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Review

Transcriptional regulation of *prolactin* in a euryhaline teleost: characterization of gene promoters through *in silico* and transcriptome analyses

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The sensitivity of prolactin (Prl) cells of the Mozambique tilapia (*Oreochromis mossambicus*) pituitary to variations in extracellular osmolality enables investigations into how osmoreception underlies patterns of hormone secretion. Through the actions of their main secretory products, Prl cells play a key role in supporting hydromineral balance of fishes by controlling the major osmoregulatory organs (i.e., gill, intestine, and kidney). The release of Prl from isolated cells of the *rostral pars distalis* (RPD) occurs in direct response to physiologically relevant reductions in extracellular osmolality. While the particular signal transduction pathways that link osmotic conditions to Prl secretion have been identified, the processes that underlie hyposmotic induction of *prl* gene expression remain unknown. In this short review, we describe two distinct tilapia gene loci that encode Prl_{177} and Prl_{188}. From our *in silico* analyses of prl_{177} and prl_{188} promoter regions (~1000 bp) and a transcriptome analysis of RPDs from fresh water (FW)- and seawater (SW)-acclimated tilapia, we propose a working model for how multiple transcription factors link osmoreceptive processes with adaptive patterns of prl_{177} and prl_{188} gene expression. We confirmed via RNA-seq and quantitative PCR that multiple transcription factors

that emerged as predicted regulators of *prl* gene expression are expressed in the RPD of tilapia. In particular, gene transcripts encoding *pou1f1*, *stat3*, *creb3l1, pbxip1a*, and *stat1a* were highly expressed; *creb3l1, pbxip1a*, and *stat1a* were elevated in fish acclimated to SW versus FW. Combined, our *in silico* and transcriptome analyses set a path for resolving how adaptive patterns of Prl secretion are achieved via the integration of osmoreceptive processes with the control of *prl* gene transcription.

Keywords: *in silico,* transcriptome, osmoreception, prolactin, promoter, tilapia, salinity

1. Introduction

Prolactin (Prl) is a pleiotropic hormone released from the pituitary gland that exhibits more biological activities within vertebrates than any other pituitary factor (1, 2). Since its discovery in the 1930s, Prl has been linked with an array of physiological processes that support reproduction, osmoregulation, growth, and development. In turn, decades of sustained investigation have focused upon how the release of Prl from the anterior pituitary is controlled (2-5). Across vertebrates, it is well established that a suite of hormones originating from central and peripheral sources participates in the complex regulation of Prl secretion (2, 6). In addition to hormones with stimulatory or inhibitory activities, the extracellular osmotic environment is an important regulator of Prl cells in euryhaline teleost fishes (6-9). This "osmosensitive" mode of regulation underlies the key role that Prl plays in coordinating teleost osmoregulatory systems (10-12).

Hydromineral balance in vertebrates, including teleost fishes, is contingent upon the tight control of solute and water movements at the macromolecular, cellular, and organismal levels. Fishes that inhabit tide-pools, rivers, and estuaries are readily subjected to changes in salinity that threaten hydromineral balance. When exposed to abrupt changes in environmental salinity, complex homeostatic control systems operate to maintain internal osmotic conditions near established set-points (270-400 mOsm/kg) (13). Deviations from extracellular osmotic set-points are detected by osmosensitive cells, denoted "osmoreceptors", that secrete hormones which act through systemic circulation to regulate organs (e.g., gill, kidney, intestine, urinary bladder, and skin) that actively transport solutes and water (14, 15, 16 , 17-19). For more than 40 years, Prl-secreting cells isolated from the *rostral pars distalis* (RPD) of Mozambique tilapia (*Oreochromis mossambicus*) have been intensely studied to resolve how perturbations in hydromineral balance (deviations of extracellular osmolality within 5 mOsm/kg) modulate the release of Prl in fashions that support a return to homeostasis (16, 20, 21). The native range

of Mozambique tilapia includes habitats with variable salinities; thus, as a model system, tilapia Prl cells allow for links to be made between aspects of cellular osmoreception and a life history strategy that imposes substantial osmoregulatory demands (22-25).

2. Tilapia prolactin cell: a model for investigating the transduction of osmotic stimuli

Given that a stable internal osmotic environment is indispensable to molecular and cellular functions across vertebrates, the systems that mediate osmoreception are likely to be conserved throughout evolution. One sees this in both mammals and teleost fishes where stretch-regulated channels control the entry of Ca^{2+} into osmoreceptive cells (26). The operation of stretch-regulated channels seemingly occurs whether an osmoreceptor is activated by a rise or fall in extracellular osmolality. A rise in osmolality, for example, is the primary stimulus by which osmoreceptive vasopressin-secreting neurons are activated in mammals; reduced cell volume leads to the generation of action potentials via stretch-inactivated cation channels (27). By contrast, tilapia Prl cells are stimulated by a fall in osmolality via stretch-activated cation channels that are activated following an increase in cell volume. Tilapia Prl cells are suppressed by an increase in osmolality. Together, these responses are consistent with Prl promoting survival in over-hydrating conditions such as freshwater (FW) habitats.

The tilapia Prl cell exhibits several attributes that provide distinct advantages for studying osmoreception. Tilapia Prl cells can be isolated as a primary culture and studied *in vitro* because they comprise >99% of the RPD (28). Moreover, the tilapia Prl cell model allows for the simultaneous quantification of gene expression and hormone secretion with other key parameters linked with osmoreception, such as cell volume, intracellular [Ca^{2+}], and cAMP levels (16). Both *in vivo* and *in vitro*, *prl* gene expression and Prl release from the tilapia pituitary are inversely related to extracellular osmolality (20, 21, 29-32). Hyposmotically driven increases in cell volume, mediated by aquaporin 3 (Aqp3), are coupled with the rapid influx of Ca^{2+} through transient receptor potential vanilloid 4 (Trpv4) channels. The increase in intracellular [Ca^{2+}] activates Prl secretion (33-37). Moreover, cAMP also accumulates in Prl cells in response to reduced extracellular osmolality and is dependent on the entry of extracellular Ca^{2+} (38-41). While some of the signaling events that occur in response to hyposmotic stimulation have been well characterized, other aspects of Prl cell physiology remain unclear, especially how extracellular osmotic conditions are linked with appropriate *prl* gene expression (Figure 1).

The tilapia pituitary secretes two Prls, Prl_{188} and Prl_{177} (previously referred to as Prl I and Prl II, respectively), which are encoded by separate genes and share 30-40% protein homology with

mammalian Prl (42, 43). While both Prl_{177} and Prl_{188} respond to reductions in extracellular osmolality and exert similar ion-retaining effects (42), their release is differentially osmosensitive (31). *prl* mRNA levels are also differentially osmosensitive, with more robust expression of prl_{188} relative to prl_{177} in response to the same hyposmotic stimulus (31). Interestingly, the salinity acclimation history of fish also influences the osmotic responsiveness of isolated Prl cells. The baseline expression of prl_{177} and prl_{188} mRNAs is 30-fold higher in Prl cells of fish acclimated to FW versus seawater (SW). Consequently, Prl cells from FW-acclimated fish are not as responsive to hyposmotic stimulation as Prl cells from SW-acclimated fish with regard to *prl* mRNA levels (32, 44). We have described additional instances of the distinct regulation of prl_{177} and prl_{188} levels by extracellular osmolality, such as during autocrine stimulation (45) and between *O. mossambicus* and its congener, *O. niloticus*, with a more narrow salinity tolerance (46). In the latter study, differences in responses of Prls and *prl*s to extracellular osmolality between both species may be tied, at least in part, to the observed inter-specific difference in salinity tolerance.

Previous investigations uncovered additional osmosensitive genes in tilapia Prl cells such as *aqp3* and *trpv4* (32, 37). As described above, these two genes encode proteins essential to the pathway mediating hyposmotically-induced Prl release. While *aqp3* levels are higher in Prl cells of fish acclimated to FW versus SW (37), *trpv4* is induced by hyperosmotic conditions (32). We reported that *osmotic stress transcription factor 1* (*ostf1*) mRNA levels in Prl cells increased in response to hyperosmotic stimulation (31); however, the role of Ostf1 in osmoreception remains to be clarified (31, 32, 37, 47, 48). Prl_{177} and Prl_{188} exert their actions through two Prl receptors, denoted Prlr1 and Prlr2 (49, 50). The expression of both *prlr* mRNAs is also osmosensitive, both in gill, a target of Prl signaling, and in pituitary (31, 51). For instance, we observed that *prlr2* expression is enhanced in Prl cells exposed to hyperosmotic conditions *in vivo* and *in vitro* (31). The two tilapia Prlrs activate divergent downstream targets upon ligand binding; expression of Prlr2, but not Prlr1, improves the tolerance of HEK293 cells to osmotic challenges (50). Importantly, Fiol et al. (50) showed that the osmotic responsiveness of tilapia Prlr2 was retained when expressed in mammalian cells (HEK293). Thus, several osmosensitive genes expressed in tilapia Prl cells are upregulated in response to hyperosmotic conditions.

3. *In silico* and *in vitro* identification of transcriptional regulators

Many layers of control are involved in transcriptional regulation, including transcription factors

(TFs; 52, 53) that bind DNA at specific TF binding sites (TFBSs) to either activate or repress transcription. TFs may act alone, or synergistically, in coordinated fashions with other TFs situated in close proximity to form a TF-module (TFM; 54). The composition and organization of TFBSs and other *cis*-regulatory elements within a gene promoter defines the gene promoter context, which may be present within the promoters of multiple genes. This context provides the major means by which gene transcription is regulated. On the other hand, different TFs may compete for the same binding site and act in fashions antagonistic to one another, ultimately initiating, repressing, or modulating expression of the regulated gene. The interplay among TFs allows for fine-tuned responses to a wide array of intra- and extracellular stimuli. Bioinformatics allows for the characterization of shared promoter structures to closely examine the regulatory characteristics of genes that respond to common stimuli. Accordingly, *in silico* promoter analyses are widely employed to reveal gene-regulatory networks that are co-regulated without *a priori* knowledge of their associations (54-57). Then, RNA-seq and qPCR can be subsequently carried out to validate the expression of TFs predicted via *in silico* promoter analysis. In the case of tilapia RPDs, the expression of putative targets can then be compared under different physiological conditions (e.g., hypo- versus hyperosmotic extracellular conditions). In a broader sense, transcriptome analyses in teleost fishes continue to facilitate the identification of novel genes involved in osmoregulation (58-63).

In mammalian kidney, a functional hyperosmotic *cis*-response element was identified in cells of the renal medulla exposed to dramatic changes in extracellular osmolality (64). This osmotic-response element (ORE), also called tonicity-responsive enhancer (TonE), regulates genes involved in the accumulation of compatible osmolytes (i.e., sorbitol, betaine, and inositol) to mitigate hyperosmotic stress (65). Following the initial characterization of an ORE in the *aldose reductase* gene, which supports the conversion of glucose to sorbitol in response to hyperosmotic stress, other ORE-containing sequences that regulate osmolyte accumulation/transport were subsequently identified. These sequences regulate the *Na^+/Cl^- coupled betaine transporter* and *Na^+/myo-inositol cotransporter* genes. These hyperosmotically-induced genes share homologous sequences in their OREs, which in turn allowed for the functional characterization of a consensus mammalian ORE (66). In a cell line derived from Mozambique tilapia brain, osmolality/salinity-responsive elements (OSREs) were identified that mediate transcriptional responses to hyperosmotic stimuli (67). By contrast, less is known about the transcriptional regulation of vertebrate genes that are induced by hyposmotic conditions. Hence, a wider

perspective on the molecular mechanisms that operate within osmoreceptive cells will be gained by characterizing the promoter regions of genes that respond to hyposmotic stimuli.

4. Transcriptional regulation of *prolactin* in tilapia

Across vertebrates, *prl* genes are comprised of five exons and four introns (68). Unlike mammalian *prl* genes that are 10-12 kilobases (kb) long, the lengths of teleost *prl* genes vary between 2.6 and 3.7 kb; the tilapia prl_{188} gene spans ~3.7 kb (68, 69). The varying *prl* gene lengths are solely attributed to differently sized introns. Early studies in mice identified two regulatory regions associated with pituitary *prl* expression that interact with pituitary-specific transcription factor 1 (Pit1; 70). In fishes, sequences upstream of *prl* genes also possess Pit1 binding sites. Mutational analyses revealed that a Pit1 binding site most proximal to the transcriptional start site (TSS) was sufficient alone to confer submaximal transcription of the rainbow trout (*Oncorhynchus mykiss*) *prl* gene (71). DNase I footprinting experiments and electrophoretic mobility-shift assays identified three regulatory regions within the 5'-flanking region of the tilapia prl_{188} gene homologous to mammalian binding sites for Pit1 (72). Accordingly, rat Pit1 specifically bound to Pit1 binding sites in the flanking region of the tilapia prl_{188} gene. The tilapia prl_{188} promoter includes two microsatellite regions consisting of CA/GT repeats found between the putative binding sites for Pit1 (68). Naylor and Clark (73) demonstrated that CA/GT repeats formed left-handed zDNA that repressed *prl* expression in rat. Moreover, zDNA regions within the tilapia prl_{188} promoter were associated with differences in prl_{188} expression in fish exposed to different salinities (74). Truncation analyses of the tilapia prl_{188} promoter in transient expression assays confirmed the functionality of the promoter in driving transcription and revealed three regulatory regions, two with stimulatory effects, and one with an inhibitory effect (72). Collectively, these investigations suggest that regulatory regions responsible for pituitary *prl* expression are conserved from fishes to mammals, thereby suggesting that common transcription factors drive pituitary *prl* expression across vertebrate clades. To shed light into the molecular mechanisms underlying osmotic regulation of *prl* genes, we first identified sequences within the promoter regions of tilapia *prl* genes that may play a role in regulating transcription in response to hyposmotic stimulation through *in silico* analysis of putative TFs with predicted TFBSs and TFMs. We then identified genes encoding TFs within the RPDs of Mozambique tilapia and compared expression levels between fish acclimated to FW versus SW. The most highly expressed genes were validated by qPCR.

5. *In silico* model of *prolactin* regulation

First, *in silico* searches were performed to screen for putative regulatory elements within the ~3.3 kb promoter regions of prl_{177} and prl_{188}. Our analysis was guided by the previous identification of three DNase protection regions, -643 to -593, -160 to -111, and -73 to -46 bp, in tilapia Prl cells (72). We first extracted the putative regulatory elements for the prl_{177} and prl_{188} promoters and identified a suite of TFMs predicted to control the expression of both genes (Figure 2). We found that only the ~0.25 kb regions flanking the TSSs share similarity; this similarity reflects the use of general transcriptional machinery factors such as TATA binding protein factor (TBP) and CCAAT-enhancer-binding proteins (CEBPs). We identified a putative noncoding RNA (ncRNA) that overlaps with the prl_{188} promoter (-1.866 to -1.985 kb). ncRNAs are known to play a role in silencing or modulating transcription by regulating the chromatin structure and by enhancing or suppressing TF binding (75-77). Thus, ncRNA may affect the chromatin structure or interfere with the binding of TFs in the prl_{188} promoter. In the prl_{188} promoter, we identified putative erythroblast transformation specific (ETSF/ETSF) and CEBP/CEBP TFM sites at -1.9 to -1.935 kb, suggesting possible competition with the ncRNA for promoter binding. Further studies are needed to characterize how ncRNAs may interact with chromatin structure to modulate prl_{188} in response to changing salinities. We found that SORY/paired box (PAX) and AP1F/SMAD are TFMs common to both the prl_{177} and prl_{188} promoters (Figure 2). On the other hand, although CEBP, GATA, and specifity protein 1 (SP1F) binding sites were found in both the prl_{177} and prl_{188} promoters, they were in different positions and/or distinctly associated with other TFs. Lastly, a melanocyte inducing transcription factor (MITF) binding site was unique to the prl_{177} promoter, while brain-derived neurotrophic factor (BRNF)/retinoic acid receptor (RXR) sites were unique to the prl_{188} promoter (Figure 2).

We identified cAMP-response element-binding protein (CREB) binding sites at -2.9 kb and -1.8 kb, and a CEBP site at -1.7 kb, of the prl_{188} promoter fragment. The prediction that CREB regulates prl_{188} expression is particularly noteworthy given that cAMP and Ca^{2+} second messengers play key roles in mediating hyposmotically-induced Prl release (34, 39). CREB is a TF that binds to highly conserved cAMP response elements (CRE) formed by the sequence, 5'-TGACGTCA-3', and is activated by phosphorylation from various kinases, including protein kinase A (PKA) and Ca^{2+}/calmodulin dependent protein kinases (78). Moreover, the -2.9 kb site is a potential contributor to the activation of prl_{188} because it falls within the region (-2.6 to -3.0 kb) previously found to induce transcription by 34% (68). We also found that predicted zDNA regions of the prl_{177} promoter were

247 separated by 2.9 kb, while in the *prl*$_{188}$ promoter, the separation was only 0.54 kb and the number and
248 orientation of the CA/GT repeats differed. Together, the differences in predicted promoter regulation
249 between *prl*$_{177}$ and *prl*$_{188}$ seemingly underlie observed differences in their mRNA expression patterns
250 (30).

251 We next focused our analysis on the proximal promoter region, between -1 and -860 bp, of *prl*$_{188}$
252 because it contains a functional Pit1 binding site (68), three DNAse protection regions (72), and two
253 microsatellite regions (74). We identified putative TFMs that overlap with three DNase protection
254 regions reported by Poncelet et al. (72) and identified Pit1/octamer (Oct1) binding sites encompassed by
255 region III (Figure 3). Moreover, the BRNF/RXR TFM overlaps with the Pit1/Oct1 binding sites, and
256 both Brn and Pit1 factors share the POU (Pit-Oct-Unc) DNA binding domain, which consists of two
257 highly conserved regions, the POU-specific domain and the POU homeodomain. The POU domain is
258 derived from the names of three TFs with well-conserved homeodomains: Pit1; the octamer
259 transcription factor proteins, Oct-1 and Oct-2; and the neural Unc-86 transcription factor originally
260 identified in *C. elegans* (79, 80). Brn factors (brain-specific homeobox/POU domain protein, also known
261 as POU domain transcription factor) are the mammalian TFs most closely related to Unc-86 that were
262 isolated after the original POU domain factors. Brn-3 factors, for example, are expressed in the pituitary
263 where they play critical roles in the development and function of the nervous system (81, 82). Brn
264 factors also interact with estrogen receptors to regulate gene transcription (83). Tilapia Prl cells respond
265 to 17β-estradiol (E_2) (84), which further potentiates the agonistic activities of other hormones, such as
266 gonadotropin releasing hormone and prolactin-releasing peptide, on Prl release (85, 86). All these TFs,
267 however, cannot simultaneously occupy the same DNA sequence, hence we hypothesize that
268 BRNF/RXR may repress *prl*$_{188}$ transcription in the pituitary. RXR is a member of the steroid/thyroid
269 hormone superfamily of nuclear receptors that bind a variety of ligands including agonists, antagonists,
270 and synergists of gene transcription. In the nucleus, RXR functions as a TF that binds to gene promoter
271 regions by either forming a homo- or heterodimer with another nuclear receptor (87). Since BRNF/RXR
272 may suppress *prl* transcription in tilapia, it may operate in Prl cells of tilapia acclimated to SW when Prl
273 secretion is minimal (88). When extracellular osmolality decreases following the transfer of an animal
274 from SW to FW (21), BRNF/RXR would ostensibly be released from the promoter and allow for
275 binding of Pit1/Oct1 TFs, which in turn induce *prl* expression.

276 In the DNase protection region II, we identified overlapping binding sites for SORY/PAX3 and
277 ESTF/AP1 TFs. SORY is an abbreviation generated by Genomatix (see Section 6) to denote the Sry and

Hox7 TFs. The sex-determining *sry* gene is found on Y chromosomes leading to the development of male phenotypes (89). *Hox* genes form a subset of homeobox genes that direct embryonic development along the head to tail axis. A number of hormones, including E_2, also regulate *hox* expression (90). In this promoter region, we hypothesize that SORY/PAX3 TFs repress the expression of prl_{188} in gonadal tissues to ensure tight regulation of its expression. The ETSF1/AP1 TFs are typically activators of gene transcription and may stimulate prl_{188} transcription in the tissues where the SORY/PAX3 TFs are not expressed. The region with the greatest number of TFBSs as predicted by *in silico* analyses was consistent with previous luciferase assays with the prl_{188} promoter where the highest activity was found at -0.55 kb followed by the -0.8 and -3.4 kb regions (65). At ~0.5 kb of the prl_{188} promoter region, we found putative binding sites for BRNF/RXR, ETSF/ETSF, OCT1/PIT1, and interferon regulatory factor family (IRFF)/activator protein 1 family (AP1F). Furthermore, the microsatellite regions that encompass the DNase protection region (-643 to -593 bp) also affect prl_{188} transcription (74). Near the DNase region, closest to the TSS (~60 base pairs upstream), we also found the pre-β-cell leukemia homeobox (PBXC)/pancreatic duodenal homeobox 1 (PDX1) TFM. While PDX1, also known as insulin promoter factor 1, is a TF in the ParaHox gene cluster (91), further study is required to assign putative roles to both PDX1 and PBXC for the regulation of prl_{188} transcription.

6. *In vitro* identification of transcription factors from Prl cells

To verify the expression of transcripts encoding the TFs described above, and to assess their expression in response to environmental salinity, we then analyzed the transcriptome of tilapia RPDs collected from animals acclimated to either FW or SW. Consistent with previous studies (31, 32), the expression of prl_{188} was higher in fish acclimated to FW versus SW (1,460,057 and 705,155 CPM, respectively). We specifically targeted transcripts corresponding to TFs identified by our *in silico* analysis, and of the 192 TFs identified within tilapia RPDs, 51% corresponded to TFs predicted to bind to prl_{177} and prl_{188} promoter regions. Conversely, all TFs predicted to bind the prl_{177} and prl_{188} promoter regions by our *in silico* approach were confirmed to be present in tilapia RPDs. In Table 1, we list the TFs with highest copy number within each TF-family predicted to bind *prl* promoter regions based on the *in silico* map shown in Figure 2. The vast majority (186 of 192) of TF transcripts identified had higher copy numbers in SW- versus FW-acclimated fish. Of the TFs also predicted to possess binding sites on the promoter regions of prl_{177} and prl_{188}, only the *myeloblastosis viral oncogene homolog 1* (*mybl1*) gene transcript was upregulated in FW-acclimated fish (Table 1). In addition to Pit1 (*pou1f1*),

CREB and STATs have also been implicated in the control of Prl cells (Table 1). Relative expression levels of *pou1f1*, *stat3*, *creb3l1, pbxip1a*, and *stat1a* were assessed by qPCR (Figure 4). While there were no differences in *pou1f1* and *stat3* expression in the RPDs of FW- versus SW-acclimated tilapia, patterns of elevated expression in SW fish were confirmed for *creb3l1, pbxip1a*, and *stat1a* (Figure 4C-E). CREB proteins that bind to the CRE region are typically activated by protein kinases elevated in response to cAMP and/or Ca^{2+}. While both of these second messengers play a role in hyposmotically-induced Prl release (39, 41), they are also involved in the inhibition of Prl (92). It is worth noting that while FW-acclimated tilapia exhibit higher *prl* mRNA levels than SW-acclimated counterparts, fish that are acclimated to SW induce a greater increase in *prl* gene expression in response to a hypoosmotic stimulus. The differing osmosensitivity based upon acclimation history may presumably occur because of the much lower mRNA levels of *prl* in SW (44) and is corroborated by the observation that many of the TFs responsive to Ca^{2+} and cAMP, especially CREB, are upregulated in SW. With greater expression in SW, multiple TFs seemingly suppress *prl* genes. Inasmuch as the JAK/STAT pathway is a known mediator of Prl signaling, the presence of two STAT isoforms among the genes with the highest copy numbers in the RPD transcriptome is consistent with the autocrine effects of Prl_{177} and Prl_{188} on tilapia Prl cells (45). Leptin similarly works through STAT signaling and this cytokine rises with SW acclimation and is a potent regulator of Prl release and gene expression and cellular glycolysis in tilapia (93-96). From our previous studies (6), it is apparent that the regulation of Prl release is multi-faceted, with a large number of agonists and inhibitors adding complexity to the physiological regulation of this pleiotropic hormone. With the unveiling of both the *in silico* regulatory model and the *in vitro* transcriptome of TFs, it is increasingly evident that osmotic regulation of the *prl* gene is complex. While the high number of TFs upregulated in SW-acclimated fish may suggest that *prls* are under inhibitory control, further investigation on the regulation and function of the most predominant TFs is warranted. Further analyses are required to unravel the responses of TFs associated with the *prl* promoter to changes in salinity *in vivo* and the interactions of predicted TFs, TFMs, and zDNA regions that underlie osmotic regulation of prl_{177} and prl_{188} expression and their dependency on cAMP and Ca^{2+} second messengers.

In conclusion, the suite of TFs and their associated TFMs predicted through *in silico* analyses and confirmed by RNAseq/qPCR highlights the complex nature of *prl* transcriptional regulation. These analyses have only begun to unravel how differences between prl_{177} and prl_{188} expression are generated in response to a common hyposmotic stimulus. The contrasting regulation of tilapia prl_{177} versus prl_{188}

reflects differences in how their hormone products mediate processes related to osmoregulation and growth (15, 41). While Prl_{188} is more robustly synthesized and released in response to a fall in extracellular osmolality compared with Prl_{177}, the latter exerts somatotropic activity (97). Ultimately, the regulatory mechanisms that underlie the transcription, translation, and secretion of both *prls*/Prls are fundamental to the capacity of euryhaline tilapia to thrive in a range of environmental salinities.

7. Methods

7.1. Animals

Mature Mozambique tilapia of both sexes (ranging between 33-166 g for RNA-seq samples and 150-1200 g for qPCR), were reared in outdoor tanks with a continuous flow of either FW or SW under natural photoperiod. SW-acclimated tilapia employed in this experiment were spawned and reared in SW, having never been previously exposed to FW. FW-acclimated tilapia, on the other hand, were spawned and reared in FW, having never been previously exposed to SW. Water temperature was maintained at 24-26 °C. Animals were fed approximately 5% of their body weight per day with Silver Cup Trout Chow (Nelson and Sons, Murray, UT). All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

7.2. Bioinformatics

Among two PRL isoforms of *O. mossambicus*, only the sequence of prl_{188} gene, including the promoter region, was previously reported (X92380). In *O. niloticus*, the prl_{177} gene is located at LG4 (LOC100534523) and arrayed tandemly with the upstream prl_{188} gene (LOC100534522). To obtain the promoter sequence of *O. mossambicus* prl_{177}, a genomic fragment (approximately 14.7 kb; prediction based on *O. niloticus* data) spanning between the *O. mossambicus* prl_{188} and prl_{177} genes was amplified using a pair of primers designed in the downstream region of the last exon of the *O. mossambicus* prl_{188} gene (X92380; forward) and in the first exon of the *O. niloticus* prl_{177} (NM_001279792; reverse) gene. The primers used were: (forward) cggtacccggggatccAAGACATAAAGACCTGGATGACTGACTGCT and (reverse) cgactctagaggatccTGAGTTTGCTTCCACTGATTCTTCTCTGAG, lower-case and capital letters represent nucleotides specific to the pUC19 vector and those specific to *O. mossambicus* (forward) or *O. niloticus* (reverse), respectively. Genomic DNA template was prepared from the liver of a female tilapia (32 g) by the salting-out method. 100 mg of tissue was minced and digested overnight at

37 °C in 2 mL of lysis buffer (0.5 mg/mL proteinase K, 10 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 0.5% SDS; pH 8.0). Then, the protein portion was precipitated and removed by adding 500 µL of 6 M NaCl to 1 mL aliquot of lysate. Genomic DNA was harvested by adding one volume of isopropanol to the supernatant. 500 ng of DNA was subjected to PCR with PrimeSTAR GXL DNA Polymerase (Takara Bio, Mountain View, CA) using the primers described above. The amplified genomic fragment was separated by agarose gel electrophoresis and purified using the UltraClean GelSpin DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA). The purified fragment was cloned into the pUC19 vector using In-Fusion HD Cloning Plus (Takara Bio) prior to sequencing of the 3' region. Sequencing was performed at the Advanced Studies in Genomics, Proteomics and Bioinformatics facility (ASGPB), University of Hawaii at Mānoa. The sequence of the *O. mossambicus* prl_{177} promoter (3.344 kb) is provided in Supplemental Materials (Figure S1). The ~3.4 kb promoters of *O. niloticus* and *O. mossambicus* prl_{177} share ~94 % identity. The putative TFBSs, TFs, and TFMs, in the prl_{177} and prl_{188} promoter sequences were identified and mapped using the Matinspector and ModelInspector tools of the Genomatix Software Suite (Matrix Family Library Version 11.0, and Module Library Version 6.3, Munich, Germany). This software version contains binding site descriptions for 9,968 transcription factors and 839 *H. sapiens*, 818 *M. musculus*, 618 *X. tropicalis*, and 612 *D. rerio* weight matrices. The promoter module library used for the ModelInspector tool contained 919 regulatory modules.

7.3. RNA-seq and analyses of the RPD transcriptome

Fish residing in FW or SW (30 per salinity) were anesthetized with 2-phenoxyethanol (0.3 ml/l, Sigma, St. Louis, MO). Fish were then euthanized by rapid decapitation, pituitaries extracted, and the RPD dissected. The RPDs were pooled by acclimation salinity and then transferred to tubes containing 1 mL Tri-Reagent (Molecular Research Center, Cincinnati, OH) at 10 RPDs/tube (i.e., 3 replicates/treatment). Total RNA was isolated from RPDs using Tri-Reagent coupled with on-column affinity purification, and DNase treatment (Direct-zol minipreps, Zymo Research Corporation, Irvine, CA) as described previously (95). RPD total RNA (10 µg) was submitted to North Carolina State University Genomic Sciences Laboratory (Raleigh, NC) for mRNA enrichment, cDNA synthesis, and Illumina library construction utilizing a Truseq RNA library prep kit v1 (Illumina, USA) in conjunction with kit-provided oligo dt capture of mRNAs from sample total RNA. Illumina libraries (n = 3; see above) were prepared for each treatment (FW and SW). Sequencing was performed on an Illumina

MiSeq platform at the Hawaii Institute of Marine Biology Genetics Core Facility with 100x2 bp paired-end protocol and with a mean cluster yield of 2.6 Million paired reads per library (9.7 Gb total).

The FastQC (98) and Trimmomatic (99) programs were used as quality control tools to inspect the data and trim adapters or low quality reads. Bowtie (ver. 1.0; 100) was used to map the reads to the *O. niloticus* reference genome (OreNil 1.0 Broad Institute; 101). Then, RNA-Seq by Expectation-Maximization (RSEM; 102) was used to quantify transcripts as counts per million (CPM, i.e., copy number); EBseq (103) was employed to analyze differential expression between the two treatments. The BioMart tool (104) was used to identify tilapia TF transcripts from the Nile tilapia genome (Ensembl).

7.4. Quantitative real-time PCR (qPCR)

RPDs were dissected out from 12 FW and 12 SW-reared tilapia. Total RNA was extracted using TRI Reagent according to the manufacturer's protocols. The concentration and purity of extracted RNA were assessed using a NanoDrop (NanoDrop One, Thermo Scientific). Total RNA (400 ng) was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). The mRNA levels of reference and target genes were determined by the relative quantification method using a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The qPCR reaction mix (15 µL) contained Power SYBR Green PCR Master Mix (Thermo Fisher Scientific), 200 nM of forward and reverse primers, and 1 µL cDNA. Dilution of experimental cDNA from RPDs ranged from 1- to 10-fold. PCR cycling parameters were: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Gene specific primers were designed using NCBI Primer-BLAST; non-specific product amplification and primer-dimer formation were assessed by melt curve analyses. Primer sequences, amplification efficiencies, and amplicon sizes are provided in Supplemental Table 1. After verification that levels did not vary across treatments, 18S ribosomal RNA (18S) was used to normalize target genes. Data are expressed as a fold-change relative to the FW group. Group comparisons were performed by two-tailed Student's t-test. Data were log-transformed when necessary to meet assumptions of normality (assessed by Shapiro-Wilk test). Statistical calculations were performed using Prism 8.0 (GraphPad, La Jolla, CA). Significance for all tests was set at $P < 0.05$.

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Conflict of interest statement

There is no conflict of interest that could be perceived as prejudicing the impartiality of this report.

Figure legends

Figure 1: Illustration depicting steps involved in the transduction of a hyposmotic stimulus into Prl release by tilapia pituitary, modified from Seale et al. (6). Prl cells of the *rostral pars distalis* (RPD) synthesize and release Prl_{177} and Prl_{188} in response to a fall in extracellular osmolality. Hyposmotic stimulation leads to an Aqp3-dependent increase in cell volume that triggers the entry of Ca^{2+} through stretch-activated Trpv4 channels. While Ca^{2+} and cAMP secondary messengers mediate Prl release, it is unknown how these intracellular signals participate in the transcriptional regulation of prl_{177} and prl_{188}.

Figure 2: Comparison between predicted transcription factor modules in prl_{177} (orange band) and prl_{188} (green band) promoter regions up to -1.3 kb. TFMs represented by white boxes are unique to either prl_{177} or prl_{188}; TFMs represented by gray ovals are common to both prl_{177} and prl_{188}. TRMs predicted to bind to the (–) strand are indicated with blue text; TFMs predicted to bind to the (+) stand are indicated with red text. Numbers below the promoter regions depict approximate base pair positions (bp) of putative TFMs and their respective binding sites relative to the transcriptional start site (TSS). Red arrows on each promoter indicate the TSS. TFMs shown in horizontal stacks compete for binding to the same region.

Figure 3: DNA sequence and regulatory elements of the proximal prl_{188} promoter (-0.88 kb). Nucleotide positions are indicated above the sequence. Both DNA strands are shown for regions -1 to -220 and -551

to -660 bp; only the coding strand is shown for the remainder of the sequence. The transcriptional start site (TSS) is indicated by the red bent arrow; the first exon of the prl_{188} gene is highlighted in yellow and the translation start site is highlighted in green. The dark purple box marks the region (-1 to -40 bp) where general TFs are predicted to bind. Previously identified DNase protection regions (65) are indicated by grey boxes. The underlined red regions represent microsatellite repeat regions (zDNA). Colored boxes indicate putative TFMs and their respective binding sites. TFMs shown above and below the sequence are predicted for the (+) and (–) strands, respectively. The purple box from -142 to -151 bp represents the overlap of the predicted sites for SORY/PAX3 and ETSF/AP1F on the (–) strand. Additional TFMs are color coded to indicate their corresponding binding sequences.

Figure 4: Gene expression of *pou1f1* (A), *stat3* (B), *creb3l1* (C), *pbxip1a* (D) and *stat1a* (E) in the *rostral pars distalis* (RPD) of Mozambique tilapia acclimated to FW (solid bars) and SW (shaded bars). mRNA levels are presented as a fold-change from the FW group. Means ± S.E.M. (n = 12). *P <0.05, **P < 0.01 and ***P < 0.001 (Student's t-test).

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Table 1: List of major transcription factor genes and related transcripts identified in the RPDs of FW- and SW-acclimated Mozambique tilapia.

Accession number (NCBI or ZFIN)	Gene	Description	TF family	Copy number (CPM)		FC	*P*	FDR
				FW	SW			
NCBI:100698135	*pou1f1*	POU class 1 homeobox 1	PIT1	2396.0±614	3564.4±376	1.5	0.194	0.397
NCBI:100692602	*stat3*	signal transducer and activator of transcription 3	STAT	2154.8±399	3776.5±554	1.8	0.083	0.339
NCBI:100703380	*creb3l1*	cAMP responsive element binding protein 3 like 1	CREB	484.1±39	851.5±157	1.8	0.136	0.355
NCBI:100709266	*pbxip1a*	pre-B-cell leukemia transcription factor-interacting protein 1	PBXC	453.5±94	658.8±58	1.5	0.152	0.358
NCBI:100696359	*stat1a*	signal transducer and activator of transcription 1	STAT	440.1±76	750.2±116	1.7	0.100	0.340
NCBI:100707472	*ap2b1*	AP-1 complex subunit beta-1	AP2F	408.6±71	625.6±92	1.5	0.140	0.355
ZFIN:ZDB-GENE-020111-3	*cebpb*	CCAAT enhancer binding protein beta	CEBP	321.3±76	486.7±133	1.5	0.357	0.545
NCBI:100706939	*foxo1a*	forkhead box protein O1-A	FKHD	222.0±42	487.7±88	2.2	0.076	0.339
RefSeq:NM_001311335	*foxp2*	Oreochromis niloticus forkhead box P2	FKHD	200.8±50	380.3±77	1.9	0.133	0.355
NCBI:100705959	*ap1b1*	adaptor related protein complex 1 subunit beta 1	AP1F	188.1±32	444.1±87	2.4	0.085	0.339
NCBI:100695946	*irf2*	interferon regulatory factor 2	IRF	155.0±27	230.0±15	1.5	0.092	0.340

ZFIN:ZDB-GENE-001031-1	*pax6b*	paired box 6b	PAX6	148.0±25	198.6±31	1.3	0.280	0.460
NCBI:100711701	*stat4*	signal transducer and activator of transcription 4	STAT	145.3±22	311.2±44	2.1	0.046	0.339
NCBI:100701775	*crebzf*	CREB/ATF bZIP transcription factor	CREB	141.3±26	242.6±6	1.7	0.054	0.339
NCBI:100711301	*nfatc1*	nuclear factor of activated T-cells, cytoplasmic 1	NFAT	139.7±14	292.1±66	2.1	0.143	0.355
NCBI:100701828	*ebox*	zinc finger E-box-binding homeobox 1	EBOX	109.3±17	229.7±16	2.1	0.006	0.244
ZFIN:ZDB-GENE-091111-4	*nf1b*	neurofibromin 1b	NF1F	102.3±16	236.0±51	2.3	0.107	0.344
NCBI:100690945	*stat5b*	signal transducer and activator of transcription 5B	STAT	78.0±6	125.0±15	1.6	0.076	0.339
NCBI:30486	*rxrbb*	retinoid x receptor, beta b	RXRF	63.0±16	104.3±17	1.7	0.146	0.355
NCBI:100707575	*brf1*	BRF1 RNA polymerase III transcription initiation factor subunit	BRNF	53.3±10	101.7±14	1.9	0.056	0.339
NCBI:100690316	*sp1*	Sp1 transcription factor	SP1F	43.5±7	79.4±11	1.8	0.061	0.339
NCBI:100704116	*smad9*	SMAD family member 9	SMAD	31.8±9	77.7±15	2.4	0.072	0.339
NCBI:100707537	*sox5*	SRY-box transcription factor 5	SORY	24.0±7	54.7±11	2.3	0.084	0.339
NCBI:100711278	*ets1*	ETS proto-oncogene 1, transcription factor	ETSF	21.7±4	40.3±5	1.9	0.052	0.339
NCBI:100705840	*gata2a*	GATA-binding factor 2	GATA	14.7±3	48.0±17	3.3	0.177	0.390
NCBI:100699256	*mitfb*	microphthalmia-associated transcription factor	MITF	12.7±2	10.7±2	0.8	0.554	0.695
ZFIN:ZDB-GENE-030131-2422	*pou2f1b*	POU class 2 homeobox 1b	OCT1	11.7±3	26.3±3	2.3	0.034	0.339
ZFIN:ZDB-GENE-041111-281	*mybl1*	v-myb avian myeloblastosis viral oncogene homolog-like 1	MYBL	2.3±0	0.7±1	0.3	0.113	0.346
NCBI:100699915	*pdx1*	pancreatic and duodenal homeobox 1	PDX1	1.7±1	2.7±0	1.6	0.274	0.455

812 Abbreviations: TF = transcription factor; FW = copy number from freshwater-acclimated fish (mean ± S.E.M., *n* = 3); SW = copy number
813 from seawater-acclimated fish (mean ± S.E.M, *n* = 3); FC = fold-change in SW relative to FW; *P* = *P*-value; FDR = False discovery rate.

Figure 1

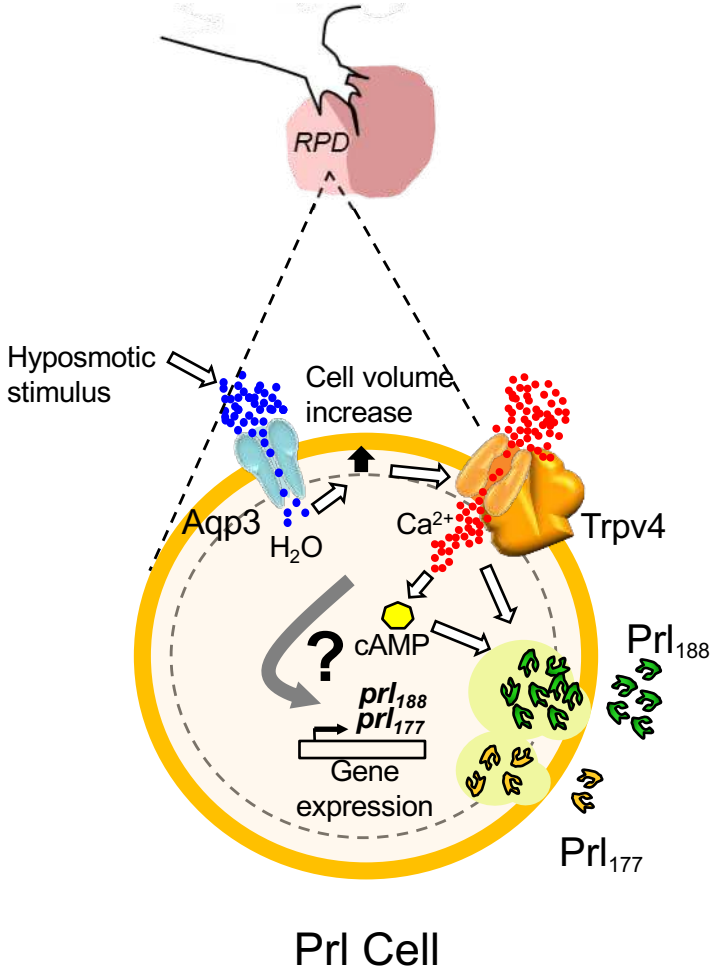

Figure 2

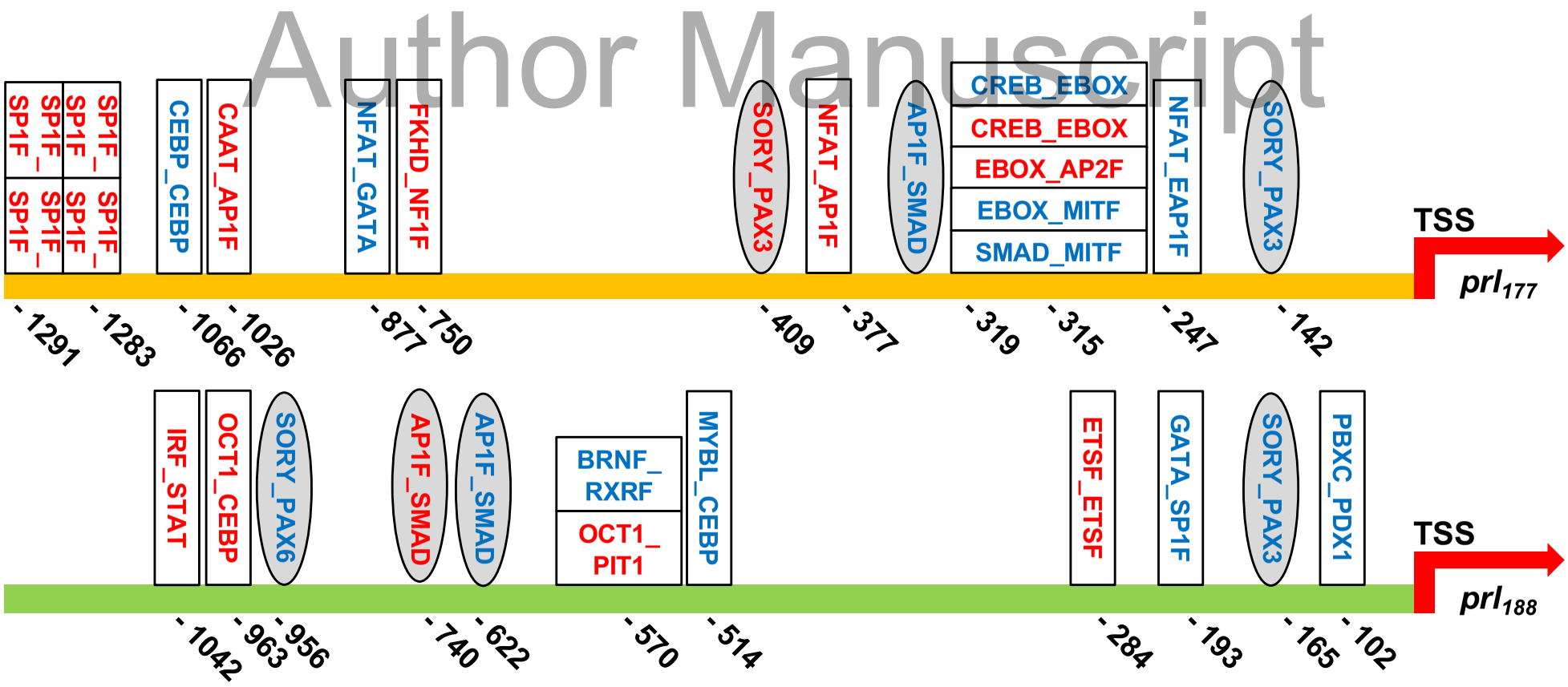

Figure 3

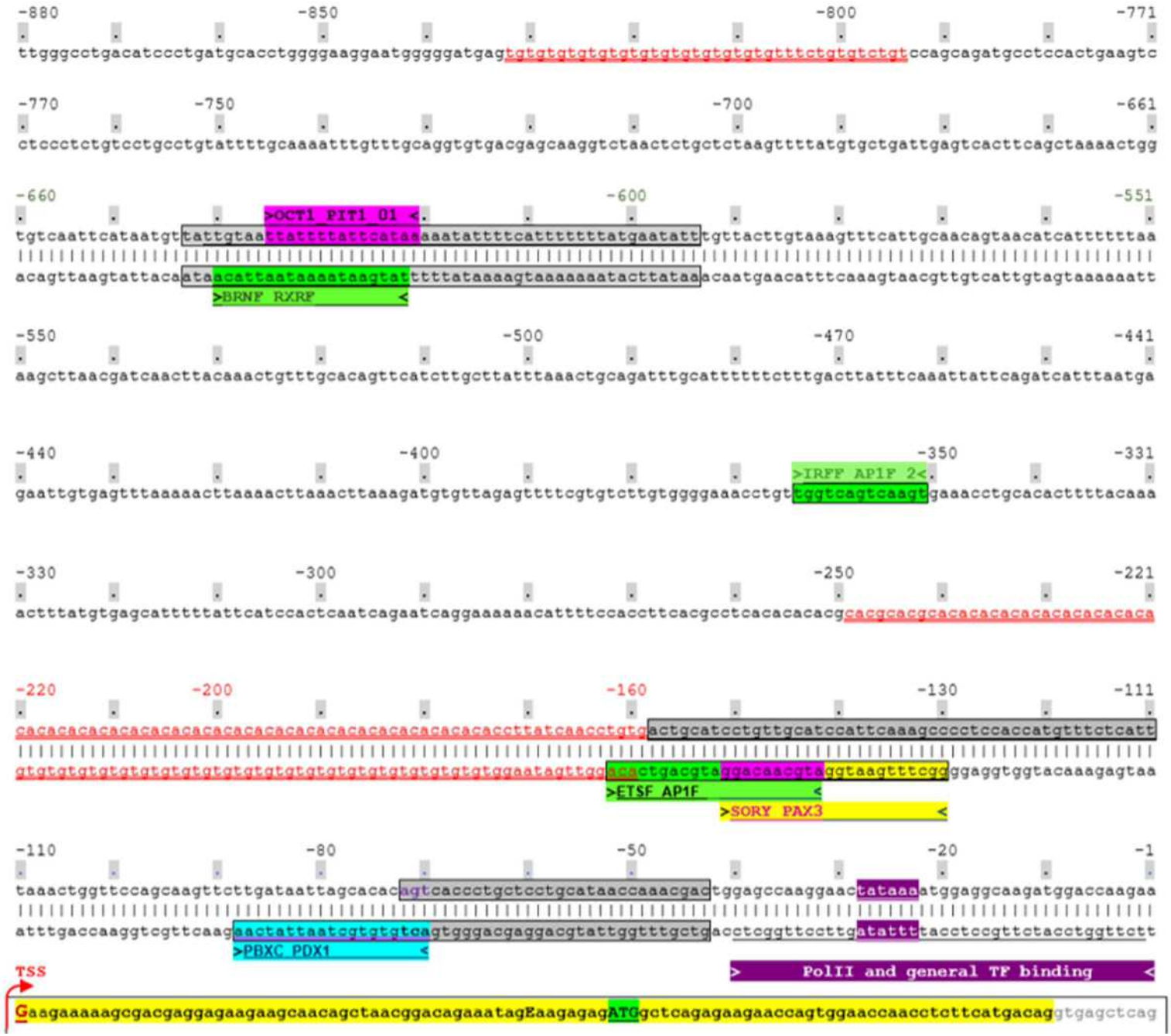

Figure 4