoreponsive CREs in intron 1 represents an intriguing future task to understand the process by which osmotic stress signals are perceived and transduced to regulate the expression of genes that compensate for salinity stress in euryhaline fish.

Methods

Cell culture. The tilapia OmB cell line was used for all experiments and luciferase reporter assays. OmB cells were maintained in L-15 medium containing 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) penicillin–streptomycin at 26 °C and 2% CO2. The purpose of FBS supplement is to support sufficient and reproducible OmB cell growth and potential variability issues derived from FBS (e.g. unknown components in FBS can interact with OmB cells or treatments) were minimized/resolved by employing proper controls in parallel with all treatments to isolate osmolality as the only variable factor. Even though FBS is not normally present in fish and has the potential to alter OmB cell responses to hyperosmolality, there is currently no alternative to the use
of serum supplement for Omb cell culture and the vast majority of studies on virtually all vertebrate cell lines use FBS or, less commonly, a similar serum such as horse serum. Using a large supply of Omb cell superstock (passage 15; P15), all experiments were carried out on Omb cells between P18 to P26. Cells were passaged every 3–4 d using a 1:5 splitting ratio. For applying hyperosmotic stress to Omb cells, hyperosmotic (650 mOsm/kg) medium was prepared using hypersaline stock solution (osmolality: 2,820 mOsm/kg). This stock solution was made by adding an appropriate amount of NaCl to regular isosmotic (315 mOsm/kg) L-15 medium. The hypersaline stock solution was then diluted with isosmotic medium to obtain hyperosmotic medium of 650 mOsm/kg. Medium osmolality was always confirmed using a freezing point micro-osmometer (Advanced Instruments).

**Proteomics.** Sample preparation by tryptic in solution digestion, data-independent acquisition (DIA) and targeted proteomics were performed as previously described using a nanoAcquity UPLC (Waters), an Impact HD mass spectrometer (Bruker), and Skyline targeted proteomics software as previously described18. Three peptides of GS that are identical in sequence in *O. mossambicus* and *O. niloticus* (NCBI Accession # XP_003444352.1) were used for quantitation (Supplementary Fig. S1). Three proteins, represented by at least three peptides each, were used for normalization (fatty acid-binding protein, NCBI Accession # XP_003444095.3, beta-tubulin, NCBI Accession # XP_003455078.1, and actin 2, NCBI Accession # XP_003455997.3).

**Cloning.** Total genomic DNA was extracted from spleen tissue of Mozambique tilapia (*O. mossambicus*) using the PureLink Genomic DNA mini Kit (Invitrogen). Fish were maintained and euthanized before obtaining spleen tissue according to UC Davis approved Institutional Animal Care and Use Committee (IACUC) protocol # 19,992. PCR primers were designed with Geneious 11.0.3 (Biomatters, https://www.geneious.com) using the *O. niloticus* glutamine synthetase (NCBI Accession # XM_003444304.4 and XP_003444352.1) genomic sequence as a template. A CCCCCC spacer followed by a restriction enzyme recognition site was added to the 5′ end of each primer. The restriction enzymes KpnI, SacI, HindIII, and NcoI (New England Biolabs) were used to clone PCR amplicons representing genomic regions of the GS gene into pGL4.23 vector. Platinum PCR SuperMix (Thermo Fisher Scientific) and/or Q5 High-Fidelity DNA Polymerase (New England Biolabs) were used to amplify DNA fragments longer than 2 kb. For fragments < 2 kb, PCR Master Mix 2x (Promega) was used. PCR was conducted as follows: initial denaturation at 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, annealing: 48°–60° for 30 s, elongation: 72 °C for 0.5–2 min, and 72 °C for 15 min. Annealing temperature and extension time were set according to the chemical features of the primers and the lengths of amplicons. PCR products were confirmed by agarose gel electrophoresis and sequentially either purified using the PureLink PCR Purification Kit (Thermo Fisher Scientific) or gel-extracted using the QIAquick Gel Extraction Kit (Qiagen). Specific primers were designed for the translation start site (start codon, SC, + 499) and the 3′ end of exon 1 (Ex1_3′, +131). The SC and Ex1_3′ primers included a NcoI restriction site that was already present in the wildtype and Ex1_3′ sites could be cloned without changing any wildtype sequence. All amplified GS gene fragments were double-digested with two enzymes (combinations of KpnI, SacI, HindIII, and NcoI). Restriction enzyme digestion was conducted in 10 μL reaction buffer (CutSmartBuffer and NEBuffer1.1) containing 2 μL (10 U/μL) of each restriction enzyme, 0.5–2 μg of purified PCR product, and nuclease-free H2O ad 100 μL. After overnight incubation at 37 °C, reactions were stopped by 20 min incubation at 80 °C. Digested inserts and vectors were purified using the PureLink Quick PCR Purification Kit (Thermo Fisher Scientific) and ligated to produce recombinant constructs using T4 DNA ligase (Thermo Fisher Scientific). Ligation reactions contained 50 ng of vector, 10–20 ng of insert (depending on its size to yield a 1:3 or 1:5 molar ratio), 2 μL of ligase buffer, 1 μL of T4 ligase (1 U/μL) and nuclease-free H2O ad 20 μL. Ligation proceeded at 25 °C for 6 h. The ligation products were double-digested with two enzymes (combinations of KpnI, SacI, HindIII, and NcoI). Colony PCR conditions were the same as described above and amplicons were checked by agarose gel electrophoresis. Colonies that contained an insert of the expected size were chosen for plasmid purification. Each bacterial colony was inoculated into L-15 medium and grown for 16–18 h to maximize plasmid yield. Liquid cultures were harvested and purified according to manufacturer’s protocol using endotoxin-free PureLink Quick Plasmid Miniprep Kit (Thermo Fisher Scientific). Insert sequences in purified DNA constructs were verified by Sanger sequencing at the University of California, Davis DNA Sequencing Facility before using the corresponding constructs for transient transfection into tilapia Omb cells.

**Enhancer trap reporter assays.** Enhancer trapping assays were performed according to the protocol previously reported by our laboratory18. To produce a backbone luciferase vector harboring the endogenous functional promoter of the GS, the functional GS core promoter region (GS-CP, −257 to +131, Fig. 3a) was cloned into upstream of the firefly (*Phoebus pyralis*) luciferase gene in pGL4.23 vector (GenBank Accession Number: DQ904455.1, Promega) and verified that it has constitutive activity but is not hyperosmotically inducible. The resulting reporter plasmid was named GS-CP+4.23. The GS-CP region was amplified using a forward primer
that included a HindIII restriction site and a reverse primer that included a NcoI restriction site. The GS-CP region and pGL4.23 plasmid were digested with the same pair of restriction enzymes and followed by ligation. Cloning, purification, and sequence-validation were conducted as described in the cloning procedure.

The GS-CP + 4.23 plasmid was used in combination with hRluc (Renilla reniformis) luciferase control plasmid pGL4.73 (GenBank Accession Number: AT7382291, Promega). Co-transfection of tilapia OmB cells with this control plasmid was used to normalize for variability of transfection efficiency and cell number. One day prior to co-transfection OmB cells were seeded in 96-well plates (Thermo Fisher Scientific) at a density of 2 x 10^4 cells per well. Co-transfection was performed when cells reached 80% to 90% confluency. Co-transfection was performed with ViaFect (Promega) reagent using previously optimized conditions. Cells were allowed to recover for 24 h after transfection before being dosed in either isosmotic (315 mOsM/kg) or hyperosmotic (650 mOsM/kg) media for 72 h. Dual luciferase activity was measured in 96-well plates using a GloMax Navigator microplate luminometer (Promega). Four biological replicates were used for each experimental condition. All luciferase raw measurements were adjusted for transfection efficiency by normalizing the firefly luciferase activity to Renilla luciferase activity. They were expressed as fold-change in hyperosmotic media relative to isosmotic controls. One-way ANOVA was performed to assess statistical significance of the data and calculate p values using the R Stats package software.

**Bioinformatics sequence analysis.** Intron 1 was searched for the occurrence of an OSRE1 consensus motif using a bioinformatics approach. For this purpose, Geneious 11.0.3 (Biomatters, https://www.geneious.com) was used. Both strands, sense and antisense, were searched. Sequence similarity searches were conducted by using the overall OSRE1-consensus sequence (DDKGGAAWDWWYDNRB) as well as several experimentally validated and previously identified variants of OSRE1 sequences, including the 17 bp sequence AGTGGA AAAATACTAAG (IMPA1.1-OSRE1), as templates.

**Synthetic oligonucleotide annealing and GeneStrands synthesis.** The effect of GS-OSRE1 copy number variation and GS-OSRE1 deletion on hyperosmotic reporter activity was analyzed. Synthetic oligonucleotides containing different copy numbers of GS-OSRE1 were produced by oligonucleotide annealing (Eurofins Genomics). GS-OSRE1 constructs containing one, two, three, four and five copies were generated. Forward and reverse PCR primers for amplifying each synthetic oligonucleotide were designed to contain SacI and HindIII restriction sites to enable subsequent cloning into GS-CP + 4.23 vector (Supplementary Table S1). Synthetic oligonucleotides harboring more than three copies of GS-OSRE1 or mutated intron 1 (Intron 1 & GS-OSRE1) were longer than 100 bp. These longer inserts were synthesized using the GeneStrands method (Eurofins Genomics). Subsequently, each insert was separately cloned into GS-CP + 4.23 luciferase reporter vector. After cloning into the reporter plasmid, the proper sequences of all inserts were verified by Sanger sequencing. These constructs were used to assess the effect of GS-OSRE1 copy number and deletion of GS-OSRE1 from intron 1 on reporter activity under hyperosmotic (650 mOsM/kg) conditions relative to isosmotic controls (315 mOsM/kg).

**Data availability**

All data generated or analyzed during this study are included in this manuscript and its Supplementary information files. Sequence data for the 5′ R5 of the O. mossambicus GS gene investigated in this study can be found in GenBank with Accession Number: MN631059. The DIA assay library, results, and metadata for glutamine synthetase quantitation are publicly accessible in the targeted proteomics database Panorama Public at the following link: https://panoramaweb.org/IUknq6.urL.

Received: 16 March 2020; Accepted: 11 June 2020
Published online: 21 July 2020

**References**

1. Fiol, D. F. & Kültz, D. Osmotic stress sensing and signaling in fishes. FEBS J. 274, 5790–5798 (2007).
2. Brawand, D. et al. The genomic substrate for adaptive radiation in African cichlid fish. Nature 513, 375–381 (2014).
3. Turner, G. F. Adaptive radiation of cichlid fish. Curr. Biol. 17, R827–R831 (2007).
4. Fiess, J. C. et al. Effects of environmental salinity and temperature on osmoregulatory ability, organic osmoles, and plasma hormone profiles in the Mozambique tilapia (Oreochromis mossambicus). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 146, 252–264 (2007).
5. Kültz, D. & Orken, H. Long-term acclimation of the teleost Oreochromis mossambicus to various salinities: two different strategies in mastering hypertonic stress. Mar. Biol. 117, 527–533 (1993).
6. Moorman, B. P., Lerner, D. T., Grau, E. G. & Seale, A. P. The effects of acute salinity challenges on osmoregulation in Mozambique tilapia reared in a tidally changing salinity. J. Exp. Biol. 218, 731–739 (2015).
7. Brevès, J. P. et al. Acute salinity challenges in Mozambique and Nile tilapia: Differential responses of plasma prolactin, growth hormone and branchial expression of ion transporters. Gen. Comp. Endocrinol. 167, 135–142 (2010).
8. Gardell, A. M. et al. Tilapia (Oreochromis mossambicus) brain cells respond to hyperosmotic challenge by inducing myo-inositol biosynthesis. J. Exp. Biol. 216, 4615–4625 (2013).
9. Kammerer, B. D., Ceich, J. I. & Kültz, D. Rapid changes in plasma cortisol, osmolality, and respiration in response to salinity stress in tilapia (Oreochromis mossambicus). Comp. Biochem. Physiol. A Mol. Integr. Physiol. 157, 260–265 (2010).
10. Kültz, D., Bastrop, R., Jürg, K. & Siebers, D. Mitochondria-rich (MR) cells and the activities of the Na+K+-ATPase and carbonic anhydrase in the gill and opercular epithelium of Oreochromis mossambicus adapted to various salinities. Comp. Biochem. Physiol. B Comp. Biochem. 102, 293–301 (1992).
11. Sacchi, R., Gardell, A. M., Chang, N. & Kültz, D. Osmotic regulation and tissue localization of the myo-inositol biosynthesis pathway in tilapia (Oreochromis mossambicus) larvae. J. Exp. Zool. A Ecol. Genet. Physiol. 321, 457–466 (2014).
12. Wray, G. A. The evolutionary significance of cis-regulatory mutations. Nat. Rev. Genet. 8, 206–216 (2007).
13. Wittkopp, P. J. & Kalay, G. Cis-regulatory elements: molecular mechanisms and evolutionary processes underlying divergence. *Nat. Rev. Genet.* **13**, 59–68 (2011).

14. Ong, C.-T. & Corces, V. G. Enhancer function: new insights into the regulation of tissue-specific gene expression. *Nat. Rev. Genet.* **12**, 283–293 (2011).

15. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* **457**, 854–858 (2009).

16. Dickel, D. E., Visel, A. & Pennacchio, L. A. Functional anatomy of distant-acting mammalian enhancers. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **368**, 20120359 (2013).

17. Rim, J. S. et al. Transcription of the sodium/myo-inositol cotransporter gene is regulated by multiple tonicity-responsive enhancers spread over 50 kilobase pairs in the 5′-flanking region. *J. Biol. Chem.* **273**, 20615–20621 (1998).

18. Wang, X. & Kültz, D. Osmolality/salinity-responsive enhancers (OSREs) control induction of osmoprotective genes in euryhaline fish. *Proc. Natl. Acad. Sci. USA* **114**, E2729–E2738 (2017).

19. Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A. & Bejerano, G. Enhancers: five essential questions. *Nat. Rev. Genet.* **14**, 288–295 (2013).

20. Smith, A. N. Some properties and occurrence of glutamine synthetase in fish. *J. Biol. Chem.* 269, 171–175 (1976).

21. Chew, S. F. et al. Intestinal osmoregulatory acclimation and nitrogen metabolism in juveniles of the freshwater marble goby exposed to seawater. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **180**, 511–520 (2010).

22. Yancey, P. H. Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.* **208**, 2819–2830 (2005).

23. Tok, C. Y. et al. Glutamine accumulation and up-regulation of glutamine synthetase activity in the swamp eel, *Monopterus albus* ( Zaiew), exposed to brackish water. *J. Exp. Biol.* **212**, 1248–1258 (2009).

24. Webb, J. T. & Brown, G. W. Some properties and occurrence of glutamine synthetase in fish. *Comp. Biochem. Physiol. B Physiol.* **54**, 171–175 (1976).

25. Marshall, W. S. Osmoregulation in estuarine and intertidal fishes. In *Fish Physiology*, Vol. 32 (eds McCormick, S. D. et al.) pp. 395–434 (Academic Press, Boca Raton, 2012).

26. Visel, A., Rubin, E. M. & Pennacchio, L. A. Genomic views of distant-acting enhancers.

27. Visel, A. et al. ChIP-seq accurately predicts tissue-specific activity of enhancers. *Nature* **457**, 854–858 (2009).

28. Wang, X. & Kültz, D. Osmolality/salinity-responsive enhancers (OSREs) control induction of osmoprotective genes in euryhaline fish. *Proc. Natl. Acad. Sci. USA* **114**, E2729–E2738 (2017).

29. Pennacchio, L. A., Bickmore, W., Dean, A., Nobrega, M. A. & Bejerano, G. Enhancers: five essential questions. *Nat. Rev. Genet.* **14**, 288–295 (2013).

30. Kalujnaia, S. et al. A regulatory element in intron 1 of the cystic fibrosis conductance regulator gene. *Nat. Rev. Genet.* **14**, 288–295 (2013).

31. Webb, J. T. & Brown, G. W. Some properties and occurrence of glutamine synthetase in fish. *Comp. Biochem. Physiol. B Physiol.* **54**, 171–175 (1976).

32. Cañedo-Argüelles, M., Kefford, B. & Schäfer, R. Salt in freshwaters: causes, effects and prospects - introduction to the theme issue.

33. Aquaculture Genomics, Genetics and Breeding Workshop et al. Aquaculture genomics, genetics and breeding in the United States: current status, challenges, and priorities for future research. *BMC Genom.* **18**, 191 (2017).

34. Shaul, O. How introns enhance gene expression. *Nat. Rev. Genet.* **15**, 145–155 (2015).

35. Kutach, A. K. & Kadonaga, J. T. The downstream promoter element DPE appears to be as widely used as the TATA box in *Drosophila* core promoters. *Mol. Cell. Biol.* **14**, 9947–9954 (1996).

36. Visel, A., Rubin, E. M. & Pennacchio, L. A. Genomic views of distant-acting enhancers.

37. Visel, A., Rubin, E. M. & Pennacchio, L. A. Genomic views of distant-acting enhancers. *Nature* **461**, 199–205 (2009).

38. Zomorodipour, A., Jahromi, E. M., Ataei, F. & Valimehr, S. Position dependence of an enhancer activity of the human beta-globin intron-ii, within a heterologous gene. *J. Mol. Med. Ther.* **1**, 19–24 (2017).

39. Marshall, W. S. Osmoregulation in estuarine and intertidal fishes. In *Fish Physiology*, Vol. 32 (eds McCormick, S. D. et al.) pp. 395–434 (Academic Press, Boca Raton, 2012).

40. Shlyueva, D., Stampfel, G. & Stark, A. Transcriptional enhancers: from properties to genome-wide predictions.

41. Marshall, W. S. Osmoregulation in estuarine and intertidal fishes. In *Fish Physiology*, Vol. 32 (eds McCormick, S. D. et al.) pp. 395–434 (Academic Press, Boca Raton, 2012).

42. Gardell, A. M., Qin, Q., Rice, R. H., Li, J. & Kültz, D. Derivation and osmotolerance characterization of three immortalized tilapia (Oreochromis mossambicus) cell lines. *PLoS ONE* **9**, e9519 (2014).

43. Diamond, J. Quantitative evolutionary design. *J. Physiol.* **542**, 337–345 (2002).

44. Takenaka, M., Preston, A. S., Kwon, H. M. & Handler, J. S. The toxicity-sensitive element that mediates increased transcription of the betaine transporter gene in response to hypertonic stress. *J. Biol. Chem.* **269**, 29379–29381 (1994).

45. Ferraris, J. D. et al. ORE, a eukaryotic minimal essential osmotic response element the aldose reductase gene in hyperosmotic stress. *J. Biol. Chem.* **271**, 18318–18321 (1996).

46. Bai, L. et al. Characterization of cis-elements required for osmotic response of rat Na(+)/H(+) exchanger-2 (NHE-2) gene. *Am. J. Physiol.* **277**, R1112–R1119 (1999).

47. Ko, B. C. B., Ruepp, R., Bohren, K. M., Gabbay, K. H. & Chung, S. S. M. Identification and characterization of multiple osmotic stress response sequences in the human aldose reductase gene. *J. Biol. Chem.* **272**, 16431–16437 (1997).

48. Takeuchi, K., Koyohara, H., Kinosita, M. & Sakaguchi, M. Role of taurine in hyperosmotic stress response of fish cells. *Fish. Sci.* **68**, 1177–1180 (2002).

49. Takeuchi, K., Koyohara, H., Kinosita, M. & Sakaguchi, M. Ubiquitous increase in taurine transporter mRNA in tissues of tilapia (*Oreochromis mossambicus*) during high-salinity adaptation. *Fish Physiol. Biochem.* **23**, 173–182 (2000).

50. Otazu, H. & Gould, K. G. Protective effect of taurine from osmotic stress on chimpanszee ostitisporosis. *Arch. Androl.* **9**, 121–126 (1982).

51. Fossett, J. K., Bern, H. A., Machen, T. E. & Conner, M. Chloride cells and the hormonal control of teleost fish osmoregulation. *J. Exp. Biol.* **106**, 255–281 (1983).

52. Rose, A. B. Requirements for intron-mediated enhancement of gene expression in *Arabidopsis*. *RNA* **8**, 1444–1453 (2002).

53. Wang, B. et al. Functional analysis of the promoter region of Japanese flounder (*Paralichthys olivaceus*) P-actin gene: a useful tool for gene research in marine fish. *Int. J. Mol. Sci.* **19**, 1401 (2018).

54. Bates, N. P. & Hurst, H. C. An intron 1 enhancer element mediates oestrogen-induced suppression of ERB2 expression. *Oncogene* **15**, 473–481 (1997).

55. Bruhat, A. et al. Regulatory elements in the first intron contribute to transcriptional regulation of the bet-3 tubulin gene by 20-hydroxyecdysone in *Drosophila* Kc cells. *Nucleic Acids Res.* **18**, 2861–2867 (1990).

56. Gallegos, J. E. & Rose, A. B. The enduring mystery of intron-mediated enhancement. *Plant Sci.* **237**, 8–15 (2015).

57. Tourmente, S. et al. Enhancer and silencer elements within the first intron mediate the transcriptional regulation of the beta 3 tubulin gene by 20-hydroxyecdysone in *Drosophila* Kc cells. *Insect Biochem. Mol. Biol.* **23**, 137–143 (1993).
58. Lis, M. & Walther, D. The orientation of transcription factor binding site motifs in gene promoter regions: does it matter?. *BMC Genom.* 17, 185 (2016).
59. Kumada, Y. et al. Evolution of the glutamine synthetase gene, one of the oldest existing and functioning genes. *Proc. Natl. Acad. Sci. USA* 90, 3009–3013 (1993).
60. Woo, S. K., Dahl, S. C., Handler, J. S. & Kwon, H. M. How Salt Regulates Genes: Function of a Rel-like Transcription Factor TonEBP. *Am. J. Physiol. Ren. Physiol.* 278, F1006–1012 (2000).
61. Cheung, C. Y. & Ko, B. C. NFAT5 in cellular adaptation to hypertonic stress—regulations and functional significance. *J. Mol. Signal* 8, 5 (2013).
62. Lorgen, M., Jorgensen, E. H., Jordan, W. C., Martin, S. A. M. & Hazlerigg, D. G. NFAT5 genes are part of the osmotic regulatory system in Atlantic salmon (*Salmo salar*). *Mar. Genom.* 31, 25–31 (2017).
63. López-Rodríguez, C. et al. Bridging the NFAT and NF-κB families: NFAT5 dimerization regulates cytokine gene transcription in response to osmotic stress. *Immunity* 15, 47–58 (2001).
64. Fiol, D. F. & Kültz, D. Rapid hyperosmotic coinduction of two tilapia (*Oreochromis mossambicus*) transcription factors in gill cells. *Proc. Natl. Acad. Sci. USA* 102, 927–932 (2005).
65. Gelev, V. et al. A new paradigm for transcription factor TFIIB functionality. *Sci. Rep.* 4, 3664 (2014).
66. Kato, M., Hata, N., Banerjee, N., Futcher, B. & Zhang, M. Q. Identifying combinatorial regulation of transcription factors and binding motifs. *Genome Biol.* 5, R56 (2004).
67. Mandriani, B. et al. Identification of p53-target genes in *Danio rerio*. *Sci. Rep.* 6, 32474 (2016).
68. Pino, L. K. et al. The Skyline ecosystem: Informatics for quantitative mass spectrometry proteomics. *Mass Spectrom. Rev.* 39, 229–244 (2017).
69. R Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria (2013). https://www.R-project.org.
70. Sharma, V. et al. Panorama: a targeted proteomics knowledge base. *J. Proteome Res.* 13, 4205–4210 (2014).

**Acknowledgements**

This investigation was supported by National Science Foundation (NSF) Grant IOS-1656371.

**Author contributions**

D.K. conceived the project, C.K. performed the experiments, conducted the bioinformatics analyses, and analyzed the results and C.K. and D.K. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** is available for this paper at https://doi.org/10.1038/s41598-020-69090-z.

**Correspondence** and requests for materials should be addressed to D.K.

**Reprints and permissions information** is available at www.nature.com/reprints.

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2020