Allelic diversification after transposable element exaptation promoted *gsdf* as the master sex determining gene of sablefish

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Concepts of evolutionary biology suggest that morphological change may occur by rare punctual but rather large changes, or by more steady and gradual transformations. It can therefore be asked whether genetic changes underlying morphological, physiological, and/or behavioral innovations during evolution occur in a punctual manner, whereby a single mutational event has prominent phenotypic consequences, or if many consecutive alterations in the DNA over longer time periods lead to phenotypic divergence. In the marine teleost, sablefish (*Anoplopoma fimbria*), complementary genomic and genetic studies led to the identification of a sex locus on the Y Chromosome. Further characterization of this locus resulted in identification of the *transforming growth factor, beta receptor Ia* (*tgfbr1a*) gene, *gonadal somatic cell derived factor* (*gsdf*), as the main candidate for fulfilling the master sex determining (MSD) function. The presence of different X and Y Chromosome copies of this gene indicated that the male heterogametic (XY) system of sex determination in sablefish arose by allelic diversification. The *gsdfY* gene has a spatio-temporal expression profile characteristic of a male MSD gene. We provide experimental evidence demonstrating a pivotal role of a transposable element (TE) for the divergent function of *gsdfY*. By insertion within the *gsdfY* promoter region, this TE generated allelic diversification by bringing *cis*-regulatory modules that led to transcriptional rewiring and thus creation of a new MSD gene. This points out, for the first time in the scenario of MSD gene evolution by allelic diversification, a single, punctual molecular event in the appearance of a new trigger for male development.

[Supplemental material is available for this article.]

Concepts of evolutionary biology suggest that morphological change may occur by rare punctual but rather large changes, or by more steady and gradual transformations (Eldredge and Gould 1972; Rhodes 1983; Mayr 1997). Thus, by analogy, the question can be asked whether genetic changes underlying morphological, physiological, or behavioral innovations during evolution occur in a punctual manner, whereby a single mutational event has a prominent phenotypic consequence, or if many consecutive alterations in DNA sequences over longer time periods lead to phenotypic divergence.

To investigate this question, the evolution of sex determination systems offers a very favorable situation because of the peculiarly high turnover rate of its genetic control in certain groups (Charlesworth and Charlesworth 2005; Herpin and Schartl 2008). Among vertebrates, teleost fishes display by far the highest diversity of sex determination systems and sex differentiation mechanisms (Herpin and Schartl 2015; Guiguen et al. 2018). Identification of master sex determining (MSD) genes on sex chromosomes from several fish species confirmed the concept that genetic triggers at the top of the regulatory hierarchy have changed

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radically as new species have evolved (Pan et al. 2016, 2019, 2021), whereas downstream regulatory networks remained more stable, generally exerting similar functions in driving testicular or ovarian differentiation in different species (Myosho et al. 2012; Kaneko et al. 2015; Zhang et al. 2016). The biological meaning and evolutionary processes of this outstanding molecular diversity to trigger the development of either testes or ovaries remain largely unknown. New MSD genes primarily emanate from one of two evolutionarily conserved processes: (1) sporadic gene duplication and insertion, followed by sub- and/or neo-functionalization; or (2) allelic diversification of a pre-existing locus (Kikuchi and Hamaguchi 2013; Guiguen et al. 2018). However, the molecular changes that allow new MSD genes to exert a novel function are not well understood aside from a few enigmatic studies in model species (for review, Herpin and Schartl 2008, 2015). A much broader knowledge is necessary to conclude about the evolutionary processes at work that bring about the great variety of fundamental steps in development, reproduction, and speciation.

In the marine teleost, sablefish (*Anoplopoma fimbria*), complementary genomic and genetic studies recently led to the identification of a sex locus on the Y Chromosome (Rondeau et al. 2013). Further characterization of this locus resulted in identification of the *transforming growth factor, beta receptor 1a* (*tgfbr1a*) gene, *gonadal somatic cell derived factor* (*gsdf*), as the main candidate for fulfilling the MSD function. The presence of different X and Y Chromosome copies of this gene indicated that the male heterogametic (XY) system of sex determination in sablefish arose by allelic diversification.

This study aims to determine what gave rise to diversification of the *gsdfX* and *gsdfY* paralogs in sablefish and whether X- and Y-specific DNA inserts in the promoter region upstream of these genes harbor elements that influence their expression. Spatio-temporal expression of the *gsdf* genes and other key genes associated with sex determination and gonadal differentiation were analyzed to determine if *gsdfY* exhibits characteristics of a male MSD gene. Moreover, a series of experiments was conducted to examine whether a transposable element (TE) from the hAT family played a critical role in the diversification of *gsdfY* via introduction of transcription factor-binding sites associated with the initiation and regulation of testicular development. This study is the first in the scenario of MSD gene evolution via allelic diversification to identify a single, punctual molecular event giving rise to a new trigger for male development.

Results

Allelic diversification gave rise to *gsdfX* and *gsdfY* with strict gonadal expression

Recent development of genetic tools in sablefish, including polymorphic markers and high-resolution linkage maps, have allowed for the successful mapping of different phenotypes of interest, including sex (Rondeau et al. 2013). Single nucleotide polymorphic (SNP) and microsatellite markers obtained from 35,000 assembled transcript sequences and 360 transcribed polymorphic loci from two families of sablefish permited the production of a map of 24 linkage groups (Rondeau et al. 2013). Although comparative mapping was unsuccessful in linking sex to the female genetic map, this trait clearly mapped to the male linkage group 14 of sablefish, indicating a male heterogametic system (Rondeau et al. 2013), which was subsquently verified by steroid-induced sex reversal and targeted breeding crosses (Luckenbach et al. 2017). The sex-specific regions were located in the vicinity of the *gsdf* gene. Gsdf has been described as an important component of the male pathway in gonad development in some other fish species (Myosho et al. 2012; Kaneko et al. 2015; Zhang et al. 2016). Several SNPs in the upstream and intronic portions of the sablefish *gsdf* gene appeared to be linked to sex. Of note, two exonic SNPs were identified within the *gsdf* coding sequence (CDS), one of which is missense and causes a phenylalanine (F) to leucine (L) change between the X and Y Chromosome copies (Fig. 1A; Rondeau et al. 2013). Otherwise, the *gsdfX* and *gsdfY* open reading frames (ORFs) are identical. Determination of the complete *gsdfX* and *gsdfY* mRNAs showed that they were each 2182 bp in length and had a 5′ untranslated region (UTR) of 145 and 3′ UTR of 1364 bp. Each mRNA also had an alternate polyadenylation site that reduced the 3′ UTR to 896 bp. Several SNPs were identified in the UTRs, with four being unique to the Y-Chromosomal copy (Fig. 1A). However, the most striking allele-specific features are located upstream of the translated region. The Y-copy has an insertion of 936 bp located 482 bp upstream of the *gsdfY* start codon, and the X-copy has an insertion of 412 bp located 1298 bp upstream of the *gsdfX* start codon (Fig. 1B). PCR amplification using primers designed to target these two regions confirmed that these X- and Y-allele-specific insertions segregate in agreement with the expected male and female genotypic sex (Fig. 1B).

gsdfY is expressed during the sex-determining period only in male sablefish and prior to other genes associated with gonadal sex differentiation

Semiquantitative analysis of the tissue distribution of the two *gsdf* allelic variant mRNAs (PCR targeting both *gsdf* mRNAs) revealed exclusive gonadal expression in juvenile sablefish (Fig. 2A). To better characterize temporal and spatial expression of the two sex-specific *gsdf* allelic variants in relation to the molecular and morphological development of male and female gonad primordia, ontogenetic gene expression profiles for *gsdfX*, *gsdfY*, and other key markers of gonadal sex differentiation were established (Fig. 2). The *gsdfX* variant was expressed at very low levels in both XX- and XY-genotype fish from hatching to 40-mm-sized fry (Fig. 2B). However, the *gsdfY* variant was particularly highly expressed in XY individuals beginning around the time of hatching. During later ontogenetic development, expression of *gsdf* in XX fish remained low relative to that of XY fish, which exhibited increases during testicular differentiation and development (Fig. 2B). In juvenile gonads, in situ hybridizations (ISHs) using a probe that does not discriminate between the two *gsdf* allelic variants revealed that, in both testis and ovary, *gsdf* transcripts are expressed in the somatic supporting cells that surround the germ cells (Fig. 2C–H). Early markers for gonadal sex differentiation and development, such as the transcription factors *wt1a*, *dmrt1*, and *foxl2*, and the steroidogenic enzyme *cyp19a1a*, start to be expressed at hatching, reaching an initial peak at 5 to 10 mm, at which point none of the genes displayed a sexually dimorphic pattern of expression (Fig. 2I–L), unlike *gsdfY* as noted above (Fig. 2B). Only during later ontogenetic development, corresponding with the period of gonadal sex differentiation, sexually dimorphic expression was apparent, with *dmrt1* being overexpressed in XY fish (Fig. 2J) and *cyp19a1a* and *foxl2* being overexpressed in XX fish (Fig. 2K,L). Transcripts for *wt1a*, on the other hand, did not exhibit sexually dimorphic expression during ontogeny (Fig. 2I).

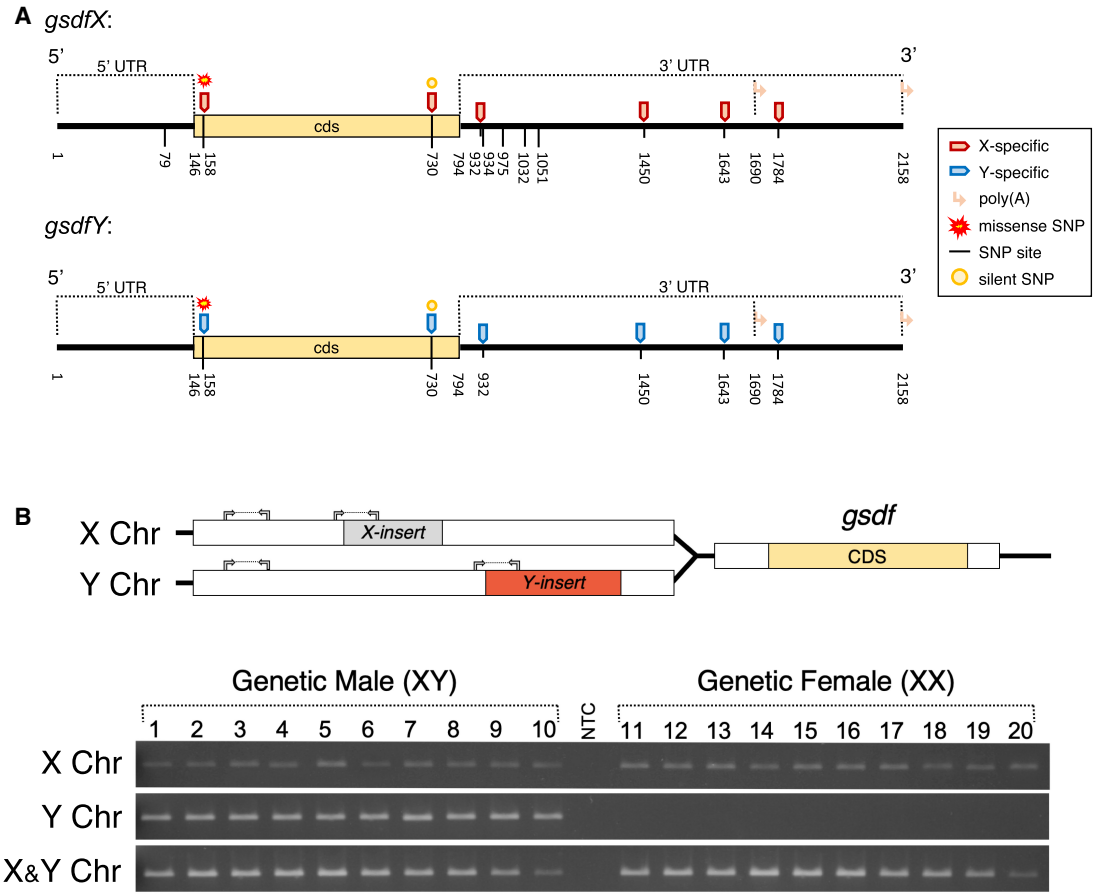


Figure 1. Allelic diversification gave rise to the sablefish (*A. fimbria*) *gsdfX* and *gsdfY* genes with sexually dimorphic expression. (*A*) Maps of the full-length sablefish *gsdfX* and *gsdfY* mRNAs, including polymorphisms and alternative poly-adenylation sites. (CDS) Coding sequence, (UTR) untranslated region, (SNP) single nucleotide polymorphism. (*B*) PCR amplification of *gsdfX* and *gsdfY* on X and Y Chromosomes, respectively, based on specific inserts upstream of the coding sequences. Amplification of an upstream sequence common to the two *gsdf* paralogs was used as a control. Bands represent amplification from 10 genotypic males (XY) and 10 genotypic females (XX). The depiction shows the general gene structure and placement of assay primers.

Functional assays reveal no difference in *gsdfX* and *gsdfY* biochemical activity/signaling properties

Within their respective CDS, *gsdfX* and *gsdfY* allelic variants exhibit a unique missense SNP resulting in a phenylalanine (F) to leucine (L) change at position 5 (pF5L) (see Fig. 1A; Supplemental Fig. S1). Although this residue is not conserved throughout evolution (Gautier et al. 2011) and apparently does not impair the quality of the signal peptide (Supplemental Fig. S1B), we nevertheless explored whether this unique mutation, nested within the signal peptide of the pro-domain of Gsdf, could result in differences in downstream signal transduction of the Gsdf ligand variants after receptor binding. To this end, we used a reporter assay to identify differential activation of Smad effectors (Fig. 3A). A luciferase reporter and transactivator plasmids (for Smad1, 2, 3, 5, or 8) were cotransfected with sablefish GsdfX and GsdfY expression plasmids in medaka fibroblast cells (Fig. 3A) to quantify, after binding to endogenous receptors, the relative differential Smad phosphorylation states using a luciferase activity assay. Although basal phosphorylation states of Smad1, 5, and 8 were not significantly impacted by either GsdfX or GsdfY expression, phosphorylation of Smad2 and 3 significantly increased for both Gsdf variants (Fig. 3B,C). The degree of stimulation of the two Gsdf variants was similar (around three times the basal activity) with respect to activation of Smad2 and 3. In conclusion, the single amino-acid difference between the X- and the Y-encoded proteins does not appear to impact the biochemical signaling function of the sablefish Gsdf proteins with regard to their receptor binding and subsequent Smad activation in the cellular environment tested (i.e., medaka fibroblast cells).

Evolution of the *gsdfX/Y* promoter sequences

During the process of allelic diversification, the transcriptional context clearly changed between the two *gsdf* variants (see Fig. 2B). To obtain insights into the sequence evolution of the *cis*-regulatory regions of the *gsdfX* and *gsdfY* genes, their upstream regions were analyzed in detail (Fig. 4A).

Comparison of the promoter regions of the sablefish *gsdf* variants upstream of the transcription start sites revealed a clear size difference of about 500 bp in length. This size difference between the *gsdfX* and *gsdfY* promoters is due to unique Y- and X-specific insertions (Fig. 4A). Located 482 bp upstream of the transcription start, the Y-specific insertion is 936 bp in length (Fig. 4A). An X-specific insertion of 412 bp is located 1298 bp upstream of the transcription start (Fig. 4A).

Sequence analyses of both inserted sequences revealed that they correspond to repeated TE derivatives (Fig. 4B). Four copies

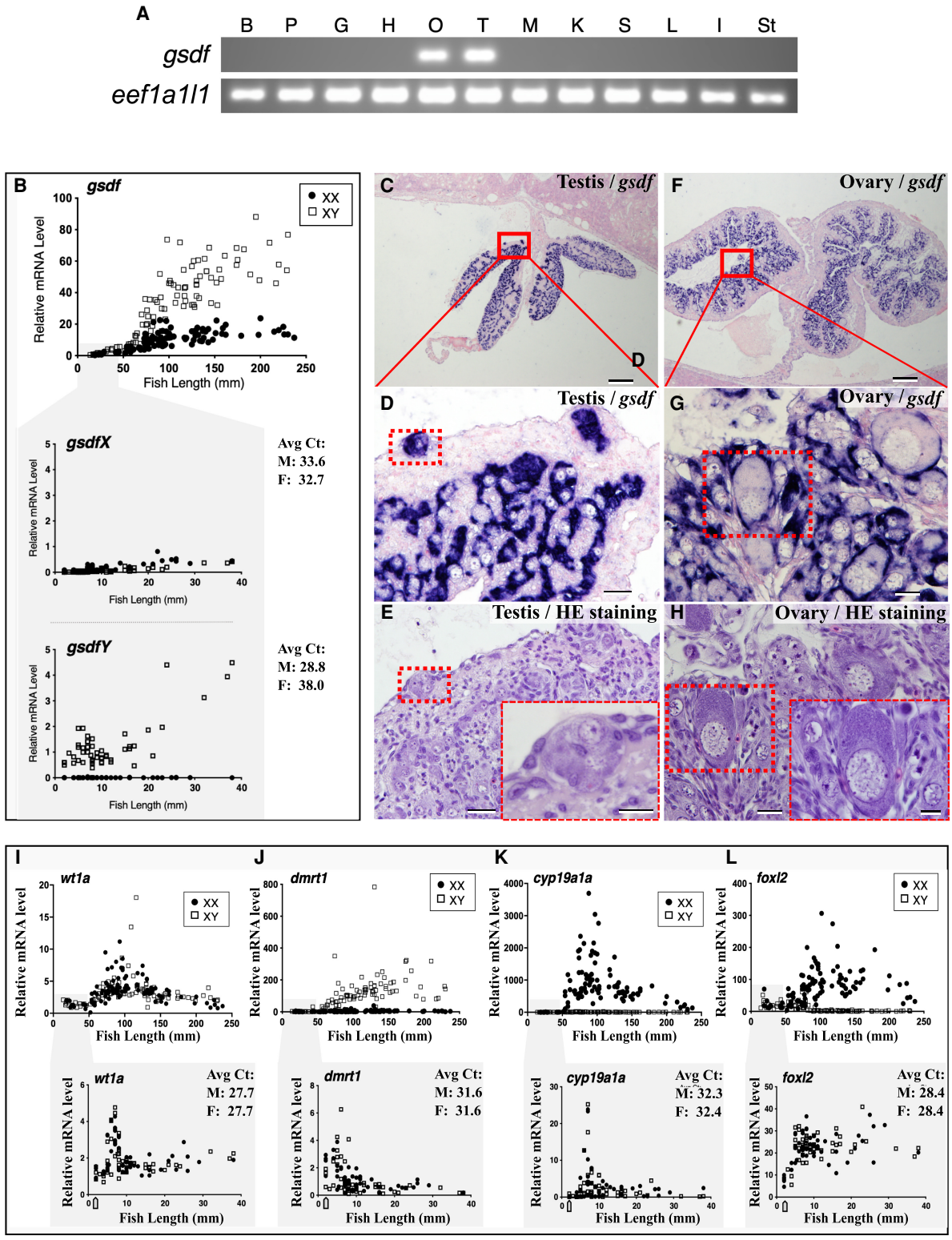


Figure 2. Temporal and spatial expression of *gsdfX*, *gsdfY*, and other early gonadal sex marker mRNAs in male and female developing gonads of sablefish. (*A*) Semiquantitative tissue distribution of *gsdf* (assay targeting both *gsdf* variants) in brain (B), pituitary (P), gill (G), heart (H), ovary (O), testis (T), muscle (M), kidney (K), spleen (S), liver (L), intestine (I), and stomach (St). Transcript levels of *eef1a1l1* were assessed to verify quality and loading of cDNAs. (*B*) Ontogenetic gene expression profiles of *gsdfX* and *gsdfY* sex-specific allelic variants during early gonadal primordium development and sex differentiation in genotypic female (XX) and male (XY) sablefish. The *upper* panel in *B* shows gonadal *gsdf* expression (nonvariant specific assay) during ontogenetic development. The *lower* panel (gray shaded) shows gene expression results for assays targeting both X and Y Chromosomal transcripts during early larval development. (*C–H*) In situ hybridization (ISH) localization of *gsdf* mRNA in juvenile sablefish gonads. (*C,D*) ISH, (*E*) hematoxylin/eosin (HE) staining of sablefish testes. (*F,G*) ISH, (*H*) HE staining of sablefish ovaries. (*I–L*) Ontogenetic expression profiles for *wt1a*, *dmrt1*, *cyp19a1a*, and *foxl2*, across early gonadal development between genotypic females and males. (*B,I–L*) Filled circles denote XX-genotype fish and open squares denote XY-genotype fish. See Hayman et al. (2021) for additional detail on gonadal gene expression data. Scale bars: *C*, *F*, 200 µm; *D*, *G*, *E*, and *H*, 20 µm; inserts in *E* and *H*, 10 µm. See also Supplemental Data S1 for raw data.

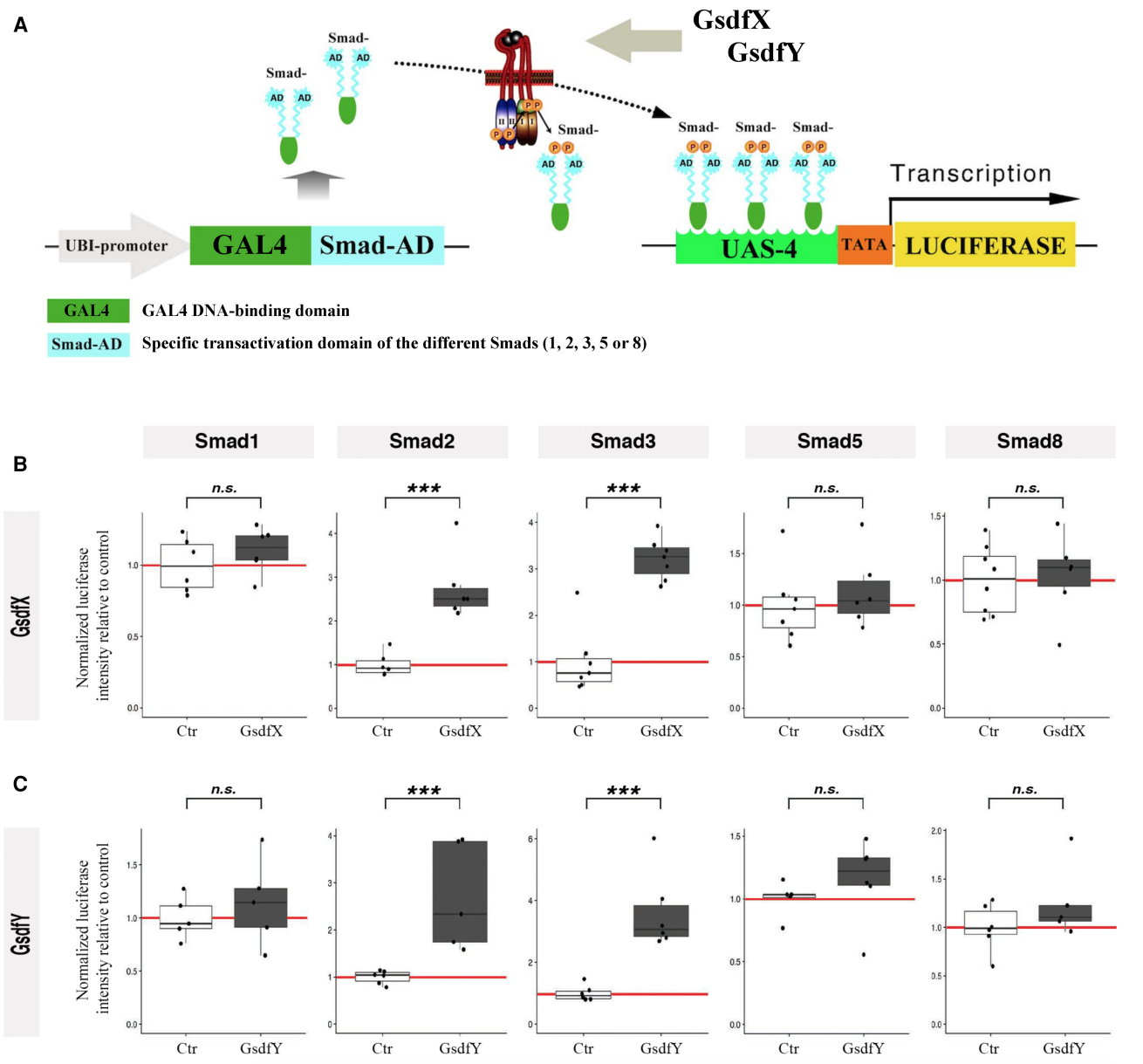
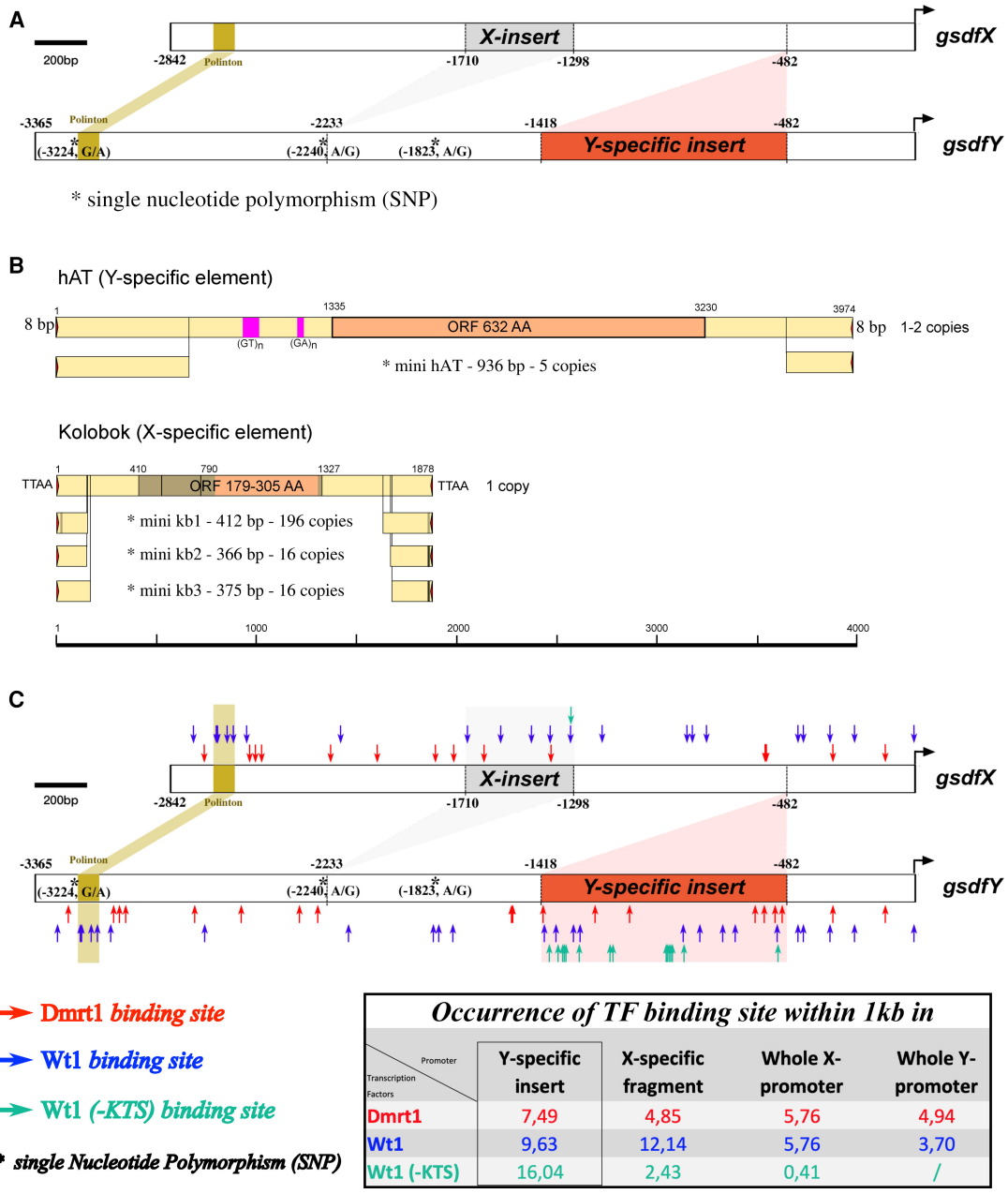


Figure 3. Differential activation of downstream Gsdf signaling pathway components upon selective GsdfX or GsdfY expression. (*A*) Medaka fibroblast cells (OLF cell line) were cotransfected with a luciferase reporter construct (*UAS-luc*) and different combinations of Smad-phosphorylation-dependent transactivating GAL4 constructs (Smad1, 2, 3, 5, or 8 -GAL4). Cells were either stimulated (cotransfection) or not (control) with GsdfX or GsdfY. In the absence of any induced signaling, the fusion proteins Smads-GAL4 remain in the cytoplasm and the luciferase reporter is only activated at a basal level. If activated, the Smads-GAL4 proteins are phosphorylated, translocate into the nucleus, and an increased luciferase expression is recorded. Results are expressed as the relative stimulation of Smad phosphorylation after either GsdfX or GsdfY stimulation compared to control (no stimulation). Irrespective of the Gsdf variants employed, monitoring of Smad1, 2, 3, 5, and 8 phosphorylation states (relative luciferase activity) upon stimulation with either GsdfX (*B*) or GsdfY (*C*) revealed that only Smads 2 and 3 were activated in both situations, whereas Smads 1/5/8 always remained unresponsive. See also Supplemental Data S1 for raw data. (n.s.) $P > 0.05$; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$.

of the Y-specific element were detected at other positions than the *gsdfY* locus in the *A. fimbria* genome assembly (NCBI BioProject [https://www.ncbi.nlm.nih.gov/bioproject/] PRJNA656728; GCA_000499045.2). The insertion present in *gsdfY* displays some specific small indels relative to those copies. This element, 927 bp long, does not code for a protein but presents two terminal inverted repeats (TIR) of 12 bp and is inserted in a target site duplication (TSD) of 8 bp. Two other, longer sequences showing homology with this element are present in the genome and contain an internal ORF encoding a putative protein with homology to a hAT Class II element, which is not interrupted by frameshifts or stop codons (Fig. 4B). Hence, this TE family could potentially be active, although the copy number in the assembled genome is very low. The shorter copies correspond to MITEs (miniature inverted transposable elements), resulting from an internal deletion of the longest element, keeping only 595 nt from the 5′ side and 340 nt from the 3′ side.

The X-specific element, on the other hand, has a size of 408 bp and is found in the genome with at least 228 homologous



Occurrence of TF binding site within 1kb in

Transcription Factors \ Promoter	Y-specific insert	X-specific fragment	Whole X-promoter	Whole Y-promoter
Dmrt1	7,49	4,85	5,76	4,94
Wt1	9,63	12,14	5,76	3,70
Wt1 (-KTS)	16,04	2,43	0,41	/

Figure 4. Comparative analysis of the *gsdfX* and *gsdfY* promoters and their transcription factor binding sites. (*A*) The analyzed promoter region of *gsdfX* in comparison to its *gsdfY* paralog. Length differences between the two *gsdfX* and *gsdfY* promoters are due to Y- and X- specific regions of which unique Y- (936 bp) and X- (412 bp) inserts have been, respectively, added and lost concomitantly during the allelic diversification event. The Y-specific insert is made of a transposable element of the hAT family. (*B*) Characteristics of Y- and X-specific elements in the *gsdf* promoter and their relatives. The scale and positions are in nucleotides. For each structure, the copy number identified in the new assembly is indicated. Typical target-site duplication is shown (in size or in sequence) at both ends of the longest elements. For both elements, the copy inserted in the *gsdf* promoter is an internally deleted version of a longer coding element, present in one or two copies. The ORF is indicated in orange and terminal inverted repeats in pink. For Kolobok, possible extension of the ORF is shown in brown (this would include two frameshift/stop codons). The asterisk indicates the MITE subfamily to which the *gsdf*-inserted copy belongs. The average number of hits per assembly (with positive hits) is indicated. Please note that the number of detected hits is highly dependent on the assembly quality (N50), so that absence of a hit is not proof that the element is not present in the species. (*C*) Analysis of the hAT transposable element revealed that its sequence contains an overrepresentation of Dmrt1 and Wt1(-KTS) binding sites compared to the remaining sequences of either the X-specific fragment or the whole X- and Y-promoters.

copies shorter than 500 bp. Its TIRs are 12 bp long, and the element is inserted into a TTAA TSD. Eight copies longer than 500 bp were found in the genome, from which two have homology to transposases of the Kolobok superfamily, which is characterized by short TIRs and insertion into TTAA TSD as well (Kapitonov and Jurka 2007). Hence, the X-specific element also corresponds to a MITE. Further detailed analyses of the distribution of the hAT and Kolobok elements in *Actinopterygii* and

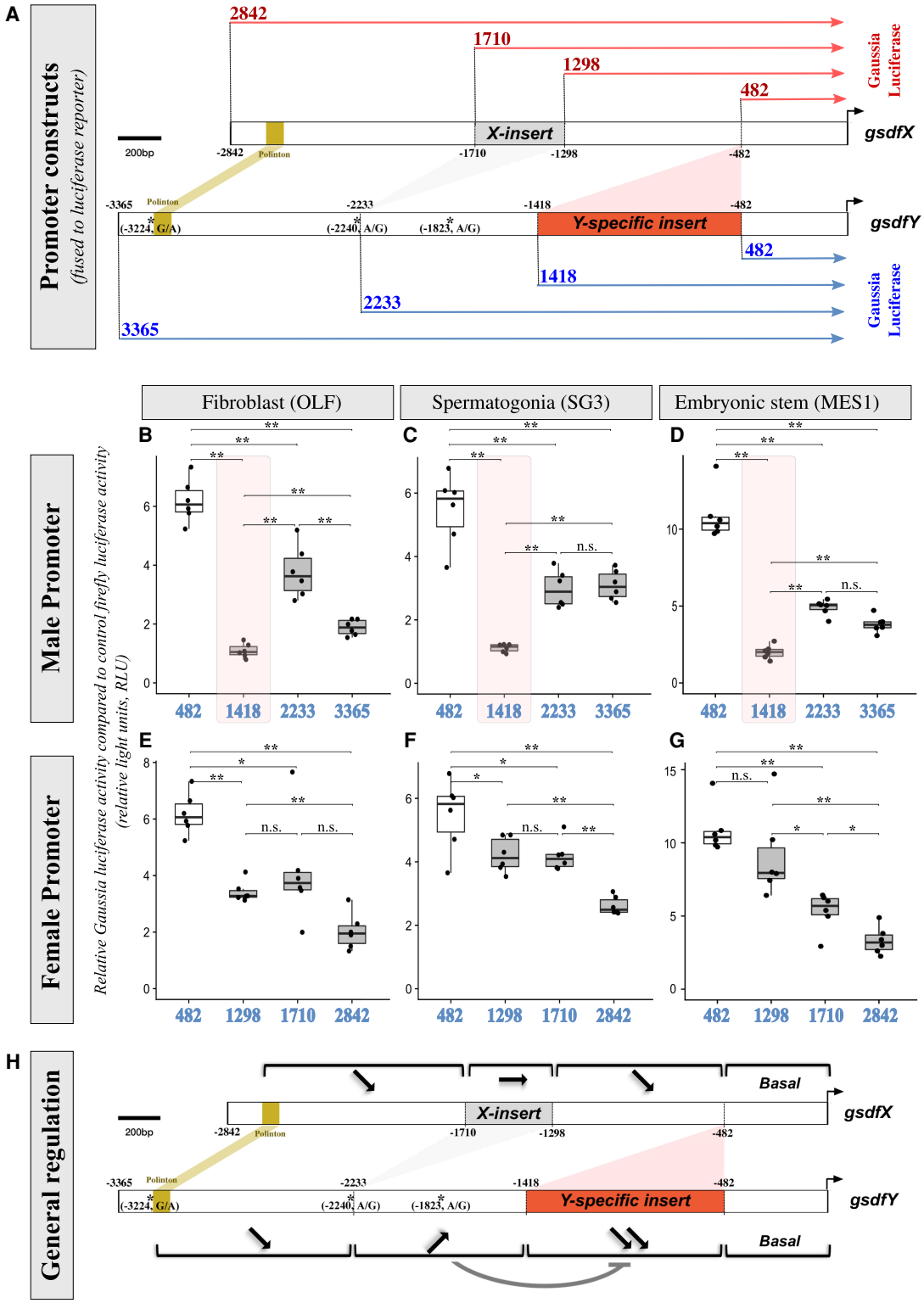


Figure 5. Transient transfection analysis of proximal *gsdfX* and *gsdfY* promoter activities. (*A*) Different deletions of the 5′ *gsdf* promoters were generated (482, 1298, 1418, 1710, 2233, 2842, and 3365 bp), fused to a luciferase reporter, and analyzed for transcriptional activity after transient transfection in different medaka cell lines (*B*–*G*). The data are presented as normalized recorded *Gaussia*/firefly luciferase activity (see the Methods section for detailed information). For every construct, transfection was repeated six times. Error bars represent the standard deviation of the means. Light-pink shaded areas (*B*–*D*) emphasize the hAT- induced modulation of transcriptional activity. (*H*) Model for the sequential regulation of *gsdfX* and *gsdfY* promoter activities. See also Supplemental Data S1 for raw data.

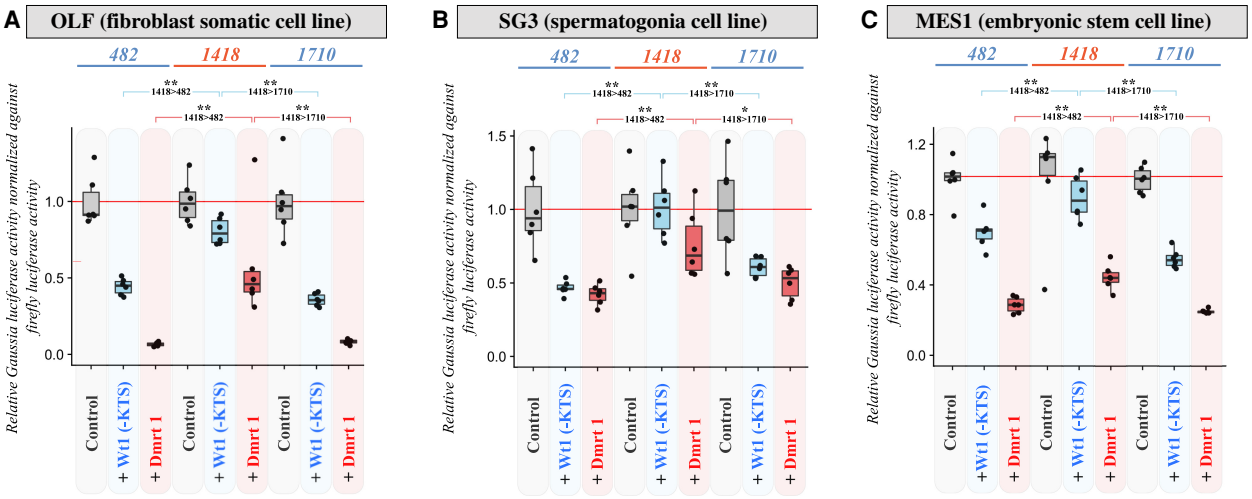


Figure 6. hAT-mediated male-specific transcriptional regulation of the *gsdfY* promoter by Dmrt1 and Wt1(-KTS). (*A*–*C*) In vitro quantification of proximal *gsdfX* and *gsdfY* promoter activities (luciferase reporters), after Dmrt1 and Wt1(-KTS) transient transfection in OLF fibroblast (*A*), SG3 spermatogonia (*B*), and MES1 embryonic stem (C) cell lines from medaka (*Oryzias latipes*). For every construct, transfection was repeated six times. Error bars represent the standard deviation of the means. See the Methods section for detailed information about the luciferase constructs. See also Supplemental Data S1 for raw data.

phylogenetic analyses are provided in Supplemental Figure S2 and Supplemental Table S2.

Identification of putative transcription factor binding sites within the *gsdfX* and *gsdfY* promoters

The sequences of the *gsdfX* and *gsdfY* promoters (–2842 and –3365 bp, respectively) (Fig. 4C) were then analyzed for the presence of putative binding sites for transcription factors. The Y-specific insert (hAT transposable element) is characterized by an overrepresentation of putative DNA-binding sites for Wt1 and Dmrt1 (Fig. 4C). Dmrt1 and Wt1, and more specifically the Wt1(-KTS) splice form, are evolutionarily conserved, dose-sensitive transcription factor proteins that are key regulators of male development. Being critical for male sex determination, both *dmrt1* and *wt1a/b* typically exhibit early and male-biased sexually dimorphic gene expression patterns (Suzuki et al. 2002; Klüver et al. 2009; Herpin and Schartl 2011, 2015). We found that the sablefish Y-specific insert upstream of *gsdf* displays 7, 9, and 16 binding sites for Dmrt1, Wt1, and Wt1(-KTS), respectively (Fig. 4C). Hence, the number of Dmrt1 and Wt1(-KTS) binding sites within the Y-specific insert are up to 39 times higher than in the whole X promoter (Fig. 4C). Nearly all of the Wt1(-KTS) binding sites within the Y promoter, in comparison to the X promoter (15 vs. 1, respectively), were delivered by this Y-specific TE insertion (Fig. 4C).

This, together with the fact that early expression patterns of *dmrt1* and *wt1* largely overlap with *gsdfY* expression during the sex determination period (Fig. 2), suggests that the Y-specific region is of primary relevance for controlling *gsdfY* transcriptional regulation.

Transcriptional activities directed by the *gsdfX* and *gsdfY* promoters in different cell lines

To evaluate the mechanisms possibly regulating differential *gsdfX* and *gsdfY* transcription, diverse portions of the *gsdfX* and *gsdfY* promoters upstream of the transcriptional start sites were cloned and used for promoter mutagenesis (or promoter bashing) luciferase assays (Fig. 5A–G) using three different medaka cell lines (OLF, MES1, and SG3) (see the Methods section for more information). Basal promoter activity was detectable with the minimal 482-bp proximal region (Fig. 5B–G). Adding more distal portions of the promoter (1298, 2233, 2842, or 3365) (see Fig. 5B–G) to the proximal region resulted in moderate negative modulation of promoter activity. In particular, in all cell lines, a drop in promoter activity was observed when the region encompassing the Y-specific insert was added (Fig. 5B–D, pink shading). Of note, addition of the X-specific region (1298 compared to 1710 in Fig. 5E–G) did not result in further regulation of promoter activity in fibroblast or spermatogonia cells (Fig. 5E,F), whereas a modest repression was recorded in embryonic stem cells (Fig. 5G). Finally, adding the most distal portion of the male promoter (3365) resulted in additional negative regulation of promoter activity in fibroblast cells (Fig. 5B), whereas no further regulation was apparent in either spermatogonia (Fig. 5C) or embryonic stem cells (Fig. 5D). This punctual discrepancy between cell types may point to the importance of the cell's identity (fully differentiated vs. stem cells) for integrating such regulations. Figure 5H recapitulates the observed changes in promoter activity determined by the promoter mutagenesis assays.

Male-specific transcriptional regulation of *gsdfY* by Dmrtl and Wtl (-KTS)

We then sought to determine if the function of the Y- and X- specific inserts in modulating *gsdfY* and *gsdfX* transcriptional regulation might be mediated by Dmrt1 and Wt1(-KTS). For this purpose, cotransfections and luciferase assays using either the 482-bp (minimal promoter), the 1418-bp (minimal promoter plus the Y-specific insert), or the 1710-bp (minimal promoter plus the X-specific insert) promoter:luciferase constructs (see Fig. 5A) were cotransfected with either Dmrt1- or Wt1(-KTS)-expressing plasmids in different cell lines (Fig. 6A–C). In the presence of either Dmrt1- or Wt1(-KTS)-expressing constructs, basal transcriptional expression (minimal promoter, 482-bp construct) was reduced in all cell types tested. With the addition of the X-specific insert to the minimal promoter (1710-bp construct), we saw no

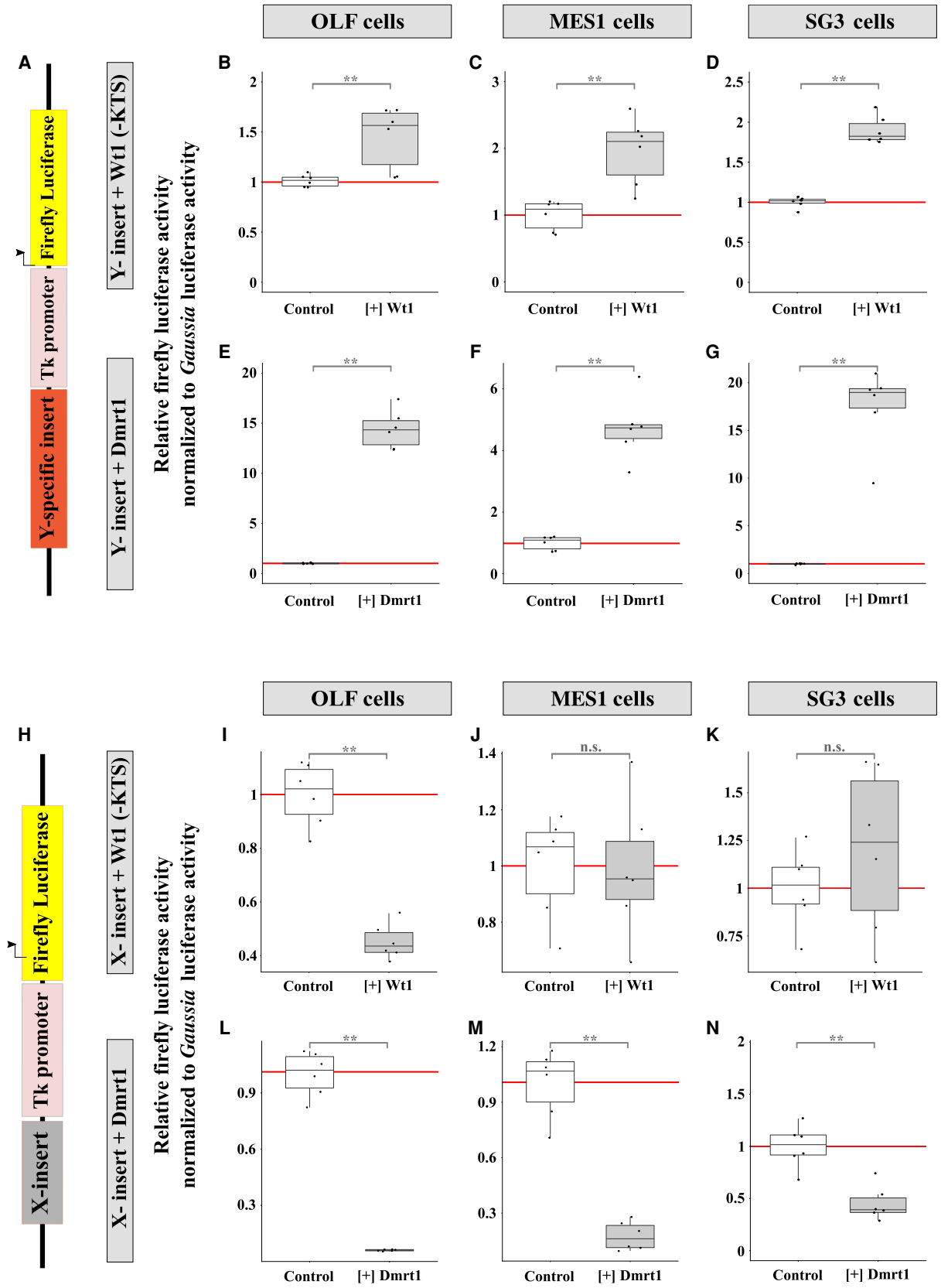


Figure 7. hAT- (Y-specific insert) and Kolobok- (X-insert) induced modulation of transcriptional regulation. (*A–G*) hAT-specific (Y-specific insert) induced modulation of transcriptional activity (luciferase reporter) of a thymidine kinase minimal promoter (*A*) upon transient transfection of *dmrt1* or *wt1*(-KTS). (*H–N*) Kolobok-specific (X-insert) induced modulation of transcriptional activity (luciferase reporter) of a thymidine kinase minimal promoter (*H*) upon transient transfection of *dmrt1* or *wt1*(-KTS). For every construct, transfection was repeated six times. Error bars represent the standard deviation of the means. See the Methods section for detailed information about the luciferase constructs. See also Supplemental Data S1 for raw data.

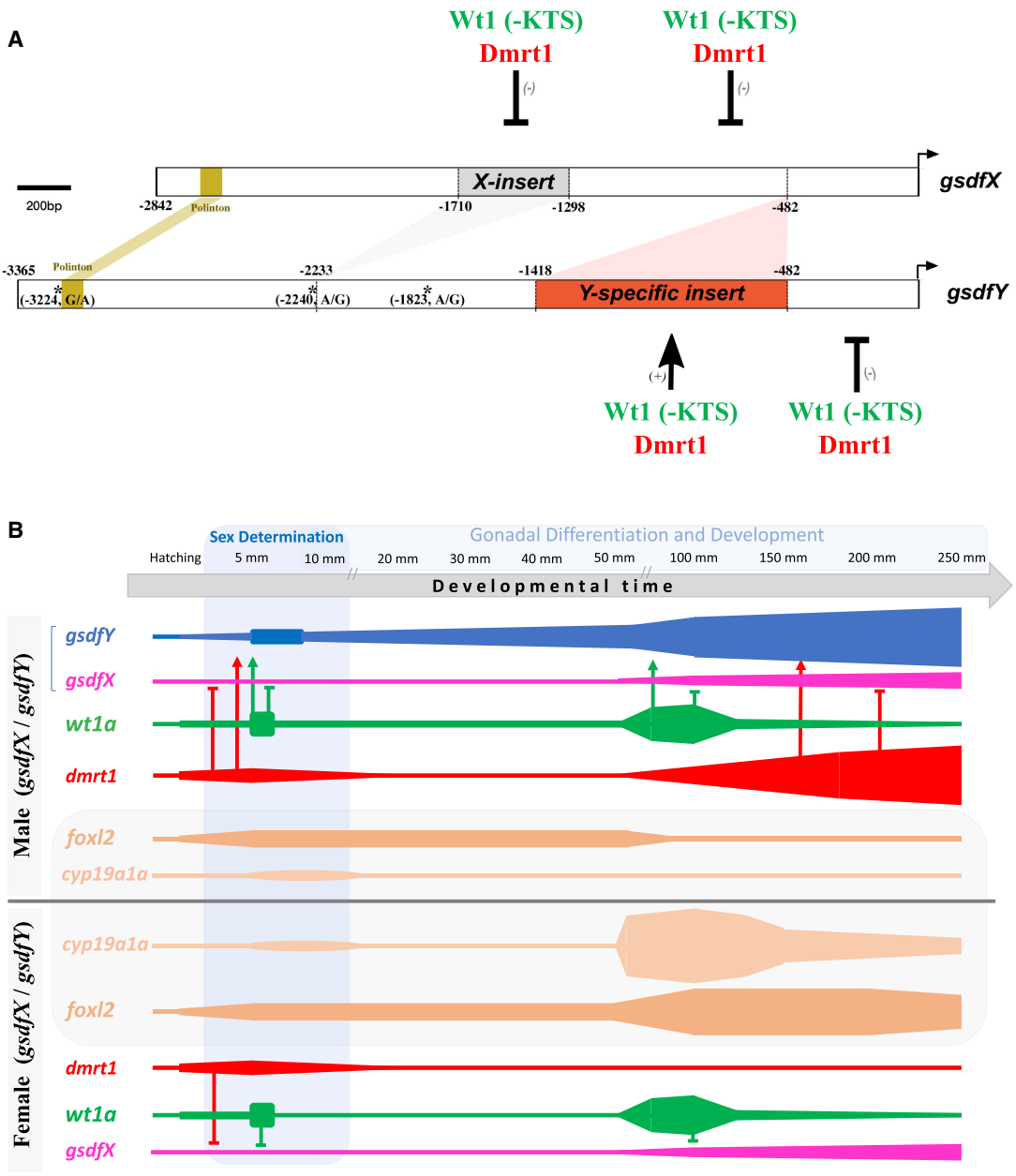


Figure 8. Models for *gsdfX* and *gsdfY* transcriptional modulation by Dmrt1 and Wt1(-KTS). Due to opposite responsiveness to Dmrt1 and Wt1(-KTS) after the Y-specific hAT element insertion into the *gsdfY* promoter (*A*), *gsdfX* and *gsdfY* exhibit very early sexually dimorphic expression patterns (*B*). This example illustrates how sexually dimorphic expression of *gsdfY* and *gsdfX* are under the dependence of non-dimorphically expressed regulators (Dmrt1 and Wt1a, for instance). Hence, sexually dimorphic expression of *gsdfY* and *gsdfX* could depend on regulators that are not themselves dimorphically expressed.

further effect on the initially observed Dmrt1- and Wt1(-KTS)-induced down-regulation. However, when the Y-specific insert was added to the minimal promoter (1418-bp construct), a clear up-regulation of basal transcriptional activity was apparent in the presence of either Dmrt1 or Wt1(-KTS) in all cell lines tested (Fig. 6A–C).

To further evaluate the ability of the Y-specific insert to mediate transcriptional regulation on its own and whether it could be modulated by expression of Dmrt1 or Wt1(-KTS), the Y-specific insert alone was fused to the basal thymidine kinase (Tk) promoter, which confers only low transcription of the reporter, and placed in front of a luciferase reporter gene (Fig. 7A). In all cell types tested (Fig. 7B–G), the Y-specific insert conferred strong up-regulation of transcriptional activity—up to 18 times higher—compared to the minimal Tk promoter in the presence of either Wt1(-KTS) (Fig. 7B–D) or Dmrt1 (Fig. 7E–G). When the X-specific insert was fused (Fig. 7H), transcriptional activity of the minimal Tk promoter was either unaffected (Fig. 7J,K) or repressed (Fig. 7I,L–N) when in the presence of Wt1(-KTS) or Dmrt1 (Fig. 7I–N). As noted earlier, differences in the cell type (differentiated vs. stem cells) in the X-specific construct may be responsible for down-regulation in OLF (differentiated) cells when exposed to Wt1(-KTS) compared

to MES1 and SG3 cells (stem cells); however, this effect was not apparent when exposed to Dmrt1. Figure 8, A and B, summarizes the above-described regulations.

Discussion

Potent innovations within regulatory networks can occur at the protein sequence level or produce alterations of *cis*-regulatory sequences defining transcription factor binding sites. After the ubiquitousness of repeated sequences was recognized, a longstanding hypothesis proposed that repeated sequences can take functional roles, for instance, in the 5′ regions of genes, by controlling transcription (Britten and Davidson 1969). This would link them to evolutionary variations and even novelties (Britten and Davidson 1971). Co-option of DNA sequences introduced during the invasion of TEs can generate, ex nihilo, new regulatory units, including enhancers (Glinsky 2015; Lynch et al. 2015; Notwell et al. 2015), repressors (Herpin et al. 2010), insulators (Wang et al. 2015), or even whole alternate gene promoters (Emera et al. 2012; Kapusta et al. 2013), substantially faster than single-point mutations. It is then postulated that co-option of ready-made *cis*-regulatory motifs nested within TEs facilitated substantial shifts in lineage-specific patterns of gene regulation over short evolutionary timescales (Rebollo et al. 2012).

Although the complex life history of sablefish (e.g., living 100 + yr and reaching sexual maturity at ~5 yr old and 55 cm long [Mason et al. 1983]) precluded our ability to conduct classical gain- and loss-of-function experiments, our systematic examination of transposon-derived *gsdf* inserts and their transcriptional regulation provides compelling evidence that *gsdfY* is the male MSD gene in this species. Comparative mapping of the sablefish genome to that of the three-spined stickleback (*Gasterosteus aculeatus*) generated linkage maps successfully identifying the locus for sex on the male map (Rondeau et al. 2013). The three-spined stickleback is one of the most closely related species to sablefish (estimated divergence of 150 mya or less) with a fully sequenced and well-annotated genome. These comparative analyses specifically anchored the sablefish MSD region to linkage group 14, corresponding to Chromosome XIV of the three-spined stickleback genome, a region of approximately 2.4 Mbs for the X and Y Chromosomes. However, the sablefish MSD region does not correspond to any of the Y-specific regions of either the three-spined (Chr XIX [Peichel et al. 2004, 2020]) or nine-spined (Chr XII [Ross et al. 2009]) stickleback, which implies that their sex chromosomes evolved independently.

Screening a genotyping-by-sequencing (GBS) library allowed narrowing down the sablefish MSD region and isolating a restricted number of SNPs unequivocally linked to sex in the vicinity of the *gsdf* gene. Gsdf is a growth factor that displays key features of the Gsdf superfamily (Gautier et al. 2011). During vertebrate evolution, it has been lost in tetrapods (Forconi et al. 2013), and its biochemical function is not well studied. Gsdf protein is nevertheless assumed to have a major role in male gonadal development due to its expression in the early differentiating testis of all fish analyzed so far. In medaka, *gsdf* plays a critical role in testis development (Chakraborty et al. 2016; Zhang et al. 2016). Besides its proposed role in the gonadal downstream regulatory network in *Oryzias latipes* (Zhang et al. 2016), *gsdf* has made it to the top of the sex determining regulatory network in *Oryzias luzonensis* (Myosho et al. 2015), a sister species to medaka, where it serves as the male sex determining gene on the Y Chromosome.

In sablefish, *gsdfY*, in contrast to its X-linked counterpart, is specifically expressed in male fry earlier than other male or female sex-related genes and prior to both molecular and morphological sexual differentiation of the gonads (Fig. 2; also Hayman et al. 2021). Comparative analysis of the *gsdfX* and *gsdfY* expression patterns clearly showed that *gsdfY*, which is expressed much earlier than *gsdfX*, experienced transcriptional rewiring during the process of allelic diversification, ultimately giving rise to the X and Y Chromosomes of sablefish. Such acquisition of a new transcriptional context resulting in a different spatio-temporal expression pattern, compatible with a sex-determining function, seems to be the main prerequisite in the process of establishment and fixation of a new MSD gene. In the two sister species, *Oryzias latipes* and *O. dancena*, and in mammals, either *dmrt1* or *sox3* genes, respectively, were subjected to profound transcriptional rewiring for establishing either *dmrt1bY* (*O. latipes*; duplication/insertion [Herpin and Schartl 2009; Herpin et al. 2009, 2010]), *sox3Y* (*O. dancena*; allelic diversification [Takehana et al. 2014]), or *SRY* (most mammals; allelic diversification [Sekido and Lovell-Badge 2008]) as MSD genes (for review, see Herpin and Schartl 2015).

In sablefish, because the unique missense mutation between the two *gsdf* variants does not appear to drastically impact their physiological activity with regard to downstream activation of Smads, any processes of functional divergence of the protein variants after allelic diversification may be reasonably excluded. Uniquely, the newly acquired MSD function of the *gsdfY* gene seems to be entirely ascribable to its new pattern of expression, indicating a neo-functionalization process.

Here, we report that the high expression of the *gsdfY* allelic copy during gonadal sex differentiation is largely imputable to a Y-specific insert derived from a TE of the hAT family incorporated into its promoter. Gsdf is an important downstream component of the male sex determination regulatory network, which in sablefish, like in *Oryzias luzonensis* (Myosho et al. 2012), acquired the role of the MSD gene. Co-option of this TE within the "*neo*"-*gsdfY* promoter was likely sufficient for transforming and elevating a protein acting downstream in the sex determination network to a MSD gene being expressed at the right time and the right place. Deciphering the mechanism by which the *neo-gsdfY* is now transcriptionally controlled and up-regulated by *dmrt1* and/or *wt1a* expression demonstrates the true exaptation of a TE into a regulatory region, thereby creating, de novo, a MSD gene.

Co-option of TEs, or TE-derived enhancers, bringing ready-made regulatory elements in a single step, in contrast to the stepwise accumulation of single mutations, might substantially facilitate immediate shifts in gene regulation over very short timescales. TEs have received particular attention in physiological processes that need rapid adaptation over evolutionary timescales, including reproduction and sex determination (Herpin et al. 2010; Lynch et al. 2011, 2015; Chuong 2013; Schartl et al. 2018). Particularly in medaka, it has been shown that, after local duplication of the *dmrt1* gene, a new hierarchy was established following insertion of TEs into the regulatory region of the *dmrt1bY* gene on the sex chromosome (Herpin et al. 2010; Schartl et al. 2018).

Altogether, our results demonstrate that allelic diversification of the *gsdf* gene gave rise to the sex determination system in sablefish. Because sablefish is widely considered a panmictic species with no apparent population genetic structure (Jasonowicz et al. 2017), *gsdfY* is likely conserved as the MSD gene across the entire species. Importantly, the MSD function of *gsdfY* was not attained by acquisition of a new function of the protein itself but rather through the acquisition of elements in the promotor region.

This resulted in a unique expression profile, which relocated *gsdfY* to the most upstream position in the sex-determining network.

The sablefish Y Chromosome provides an example of how an evolutionary novelty, which is predicted to require transcriptional rewiring of the regulatory network, was brought about after co-option of ready-made *cis*-regulatory sequences carried by a TE. Bringing another layer of transcriptional modulation via Dmrt1 and Wt1(-KTS) regulation, a unique TE exaptation into the *gsdfY* promoter appears to have created the MSD gene of sablefish.

Although direct causality between accumulation of Dmrt1 and Wt1(-KTS) binding sites and regulation remains to be investigated in greater detail, the hAT-type TE, in its entirety, confers early up-regulation of *gsdfY* expression by Dmrt1 and Wt1(-KTS), two key proteins of the canonical gonadal gene regulatory network. Thus, preventing any expression pattern redundancy between the two *gsdf* allelic copies, such divergent expression regulation might constitute a reasonable evolutionary scenario for the preservation of both *gsdf* gene copies (X and Y), protecting them from any purification/degeneration processes after allelic diversification. Finally, our data provide strong evidence for an efficient role(s) of TEs in the rewiring of gene regulatory networks in the particular context of establishing new master sex determinants over a very short timescale. An interesting future question regarding the evolutionary timescale of this occurence is whether the rare skilfish (*Erilepis zonifer*), the only other species with sablefish in the family *Anoplopomatidae*, also possesses the hAT and Kolobok TEs, particularly in the region upstream of *gsdf*.

Evolution of new sex determining genes by allelic diversification has often intuitively been associated with gradual processes that occur slowly over evolutionary timescales (Charlesworth 1991; Charlesworth and Charlesworth 2005). We, however, found that in sablefish, allelic diversification of a sex determination gene, initiated by the exaptation of a TE, led to complete transcriptional rewiring of the allele on the proto-Y Chromosome. This provides a unique functional example of a bona fide punctual process as an efficient alternative to the phyletic gradualism model (Sheldon 2001) for the molecular evolution of a master sex determining gene.

Methods

Bioinformatic analyses

Binding sites for Dmrt1, Wt1s, and other transcription factors were identified using MatInspector from the Genomatix portal (http ://www.genomatix.de). High molecular weight DNA was extracted using the Qiagen midi prep kit (Qiagen). DNA was used to prepare libraries for three approaches for genome sequence data: (1) overlapping read pair shotgun data generated on the HiSeq X Ten platform; (2) 3-, 8-, and 20-kb mate pair libraries; and (3) Pacific Biosciences (PacBio) long-read data. For the mate pair libraries, the Lucigen NxSeq Clone Free Mate Pair chemistry (Lucigen) was used; each of these libraries was sequenced on the Illumina MiSeq. The data were trimmed for adapters, and then mate read pairs were scanned for the mate pair junction code sequence, and read pairs were split. The HiSeq X Ten data were trimmed and adapters removed using cutadapt v. 1.8.3 (Martin 2011). Data from a prior genome assembly (GCA_000499045.1) were combined with trimmed HiSeq X Ten and mate pair libraries using ALLPATHS-LG 52488 (Gnerre et al. 2011). PacBio data were error-corrected with proovread (Hackl et al. 2014) using the HiSeq X Ten data. The ALLPATHS assembly produced from all Illumina data was then combined with the error-corrected PacBio reads for a hybrid assembly using PBJelly2 (PBSuite v15.8.24 [English et al. 2012]). The completed hybrid assembly was evaluated for completeness using BUSCO (Simão et al. 2015) and the vertebrata_odb9 conserved gene database.

BLAST searches in the *Anoplopoma fimbria* assembled genome

Sequences for the X-specific and the Y-specific elements were used in a megaBLASTN search (BLAST 2.6.0+ [Camacho et al. 2009], default parameters) against the *A. fimbria* genome assembly (GCA_000499045.2). Hits present on the same scaffold or contig with a distance of <2000 bp were reassembled into a single copy. We further discarded incomplete or truncated copies (missing one end), or copies that possessed more than 100 Ns within the sequence. Copies longer than 1000 bp were used in a BLASTX search (evalue -1E-10) against the Repbase protein database (Repbase20.05_REPET edition, https://www.girinst.org/) in order to identify the elements and extract the ORF sequences.

Homology searches in the Perciformes and Actinopterygii genomes

For both elements, MITE and ORF sequences were used as queries in BLASTN searches (evalue -1E-10) against all Actinopterygii genomes present in the NCBI genome databases (932 assemblies, 672 species, https://www.ncbi.nlm.nih.gov/genome/), including Perciformes (38 species). Only hits longer than 200 bp (for search with ORFs) or longer than 100 bp (for search with MITE sequences) were kept. Distributions in the different Actinopterygii orders are shown in Supplemental Figure S2 and Supplemental Table S2.

Phylogenetic analysis

Sequences presenting homology with coding sequences were then used in a BLASTX search against the Repbase protein database (-evalue 1E-10, -max_targets_seqs 1). For each assembly, we then selected the sequence presenting the highest homology. Sequences were aligned according to codon sequences and then translated and aligned along the Repbase protein sequences using MAFFTv7.2 (Katoh and Standley 2013). We further removed close sequences from the same species and sequences presenting long internal deletions or insertions. Amino-acid alignments were trimmed in order to keep only conserved regions. Phylogenies (Supplemental Fig. S2) were reconstructed using FastTree v2.1.7 (Price et al. 2009). Robustness was assessed by the Shimodaira-Hasegawa (SH) test implemented in FastTree.

Cell lines and cell transfections

Medaka fibroblast-like (OLF), spermatogonia (SG3), and embryonic stem (MES1) cells were cultured as previously described (Hong et al. 2004; Thoma et al. 2011; Zhang et al. 2016). For transfection, cells were grown to 80% confluency in six-well plates and subsequently transfected with 5 µg expression vectors using either Lipofectamine (Invitrogen) or FuGENE (Roche) reagents as described by the manufacturers.

Luciferase assays

For promoter analyses (Figs. 5, 6A–C), 482-, 1298-, 1418-, 1710-, 2233-, 2842-, and 3365-bp fragments upstream of *gsdfX* and *gsdfY* promoters (see Fig. 5A for the map) were isolated by PCR from genomic DNA of sablefish, sequenced, and cloned into the pGLuc-basic plasmid (*Gaussia* luciferase; New England Biolabs). Differential *Gaussia* luciferase activities were then quantified using the Dual Luciferase Reporter Assay System from Promega and normalized against the cotransfected firefly luciferase-expressing

control plasmid ptkLUC+ (accession number AF027128). For Figure 6D–H, the hAT Y-specific insert was cloned into the ptkLUC+ plasmid (firefly luciferase), upstream of the thymidine kinase minimal promoter, and (firefly) luciferase activities normalized using the *Gaussia* luciferase expressing pCMV-GLuc plasmid (New England Biolabs). Experiments for which error bars are shown resulted from at least six replicates and represent the standard deviation of the mean. Statistical significance was assessed by means of the Mann-Whitney *U* test.

UAS-GAL4-Smad-AD assay

For monitoring differential activation of Smads upon selective GsdfX or GsdfY expression, medaka fibroblast cells were seeded in six-well plates and cotransfected with a combination of four kinds of plasmids (see also Fig. 3): (1) an expression plasmid which encodes a fusion protein (Smad1-AD-GAL4-DBD, Smad2-AD-GAL4-DBD, Smad3-AD-GAL4-DBD, Smad5-AD-GAL4-DBD, or Smad8-AD-GAL4-DBD; 300 ng per well) that will translocate to the nucleus upon phosphorylation by the different Gsdfs and in return transactivate the UAS-4 promoter through its GAL4 DNA-binding domain; (2) a reporter plasmid, which codes for luciferase under the control of a minimal promoter, which contains UAS sequences (UAS-luc, firefly luciferase; 300 ng per well); (3) plasmids coding for the different Gsdf ligands to be tested for signaling activity (either *pCMV-gsdf-X*, *pCMV-gsdf-Y*, or a control plasmid; 400 ng per well); and (4) a *Gaussia* luciferase expression plasmid for normalization (*pCMV-Gluc*; 5 ng per well). After 24 h, cells were washed with 2× phosphate-buffered saline solution (PBS) and lysed with 75 µL of passive lysis buffer (Dual Luciferase Reporter Kit Assay; Promega), and then subjected to luciferase assay. Firefly luciferase activity (*UAS-luc* reporter constructs) was quantified using the Dual Luciferase Reporter Assay System (Promega) and normalized against cotransfected *Gaussia* luciferase-expressing plasmid. Data sets are the result of at least four independent cell transfections and luciferase measurements. Statistical significance was assessed by means of the Mann-Whitney *U* test (n = 4 or 8).

Nucleotide isolation and cDNA synthesis

Sablefish tissues used for various analyses (*gsdf* mRNA cloning, PCR, tissue distribution, and ontogenetic expression) were collected from animals cultured at the Northwest Fisheries Science Center, Manchester Research Station. All fish sampled were produced by in vitro fertilization using wild sablefish broodstock captured off the coast of Washington State, USA (Cook et al. 2015). Genomic DNA was isolated from fin clips using the DNeasy Blood and Tissue kit (Qiagen) or gonadal tissue following the Tri-Reagent (Molecular Research Center) DNA isolation protocol. RNA isolation and cDNA synthesis generally followed previous work (Luckenbach et al. 2011; Hayman et al. 2021). In brief, tissues were preserved in RNAlater (Ambion) or quickly frozen in liquid nitrogen and total RNA later extracted using 1 mL Tri-Reagent following the manufacturer's protocol including the optional spin step after homogenization. Total RNA was then DNase-treated using the TURBO DNA-free kit (Invitrogen) and quality assessed by spectrophotometry (NanoDrop). Reverse transcription (RT) of 500 ng (tissue distribution) or 250 ng (gene expression) of RNA per 10 µL reaction was done using SuperScript II (Invitrogen).

Full-length gsdfX *and* gsdfY *mRNA sequences*

To obtain full-length *gsdfX* and *gsdfY* mRNA sequences, DNase-treated RNA samples from immature testis and ovary were used to generate RACE-ready first-strand cDNA (GeneRacer kit; Invitrogen) following the manufacturer's protocol. GeneRacer and gene-specific primers were used for RACE PCR (Supplemental Table S1). Subsequent 5′ and 3′ products were run on agarose gels, bands were punched and purified (Qiagen Gel Extraction Kit) and then cloned using the Zero Blunt TOPO PCR Cloning kit (Invitrogen) with at least three clones per amplicon sequenced in both directions (GenScript). Full-length sequences for *gsdfX* and *gsdfY* were aligned using MacVector software version 12.6 (Accelrys) and deposited in the NCBI GenBank database (see Data access).

Genotypic sexing assays

For determination of genotypic sex in sablefish, PCR assays were conducted that targeted the X-insert in the *gsdf* promoter (Rondeau et al. 2013; Luckenbach et al. 2017). To demonstrate that the *gsdfX* and *gsdfY* alleles were in accordance with phenotypic sex, PCRs were developed targeting chromosome-specific inserts in the regions upstream of *gsdf* (Supplemental Table S1). Primer pairs amplified sequences upstream of and into target X and Y inserts. As a positive control, an upstream sequence common to both *gsdf* paralogs was also amplified. PCR products were resolved on a 1.5% agarose gel with GelRed stain (Biotium).

Tissue distribution and ontogenetic expression

For tissue distribution and ontogenetic gene expression analysis, some data from a previous study characterizing sablefish sex differentiation (Hayman et al. 2021) were incorporated. In addition, an early developmental series of samples was collected that targeted the period of sex determination. For this, cultured sablefish embryos were collected beginning 5 d prior to hatching, and whole larvae were collected at hatch and then sampled weekly through ~40 mm fork length (FL).

Tissue distribution and gene expression methods were reported previously or as generally described (Luckenbach et al. 2017; Hayman et al. 2021). In brief, tissue distribution semiquantitative PCRs were conducted across 11 tissues from an immature female sablefish, with the exception of testis tissue, which was collected from an immature male (both fish were 251 mm FL). Equal amounts of cDNA (2 ng) were amplified over 32 cycles and resolved on a 1.5% agarose gel. For gene expression analyses, RT-quantitative PCR (RT-qPCR) assays were conducted in 384-well plates run on a 7900HT Fast Real-Time PCR machine (Applied Biosystems). Reactions contained 1× Power SYBR Green Master Mix (Applied Biosystems), 150 nM of each gene-specific primer (Supplemental Table S1), and 2 ng of cDNA template in 12.5-µL volumes. Standards were serially diluted and run in triplicate with pooled cDNA amounts of 5, 1, 0.25, or 0.05 ng per reaction. No amplification and no template controls (NACs and NTCs) were included in all assays and had no amplification. To confirm amplification of a single PCR and correct target in experimental samples, melt curves were included and PCR products were directly sequenced for each targeted gene. All RT-qPCR data were normalized to the geometric mean of three established reference genes (*actb1, btf3,* and *rpl4*) (Hayman et al. 2021).

In situ hybridization

Testis and ovary samples collected from juvenile sablefish (male: 235 mm FL, 125g body weight; female: 235 mm FL, 130g) were fixed in Bouin's fixative at 4°C. After fixation, the tissues were dehydrated, embedded in paraffin, and sectioned at a depth of 4 µm. A subset of the paraffin sections was stained with hematoxylin and eosin (HE) stains. The localization of sablefish *gsdf* mRNA was assessed by in situ hibridization. The full *gsdf* CDS (648 bp) was

amplified using SF-GSDF-ISH-F: 5′-ATGTCCTTTACCCTCGTTGT CACGACGATG-3′ and SF-GSDF-ISH-R: 5′-TTACTCTCTGCTGGG TGGCTGGAGGTT-3′ primers (GenBank Accession # MT900066) and subcloned into the pGEM T-easy vector (Promega). Sense- and antisense-RNA probes were transcribed in vitro using digoxigenin-labeled uridine triphosphate (UTP; Roche) and SP6 or T7 RNA polymerase (Promega). ISH was performed as described by Octavera and Yoshizaki (2019).

Ethics statement

All sablefish were handled by NOAA Northwest Fisheries Science Center staff in accordance with National Research Council guidelines for aquatic animals (National Research Council 2011) and the American Veterinary Medical Association (https://olaw.nih.gov/sites/default/files/Euthanasia2007.pdf). For sampling, fish were first euthanized using a lethal dose of Tricaine-S (200 mL/L; Western Chemical) and then decapitated prior to tissue collections.

Data access

The original sablefish genome assembly (GCA_000499045.1) presented in Rondeau et al. (2013) was updated with the collection of additional sequence data from the same individual (a male) and was submitted to the NCBI Assembly database (https://www.ncbi.nlm.nih.gov/assembly) under accession number GCA_000499045.2. The updated Whole Genome Shotgun project was submitted to the Assembly database under accession number AWGY00000000.2. Full-length mRNA sequences for *gsdfX* and *gsdfY* were submitted to the NCBI GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers MT900065 and MT900066, respectively. All relevant data are within the paper or as Supplemental Material (Supplemental Data S1).

Competing interest statement

The authors declare no competing interests.

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