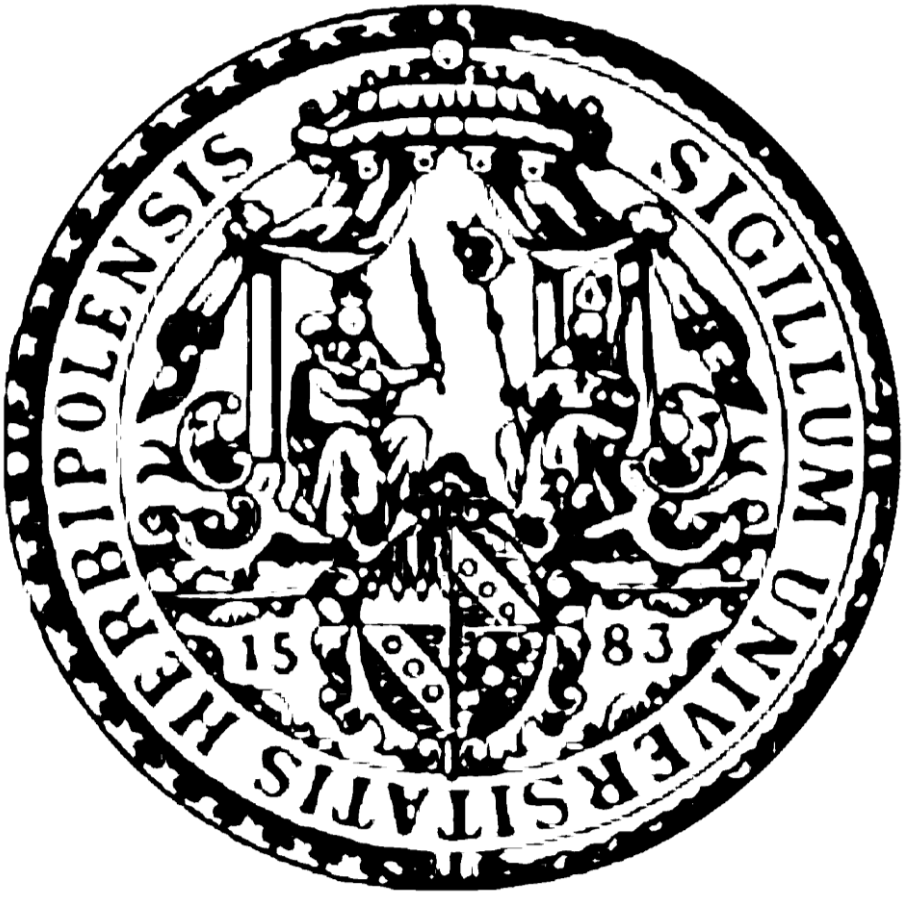
BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF AN ORIGINAL MASTER SEX DETERMINING GENE IN SALMONIDS



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Affidavit

I hereby declare that my thesis entitled: "Biochemical and molecular characterization of an original master sex determining gene in salmonids" is the result of my own work.

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Hiermit erkläre ich an Eides statt, die Dissertation: „Biochemical and molecular characterization of an original master sex determining gene in salmonids" eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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Abstract

Sexual development is a fundamental and versatile process that shapes animal morphology, physiology and behavior. The underlying developmental process is composed of the sex determination and the sex differentiation. Sex determination mechanisms are extremely labile among taxa. The initial triggers of the sex determination process are often genetics called sex determining genes. These genes are expressed in the bipotential gonad and tilt the balance to a developmental program allowing the differentiation of either a testis or an ovary. Fish represent a large and fascinating vertebrate group to study both sex determination and sex differentiation mechanisms. To date, among the known sex determining genes, three gene families namely sox, dmrt and TGF-β factors govern this developmental program. As exception to this rule, *sdY* "sexually dimorphic on the Y" does not belong to one of these families as it comes from the duplication / evolution of an ancestor gene related to immunity, i.e., the interferon related factor 9, *irf9*. *sdY* is the master sex determining gene in salmonids, a group of fishes that include species such as rainbow trout and Atlantic salmon. The present study was aimed to firstly characterize the features of SdY protein. Results indicate that SdY is predominantly localized in the cytoplasm tested in various fish and mammalian cell lines and confirmed by different methods. Predictive *in silico* analysis revealed that SdY is composed of a β-sandwich core surrounded by three α-helices as well specific characteristics conferring a putative protein-protein interaction site. Secondly, the study was aimed to understand how SdY could trigger testicular differentiation. SdY is a truncated divergent version of Irf9 that has a conserved protein-protein domain but lost the DNA interaction domain of its ancestor gene. It was then hypothesized that SdY could initiate testicular differentiation by protein-protein interactions. To evaluate this we first conducted a yeast-two-hybrid screen that revealed a high proportion of transcription factors including fox proteins. Using various biochemical and cellular methods we confirm an interaction between SdY and Foxl2, a major transcription factor involved in ovarian differentiation and identity maintenance. Interestingly, the interaction of SdY with Foxl2 leads to nuclear translocation of SdY from the cytoplasm. Furthermore, this SdY translocation mechanism was found to be specific to fish Foxl2 and to a lesser extend Foxl3 and not other Fox proteins or mammalian FoxL2. In addition, we found that this interaction allows the stabilization of SdY and prevents its degradation. Finally, to better decipher SdY action we used as a model a mutated version of SdY that was identified in XY females of Chinook salmon natural population. Results show that this mutation induces a local conformation defect obviously leading to a misfolded protein and a quick degradation. Moreover, the mutated version compromised the interaction with Foxl2 defining a minimal threshold to induce testicular differentiation. Altogether results from my thesis propose that SdY would trigger testicular differentiation in salmonids by

preventing Foxl2 to promote ovarian differentiation. Further research should be now carried out on how this interaction of SdY and Foxl2 acts *in-vivo*.

Résumé

Le développement du sexe est un processus fondamental et versatile qui forme la morphologie, la physiologie et le comportement des animaux. Le processus de développement sous-jacent est composé de la détermination et de la différentiation du sexe. Les mécanismes de détermination du sexe sont extrêment labile parmi les taxons. Les signaux initiaux du processus de détermination du sexe sont souvent génétiques et nommés gènes de détermination du sexe. Ces gènes sont exprimés dans la gonade bipotente et font pencher l'équilibre vers un programme de développement permettant la formation soit d'un testicule soit d'un ovaire. Les poissons représentent un large et fascinant groupe de vertébrés pour étudier les processus de détermination et de différentiation du sexe. A l'heure actuelle, parmi les gènes de détermination connus, trois familles de gènes nommément sox, dmrt and les facteurs TGF-β gouvernent ce processus de développement. Comme exception à cette règle, *sdY* « sexually dimorphic on the Y » n'appartient à aucune de ces familles puisqu'il provient d'une duplication/évolution d'un gène ancestral de l'immunité, c'est-à-dire d'un facteur lié à l'interféron, *irf9*. *sdY* est le gène maître de la détermination du sexe chez les salmonidés, un groupe de poissons incluant des espèces tel que la truite arc-en-ciel et le saumon Altantique. L'étude présentée avait pour but de premièrement caractériser les propriétés de la protéine SdY. Les résultats indiquent que SdY est localisée de façon prédominante dans le cytoplasme testés dans diverses cellules de poissons et de mammifères et confirmé par des différentes méthodes. Une analyse *in silico* prédictive a révélé que SdY est composé d'un core β-sandwich entouré par trois hélices-α ainsi que des caractéristiques lui conférant un site d'interaction protéine-protéine. Deuxièment, l'étude avait pour but de comprendre comment SdY pouvait entraîner la différentiation testiculaire. SdY est une version tronquée divergente de Irf9 qui a conservé le domaine protéine-protéine mais a perdu le domaine d'interaction à l'ADN présent dans le gène ancestral. Il a été proposé que SdY entraîne la différentiation testiculaire par interaction(s) protéine-protéine. Afin d'évaluer cette hypothèse, un crible double-hybride en système levure a révélé une forte proportion de facteurs de transcription incluant les protéines fox. En utilisant de nombreuses méthodes au niveau cellulaire et biochimique, nous avons confirmé une interaction entre SdY et Foxl2, un facteur majeur impliqué dans la différentiation ovarienne et gardien de son identité. De façon intéressante, l'interaction de SdY avec Foxl2 conduit à une translocation nucléaire de SdY à partir du cytoplasme. De plus, le mécanisme de translocation de SdY est spécifique à la protéine Foxl2 et dans une moindre mesure à Foxl3 parmi les protéines Fox

de poissons ou bien des protéines FoxL2 de mammifères. Puis, nous avons montré que cette interaction permet la stabilisation de SdY et empêche sa dégradation. Enfin, pour mieux décrypter l'action de SdY, nous avons utilisé comme modèle une version mutée qui a été identifiée dans une population naturelle de saumon Chinook avec des individus XY femelles. Les résultats montrent que la mutation induit un défaut de conformation local menant à une protéine mal-repliée et à sa dégradation. De plus, la version mutée compromet l'interaction avec Foxl2 définissant un seuil minimal d'induction de la différentiation testiculaire. Les résultats de ma thèse pris dans leur ensemble proposent que SdY pourrait entraîner la différentiation testiculaire chez les salmonidés en empêchant Foxl2 d'induire la différentiation ovarienne. Les recherches doivent se poursuivre dans le but de comprendre comment l'interaction SdY avec Foxl2 fonctionne *in vivo*.

Zusammenfassung

Sexuelle Entwicklung ist ein grundlegender und vielfältiger Prozess, der die Morphologie, Physiologie und das Verhalten von Tieren gestaltet. Der zugrundeliegende Entwicklungsprozess besteht aus der Geschlechtsbestimmung und der Geschlechtsdifferenzierung. Die Mechanismen der Geschlechtsbestimmung sind sehr instabil zwischen verschiedenen Arten. Die Auslöser des Prozesses der Geschlechtsbestimmung sind oft genetischen Ursprungs wie geschlechtsbestimmende Gene. Diese Gene werden in den bipotentialen Gonaden exprimiert und steuern die Balance eines entwicklungsgemäßen Programms, das die Differenzierung zum Testis oder Ovar erlaubt. Fische repräsentieren eine umfangreiche und faszinierende Gruppe von Vertebraten, um die Mechanismen der Geschlechtsbestimmung und –differenzierung zu untersuchen. Bislang ist bekannt, dass –unter den bekannten geschlechtsbestimmenden Genen- die drei Gen-Familien sox, dmrt und die TGFß-Faktoren dieses Entwicklungsprogramm steuern. Als Ausnahme von dieser Regel ist *sdY* „sexually dimorphic on the Y" keiner dieser Familien zugehörig da es von der Duplikation / Evolution eines Vorgänger-Gens, das mit Immunität wie z.B. interferon related factor9, *irf9*, in Verbindung steht, herrührt. *sdY* ist das Mastergen der Geschlechtsbestimmung in Salmoniden, die als Gruppe von Fischen Arten wie die Regenbogenforelle und den Atlantischen Lachs umfassen. Das Ziel der vorliegenden Arbeit war es zunächst die Eigenschaften des SdY Proteins zu charakterisieren. Die Ergebnisse zeigen, dass SdY vor allem im Zytoplasma lokalisiert ist. Dies wurde in verschiedenen Fischen und Säugetier Zelllinien untersucht und mit Hilfe verschiedener Methoden bestätigt. Prädiktive *in silico* Analysen zeigten, dass SdY aus einem ß-sandwich Kern besteht, der von drei α-Helices umgeben ist sowie spezifischen Eigenschaften für eine putative Protein-Protein Interaktion Stelle. Das zweite Ziel der

vorliegenden Arbeit war es, zu verstehen, wie SdY die testikuläre Differenzierung auslösen könnte. SdY ist eine verkürzte, divergente Version von Irf9, das eine konservierte Protein-Protein Domäne aufweist, jedoch seine DNA Interaktion Domäne a seines Vorläufer Gens verloren hat. Daher wurde angenommen, dass SdY die testikuläre Differenzierung durch Protein-Protein Interaktion initiieren könnte. Um diese Hypothese zu bestätigen führten wir zuerst einen Yeast Two-Hybrid Screen durch, der einen hohen Anteil an Transkriptionsfaktoren darunter fox Proteine zeigte. Unter Einsatz verschiedener biochemischer und zellulärer Methoden bestätigten wir eine Interaktion zwischen SdY und Foxl2, einem wesentlichen Transkriptionsfaktor, der in die Differenzierung und die Erhaltung der Identität der Ovarien involviert ist. Interessanterweise führt die Interaktion von SdY mit Foxl2 zu einer nukleären Translokation von SdY aus dem Zytoplasma. Außerdem wurde festgestellt, dass dieser SdY Translokations-Mechanismus für das Fisch Foxl2 und in einem geringerem Maße für Foxl3 spezifisch ist aber nicht für andere Fox Proteine oder Säuger FoxL2. Des Weiteren haben wir herausgefunden, dass diese Interaktion die Stabilisierung von SdY ermöglicht und sein Abbau verhindert. Zuletzt haben wir ein Modell einer mutierten Version von SdY benutzt, die in XY Weibchen der natürlichen Population der Königslachse identifiziert wurde, um die Wirkung von SdY besser zu entschlüsseln. Die Ergebnisse zeigen, dass diese Mutation einen lokalen Konformationsdefekt verursacht, der zu fehlgefalteten Proteinen und einem raschen Abbau führt. Darüber hinaus beeinträchtigt die mutierte Version die Interaktion mit FoxL2 und definiert einen minimalen Grenzwert, um die testikuläre Differenzierung zu induzieren. Insgesamt deuten die Ergebnisse meiner Dissertation darauf hin, dass SdY die testikuläre Differenzierung in Salmoniden auslöst, indem es verhindert, dass Foxl2 die Differenzierung der Ovarien fördert. In Zukunft soll erforscht werden, wie sich die Interaktion von SdY und Foxl2 *in-vivo* auswirkt.

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Introduction

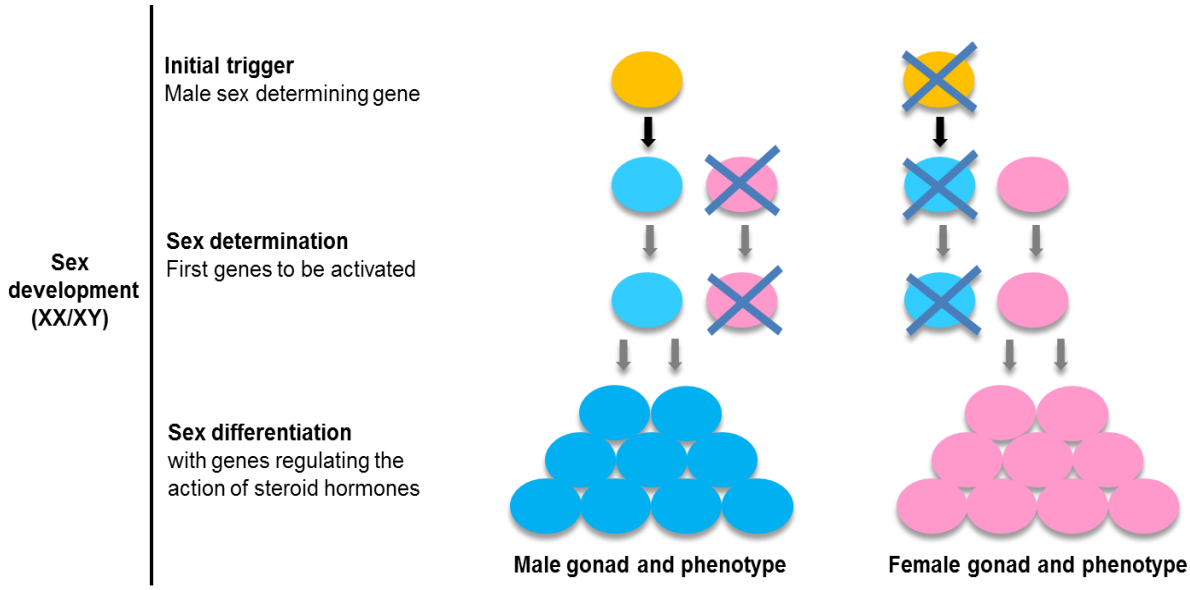


Figure 1. A view on sex development in a XX/XY system. The initial trigger (male sex determining gene) switch on the cascade and the sex develops as a male. In absence of the male sex determining gene, the female pathway is established. (Adaptated from Heule, 2013)

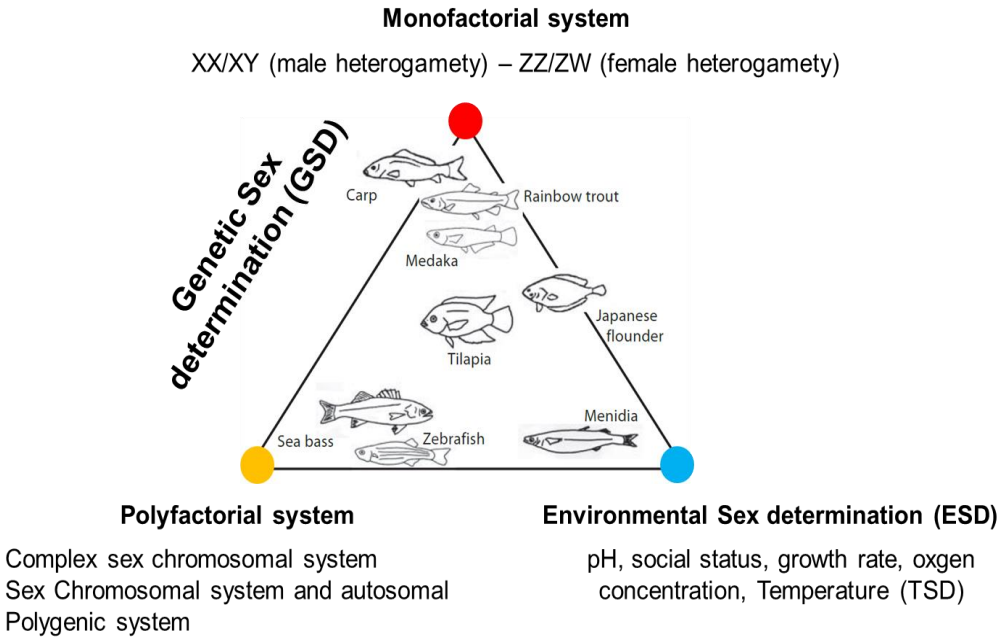


Figure 2. Shematic representation of sex determining mechanisms in fish. Two main mechanisms establish the sex determination: Genetic sex determination classified in monofactorial sytem and polyfactorial sytem and the environmental sex determination. Each category is not exclusive (Adapted from Baroillier, 2009 and Heule, 2013)

1. Sex determination

1.1 General overview of the sex determination

Sexual development is one of the most fundamental and intriguing developmental processes shaping the life history of the vast majority of individuals. Sexual development is classically composed of two processes, sex determination and sex differentiation. The sex determination is defined as the master switch or primary mechanism governing the fate of the phenotypic sex and the sex differentiation as the developmental consequence of the sex determination process (***Herpin and Schartl, 2015***). Both processes involve a highly organized gene regulatory network resulting in the formation of the gonad named testis in male and ovary in female (***Figure 1***). Sex determination is mostly triggered by the genome (genetic sex determination) or by the environment (environmental sex determination, mainly the temperature) (***Figure 2***). Contrary to many other developmental processes, the mechanisms of sex determination exhibit a very large diversity at the top of the cascade among various organismic groups and even in closely related group. This suggests that the events triggering sex determination have evolved quickly, repeatedly and independently during evolution.

Fish represents more than the half of vertebrates and show an extreme diversity and this is especially true for sexual development. Besides having different strategies of reproduction, fish exhibit both genetic sex determination and environmental sex determination offering an incredible playground to study the various mechanisms involved in this process. Many environmental factors can influence the sex establishment in fish such as water pH, oxygen concentration, growth rate, density, social state, and most commonly the water temperature (***Devlin and Nagahama, 2002; Ospina-Alvarez and Piferrer, 2008; Penman and Piferrer, 2008***). Many fish also have pure genetic sex determination systems (***Devlin and Nagahama, 2002***) or a combination of genetic sex determination and environmental sex determination like in the case of thermal effects of genetic sex determination that has been described in many species (***Devlin and Nagahama, 2002; Ospina-Alvarez and Piferrer, 2008; Penman and Piferrer, 2008***).

1.2 Genetic sex determination

Genetic sex determination (GSD) involves an inherited master switch present on sex chromosomes. This master switch is named sex determining gene or master sex determining gene. From a cytological point of view, sex chromosomes are either morphologically

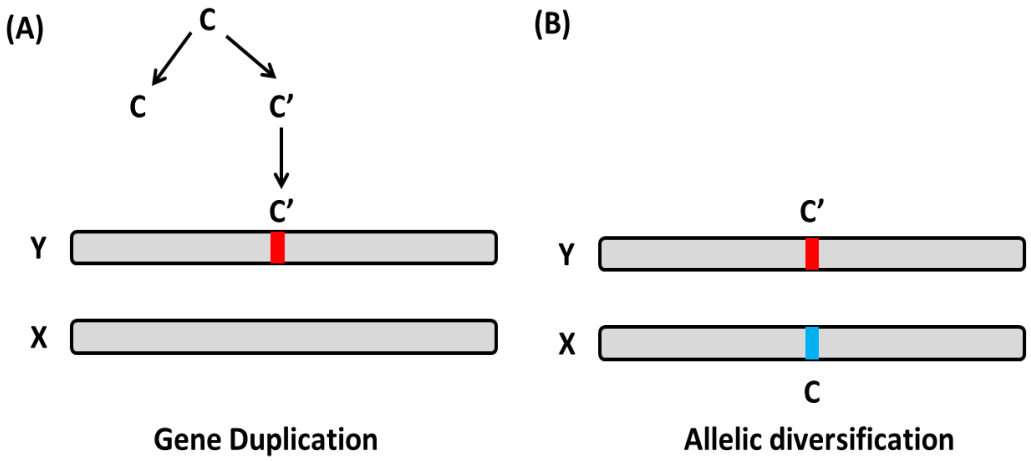


Figure 3. Schematic representation of the two main mechanisms leading to monofactorial genetic sex determination. (**A**) The duplicated gene C give rise to gene C and C'. The gene C' is inserted in the Y chromosome.(**B**) The gene C is present on the X chromosome that give rise to the gene C' inserted in the Y chromosome (Adapted from Kikuchi, 2013) .

distinguishable (heteromorphic chromosomes) or morphologically identical (homomorphic). In both cases, in simple genetic monofactorial systems, one sex is heterogametic (possessing two different sex chromosomes and hence producing two types of gametes) and the other one homogametic (a genotype with two copies of the same sex chromosome, producing only one type of gamete). A male heterogametic system is called XX-XY system (where the Y chromosome determines the male sex) and a female heterogametic system is denoted ZZ-ZW (where the W chromosome determine the female sex) (**Heule et al., 2014**). In fish, both systems are present. In mammals, the male heterogametic system drives sex determination whereas in birds and in the African clawed frog *Xenopus laevis* a female heterogametic system is present. In fish, the sex chromosomes are often homomorphic and considered not differentiated (young sex chromosomes). The master switch or master sex determining gene acts as a presence/absence signal. The presence of this sex determining gene initiates a cascade that will ultimately lead to the development of one sex, whereas its absence will lead to the other sexual differentiation program (**Figure 1**). GSD includes monofactorial systems that involve the initial switch of a single sex determining gene, like for instance the "*sexually dimorphic on the Y chromosome*" gene (*sdY*) in salmonids; or polygenic or polyfactorial systems implicating multiple genes on multiple sex chromosomes such as in laboratory zebrafish (**Anderson et al., 2012; Kikuchi and Hamaguchi, 2013; Penman and Piferrer, 2008**) or in some Malawi lake cichlids (**Moore and Roberts, 2013; Parnell and Streelman, 2013**). To date, all known master sex determining genes belong to species with a monofactorial system. Thus, the focus in this introductory chapter will be mainly on these monofactorial systems.

1.3 Sex determining genes: From fish to mammals

Sex determining genes arose from two main independent mechanisms: gene duplication and allelic diversification. A duplicated copy of an ancestral gene and its insertion in the proto-sex chromosome constitute the gene duplication mechanism. Allelic diversification comes from a stepwise diversification of one or two loci of a sex chromosome pair, where one allele became the sex determiner while the other allele at the same locus retained a non sex determination function or favored the development of the opposite sex (**Kikuchi and Hamaguchi, 2013**) (**Figure 3**). A sex determining gene in a XX/XY simple monofactorial sex determination system should fulfill four requirements: the male sex determining gene should be present on the Y chromosome, expressed at the right time (before the sex differentiation period), at the right place (in the gonad), and should be necessary and sufficient to trigger testicular differentiation. Despite a high diversity of sex determining genes, three independent families of proteins namely the Sox, Dmrt and

Table 1. Master sex determining genes in Vertebrates (adapted from Herpin and Schartl, 2015).

Master SD gene	Organism	SD system	SD gene ancestor	SD gene generated from ancestor by	Gene ancestor function
SRY	Therian mammals	XY	sox3	Allelic diversification	Transcription factor, required in formation of the hypothalamo–pituitary axis, functions in neuronal differentiation, expressed in developing gonads
Dmrt1	Birds	WZ	Dmrt1	Allelic diversification	Transcription factor, key role in male sex dermination and differentiation
DM-W	Frog *Xenopus laevis*	WZ	Dmrt1	Gene duplication	Transcription factor, key role in male sex dermination and differentiation
Dmrt1bY	Medaka (*Oryzias latipes* , *O. curvinotus*)	XY	*Dmrt1*	Gene duplication	Transcription factor, key role in male sex dermination and differentiation
sdY	**Rainbow trout (*Onchorynchus mykiss*)**	**XY**	***Irf9***	**Gene duplication**	**Interfereron response factor, no gonadal function known**
gsdfY	Luzon ricefish (*Oryzias luzonensis*)	XY	*gsdf*	Allelic diversification	TGF-b factor, important role in fish gonad development
sox3Y	Indian ricefish (*Oryzias dancena*)	XY	*sox3*	Allelic diversification	Transcription factor, required in formation of the hypothalamo–pituitary axis, functions in neuronal differentiation, expressed in developing gonads
amhY	Perjerrey (*Odontheshes hatcheri*)	XY	*Amh*	Gene duplication	Anti-Muellerian hormone, growth factor
amhY	Nile Tilapia (*Oreochromis niloticus*)	XY	*Amh*	Gene duplication	Anti-Muellerian hormone, growth factor
amhr2Y	Fugu (*Takifugu rubripes*)	WZ	*Amh receptor 2*	Allelic diversification	Type II receptor for Amh, important function in gonad development
Dmrt1	Chinese tongue sole (*Cynoglossus semilaevis*)	XY	*Dmrt1*	Allelic diversification	Transcription factor, key role in male sex dermination and differentiation

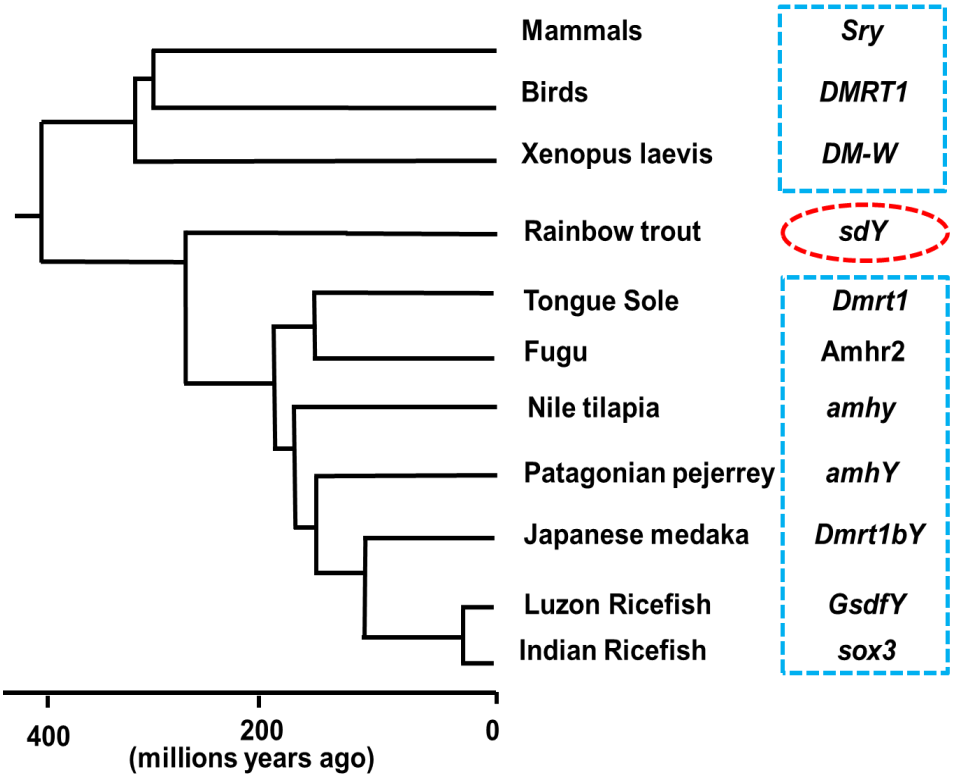


Figure 4. Schematic phylogenetic tree with the different master sex determining genes. The master sex determining gene that come from an ancestral gene implicated in sex development are in the blue square. *sdY* is surrounded in red.

Transforming Growth Factors (TGF-β) families emerged to govern the underlying developmental pathway. The single exception to this rule in vertebrate is the salmonid sex determining gene, *sdY* that does not belong to any of these three families (***Table 1, Figure 5***).

1.3.1 *Dmrt1 related sex determining genes*

dmrt1bY was the first sex determining gene found in fish. *dmrt1bY* acts as a sex determining gene in two ricefishes species of the genus *Oryzias,* the Japanese medaka (*Oryzias latipes*) and the Malabar ricefish (*Oryzias curvinotus*) (***Matsuda et al., 2002; Matsuda et al., 2003; Nanda et al., 2002***). *dmrt1bY* arose from a gene duplication of the autosomal *dmrt1a* (*dmrt1* autosomal) gene and was inserted in the Y chromosome. The Dmrt (*doublesex and mab-3 related transcription factor)* family is an evolutionary conserved gene family involved in sexual development. This gene family is characterized by a highly conserved DNA binding core motif, the DM (doublesex and Mab-3) domain and acts as transcriptions factors (Herpin and Schartl, 2011a; Murphy et al., 2015). *dmrt1bY* is expressed in the somatic cells surrounding the primordial germ cells (PGC) and later on exclusively in Sertoli cells in males at stage 34, few days before the hatching stage (***Kobayashi et al., 2004***). One to two days after the expression of *dmrt1bY*, females exhibit an increased PGC proliferation compared to male. Consistent with this, the knockdown of Dmrt1bY using gripNA methodology in XY males induces mitotic and meiotic activities similar to XX female (***Paul-Prasanth et al., 2006***). Then, it has been shown that *dmrt1bY* mediates a PGC mitotic arrest (G2/M phase of the cell cycle) in males prior testis differentiation possibly via a crosstalk between Sertoli cells and PGCs (***Herpin et al., 2007***). The overexpression of a 117 kb genomic fragment containing *dmrt1bY* and the overexpression of Dmrt1bY cDNA under the control of cytomegalovirus (CMV) induced a sex reversal (phenotypic female sex to phenotypic male sex) in XX gonads (***Matsuda et al., 2007***). Frameshift in the coding sequence of *dmrt1bY* using transcription activator-like effector nucleases (TALEN) method in XY medaka fish leads to a sex reversal (male to female) accompanied by an upregulation of wnt signaling pathway genes, a signaling pathway acting in female (***Luo et al., 2015***).

In the flatfish half-smooth tongue sole, (*Cynoglossus semilaevis*), a female heterogametic system triggers sex determination (ZZ-ZW). Dmrt1 is located in the Z chromosome and lost its function in the W chromosome. The presence of two copies of dmrt1 triggers the male sex whereas one copy (haploinsuffiency) leads to the female sex (***Chen et al., 2014***). Interestingly, the same mechanism occurs in birds (***Smith et al., 2009***).

In addition, a gene duplication of *dmrt1* followed by a truncation and insertion in the W chromosome acts as a dominant negative version and rules the sex determination system in the frog *Xenopus leavis* (**Yoshimoto et al., 2008**).

1.3.2 Sox related sex determining genes

Sox3

In the genus *Oryzias*, an allelic diversification of the *sox3* gene triggers the sex determination in *Oryzias dancena*. The male sex determining gene was named *sox3Y* but this gene is not different from its X allelic copy. However, on the Y chromosome, a specific cis regulatory DNA segment downstream of *sox3Y* is able to induce male differentiation. As this cis regulatory DNA segment is absent from the *sox3X* locus, ovarian differentiation proceeds. It has also been found that *sox3Y* initiates testicular differentiation by up-regulating Gsdf (Gonadal Soma Derived growth Factor, a TGF-ß family member absent in tetrapods). Of interest, overexpression of *sox3* in the *Oryzias latipes* species that has *dmrt1bY* as a master sex determining gene does not lead to male development. This suggests that *sox3* acquired its sex determination function concomitantly with major changes in the downstream gonadal gene regulatory network (**Herpin and Schartl, 2015; Takehana et al., 2014**).

SRY (sex determining region on the Y chromosome)

SRY, the universal male sex-determining gene in all therian mammals except the mole vole and the spiny rat, arose from an ancestral *sox3* allelic diversification. A dominant mutati the Y-chromosomal *sox3* allele and a gene fusion with regulatory sequences from another gene (DiGeorge syndrome chromosomal [or critical] region 8, DGCR8) present on the X chromosome triggered the emergence of SRY (**Koopman et al., 1991; Lovell-Badge and Robertson, 1990**; **Sato et al., 2010**; **Stevanovic et al., 1993**). It has been demonstrated that SRY protein binds to a specific enhancer named testis-specific enhancer of *Sox9* core (TESCO) on *Sox9* promoter (*Sox9* described later) and activated downstream targets genes for testis development (**Li et al., 2014b; Sekido and Lovell-Badge, 2008**). At the protein level, SRY is composed of an evolutionary conserved HMG (high-mobility group) DNA-binding domain and two less conserved flanking domains in both N-terminal and C-terminal parts respectively named NTD and CTD. Among SRY proteins, both mouse and rat SRY proteins conserved the HMG domain but lack almost all of both NTD and CTD domains. The latter replaced by a bridge domain followed by a polyglutamine rich region (Q-rich region). The HMG domain (79 aminocids) enables the binding and the bending to the DNA and the nuclear localization (presence of two nuclear localization signals (NLS) in the HMG domain)

(***Zhao and Koopman, 2012***). SRY lacks a transactivation domain (present in most of SOX proteins) and needs to recruit coactivator/corepressor to achieve its transcriptional function. Various SRY binding partners have been found such as transactivators (NR5A1(nuclear receptor subfamily 5, group A, member 1), SP1 (specificity protein 1)), transcription factors (homeodomain proteins, zinc fingers proteins, helix-loop-helix proteins, leucine zipper proteins), epigenetic regulator (Krüppel-associated box-only (KRAB-O), histone deacetylase (HDAC)), also with Poly [ADP-ribose] polymerase 1 to modulate its affinity on the DNA sequence and a wnt signaling factor (nuclear β-catenin) to inhibit the female pathway (***Bernard et al., 2008; Lau and Li, 2009; Li et al., 2006; Peng et al., 2009; Thevenet et al., 2004; Wissmuller et al., 2006***). Nevertheless, the underlying mechanisms are not fully elucidated. In the case of rodent, Zhao et al demonstrated that the polyglutamine rich region prevent the proteasomal degradation of SRY and acts as a transactivation domain (***Zhao et al., 2014***). From a series of chromatin immunoprecipitation, it has been suggested that SRY acts on sox9 to initiate early Sertoli cells to form testis cord and repress ovarian differentiating genes (***Li et al., 2014b***).

1.3.3 TGF-β related sex determining genes

Gsdf^{Y}

In the genus *Oryzias O. luzonensis* has lost *dmrt1bY*, and a TGF-β related growth factor, Gsdf (Gonadal soma derived growth factor) was identified as sex determining gene (***Myosho et al., 2012***). Gsdf was originally found as a somatic factor controlling the proliferation of primordial germ cells and spermatogonia in rainbow trout (***Sawatari et al., 2007***). This factor seems to be fish specific (***Gautier et al., 2011; Sawatari et al., 2007***). In that species, *gsdf* was brought as a master sex-determining gene by an allelic diversification process. Both the X and the Y chromosomes have the *gsdf* gene but each sex chromosome possess specific alleles i.e., *gsdf^{X}* and *gsdf^{Y}*. During the sex-determining period, the *gsdf^{Y}* allelic copy is overexpressed. The two allelic copies differ in the promoter sequence. An overexpression of Gsdf^{Y} in XX female *Oryzias latipes* leads to sex reversal (female to male). It has been suggested that *gsdf^{Y}* takes over the function of *dmrt1bY* (***Myosho et al., 2012***). *Gsdf^{Y}* is also a putative sex determining gene in another fish species, the sablefish, *Anoplopoma fimbria* (***Rondeau et al., 2013***).

amhY

In the Patagonian pejerrey, *Odontesthes hatcheri*, a fish species living in southern South America, Hattori et al discovered that the sex determining gene is the anti mullerian hormone gene (*amh*), a member of TGF-β family, (***Hattori et al., 2012***). Amh is a secreted glycoprotein implicated in the regulation of primordial germ cell proliferation and spermatogenesis (***Miura et al., 2002; Shiraishi et al., 2008***). This sex determining gene arose from a duplicated copy of *amha* (*amh* autosomal), named *amhY*. On the Y chromosome, this gene has a specific 557-bp long insertion in the third intron but the rest of the sequencing is quite identical and for instance the two proteins amha and amhbY, shares more than 90% of sequence identity at the amino acid level. *amhY* expression presents a typical pattern of a sex determiner characterized by a presence in somatic cells surrounding the germ cell and expression during gonadal development.

In Nile tilapia (*Oreochromis niloticus*), a XX-XY specie commercially important in which the sex is mainly controlled genetically, a tandem duplication of *amh* located on the Y was recently discovered. The tandem duplicate consists of *amh*-Δ*y* gene and immediately downstream *amhy* gene. *amh*-Δ*y* gene contains insertions-deletions in the promoter region and also an insertion of 5bp in the exon VI resulting to a premature stop codon leading to a truncated protein lacking the TGF-β domain. However, *amhy* differs from the X-linked *amh* of a promoter part and in the coding region of a single missense mutation (C/T) leading to an aminoacid substitution (S92L). Both genes are uniquely expressed in XY male fish at the onset of the sex determination period (5 days after hatching). Using CRISPR-Cas9 methodology, solely *amhy* loss-of-function triggers a male to female sex reversal in XY fish, while *amh*-Δ*y* alone could not. The overexpression of *amhy* using a fosmid or a plasmid containg *amhy* under a CMV promoter in XX female fish leads to a female to male sex reversal. All these experiments suggest that amhy is a good candidate to trigger the sex determination in Nile tilapia (Li et al., 2015a).

Amhr2Y – (amh receptor 2)

In the tiger pufferfish (fugu), a marine fish, a missense single-nucleotide polymorphism (SNP) determines sex (***Kamiya et al., 2012***). This missense mutation is localized in the kinase domain of the Amh receptor type 2 (AmhR2, member of the TGF-β type II receptor superfamily) leading to an amino acid substitution (Aspartic acid 384 changed into Histidine) that confers an attenuated function to the female AmhR2 (H384) when compared to the male specific allelic copy of Amhr2 (D384). The males are heterozygous (X and Y allelic copies) and females homozygous (only the X copy) for the

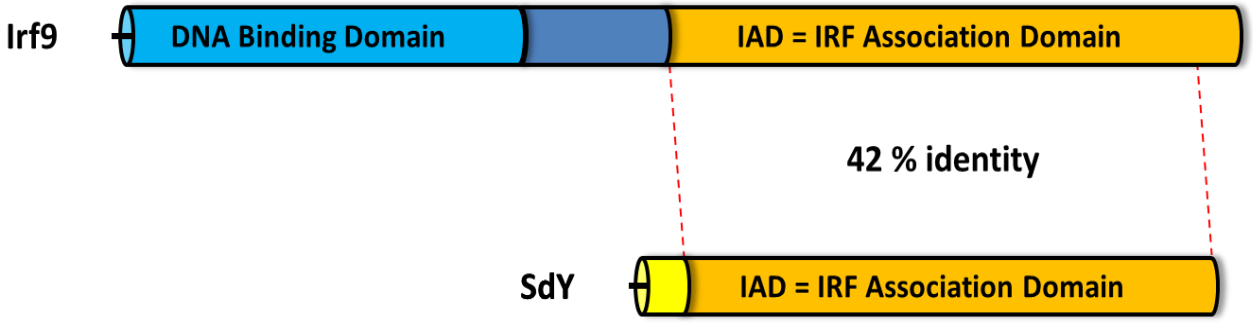


Figure 5. SdY comes from a truncated divergent copy of Interferon regulatory factor 9 and conserved the protein-protein interaction domain. SdY shares 42% of identity with the Interferon Associated Domain (IAD).

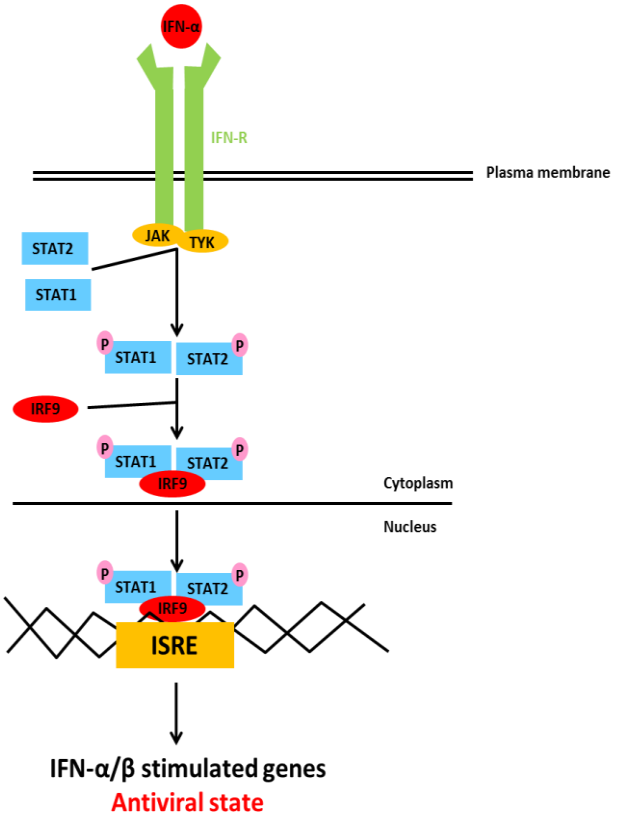


Figure 6. IRF9 triggers antiviral response through JAK-STAT signaling pathway. Upon virus infection, interferon-α (IFN-α) binds to its receptors. In turns, receptors phosphorylate both STAT1 and STAT2. IRF9 associates with both STAT and the complex binds to interferon specific responsive element (ISRE) stimulating the immune response.

mutation and this sex specific SNP is conserved over 10 million years of evolution as it is also present in two other *Takifugu* species (*T. pardalis* and *T. poecilonotus*).

All sex-determining genes described above belong to *dmrt*, *sox* or *TGF-β* gene families and these sex-determining genes are often referred as “usual suspects” as they are issued from genes well-known for their implication in the sex determination / sex differentiation pathways (***Herpin and Schartl, 2015***). However, *de novo* evolution of new sex determining genes in fish is also possible as demonstrated by the discovery of *sdY* (***Table 1, Figure 4***).

1.3.4 Sex determining gene not related to sex pathway

sdY (sexually dimorphic on the Y)

The rainbow trout (*Oncorhynchus mykiss*) belongs to the salmonid family in which an embryonic testicular specific gene was discovered at the onset of the sex determination (***Yano et al., 2012***). This gene named *sdY* for *sexually dimorphic on the Y* is only present in salmonid male genomes and was shown to be necessary and sufficient to trigger testicular differentiation (***Yano et al., 2012; Yano et al., 2013***). It encodes a protein of 215 amino acids that has homologies with interferon regulatory factor 9 (Irf9). The *sdY* gene has been then hypothesized to arose from the duplication of *irf9* followed by a truncation and variation of the DNA sequence (***Yano et al., 2012***) (***Figure 5***). The appearance of sdY constitutes an innovation compared to other sex determining genes because Irf9 (also named p48) is a member of the Interferon Regulatory Factor (IRF) family involved in innate immunity and viral response (***Zhao et al., 2015a***). During viral infection, Interferon-α/β (cytokines) activates the JAK-STAT signaling pathway that directs an Irf9-STATs complex to the nucleus to ultimately stimulate immune response genes (***Figure 6***). Irf9 is ubiquitously expressed in tissues including adult gonads (sex not indicated) in Atlantic salmon and Japanese flounder but Irf9 function has to be elucidated in this tissue (***Hu et al., 2014; Sobhkhez et al., 2014***). At the protein level, Irf9 is composed of a DNA binding domain and protein-protein interaction domain named Interferon Associated Domain (IAD). SdY shares 34.7-47.2% identity and a highly conserved stretch with Irf9 in their common protein-protein interaction domain (IAD) (***Yano et al., 2013***). Targeted deletion of sdY using two different pairs of zinc-fingers nucleases triggered on one hand the leucine 43 (L43) amino acid deletion resulting in no sex reversal for the first pair and on the other hand insertion-deletion in the 97-108 amino acids region with the second pair induced a sex reversal. The single amino acid glycine 103 (G103) loss induced a sex reversal. Both L43 and G103 are conserved in trout Irf9. A comparison of twelve amino acids among salmonids SdY sequences around the leucine 43

region revealed 66.6% of amino acid identities and in the region surrounding glycine 103 revealed 99.6% of amino acid conservation. This study showed some crucial amino acids for SdY action (**Yano et al., 2014**).

In rainbow trout, *sdY* was exclusively detected in somatic epithelial dorsal cells and also in somatic cells surrounding the germ cells in the differentiating testis and its expression begins just after hatching (32 dpf) to reach a sustained high expression during the sex differentiation period (50-90 dpf), followed by a subsequent decrease. The same kinetics is observed in Atlantic salmon (**Lubieniecki et al., 2015a**). In adult testis, *sdY* expression decreases during the completion of spermatogenesis.

A study on salmoninae (a subfamily of salmonids) sex chromosomes revealed that sex specific genetic markers linked to the sex determination locus termed as SEX locus was located on different sex chromosomes and also in different positions in the sex chromosome of four different species (**Woram et al., 2003**). Indeed, genetic maps located the SEX locus in the telomeric region for three species (Artic charr, Atlantic salmon, brown trout) but in the centromeric region for rainbow trout (**Woram et al., 2003**). In addition, a set of sex specific markers linked to the SEX locus in one species was not spatially correlated to the same set of sex specific markers in the SEX locus in other species. It has been suggested that either a short chromosome arm could transpose or several sex determining genes was present in salmonids or a single sex determining gene could move around the chromosomes by transposition (**Woram et al., 2003**). The presence of *sdY* in several salmonids species revealed firstly that the sex is determined by a single gene. An analysis of the *sdY* locus (800 kb) in rainbow trout suggested the presence of transposons, ribosomal DNA, repetitive elements and few single copy genes such as *CREB-regulated transcription activator*, *cAMP responsive element binding* (**Phillips et al., 2013**). Then, a deeper comparative analysis of the sex-determining region shared by three salmonids (rainbow trout, Chinook salmon and Atlantic salmon) revealed that only 4.1 kb of the sdY locus is conserved within species suggesting that this is the minimal region sufficient to trigger masculinization. This minimal region also contains potential elements necessary for transposition such as transposase and RNA-directed DNA polymerase (**Faber-Hammond et al., 2015**). These studies revealed that a single master sex determining gene (sdY) is present and may transpose between the different chromosomes and behave as a jumping gene (**Faber-Hammond et al., 2015; Lubieniecki et al., 2015b**).

1.4 Turn-over of the sex determination system and evolution

Sex determination signals and mechanisms can evolve rapidly resulting in a quick turnover of master sex determining genes. This turnover of sex determining genes is particularly visible in fish. Many processes have been proposed to explain how genes can take over a sex determining role. Some of the processes are purely mechanistic (gene duplication, mutations, gene dosage compensation) while other mechanisms implicated more theoretical approaches such as sexual antagonism or sexual conflict (selection for a trait that benefits one sex to the detriment of the other sex), random genetic drift, sex ratio selection (***Grossen et al., 2011; Kozielska et al., 2006; Roberts et al., 2009; van Doorn and Kirkpatrick, 2007***) (For review, (***Bachtrog et al., 2014; Herpin and Schartl, 2015***). Some suggested that the adoption of a new sex determination system would be easier in fish and poikilothermic species in general compared to thermo-regulated animals due to the great impact of the temperature (***Kikuchi and Hamaguchi, 2013***).

Why the sex determination systems are so labile? Why a high turn-over between the species? Several of these questions remain obscure. Ultimately, the apparition and the selection of a new sex determining gene drive the speciation (***Kitano and Peichel, 2012***). In addition, it is important to note that the precise molecular mechanisms of each of the known sex determining genes leading the sex differentiation are unknown.

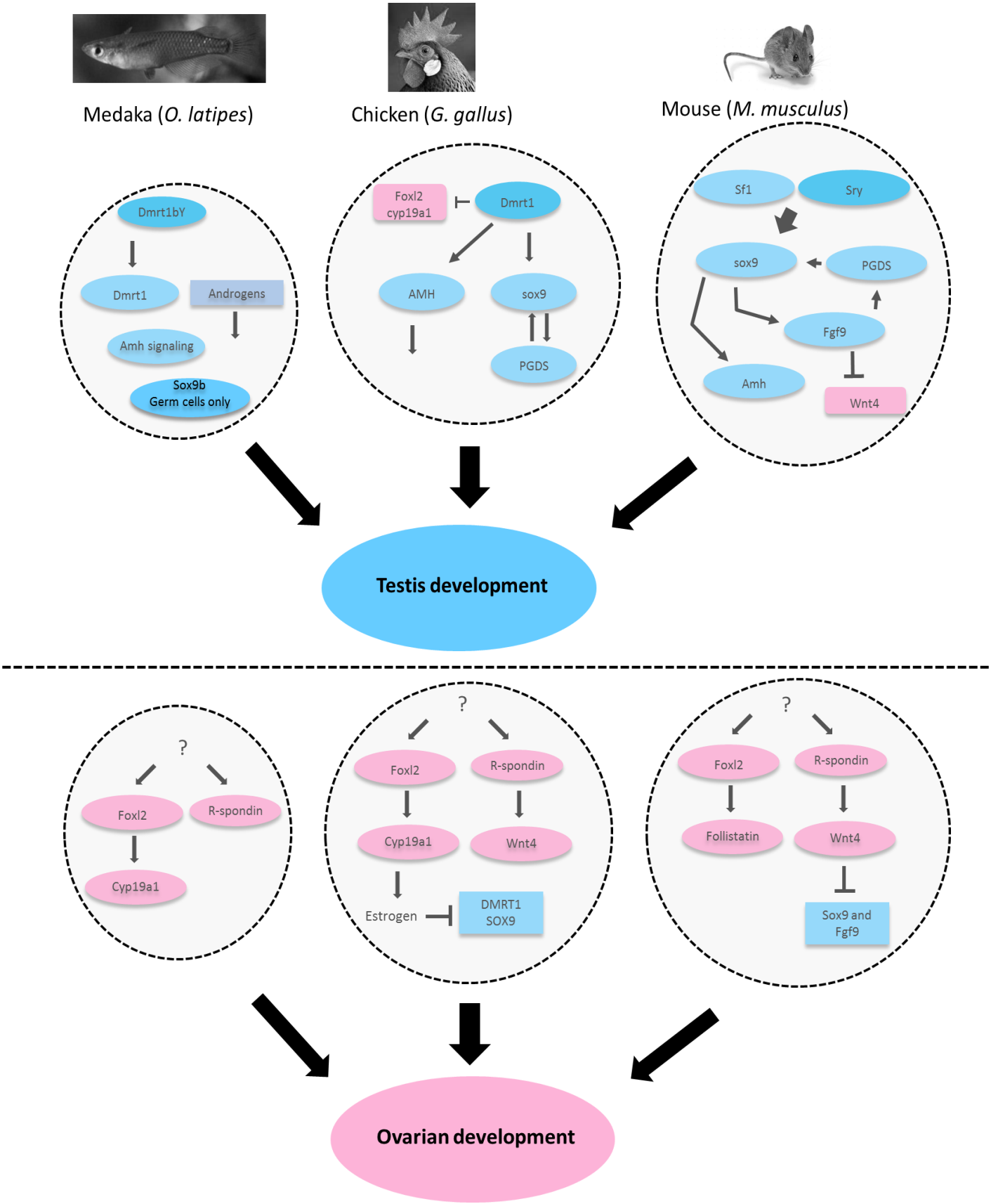


Figure 7. Overview and comparison of genes expressed during sex development of medaka (fish), chicken (birds) and mouse (mammals). Females genes are colored in pink and male genes in light blue. The arrows indicate an activation and the broken bars show a repression. This figure points out the conservation of some genes such as Amh FoxL2 and R-spondin. The underlying regulation of each species is not equivalent. Somatic sox9 have been co-opted to testis development during evolution. Cyp19a1 have its importance in non-mammalian species. (Adapted from Cutting, 2013)

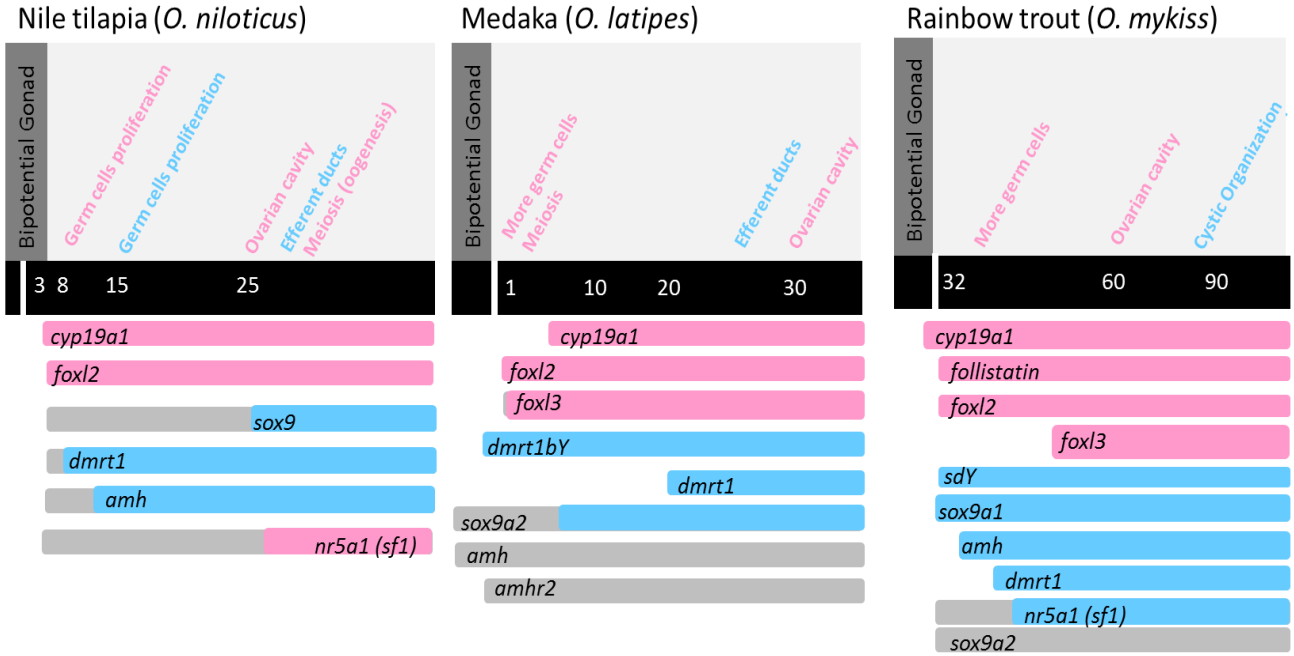


Figure 8. Comparison of gene expression during sex development of three fishes Nile tilapia, medaka, rainbow trout. The black line represents a time line of developmental age, given in days post hatching (dph) for Nile tilapia and medaka, and in days post fertilization (dpf) in rainbow trout. Hatching is indicated by a white line. The darker grey area represents time of undifferentiated (bipotential) gonad. The ovary enriched genes are in pink and testis specific genes are in light blue. Concomitant genes are in grey. (Adapted from Siegfried, 2012)

2. Sex differentiation

Gonadal sex differentiation is defined as a consequence of the sex determination process that allows the development of the internal genitalia and the secondary sex characters (***Herpin and Schartl, 2015***). Despite a differential temporal expression of the main actors observed between the species, the downstream gene regulatory network promoting the gonadal sex differentiation is more conserved during the evolution compared to the sex determination (***Figure 7 and 8***). Sex differentiation involves a complex balance between the activation of the downstream regulators of one sex and the repression of the opposite sex actors (***Figure1***).

Most of the fish species are gonochoristics meaning that each individual develops from an undifferentiated gonad either as male or as female and remains with the same functional sex throughout their life spans (***Devlin and Nagahama, 2002***). The undifferentiated gonads are composed of somatic cells and primordial germ cells (PGC). Primordial germ cells become the spermatogonia or the ooogonia, the future gametes in testis or in ovary, respectively. In teleost fish, a pool of somatic cells give rise to two main distinct cell lineages: the supporting cell lineage and the steroidogenic cell lineage. The supporting cell lineage gives rise to Sertoli cells in testis and the granulosa cells in ovary. Their main function is to nurture and physically maintain the germ cells. The interstitial cell lineage gives rise to Leydig cells and theca cells in testis and ovary respectively. These two cell types produce steroids notably the testosterone in Leydig cells and estrogens in theca cells. All types of cells interact and are spatio-temporally coordinated to ensure the proper development of the testis or the ovary to ultimately conveying the genetic information to the next generation. In fish, the main actors are identified but generally the function remains poorly elucidated.

2.1 Main players during sex differentiation

2.1.1 Male actors

2.1.1.1 Sox9 – SRY box 9

Sox9 genes are evolutionary conserved transcription factors belonging to the sox the family that is characterized by a high mobility group (HMG) box as DNA binding domain (***Kent et al., 1996***). Sox9 is expressed in the developing testes especially in pre-Sertoli cells of all vertebrate embryos that have been examined so far (Kent et al., 1996; Kobayashi et al., 2005; Moreno-Mendoza et al., 2004; Rodriguez-Mari et al., 2005; Shoemaker et al., 2007a; Western et al., 1999). In mammals, sox9 is a downstream target of SRY and NR5A1 (NR5A1, described later). Both SRY and NR5A1 will allow the expression of sox9 by binding

to a testis-specific enhancer named TES (***Sekido and Lovell-Badge, 2008***). The expression of sox9 will activate the expression of the fibroblast growth factor 9 (FGF9) and prostaglandin D synthase, indispensable elements for the proper development of the testes. In turn, the product of prostaglandin D synthase (PGD2) will regulate positively Sox9 expression (***Colvin et al., 2001; Wilhelm et al., 2005***). Hence, a positive feedback loop triggers testicular differentiation. In absence of SRY, it has been suggested that the dmrt1 gene could do the job. In chicken, *dmrt1* precedes the expression of *sox9*. In fish, *sox9* genes have been identified and described in many species including rainbow trout, medaka, zebrafish ***(Chiang et al., 2001; Vizziano et al., 2007; Yokoi et al., 2002***). Two *sox9* genes have been identified in the rainbow trout: *sox9a1* and *sox9a2*. *sox9a1* acts specifically in males and *sox9a2* is present in both male and female gonads (***Takamatsu et al., 1997; Vizziano et al., 2007***). In medaka, *sox9a2* controls the proliferation and the survival of the primordial germ cells (***Nakamoto et al., 2005; Nakamura et al., 2012***).

2.1.1.2 NR5A1 (nuclear receptor subfamily 5, group A, member 1*)(also named Steroidogenic Factor 1 (SF1) or Ad4BP (Adrenal 4-binding protein)(also named NR5A1*

NR5A1 encodes a nuclear receptor considered as a master regulator of steroidogenesis. NR5A1 is characterized by a two zinc fingers domain forming the DNA binding domain (DBD), a hinge and a ligand binding domain (LBD). From *in vitro* and *in vivo* studies, it has been shown that NR5A1 acts as a pleiotropic transcription factor that regulates the corticotropic and gonadotropic axes and central nervous system and indispensable for the formation of mammalian primary steroidogenic organs (adrenal gland and gonad) (***Luo et al., 1994; Shinoda et al., 1995; Suntharalingham et al., 2015; Valenzuela et al., 2013***). The expression pattern of Nr5a1 differs among the species. *Nr5a1* is an early expressed gene in the undifferentiated gonad in mammals and constantly maintained in the somatic cells of the early developing testis to act with SRY in human and mouse. In Sertoli cells, NR5A1 induces the expression of the anti mullerian hormone Amh (Amh, described later). In Leydig cells, NR5A1 activates the expression of steroidogenic enzyme promoting the androgeneization of external genitalia. In female, in human, NR5A1 persist during early ovarian development while the expression declines in mouse developing ovary (***Lin and Achermann, 2008; Luo et al., 1994)***. In chicken, a high expression was detected in embryonic testis although during the development the expression is higher in female (***Yamamoto et al., 2003***).

In rainbow trout, *nr5a1* expression increases in both developing gonads and later *nr5a1* displays a male specific expression (**Baron et al., 2005b; Hale et al., 2011; Vizziano et al., 2007**) while in Nile tilapia and catfish (*C. cariepinus*), the expression is higher during female development (**Ijiri et al., 2008; Raghuveer et al., 2011**).

2.1.1.3 Amh (anti mullerian hormone or mis, mullerian inhibiting substance)

Amh is a secreted glycoprotein belonging to TGF-β family which is expressed in Sertoli cells and in post-natal granulosa cells in mammals (**Josso et al., 2001; Josso et al., 1998; Munsterberg and Lovellbadge, 1991**). In mammals, its function is to promote the regression of Müllerian ducts in embryonic testis but Amh is also implicated in testicular differentiation, suppresses *Cyp19a1* (aromatase, estrogen synthase) in both male and female somatic cells in the fetal gonad (**Behringer et al., 1994; Diclemente et al., 1992; Vigier et al., 1989; Vigier et al., 1987)**. In women and in knockout female mice, the lack of *Amh* induces a precocious folliculogenesis and inhibits meiosis in fetal ovaries (**Durlinger et al., 2002; Josso et al., 1998; Visser et al., 2006**). In chicken, Amh does not contribute directly to testicular or ovarian differentiation but is required in a sex-independent manner for the proper cell proliferation and the urogenital system (**Lambeth et al., 2015**). Nevertheless, Amh plays a major role during testicular development by repressing *Cyp19a1* (**Diclemente et al., 1992; Nishikimi et al., 2000**). In fish, *amh* expression has been detected in several species such as rainbow trout, medaka, Nile tilapia that showed a higher expression in male and localized preferentially in the somatic cells surrounding the germ cells in both male and female (**Ijiri et al., 2008; Kluver et al., 2007; Nakamura et al., 2006; Pfennig et al., 2015; Vizziano et al., 2007**). One of the Amh functions in fish could be the regulation of proliferation and differentiation of primordial germ cells. In fact, *amh* expression is spatio-temporally correlated with the onset on meiosis in both male and female. In males, the expression declines during the onset of meiosis and also in Sertoli cells for instance in male rainbow trout and Atlantic salmon (**Jamin et al., 2008; Lubieniecki et al., 2015a; Vizziano et al., 2008; Vizziano et al., 2007**). The knockout of *amh* and/or its putative receptor *amhr2* triggers male to female sex reversal in Nile tilapia and fugu (Kamiya et al., 2012; Li et al., 2015a). In medaka, 50 % of the mutated *amhr2* homozygous fishes underwent a male to female sex reversal, the mutation is named *hotei* (**Morinaga et al., 2007**). In female, the knockout of *amh* in XX Nile tilapia female results in blockage of oogenesis and a follicular development is arrested at early stages in *hotei* medaka (Li et al., 2015a; Morinaga et al., 2007). Furthermore, some experimental data showed that spermatogonial proliferation

induced by 11-ketotestosterone (major androgen in teleosts) was inhibited by an active recombinant form of Amh in Japanese eel and type A spermatogonia remains undifferentiated in zebrafish (**Miura et al., 2002; Skaar et al., 2011**). The *amhr2* medaka mutants display also a hyperproliferation of undifferentiated type A spermatogonia (**Morinaga et al., 2007**).

In addition, Amh is linked to steroidogenesis. During the testis development, *amh* transcripts increase temporally with genes implicated in steroidogenesis commonly to different species (*Cyp17*, *Cyp11*, *hsd3-β1*, *nr5a1*) (**Baron et al., 2008; Ijiri et al., 2008; Vizziano et al., 2008; Wang and Orban, 2007**). However, Skar et al. showed in adult zebrafish testis culture that the recombinant Amh represses some steroidogenic genes such as steroidogenenic acute regulatory protein (*star*), a variant of insulin (*insl3*), *cyp17a1*. Beside this, the link between *amh* and *cyp19a1* (*aromatase*) is unclear. The knockdown of *amhY* by morpholino leads to an upregulation of two female factors *foxl2* and *cyp19a1* in pejerrey (**Hattori et al., 2012**). Similarly, *cyp19a1* upregulation is observed when *amhy* knockout in XY male Nile tilapia and the expression level is comparable as in XX female (Li et al., 2015a) but *amh* overexpression in XX female does not induce a female to male sex reversal. Nevertheless, a link could be established. Additionally, in rainbow trout, the amh promoter is characterized by a highly conserved binding site for Nr5a1 and also by the absence of putative sox binding sites whereas in mouse, Sox9 activates the promoter (**Arango et al., 1999; Jamin et al., 2008**)

2.1.1.4 Dmrt1 – Double sex/Male-abnormal-3 Related Transcription factor

Dmrt1 is a transcription factor implicated in testis differentiation and evolutionary conserved from cnidarian to mammals (For review (**Herpin and Schartl, 2011b, 2015; Matson and Zarkower, 2012**). Dmrt1 is present during embryogenesis and also at adult stage. Dmrt1 holds a key position in sex differentiation. Dmrt1 directs the cellular fate of male somatic cells by promoting the male specific pathway and meanwhile repressing female genes such as *Foxl2*. In addition, dmrt1 maintains the identity of the male somatic cells (**Kim et al., 2007; Lindeman et al., 2015; Matson et al., 2011; Minkina et al., 2014; Murphy et al., 2010; Zhao et al., 2015b**).

2.1.2 Female actors

2.1.2.1 R-Spondin1 (Roof plate specific spondin) (R-SPO1) / Wnt4 (Wingless-type MMTV integration site family, member 4) / β-catenin pathway/ Follistatin

Ovarian development requires the canonical β-catenin signaling pathway. In mammals, Wnt4 and R-spondin1 that are two members of the wnt signaling pathway both drive the stabilization of β-catenin and its import to the nucleus to activate the female sex differentiation (***Chassot et al., 2012; Chassot et al., 2008; Liu et al., 2009; Parma et al., 2006; Vainio et al., 1999***). Meanwhile, ectopic expression of β-catenin in somatic cells of mouse XY gonads triggered a male to female sex reversal characterized by a testicular fate disruption and ovarian development (***Maatouk et al., 2008***). Furthermore, *in vitro* studies showed that human and mouse SRY interacts with the nuclear β-catenin leading to its degradation in nuclear bodies preventing any activation of wnt signaling dependent genes (***Bernard et al., 2008; Lau and Li, 2009; Tamashiro et al., 2008***).

In fish, wnt4 transcripts are detected in somatic cells surrounding the germ cells in rainbow trout and Rspo-1 and Rspo-2 in both somatic and germ cells of medaka (***Herpin et al., 2013; Nicol et al., 2012; Zhou et al., 2012***). Then, in rainbow trout, wnt4 is not preferentially expressed in one sex during gonad differentiation (***Nicol et al., 2012***). However, other wnt members display a more contrasted sex-specific expression (***Nicol and Guiguen, 2011; Nicol et al., 2013***). The wnt-R-spondin1-β-catenin pathway seems to be conserved across the taxa to induce female development and should be repressed during male differentiation as shown in mammals, red eared slider turtle and zebrafish, chicken (***Chassot et al., 2008; Lambeth et al., 2013; Mork and Capel, 2013; Sreenivasan et al., 2014***)

2.1.2.2 Follistatin (Fst)

Follistatin is an antagonist of some TGF-ß members exclusively expressed during gonadal differentiation in females (***Trombly et al., 2009***) and acting downstream of wnt signaling pathway (***Chassot et al., 2014; Menke and Page, 2002; Yao et al., 2004***). In mouse, follistatin prevent the formation of testis-like vasculature (***Yao et al., 2004***). In trout, *follistatin* (*fst*) is an early marker that colocalizes with *cyp19a1a* transcript and regulated by the wnt signaling pathway (***Nicol et al., 2013***). Despite a co-expression *r-spo-1* and *fst* before the induction of dmrt1bY in medaka, *fst* was absent in male and not detected during the induction and development period (***Herpin et al., 2013***).

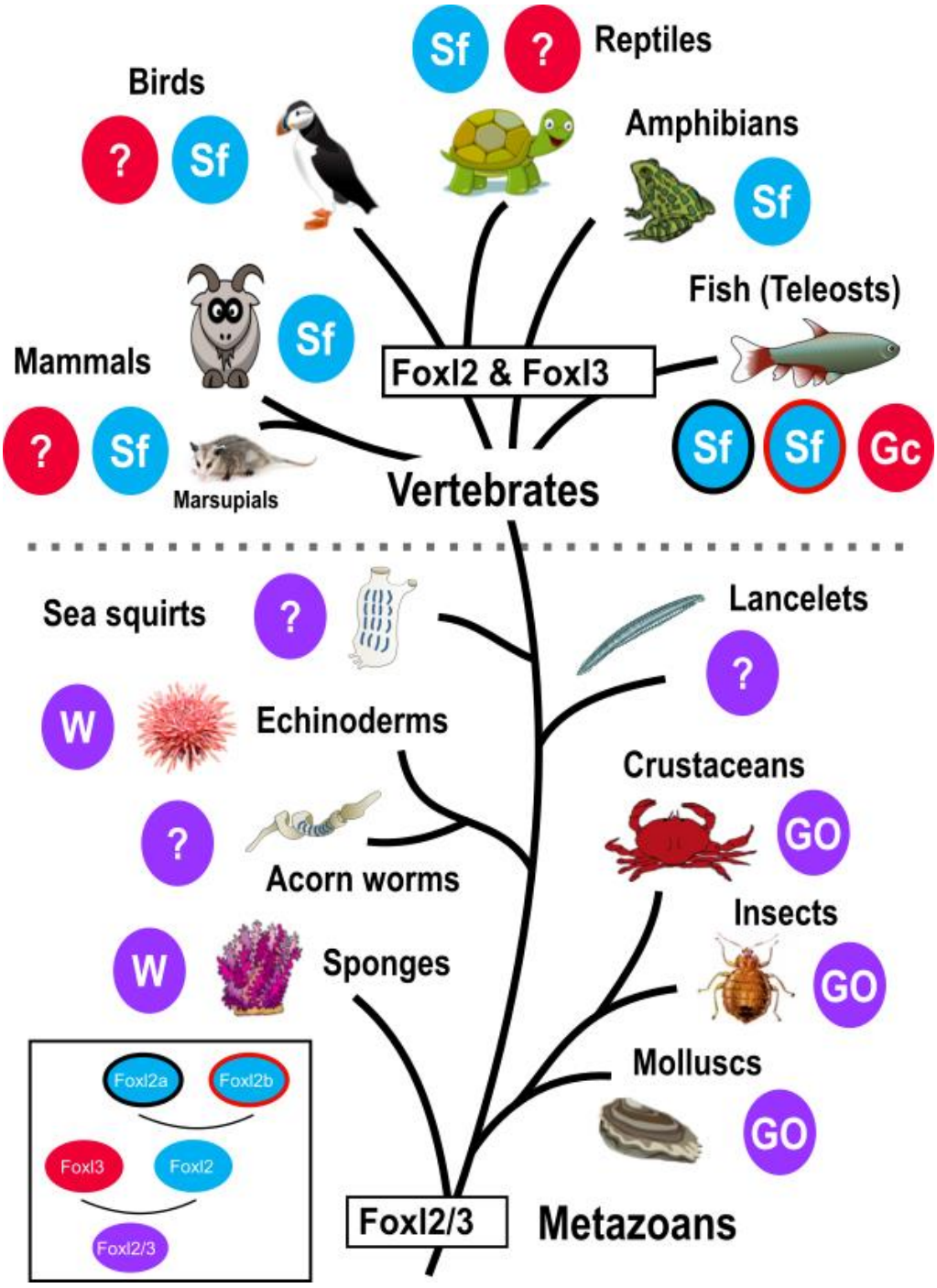


Figure 9. Schematic representation of the evolution of gonadal expression of Foxl2 and its relatives (Foxl2/3 and Foxl3) in Metazoa. Foxl2/3 is represented in blue, Foxl2 in purple and Foxl3 in red. Following the teleost fish genome duplication (TGD), some species retained two foxl2 genes, Foxl2a (purple with a black circle) and Foxl2b (purple with a red circle). “W”: Whole animal (no restricted expression), “GO”: expression in gonads, “Sf”: predominant gonadal expression in female somatic tissue, “Gc”: predominant gonadal expression in germ cells, “?”: expression pattern not described.

2.1.2.3 Fox genes

The Fox (Forkhead Box) genes belong to the winged helix transcription factor family characterized by the presence of an evolutionary well conserved DNA binding domain (DBD), the Forkhead domain. The Forkhead / hepatocyte nuclear factor 3 gene family (FKH/HNF3) was firstly discovered in Drosophila (***Weigel et al., 1989***). The fox genes are present along the Opisthokonts (a phylogenetic group gathering all descendants of the last common ancestor of animals and fungi) (***Nakagawa et al., 2013***). The Forkhead domain amino acid sequence ranges between 80-100 amino acids. In mammals, the Fox family has been subdivides into 19 subfamilies (A-S) according to the degree of conservation of the amino acid sequences. In fish, the S subfamily has not been found (***Shen et al., 2011; Shen et al., 2012; Yuan et al., 2014***). Moreover, structural studies from different Forkhead domain-DNA complexes revealed that Forkhead domain is composed of three α-helices (H1-3), linked with a β-sheet and two loops or wings (W1-2) (***Clark et al., 1993; Obsil and Obsilova, 2008***). The Forkhead domain binds to the major groove of the DNA through its H3 helix (recognition helix) (***Clark et al., 1993***). The binding specificity of the Forkhead proteins relies on the non-conserved basic region of the wings, which interact with the adjacent DNA backbone (***Obsil and Obsilova, 2008***). In addition, *in vitro* studies revealed that the forkhead domain targets a core consensus sequence (5'RYAAAYA-3') where R=A or G; Y=C or T; (***Georges et al., 2010***). Inside the Forkhead domain, a monopartite or bipartite nuclear localization signal (NLS) triggers its import into the nucleus. Being involved in main signaling pathways such as TGF-ß, wnt/β-catenin, hedgehog, MAPK, and insulin/IGF, the Forkhead proteins regulate key processes such as embryonic development, cell cycle regulation, cell survival, immunoregulation, metabolism, tumorigenesis, and ageing (***Benayoun et al., 2011a; Lehmann et al., 2003; Wijchers et al., 2006***). Besides Foxl2, only a few fox genes (*Foxc1* is implicated in the primordial germ cell migration and folliculogenesis, *Foxo3* is implicated in primordial follicle activation) have suggested to be implicated during the sex development (***Uhlenhaut and Treier, 2011***).

2.1.2.3.1 Foxl2 (*Foxl2 and its relatives are evolutionary conserved players in gonadal sex differentiation, Bertho et al, review in sexual development journal, appendix)*

Evolution of Foxl2

Foxl2 is a transcription factor evolutionary conserved from sponges (*Suberites domuncula*) to mammals (***Figure 9***). Apart from its role in craniofacial and skeletal development and uterine maturation

(**Bellessort et al., 2015; Marongiu et al., 2015**), FOXL2 has been also shown to be implicated in ovarian differentiation, follicle development and maintenance of the ovarian cellular fate (**Baron et al., 2005a; Baron et al., 2004; Boulanger et al., 2014; Caburet et al., 2012; Georges et al., 2014a; Govoroun et al., 2004; Ottolenghi et al., 2005; Uhlenhaut et al., 2009**). This transcription factor achieves its function through the recognition and the binding to a DNA conserved core sequence or a specific high-affinity FOXL2 binding element (FLRE) (**Benayoun et al., 2008**). Sequence analysis revealed that the Forkhead domain (110 amino acid) and the C-terminal part are well conserved among species (human, mouse, goat, pufferfish, rainbow trout). Nevertheless, the N-terminal part of the protein is more divergent, obviously due to lesser evolutionary constraints (Cocquet et al., 2003b; Cocquet et al., 2002).

Functions and regulation of FOXL2 in mammals

FOXL2 has been extensively studied and its gonadal expression in mammals was mainly detected both during early stages of ovarian differentiation and in the adult ovary (Cocquet et al., 2002; Crisponi et al., 2001; Pailhoux et al., 2001b; Schmidt et al., 2004; Uda et al., 2004) where the FOXL2 protein is localized in the somatic part, especially in granulosa cells (**Cocquet et al., 2003b; Cocquet et al., 2002; Pannetier et al., 2003**). In developing and adult testis, *Foxl2* RNA has been detected except in human adult testis at very low level but the presence of the protein has not been observed (Cocquet et al., 2003a; Cocquet et al., 2002; Collet and Lester, 2011a; Crisponi et al., 2001; Pannetier et al., 2003). Apart from the gonad, *FOXL2* is also expressed in the primordial mesenchyma of the developing eyelids in mammals (human, goat, mouse) (**Boulanger et al., 2014; Cocquet et al., 2002; Crisponi et al., 2001; Pannetier et al., 2003**); in the developing pituitary gland where it should be responsible for its organogenesis and as well in adult pituitary gland especially in the gonadotrope and thyrotrope cells (Ellsworth et al., 2006; Kioussi et al., 1999b; Treier et al., 1998) and in macrophage, blood, reticulocytes, hepatocytes, colon, and heart where its function still remains unknown (**Caburet et al., 2012; Moumne et al., 2008a**).

To better understand the function of FOXL2, independent knockout experiments of *FOXL2* have been performed in two species, i.e., mouse and goat (**Boulanger et al., 2014; Schmidt et al., 2004; Uda et al., 2004**). Heterozygote *Foxl2* deletion (*Foxl2* $^{(+/-)}$) in mice resulted in no dramatic morphological or functional change (**Uda et al., 2004**). Despite a strong perinatal lethality (50-95%), both models of mice lacking *FoxL2* (FoxL2 $^{(-/-)}$) develop normally until birth and no perinatal sex reversal is observed (**Schmidt et al., 2004; Uda et al., 2004**). However

Foxl2 loss of function in female mice triggers a correct formation of primordial follicles but the transition squamous to cuboidal failed in granulosa cells (model 1) (**Schmidt et al., 2004**). In the second model, primordial follicle was not observed leading to disorganized ovaries. Both models showed that at perinatal stage a progressive follicular depletion and early oocyte atresia triggers sterility (**Uda et al., 2004**). During granulosa cell formation in Foxl2$^{-/-}$ mice , these cells acquires Sertoli like cell fate and male characteristics including the expression of *Sox9*, *Amh* and other genes involved in testicular fate (**Ottolenghi et al., 2005**). The simultaneous loss-of-function of *Foxl2* and *Wnt4* or *FoxL2* (Foxl2$^{-/-}$) and *Rspo1* (*Rspo1*$^{-/-}$) results both in early sex reversal and similar features (testis like structure) suggesting a complementary role of *Foxl2* and *Wnt4*/*Rspo1* (**Auguste et al., 2011; Ottolenghi et al., 2007**). The overexpression of FoxL2 in XY mice leads to seminiferous tubules disorganization and the development of ovotestis like gonads (**Ottolenghi et al., 2007**). In adult mice, Uhlenhaut *et al*, demonstrated that FOXL2 prevents the transdifferentiation of granulosa cells into Sertoli like cells and theca cells to Leydig like cells by continuously repressing the key testis gene *Sox9* in adult ovaries. Then, the authors demonstrated that FOXL2 and estrogen receptor (ESR1) synergistically interact and repress the gonad specific enhancer TESCO element in the sox9 promoter (**Uhlenhaut et al., 2009**). Recently, it has been reported that FOXL2 directly binds to estrogen receptor 2 (esr2) promoter regulating the estrogen signaling (estrogen receptor, estradiol) in granulosa cells confirming a positive feed-forward loop of estrogen regulation. Moreover, this study showed that FOXL2 by regulating the estrogen signaling reinforces indirectly the repression of Sox9 (**Georges et al., 2014b**).

In goat, *FOXL2* loss of function leads to a complete XX female-to-male sex reversal, also marked by an agenesis of the eyelid (Boulanger et al., 2014; Pailhoux et al., 2001b). The sex reversal is characterized morphologically by Sertoli-like cells arranged in seminiferous cords, presence of Leydig cell and interstitial cells. The fetuses are also marked by the presence of testosterone and complete male genitalia. Moreover, male markers such as *Dmrt1*, *Sox9* are abundantly expressed (**Boulanger et al., 2014**). Consistent with this, FOXL2 is necessary for the development of the ovary and for maintaining its fate throughout the life preventing any male cues. Sex fate maintenance involves an antagonism between FOXL2 and DMRT1. In fact, DMRT1 directly represses *FOXL2* among others and prevent any transdifferentiation in postnatal testis (**Matson et al., 2011**). Moreover, DMRT1 prevents the induction by retinoic acid of female specific genes such as *FoxL2* in adult testis (**Minkina et al., 2014**). In addition, ectopic expression of DMRT1 silenced FoxL2 in granulosa cells independently of SOX9 expression (**Lindeman et al., 2015**). In human, mutations in *FOXL2* cause an autosomal dominant disease named Blepharophimosis/ptosis/epicanthus inversus

syndrome (BPES) accompanied by a premature ovarian failure (POF) (BPES type I) (***Crisponi et al., 2001***). Intragenic mutations are found in most of the case (71%). Missense mutation in the Forkhead domain leads to protein mislocalization and aggregation impacting the transactivation domain (***Crisponi et al., 2001; De Baere et al., 2001; Verdin and De Baere, 2012***).

Foxl2 loss of function affects differentially gonad development in goat and mouse. In mouse, a complete sex reversal is not observed. Boulanger et al suggest that during gonadal switch the presence of estrogen is indispensable to achieve the complete feminization in goat but also in humans and non-mammalian species (including fish) (***Boulanger et al., 2014***). During this period, FOXL2 through its action on *Cyp19a1* promoter leads to the synthesis of estrogens. In mice, neither estrogen receptors nor aromatase are present during the fetal period (***Couse et al., 1999; Fisher et al., 1998***). From all the studies mentioned above, it clearly appears that *Foxl2* acts as an anti-testis gene from fetal to adult life (Georges et al., 2014b; Ottolenghi et al., 2005; Pailhoux et al., 2001b; Schmidt et al., 2004; Uda et al., 2004; Uhlenhaut et al., 2009).

The major knowledge about FOXL2 modulation by posttranslational modifications and protein-protein interaction as well for its targeted genes comes from mouse and mammalian granulosa cell lines (***Caburet et al., 2012; Georges et al., 2014a; L'Hote et al., 2012; Moumne et al., 2008a)***. FOXL2 exerts a pleiotropic action on gonad determination and cell identity maintenance, pituitary function, steroidogenesis, ovulation, reactive oxygens species detoxification, apoptosis, and cell cycle control (***Caburet et al., 2012***). Furthermore, the posttranslational modifications by phosphorylation, SUMOylation and acetylation affect the behavior of FOXL2. Its phosphorylation increases the repression of a target gene named StAR (steroidogenesis acute repressor) (***Pisarska et al., 2004; Pisarska et al., 2010***). SUMOylation triggers the stabilization and activation of FOXL2 (***Georges et al., 2011; Kuo et al., 2009; Marongiu et al., 2010***). Then, deacetylation suppresses FOXL2 activity on targets linked to cell-cycle and DNA repair in a dose-dependent manner (***Benayoun et al., 2011b***).

Besides the modulation of FOXL2 by posttranslational modifications, FOXL2 can also accomplish its role by direct protein-protein interaction either with the Forkhead domain or with the transactivation domain.

One of the first characterized FOXL2 interactants was a transcriptional regulator of the DEAD-box RNA helicase family (DDX20, also known as DP103 or Gemin-3). FOXL2 in association with DDX20 amplifies the pro-apoptotic effect of FOXL2 in ovarian CHO and primary granulosa cell lines. It was suggested that this apoptotic effect may regulates the follicle stock (***Lee et al., 2005***). Recently, the transcription factor Newborn oogenesis

homeobox NOBOX involved in folliculogenesis and belonging to the homeodomain protein family was identified as a key FOXL2 partner. NOBOX interacts with FOXL2 via their respective DNA-binding domains. *In vitro*, NOBOX or FOXL2 activates independently two different promoters (oct4 and DK3) but the association NOBOX-FOXL2 induces a reciprocal inhibitory effect on both promoters (***Bouilly et al., 2014***). Moreover, the Forkhead domain of FOXL2 interacts with the MH2 (mad homology 2) protein-protein interaction domain of SMAD3, a TGF-β mediator activated by phosphorylation through activin and TGF-β receptors to regulate transcriptionally gonadotropin hormone secretion (Gonadotropin releasing hormone (GnRH), Follicle Stimulating Hormone (FSH), gonadotropin subunit-α (α-GSU)) in the pituitary–ovary axis (***Blount et al., 2009; Ellsworth et al., 2003; Ellsworth et al., 2006; Lamba et al., 2009***). Additional experiments demonstrate that GATA4 (a zinc-finger transcription factor implicated in the regulation of sex determination and sexual differentiation) interacts physically with FOXL2 and SMAD3 in ovarian granulosa cell tumors (***Anttonen et al., 2014***). In addition, FOXL2 physically interacts with nuclear receptors NR5A1 (SF-1), NR5A2, estrogens receptors (ESR1 and ESR2 alias ERα and ERβ), retinoic-acid-sensitive receptor NR2C1 and the progesterone receptor (PGR) important for the regulation of steroidogenesis and ovarian development (***Georges et al., 2014b; Ghochani et al., 2012; Kim et al., 2009; L'Hote et al., 2012; Park et al., 2010; Uhlenhaut et al., 2009***). In addition to a clearly demonstrated role in steroidogenesis, FOXL2-NR5A1 interaction directly or indirectly with the complex FOXL2-ESR1/ESR2 synergistically repress *Sox9* in granulosa cells postnatally (***Georges et al., 2014b; Uhlenhaut et al., 2009***). L'hôte *et al* also identified new partners of FOXL2 through a yeast-two-hybrid screening including co-regulators of nuclear receptors GMEB1 (Glucocorticoid Modulatory Element Binding 1) and the transcription factor CREM (cAMP responsive element modulator), the chromatin modulators BANF1 (barrier to autointegration factor 1) and KU70 product of *XRCC6* (X-ray repair cross-complementing 6), the histone deacetylase SIRT1 and two CXXC zinc finger domain-containing proteins (CXXC4 and CXXC5) modulating the wnt signaling pathway (***L'Hote et al., 2012***). FOXL2 as other Fox proteins could also forms homodimers but a clear effect on transcriptional activity has not been demonstrated as only monomeric binding sites have been reported (***Benayoun et al., 2008; Kuo et al., 2011; Lamba et al., 2009***). During eutherian mammals evolution, FOXL2 acquired a polyalanine (polyA) stretch of 14 residues considered as a repressor domain (***Pisarska et al., 2004***). This polyA tract extension ranges from 14 to 24 alanine residues and leads to pathogenicity. PolyA expansions in a length dependent manner has been demonstrated to trigger protein mislocalization, aggregation and an altered transactivation that are often responsible of

BPES syndrome (***Benayoun et al., 2008; Beysen et al., 2008; Caburet et al., 2004; Caburet et al., 2012; Moumne et al., 2008a; Moumne et al., 2008b; Moumne et al., 2005***).

Localization and expression of foxl2 in fish gonads

The fish Foxl2 proteins that lack the polyalanine stretch share 62% of identities with eutherians mammals FOXL2 and sequence identities within teleost fish are generally high (82-92%) (Smith et al., 2013a). *foxl2* displays a clear dimorphic expression in differentiating and adult gonads with a higher expression in ovary compared to testis in many species including rainbow trout, medaka, Nile tilapia (Baron et al., 2004; Nakamoto et al., 2006a; Wang et al., 2004b). Foxl2 has been found to localize in somatic cells in the female developing gonads (Nakamoto et al., 2006a; Wang et al., 2007b). At adult stages, in ovary, the protein is mainly present in follicular cells (granulosa and theca) surrounding the oocytes. In male, during sexual development, a low but significant *foxl2* expression is detected by sensitive quantitative methods but the mRNA and/or the protein have been not visualized yet. In the adult testis, Foxl2 has been detected in Leydig and germ cells of zebrafish (Caulier et al., 2015b). It worth to note that *foxl2* is absent from the developing and adult medaka testis (Nakamoto et al., 2006a). The testicular function of *foxl2*/Foxl2 is unknown.

Functions of foxl2 in fish

Up to now, the only fish model where a *foxl2* knock-out has been reported is the Nile tilapia in which loss of function of *foxl2* was obtained by gene targeted deletion using TALEN and CRISPR/Cas9 methods. This inactivation in XX female tilapia leads to female-to-male sex reversal (***Li et al., 2014a; Li et al., 2013***) with a gonadal phenotype characterized by various degrees of oocyte degeneration, and granulosa cells remaining in their original location, but expressing the Sertoli cell marker *dmrt1*. The same phenotype is observed after overexpression of a dominant negative form of Foxl2 (with a deletion of the transactivation domain) in XX embryonic gonads. This gonadal phenotype is also characterized by a down-regulation of *cyp19a1a* expression resulting in an absence of estrogen production. Overexpression of *foxl2* cDNA coupled with GFP in XY gonads under a CMV promoter at embryonic stage does not lead to a complete sex reversal but only to degeneration of testis which is replaced by ovarian-like structure confirmed by histological sections. In addition, interstitial cells and spermatogenic cysts were enlarged suggesting a role of Foxl2 in somatic cell proliferation. This transition is accompanied by an up-regulation of *cyp19a1a* resulting in

similar levels of estradiol as in the control female gonad (***Li et al., 2013; Wang et al., 2007a***). From those studies, Foxl2 seems to be an indispensable factor to complete female gonad formation by promoting ovarian differentiation and for the maintenance of the ovarian cell fate in fish.

2.1.2.3.2 Foxl3 (Forkhead box L2b, ancient Foxl2b in fish)

foxl3 was first described as a foxl2 divergent paralog (*foxl2b*) in the rainbow trout and at that time was thought to be the result of a teleosts or a salmonid specific duplication of the foxl2 gene *(**Baron et al., 2004**)*. Many fish *foxl2b* genes have since then been described and recent phylogeny analysis demonstrated that these two genes, *foxl2* and *foxl3* ancient paralogue genes (***Baron et al., 2004; Crespo et al., 2013; Geraldo et al., 2013***) already present well before the teleost specific genome duplication. Foxl3 has been lost repeatedly in the tetrapod lineage as evidenced by its presence in birds and turtles but absence in placental mammals (**Crespo et al., 2013; Geraldo et al., 2013**). Both *Foxl2* and *Foxl3* genes code for functional transcription factors that share barely similar Forkhead domain sequences suggesting same DNA targets but the C-terminal domain is strongly divergent (***Crespo et al., 2013***).

In rainbow trout during embryonic stages, *foxl3* is expressed in the female gonad peaking transiently just before and during the first oocyte meiosis whereas its expression remains undetectable in males. The precise localization of *foxl3* in trout is not known yet. In medaka, *foxl3* is specifically expressed in both female and male germ cells during the sex determination period with a lower expression in male solely during the first few days just after sex determination. At adult stage, the expression pattern is inversed and *foxl3* transcripts are predominantly expressed in the testis in rainbow trout, Atlantic salmon and European sea bass than in ovaries (***Crespo et al., 2013; von Schalburg et al., 2010; von Schalburg et al., 2011***). Furthermore, *foxl3* is highly expressed in male gills compared to a slight expression in females, a weak expression is also observed in male spleen and in female hypothalamus examined in European sea bass (***Crespo et al., 2013***). Recently, the Foxl3 function was described in the medaka in which Foxl3 serves as germ cell-intrinsic cue for sperm-egg decision. Targeted inactivation of *foxl3* in XX female gonads triggers the loss of oocytes that are replaced by the development of functional sperm and the somatic cells are not affected. In male, *foxl3* inactivation leads to a severe delay of spermatogenesis. From these results, the authors stipulate that *foxl3* suppress the spermatogenesis in female (***Nishimura et al., 2015***).

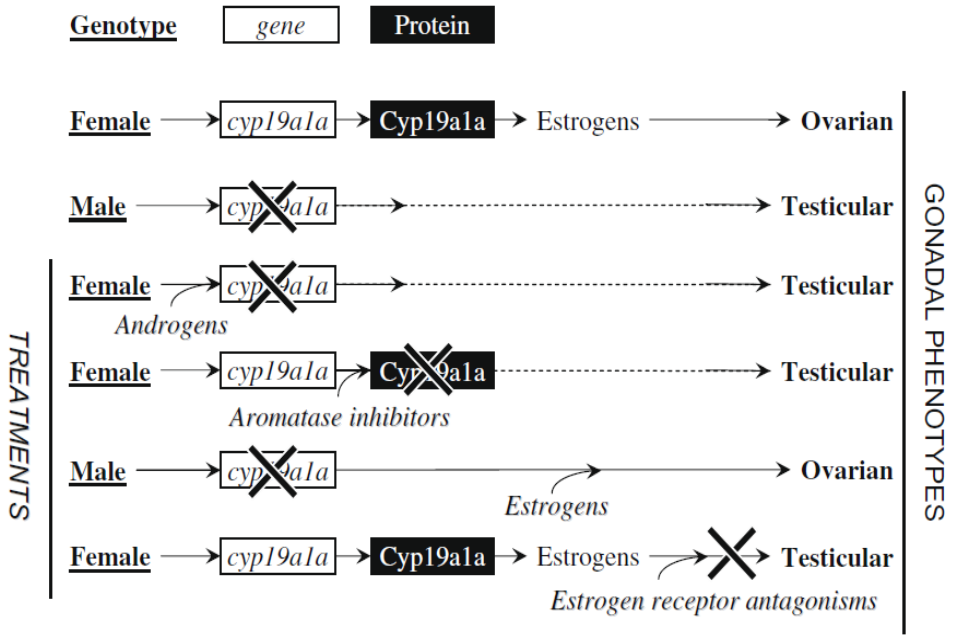


Figure 10. Pivotal role of aromatase in female gonad differentiation. Inhibition of cyp19a1a expression leads to a sex reversal. Cyp19a1 achieves its function through estrogens.

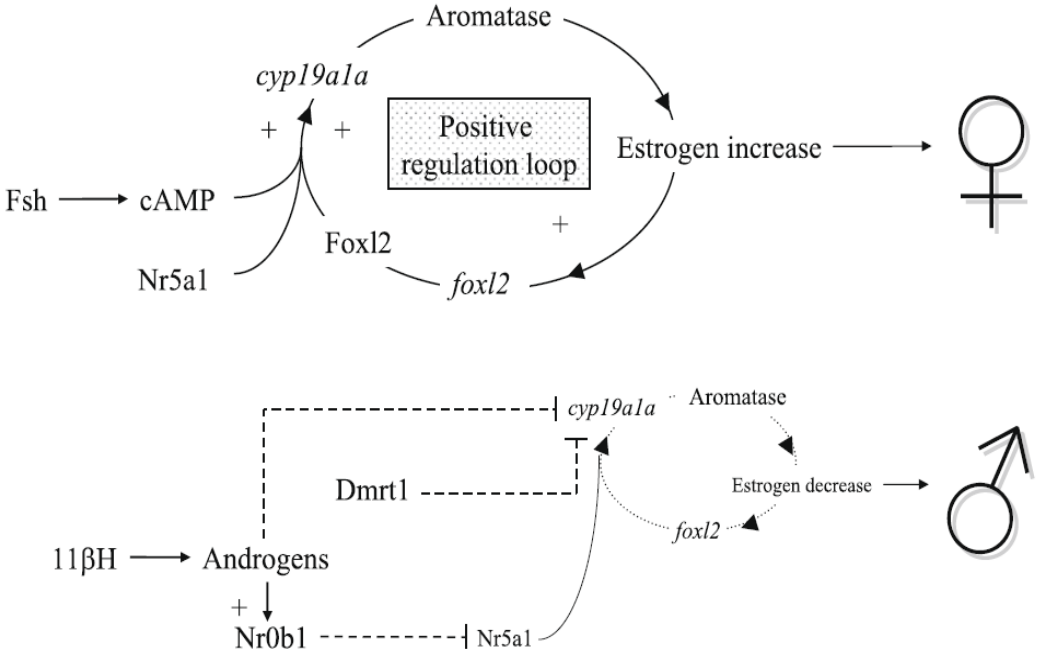


Figure 11. Regulation of aromatase in female and male. Known interactions are indicated with arrows. Suggested interactions by literature are symbolized with dashes.

2.1.2.4 Cyp19a1 – (Cytochrome P450, family 19, subfamily A, polypeptide 1a) Cyp19a1 = gene name, aromatase = protein name

Steroids such as estrogens (E2) play an important role in ovarian differentiation and maintenance especially in fish (***Guiguen et al., 2010; Paul-Prasanth et al., 2013; Piferrer and Blazquez, 2005***). The product of the CYP19a1 gene is called aromatase, a key microsomal enzyme that catalyzes the synthesis of estrogens from androgens in the smooth endoplasmic reticulum of steroidogenic cells. In fish two *cyp19a1* genes have been generated by the teleost specific whole genome duplication with *cyp19a1a* known as the ovarian form and *cyp19a1b* as the brain form. In fish, *cyp19a1a* expression is female specific and occurs prior sex differentiation in most fish species studied so far (***D'Cotta et al., 2001; Devlin and Nagahama, 2002; Suzuki et al., 2004; Vizziano et al., 2007***). During the early stage of differentiation, *cyp19a1a* localizes in a subset of unknown lineage somatic cells and thereafter is found in both granulosa and theca cells (***Sakai et al., 2008; Tanaka et al., 2008***). *cyp19a1a* gene disruption by TALEN leads to testis formation in Nile tilapia (***Li et al., 2013***) (***Figure 10***) and aromatase inhibitors treatment induces masculinization in several fish species such as Chinook salmon (*Oncorhynchus tshawytscha*) (***Piferrer et al., 1994***), rainbow trout (***Guiguen et al., 1999b***), Nile tilapia (***Guiguen et al., 1999b***), fugu (***Rashid et al., 2007***), zebrafish (***McAllister and Kime, 2003***) and this confirmed the previously well-known important role of estrogen for ovarian differentiation in fish. The regulation of the *cyp19a1a* promoter involves many factors such as activators Foxl2, Nr5a1, CREB (cAMP responsive element Binding), repressor Nrb01 (Dax1). Foxl2 regulates steroidogenesis especially via its binding to cyp19a1 promoter (***Bentsi-Barnes et al., 2010; Pannetier et al., 2006; Wang et al., 2007a***). Expression levels of *foxl2* and *cyp19a1a* mRNA are often positively correlated in different fish species such for instance in the rainbow trout, medaka, Nile tilapia, European sea bass, catfish, Japanese flounder, spotted scat (Baron et al., 2004; Crespo et al., 2013; Liu et al., 2015; Nakamoto et al., 2006a; Sridevi and Senthilkumaran, 2011; Vizziano et al., 2007; Wang et al., 2007a). The regulation could be independent or synergistic via protein-protein interaction with the nuclear receptor nr5a1 (***Kanda et al., 2006; Sridevi et al., 2012; Wang et al., 2007a; Yamaguchi et al., 2007***). Guiguen et al proposed a positive regulation loop where Foxl2 activates the *cyp19a1a* promoter, in turn the enzyme produce estrogens (E2) and the product induces the synthesis of Foxl2 (***Guiguen et al., 2010***) (***Figure 10-11***)

2.2 Sex differentiation plasticity

The different genes presented above are the major players involved in gonadal sex differentiation. Until recently, the cascade driving this sex differentiation process was thought to be well conserved as the same actors were repeatedly found (***Figure 7 and 8***). Graham et al suggested 'masters change, slaves remains' (***Graham et al., 2003***) suggesting that master sex determining genes could be more prone to variation than sex differentiating genes. However, recent data in fish support that in addition to a high turnover at the top of the hierarchy, a flexibility could be also observed downstream (***Herpin and Schartl, 2015; Heule et al., 2014***). This is evidenced by the many species-specific dimorphic expression patterns seen throughout the development (***Bohne et al., 2013; Herpin et al., 2013***). In addition, it seems that the sex differentiating pathway in male appears more labile between the species but implicates more players than in female allowing probably a more strict control (***Cutting et al., 2013***). This suggestion fits also with the evidence that it is more difficult to observe a complete male to female sex reversal by gene targeted disruption in XY males than a complete a complete female to male sex reversal male in XX females (***Li et al., 2013***). The molecular downstream sex differentiation pathway reflects also a level of divergence in functions, regulations and interplays among the species probably by the regulatory putsches triggered by the sex determining genes (***Herpin and Schartl, 2008***). In line with this, Herpin and Schartl, suggest a new paradigm "When masters change, some slaves remain, others are dismissed or acquire new tasks, and new ones can be hired" (***Herpin and Schartl, 2015***)

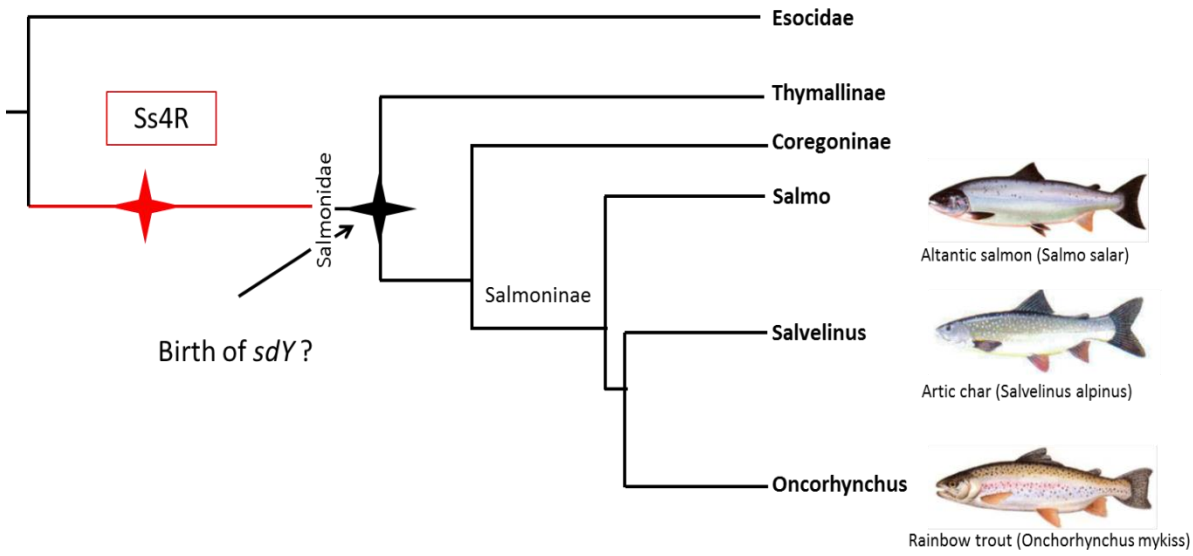


Figure 12. Schematic phylogenetic tree of salmonids. Three emblematic species of salmoninae are represented. The specific salmonids genome duplication (Ss4R) is indicated in red. Note that all the species are not shown (Adapted from Yano et al, 2013)

3- Salmonid family

The teleost family Salmonidae is a small taxon of 65-70 different species included in 3 sub families i.e., the Coregoninae (whitefishes), the Thymallinae (grayling), and salmoninae (salmon, trout and charr) (***Figure 12***). These fish are further classified into 11 genera and approximately 70 species (***Koop et al., 2008***). Most of the salmonids are anadromous species meaning that they move from the sea to fresh water to spawn. Generally, the spawning place is the same river where they were born. Salmonids sex determination has been described as being male heterogametic (XX/XY) (***Davidson et al., 2009***).

Many members of the Salmoninae, including Atlantic salmon *(Salmo salar),* Pacific salmon and rainbow trout (*Oncorhynchus* sp.) and Arctic charr *(Salvelinus alpinus),* are important for aquaculture, wild stock fisheries and recreational sport fisheries. Besides their great economic and societal importance, the salmonids are also of considerable scientific importance in research fields like evolutionary biology, ecology, physiology, genetics, immunology, nutrition and environmental toxicology (***Thorgaard et al., 2002***). No other group of fish receives such comprehensive commercial and scientific attention. There is a large body of work on salmonid sex determination and sex manipulation, fueled in part by the desire to produce all female stocks for aquaculture (***Davidson et al., 2010***). Male rainbow trout mature earlier than females and females are then more valuable for aquaculture production because of their delayed age at maturity that prevent them from an early reduced growth rate during gonad maturation (***Piferrer et al., 2012***).

Extant Salmonidae emerged from a last common ancestor around 60 million years ago and they all have a genome that experienced an additional whole genome duplication (WGD) event estimated around 100 million years ago, named the salmonid-specific 4^{th} WGD or Ss4R. Such whole genome duplication events are rare but have dramatic effect resulting from the doubling of their complete genome sequence (***Berthelot et al., 2014; Davidson et al., 2010; Macqueen and Johnston, 2014***)

3.1 Rainbow trout as model specie to study sex determination-differentiation

Rainbow trout (*Oncorhynchus mykiss*) is well-studied fish and has been extensively used as a research model. This fish is present worldwide in both hemispheres with important ecologic and economic values. Rainbow trout is a freshwater fish inhabiting cold water and is able to resist to high variation in temperatures (0-21°C). In aquaculture, the ideal temperature is around 10°C before hatching and comprise between 15-17°C after hatching. The water

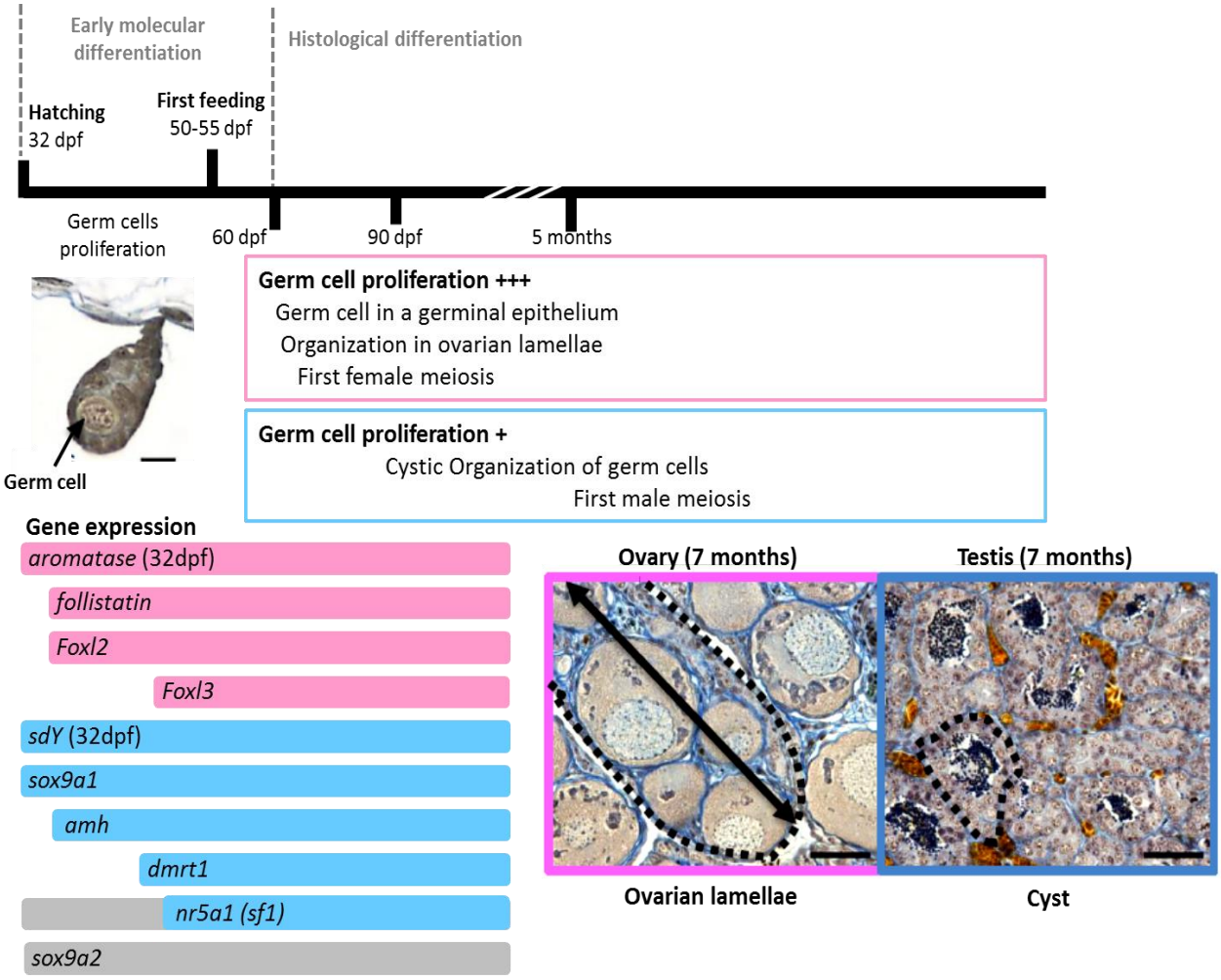


Figure 13. Kinetics of sex differentiation in rainbow trout. After hatching, the early molecular differentiation occurs followed by the histological differentiation. Gene expression and main events are shown. Female (pink) and male (blue).(Adaptated from Valdivia, K, 2012)

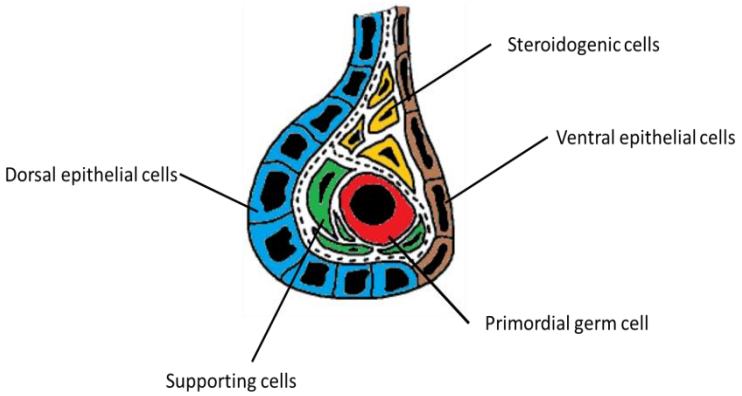


Figure 14. Schematic representation of a developing gonad. The dorsal (blue) and ventral (brown) epithelial cells, supporting cells (green) and steroidogenic cells are represented (yellow).

temperature variation between the different seasons and the photoperiod influence the development of the trout.

The production of rainbow trout is around 730,000 tons in 2010 (FAO source) and this species is first aquaculture fish production in France. Besides its importance for aquaculture, rainbow trout is also a model species to study sex differentiation. The embryonic development is slow favoring a better description of the underlying mechanism of sex differentiation. Genomic resources are available including many cDNA libraries and a whole genome sequence that facilitates the identification of new players implicated the sex differentiation (***Berthelot et al., 2014; Le Cam et al., 2012***). Another advantage is also that trout is bigger than many other fish models such as zebrafish and medaka facilitating biological exploration and measurements such as hormone blood levels. Moreover, in laboratories, the production of genetic male (XY) or female (XX) monosex population simplifies the study of sex differentiation (***Chevassus et al., 1979***). The induction of sex reversal by hormone treatment is also well established (***Guiguen et al., 1999a***). More and more genetic tools to knock out a gene such as zinc finger nucleases, CRISPR-Cas9 tools or to induce gene overexpression by transgenic methods are available. However, the long reproductive life cycle (3 years) and its big size is a major constraint to implement functional genomic as routine laboratory experiments (***Edvardsen et al., 2014; Yano et al., 2012; Yano et al., 2014***).

3.2 Sex differentiation in trout

In the wild spawning and hatching of rainbow trout begins during winter in cold water. The first signs of sex development start just after hatching around 30 days post-fertilization (30 dpf) at 10°c. At this stage, trout sex cannot be distinguished morphologically or histologically. The difference between male and female is only detectable by specific pattern of gene expression linked to the corresponding sex (molecular differentiation period) during almost one month. Then, the morphology of the gonads changes in female at the histological level (histological differentiation period) (***Figure 13***).

3.2.1 Formation of the gonad - early molecular differentiation

During the first steps of development between 28 dpf to 35 dpf, the undifferentiated gonad is formed by a continuum of somatic cells and by a few scattered primordial germ cells (PGC) identifiable by their large, pale, round nuclei with a single nucleolus (***Lebrun et al., 1982***) (***Figure 14***). The somatic cells are highly organized and composed of four different cell

lineages: supporting somatic cells surrounding the germ cell, steroidogenic somatic cells and two types of dorsal (cuboid) and ventral (flat) epithelial cells. The cellular fate of the supporting somatic cells will be the Sertoli cells in male or the granulosa cells in female. The stereoidogenic cell will be the future Leydig cells or the theca cells. A specific gene network is established in the undifferentiated gonad for both male and female. The expression male sex-determining gene, *sdY*, tilts the balance triggering the male development. As a consequence, in the absence of the male sex-determining gene, the female pathway is engaged. The expression of *sdY* starts around 30 dpf in dorsal epithelial cells and in an unknown type of cells surrounding the germ cells (**Yano et al., 2012**). Meanwhile, in male, *sox9a1*, *sox9a2* and *amh* expression begins in the somatic cells surrounding the germ cells. Later on, around 40-45 dpf *dmrt1* starts to be expressed. In female, around 30 dpf, *cyp19a1a* and *foxl2a* are expressed. A spatiotemporal coexpression of *cyp19a1a* and *foxl2* mRNA is found in the steroidogenic cells and strengthens the hypothesis of a positive loop of regulation (**Guiguen et al., 2010**). *foxl2* localize also in the supporting cells. During the early differentiating period, *nr5a1* is expressed in both sexes. Then, around 40-45 dpf, a clear sexually dimorphic expression appears such as the expression of *dmrt1*, *amh*, in male and *cyp19a1, foxl2*, *fst* in female. This period is followed by the expression of *foxl3* and most of the steroidogenic enzymes. During the period of molecular differentiation, the yolk (vitellus reserve) will regress and the young trout will feed itself (first feeding period, 55-60 dpf) and the size of the gonads increase. During the first feeding period, at 56 dpf, the female gonad acquires a larger size than the male, but no clear histological difference are observable (**Vizziano et al., 2007**)

3.2.2 Formation of the gonad - Histological differentiation

At 60-65 dpf, a clear histological dimorphism is observed in female whereas in male no visible morphological changes is perceptible. The germ cell proliferation will be the first histological sign of sexual dimorphism (**Lebrun et al., 1982**). Proliferative germ cells (oogonia in female) form a cluster in the dorsal part of the gonad embedded in a germinal epithelium delimited by a basal membrane. The oogonia then quickly enter into an asynchronous meiosis and are named ovocytes (**Lebrun et al., 1982**). Then, the germinal epithelium will invaginate to form ovarian lamellae. In male, the histological differentiation appears later around 90 dpf characterized a cystic organization of the spermatogonia surrounded by Sertoli cells. Several months later, at the puberty stage (1 or 2 years old) a synchronous meiosis is observed in the cyst providing the future male gametes.

Aims and outline of the thesis

Most of the vertebrates including fishes adopted two sexes (female and male) to maintain genetic variation and survival. Sex determination and sex differentiation mechanisms trigger the sex formation and sexual characteristics of an organism. Despite the common endpoint, it seems that there are endless ways to determine the sex. At the top of the hierarchical developmental cascade, a huge diversity of factors drives the sex determination pathway. In most of animal lineage, the triggers for the sex determining pathway is a gene or several genes and this type of sex determination is named genetic sex determination. Only few sex determining genes has been discovered yet that belongs to three gene families namely sox, dmrt and TGF-β. All of these sex determining genes originated from the sex developmental pathway. One exception to this rule, examining the rainbow trout, Yano et al. identified the master sex determining gene called *sdY* for "Sexually Dimorphic on the Y" which originated from an immune-related gene, irf9 (interferon regulatory factor 9). Moreover, *sdY* is a male sex determining gene detected in all the salmonids species examined so far (***Yano et al., 2012***). In the undifferentiated gonad, the sex determining gene toggles the balance to the male sex. To date, how a sex determining factor acts is not fully understood. Commonly, the underlying sex differentiation program leads to the activation of one sex and the repression of the opposite one. This program is triggered by a same set of genes. Nevertheless, depending on specific spatio-temporal gene expression, the process could be highly divergent among the species in both sexes.

In this context, the global aim of my thesis was to shed light on the biological function of SdY. In other words, understand how SdY can triggers sex differentiation in salmonids.

sdY gene was recently discovered and described in my host laboratory (Yann Guiguen's lab) (***Yano et al., 2012; Yano et al., 2014; Yano et al., 2013***). In a first part, the main objectives were to describe and to characterize the protein SdY at different levels. Using various and complementary strategies, I analyzed different features of SdY such as expression and localization. Moreover, from *sdY* gene description, it indicated that SdY could interact with a partner. Following the discovery of a putative binding partner, the great and exciting challenge was to elucidate the molecular mechanism underlying this interaction. In a second part, the aim was to investigate the features of a SdY mutation in a salmonid species named Chinook salmon (*Oncorhynchus tshawytscha*). For this purpose, using same strategies as in chapter 1, the analysis of the mutated version of SdY and in comparison with the wild-type version revealed some specific characteristics.

Materials and Methods

Cell Culture

Cell line	Supplier	Type
RTG2	-	Rainbow trout gonadal cell line
ASK-TOF	B. Collet (Aberdeen)	Altantic salmon kidney cell line
CHSE-TOF	B. Collet (Aberdeen)	Chinook embryonic cell line
HEK 293T	M. Gessler (Würzburg)	human embryonic kidney cells (transformed with large T antigen)
TM3	-	Mouse Leydig cell line

Human embryonic kidney (HEK 293) cell line (Gift from Prof. Gessler, University of Wuerzburg) and mouse Leydig cell line TM3 cells were cultured and maintained in DMEM medium (PAN Biotech), supplemented with 10% FCS (PAN Biotech) and 1% Penicillin-Streptomycin (PAN Biotech) at 37°C with 5% CO_2. Transfections for HEK 293 cells were performed by incubating cells with Polyethylenimine (PEI) (100 mg/mL PEI diluted 1:100 in 150mM NaCl) and respective plasmids (10 µg for 10 cm dishes, 2 µg for 6 wells plates) for 6-8 hours into fresh medium. Then, the medium was discarded and fresh medium was added transfection.

Rainbow trout gonadal (RTG2) cells were cultured and maintained in L15 medium, 20mM glutamine (PAN Biotech), supplemented with 10% FCS (PAN Biotech) and 1% Penicillin-Streptomycin (PAN Biotech), at 20°C, in atmosphere of air. Similar conditions have been used for CHSE-TOF and ASK-TOF (***Collet and Lester (2011a***); (***Collet and Lester, 2011b***). For transfection, RTG2 cells were detached by Trypsin-EDTA (Sigma-Aldrich, P0781) and pelleted by centrifugation at 1000g for 5 min, washed once with medium and once with Phosphate Buffered Saline (PBS). The pellet was drained and re-suspended in solution V (Amaxa Kit) at a density of 10^6 cells/ml. 2 µg of plasmid was added to the suspension. After mix, the suspension was transferred to a cuvette (Kit V, Amaxa). After optimization, the program D-23 was used to electroporate the cells. After transfection, cells were immediately transferred to 6 wells plates filled with medium. Experiments were performed 72h after transfection.

Cloning

Plasmids and primers used are listed in the tables. A fragment corresponding to the coding sequence of SdY was amplified from the psdy:sdy-pcry:cfp plasmid (***Yano et al., 2012***) and inserted into pCS2+. The PCR-amplified fragment was digested by EcoR1 and Xho1

restriction enzymes and inserted into HA:mcherry-pCS2+, (gift from Manfred Gessler), pemGFP-pCS2+,3xHA-pCS2+,pGEX-4T1(gift from Martin Eilers) expression vectors.
The mutated SdY I183N was obtained by PCR amplification with a reverse primer containing the base change. Both rainbow trout Foxl2a (tFoxl2a) and Foxl2b (tFoxl2b) and both medaka Foxl2 (OlaFoxl2) and Foxl3 (OlaFoxl3) were obtained from gDNA by PCR amplification and inserted in pCS2+, HA-mcherry-pCS2+, and 3xFLAG-pCS2+ between the EcoRI-XhoI restriction sites. The constructions 3xHA-pCS2+ and 3xFLAG-pCS2+ were obtained by cloning the sequence 3xHA or 3xFLAG flanked by HindIII restrictions sites. pemGFP:SdY-pCS2+ was obtained by inserting a PCR-amplified fragment corresponding to emGFP in-frame into the EcoRI site. 4xFoxl2-Dmrt1-Foxl2 was constructed (Eurogentec, Germany) as follows: 4 copies of the foxl2-dmrt1-foxl2 binding site were concatemerized and ligated into pminiTK-Firefly Luciferase (Promega) between the HindIII and BamHI sites. Rainbow trout pCMV-Sport6 plasmids containing cDNAs of tFoxd2 (1RT36L02_D_F01), ftoxd3 (1RT14E13_A_C07), tfoxl3 (1RT78J09_B_E05), tfoxn3 (1RT40M02_C_G01), tfoxn2 like (1RT120O15_A_H08), tfoxo3 (1RT148E11_A_C06 ou 1RT149F12_D_C06) were obtained from an expressed sequence tags plasmid arrayed collection. All plasmid were entirely sequenced to verify that they contain a full coding sequence and to check for the absence of any mutations.

Backbone	Insert	Promoter	Source
pCS2$^+$	SdY	CMV	this work
pCS2$^+$	FLAG-SdY	CMV	this work
pCS2$^+$	SdY-FLAG	CMV	this work
pCS2$^+$	emerald GFP-SdY (GFP-SdY)	CMV	this work
pCS2$^+$	SdY-2A-GFP	CMV	this work
3xHA-pCS2$^+$	3xHA-SdY	CMV	this work
3xFLAG-pCS2$^+$	3xFLAG-SdY	CMV	this work
HA-mCherry-pCS2$^+$	HA-mCherry-SdY	CMV	this work
pGEX-4T1	GST-SdY	CMV	this work
pCS2$^+$	SDY I183N	CMV	this work
pCS2$^+$	FLAG-SDY I183N	CMV	this work
3XFLAG-PCS2$^+$	3xFLAG-SDY I183N	CMV	this work
pCS2$^+$	meGFP-SDY I183N	CMV	this work
HA-mCherry-pCS2$^+$	HA-mCherry-SdY I183N	CMV	this work
pGEX-4T1	GST-SdY I183N	CMV	this work
pCS2$^+$	Foxl2a (rainbow trout)	CMV	this work
3XFLAG-pCS2$^+$	3xFLAG-Foxl2a	CMV	this work

HA-mCherry-pCS2$^+$	**HA-mCherry-Foxl2a**	**CMV**	**this work**
pCS2$^+$	Foxl2b (rainbow trout)	CMV	this work
3xFLAG-pCS2$^+$	3xFLAG-Foxl2b	CMV	this work
HA-mCherry-pCS2$^+$	HA-mCherry-Foxl2b	CMV	this work
pCS2$^+$	Foxl2 (medaka)	CMV	M.Adolfi
3xFLAG-pCS2$^+$	3XFLAG-Foxl2	CMV	this work
HA-mCherry-pCS2$^+$	HA-mCherry-Foxl2	CMV	this work
pcDNA3.1	meGFP-Irf9a	CMV	B. Collet (Aberdeen)
pVP16	VP16-FoxL2 (mouse)	CMV	E. Pailhoux (Jouy-en-josas)
pTK-LUC	4xFoxL2-Dmrt1-FoxL2-Luc	thymidine kinase	this work
pGL3 basic	3xGRAS-LUC	-	E. Pailhoux (Jouy-en-josas)
pCS2$^+$	3xFLAG	CMV	this work
pCS2$^+$	3xHA	CMV	this work
pCS2$^+$	HA-mCherry	CMV	M. Gessler (Wuerzburg)
pCS2$^+$	Foxl3 (medaka)	CMV	this work
pCMV Sport6	Foxd2 like (1RT36L02_D_F01)	CMV	this work
pCMV Sport6	Foxd3 (1RT14E13_A_C07)	CMV	this work
pCMV Sport6	Foxl3 (1RT78J09_B_E05)	CMV	this work
pCMV Sport6	Foxn2-like (1RT120O15_A_H08)	CMV	this work
pCMV Sport6	Foxn3 (1RT40M02_C_G01)	CMV	this work
pCMV Sport6	Foxo3 (1RT148E11_A_C06)	CMV	this work
pSG5	*FOXL2 (*goat*)*	SV40	E. Pailhoux (Jouy-en-josas)
psdy:SdY-pcry:CFP	-	-	Y. Guiguen (Rennes)

Primers for cloning		
Primers	**Sequence (5`→3`)**	**Purpose**
SdY-EcoR1-Fwd	ATAGAATTCACCATGCTCATAAAAAACTCCAGC	Cloning of SdY between EcoR1/Xho1
SdY-Xho1-Rev	AACTCGAGTCAGACTCCAGGAGAGACAGGG	
FLAG-SdY-EcoR1-Fwd	AAGAATTCACCATGGACTACAAAGACGATGACGACCTCATAAAGAACTCCAGC	Cloning of FLAG-SdY between EcoR1/Xho1

SdY-Xho1-Rev	**AAGCGGCCGCTCAGACTCCA GGAGAGACAGGG**	
meGFP-EcoR1-Fwd	ATAGAATTCACCATGGTGAGC AAGGGCGAGGAG	Cloning of meGFP between EcoR1/EcoR1
meGFP-EcoR1-Rev	ATAGAATTCCTTGTACAGCTCG TCCATG	
SdY-2A-GFP-EcoRI-Fwd	TTCTCGAGTTATTGATATCAGG GCCGGGATTCTCCTCCACGTC ACCGCATGTTAGACTTCCTCTG CCCTCACCGCTACCGACTCCA GGAGAGACAGGG	Cloning of SdY between EcoR1/EcoR5
SdY-2A-GFP-EcoR5-Rev	AACTCGAGTCAGACTCCAGGA GAGACAGGG	
SdY I183N-EcoR1-Fwd	ATAGAATTCACCATGCTCATAA A AAACTCCAGC	Cloning of SdY I183N between EcoR1/Xho1
SdY I183N-Xho1-Rev	AACTCGAGTCAGACTCCAGGA GAGACAGGG	
FLAG-SdY I183N-Xcm1-Fwd	ATGGCTCCCAACCTCAATGTG GGTTCAGCCTATGGTTTGGAC AAGACTCATCACTCAGTGCAC CAAACTTTATATCG	Mutation and cloning of SdY I183N between Xcm1/Xho1
SdY I183N-Xho1-Rev	AACTCGAGTCAGACTCCAGGA GAGACAGGG	
Foxl2a-EcoR1-Fwd	ATAGAATTCACCATGATGGACA CTTACCAAAACC	Cloning of Foxl2a between EcoR1/Xho1
Foxl2a-Xho1-Rev	AACTCGAGTTATATATCAATCC GCGC	
Foxl2b-EcoR1-Fwd	ATAGAATTCACCATGATGGACA CTTACCAAAACC	Cloning of Foxl2b between EcoR1/Xho1
Foxl2b-Xho1-Rev	AACTCGAGTTATATATCAATCC GCGC	
Foxl2-EcoR1-Fwd	ATGAATTCACCATGATGGCCA CTTACCAAA	Cloning of Foxl2 between EcoR1/Xho1
Foxl2-Xho1-Rev	AACTCGAGTCAAATATCA ATC CTCGTGTGCAAAGCG G	
HA-Hind3-Fwd	ATTAAGCTTGCCACCATGGACT ACAAAGACCATGACGGTGA	Cloning of HA between Hind3-EcoR1
HA-EcoR1-Rev	ATAGAATTCCTTGTCATCGTCA TCCTTGTAG	
FLAG-Hind3-Fwd	ATGAATTCACCATGATGGCCA CTTACCAAA	Cloning of FLAG between Hind3-EcoR1

The following plasmids were obtained by copy-paste method (insert cut from the original plasmid and paste in the wanted plasmid). For HA-SdY, FLAG-SdY, HA-mCherry-SdY, GST-SdY, the original plasmid was pCS2^{+}-SdY. The insert was cut between EcoR1 and Xho1. For 3xFLAG-Foxl2a, HA-mCherry-Foxl2a, the original plasmid was pCS2^{+}-Foxl2a. For 3xFLAG-Foxl2b, HA-mCherry-Foxl2b, the original plasmid was pCS2^{+}-Foxl2b. For 3xFLAG-Foxl2, HA-mCherry-Foxl2, the original plasmid was pCS2^{+}-Foxl2.

Quantitative real-time PCR

The expression of *sdY, foxl2a, foxl2b, cyp19a1a* was measured by qPCR using the Step One Plus real-time PCR system (Applied Biosystems/ Life Technologies, Ltd.), as previously described (Baron et al., 2005c). The early gonadal differentiation expression profiles were measured from 33 to 125 days post fertilization (dpf) on pools of male and female gonads (***Vizziano et al., 2007***). All expression levels were normalized using *18S* primers.

Primers for Real-time-PCR	
Primers	**Sequence**
qsdY_Fwd	GTGGTTTTAAGCTCTAGGGAGGA
qsdY_Rev	GAGTGATGAGTCTTGTCCAAAC
qfoxl2a-Fwd	TGTGCTGGATTTGTTTTTTGTT
qfoxl2a-Rev	GTGTCGTGGACCATCAGGGCCA
qcyp19a1-Fwd	CTCTCCTCTCATACCTCAGGTT
qcyp19a1-Rev	AGAGGAACTGCTGAGTATGAAT
q18S-Fwd	ATCCGGCGGCGTTATTCCCATGA
q18S-Rev	CCCCSGACATCTAAGGGCATC

Western Blot

Cells were lysed in HEPES-based lysis buffer (20 mM HEPES (pH 7.8), 500 mM NaCl, 5 mM MgCl2, 5 mM KCl, 0.1% deoxycholate, 0.5% Nonidet-P40, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 200 mM Na_3VO_4, 1 mM phenylmethanesulphonylfluoride and 100 mM NaF) [illegible] 3 h. Cells debris were pelleted during 15 min at 16000 g. Cell lystate concentration was measured via Bradford assay (Cary 50 Spectrophotometer, Varian). The totality of the protein lysate (30–50 µg) was resolved by SDS-PAGE on 12% Tris-glycine gels followed by transfer to nitrocellulose membranes. Unspecific binding was blocked with 5% BSA in TBST (10 mM Tris pH 7.9; 150 mM NaCl; 0.1% Tween) for 1h at room temperature. Incubation with primary antibodies was performed overnight at 4°C. After three washes with TBST, HRP conjugated antibodies were incubated with the blocking solution during 1h. Following the washes, membranes were incubated with the SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) during 1 min. The signal from the membrane was detected using the Photo Image Station 4000MM (Kodak). Experiments were always repeated and most of the time, protein blot images are representative for 2–3 independent experiments. Quantitative analysis was performed with ImageJ 1.48v software (www.imagej.nih.gov).

Antibodies

Antibodies were purchased from different companies, HA (Sigma-Aldrich), FLAG (Sigma-Aldrich), β-actin (Santa Cruz Biotechnology), α-tubulin (Sigma-Aldrich), mouse IgG (Sigma-Aldrich).The rabbit anti-Foxl2 polyclonal antibody is directed against the N-terminal 15-amino acid peptide as described previously (Caulier et al., 2015a). Two mouse anti-SdY monoclonal antibodies were generated by Proteogenix, France.

Cycloheximide treatment

HEK 293 T cells were transfected either with 3xHA-SdY or with 3xHA-SdY I183N or in combination with 3xFLAG-tFoxl2b expression vector. 48 h post-transfection, cells were treated with 50 µM cycloheximide (protein synthesis inhibitor (calbiochem) or ethanol (vehicle control) during 4 h or 8 h. Untreated cells (0 hour) and treated cells were harvested and subjected to cell lysis followed by SDS-PAGE and Western Blot as described above.

MG132 treatment

HEK 293 T cells were transfected either with 3xHA-SdY or with 3xHA-SdY I183N or in combination with 3xFLAG-tFoxl2b expression vector. 48 hours post-transfection, cells were

treated with 20 µM MG132 (proteasome inhibitor) (Merck) or DMSO (vehicle control) during 8 h. Untreated cells (0 hours) and treated cells were harvested and subjected to cell lysis followed by SDS-PAGE and western Blot as described above.

Co-Immunoprecipitation

HEK-293T cells were transfected with 3xHA-SdY and 3xFLAG-tFoxl2b (or 3xFLAG-tFoxl2a or 3xFLAG-OlaFoxl2) constructs to be assessed for ability to coimmunoprecipitate. After 48 h, cells were scraped and resuspended in 50 µl lysis buffer (20 mM HEPES (pH 7.8), 500 mM NaCl, 5 mM MgCl2, 5 mM KCl, 0.1% deoxycholate, 0.5% Nonidet-P40, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 200 mM Na_3VO_4, 1 mM phenylmethanesulphonylfluoride and 100 mM NaF). Cells were incubated in lysis buffer for 30 min at 4 °C and then cleared by high-speed centrifugation during 20 min. After Bradford protein concentration measurement, HNTG Buffer (20 mM HEPES pH 7.5; 150 mM NaCl; 10% glycerol; 0.1% Triton X-100) was added (1:1) to 250 µg of the whole cell lysate. After pre-clearing with IgG antibodies for 1 h at 4 °C, whole-cell lysates were used for immunoprecipitation with the corresponding antibodies. 1 µg of anti-FLAG, anti-HA or IgG antibody was added to 500 µL cell lysate or 5 µg of anti-SdY or anti-FoxL2, which were incubated at 4 °C overnight. After addition of washed protein G agarose beads (Pierce, 20398), incubation in HNTG buffer was continued for another 2 h. Immunoprecipitates were washed (five rounds of and centrifugation (1000g), supernatant discarded, HNTG lysis buffer added) and eluted with SDS-PAGE loading buffer by boiling for 10 min. Coimmunoprecipitation was detected by standard Western blot analysis procedure.

GST pull down

GST or GST-fusion proteins were induced with 1 mM IPTG in BL21 E. coli at 15°C, overnight. Bacteria were resuspended in cold STE Buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, and 1 mM EDTA), treated during 1h with 1 mg/mL lysozyme and 1.5% Sarkosyl. After sonication (4-6 cycles, 45s pulses, maximum amplification) and addition of 1% Triton X-100, insoluble material was pelleted by centrifugation (25000g, 30 min). Then, GST and recombinant proteins were bound to glutathione Sepharose-4B beads (GE Healthcare) overnight. After extensive washing, beads were eluted with 20 mM glutathione in TBS Buffer (50 mM Tris, 200 mM NaCl, 5 mM DTT, pH 8.5). Following the elution step, proteins were subjected to dialysis and protein concentration using an Amicon Ultra-15 Centrifugal Filter Unit (MW cut off 30 kDa). The purity of each protein preparation was confirmed by SDS-PAGE/Coomassie staining and quantified by the Bradford method.

For the pull-down assay, 20 µg of the purified GST fusion protein was immobilized on the glutathione-Sepharose 4B beads (GE healthcare Bio-Sciences AB). GST-SdY or GST-SdY I183N proteins linked to the beads were incubated with 3xFLAG-tFoxL2b-transfected HEK 293T cell lysates in TBS buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100) supplemented with protease inhibitor) (10 mg/ml aprotinin, 10 mg/ml leupeptin, 200 mM Na_3VO_4, 1 mM phenylmethanesulphonylfluoride and 100 mM NaF cocktail) for 2 h at 4 °C. After extensive washing, bound proteins were eluted in SDS loading buffer and analyzed by Western-blotting. GST protein was used as the negative control under the same conditions. These samples were analyzed by Western blot using anti-FLAG, anti-GST and anti-α-tubulin antibodies.

Yeast two-hybrid screen

Yeast two-hybrid screening was performed by Hybrigenics Services, SAS, Paris, France (http://www.hybrigenics-services.com). The coding sequence for SdY protein (aa 1-215) (GenBank accession number GI:392583258) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (N-LexA-SdY-C) and into pB66 as a C-terminal fusion to Gal4 DNA-binding domain (N-Gal4-SdY-C). The constructs were checked by sequencing and used as a bait to screen a random-primed *Onchorynchus mykiss* immature male gonad (sampled 75 days post fertilization) cDNA library. The prey fragments of 202 positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. Each fragments corresponding to interacting proteins were identified using GenBank database (NCBI). The common sequence shared by all prey fragments of the same protein defines the Selected Interacting Domain (SID) containing all the structural determinants required for a given interaction to occur. A confidence score (PBS, Predicted Biological Score) that outlines the reliability of the interaction is given to each interaction as previously described (***Formstecher et al., 2005***). PBS scores were divided into four categories, from A (highest confidence) to D (lowest confidence).

Protein structure prediction

The predicted structural model of SdY was produced on the basis of the known human IRF-3 domain. Different instances of the IRF3 domain were used (Protein database bank (PDB) code 3dsh, 3a77, 1zoq). The three dimensional views of SdY, consurf and electrostatic potential analysis were made with the software PyMOL Molecular Graphics System, Version 1.7.4 Schrödinger, LLC. Other prediction software used for analysis predict protein (https://www.predictprotein.org/) and raptorX (***Kallberg et al., 2012***)

Immunofluorescence

HEK 293T cells were seeded in a 6-well plate containing coverslips. After mEGFP-SdY and mCherry-tFoxl2b cotransfection for 48 h, cells were fixed in 4% fresh paraformaldehyde (PFA) for 15 min, extensively washed, and permeabilized with 0.1% Triton X-100 in PB
10 min. 100 mM glycine was added to quench the autofluorescence of the PFA. Then cells were blocked with 1% BSA during 20 min. Primary antibody was incubated overnight at 4°C. After extensive washes with PBS, cells were incubated with Alexa 488 or Alexa 594 conjugated secondary antibodies in 1% BSA for 1 h, followed by Hoechst 33342 (Invitrogen) staining for 5 min (1 µg/mL final concentration). Cells were mounted using Mowiol 4-88 (Roth).
Confocal images were acquired using a Nikon Eclipse C1 laser-scanning microscope (Nikon), fitted with a 60x Nikon objective (PL APO, 1.4 NA), and Nikon image software. Images were collected at 1024x1024 pixel resolution. The stained cells were optically sectioned in the z axis. The step size in the z axis varied from 0.2 to 0.25 mm to obtain 50 slices per imaged file. All experiments were independently repeated several times.

Colocalization

The Nikon NIS-Elements imaging analysis software was used for the colocalization analyses. In this analysis, confocal images of double-stained sections were first subjected to background correction. Mander's overlap coefficients and Pearson's correlation were calculated and used to obtain the co-localization values as percentages of SdY overlapping with tFoxl2b for a minimum of 10 cells. The Pearson correlation coefficient (PCC) and the Mander's overlap coefficient (MOC) were used to quantify the degree of colocalization between fluorophores. The two coefficients are mathematically similar, differing in the use of either the absolute intensities (MOC) or of the deviation from the mean (PCC) (***Adler and Parmryd, 2010***). The Pearson's coefficient values range from 1.0, an indication of complete colocalization of two structures, over 0, which indicates no significant correlation, to –1, which indicates complete separation of two signals (Zinchuk et al., 2007). The degree of colocalization from the Pearson's coefficient values was categorized as very strong (0.85–1.0), strong (0.49–0.84), moderate (0.1–0.48), weak (–0.26 to 0.09), and very weak (–1 to –0.27) based on a previously published description (***Zinchuk et al., 2013***). MOC values range from 0, which indicates no correlation and 1, which indicates positive correlation.

Luciferase assay

HEK293 and TM3 cells were transfected using PEI or Lipofectamine 3000 (Invitrogen) respectively with the following plasmids: 1) 0.3 µg of 3xGRAS sequence (kindly provided by E. Pailhoux lab) cloned into pGL3 basic or 4xFoxl2-Dmrt1-Foxl2 binding site of trout *cyp19a1* promoter cloned into pminiTK-*Firefly* luciferase reporter plasmids; 2) 0.05 µg-0.5 µg of pCS2+ expression plasmid (Invitrogen), containing the CDS encoding SdY or Foxl2 (rainbow trout or medaka); and 3) pRL-TK (Promega Corp.), 100 ng/well, *Renilla* luciferase employed as an internal control. 0.6 µg of pEGFP was used as a transfection control. Each experiment was performed with 1.0 µg final. The ajustement was made with empty plasmids (pCS2+, pVP16, pGL3) according to the experiments. The day before transfection, cells were seeded into 12-well plates. To optimize the transfection, HEK293 cells and TM3 cells were 80% and 65% confluent, respectively. The transfection solution was made of 100 µl of Opti-MEM I without serum containing precomplexed DNA, and 2 µl of Lipofectamine reagent. Cells were washed in PBS 48 h after transfection and lysed in 100 µl luciferase lysis buffer. Firefly luciferase and *Renilla* luciferase readings were obtained using the Dual-Luciferase Reporter Assay System (Promega) and LUMAT LB 9501 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

Genomic DNA extraction

Extraction of trout genomic DNA was previously described (***Collet and Secombes, 2001***). Briefly, genomic DNA was extracted from liver tissue with 25 mL of lysis buffer (10 mM Tris/HCl 100 mm EDTA, 0.5% SDS, and 20 µg/mL RNase A). Proteinase K was added to 150 mg/mL and the sample was incubated at 56 °C overnight. A double extraction phenol-chloroform (1:1) followed by a choloroform-isoamyl alcohol (24:1) extraction was executed. DNA precipitation was performed with Isopropanol (1:1). The precipitate was pellet by centrifugation at 16000 g and washed twice in 70% ethanol , dried at room temperature and dissolved in 2 mL of distilled water.

Fish maintenance and breeding

Medaka were taken from closed breeding stocks of the Carbio (Carolina Biological Supplies) strain (WLC) and kept under standard conditions. Medaka embryos were staged according to Iwamatsu (Iwamatsu, 2004)

RNA injections

Capped RNA (*GFP-sdY*; *mCherry-tFoxL2b*, *Olafoxl2*) for injections was transcribed from linearized pCS2+ vector using the SP6/T3/T7 m MESSAGE mMACHINE Kit (Ambion). One nL was injected into the cytoplasm of one-cell stage Medaka embryos as described (***Koster et al., 1997***).

Statistical analysis

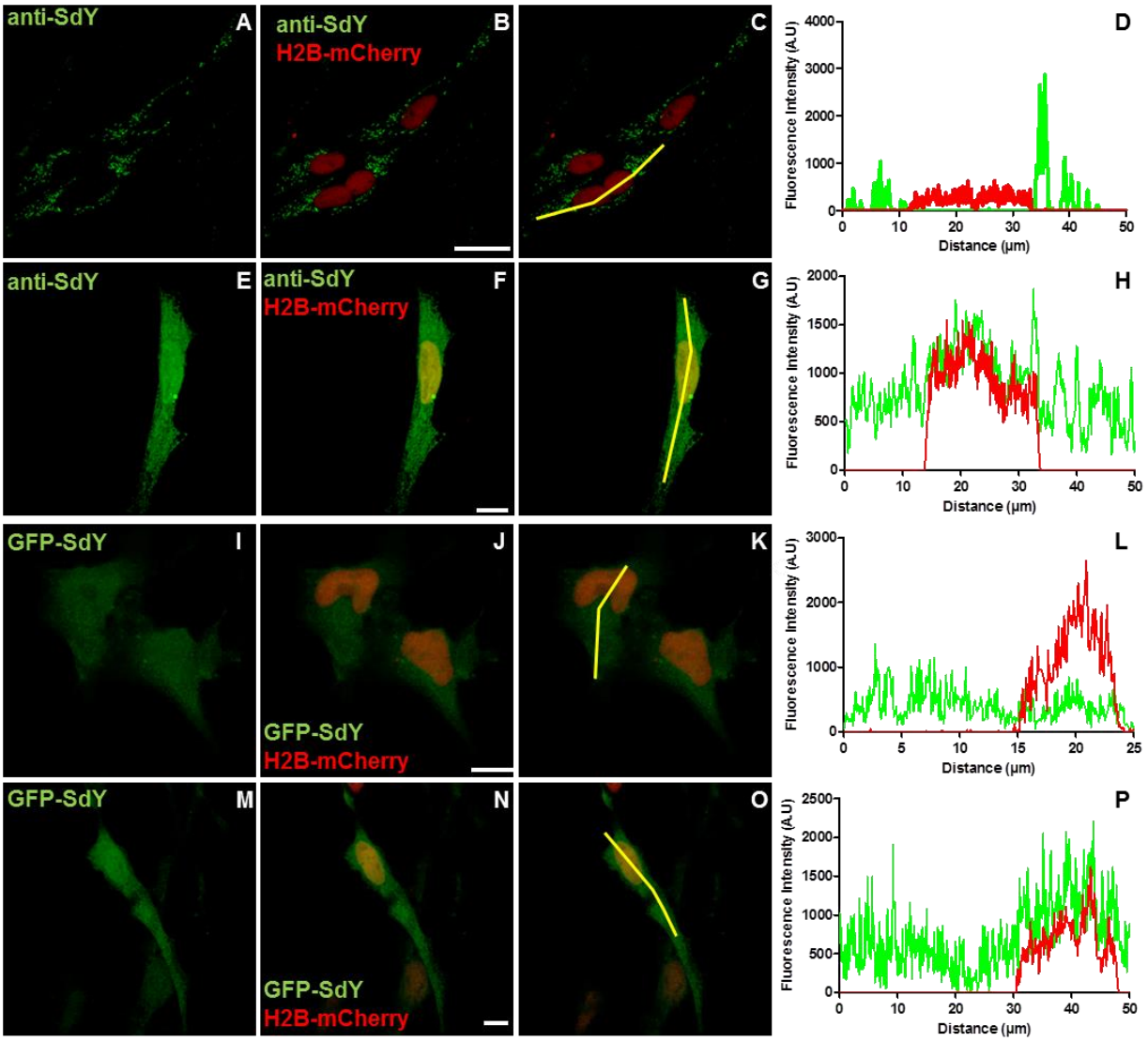
Statistical differences were analysed using a paired Student's t-test. Significant differences are symbolized in figures by asterisks if $p<0.001$ (***), $p<0.05$ (**), $p<0.01$ (*) or n.s. if not significant.

In situ hybridization

In situ hybrizidation was performed as previously described (Yano et al., 2012). RNA probes were produced from PCR product obtained by amplification of *tfoxl2*. 10ng of the PCR product was used as template for digoxygenin-labeled RNA probe synthesis using digoxygenin 11-UTP (Roche Diagnostics Corp) and T3 or T7 RNA polymerase (Promega, Charbonnières, France) following standard protocols. Whole mount *in situ* hybridization was carried out using an In situ Pro, Intavis AG robotic station. Male and female embryos were fixed overnight in 4% paraformaldehyde at 4°C, and dehydrated in 100% methanol and stored at -20°C. Before In situ hybrizidation they were rehydrated, permeabilised by proteinase K treatment (25 µg/ml, 30 min, at room temperature), and postfixed (4% paraformaldehyde and glutaraldehyde 0.2 %, during 20 min). Pre-hybridization and hybridization medium contained 50% formamide, 5XSSC, 0.1 % tween 20, 0.005 % heparine, 0.1mg/ml tRNA. Hybridization was carried out at 65°C for 16 hours. After posthybridization washes, embryos were incubated in blocking buffer (PBS / Triton 0.1 % / Tween 20 0.2%, containing 2% serum) for 2 hours before addition of the alkaline phosphatase coupled anti-digoxygenin antibody (1:2000, Roche Diagnostics Corp) for 6 hours. After washes, color reaction was performed in the presence of NBT/BCIP (Roche). Briefly, dehydration and paraffin infiltration were performed in a Citadel 1000 tissue processor (Shandon, Pittsburgh, PA). Dehydrated tissues were embedded in plastic molds in paraffin using a HistoEmbedder (TBS88; Medite, Burgdorf, Germany). Each embedded samples was sectioned 5µm thick on a MICRO HM355 (Thermo Fisher Scientific, Walldorf, Germany).

Results

In the family Salmonidae, a novel master sex determining gene has been discovered. Contrary to other sex determining genes, sexually dimorphic on the Y (*sdY*) has the particularity that it derives from a non-sex related gene. *sdY* arose by a local gene duplication event of the immune related gene, interferon regulatory factor 9 (*irf9)*. This latter is a transcription regulatory factor implicated in type I interferon signaling. Despite the lack of the DNA binding domain, SdY shares homologies with the carboxy-terminal part of Irf9. From the previous information, SdY could exert its function related to interferon signaling pathway or acquire a totally new function. However, the question how does SdY trigger testicular differentiation, is totally unanswered? This chapter aims to characterize the features of SdY.



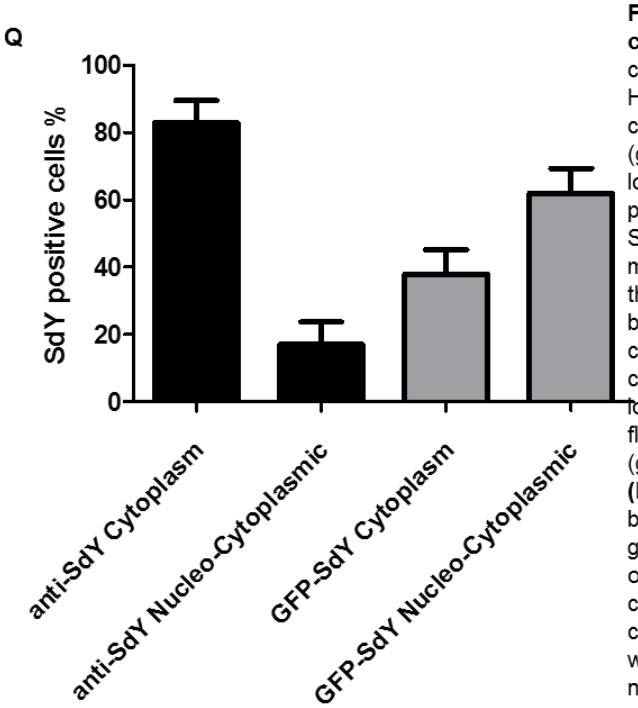


Figure 1. SdY localizes predominantly into the cytoplasm of RTG2 cells. (A-H) Rainbow trout gonadal cells (RTG2) were transiently co-transfected with SdY and Histone H2B-mCherry (nucleus marker) expression constructs. SdY was detected with anti-SdY antibody (green) displaying a cytoplasmic and perinuclear localization **(A,B)** confirmed by the fluorescence intensity profile **(D)** established from the yellow drawn line in **(C)**. SdY can be also distributed in a nucleo-cytoplasmic manner **(C,D)**. Analysis of the fluorescence intensity from the drawed yellow line in E shows the same pattern between SdY (green) and H2B-mcherry (red). **(I-Q)** SdY coupled to a green fluorescent protein GFP was transiently cotransfected with H2B-mcherry. **(I-M)** Cytoplasmic localization of SdY in RTG2 cells **(I)**, confirmed by the fluorescence intensity analysis **(L)** from both GFP-SdY (green) and H2B-mcherry (red) signal along the yellow line **(K)**. **(M-P)** Nucleo-cytoplasmic localization of SdY has been also observed revealed by the fluorescence intensity graph **(P)** according the yellow line **(O)**. **(Q)** Quantification of observed pattern. A cytoplasmic localization was counted when the majority of signal comes from the cytoplasm. A nucleo-cytoplasmic localization was counted when a strong signal was detected in both cytoplasm and nucleus. (Scale Bar, 10 µm.)

SdY localizes predominantly in the cytoplasm

To explore SdY function, I first examined its subcellular localization. Rainbow trout gonadal cell line (RTG2) was cotransfected with a plasmid coding for SdY and a histone H2B-mcherry plasmid to label the nucleus in red. SdY was detected with a monoclonal antibody (***Figure 1A-H***). Immunofluorescence analysis revealed that SdY (green) localizes predominantly in the cytoplasm forming punctuate structures around the nucleus (red) (***Figure 1A-C***). To confirm this pattern, the fluorescence intensity was measured along the line drawn in light blue in ***Figure 1C***. The fluorescence intensity profile clearly demonstrates the presence of SdY in the cytoplasm surrounding the nucleus (***Figure 1D***). This representative expression pattern was detected for more than 80% of SdY positive cells (***Figure 1Q***). Moreover, a nucleo-cytoplasmic localization was observed and image superposition confirmed the presence of SdY in the nucleus (***Figure 1 E-G***). Punctuate structures are also present in the cytoplasm. However this feature is not seen in the nucleus. This result indicated that SdY can be present in the nucleus as well. In the same line as above fluorescence intensity was measured for both SdY and H2B-mcherry and reported in the ***Figure 1H***. The profile showed a nucleo-cytoplasmic localization of SdY seen for less than 20% SdY positive cells (***Figure 1Q***). To independently confirm both localizations, a genetically modified green fluorescent protein mEmerald-GFP (meGFP) was fused with SdY in the N-terminal part. mEmerald-GFP is more suitable to use than the well-known GFP because meGFP is characterized by a monomeric form, a brighter signal, and thus can avoid mislocalization. Using co-transfected RTG2 cells with GFP-SdY and H2B-mcherry expression plasmid, I mainly detected a signal in the cytoplasm and only a low signal in the nucleus (***Figure I-J***). Measuring the fluorescence intensity illustrated in ***Figure 1K***, the profile revealed that SdY is found mostly in the cytoplasm. This phenotype was observed in one-third of all the analyzed cells (***Figure 1Q***). Additionally, a nucleo-cytoplamic localization was also visualized (***Figure 1M-N***). The quantification of fluorescence intensity for both channels demonstrated the nucleo-cytoplasmic localization of GFP-SDY (***Figure 1O-P***) observed in more than 65 % of the positive cells (***Figure 1Q***). It is worth to note that GFP-SdY forms also punctuate structure and recapitulates the pattern found with the antibody. To validate the localization data, two other salmonids cell lines (Atlantic Salmon Kidney (ASK) and CHSE (CHinook Salmon Embryo cells)), were each cotransfected with SdY and Histone H2B-mcherry expression plasmid. Using immunofluorescence, SdY protein was detected with an antibody. In both cell lines, SdY (green) was visualized in the cytoplasm surrounding the nucleus (red) (***Figure 2 A-B and E-F***). The fluorescence intensity was measured in both cases (***Figure 2C and 2G***). A similar profile was observed in both ASK and CHSE cell lines revealing the presence of

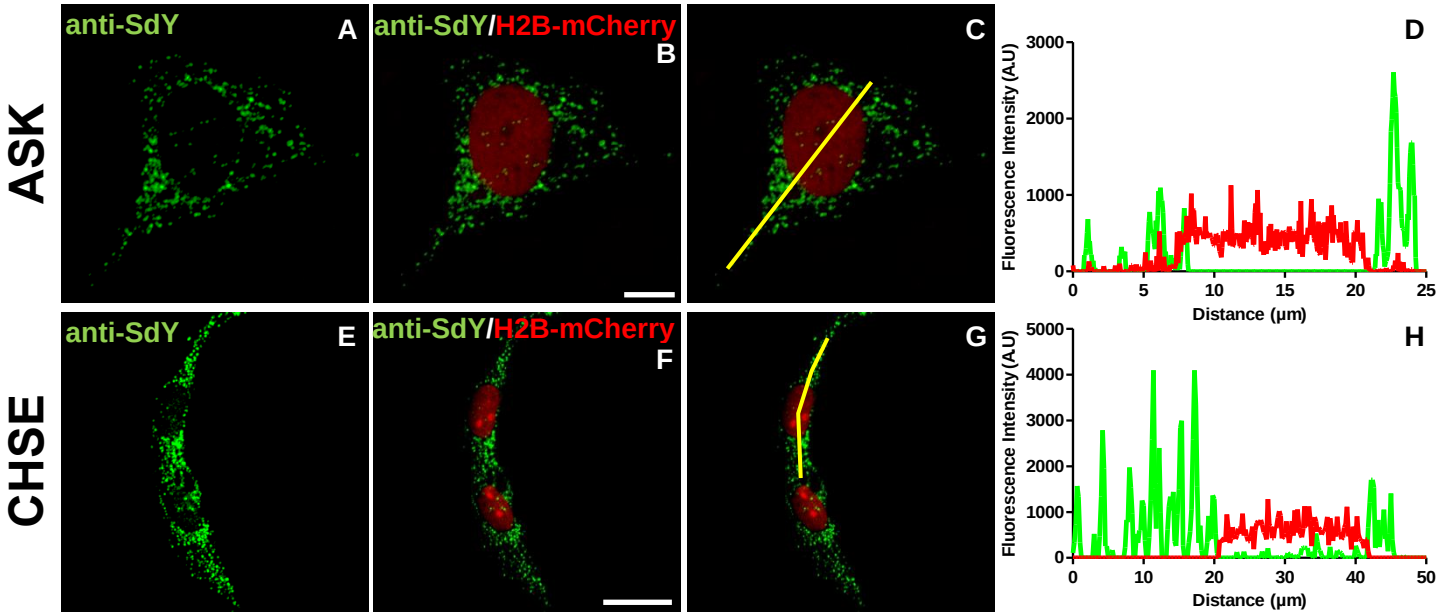


Figure 2. SdY localizes predominantly in the cytoplasm in both ASK and CHSE cell lines. (A-H) Atlantic Salmon Kidney cell line (ASK) **(A-D)** and CHinook Salmon Embryonic cell line (CHSE) **(E-H)** were transiently co-transfected with both SdY and Histone H2B-mCherry (nucleus marker, in red) expression constructs. SdY was detected with an anti-SdY antibody (green). SdY displays a cytoplasmic and perinuclear localization in both ASK **(A, B)** and CHSE **(C,D)** cell lines. The yellow line in the image **C** and **G** indicates the area selected for fluorescence intensity profile analysis, which are shown in figure **D** and **H**. The x axis shows the position along the line, and the y axis shows the fluorescence intensity. Both figures revealed that SdY localizes predominantly in the cytoplasm in the form of punctuate. (Scale Bar, 10 µm.)

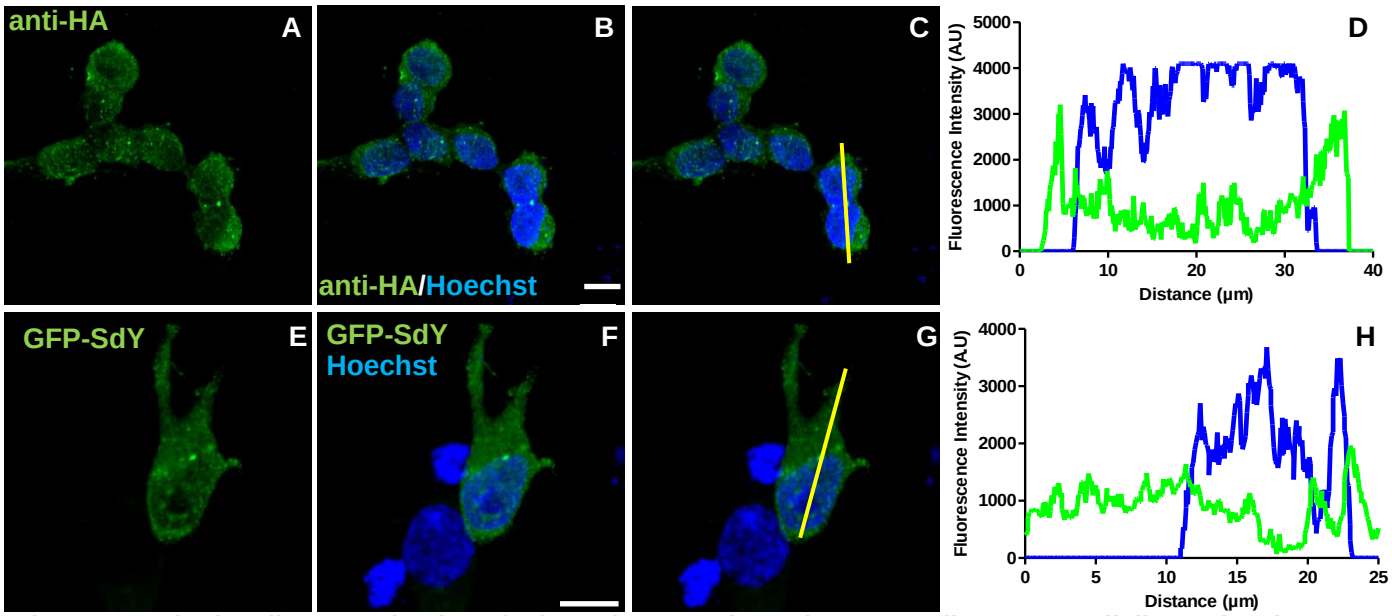


Figure 3. SdY localizes predominantly into the cytoplasm in mammalian HEK cell lines. (A-H) Human Embryonic Kidney cell line (HEK) was transiently co-transfected with either 3xHA-SdY **(A-D)** or with GFP-SdY **(E-H)**, stained with Hoechst (Blue, showing the nucleus). **(A-D)** SdY was detected with an anti-HA antibody (green). SdY is distributed mainly into the cytoplasm characterized by a perinuclear localization. A very small fraction is contained into the nucleus confirmed by the fluorescence intensities **(A-D)**. The yellow line in the image **C** and **G** indicates the area selected for fluorescence intensity profile analysis, which are shown in figure **D** and **H**. The x axis shows the position along the line, and the y axis shows the fluorescence intensity for SdY (green) and Hoechst (blue). (Scale Bar, 10 µm.)

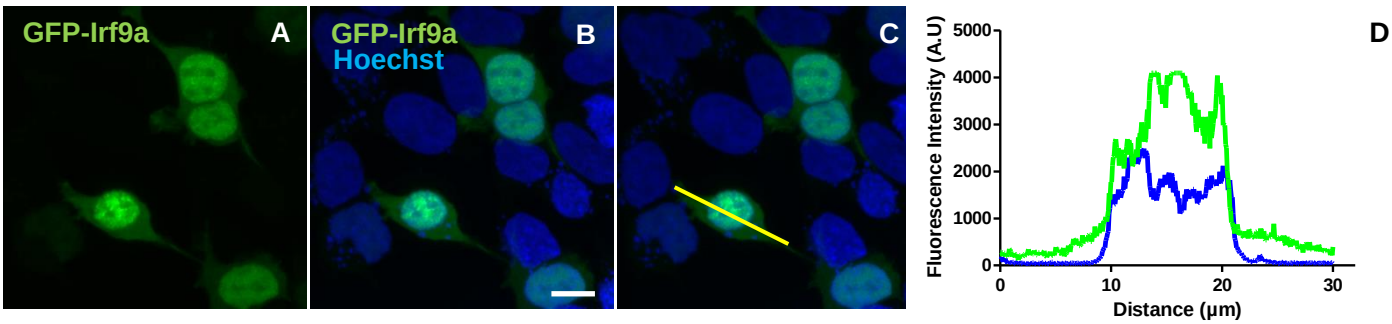


Figure 4. Irf9 localizes predominantly in the nucleus in mammalian HEK cell lines. (A-C) Human Embryonic Kidney cell line (HEK) was transiently transfected with either GFP-Irf9a **(E-H)**, stained with Hoechst (Blue, showing the nucleus). **(A-D)** SdY was detected with an anti-HA antibody (green). Irf9a is mainly distributed in nucleus confirmed by the fluorescence intensities **(D)**. The yellow line in the image **C** the area selected for fluorescence intensity profile analysis, which are shown in figure **D**. The x axis shows the position along the line, and the y axis shows the fluorescence intensity for Irf9a (green) and Hoechst (blue). (Scale Bar, 10 µm.)

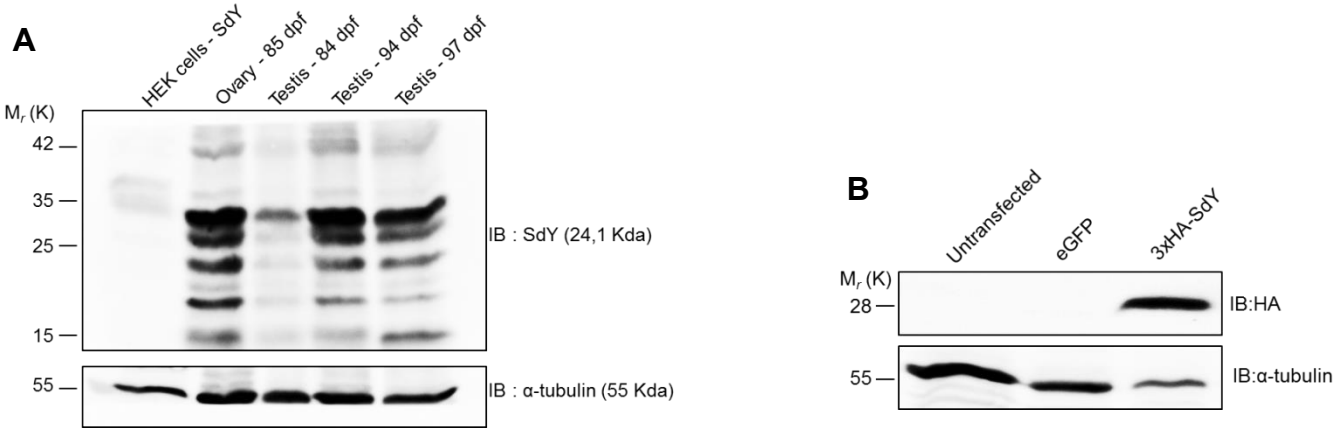


Figure 5. Undetectable SdY protein with anti-SdY antibodies. A. Western blot analysis of transiently transfected HEK cells with an expression plasmid encoding for SdY (lane1, positive control)) and gonads (ovary (85dpf), testis (84 dpf, 94 dpf, 97 dpf) (lane 2 to 5). 50 µg of the whole cell lysate are loaded followed by immunoblotting (IB). Despite a major band detected about 30 kDa, this band is also found in the ovary lane (negative contol). Two monoclonal antibodies against SdY have been tested and the result shown is representative of both antibodies. **B.** Detection of SdY with an anti-HA antibody. Cells (HEK293T) were transiently transfected with an expression vector encoding for eGFP (lane 2) or 3xHA-SdY (lane3). After whole cell lysis and immunoblotting against HA epitope, a single band corresponding to SdY appeared at 28 Kda and solely this band in 3xHA-SdY lane. α–tubulin was used as a loading control. Results shown are representative at least of three independent experiments.

SdY (green) in the cytoplasm and H2B-mcherry (red) in the nucleus (***Figure 2D and 2H***). Both cell lines showed a cytoplasmic localization of SdY. No nucleo-cytoplasmic and nuclear localization was observed. To further analyze the localization of SdY a human em kidney cell line (HEK) was transfected with HA tagged SdY. Immunofluorescence aga tag shows the cytoplasmic localization of SdY (***Figure 3A***). Nuclei are stained with Hoechst (blue) (***Figure 3B***). The fluorescence intensity profile established from the yellow line (***Figure 3C***) demonstrates the cytoplasmic localization (***Figure 3D***). To validate this pattern, GFP-SdY construct was transfected in HEK cells followed by nucleus staining. GFP-SdY localizes in the cytoplasm in form of dots (***Figure 3 E-F***). Both GFP-SdY and Hoechst intensity were measured and depicted in ***Figure 3H***. The intensity profile shows a cytoplasmic localization of SdY. No nucleo-cytoplasmic and nuclear localization were observed. To have a clear picture about SdY and Irf9a localization, GFP-Irf9a from Atlantic salmon was transfected in HEK cells and the nucleus stained with Hoechst. GFP-Irf9a was mainly visualized in the nucleus (***Figure 4A***) confirmed by the fluorescence intensity profile (***Figure 4D***) established from the yellow line in ***Figure 4C***. Altogether, these results suggest that SdY localizes predominantly in the cytoplasm around the nucleus and in few cases is able to shuttle between the cytoplasm and the nucleus. From a technical point of view, it is worth to note that one of the two SdY antibodies recapitulated the same pattern of expression seen for GFP-SdY or the antibody against HA suggesting that this antibody recognized the native form of SdY.

SdY does not show any post-translational modifications

To further investigate the characteristics of SdY, I wanted to detect endogenous SdY *in vitro* and *in vivo* by immunoblotting. Using mammalian HEK cells overexpressing SdY coding part and 85, 94 and 97 days post fertilization (dpf) testes when SdY reached its maximal expression; several bands were detected by the two different monoclonal antibodies against SdY. A representative immunoblot is shown in ***Figure 5A***. 85 dpf ovaries were used as a negative control and α–tubulin as a loading control. The size of SdY is estimated to be 24.1 kDa according to the aminoacid sequence. Same bands are observed in ovaries at the same size for both overexpressed cells and testes. This result suggested that both antibodies do not detect specifically the presence of SdY. To tackle this technical problem, SdY was tagged with a triple optimized sequence encoding for Human influenza hemagglutinin (3xHA, 3.5 kDa). Following immunoblotting, specific presence of SdY was recognized by a monoclonal antibody against the tag HA at 28 kDa. No extra bands suggesting post-translati

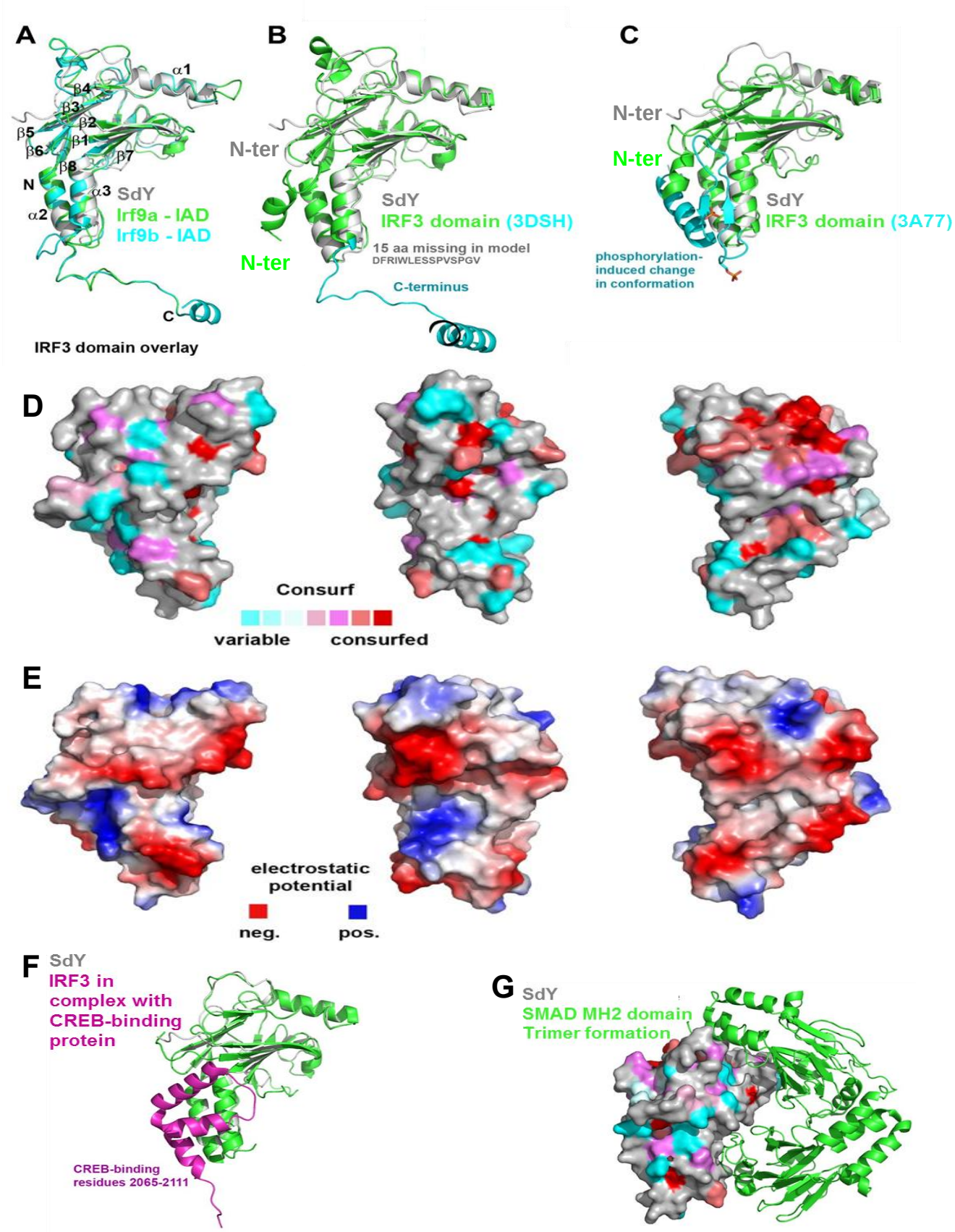


Figure6. SdY shares structural homologies with Irf9. A The structure of SdY (grey), Interferon Associated Domain (IAD) from tIrf9a (green), IAD from tIrf9b (blue) were modeled from the template IRF3 (PDB 3DSH). The three α-helix and 8 β-sheets forming a β sandwich are represented. Note the superposition of the three α-helices. **B** A close-up shows SdY and the IRF3 domain. **C** The structure of phosphorylated IAD domain of IRF3 (green-blue) (PDB 3A77) was used as a template for modeling SdY (gray). **D** Consurf analysis of SdY. The most variable amino acid between the sequence are colored in blue and the most conserved aminoacid are colored in red. The same model is shown in three different views in clockwise manner (+90°). **E** The molecular surface of SdY is colored according to the electrostatic potential (red, blue and white are negative, positive and neutral electrostatic potential values, respectively). The same model is shown in three different views in clockwise manner. **F** Model of SdY (grey) from IRF-3 (green) in complex with CREB Binding protein (purple). **G** Putative trimer formation with SdY (grey) and two MH2 domain from SMAD protein (green).

-onal modifications were observed. Additionally, no bands corresponding to 3xHA-SdY were visualized in untransfected and GFP transfected cells (***Figure 5B***). Collectively, the data revealed that up-to-now SdY specific recognition by immunoblot is only possible by a tagged version of SdY and also SdY do not revealed any observable post-translational modifications such as phosphorylation under the conditions of ectopic expression.

Bioinformatic tools suggest that SdY is a monomer and constituted of an interaction platform

To better characterize SdY, I tried to get more information about its 3D structure. SdY shares 42% of homology with Interferon Associated Domain of Irf-9, a protein-protein interaction domain (***Yano et al., 2012***). Using bioinformatics tools, the amino acid sequence was analyzed. According to amino acid identities, the data converged to model SdY using IRF-3 domain as template. This domain represents the common structural domain attributed to Interferon Associated Domain (IAD). SdY was modeled by Prof. T. Mueller (University of Wuerzburg) from the crystal of the human IRF-5 dimer studied by X-ray diffraction referenced as template 3dsh (Protein Data Bank code, 3dsh) (***Chen et al., 2008***). This template represents the best predicted model found in the PDB database. More than 20% of amino acid identities are found between SdY and the template. ***Figure 6A*** shows the SdY (grey) secondary structure in association with the deduced structure of trout Irf9a (green) and trout Irf9b (light blue). SdY and trout Irfs exhibited three α-helix surrounded by 8 anti-parallel β-sheets forming a β-sandwich. Each secondary structure is depicted. A superposition between the different proteins is shown. The last 15 amino-acid are missing in the original model due to their absence in the crystal (the residues were not visible in the density electron maps and not included in the model (***Chen et al., 2008***). In ***Figure 6B***, IRF3 domain (green) and SdY (grey) models superimposed together in term of secondary structure. It is worth to note that the C-terminal part of IRF-3 showed an open conformation indicating its relative importance. Interestingly, a better superposition between SdY (grey) and IRF3 domain (green, light blue) was observed using template 3a77 (PDB code, 3a77), the activated form of IRF3 after phosphorylation (***Figure 6C***) (***Takahasi et al., 2010***). Additionally, bioinformatics analysis using different softwares on the SdY protein predicted no strict phosphorylation site or posttranslational modifications. A Consurf analysis showed aminoacid conservation between the IAD of IRF7, IRF8 and IRF9 from different species. The coloration indicates from variable (light blue) to conserved (red) the degree of conservation. In this panel, the same model is represented with a rotation of 90° (clockwise). The model on the right, on the upper part,showed a highly conserved patch (in red) (***Figure 6D***). Next, the electrostatic potential

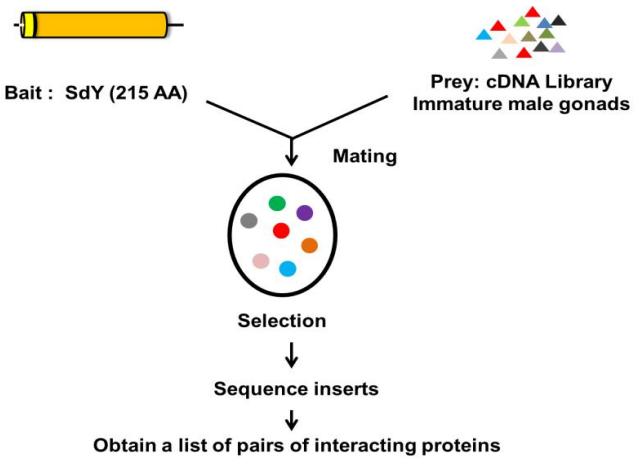


Figure 6. SdY interacts with Forkhead box proteins. Flowchart detailing the Yeast two Hybrid process. SdY has been chosen as a bait and a cDNA library from immature male gonad as a prey. Each prey yeast has been mated with SdY yeast. Positive clone are selected on deficient media and sequenced. From the sequence, a list of SdY interacting partners is obtained.

Table 1. General features of the SdY interaction

	Value
Screens	2
Analysed interaction	183,3 x10 ^{6}
Processed clones	202
Clones identified	46 (PBS A to D)
Selected Interacting Domain identified	26

Table 2. General description of prey molecular function

Prey Molecular Function		Value (%)	Selected Interacting Domain	Value (%)
Transcription factors		64,9		
	Fox Protein	82, 8	Forkhead	26
	Zinc Finger	9,9	Zn Finger	9
	Homeobox	2,7	Homeobox	4
	bHLH	1,8	bHLH	2
	Nuclear Hormone receptor	1,8	Nuclear Hormone receptor	2
	HMG	0,9	HMG	4
Other functions		35,1	Other domains	53

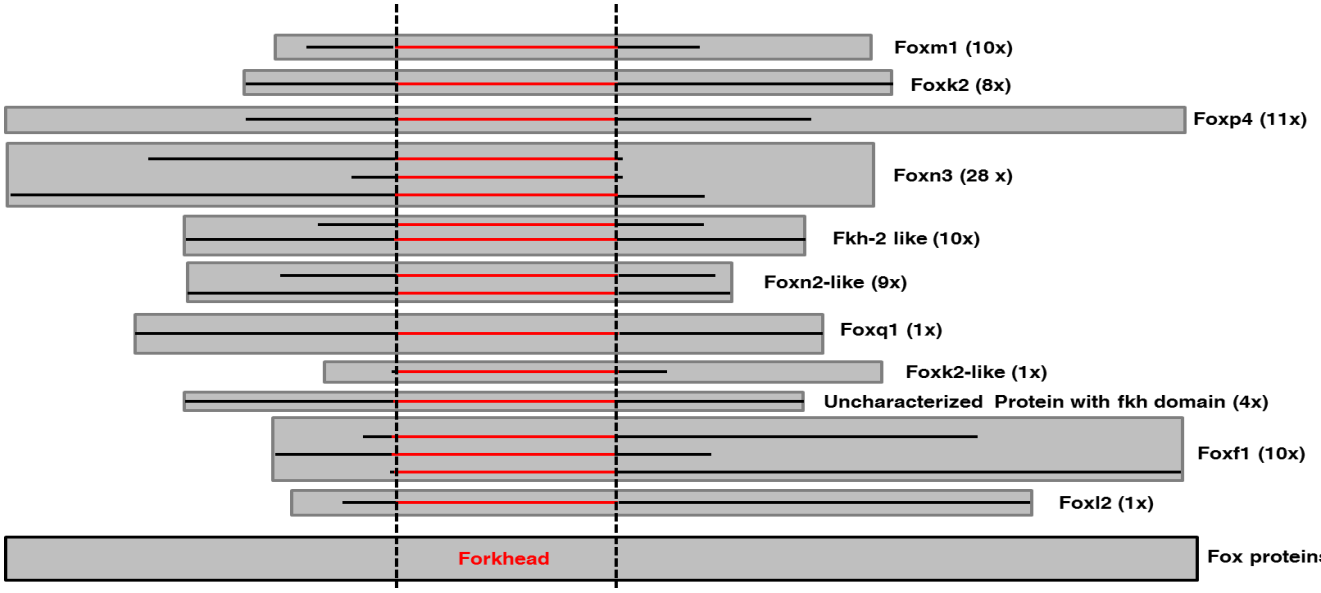


Figure 7. Determination of the Forkhead domain as a binding domain for SdY. Each grey boxes represent a Fox protein and on the right the corresponding associated number of clones obtained during the screen. For each protein, all fragments encoding for the same target ORF are aligned. This yields to a selected interacting domain (SID) represented by the black line including the forkhead domain (red line). An alignment of all SID was performed and narrows the Forkhead domain.

was studied. Red to blue color saturation showed negative and positive charges, respectively. Interestingly, the highly conserved patch exhibited positive charges due to the presence of arginines indicating a putative binding site (***Figure 5E***). In this context, a X-ray diffraction of the IRF3 domain associated with a CREB binding protein (PDB code, 1zoq) was merged to the SdY model showing a possible localization of a putative binding partner. It is worth to note that the interaction site is not located on the conserved patch (***Figure 6F***).
Moreover, at the structural level, the IRF3 domain shares similarities with the protein-protein interaction domain MH2 (Mad homology 2) of SMAD protein especially in the basic subdomain domain (***Qin et al., 2003; Takahasi et al., 2003***). Both of them are able to form a heterotrimeric complex (***Derynck et al., 1998; Qureshi et al., 1995***). Structural analysis of a homotrimer formation with two MH2 domains (PDB code 3gmj) and SdY does not show any overlap between both domains suggesting that SdY is a monomer (***Figure 6G***) (***Wang et al., 2009***). Collectively, the comparative structural analysis indicated that SdY should be a monomer and that conserved β-sandwich and basic surface area elements would provide an interaction core for the recruitment of a binding partner.

SdY interacts with Forkhead box proteins

From the previous work of Yano et al and the structural information about SdY, we hypothesize that SdY may act via protein-protein interaction to trigger testicular differentiation. To investigate the putative partners of SdY, the method of choice was a yeast two hybrid (Y2H) screen (***Figure 6***). Over more than 180 million interactions were tested and 202 positive clones were processed. Positive clones were isolated and the corresponding prey fragments and identified using NCBI database which generated a list of 46 putative interacting partners (***Table 1***). In order to be more consistent, a predicted biological score (PBS) was calculated to assess the reliability of each interaction **(*Appendix*)**. A third step consisted to overlap prey fragments originating from the same gene and gathered together in a cluster. The translated amino acid sequences of the fragments were aligned and superimposed onto the open reading frame. Overlapping regions shared by all fragments was named selected interacting domain (SID) (***Formstecher et al., 2005***).The yeast-two-hybrid revealed that more than two-third of the candidate represents transcription factors. Interestingly, inside the transcription factors group, an abundant fraction (80%) of FOX proteins was identified (***Table 2***). Among these Fox proteins, the screen identified Foxn3, Foxp4, Foxk2, Foxm1, Foxf1 and Foxl2 according to the predicted biological score (PBS) ranking (***Figure 7 and Appendix 1***). Moreover, the most representative Selected Interacting Domain (SID) shared by all the clones was the Forkhead domain (more than 27 %) (***Table***

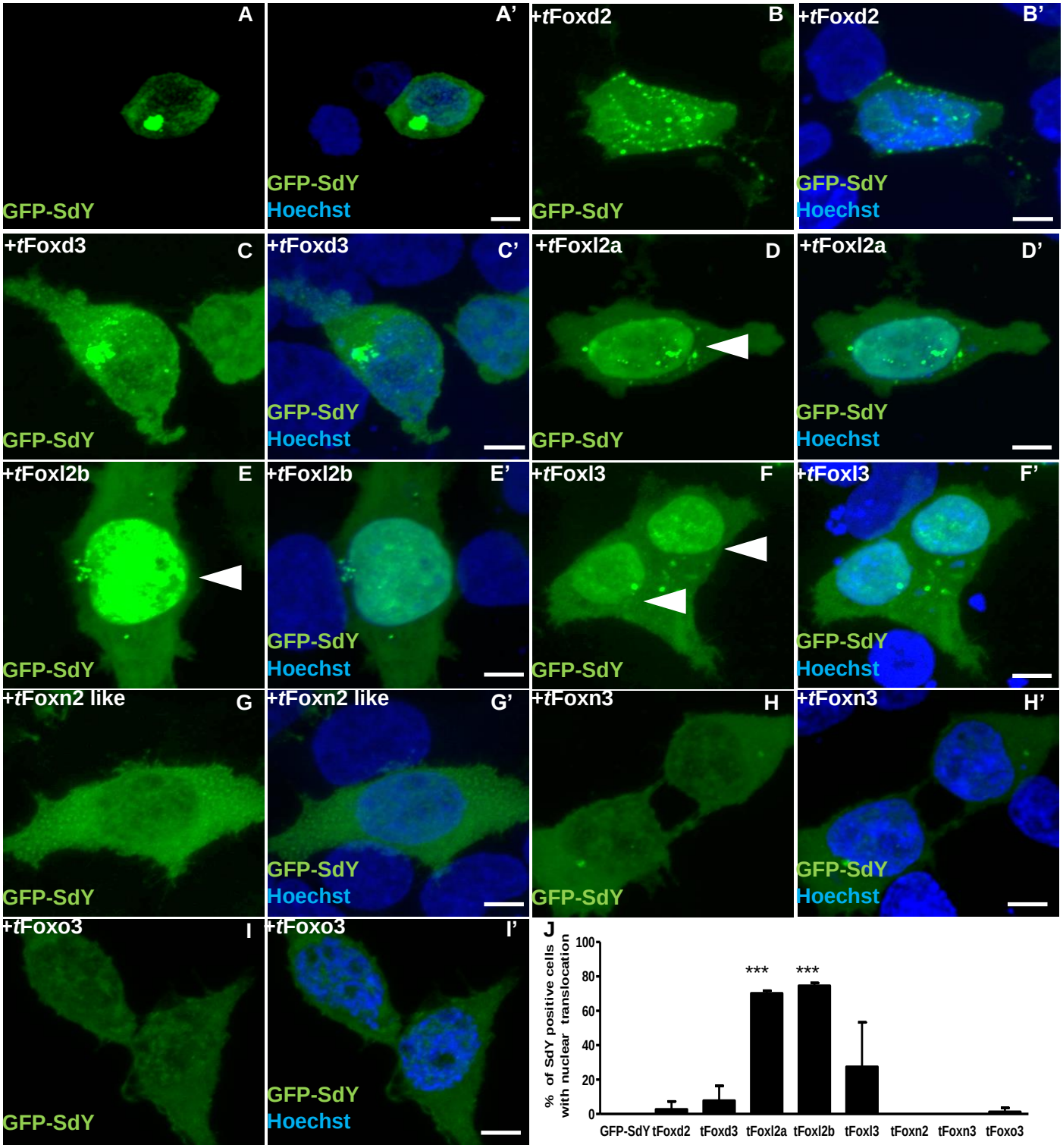


Figure 8. **Specific Nuclear translocation of SdY in presence of Foxl2a and Foxl2b**. **(A-A')** HEK 293T cells were transiently transfected with an expression vector encoding GFP-SdY **(A)** and the nucleus stained with Hoechst. **(A')** **(B-M')** GFP-SdY and various trout Fox (tFoxd2 like, tFoxd3, tFoxl2a, tFoxl2b, tFoxl3, tFoxn3, tFoxo3) were cotransfected in HEK cells **(B, C, D, E, F, G, H, I)** counterstained with Hoechst **(B, C',D', E', F', G', H', I')**. In presence of tFoxl2a, tFoxl2b, tFoxl3, SdY translocates into the nucleus. Nuclear translocation of SdY are depicted with white arrows in D, E, F. **(J)** Quantification of SdY nuclear translocation showing that tFoxl2a, tFoxl2b, tFoxl3 specifically trigger cytoplasmic–nucleus movement of SdY. Data are the mean ±s.d.; n=200 cells for each group from three independent. Statistical significance was calculated using Student's t -test P< 0,001 (***). (Scale Bar, 10 µm). t (trout)

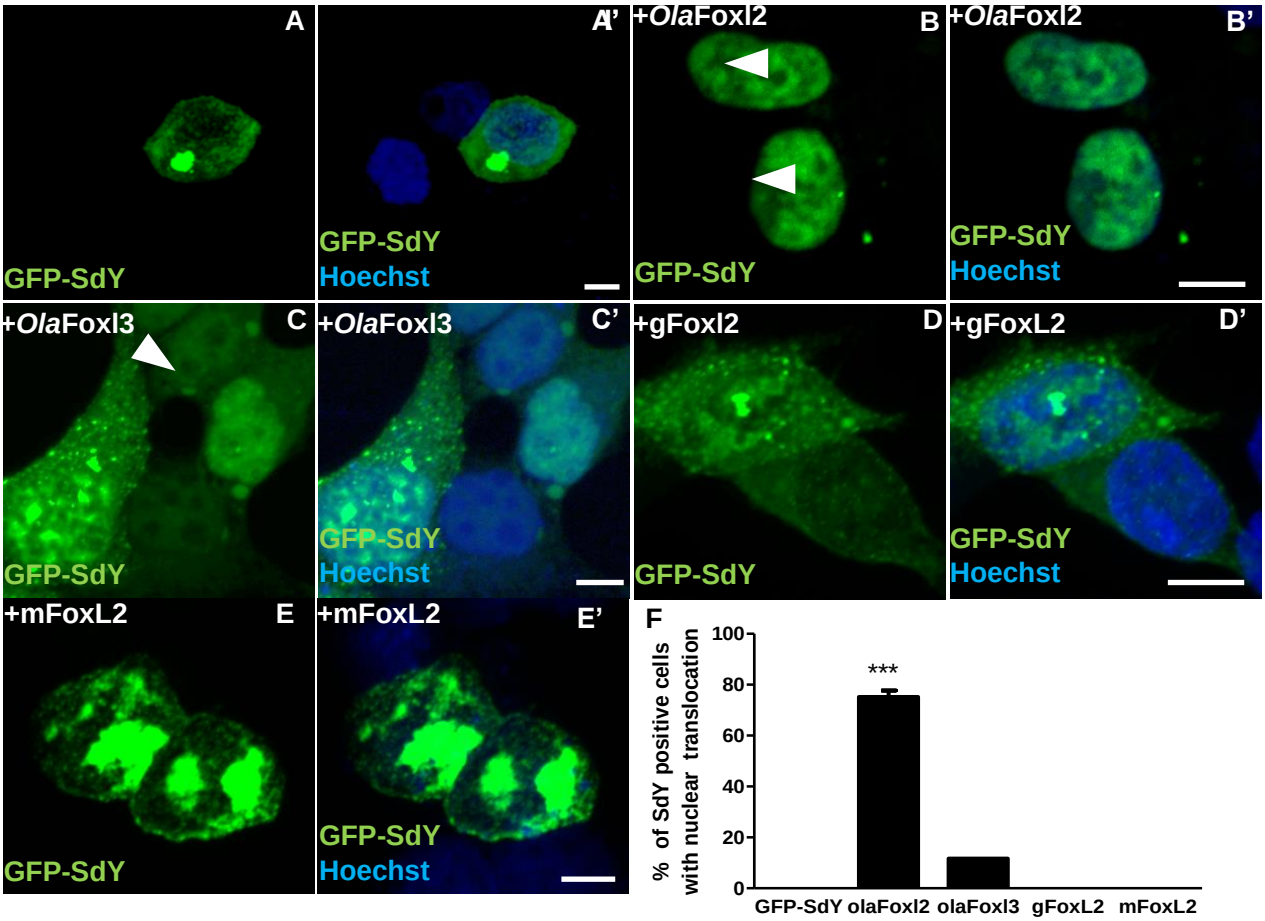


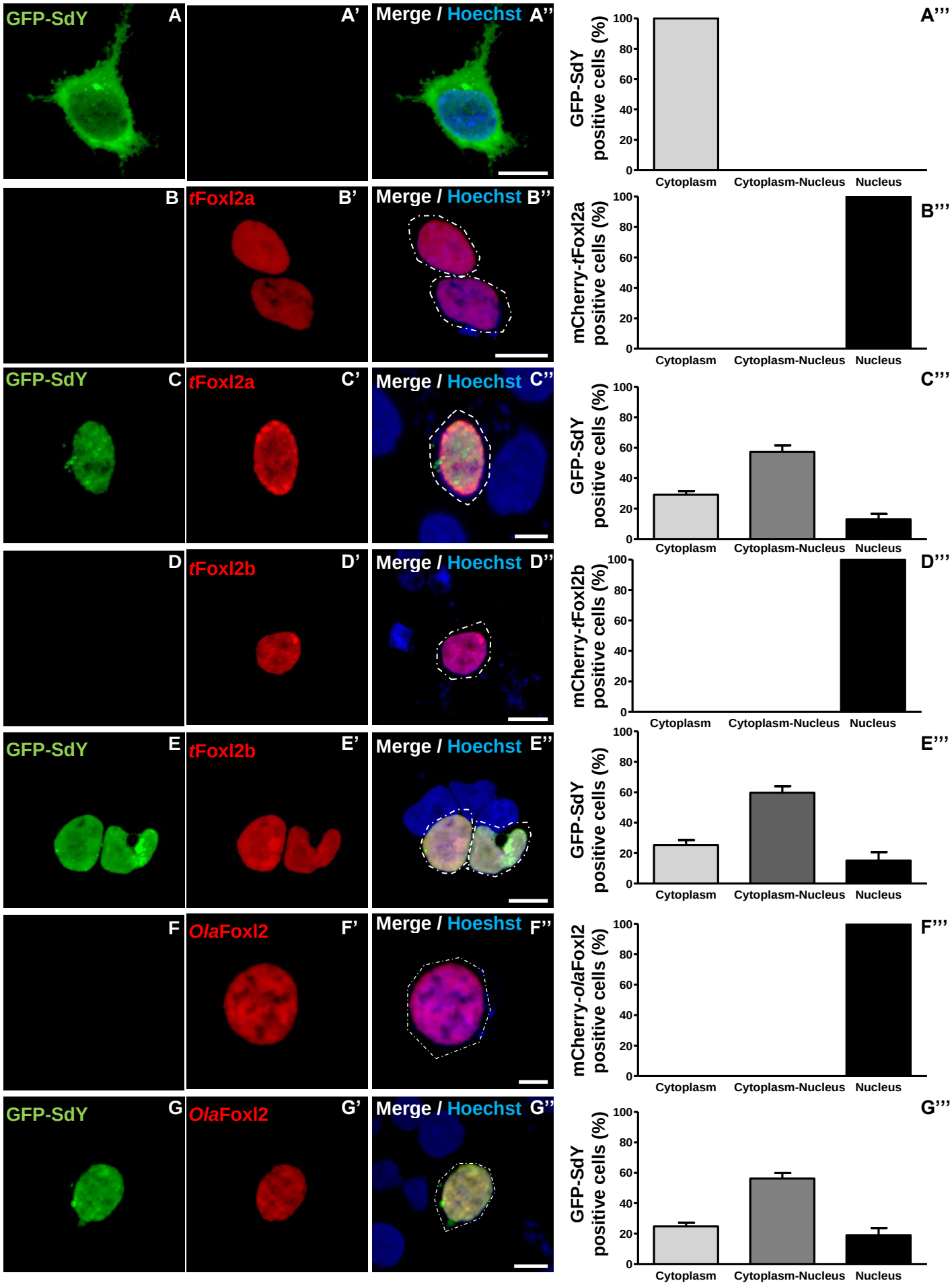
Figure 9: Fish Foxl2 specifically translocate SdY. **(A-A')** HEK 293T cells were transiently transfected with an expression vector encoding GFP-SdY **(A)** and the nucleus stained with hoechst. **(A')** **(B-E')** GFP-SdY and various FoxL2 from different species (OlaFoxl2, Olafoxl3, gFoxL2, mFoxL2) were cotransfected in HEK cells **(B, C, D, E)** counterstained with Hoechst **(B, C',D', E')**. In presence of medaka olaFoxl2 and olaFoxl3, SdY translocated into the nucleus. Nuclear translocation of SdY are depicted with white arrows in **B and C**. **(F)** Quantification of SdY nuclear translocation showing that olaFoxl2 specifically trigger cytoplasmic–nucleus movement of SdY and in a lesser extent for Foxl3. Data are the mean ±s.d.; n=200 cells. Only one experiment was performed for olaFoxl3, gFoxL2, mFoxL2 except for olaFoxL2, three independent were performed. Statistical significance was calculated using Student's t -test P< 0,001. (Scale Bar, 10 µm.) ola (medaka), g(goat), m (mouse)

2). Next, the alignment based on the SID among the fox candidates defined the Forkhead as the SdY interacting domain (***Figure 7***). In addition, Kansl1, which encodes a nuclear protein as part of the Non Sex Lethal (NSL) complex, was found in the Y2H screen. It is involved in acetylation of nucleosomal histone H4 on several lysine residues and therefore may be involved in the regulation of transcription *(**Cai et al., 2010; Huang et al., 2012**)*. The remainder of the putative binding partners was cytoplasmic protein. Most of them have a low confidence PBS (rank D). This category includes usually mainly false positive candidates or possible interaction. Known false positive such as HSP, ribosomal protein, collagen related protein were not subjected to a deep analysis so far (***Serebriiskii et al., 2000***). Altogether, the data revealed that SdY could interact with a fox transcription factor through its Forkhead domain.

SdY translocates in the nucleus in presence of Foxl2

To confirm an interaction between SdY and the different Fox proteins, SdY and the putative partner should at least localize in the same cellular compartment. To test this hypothesis, co-transfection in HEK cells during 48 hours was performed with GFP-SdY together with several trout fox proteins (Foxd2, Foxd3, Foxl2a, Foxl2b, Foxl3, Foxn2-like, Foxn3, Foxo3) followed by confocal microscopy to visualize the distribution of GFP-SdY. Fox proteins are composed of bipartite or monopartite nuclear localization signal, triggering the nuclear localization (see Introduction). However, Foxo3 proteins have a nucleo-cytoplasmic localization (***Brunet et al., 1999***). For each experiment, representative pictures taken with similar settings and showing GFP-SdY (left) and a merged GFP-SdY/Hoechst nucleus stained in blue (right) are depicted (***Figure 8***). Most of the cotransfected cells showed a cytoplasmic localization of SdY, but a clear nuclear translocation of GFP-SdY was observed in presence of tFoxl2a and tFoxl2b (***Figure 8D-E'***). More than 75 % observed cells presented this phenotype (***Figure 8J***). tFoxl3 also exhibits this pattern but to a lesser extent (***Figure 8F-F'***). HEK cells do not endogenously express SdY, neither FOXL2 nor FOXL3 suggesting that the observed effect is specific to SDY and Foxl2 or Foxl3 (***Blount et al., 2009***). This experiment revealed that SdY physically and specifically interact with tFoxl2a and tFoxl2b leading a relocalization of SdY to form a protein complex with only some trout Fox proteins (l2 and l3) in the nucleus.

Next, I wonder if this interaction is species specific. Using the same co-transfection experiments, GFP-SdY and Foxl2 from fish (medaka) and mammals (goat and mouse) were co-expressed. Nucleus was stained with Hoechst (blue) and cells were observed by confocal microscopy. Experimental data revealed that GFP-SdY strongly translocated in the nucleus only in presence of medaka Foxl2 (OlaFoxl2) (***Figure 9 B-B'***). Quantitative analysis


GFP-SdY
A
A'
Merge / Hoechst
A''
A'''
GFP-SdY positive cells (%)
B
tFoxl2a
B'
B''
B'''
mCherry-tFoxl2a positive cells (%)
C
C'
C''
C'''
D
tFoxl2b
D'
D''
D'''
mCherry-tFoxl2b positive cells (%)
E
E'
E''
E'''
F
OlaFoxl2
F'
Merge / Hoeshst
F''
F'''
mCherry-olaFoxl2 positive cells (%)
G
G'
G''
G'''
0
20
40
60
80
100
Cytoplasm
Cytoplasm-Nucleus
Nucleus

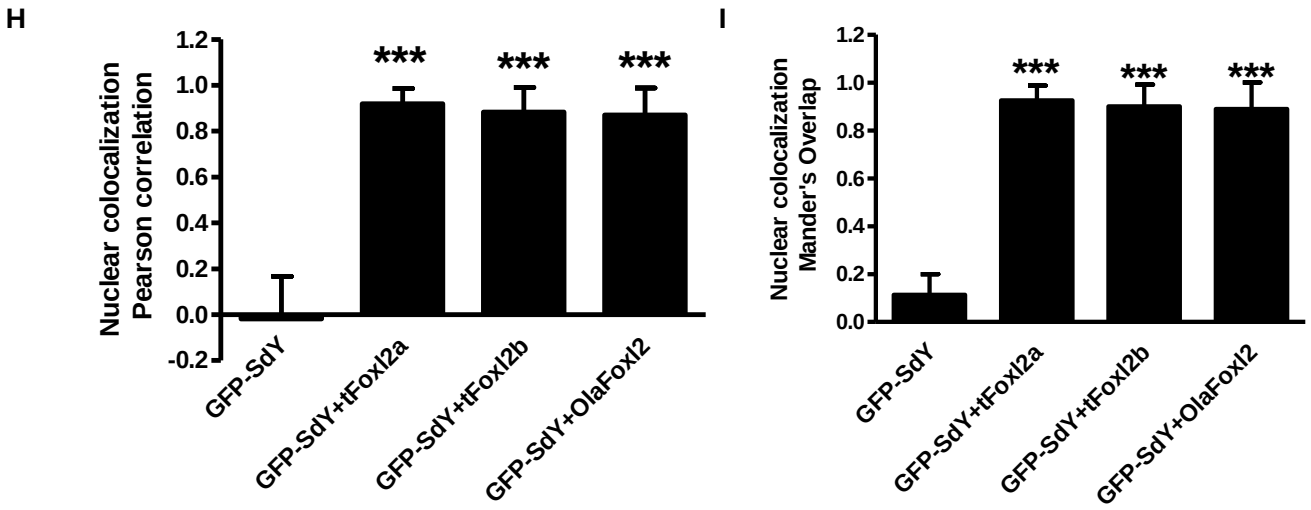


Figure 10. SdY translocates into the nucleus in presence of FoxL2. **(A-G''') Subcellular localization of SdY in presence or not of tFoxl2a, tFoxl2b, OlaFoxl2.** HEK 293T cells were transiently transfected with an expression vector encoding for GFP-SdY **(A-A''')**, tFoxl2a **(B-B''')**, GFP-SdY and tFoxl2a **(C-C''')**, tFoxl2b **(D-D''')**, GFP-SdY and tFoxl2b **(E-E''')**, OlaFoxl2 **(F-F''')**, GFP-SdY and OlaFoxl2 **(G-G''')** during 48 H, and the subcellular localization was analyzed by confocal microscopy. **A** Cytoplasmic localization of SdY. **A'**. No image. **A''** GFP-SdY counterstained with Hoechst showing the nucleus (in blue). **A'''-** Quantitative analysis of subcellular localization. Percentages are representative of at least 300 SdY positive cell counts from a minimum of three independent experiments. **(B-B''') Subcellular localization of tFoxl2a. B** No image **B'** Nuclear localization of tFoxl2a. **B''** tFoxl2a counterstained with Hoechst showing the nucleus (in blue), white dots surround the cell membrane. **B'''** Quantitative analysis of subcellular localization. Percentages are representative of at least 300 tFoxl2a positive cell counts from a minimum of three independent experiments. **(C-C''') Subcellular localization of SdY in presence of tFoxl2a. C** Nucleo-Cytoplamic localization of SdY **C'** Nuclear localization of tFoxl2a. **C''** Merge of **C-C'**, nucleus counterstained with Hoechst showing the nucleus (in blue), white dots mark out the cell membrane. **C'''** Quantitative analysis of subcellular localization. Percentages are representative of at least 300 SdY and tFoxl2a positive cell counts from a minimum of three independent experiments. (**D-D''') Subcellular localization of tFoxl2a. D** No image **D'** Nuclear localization of tFoxl2b. **D''** tFoxl2b counterstained with Hoechst showing the nucleus (in blue), white dots surround the cell membrane. **D'''** Quantitative analysis of subcellular localization. Percentages are representative of at least 300 tFoxl2b positive cell counts from a minimum of three independent experiments. **(E-E''') Subcellular localization of SdY in presence of *t*Foxl2b. E** Nucleo-Cytoplamic localization of SdY **E'** Nuclear localization of tFoxl2b. **E''** Merge of **E-E'**, nucleus counterstained with Hoechst showing the nucleus (in blue), white dots mark out the cell membrane. **E'''** Quantitative analysis of subcellular localization. Percentages are representative of at least 300 SdY and tFoxl2b positive cell counts from a minimum of three independent experiments. (**F-F''') Subcellular localization of *t*Foxl2a. D** No image **F'** Nuclear localization of tFoxl2b. **F''** tFoxl2b counterstained with Hoechst showing the nucleus (in blue), white dots surround the cell membrane. **F'''** Quantitative analysis of subcellular localization. Percentages are representative of at least 300 OlaFoxl2 positive cell counts from a minimum of three independent experiments. **(G-G''') Subcellular localization of SdY in presence of *Ola*Foxl2. G** Nucleo-Cytoplamic localization of SdY **G'** Nuclear localization of OlaFoxl2. **G''** Merge of **G-G'**, nucleus counterstained with Hoechst showing the nucleus (in blue), white dots mark out the cell membrane. **G'''** Quantitative analysis of subcellular localization. Percentages are representative of at least 300 SdY and OlaFoxl2 positive cell counts from a minimum of three independent experiments. **H** Quantitative analysis of nuclear colocalization assessed for SdY, SdY and tFoxl2a, SdY and tFoxl2b, SdY and OlaFoxl2 by Pearson's correlation. **I** Quantitative analysis of nuclear colocalization assessed for SdY, SdY and tFoxl2a, SdY and tFoxl2b, SdY and OlaFoxl2 by Mander's Overlap. Statistical significance was calculated using Student's t-test p< 0,001(***). (Scale Bar, 10 µm.) t (trout), ola (medaka)

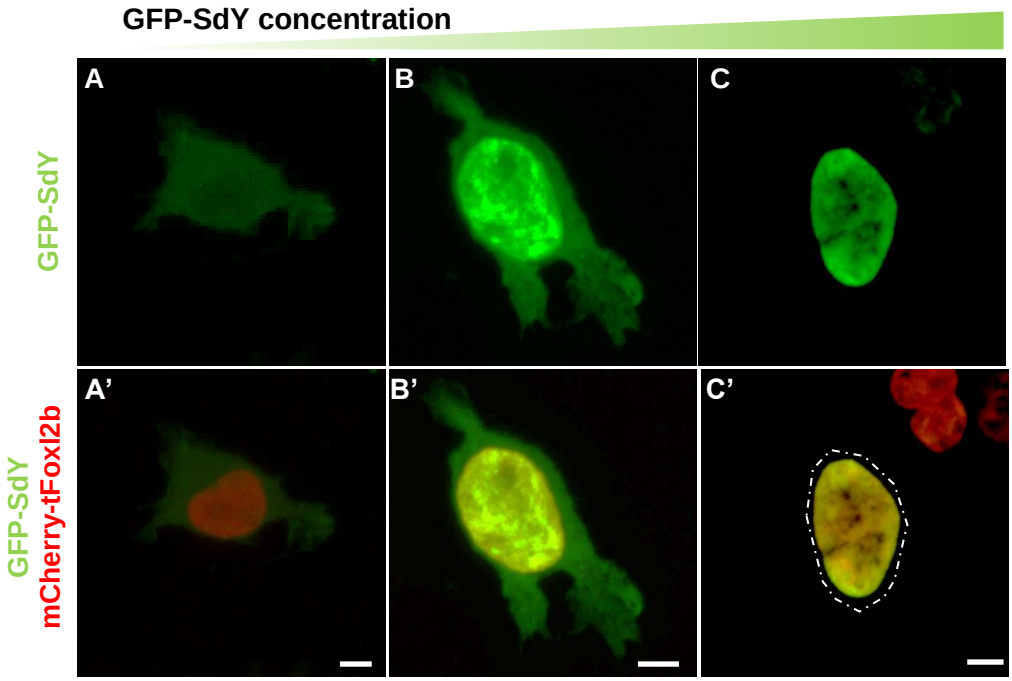


Figure 11. SdY nuclear translocation through Foxl2b is dose dependent. (A-C') Various Subcellular localization of SdY in presence of tFoxl2b. HEK 293T cells were transiently transfected with an expression vector encoding for GFP-SdY and tFoxl2b (A-C') during 48 H, and the subcellular localization was analyzed by confocal microscopy. **A** Cytoplasmic localization of SdY. **A'**. Cytoplasmic localization of SdY and nuclear localization of tFoxl2b. **B** Nucleo-Cytoplasmic localization of SdY. **B'**. Nucleo-Cytoplasmic localization of SdY and nuclear localization of tFoxl2b **C** Nuclear localization of SdY. **C'** Nuclear localization of SdY and nuclear localization of tFoxl2b. The same pattern of expression is observed for the combination GFP-SdY and tFoxl2a also GFP-SdY and *Ola*Foxl2. (Scale Bar, 10 µm.)

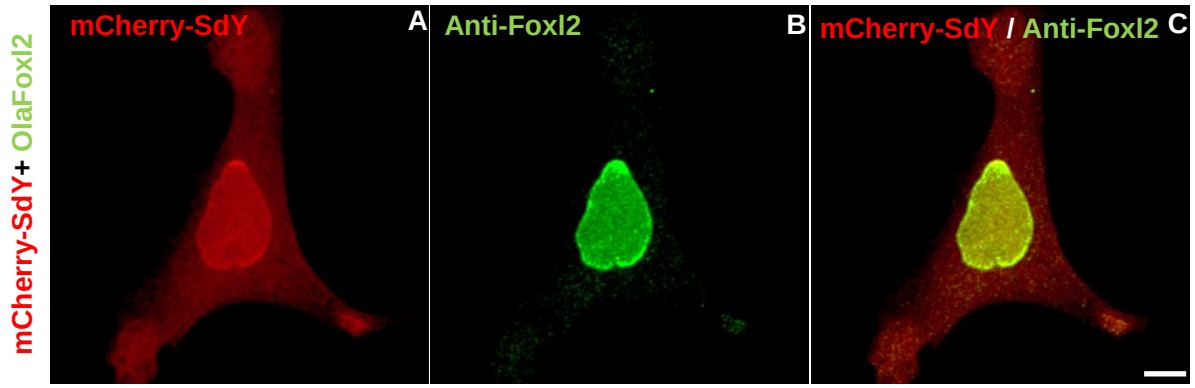


Figure 12. Strong nuclear translocation of SdY in presence of Foxl2 into RTG2 cells. (A-C) Rainbow trout gonadal cells (RTG2) were transiently co-transfected with mCherry-SdY and OlaFoxl2 expression constructs. SdY was fused to a red fluorescent protein (mCherry) (**A**) and Foxl2 was detected with an antiFoxl2 antibody (green) (**B**). The superposition for both red and green channels is showed in **C**. (Scale Bar, 10 µm)

demonstrated that more than 75 % of SdY positive cells show a nuclear translocation (***Figure 9 F***). Interestingly, a low percentage of cells presented a nuclear translocation of GFP-SdY when medaka Foxl3 was co-transfected (***Figure 9 C-C' and 9F***). An absence of GFP-SdY nuclear translocation was observed in presence of goat FOXL2 or mouse FOXL2 (***Figure 9 D-E' and 9F***). Collectively, these results demonstrate that the interaction between SdY and mammals FOXL2 is not functional suggesting that Foxl2 structural specificities are important to establish an efficient interaction with SdY.

To further validate the nuclear translocation of SdY in presence of fish Foxl2, coexpression in HEK cells was done. For this purpose, Foxl2 (tFoxl2a, tFoxl2b, olaFoxl2) were tagged with a red fluorescent protein (mcherry). Hoechst staining confirmed the nuclear localization. Localization analysis between GFP-SdY and Foxl2 was revealed by confocal microscopy (***Figure 10***). Despite a low proportion of GFP-SdY in the cytoplasm (around 25%), the majority of GFP-SdY localized in nucleo-cytoplamic (60%) manner or in the nucleus (15%). Interestingly, this phenomenon is shared by the three Foxl2 and the proportion of cellular localization is quite similar. Merged images showed a yellow color in the nucleus indicating colocalization. To go further, Pearson's correlation and Mander's Overlap colocalization coefficient was measured. Pearson's correlation and Mander's Overlap measurements showed that GFP-SdY and tFoxl2s are above 0.8 indicating a very strong correlation confirming the colocalization. GFP-SdY alone served as a control and the correlation was estimated as weak. (***Figure 10H-I***). Altogether, the data showed that GFP-SdY translocated in the nucleus in presence of mCherry-Foxl2s and colocalized.

In order to get a first idea on the quantitative aspect of the interaction in cotransfected cells, I studied the effect of GFP-SdY concentrations on the interaction. Transiently co-expressing HEK cells with GFP-SdY and mCherry-tFoxl2b were used to visualize the feature of GFP-SdY. The experiment revealed that the more GFP-SdY was expressed the more GFP-SdY translocated from the cytoplasm to the nucleus, in presence of equal amounts of mcherry-tFoxl2b (***Figure 11***). The data indicated that the nuclear translocation is dose dependent.

Next I wanted to confirm this interaction in another cell line and also to check if used of a fusion protein could lead to artefactual results. Fish RTG2 cell line was cotransfected with SdY fused to mCherry, a red fluorescent protein in N-terminal part and with OlaFoxl2 tagged with FLAG in C-terminal part. After immunolocalization, a clear nucleo-cytoplamic localization of mCherry-SdY and a nuclear localization of Foxl2 (green) were observed (***Figure 12 A-B***). It is worth to note the color superposition in the nucleus (***Figure 12 C***). This data confirm the interaction between SdY and Foxl2 and that tagged versions of both SdY and Foxl2 do not affect the interaction.

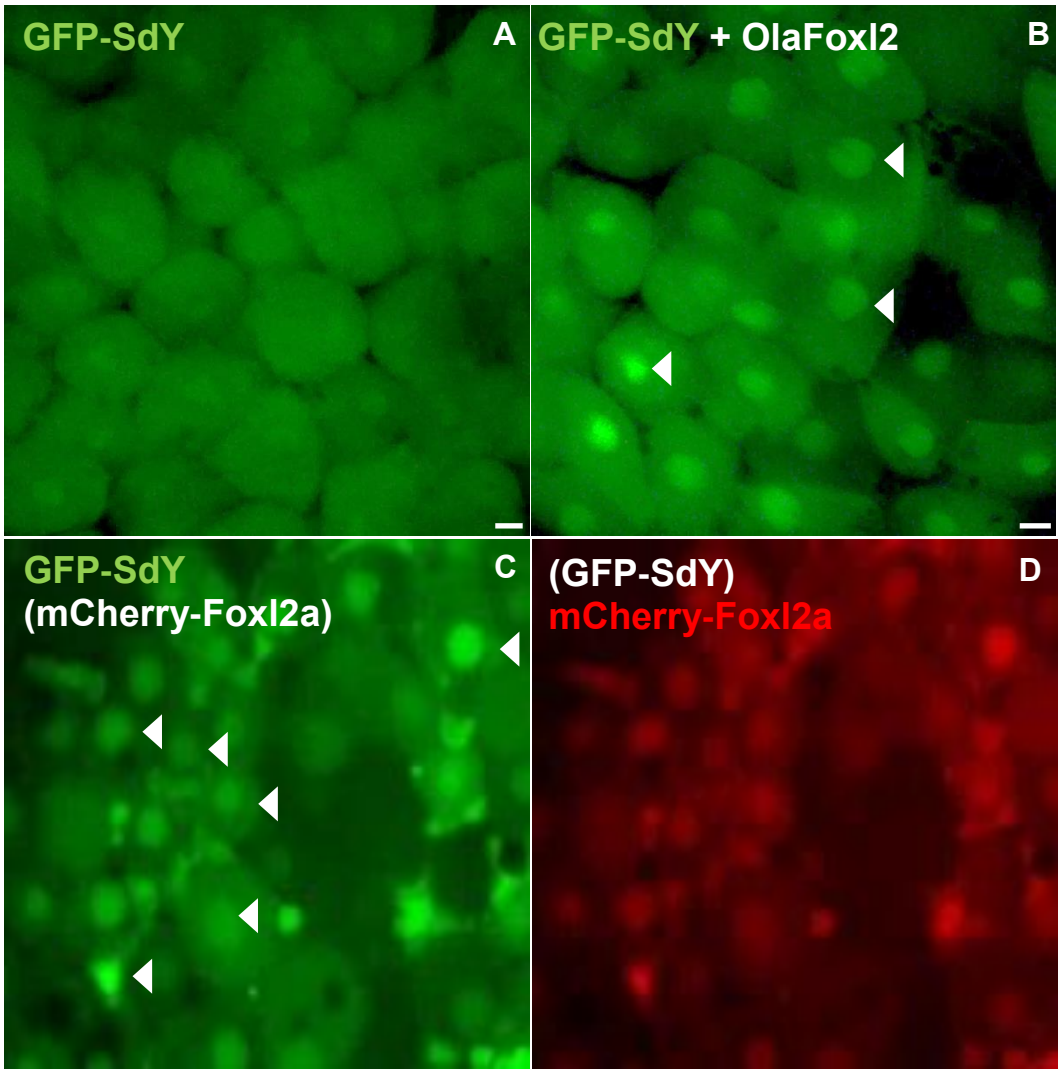


Figure 13. Nuclear translocation of SdY in presence of Foxl2 in medaka embryos. **(A-B)** Confocal microscopy analysis of GFP-SdY alone (**A**) or with medaka Foxl2 (**B**) RNA injected embryos at one cell stage. **(C-D)** Epifluorescence pictures with GFP-SdY (**C**) and mCherry-Foxl2a (**D**) RNA injected embryos at one cell stage. Note the nuclear SdY accumulation in presence of Foxl2 (**B**) or Foxl2a (**C**) indicated by white arrows. (Scale Bar , 10 µm)

In order to be more physiological, we used the medaka embryo as an *in vivo* bioreactor to visualize the interaction between SdY and olaFoxl2. One-cell stage medaka embryos were injected with GFP-*sdY* RNA as a control and either with both GFP-*sdY* and Ola*foxl2* c *sdY* and mCherry-*foxl2b*. Fluorescent proteins were observed by confocal microscopy during the gastrula period for GFP-SdY alone and OlaFoxl2 while both co-injected GFP-SdY and mCherry-tFoxl2b were observed under epifluoresence microscope. As a control, GFP-SdY injected alone in embryos predominantly showed a cytoplasmic localization of green fluorescence. Some nucleocytoplasmic localization, however, was also observed. When co injected with OlaFoxl2, most of the cells present a clear GFP-SdY accumulation in the nucleus accompanied by a weak cytoplasmic localization compared to GFP-SdY alone (***Figure 13***).The same clear nuclear GFP-SdY accumulation is observed when the GFP-SdY and mCherry-Foxl2b are co-injected. Collectively, these confirm the interaction between SdY and Foxl2, and that the complex localizes in the nucleus.

SdY physically interacts with Foxl2

To verify the physical interaction between SdY and Foxl2, biochemical experiments have been performed. First, in order to observe the presence and the pattern of Foxl2a, Foxl2b and the medaka Foxl2 in presence or in absence of SdY, the proteins were overexpressed in HEK 293T cells and subjected to western blotting. The size of SdY in absence or in presence of Foxl2 was constant to 28 kDa. A band at 38 kDa was detected for both trout and medaka Foxl2 proteins as predicted by protein sequence analysis. Then, a GST pull down assay was done. In my case, GST protein was fused with SdY in the C-terminal part. The expression of GST-SdY was induced by IPTG in two protease deficient bacteria (Rosetta and BL-21). The overexpression of GST-SdY was characterized in both Rosetta and BL-21 bacteria strains at different temperatures (15°C to 37°C) and various concentration of IPTG (0.05 to 1mM) and monitored by the presence and amount of GST-SdY in the pellet fraction. The result suggests that GST-SdY was insoluble and expressed in inclusion bodies compare to GST alone, which was always present in the supernatant fraction (soluble part). To extract GST-SdY from the inclusion bodies, sarkosyl detergent was used. After purification, both GST (26 kDa) and GST-SdY (50 kDa) were coated on glutathione beads and incubated in HEK cell lysate with the overexpressed tagged version of tFoxl2b (3xFLAG-tFoxl2b). Both pulled down proteins were subjected to immunoblotting. The presence of tFoxl2b was revealed by an antibody against the FLAG tag. As a control, the presence of both GST and GST-SdY was detected by an antibody against GST. Several bands of lower molecular weight than the GST-SdY obviously resulted from protein

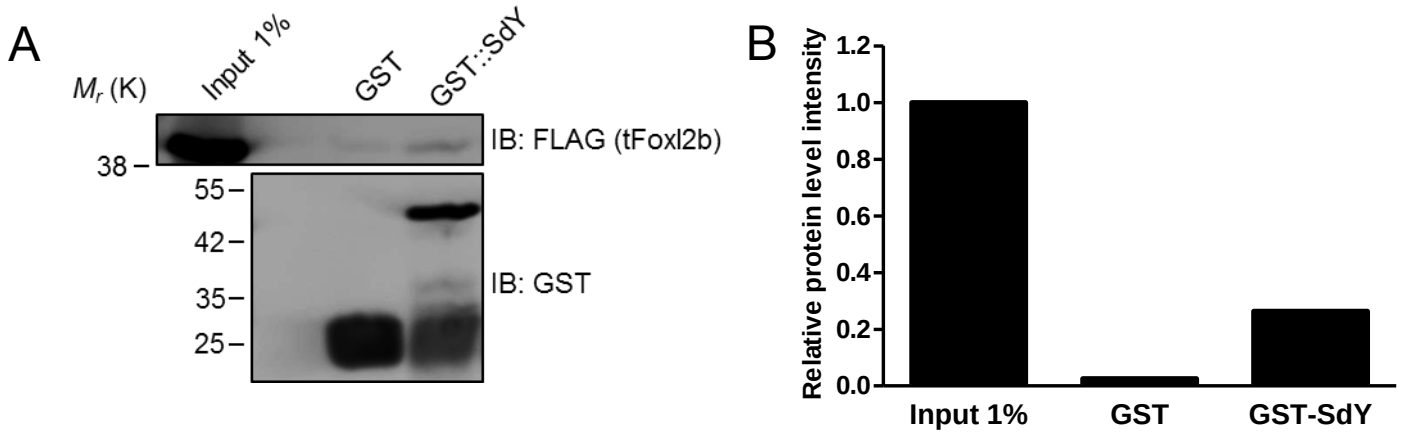


Figure14. SdY interacts with tFoxl2b. **A**. Purified GST and GST-SdY was incubated with sepharose beads. Cell extract (50 µg) from 293T cells transiently transfected with expression vector for 3xFLAG-tFoxl2b was added to the beads complex. 20 µg of GST or GST-SdY was used to pull down tFoxL2b. Membrane was re-probed with anti-GST antibody. **B.** Quantification of **(A).**

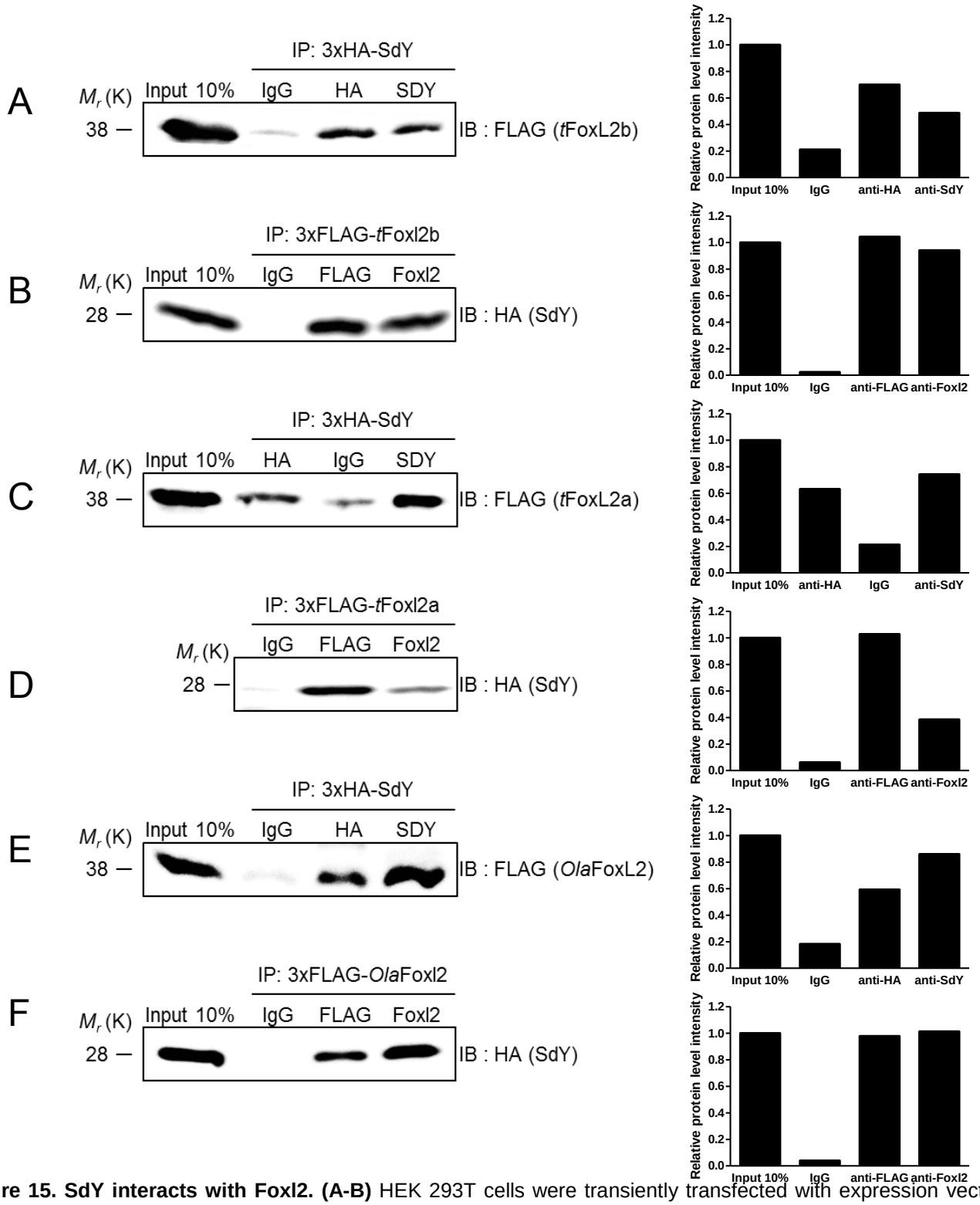


Figure 15. SdY interacts with Foxl2. (A-B) HEK 293T cells were transiently transfected with expression vectors for 3xHA-SdY and 3xFLAG-tFoxl2b for 48 H. **A** (left) SdY was immunoprecipitated with an HA or SdY antibody, followed by immunoblotting with an antibody against FLAG tag to show the presence of *t*Foxl2b. IgG mouse antibody is used as control. (right) Quantification of the co-immunoprecipitation. **B.** (Left) tFoxl2b was immunoprecipitated with either an FLAG or an FoxL2 antibodies, followed by immunoblotting with an antibody against HA tag to reveal SdY. IgG mouse antibody is used as control. (right) Quantification of the co-immunoprecipitation. **(C-D)** HEK 293T cells were transiently transfected with expression vectors for 3xHA-SdY and 3xFLAG-tFoxl2a for 48 H. **C** (left) SdY was immunoprecipitated with an HA or SdY antibody, followed by immunoblotting with an antibody against FLAG tag to show the presence of *t*Foxl2a. IgG mouse antibody is used as control. (right) Quantification of the co-immunoprecipitation. **D.** (left) tFoxl2a was immunoprecipitated with either an FLAG or an FoxL2 antibodies, followed by immunoblotting with an antibody against HA tag to reveal SdY. IgG mouse antibody is used as control. (right) Quantification of the co-immunoprecipitation. The input is missing. **(E-F)** HEK 293T cells were transiently transfected with expression vectors for 3xHA-SdY and 3xFLAG-*Ola*Foxl2 for 48 H. **E** (left) SdY was immunoprecipitated with an HA or SdY antibody, followed by immunoblotting with an antibody against FLAG tag to show the presence of *Ola*Foxl2. IgG mouse antibody is used as control. (right) Quantification of the co-immunoprecipitation. **F.** (left) OlaFoxl2a was immunoprecipitated with either an FLAG or an Foxl2 antibodies, followed by immunoblotting with an antibody against HA tag to reveal SdY. IgG mouse antibody is used as control. (right) Quantification of the co-immunoprecipitation. Input is used as positive control.

degradation. Unique detection of 3xFLAG-tFoxl2b in the GST-SdY lane confirmed the interaction between GST-SdY and 3xFLAG-tFoxl2b (***Figure 14A***). In addition, relative protein intensity was measured using ImageJ software. The analysis confirmed the detection of tFoxl2b and its absence in GST lane (***Figure 14B***). This biochemical experiment reveals a physical interaction between SdY and tFoxl2b in the cell lysate. To reinforce the idea that SdY interacts with Foxl2, a co-immunoprecipitation (co-IP) experiment was performed. Tagged versions of SdY (3xHA-SdY) and tFoxl2b (3xFLAG-tFoxl2b) were overexpressed in HEK cells. This cell lysate constitutes the input. After immunoprecipitation both proteins were subjected to immunoblotting. Immunoglobulin G antibodies were used as a control. The immunoblot shows the presence of tFoxl2b detected by an anti-Flag antibody when 3xHA-SdY was precipitated either with an anti-HA or anti-SdY antibody (***Figure 15A***). Protein intensity was measured for each band (***Figure 15A')***. Despite a weak signal in the IgG lane, the relative signal for anti-HA or anti-SdY is clearly visible. Interestingly, when 3xFLAG-tFoxl2b is precipitated with an anti-FLAG antibody or an anti-Foxl2 antibody, a band corresponding to SdY is detected by an anti-HA antibody (***Figure 15B***). The relative protein intensity confirmed the interaction (***Figure 15B'***). In the same line, the experience was made with 3xHA-SdY and 3xFLAG-tFoxl2a. The immunoblot revealed the precipitation of 3xFLAG-tFoxl2a by both HA and SdY antibodies (***Figure 15C-C'***). The precipitation of 3xFLAG-tFoxl2a triggered also 3xHA-SdY (***Figure 15D-D'***) indicating an interaction. Moreover, I tested if olaFoxl2 was able to interact physically SdY. The same experiment described above was performed with 3xHA-SdY and OlaFoxl2. The detection of Foxl2 when SdY was immunoprecipitated confirmed the interaction (***Figure 15E***). In addition, the presence of SdY is also revealed when 3xFLAG-tFoxl2 is precipitated (***Figure 15F***). For both experiments, protein intensity measurement for each band validated the interaction between SdY and OlaFoxl2 (***Figure 15F'-E'***). Collectively, these data indicated that Foxl2 can bind SdY *in vitro* after overexpression in HEK cells.

Physiological impact of the interaction

From the interaction between SdY and Foxl2 and the nuclear localization of the complex, it can be hypothesized that SdY-Foxl2 complex will act on a specific promoter to regulate gene expression during gonad differentiation. One of the Foxl2 targets is the *cyp19a1* promoter (See Introduction). Elodie Jouanno (INRA, LPGP Institute) analyzed gene expression of *sdY,* both paralogues of *foxl2*, *foxl2* and of *cyp19a1*. RNA from male and female trout gonads were extracted from 33 dpf and 125 dpf and subjected to quantitative PCR. From the hatching (33 dpf) stage to the first morphological differentiation, *sdY* reaches a peak around

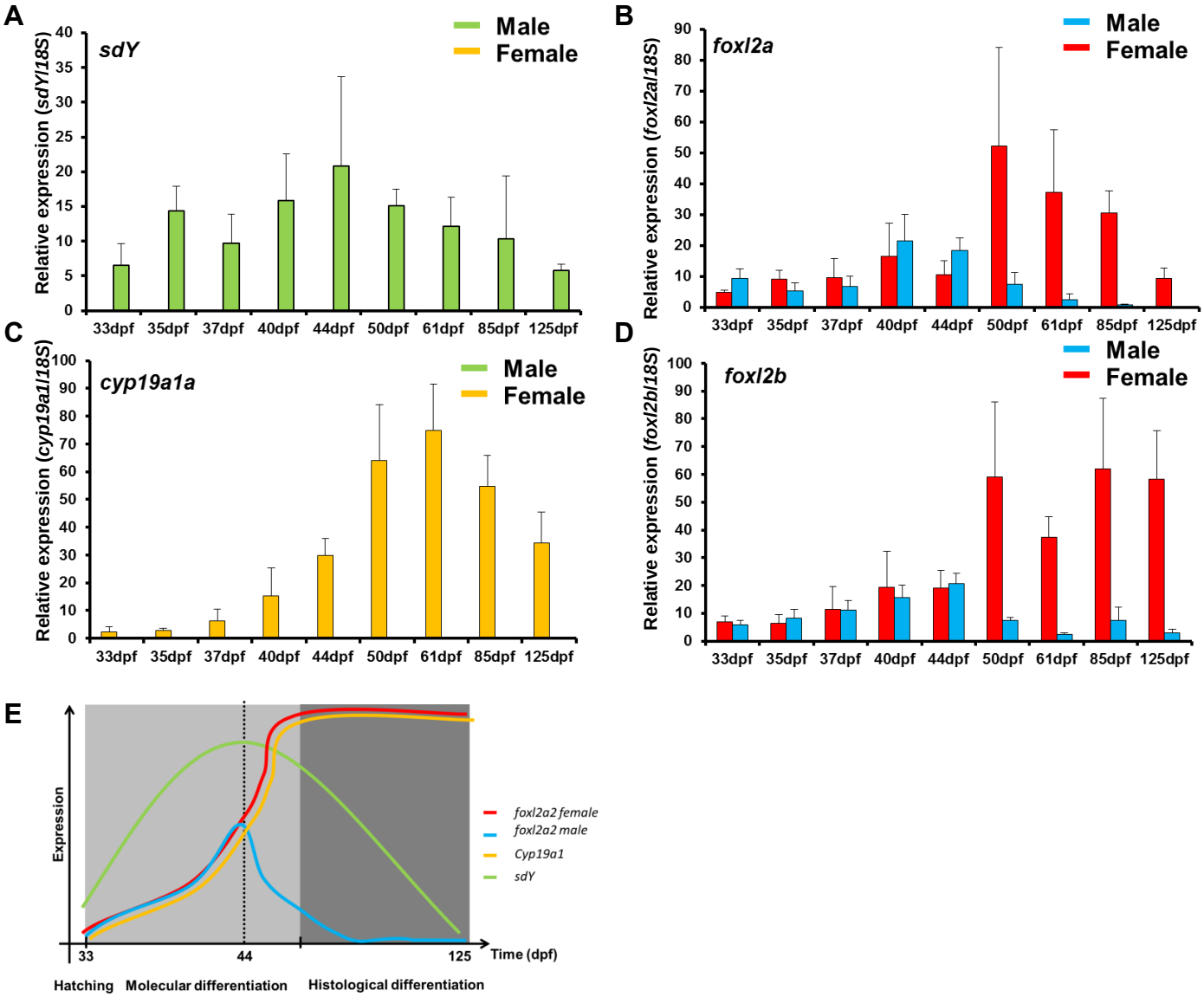


Figure 16. SdY follows the expression of male foxl2s. (**A-D**) Gene expression profile of *sdY*, *foxl2a*, *foxl2b*, *cyp19a1* in male and in female. Both female and male gonads were subjected to qPCR analysis from 33 days post-fertilization (dpf) to 125 dpf. The expression is normalized to 18S. (**E**) Schematic representation of *sdY* (green line), *foxl2b* female (red line), male (blue line) and cyp19a1(yellow line) expression pattern. All the genes showed an increase until 44dpf. Male *foxl2b* expression and *sdY* expression decreased while female *foxl2b* and *cyp19a1* expression rise to reach a plateau. Only *foxl2b* is shown but the same pattern can be observed for

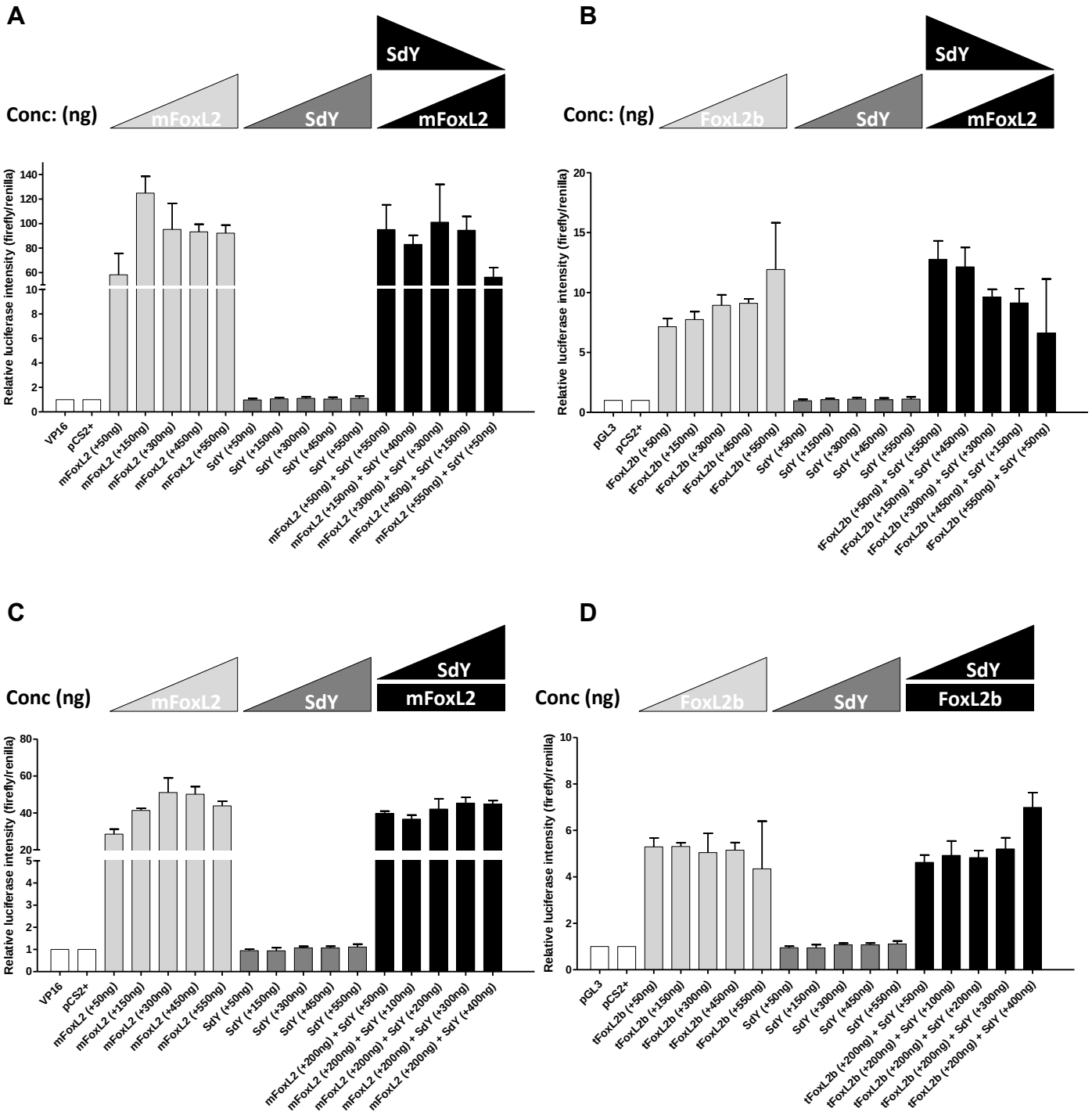


Figure 17. No effect of SdY on gonadotropin releasing hormone (GnRH) receptor activating sequence (GRAS) promoter induced by either mouse FoxL2 or trout Foxl2b. (A-B) SdY and mFoxL2 **(A)** or Foxl2b **(B)** expression vectors (50-550ng) were cotransfected in HEK 293 cells with the GRAS firefly promoter reporter (300 ng/well). **(C-D) SdY,** mFoxL2 **(C)** or Foxl2b **(D)** expression vectors (50-550ng) were cotransfected in HEK 293 cells with the GRAS firefly promoter reporter (300 ng/well). In combination, increasing concentration for SdY (50-400ng) and the concentration (200ng) for mFoxL2 **(C)** or FoxL2b **(D)** were used. VP16, pCS2+, pGL3 were used as plasmid control. The total amount of transfected plasmid, including the pRL-TK control vector (100 ng/well), was adjusted to 1.0 µg with empty vectors. Firefly and *Renilla* luciferase activities were measured 48 h after transfection. Relative luciferase activity was calculated by dividing the firefly luciferase activity with the *Renilla* luciferase activity. The value result from the average ± SD of three biological replicates of one experiment. mouse FoxL2 (mFoxL2); trout FoxL2b (FoxL2b).

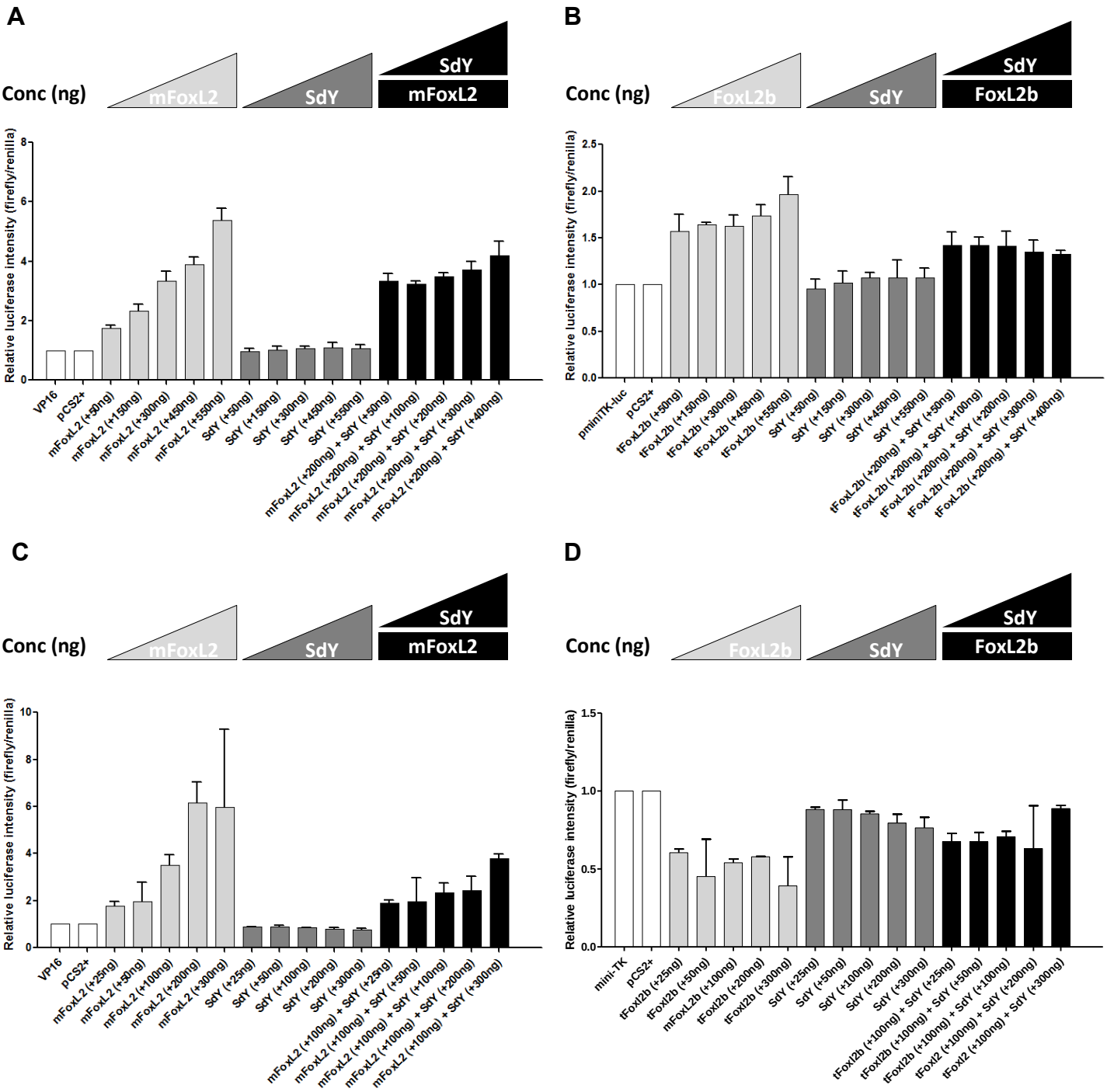


Figure 18. No effect of SdY on 4xFoxl2-Dmrt1-Foxl2 sequence promoter induced by either mouse FoxL2 or trout Foxl2b. (A-B) SdY and mFoxL2 **(A)** or Foxl2b **(B)** expression vectors (50-550ng) were cotransfected in HEK 293 cells with the GRAS firefly promoter reporter (300 ng/well). In combination, concentration gradient was used for SdY (50-400ng) and a constant concentration for both mFoxL2 or Foxl2b (200ng). **(C-D)** SdY, mFoxL2 **(C)** or Foxl2b **(D)** expression vectors (50-550ng) were cotransfected in TM3 cells with the GRAS firefly promoter reporter (300 ng/well). In combination, increasing concentration for SdY (50-400ng) and the same amount (200ng) for both mFoxL2 **(C)** or FoxL2b **(D).** VP16, pCS2+, pmini-TK were used as plasmid control. The total amount of transfected plasmid, including the pRL-TK control vector (100 ng/well), was adjusted to 1.0 µg with empty vectors. Firefly and *Renilla* luciferase activities were measured 48 h after transfection. Relative luciferase activity was calculated by dividing the firefly luciferase activity with the *Renilla* luciferase activity. The value result from the average ± SD of three biological replicates of one experiment. mouse FoxL2 (mFoxL2); trout FoxL2b (FoxL2b).

44 dpf and thereafter declines to 125 dpf (***Figure 16A***). In males for both *foxl2a* and *foxl2b*, an increase until 44 dpf followed by a decrease are observed (***Figure 16B-D***). In females, the expression of *foxl2s* and *cyp19a1 (a Foxl2 target)* are exponential (***Figure 16B-C-D***). In males, cyp19a1 expression is barely detectable (***Figure 16C***). These findings suggest that sdY and foxl2s expression in male are correlated and could act on cyp19a1 promoter. To go further on the hypothesis, I evaluated the activity of Foxl2 on different promoters (3xGRAS and 4xFoxl2-Dmrt1-Foxl2) containing Foxl2 binding sites with either mouse FOXL2 or Foxl2b in presence or absence of SdY in HEK and TM3 cells. Gradual amount of mouse FoxL2 (mFoxL2), trout Foxl2b, and SdY was assessed. mFOXL2 was used as control. Both mouse FOXL2 and trout Foxl2 alone was able to activate gene transcription (***Figure 17 ABCD*** and ***Figure 18 ABC***) except for Foxl2b in TM3 cells (***Figure 18 D***) for each promoter tested. SdY alone presented no differential activities as expected. In combination with SdY both mouse or trout FoxL2, the luciferase activities were similar to FoxL2 alone either in gradient (***Figure 17 AB***) or with a constant concentration but not marked by a repression in both HEK and TM3 cell lines (***Figure 17 CD and Figure 18 ABC***).

This second part aims to understand an intriguingly phenomenon occurring in Chinook salmon, *Oncorhynchus tshawytscha* that also has SdY and is believed to function like in rainbow trout. In this species, in some wild-caught fish, a discrepancy is observed between the genotypic and the phenotypic sex. Phenotypic female Chinook salmon that have a male genotype, as deduced from the presence of a male specific marker, are fertile and cannot be visually distinguished from genetically normal females (***Williamson and May, 2002***). Surprisingly, this observation has been reported several times and in Northwest Pacific regions Columbia river (Nagler et al., 2001b), Alaska, Idaho, and Washington (***Cavileer et al., 2015)***, Southwest California region (Williamson and May, 2002, 2005; Williamson et al., 2008a). Three independent hypotheses were proposed to elucidate the underlying mechanism. The first possibility was that salmons could have a sex reversal due to endocrine-disruptor chemicals (EDCs) or pollutants exposition (Nagler et al., 2001b). This hypothesis was rejected using artificial crosses between genotypically normal males (XY) and apparent sex-reversed males (XY females). Genotyping was performed using two different Y chromosome markers OtYl and growth hormone pseudogene (GH-Psi). The breeding results in half phenotypic female offspring of XY females which have a male genotype according to both Y-chromosome markers (Williamson and May, 2005). In addition, Williamson et al. analyzed both X and Y chromosomes of offspring produced by normal and "apparent" XY-female Chinook salmon using Fluorescence in Situ Hybridization (FISH). FISH analyses suggest that apparent XY-female Chinook salmon in California are not the product of a Y chromosome to autosome translocation (Williamson et al., 2008a). Up to now, two explanations are still subject of debate. The first one is that it could be a recombination of markers between the sex chromosomes. The second is that a Y chromosome retains a dysfunctional or missing sex-determining region (Williamson et al., 2008a). *sdY* could be a good candidate to decipher the underlying mechanism of observed incongruence between the genotypic and the phenotypic sex in Chinook salmon.

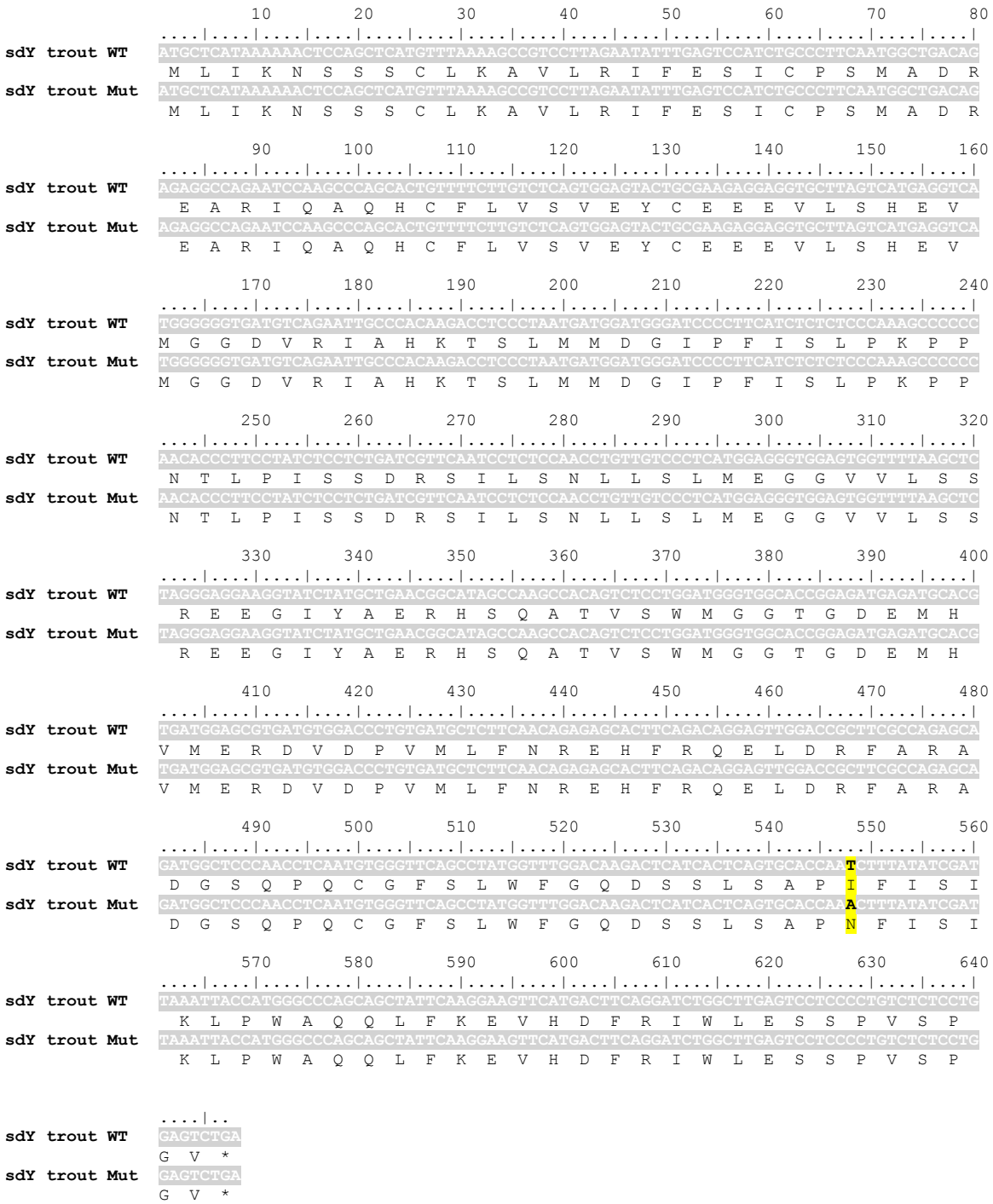


Figure 1. Sequence alignment of SdY and SdY I183N. Nucleotide and amino acid alignment of SdY and mutated SdY (SdY I183N, highlighted in grey) found in Chinook salmon.

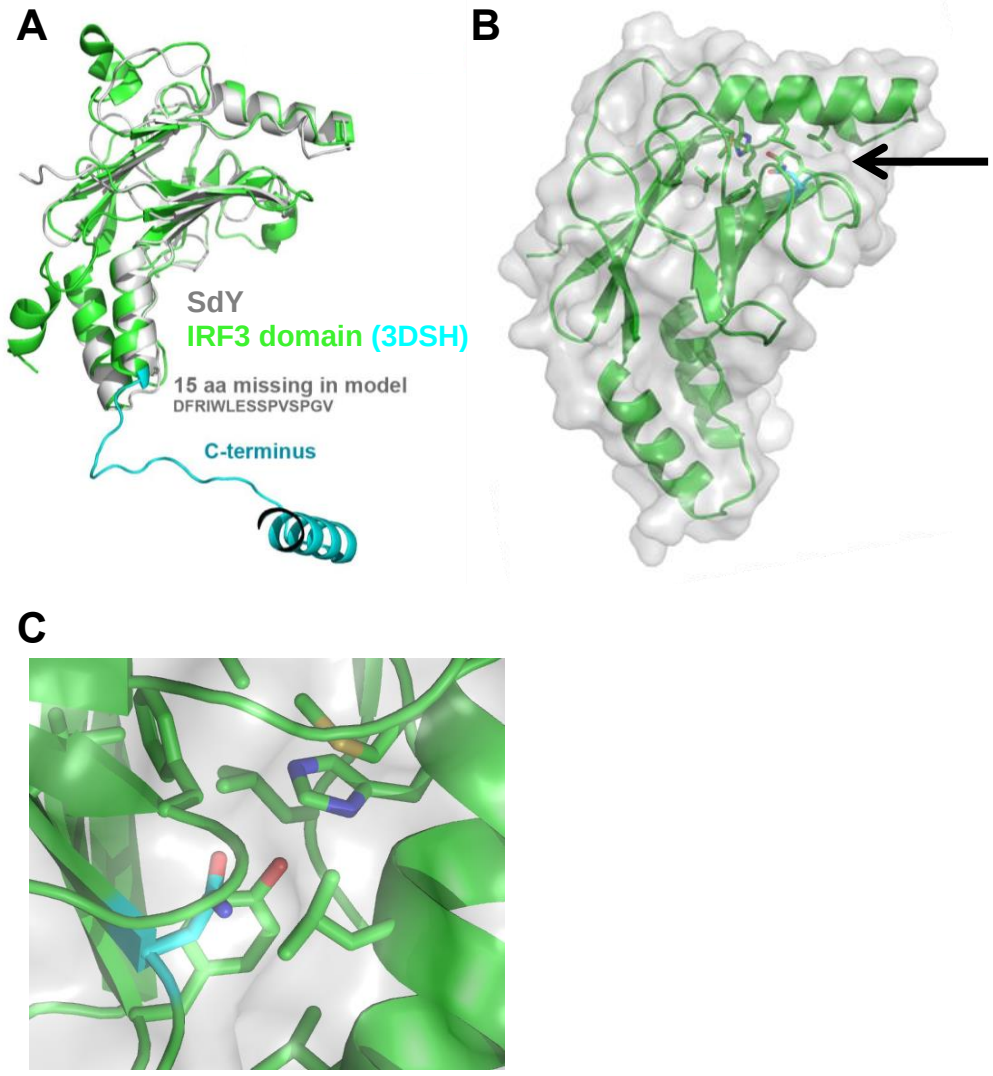


Figure 2. The mutation I183N locally affects the structure of SdY. (A) Native structure structure of SdY (grey) using IRF3 domain (green, PDB code 3dsh) as template. (**B**) The same template has been used to model SdY I183N (green). The mutation located in the core protein, shown by a black arrow. (**C**) A close-up view of the region. The mutation (N-Asparagin) is shown in cyan. Asparagin amino acid is embedded by a hydrophobic pocket leading to a local unfolding.

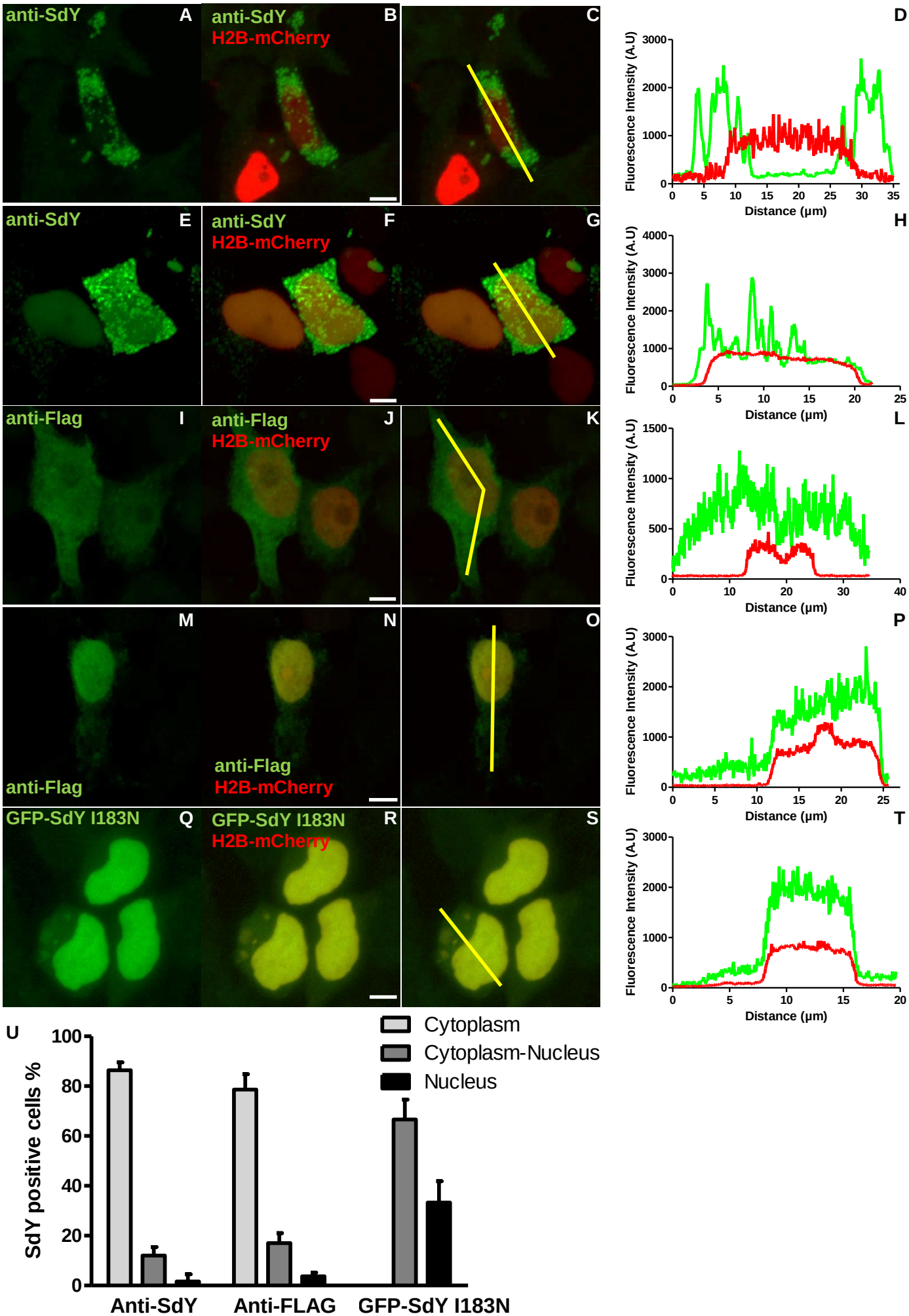

anti-SdY
A
anti-SdY
H2B-mCherry
B
C
D
Fluorescence Intensity (A.U)
Distance (μm)
anti-SdY
E
anti-SdY
H2B-mCherry
F
G
H
anti-Flag
I
anti-Flag
H2B-mCherry
J
K
L
M
anti-Flag
N
anti-Flag
H2B-mCherry
O
P
GFP-SdY I183N
Q
GFP-SdY I183N
H2B-mCherry
R
S
T
U
SdY positive cells %
Cytoplasm
Cytoplasm-Nucleus
Nucleus
Anti-SdY
Anti-FLAG
GFP-SdY I183N

Figure 3. The mutated version of SdY localizes differently. (A-H) Human Embryonic Kidney cell line (HEK) were transiently co-transfected with SdY I183N and Histone H2B-mCherry (nucleus marker) expression constructs. SdY I183N was detected with anti-SdY antibody (green) displaying a highest cytoplasmic and a weaker nuclear localization (**A,B**) along the cell confirmed by the fluorescence intensity profile **(D**) prepared from the yellow drawn line in **C**. SdY I183N can be also distributed in a nucleo-cytoplasmic manner (**E, F**). Analysis of the fluorescence intensity from the drawed yellow line in G shows the nucleo-cytoplasmic pattern between SdY (green) and H2B-mcherry (red) (**H**). (**I-P**) SdY was fused with Flag tag in N-terminal and cotransfected with H2B-mcherry in HEK cells. (**I, L**) Nucleocytoplasmic localization of SdY I183N (**I,J**) with a lighter expression of SdY I183N into the nucleus confirmed by the fluorescence intensity analysis (**L**) from (**K**). (**M,P**) Nucleocytoplasmic localization of SdY I183N (**M,N**) with a higher expression of SdY I183N in the nucleus confirmed by the fluorescence intensity analysis (**P**) from (**O**). **(Q-T)** SdY I183N coupled to a green fluorescent protein GFP was transiently cotransfected with H2B-mcherry. Nucleo-cytoplasmic localization of SdY in HEK cells (**Q, R**), confirmed by the fluorescence intensity analysis (**T**) from both GFP-SdY (green) and H2B-mcherry (red) signal along the yellow line (**S**). **(M-P)** Nucleo-cytoplasmic localization of SdY has been also observed revealed by the fluorescence intensity graph (**P**) along the yellow line (**O**). **(U)** Quantification of observed phenotype. Cytoplasmic localization was counted when the majority of signal comes from the cytoplasm. A nucleocytoplasmic localization was counted when a strong signal was detected in both cytoplasm and nucleus. In a same way, a nuclear localization was counted when the signal was detected in the nucleus and when the signal follow the pattern of fluorescence intensity. (Scale Bar, 5 µm)

To better understand the enigmatic phenomenon in Chinook salmon, the fragments corresponding to *sdY* have been amplified and sequenced using as templates animals from the mapping families described in (***Williamson and May, 2005***) . Surprisingly, XY females do have a *sdY* PCR amplification and the sequencing revealed that all these XY females have a SdY protein sequence containing a missense mutation (C to A) triggering the replacement of isoleucine 183 by asparagine (I183N) (***Figure 1***). Isoleucine at position 183 is conserved in all salmonids SdY proteins investigated right now (***Yano et al., 2013***) and in the IAD domain of Irf9 in both Rainbow trout and Atlantic salmon. Interestingly, this amino acid is also conserved in the Irf9 sequence of goldfish (*C. auratus*) suggesting its importance at the structural level. To evaluate the importance of the substitution, Prof. T Mueller first modeled the structure of SdY I183N (green) embedded in molecular surface (grey) using IRF3 domain (code PDB code, 3dsh). The mutation occurs at the beginning of the β2-strand shaping the hydrophobic core pocket important for an interaction (***Figure 2A-B***). Isoleucine substitution by asparagine leads to hydrophobic amino acid to a hydrophilic amino acid. The presence of the hydrophil amino acid in a hydrophobic environment is predicted to create a local misfolding (***Figure 2C***). Altogether the results suggest that a mutated version of SdY exists in female Chinook salmon. This mutation may lead to a local misfolding protein and could affect partner binding.

I further explore the functionality of this mutated version of SdY. The first examination was the localization. For this purpose, mammalian HEK 293 cells were transfected with different plasmids encoding for SdY I183N (untagged, a FLAG-tagged or GFP fusion). After co transfection with histone H2B-mcherry as a nuclear marker, the localization of SdY I183N was detected with either an SdY antibody, a flag antibody or detected by green fluorescence emission. Three different localizations of SdY I183N were observed and compared to the wild type situation. Using an anti-SdY antibody, SdY I183N was mainly distributed (more than 90%) in the cytoplasm in form of punctuate structures surrounding the nucleus. A nucleo-cytoplasmic localization (10%) and nuclear localization (5%) were also detected confirmed by the superposition with H2B-mCherry channel and the fluorescence intensity profile (***Figure 3A-H***). A nucleo-cytoplasmic or a nuclear localization was not observed for wild-type SdY in HEK cells. A more sensitive antibody such as FLAG antibody was used to validate the localization of the mutated version. Similarly, 3xFLAG-SdY I183N was found predominantly in the cytoplasm (75%) also both cytoplasm and nucleus (18%) or exclusively in the nucleus (7%) confirmed by the fluorescence intensity profile (***Figure 3I-P***). Furthermore, to have a clear picture of the localization, a green fluorescent protein or red fluorescent protein were coupled to SdY I183N. Unexpectedly, both fused proteins do not give a clear signal. The observation of the protein was only possible with a high magnification objective.

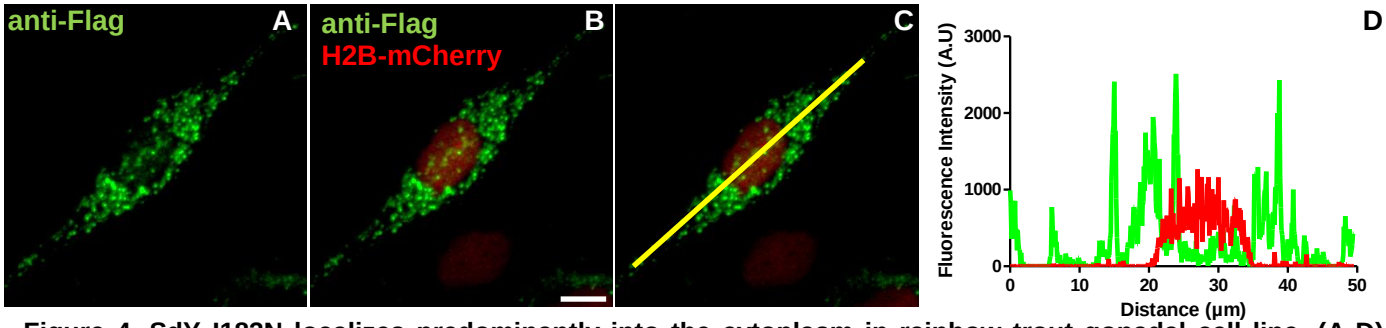
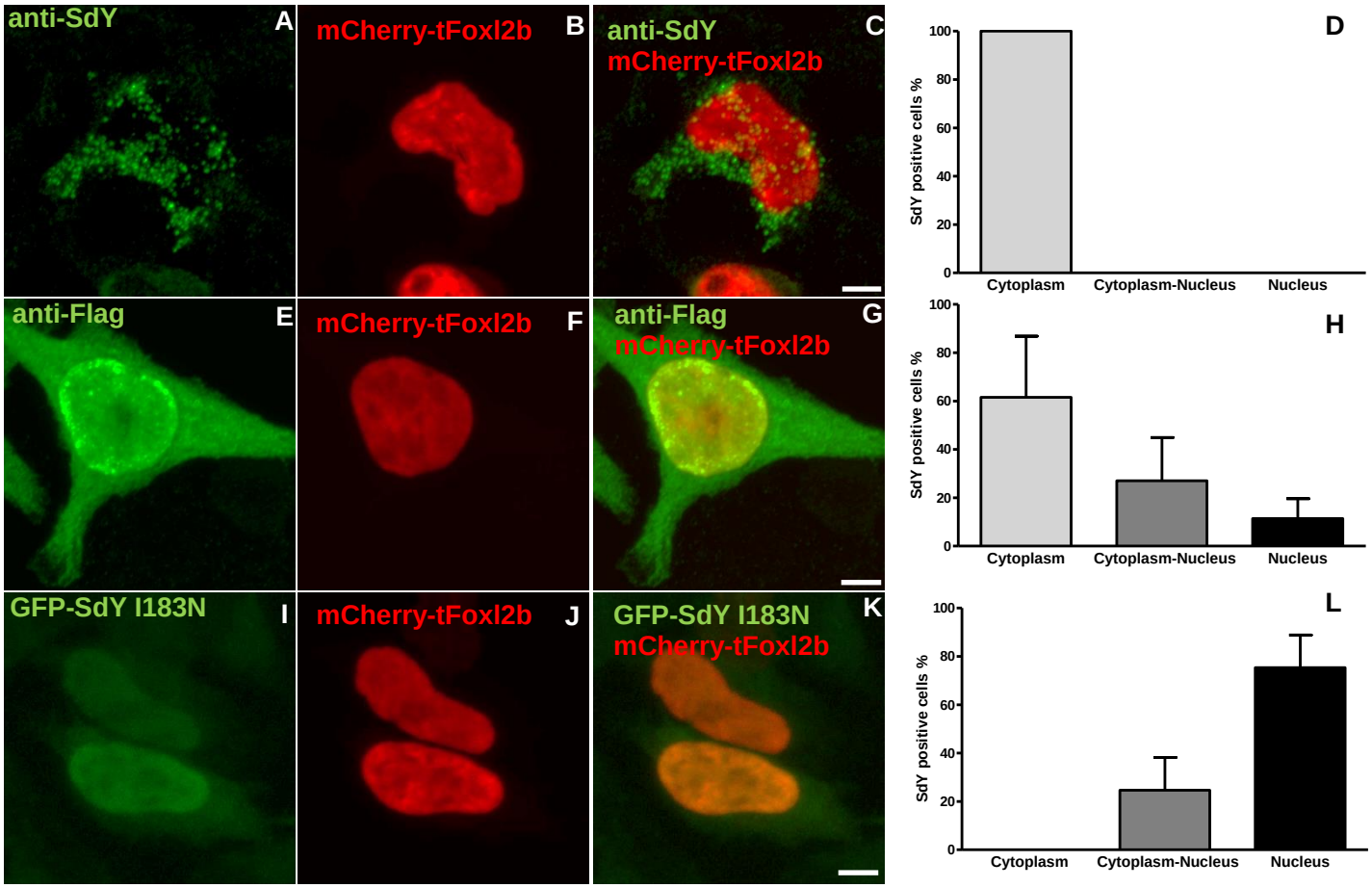


Figure 4. SdY I183N localizes predominantly into the cytoplasm in rainbow trout gonadal cell line. (A-D) Rainbow trout gonadal cell line was transiently co-transfected with both SdY I183N coupled with a Flag tag and Histone H2B-mCherry (nucleus marker, in red) expression constructs. SdY was detected with an anti-flag antibody (green). SdY displays a cytoplasmic and perinuclear localization in RTG2 cells. The yellow line in the image **C** indicates the area selected for fluorescence intensity profile analysis, which are shown in figure **D**. The x axis shows the position along the line, and the y axis shows the fluorescence intensity with SdY (green) and H2B-mcherry (red). The graph revealed that SdY localizes predominantly into the cytoplasm in the form of punctuate. The pattern observed corresponds to hundred cells from one experiment. (Scale Bar, 10 µm.)



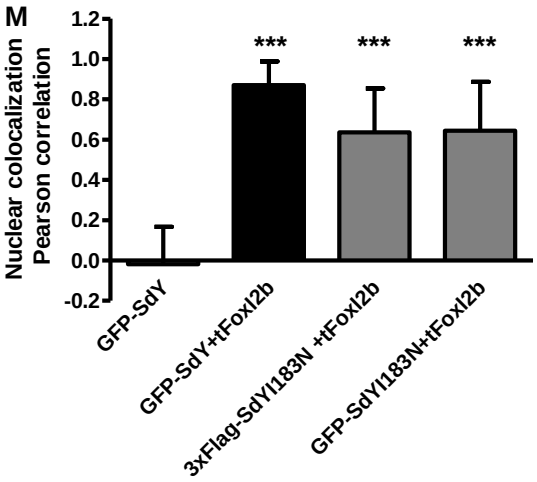


Figure 5. Nuclear translocation mediated by tFoxl2b is impaired with mutated SdY I183N. (A,M) SdY I183N **(A)** or 3xFlag-SdYI182N **(E)** GFP-SdY **(I)**, was overexpressed along with mCherry-tFoxl2b in HEK 293T cells. Mutated SdY was detected either by an SdY antibody **(A, C)** or Flag antibody **(E, G),** or by the green fluorescent protein fused **(I, K)**. **(D, H, L)** The localization of SdY was measured into the cytoplasm, cytoplasm-nucleus or nucleus**.** Each analysis was quantified and reported in **D**, **H**, **L** from the experiment **A** , **E**, **I** respectively. **(M)** Quantification of the nuclear colocalization using Pearson's correlation displays a decrease in both mutated 3xFlag-SdY I183N and GFP-SdY I183N compare to GFP-SdY and tFoxl2b. No significative difference between GFP-SdY and 3xFlag-SDY I183N and GFP-SdY I183N but a significative difference compare to GFP-SdY alone. Statistical significance was calculated using Student's t -test p< 0,001(***). (Scale Bar, 5 µm)

Nevertheless, the localization of GFP-SdY I183N was detected either in the cytoplasm and the nucleus (64%) or exclusively the nucleus (36%). The distribution of GFP-SdY I183N looked smoother than GFP-SdY (***Figure 3Q-T***). Localization was quantified (***Figure 3U***). To validate the localization of SdY I183N in salmonid cells, Rainbow trout gonadal (RTG2) cells were cotransfected with a vector expressing the construct FLAG-SdY I183N and H2B-mCherry. A cytoplasmic localization in form of punctuae distribution was detected by an anti-FLAG antibody confirmed by the fluorescence intensity profile (***Figure 4A-D***). In the RTG2 cell line, no nucleo-cytoplasmic or nuclear localization was observed. Collectively, the data suggest that SdY I183N mislocalizes compare to wild-type SdY situation and that its conformation could be affected.

SdY I183N shows reduced interaction with tFoxl2b

To find out if SdY I183N could bind to tFoxl2 in the same way as wild type SdY or in a different manner, the localization and the interaction has been tested. Under similar conditions as wild-type SdY, HEK cells were cotransfected with mCherry-tFoxl2b and untagged SdY I183N or 3xFLAG tagged SdY I183N or coupled with GFP-SdY. Untagged SdY I183N was detected with anti-SdY antibody. Immunofluorescence revealed that SdY I183N localizes in the cytoplasm forming dots structure. In this experiment, only 50 cells were detectable (***Figure 5A-D***). To confirm this localization, I next used the construct composed of FLAG sequence in the N-terminal part of the SdY I183N coding sequence. In that case, SdY I183N was detected with an anti-FLAG antibody. The cotransfection with 3xFLAG-SdY I183N and mcherry-tFoxl2b revealed that SdY I183N mainly localizes in cytoplasm (60%), in the nucleo-cytoplasm (25%) and in nucleus (15%). A representative picture of a nucleo-cytoplasmic localization is shown (***Figure 5E-H***) where SdY appears in green and tFoxl2b in red. The colocalization between SDY I183N and tFoxl2b in the nucleus has been measured using Pearson's correlation. In ***Figure M***, compared to SdY alone as a negative control and GFP-SdY as a positive control, the colocalization coefficient of 3xFlag-SdY I183N reaches 0.6 suggesting that the colocalization still persist but it is lesser than for wild type SdY (***Figure 5M***). A third construct has been used to explore the behavior of SdY I183N in presence of tFoxl2b. GFP-SdY I183N showed either a nucleo-cytoplasmic (33%) or a nuclear localization (66%) (***Figure 5I-L***). The nuclear colocalization between GFP-SdY I183N and mcherry-tFoxl2b was estimated at 0.65. Comparing to 3xFlag-SdY I183N, the colocalization coefficient number are quite similar. A t-test revealed that the difference is significant between GFP-SdY alone and 3xFlag-SdY I183N or GFP-SdY I183N but is not significant between GFP-SdY and either 3xFlag-SdY I183N or GFP-SdY I183N (***Figure 5M***).

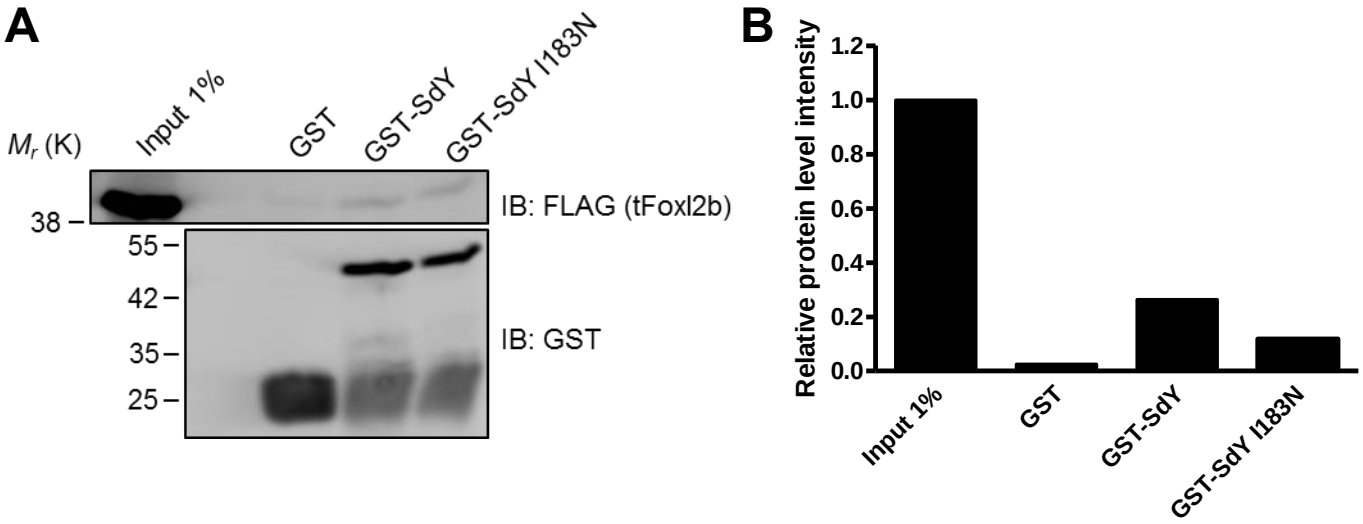


Figure 6. SdY I183N slightly interacts with tFoxl2b. A. Purified GST and GST-SdY was incubated with sepharose beads. Cell extract (50 µg) from 293T cells transiently transfected with expression vector for 3xFLAG-Foxl2b was added to the beads complex. 20 µg of GST, GST-SdY or GST-SdY I183N was used to pull down tFoxL2b. Membrane was re-probed with anti-GST antibody. **B.** Quantification of **(A).**

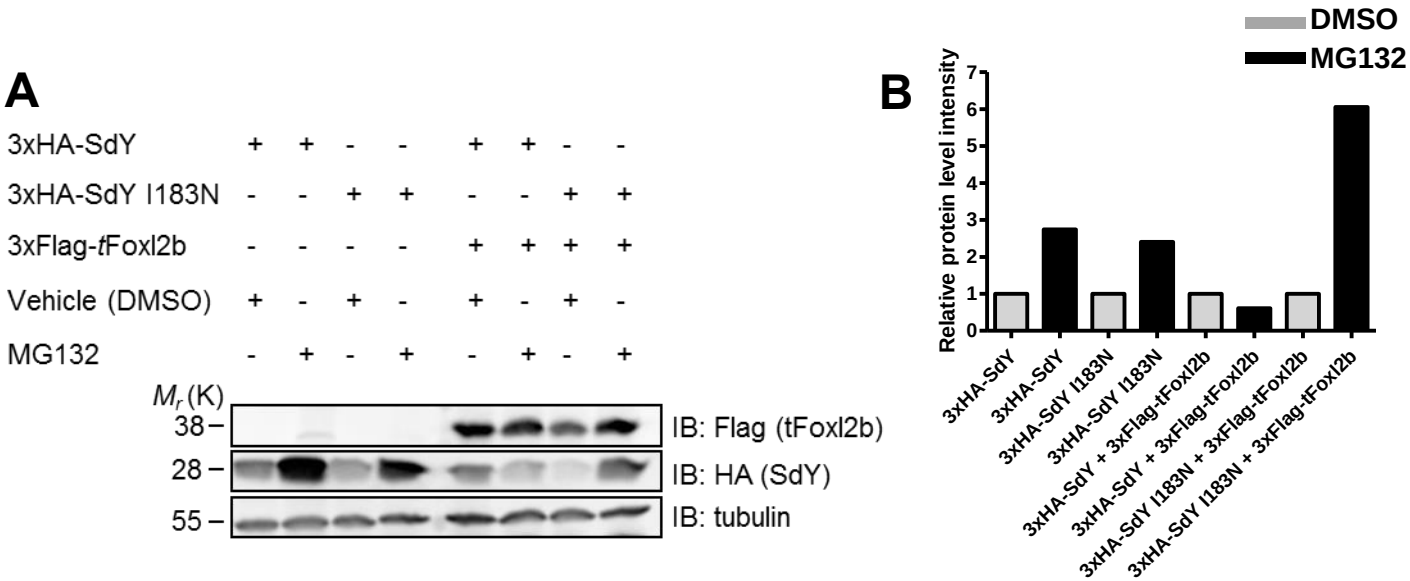


Figure 7. SdY is stabilized with tFoxl2b but not SdY I183N. **A**.Western blot analysis of SdY, SdY I182N or tFoxl2b protein levels following 8H treatment with a proteasome inhibitor MG132. 293T cells were treated with DMSO (vehicle control), MG132 (20 µm). Tubulin was blotted as a loading control. **B.** Quantification of **(A).**

Collectively, the localization of SdY I183N in presence of tFoxl2b suggest that both protein can colocalize but to a lesser extent than with wild-type protein.

Next I sought to determine if SdY I183N can bind to tFoxl2b in a similar or different manner as wildtype SdY. A GST pull down has been carried out. After overexpression in bacteria both GST-SdY and GST-SdY I183N were insoluble. Extraction of fused protein from inclusion bodies was performed by sarkosyl. Using purified GST, GST-SdY or GST-SdY I183N protein I investigated its ability to bind to tFoxl2b. Following cell lysis from HEK 293 cells transfected with 3xFLAG-tFoxl2b, a GST pull down assay has been performed. The pull-down complex was subjected to SDS-PAGE and Western blotting with antibody against FLAG peptide revealing the presence of tFoxl2b. As a control of the overexpression of 3xFLAG-tFoxl2b, input lane was characterized by a strong band at 38 kDa corresponding to tFoxl2b. GST-SdY, GST-SdY I183N were able to pull down 3xFLAG-tFoxl2b but not GST (***Figure 6A***). The relative protein level intensity was measured (***Figure 6B***). The quantification demonstrated that SdY or SdY I183N bind to 3xFLAG-tFoxl2b but with a less strong interaction for GST-SdY I183N. These data provide further evidence that SdY I183N is able to interact with tFoxl2b but to a lesser extent than the wild type SdY.

Unstable SdY I183N triggers its degradation

Local misfolding from the structural model, unclear localization and weak interaction of SdY I183N suggested that the protein could be degraded by the proteasome system. To test this hypothesis, HEK cells were transfected with the expression vector 3xHA-SdY or 3xHA-SdY I183N and were treated with MG132, a proteasome inhibitor. The degradation was followed by western blot analysis. The treatment leaded to a stronger band observed for both 3xHA-SdY and 3xHA-SdY I183N and confirmed by the relative protein level intensity analysis (***Figure 7A-B***). These data suggest that both SdY and the mutated version are prevented from degradation when treated with a proteasome inhibitor. Interestingly, in presence of tFoxl2b, the expression of wild type SdY was not altered by the treatment. In contrast, SdY I183N was degraded, confirmed in the quantification (***Figure 7A-B***). Collectively, the results demonstrate that both SdY and SdY I183N are degraded in absence of Foxl2b. One possible cause of SdY or SdY I183N degradation could be its instability as a monomer, stability that would be enhance as a SdY:Foxl2 heterodimer. I further investigate the stability of both SdY and SdY I183N by measuring the protein level in HEK 293 incubated for different time periods in the presence of protein synthesis inhibitor cycloheximide (CHX). A sharp decrease was observed for the SdY protein level after 4 hours and then it remained stable during the

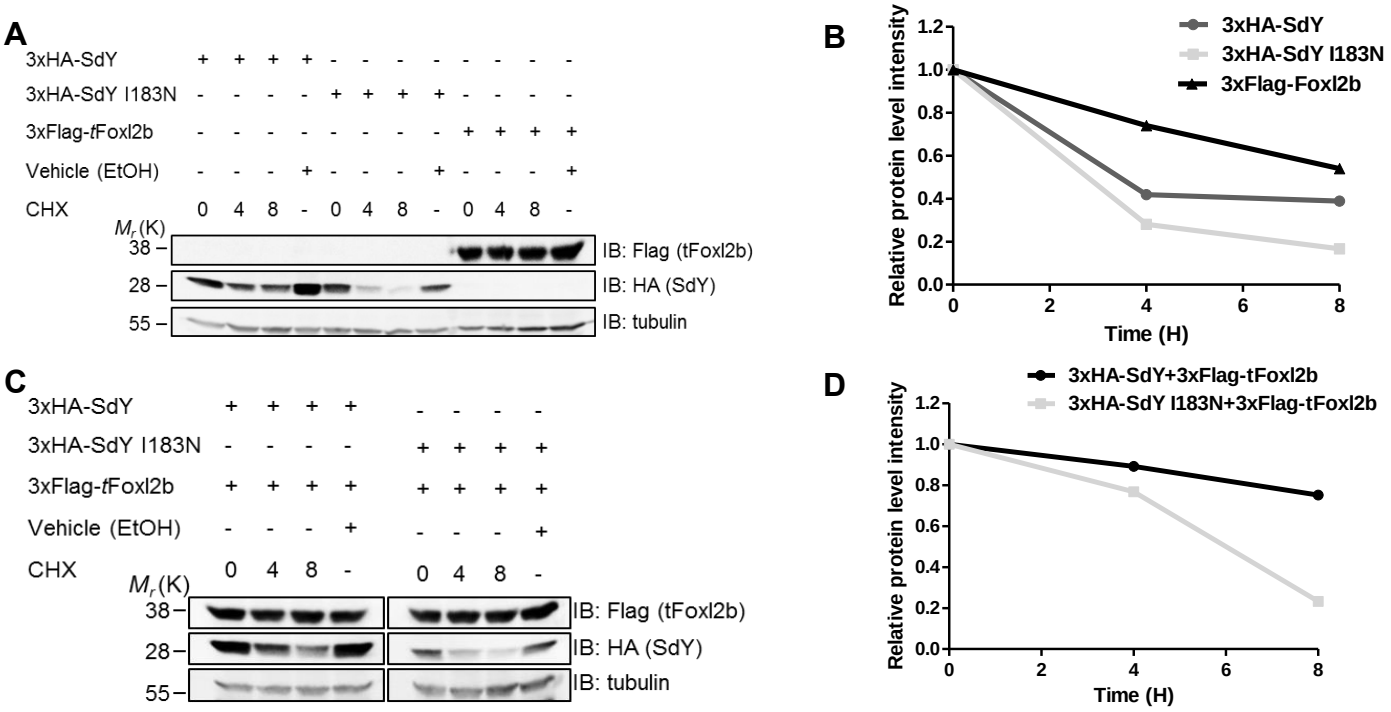


Figure 8. tFoxl2b stabilizes SdY but not SdY I183N. (**A)** cycloheximide (CHX) time course were performed to assess SdY or SdY I183N stability in presence or absence of tFoxl2b. HEK293T cells were transiently transfected with 3xHA-SdY (**A**), 3xHA-SdY I183N or 3xFlag-tFoxL2b (**A**) or in combination (**B**). Cells were treated with 50 µm of CHX and harvest for indicated time (**A** and **B**). Lysates were standardized for total protein concentration and expression levels of 3xHA-SdY (**A**), 3xHA-SdY I183N or 3xFlag-tFoxL2b were detected by Western blotting. Tubulin was blotted as a loading control. tFoxl2b increased SdY, but not I183N stability confirmed by the analysis in **B** and **D**. Only one experiment has been performed.

8H of treatment. In the case of SdY I183N, a severe reduction of protein level was observed confirmed also by the measurement of the relative protein level intensity. Moreover, the cycloheximide treatment slightly affected tFoxl2b. The results showed that SdY and SdY I183N have a turn-over estimated around 3 hours (***Figure 8A-B***). Surprisingly, in combination with tFoxl2b, both SdY and SdY I183N present a high expression protein level during the treatment. This was confirmed by relative protein level intensity measurements. In the case of SdY I183N, a dramatic decrease of protein expression was observed after 4 hours of treatment. Following this observation, the level of expression is similar to the situation in absence of tFoxl2b (***Figure 8C-D***). All together, the data demonstrate that SdY is stabilized by the presence of tFoxl2b. Moreover, SdY I183N is an unstable protein despite the presence of tFoxl2b.

Discussion and perspectives

1. Characterization of SdY

1.1 Cellular localization of SdY

The function of the sex-determining factor SdY has not been characterized so far. During my PhD, I attempted to decipher how *sdY* can trigger its role as a master sex-determining gene. For this purpose, I firstly studied where SdY is localized in the cell using *in vitro* culture systems and after ectopic expression in early embryos of medaka. I demonstrated that SdY alone mainly adopts a cytoplasmic localization with only a low proportion of nuclear localization only observed in some cell dependent contexts. This suggests that SdY could trigger its action in the cytoplasm and in agreement with these results, the sequence analysis of SdY does not show any nuclear localization signal (NLS). A nucleocytoplasmic shuttling by passive diffusion or facilitated diffusion without a NLS tag is however possible when the protein size is smaller than 60 kDa , which is the case for SdY (24,1 kDa) (***Wang and Brattain, 2007***). Likely, SdY is mainly cytoplasmic but a certain fraction could shuttle between the cytoplasm and the nucleus due to its small size. SdY is a divergent version of Irf9 that conserved the IAD protein-protein interaction domain. Similarly to SdY, the IAD domain of Irf9 in the fish common carp is also found in both cytoplasmic and nuclear compartment (***Shi et al., 2012***). On the contrary, in Atlantic salmon and common carp, wildtype full length Irf9 localizes mainly in the nucleus (***Holland et al., 2008; Shi et al., 2012; Sobhkhez et al., 2014***). The same pattern is observed for fish Irf3 and Irf7 (***Holland et al., 2008***). One common feature among the rainbow trout Irf3, Irf7, and mouse IRF5 is the visualization of dots structure around the nucleus (***Holland et al., 2008; Lopez-Pelaez et al., 2014***). The origin of the punctuate structure is unknown. It is not excluded that this punctuate structure originated from degradation due to deleterious effects post transfection. The presence of these punctuate structures is not observed in medaka embryos confirming likely a post-transfection effect. To confirm the SdY localization *in vivo*, immunohistochemistry experiments were performed by Elodie Jouanno on gonad tissues. Unfortunately, SdY was not detectable mainly explained by the difficulty to work with gonad tissue and the antibody.

1.2 Is SdY can be post-translationally modified?

A posttranslational modification such as phosphorylation or a protein-protein interaction can modulate the localization and the activity of a protein. The phosphorylation state is a key modification in the IRF family, the ascendant family of SdY. The phosphorylation promotes the activation of IRF3 and IRF5 enabling the formation of homodimers or heterodimers (***Chen et al., 2008; Qin et al., 2003; Takahasi et al., 2003***) . Phosphorylated serine in IRF3

and IRF5 lie on the same amino acid sequence position (ISN) in the IAD domain. This crucial position is however not found both in SdY and the IAD sequence of Irf9. Our *in silico* model of SdY suggested that SdY is more similar to phosphorylated (activated) IRF3 and IRF5 forms. These findings stipulated that SdY could be not phosphorylated in the same manner as IRF3 and IRF5 but the shape could imitate a phosphorylation state. Further experiments will be necessary to validate or not the phosphorylation state of SdY by using antibodies against phosphorylated amino-acid. Then, the phosphorylation site could be confirmed by mutagenesis.

1.3 Based on its 3D structure reconstruction, could SdY, be involved in TGF-ß signaling?

To date, members of sox, dmrt and TGF-β families can lead the developmental pathway at the top of the male sex determination cascade in vertebrates (***Herpin and Schartl, 2015***). On the contrary, sdY is derived from the Interferon regulatory factor 9 (IRF9) family. The question on the function of *sdY* is then: is the sdY function derived from one of the functions of its ancestor Irf9 (sub-functionalization hypothesis) or is sdY evolved a totally new function (neo-functionalization hypothesis)?

The sub-functionalization hypothesis would imply a yet non-described implication of Irf9 and / or the interferon pathway in gonadal sex differentiation. This absence of published study on the interferon signaling pathway during gonad development could however be interpreted as an absence of any major implication of this pathway in that process albeit Irf9 is ubiquitously expressed in Atlantic salmon (***Sobhkhez et al., 2014***) and rainbow trout differentiating gonads (INRA lab, Ayaka Yano).

To better decipher the underlying mechanism of SdY, its protein structure has been studied in collaboration with a protein structuralist, Prof. Müller. The information deduced from the structure of IRF-3 domain (structural domain name given for interferon associated domain, IAD) and the alignment between different IRFs proteins suggested that SdY has a conserved β-core sandwich decorated with an α-helix in N and C-terminal position. These key elements provide an interaction core module. Interestingly, despite having divergent sequences, this IRF-3 domain shares some structural similarities with the Forkhead associated domain (FHA) and Mad Homology 2 (MH2) subdomain of the SMAD proteins that are signaling effectors of the TGF-β signaling pathway. IRF3 domain, FHA and MH2 domain are well studied protein-protein interaction domains (***Macias et al., 2015***). A further interrelation exists between the IRF and the SMAD protein in terms of their mode of action. Upon phosphorylation, both IRF3 and SMAD proteins oligomerize and enter in the nucleus to exert their function as

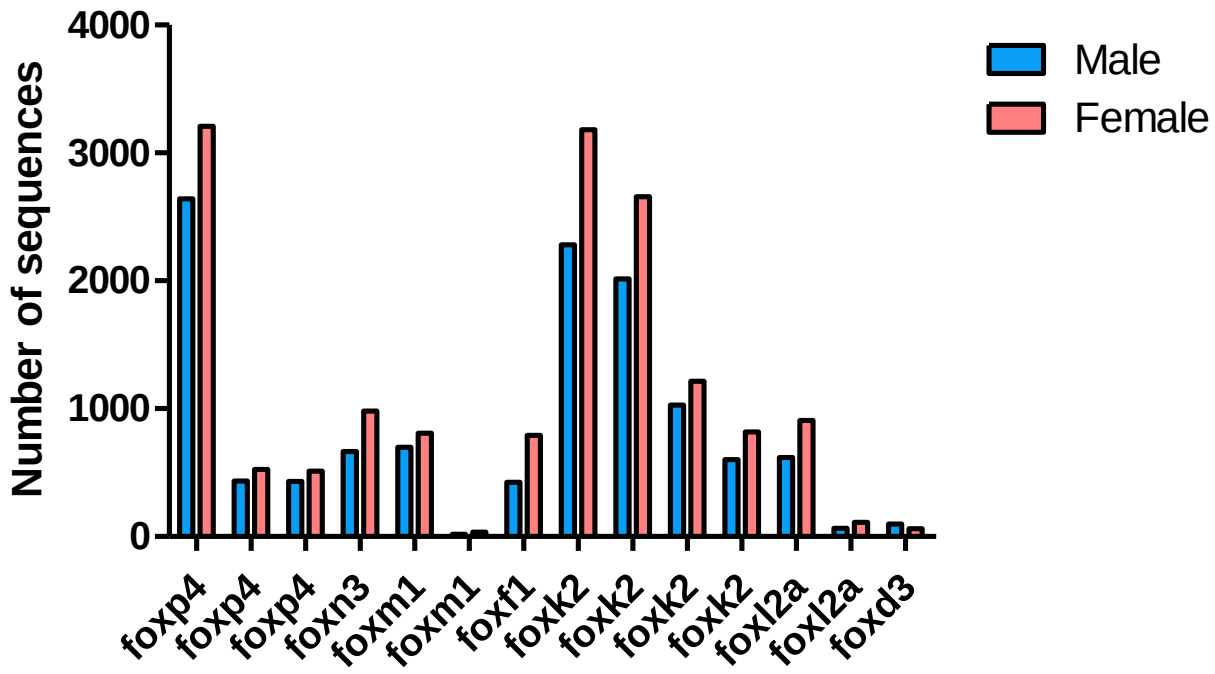


Figure 1. Number of RNA sequences corresponding to each Fox clone found in the yeast two hybrid screen during the very early phase of sex differentiation (30-36 dpf) in both male and female gonads. The number of sequences in both male (blue) and female (female) gonad is depicted according to the transcripts of gonad sample.

screen. Our results indicate that the 3D structure of SdY better fits with the phosphorylated or in other words, activated form of IRF3 domain than its non-phosphorylated form. Furthermore, MH2 domains assemble in a homotrimer structure. However, SdY could not recapitulate the homotrimer association formed with MH2 domains predicting that SdY is a monomer. Nevertheless, SdY undoubtedly conserved structural core domain such as the β-sandwich and the basic domain on the surface providing a platform of interaction. This would put SdY among the known sex determining gene families, near the TGF-β family genes suggesting a possible role in this pathway. It is tempting to hypothesize that during the evolution, a protein rewiring occurred meaning that instead of being involved in the interferon signaling pathway, SdY switched to TGF-β signaling pathway. Further experiments should rely on a possible physical link between SdY and SMAD proteins and ultimately on how it could impact sex determination/differentiation. For example, SdY and different SMAD interactions could be easily performed with biochemical or immunolocalization assays.

2. SdY interactome

Based on the fact that SdY lack the transactivation domain of its ancestor protein, Irf9, we hypothesized that it could trigger its effects through protein-protein interactions and investigated these potential SdY protein partners using a yeast-two-hybrid (Y2H) screen. To date, the interactome of most vertebrate sex determining factors is unknown and no protein-protein interaction methods have been used to identify protein partners for any sex determining factors in fish. Even SRY, the mammalian sex-determining factor has been poorly investigated in that regards. It has been shown however, that the HMG domain of SRY and members of sox family can interact via their C-terminal domain with many of transcription factor domains including bHLH, homeodomain proteins, zinc finger proteins, basic helix–loop–helix and leucine zipper proteins (***Wissmuller et al., 2006***). In addition, SRY can bind with different partners such as covactivators (NR5A1), epigenetic regulators (HDAC3), and a female factor, nuclear β-catenin (See Introduction). Nevertheless, there was no common partner between SdY and SRY found in our Y2H screen for SdY, supporting that the mechanism of testicular induction is likely to be different.

The most astonishing result of your Y2H screen was that the high proportion of Fox proteins that could bind to SdY as among 46 putative binding partners we found 12 different fox proteins. The Y2H was performed when *sdY* reached its maximal expression (75 dpf). We then compare the presence and the expression of the different fox proteins found in the screen with the data obtained from a transcriptome performed during early gonad differentiation (35-40 dpf) in both male and female. Transcriptomic data revealed that the candidates *foxp4*, *foxn3*, *foxk2*, *foxm1*, *foxf1* and *foxl2* (***Figure 1***) were present during the

rainbow trout early gonadal differentiation stage but the precise expression pattern of most of these genes has not been studied. Apart from the expression of foxl2, only two studies explored the expression of Fox genes during gonadal differentiation in medaka and Nile tilapia fish (**Shen et al., 2012; Yuan et al., 2014**). In Nile tilapia, the expression level of some Fox proteins in fish was studied in both male and female differentiating gonads revealing that *foxp4* is highly expressed in male and female gonad during the onset of the sex determination period like what was suggested in rainbow trout based on transcriptomic data. Except a defined role in brain, lung and heart, *FoxP4* implication in gonads, gills in fish, liver and gut has not been deeply explored (**Teufel et al., 2003**). However, two studies demonstrated that Foxp4 acts as a transcriptional repressor and regulates cell differentiation in epithelia. The first study showed that Foxp4 control the epithelial cell differentiation and the regeneration of globet cells (mucus producing cell) by repressing the cell lineage program in the developing lung and second one demonstrate that Foxp4 mediates the suppression of N-cadherin (a component of adherent junction) regulating the neuroepithelium character and progenitor maintenance in the central nervous system (**Li et al., 2012; Rousso et al., 2012**). During mammalian heart development, the migration and the fusion of bilateral cardiac mesoderm tubes is observed to form the primitive heart tube. *Foxp4* $^{(-/-)}$ mice exhibit no tube fusion during heart development but the heart is still formed by the four chambers suggesting that the tube fusion is not required to heart development. In this context, FoxP4 triggers the specification of mesodermal pre-cardial cells (**Li et al., 2004**). A potential interaction SdY-Foxp4 could play a role in the differentiation of the epithelial cells of the rainbow trout differentiating male gonad that are strongly expressing *sdY*. A deeper analysis of Foxp4 in gonad should be done to acquire a better knowledge about gonad differentiation.
The other candidate binding partners resulting from our Y2H screen, FoxN3, FoxK2, FoxM1, have a well-demonstrated role in cell cycle regulation in mammals (**Huot et al., 2014; Marais et al., 2010; Wierstra, 2013**). All these genes are expressed during gonad differentiation in Nile tilapia with a male dimorphic expression for *foxn3* and a female dimorphic expression for *foxm1* (**Yuan et al., 2014**). However, the functions of all these Forkhead factors during gonad differentiation are unknown. However it is worth to note that *foxm1* is also highly expressed both in adult testis and ovary in medaka (**Shen et al., 2012**).

Among the Fox candidates obtain in the screen, Foxl2 is the unique partner found that has a known and important functional role in gonad differentiation and maintenance especially in female (See Introduction). The screen revealed a single *foxl2* clone. This finding was unexpected because in a technical point of view, the male immature gonad cDNA library used for the Y2H was established when *sdY* reached its maximal expression (75 dpf). But

according to our results and (**Baron et al., 2004; Vizziano et al., 2007**), at this time point, both *foxl2a* and *foxl2b* mRNA in male are not detected by a sensitive method such as RT-qPCR. This result suggested that some Foxl2a mRNAs were still expressed during this period and that the Y2H technology used was extremely sensitive. Moreover, the presence of Foxl2a is even more surprising because FOXL2 is known to be toxic in yeast system however it could explain why there is also only one clone (**L'Hote et al., 2012**). A physical interaction between SdY and Foxl2a or Foxl2b was confirmed using co-immunoprecipitation approach and SdY and Foxl2b by GST pull down. Both Foxl2a and Fox2b shares 95% of amino acid identities with 98% in the Forkhead domain. In the same line, an interaction occurs between SdY and medaka Foxl2. Both trout Foxl2a and medaka Foxl2 proteins share 92% identities with 98% in the Forkhead domain medaka Foxl2. Similar experiments should be performed using serial deletions to prove that SdY interact with the Forkhead domain.

The most incredible effect of the interaction SdY-Foxl2 is the nuclear translocation of SdY to the nucleus demonstrated after overexpression in different cell lines and *in vivo* in medaka embryos. It seems that after the synthesis of both SdY and Foxl2 proteins in the cytoplasm, the interaction occurs and Foxl2 directs SdY in the nucleus. In fact, Foxl2 is composed of a conventional lysine/arginine rich strong nuclear localization signal (NLS) and a non-conventional NLS located upstream both in the C-terminal of the Forkhead domain (**Moumne et al., 2008a**). Conventional NLS deletion triggers a nucleo-cytoplasmic localization of FoxL2 (**Moumne et al., 2008a**). The nuclear translocation of SdY confirmed on the one hand that this specific interaction occurs at least partially with the Forkhead domain and in the other hand SdY does not hide or prevent NLS recognition by importins. This nuclear translocation of SdY by Foxl2 is only found with fish Foxl2 and is not visualized with mammalian FOXL2 while all FOXL2 proteins contain both conventional and non-conventional NLS. It suggests that SdY likely has a better affinity for fish Foxl2 but the underlying mechanism is not fully understood yet. Among the known interacting partners of FOXL2, many are nuclear proteins (**Georges et al., 2011; L'Hote et al., 2012; Lee et al., 2005**). Up to now, the nuclear translocation of a Foxl2 partners by Foxl2 is only described for SdY. A translocation effect between different fox proteins has been recently demonstrated for FoxK2 and FoxK1 that specifically translocate the cytoplasmic Dishevelled protein into the nucleus to promote wnt/β-catenin signaling (**Wang et al., 2015**). Our study and this from Wang *et al* showed a nuclear translocation effect with three different Fox proteins that raise the question about the existence of a common translocation mechanism among the Fox proteins family to trigger their cytoplasmic partners into the nucleus. This Fox proteins specificity is interesting as different Fox proteins could be involved in many pathways that are vital for the cellular integrity (Benayoun et al., 2011a; Lehmann et al., 2003; Wijchers et al., 2006) and it would

have been difficult to understand how SdY would be able to triggers its specific action in the differentiating gonad without strongly disturbing cellular homeostasis through interactions with multiple Fox proteins.

I also investigated if an interaction could occur between SdY and Foxl3 because *foxl3* is a paralogue of *foxl2* and the closest Fox in the phylogeny of Fox proteins. Despite a rel high divergence in the complete amino acid sequence, these two proteins share a highly conserved Forkhead domain. My results showing that Foxl3 is also able to translocate SdY inside the nucleus albeit to a lesser extent when compared to Foxl2, suggest that Foxl3 can also interact with SdY. This interaction underpins the idea that the Forkhead domain could be the domain involved in the interaction between SdY and Foxl2. This interaction through the Forkhead domain is also suggested by the direct analysis of the results of the Y2H screening. Indeed, all fragments encoding for Fox proteins were aligned and this yield a selected interacting domain which narrow the Forkhead domain. One of the known functions of Foxl3 is to suppress spermatogenesis in female (***Nishimura et al., 2015***). In rainbow trout, Foxl3 is not detected during very early embryonic stages both in male and female gonads. I can be then be hypothesized that the undetectable expression of *foxl3* in male is the direct result of an inhibition due to its interaction with SdY that would prevent an early initiation of spermatogenesis. A likely hypothesis would be just considering that Foxl3 is absent during testicular differentiation and that this interaction with SdY only occurs *in vitro* due to structural similarities shared by Foxl2 and Foxl3 notably by their Forkhead domain, but does not reflect any real in-vivo function.

The Y2H hybrid screen also identified another protein subunit, Kat8 regulatory nonspecific lethal (KANSL), as a putative binding partner. The heptameric KANSL complex in mammals or NSL in Drosophila is a chromatin modifier regulating the expression of thousands of genes by acetylating histone H4 on lysine 16 (H4K16), lysine 5 (H4K5) and lysine 8 (H4K8). In Drosophila, 90% of housekeeping genes are regulated by KANSL (***Feller et al., 2012; Lam et al., 2012***). In mammalian embryonic stem cells, KANSL regulates enhancers and induces differentiation (***Chelmicki et al., 2014; Ravens et al., 2014)***. Mutations in the KANSL1 gene lead to a human genetic disorder named 17q21.31 microdeletion syndrome characterized mainly by intellectual disability and young boys being affected by hypospadias and cryptorchidism (***Koolen et al., 2012; Zollino et al., 2015***). More recently, it has been shown that KANSL1 regulates chromosome segregation and spindle assembly during mitosis (***Meunier et al., 2015***). It will be interesting to determine whether KANSL1 is implicated in testicular differentiation. No specific protein domain was predicted to interact with SdY. Its role related to SdY could be to open the chromatin and facilitates the expression of testis

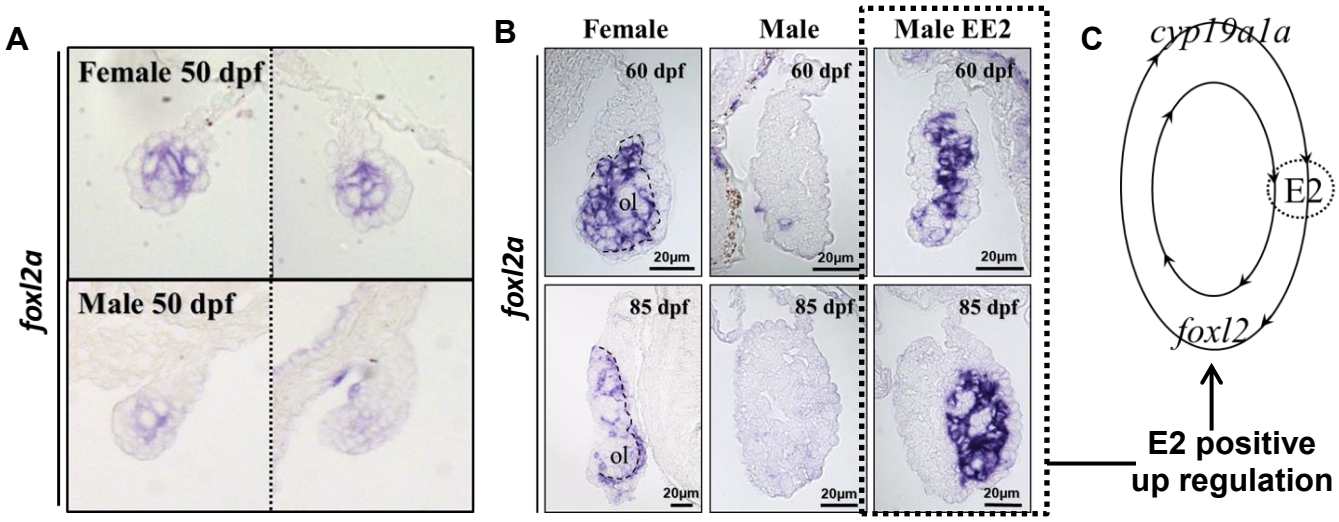


Figure 2. foxl2a localization and its relationship with cyp19a1 and estradiol(E2). (**A**) *in situ* hybridization of *foxl2a* in both female and male left-right gonads at 50 dpf. *foxl2a* is expressed in both female and male somatic cells. (**B**) *in situ* hybridization of *foxl2a* in both female and male gonads at 60 dpf (upper part) and 85 dpf (lower part) and ethinylestradiol (EE2) treated male gonad. Male gonads show strong expression of *foxl2a* after EE2 treatment at 60 and 85 dpf compare to untreated gonad. Foxl2 is largely expressed in ovarian lamellae of female gonads. (**C**) Schematic model of a positive feedforward loop of regulation including *foxl2, cyp19a1* and E2. *foxl2* binds to *cyp19a1a* promoter, in turns, Cyp19a1a protein convert androgens to estrogens (E2) that induces the expression of *foxl2*.

specific genes or likely ensures the induction of differentiating genes. The role of KANSL1 is not defined in testis and due to time constraint has been not explored during my PhD.

3. How could SdY trigger testicular differentiation?

The above-discussed experiments have shed new light on how SdY could trigger testicular differentiation and the interaction between SdY and Foxl2 provided a first very important link with a known pathway of the sex differentiation cascade. But how this interaction leads to testicular differentiation *in vivo* is still an open question?

I will present below three different scenarios that are mutually non-exclusive and could explain how this interaction between SdY and Foxl2 could lead to testicular differentiation .

Scenario 1: repression of ovarian differentiation through inhibition of *cyp19a1a* expression and estrogen production.

The first scenario is that SdY interaction with Foxl2 leads to the repression of the *cyp19a1a* promoter and the inhibition of estrogen production preventing in this way the female pathway to develop. Foxl2 is known to activate cyp19a1 gene in fish and mammals (***Fleming , 2010; Pannetier et al., 2006; Uhlenhaut et al., 2009; Wang et al., 2007a; Yamaguchi et al., 2007)*** and exogenous estradiol up-regulate *foxl2* (***Figure 2***) (***Baron et al., 2004; Wang et al., 2007a***). Moreover, it has been shown that in mammals FOXL2 induces estradiol receptor (*Esr2*) expression (***Georges et al., 2014b***). Hence, a feedforward loop exists in which FOXL2 stimulates estradiol synthesis via cyp19a1 promoter and its receptivity (Esr2 expression) in granulosa cells to maintain its identity (***Georges et al., 2014b; Guiguen et al., 2010***). However, FOXL2 is also able to repress Cyp19a1 at least in mammals (***Bentsi-Barnes et al., 2010; Dai et al., 2013; Kuo et al., 2012***) but no clear explanations are enounced. From gene expression kinetics we know that rainbow trout *foxl2* genes (*foxl2a* and *foxl2b* salmonid specific paralogs) are expressed in both male and female early differentiating gonads, and then repressed in male differentiating gonads when *sdY* reaches its maximal expression. This expression pattern of *foxl2* genes parallels the expression of *cyp19a1a* (my results and (***Baron et al., 2004; Vizziano et al., 2007***) and these expression patterns are in agreement with the hypothesis that SdY could repress both *foxl2* and *cyp19a1a*. This inhibition will ultimately lead to testicular differentiation by preventing the production of estrogen that is needed for the female developmental program to proceed. This key role of estrogens for gonadal differentiation in fish has been explored by inhibition of aromatase activity using specific anti-aromatase inhibitor in many fish species including in rainbow trout (Guiguen et al., 1999a) and this blockage of estrogen production in females

always trigger testicular differentiation even in adult females (for review see (**Guiguen et al., 2010**)). In addition it has been also shown that such an inhibition of endogenous estrogen synthesis in rainbow trout induces a much more complete and physiological testicular pattern of gene expression than the one observed following androgen-induced masculinization (**Vizziano et al., 2008**). This suggests that inhibition of estrogen production could be a physiological process needed for testicular differentiation *in vivo* and that the female pathway including early players like *cyp19a1a* and *foxl2* must be turned off during the first steps of testicular differentiation. In Patagonian pejerrey, the knockdown of the master sex determining gene *amhY* in XY male leads to a rapid increase of *foxl2* and *cyp19a1* expression (**Hattori et al., 2012; Hattori et al., 2013**). Moreover, the gene disruption of *amhy* (sex determining gene in Nile tilapia) in XY male triggers *cyp19a1* expression and similar level of estradiol as detected in female (Li et al., 2015a). Repression of *cyp19a1a* and repression of female ovarian differentiation could then be a conserved mechanism for initiation of testicular differentiation among teleost fish even if the mechanisms of that repression could be different in different species such like SdY-Foxl2 interaction in salmonids or AmhY inhibition of *cyp19a1a* in Pejerrey and Nile tilapia.

To validate this transactivation repression hypothesis, I tested different promoters, including the *cyp19a1a* promoter, containing Foxl2 binding sites coupled with a luciferase protein to evaluate the transcriptional activity of the SdY-FoxL2 complex. These preliminary experiments suggested either that SdY does not modify the transcriptional activity of FoxL2 (discussed in hypothesis 3) or that the effects of the complex were not observable by this method. The latter suggestion is possible because in HEK cells, the presence of SdY is deleterious and the proportion of Foxl2 transfected cells is higher. In TM3 cells, the transfection rate of TM3 cells was very low (around 5-10%) and this high proportion of non-transfected cells could have masked the effects on the luciferase activity. Moreover, Fox proteins alone such as FOXL2 are often not potent transcriptional factor (**Ellsworth et al., 2003**) potentially preventing the detection of clear transactivation effects.

Scenario 2: activation of the male pathway.

The second scenario of SdY-Foxl2 complex action is the activation of downstream specific genes such as *nr5a1*, TGF-β related genes *amh or gsdf* to initiate a male specific program. In the case of *nr5a1*, a sexually dimorphic pattern in favor of male occurs during gonad differentiation (**Hale et al., 2011; Vizziano et al., 2007**). It has been demonstrated in tilapia that the ligand binding domain of Nr5a1 can interact with the Forkhead domain of Foxl2 leading to a synergistic activation of the *cyp19a1a* promoter (**Wang et al., 2007a**). Recently, it has also been shown that FOXL2 can transcriptionally repress the *Nr5a1* promoter by

antagonizing Wilms tumor 1 (or WT1, a zinc finger transcription factor indispensable for urogenital development in mammals) in mouse ovaries, promoting *Nr5a1* expression in testis. The binding site of FOXL2 in *Nr5a1* promoter is highly conserved at least in mammals (**Takasawa et al., 2014**). From these findings, it could be possible that SdY-Foxl2 could bind to the *nr5a1* promoter to induce in turn testis specific genes such as *sox9a2* and simultaneously inhibit *cyp19a1a* expression.

For my theory to connect SdY to TGF-β signaling it should be noted that *amh* and *gsdf* are genes co-expressed few days after *sdY* expression in both rainbow trout and Atlantic salmon in the somatic cells surrounding the germ cells (**Lubieniecki et al., 2015a; Sawatari et al., 2007; Vizziano et al., 2007**). In rainbow trout and Atlantic salmon, Amh could play a role by inducing a PGC proliferation arrest and by inhibiting male steroidogenesis and also female development through the repression of *cyp19a1a* (**Guiguen et al., 2010; Maugars and Schmitz, 2008; Pfennig et al., 2015; Rolland et al., 2008; Vizziano et al., 2008; Vizziano et al., 2007**). SdY like Dmrt1bY in medaka could trigger a mitotic arrest of PGCs prior to the sex differentiation period in a cell non-autonomous manner (**Herpin et al., 2007**). In rainbow trout, masculinizing treatment in XX female by an inhibitor of E2 (ATD) leads to a rapid down-regulation of *cyp19a1a* and a quick up-regulation of *amh* (**Vizziano et al., 2008**). In addition, androgen treatment (11β-hydroxyandrostenedione) triggered a down-regulation of *amh* (**Baron et al., 2008**). Both of them suggest a role of amh during sex differentiation. Some putative binding sites of *Foxl2* were found in the *amh* promoter of Atlantic salmon (**von Schalburg et al., 2011**). The protein complex SdY-Foxl2 could also activate the promoter of Gsdf. This protein is necessary and sufficient to trigger testicular differentiation in various *Oryzias* species and in Nile tilapia and dispensable for testis maintenance suggesting a role upstream Dmrt1 and downstream of the sex determining genes (**Imai et al., 2015**). But the exact role of Gsdf during the sex differentiation period remains unknown.

The complex SdY-Foxl2 could induce expression testis specific genes such as *amh* and *gsdf* that may initiate the male developmental pathway early in the sex differentiation process and long term effects could be induced by the repression of the induction of *cyp19a1a*. Both scenario 1 and 2 are then not mutually exclusive. During the first period of male sex differentiation, SdY and Foxl2 could repress the *cyp19a1a* promoter and concomitantly activate *amh* or *gsdf* gene expression. To confirm either one of the two scenarios, experiments are needed to first prove that the complex binds to DNA and secondly observe an effect on both male and female specific promoters. The complex should ultimately on the one hand activate male specific and/or in the other hand inhibit female specific gene expression.

Scenario 3

My third scenario suggests that the interaction between SdY and Foxl2 would not directly affect the up or down regulation of a target gene through promoter transactivation or repression. In this case, the SdY-Foxl2 interaction would just prevent Foxl2 from binding to DNA through its Forkhead domain. In the case of the *cyp19a1a* promoter, other factors such as *dmrt1* or *dax1* (*nr0b1*) have been shown to suppress *cyp19a1a* expression (***Li et al., 2013; Vizziano et al., 2007***). The possibility that a third partner is also involved in the complex should not be excluded. One of the candidates could be Nr5a1 because a direct interaction with FoxL2 has been demonstrated in Nile tilapia. From the result of the yeast-two hybrid screen, we know that Nr5a1 was not found as a SdY partner suggesting that it would have to bind only to FoxL2. Nr5a1 is a factor in the undifferentiated gonad in male rainbow trout (***Hale et al., 2011***). The interaction with Sf1 involves the possibility that the heterotrimeric complex binds to DNA and regulates the sex specific genes mentioned above. To discover a new partner for the protein complex SdY-Foxl2, a GST pull-down followed by mass spectrometry can be monitored. Purified SdY fusion proteins (HA or GST tag) will be incubated with male gonads lysates. Then, a band corresponding to putative interacting proteins from the protein gel stained with blue Coomassie or silver stained can be studied by mass spectrometry. Ultimately, a putative interacting partner can be identified.

4. Does SdY I183N defines a threshold to induce testicular differentiation?

In Chinook salmon (*Oncorhynchus tshawytscha*), there is a small number of females in which sex phenotypes and sex genotypes do not agree (***Cavileer et al., 2015; Nagler et al., 2001a; Williamson and May, 2002, 2005; Williamson et al., 2008a***) . These females have been identified as XY females based on the detection of a male specific marker and their cytology (***Williamson and May, 2002, 2005; Williamson et al., 2008b***). These XY have been found to have a *sdY* gene but the complete sequencing of sdY in these XY females showed that a missense mutation of SdY in these animals. This single amino acid change (SdY I183N) is predicted to confer a local protein misfolding in the hydrophobic core of SdY leading to a potential cellular mislocalization compared to the native SdY, a quick degradation, and an interaction with Foxl2 with less affinity. Although a better stabilization of SdY I183N is observed during a short period (4 hours) in presence of Foxl2b compared to the absence of with Foxl2b, however after 8 hours, this stabilization is not visualized. The instability of SdY I183 leads to its degradation. Regarding the data, the hypothesis that sdY I183N may not lead to testicular differentiation seems to be confirmed. Given that, the mutated version could not play the same role as the wildtype protein as a sex-determining factor. This discrepancy between a male genotype and female phenotype also exist in wild-

caught medaka from different locations (***Otake et al., 2008; Shinomiya et al., 2004***). According to the strains, three types of mechanisms occurs in XY female, a single mutation leading to a frameshift, exon excision or no mutations but a low expression of *dmrt1bY*. In rodents and humans, independent mutations affect SRY leading to a sex reversal (***Knower et al., 2003; Zhao and Koopman, 2012***). In human, an impaired nucleocytoplasmic trafficking due to mutated hSRY causes sex reversal and defines a transcriptional threshold (***Chen et al., 2013***). Likely, similar to other species, a minimal threshold of functional SdY is required to induce testicular differentiation. In absence of the required threshold, female factors such as Foxl2 and cyp19a1 tilt the balance to the female development and take over the function of SdY I183N leading to a female phenotype.

Conclusion

The main objective of my PhD thesis was to start to decipher how SdY the male sex determining factor in salmonids could trigger testicular differentiation. Among the known sex determining genes, the underlying mechanisms of action of these sex-determining proteins are still incipient. At the onset of gonadal differentiation, the bipotential (or undifferentiated) gonad has the capacity to either develop as an ovary or as a testis. The sex-deter factor tilts the balance in favor of one of these two developmental processes. As SdY lost the transactivation domain of its ancestor gene and retained its protein-protein interaction domain, our working hypothesis was that SdY should interact with a partner to initiate testicular differentiation. My PhD results clearly demonstrated that SdY interacts specifically with Foxl2, a major transcription factor implicated in ovarian differentiation and maintenance also considered as an anti-testis gene.

My demonstration was first based on the identification of different Forkhead box proteins as being potential SdY partners using a large scale Y2H screening approach. With this knowledge I hypothesized that among these Forkhead box proteins, Foxl2 would be the best candidate protein to investigate further. Using biochemical approaches I first confirmed that SdY can bind specifically with fish Foxl2. I then demonstrated, using *in vitro* and *in vivo* experiments, that SdY alone predominantly localizes in the cytoplasm of transfected cells and can be specifically translocaled within the nucleus when co-transfected with fish Foxl2. This result confirmed at the cellular level, that SdY can specifically interact with Foxl2, and suggested that this partnership initiates in the cytoplasm, followed by a translocation of the SdY/Foxl2 complex into the nucleus. This SdY/Fox interaction is highly specific to fish Foxl2 as mammalian Foxl2 and distantly related Fox proteins cannot translocate SdY into the nucleus. However I observed that the Foxl2 closely related spermatogenesis repressor Foxl3 protein can also translocated SdY in the nucleus albeit at a much lower extent. In addition, I found that SdY is more quickly degraded in the absence of Foxl2 suggesting that this protein-protein interaction is important for SdY stabilization.

During my thesis, I also investigated why in another salmonid species, the Chinook salmon, femaleness can proceed in presence of a XY genotype and a *sdY* gene. In this species some XY females have been consistently observed in natural populations. By analyzing these animals in comparison with XY males, we found that XY females have a complete *sdY* gene containing a missense mutation in the sdY coding sequence. Three dimensional modeling of SdY suggest that this mutation (I183N) potentially triggers a local misfolding of the SdY protein structure. Using this mutated version of SdY (SdY I183N), and in vitro cellular approaches, I demonstrated that its cellular behavior is different from the “wild-type” protein with some localization defects and a quick degradation compared to the wild-type SdY. In

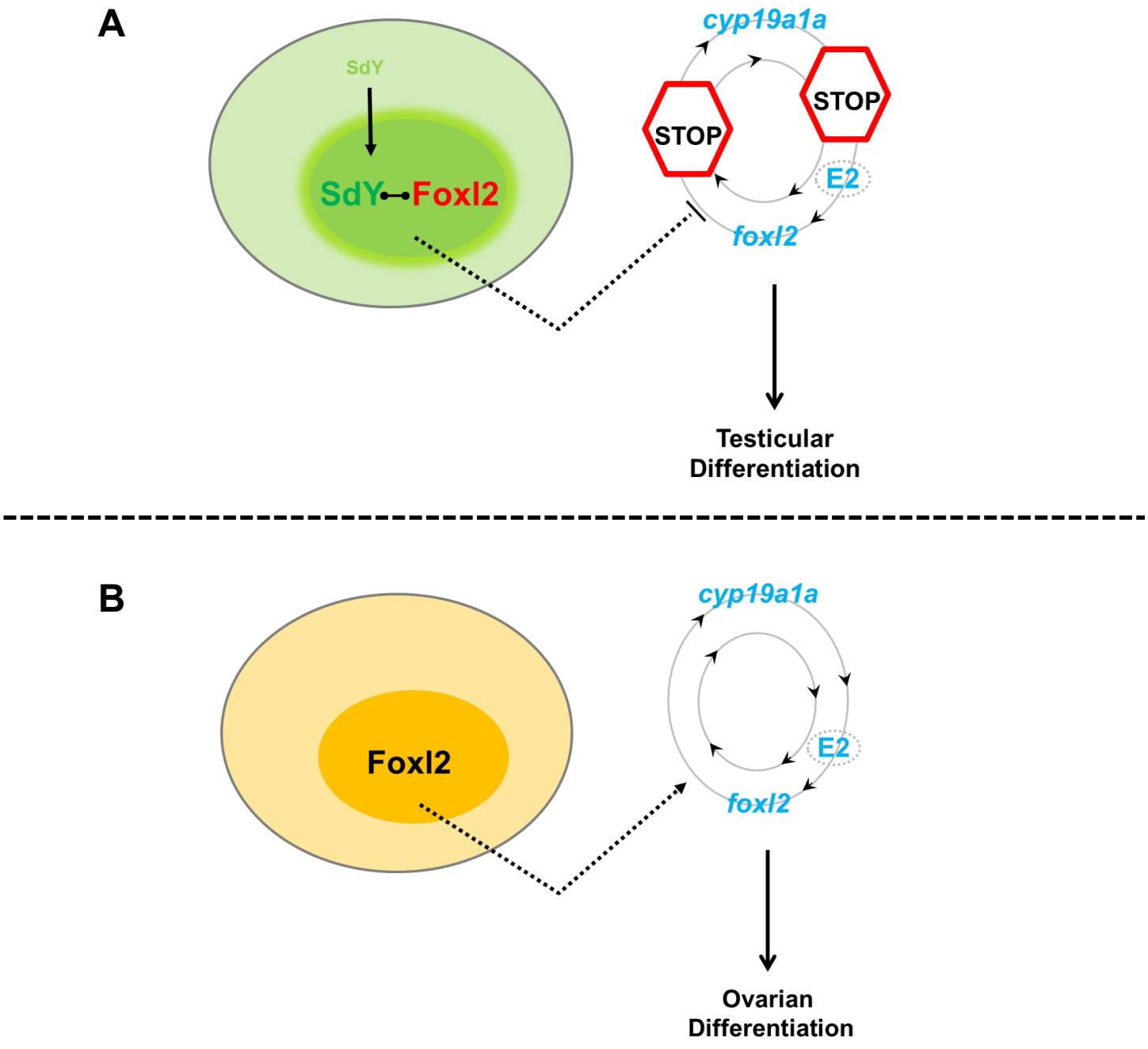


Figure 1. Model of SdY action to initiate testicular differentiation. A Cytoplasmic SdY (light green) binds to Foxl2. Then the SdY (dark green)-FoxL2 (red) complex goes to nucleus. Thus SdY would inhibit the positive feedforward loop involving *foxl2*, *cyp19a1a* and estradiol (E2). Ultimately, in absence of estradiol, the testicular differentiation occurs. **B** In absence of SdY, the feedforward loop triggers ovarian differentiation.

addition, I showed that SdY I183N has a strongly reduced interaction with Foxl2. A better stabilization of SdY I183N is observed in presence of Foxl2 but could not prevent its degradation.

The main conclusion of my work is that SdY associates with Foxl2. This interaction would trigger testicular differentiation by preventing any female cues in the differentiating testis and this could be the first key step to initiate testicular differentiation in salmonids (***Figure 1***).

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Appendix

I - Table. List of putative interacting partners found in the yeast two hybrid screen. SID (Selected Interacting Domain), PBS (Predicted Biological Score).

Clone	Clone Name	SID	Gene Ontology	Localization	PBS
1	Foxn3	FKH domain	Transcription regulator activity	Nucleus	B-D
2	Kansl	No SID	Transcription regulator activity	Nucleus	A-B-D
3	Foxp4	FKH domain	Transcription regulator activity	Nucleus	A-B
4	Foxf1	FKH domain	Transcription regulator activity	Nucleus	B-D
5	(fkh-2)-like	FKH domain	Transcription regulator activity	Nucleus	D
6	Foxm1	FKH domain	Transcription regulator activity	Nucleus	B
7	hypothetical protein with ub domain	Ub Domain	Protein tagging activity	Cytoplasm	B
8	Foxk2	FKH domain + Rel domain	Transcription regulator activity	Nucleus	D
9	Tropomyosin 4	Tropomyosin domain	Structural molecule activity	Cytoplasm	B
10	Foxn2-like	FKH domain	Transcription regulator activity	Nucleus	C-D
11	ran Binding Protein	Ryanodine receptor	Signal transducer actvity	Cytoplasm	D
12	uncharacterized protein with FH domain	FKH domain	Transcription regulator activity	Nucleus	D
13	formin binding protein 4 with WW domain	WW domain	Binding	Cytoplasm	C
14	acetyl-CoA carboxylase 2-like	Carboxyl transferase	Catalytic activity	Cytoplasm	C
15	estrogen-related receptor beta type 1	Nuclear hormone receptor	Transcription regulator activity	Nucleus	D
16	homeobox protein goosecoid isoform B-like	Homeobox domain	Transcription regulator activity	Nucleus	D
17	transcription initiation factor TFIID subunit 1-like	Bromo domain	Transcription regulator activity	Nucleus	D
18	zinc finger and BTB domain-containing protein 16-A-like	Zn and BTB domain	Transcription regulator activity	Nucleus	D
19	collagen alpha-2(V) chain-like	collagen domain	Structural molecule activity	Cytoplasm	D
20	MADD : MAP-kinase activating death domain isoform 1	No SID	Apoptosis regulator activity	Cytoplasm	D
21	zinc finger E-box-binding homeobox 2 isoform 1	Zn Finger domain	Transcription regulator activity	Nucleus	D
22	HERC2	Zn Finger domain + SP domains	Catalytic activity	Nucleus	D
23	hairy/enhancer-of-split	bHLH + Orange	Transcription regulator activity	Nucleus	D
24	Kelch domain - protein protein interaction	Kelch + BTB/POZ +SP	Binding	Cytoplasm	D
25	hypothetical protein	Ub Domain	Protein tagging activity	Cytoplasm	D
26	Foxq1-like	FKH domain	Transcription regulator activity	Nucleus	D
27	zinc finger protein 644-like	Zn Finger	Transcription regulator activity	Nucleus	D
28	human immunodeficiency virus type I enhancer-binding protein 2	Zn Finger	Transcription regulator activity	Nucleus	D
29	Foxk2-like	FKH domain	Transcription regulator activity	Nucleus	D
30	protein osteopotentia homolog	Sad1-unc domain	Nuclear enveloppe	Cytoplasm	D
31	taperin-like (SH3 domain)	No SID	Signal transducer actvity	Cytoplasm	D
32	HMG domain-containing protein 4-like	HMG domain	Transcription regulator activity	Nucleus	D
33	uncharacterized protein with DBD	Homeobox domain	Transcription regulator activity	Nucleus	D
34	latent-transforming growth factor beta-binding protein 2-like	No SID	Signal transducer actvity	Cytoplasm	D
35	mediator of RNA polymerase II transcription subunit 14-like	No SID	Transcription regulator activity	Nucleus	D
36	amyloid beta A4 protein	Coiled domain	Structural molecule activity	Cytoplasm	D
37	fez1	Coiled + Fez like domain	Transporter activity	Cytoplasm	D
38	zinc Finger	Zn Finger	Transcription regulator activity	Nucleus	D
39	Foxl2	FKH domain	Transcription regulator activity	Nucleus	D
40	Intraflagellar transport complex B protein 46 C	No SID	Transporter activity	Cytoplasm	D
41	glucose regulated protein	Coiled	Transporter activity	Cytoplasm	D
42	Signal sequence receptor	Translocon domain	Transporter activity	Cytoplasm	D
43	p53	p53	Transcription regulator activity	Nucleus	D
44	hairy/enhancer-of-split related with YRPW motif 2-like	bHLH + YRPW	Transcription regulator activity	Nucleus	D
45	zinc finger and BTB domain-containing protein 16-A-like	Zn and BTB domain	Transcription regulator activity	Nucleus	D
46	zn271	Zn Finger domain	Transcription regulator activity	Nucleus	D

Foxl2 and its relatives are evolutionary conserved players in gonadal sex differentiation.

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Abstract

Foxl2 is a member of the large family of Forkhead Box (Fox) domain transcription factors. It emerged during the last 15 years as a key player in ovarian differentiation and oogenesis in vertebrates and especially mammals. This review focuses on "*Foxl2*" genes in light of recent findings on their evolution, expression and implication in sex differentiation in animals in general. Homologs of *Foxl2* and its paralog *Foxl3* are found in all metazoans, but their gene evolution is complex, with multiple gains and losses following successive whole genome duplications events in vertebrates. This review aims to decipher the evolutionary forces that drove *foxl2/3* gene specialization through sub- and neo-functionalization during evolution. Expression data in metazoans suggests that Foxl2/3 progressively acquired a role in both somatic and germ cell gonad differentiation and that a certain degree of sub-functionalization occurred after its duplication in vertebrates. This generated a scenario where Foxl2 is predominantly expressed in ovarian somatic cells and Foxl3 in male germ cells. To support this hypothesis we provide original results showing that in the pea aphid (insects) *foxl2/3* is predominantly expressed in sexual females and showing that in bovine ovaries FOXL2 is specifically expressed in granulosa cells. Overall, current results suggest that Foxl2 and Foxl3 are evolutionary conserved players involved in somatic and germinal differentiation of gonadal sex.

Introduction

Fox genes belong to a large family of transcription factors characterized by an evolutionary well-conserved DNA binding domain known as the Forkhead Box (Fox) domain. This domain name, also sometime referred as the winged-helix domain (Lai et al., 1993), was first given based on the fork-headed phenotype observed due to the mutated version of the prototypic *fox* gene (*fkh*) that was initially identified in *Drosophila* (Weigel et al., 1989). The Forkhead domain, which contains 80-100 amino acids, is composed of three α-helices linked with a β-sheet followed by two loops or wings, and binds to the major groove of DNA through its H3 helix (Clark et al., 1993). This binding specificity of Forkhead proteins relies on non-conserved sequences in the basic region of the wings, which interact with the adjacent DNA backbone (Obsil and Obsilova, 2008) on a core consensus sequence (5'RYAAAYA-3', where R=purine (A or G); Y=pyrimidine (C or T)) (Georges et al., 2010). *Fox* genes have been identified in all descendants of the last common ancestor of animals and fungi (Nakagawa et al., 2013), with at least four *Fox* genes in yeast, 16 in *Drosophila melanogaster* (Mazet et al., 2003) and 50 in the human genome (Jackson et al., 2010). Being involved in major signaling pathways such as TGF-ß, Wnt/β-catenin, hedgehog, MAPK, and insulin/IGF, the Forkhead proteins regulate many key processes including embryonic development, cell cycle regulation, cell survival, immunoregulation, metabolism, tumorigenesis, and ageing (Benayoun et al., 2011a; Hannenhalli and Kaestner, 2009; Lehmann et al., 2003; Wijchers et al., 2006).

Since their discovery, some *Fox* genes have been shown to be implicated also in gonadal regulation and sex development with, for instance, *Foxc1*, *Foxl2* and various *Foxo* genes that have been reported to be implicated in the control of ovarian function (Uhlenhaut and Treier, 2011) and *Foxj2*, *Foxp3*, and *Foxo1* in spermatogenesis and testis function (Goertz et al., 2011; Granadino et al., 2000; Jasurda et al., 2014). Among all these genes *Foxl2*, has attracted particular attention during the last 15 years as a key player due to its involvement in sex differentiation and oogenesis (Baron et al.,

2005a; Benayoun et al., 2009; Boulanger et al., 2014; Georges et al., 2014a; Nicol and Yao, 2014; Veitia, 2010). A substantial amount of information on FOXL2 action was initially acquired from studies in humans and other mammals, and many excellent reviews have been already been published, most centered on the roles of FOXL2 in normal or pathological female development in mammals and/or vertebrates (Baron et al., 2005a; Benayoun et al., 2009; Benayoun et al., 2010; Beysen et al., 2009; Biason-Lauber, 2012; Caburet et al., 2012; De Baere et al., 2005; Fuhrer, 2002; Georges et al., 2014a; Kobel et al., 2009; Leung et al., 2016; Moumne et al., 2008a; Pisarska et al., 2011; Rosario et al., 2014; Takahashi et al., 2013; Uhlenhaut and Treier, 2011; Verdin and De Baere, 2012). The present review focuses on "*foxl2*" genes in light of recent findings on their evolution, expression and roles in sex differentiation in animals.

An introduction to Foxl2: early findings, the mammalian view.

Before mutations in *FOXL2* gene were found to be responsible for *Blepharophimosis-ptosis-epicanthus syndrome* (BPES, OMIM #110100) in humans, a condition involving eyelid malformations and premature loss of ovarian function (Crisponi et al., 2001). *FOXL2* was initially named *PFRK* for pituitary forkhead factor based on its first identification as a gene expressed in the pituitary (Kioussi et al., 1999a). In this tissue, *FOXL2* was found to be expressed in all gonadotropes and thyrotropes and a small fraction of prolactin-containing cells during pregnancy, but not in somatotropes or corticotropes. As first demonstrated in the above-mentioned BPES phenotype, FOXL2 is also involved in cranio-facial development (Crisponi et al., 2001). In BPES in humans (Beysen et al., 2008), *FOXL2* haplo-insufficiency leads to eyelid malformation, but its total loss-of-function (complete knockout leading to homozygous null mutations) in mice (Schmidt et al., 2004; Uda et al., 2004) and goats (Boulanger et al., 2014) results in a complete absence of eyelids. Moreover, in cranio-facial development, *Foxl2* is involved not only in eyelid differentiation, but also in extraocular muscle and bone differentiation (Heude et al., 2015). *Foxl2* is also expressed both by cranial neural crest cells

(CNCCs) and by cranial mesodermal cells (CMCs), which give rise to skeletal (CNCCs and CMCs) and muscular (CMCs) components of the head. *Foxl2* conditional inactivation in mice, in either CNCCs or CMCs show that *Foxl2* function in CNCCs is necessary for the development of the levator palpabrae superioris, the superior and inferior oblique muscles of the eyelid. *Foxl2* deletion in either CNCCs or CMCs prevents eyelid closure and induces subtle skeletal developmental defects (Heude et al., 2015). In addition, FOXL2 is also involved in cartilage and skeletal formation, bone mineralization and growth as demonstrated in a constitutive *Foxl2*-deficient mice model (Marongiu et al., 2015; Shi et al., 2014).

The first demonstration of the involvement of *FOXL2* in ovarian development was shown in type I BPES where affected women suffer not only from eyelid malformation, but also from premature ovarian failure (POF) (Crisponi et al., 2001). At about the same time, *Foxl2* was also shown to be involved in the polled intersex syndrome (PIS) in goats, where a natural deletion of 300kb containing the *FOXL2* gene and three long non-coding RNAs, leads to the extinction of *Foxl2* ovarian expression and triggers early testis differentiation and XX female-to-male sex-reversal (Pailhoux et al., 2001a; Pannetier et al., 2012). Since these early results, many studies have confirmed that *FOXL2* is a crucial female-reproductive factor and the recent demonstration that its complete knockout triggers XX sex-reversal in goats now brings *FOXL2* forward as a key ovarian-determining gene (Boulanger et al., 2014). In goats, *FOXL2* knockout leads to a complete XX female-to-male sex reversal accompanied by an agenesis of the eyelid. This sex reversal is characterized morphologically in the gonad by Sertoli-like cells arranged in seminiferous cords, presence of Leydig cells and interstitial cells. XX goat fetuses homozygous for *FOXL2* loss of function mutations are also marked by a more abundant presence of testosterone and complete male genitalia. Moreover, male markers such as *Dmrt1*, *Sox9* are abundantly expressed in the gonad (Boulanger et al., 2014). Consistent with this finding, it has been concluded that *FOXL2* is necessary for the development of the ovary and for maintaining its fate

throughout the life by preventing any male cues. Other *FOXL2* knockout experiments have also been performed in mouse (Schmidt et al., 2004; Uda et al., 2004). Despite a strong perinatal lethality, mice lacking *Foxl2* develop normally until birth and with no perinatal sex reversal (Schmidt et al., 2004; Uda et al., 2004). *Foxl2* loss of function in female mice, however, triggers sterility: the ovaries are small and disorganized where primary follicles are not formed and can't complete maturation. Granulosa cells transdifferentiate into Sertoli-like cells and acquire male characteristics, including the expression of *Sox9*, *Amh* and other genes involved in testicular fate (Ottolenghi et al., 2005). The overexpression of *Foxl2* in XY mice leads to disorganization of the seminiferous tubules and the development of ovotestis-like gonads while *Foxl2* ablation in XY male does not affect the testis development (Ottolenghi et al., 2005; Schmidt et al., 2004; Uda et al., 2004). In conditional adult mice, Uhlenhaut *et al*, demonstrated that FOXL2 normally acts to prevent the transdifferentiation of granulosa cells into Sertoli like cells and theca cells to Leydig like cells by continuously repressing the key testis gene *Sox9* in adult ovaries (Uhlenhaut et al., 2009).

It is interesting to note that *FOXL2* loss of function affects gonad development differently in goat and mouse. In goat, a complete sex reversal is observed while in mouse this is only partial. This difference has been suggested to be in relation with an important role of Foxl2 as a key regulator steroid synthesis, especially estrogen production (Elzaiat et al., 2014; Pannetier et al., 2006; Wang et al., 2007a). In non-mammalian vertebrates, like fish for instance, the aromatase enzyme (Cyp19a1a) needed for estrogen synthesis, is a key enzyme for gonadal sex differentiation, as the presence of estrogens is necessary for ovarian differentiation (Guiguen et al., 2010). In mice, neither estrogen receptors nor aromatase are present during the fetal period (Couse et al., 1999; Fisher et al., 1998), but in other mammalian species the presence of estrogen seems to be required to achieve a complete feminization of the gonad (Boulanger et al., 2014). During this fetal period, FOXL2, through its action on *Cyp19a1* promoter, leads to the synthesis of estrogens at least in goat and humans (Fleming et al., 2010; Pannetier et al., 2006). The absence of a complete sex reversal in *Foxl2*

knockout mice may then be the reflection of this different sensitivity of gonadal sex differentiation to estrogens (Boulanger et al., 2014).

The role of *FOXL2* in ovarian differentiation seems to be highly conserved among vertebrates, as for instance *FOXL2* is one of the earliest sexually dimorphic genes during ovarian development along with *Follistatin* and *CYP19* aromatase, two demonstrated promoter targets of FOXL2 (Auguste et al., 2011; Kashimada et al., 2011; Pannetier et al., 2006). FOXL2 has also been shown to be crucial for sex fate maintenance through its antagonism with DMRT1. In mice, DMRT1 directly represses *Foxl2,* among others, and prevents trans-differentiation in postnatal testis and adult testis (Matson et al., 2011; Minkina et al., 2014). Moreover, retinoic acid signaling promotes male gametogenesis and could trigger Sertoli to granulosa cell trans-differentiation by activating female gene such as *Foxl2* in absence of *Dmrt1* (Minkina et al., 2014). To protect the testicular fate, DMRT1 restricts retinoic acid receptor (RARα) activity in Sertoli cells preventing the induction of *Foxl2* expression (Minkina et al., 2014). In addition, ectopic expression of DMRT1 silences *Foxl2* expression in granulosa cells independent of *Sox9* expression (Lindeman et al., 2015). Recently, it has also been reported that FOXL2 directly binds to estrogen receptor 2 (*Esr2* or *Er-β*) promoter regulating estrogen signaling in granulosa cells, which confirm a positive feed-forward loop of estrogen regulation (Georges et al., 2014b). Moreover, this study suggested that FOXL2 by regulating estrogen signaling reinforces indirectly the repression of *Sox9* (Georges et al., 2014b). In a conditional deletion of Foxl2 in adult mice ovarian follicles, FOXL2 and estrogen receptor (ESR1 or ER-α) synergistically interact and repress the gonad-specific enhancer element TESCO in the *Sox9* promoter (Uhlenhaut et al., 2009).

This role of *FOXL2* in ovarian differentiation seems to be highly conserved among vertebrates, as on one hand, *FOXL2* is one of the genes expressed earliest in ovarian development along with *Follistatin* and *CYP19* aromatase, two demonstrated promoters targets of FOXL2 (Auguste et al., 2011;

Kashimada et al., 2011; Pannetier et al., 2006); on the other hand, FOXL2 has been clearly linked to control steroid synthesis, especially estrogen production representing a crucial event of sexual differentiation in non-mammalian vertebrates, where changing sex-steroid hormones induces sexual fate change into one sense or the other (Elzaiat et al., 2014; Pannetier et al., 2006; Wang et al., 2007a).

The complexity of "*foxl2*" gene evolution

Foxl2 is an evolutionary well conserved transcription factor that has been characterized in many metazoans (see Figure 1 and Table I), including sponges (*Suberites domuncula*) (Adell and Muller, 2004), mollusks (Naimi et al., 2009; Teaniniuraitemoana et al., 2014; Tong et al., 2015; Zhang et al., 2014), arthropods including insects and crustaceans (De Loof et al., 2010; Farlora et al., 2014; Li et al., 2015b; Ma et al., 2012), hemichordates (Fritzenwanker et al., 2014), echinoderms (Tu et al., 2006), cephalochordates (Yu et al., 2008) and urochordates (Yagi et al., 2003). In vertebrates, *foxl2* evolution has been recently challenged based on the existence of highly divergent *foxl2* genes that were initially identified only in some teleost fish species (Baron et al., 2004; Jiang et al., 2011). These additional teleost *foxl2* genes were initially thought to be paralogs resulting from the teleost-specific whole duplication (TGD) that occurred at the base of the teleost radiation roughly 320-400 Mya (Braasch and Postlethwait, 2012; Santini et al., 2009). However, based on the recent availability of additional and evolutionary relevant whole genome sequences in vertebrates, two independent groups (Crespo et al., 2013; Geraldo et al., 2013) revisited *Foxl2* evolution and demonstrated that *Foxl2* is the result of a duplication of an ancestral gene at the base of the vertebrate radiation leading to two vertebrate *Foxl2* paralogs that have been named *Foxl2* and *Foxl3* (Crespo et al., 2013) or *Foxl2a* and *Foxl2b* (Geraldo et al., 2013) (Figure 1). In the present review, we choose to use the gene nomenclature given by Crespo and collaborators (Crespo et al., 2013) (*Foxl2* and *Foxl3*) because this nomenclature accounts better for additional duplicated genes that are still present in some basal

teleosts (see Figure 1 and (Crespo et al., 2013)). We then reserve '*foxl2a'* and '*foxl2b'* names for the teleost-specific *foxl2* ohnologs and use '*Foxl2/foxl2'* and '*Foxl3/foxl3'* for the jawed vertebate (gnatosthomes) ohnologs resulting from the duplication of an evolutionary conserved gene found in metazoans from sponges to urochordates referred as *foxl2/3*. During one of the two rounds (VGD1 and VGD2) of whole genome duplications (WGDs) that occurred at the root of the vertebrate lineage (Dehal and Boore, 2005), the *foxl2/3* gene was duplicated, resulting in two duplicated (ohnologous) genes *foxl2* and *foxl3*. In contrast to rayfin fish (actinopterygians), which all kept a single copy of the *foxl3* gene, independent lineage losses occurred for *Foxl3* in lobefins species (sarcopterygians) because this gene is not found in placental mammals and amphibians. *Foxl3* is however present in the genomes of some birds, marsupials (Crespo et al., 2013; Geraldo et al., 2013) and turtles (Crespo et al., 2013) (see Figure 1).

In contrast to *Foxl3*, *Foxl2* has been conserved in all vertebrates, but its evolution following the TGD is a bit more complicated. The *foxl2* gene was retained as two TGD paralogs i.e., *foxl2a* and *foxl2b* in many basal teleosts (including for instance marine eels, arowanas, herrings, carps, zebrafish, catfishes and cavefish) and kept only as a single *foxl2b* gene in all other teleosts. In salmonids (trouts and salmons), which experienced another round of whole genome duplication (SaGD) around 100 Mya (Berthelot et al., 2014; Macqueen and Johnston, 2014), *foxl2b* was further duplicated, resulting in two co-orthologs of the teleost *foxl2b* gene, we call *foxl2b1* and *foxl2b2* (Figure 1). This pattern of duplication could fit with the simple and parsimonious hypothesis of a single duplication of *foxl2* after TGD leading to two ohnologs (*foxl2a* and *foxl2b*) followed by a secondary loss of *foxl2a* in the Euteleost lineage. However simple phylogeny reconstructions failed to provide a clear picture of TGD paralogy relationships, preventing any clear *foxl2a* and *foxl2b* ohnolog assignments among taxa (authors' unpublished data). This problem may suggest a more complex evolution of teleost *foxl2* genes, with multiple lineage-specific gains and losses at different levels of their evolution. More comprehensive works including for instance synteny reconstruction based on good quality genome information would be needed to get a better picture of this complex evolution.

In contrast to the shared structural features of the DNA-binding domain of Foxl2 and Foxl3, the two proteins have quite different C-terminal domains (Crespo et al., 2013) and these derived proteins retain strong similarities with the Foxl2/3 protein only in the Forkhead domain (Figure 2). During evolution of eutherian mammal, *FOXL2* acquired a polyalanine (polyAla) stretch of 14 alanines amino acid residues. This polyAla tract extension can vary among different individuals, leading to insertion of between 14 to 24 alanine residues and causing pathogenicity. Truncated FOXL2, missense mutations in the Forkhead domain or polyAla expansion in a length-dependent manner can trigger protein mislocalization, aggregation, and altered transactivation activities that are often responsible for BPES syndrome (Moumne et al., 2008a). FOXL2 proteins in non-eutherian mammals (monotremes and marsupials) generally lack this polyalanine stretch. The absence of the polyAla tract is also observed in teleost fish despite good overall similarities with Foxl2 in eutherian mammals (Smith et al., 2013b) and the presence of other homopolymer stretches of amino acid residues, including polyprolines (polyP), and polyhistidines (polyH) (Crespo et al., 2013). However, in the cavefish *Astyanax mexicanus* stretches of glutamines (polyQ), are found in one of the TGD paralogs of Foxl2 (sequence accession: XP_007241719.1). This PolyQ additional amino acid track is not found in the other cavefish TGD paralog (sequence accession: XP_007232357.1) or in other vertebrate Foxl2 sequences publicly available. This intriguing specific PolyQ expansion of cavefish Foxl2 may indicate a neo functionalization of this Foxl2 ohnolog in cavefish potentially through an altered or modified transactivation activity as shown for the polyAla expansion in eutherian mammals. Functional experiments in cavefish would be required to test this prediction.

Evolution of Foxl2 expression among metazoans: Is the predominant ovarian expression a mammalian-biased view?

In mammals, FOXL2 is mainly expressed in the somatic cells of the female gonad. Reinforcing its predominance as a female gene, reports of expression in the developing testis are still subject to debate since the presence of *FOXL2* gene products have not been unequivocally demonstrated using either sensitive quantitative methods or antibodies (Cocquet et al., 2003a; Cocquet et al., 2002; Crisponi et al., 2001; Pannetier et al., 2003) (Table I). More precisely, "*Foxl2* is expressed in a female-specific manner in the gonads from 12.5 dpc on and this expression pattern is conserved between different phyla" (Loffler et al., 2003; Schmidt et al., 2004; Wilhelm et al., 2007). Additionally it is expressed in mesenchymal pre-granulosa and later granulosa cells before its expression stops postnatally (Schmidt et al., 2004) (Figure 3). However, beside this mammalian-based (murine?) view of a universal female pattern of expression, the FOXL2 theme might be slightly different in "different phyla", although some parts of the refrain might be indeed similar or not. In detail, in the fetal mouse ovary, *in situ* hybridization revealed that *Foxl2* is expressed in somatic cells that later have the potential to become granulosa, theca, or stroma cells (Pisarska et al., 2004). Conversely, no expression of *Foxl2* could be detected in oocytes (Pisarska et al., 2004). Later, by 13 days after birth (13 dpn) *Foxl2* is expressed in the granulosa cells of all follicles while oocytes still remain devoid of any *Foxl2* transcripts (Pisarska et al., 2004) (Figure 3 and Table I). At 20 dpn, *Foxl2* is still expressed in granulosa cells surrounding small and medium follicles while interestingly it is also detected in a somatic sub-population of large follicles (Pisarska et al., 2004). In adult ovaries, FOXL2 expression is restricted to granulosa cells surrounding small and medium follicles, but it is absent in the granulosa cells of antral follicles and the corpus luteum (Pisarska et al., 2004). Contrary to the situation for somatic cells the expression of *Foxl2* transcripts and their translational control in germ cells and in oocytes is still unclear (Loffler et al., 2003; Pisarska et al., 2004; Schmidt et al., 2004). As a variation on the same theme, in goat for instance, FOXL2 protein is not detected the oocyte whereas persistent expression is clearly seen in the granulosa cells of large follicles up to ovulation although declining while maturation occurs (Figures 3 and 4).

In Reptiles *FoxL2* has been well characterized in only three reptile species that experience temperature dependent sex determination. In both red-eared slider *Trachemys scripta* and in common snapping turtle *Chelydra serpentina*, *Foxl2* is not differentially expressed in animals during incubation at female promoting temperature (FPT) or at male promoting temperature (MPT). Its expression starts to be significantly dimorphic after that thermosensitive period at FPT (Bieser and Wibbels, 2014; Loffler et al., 2003; Rhen et al., 2007) (see Table I). In addition, in *Trachemys scripta*, *in situ* hybridization experiments confirmed this expression pattern. *Foxl2* mRNA is localized in the somatic part of developing gonads at FPT and MPT. At the end of the thermosensitive period, *Foxl2* is detected in both primitive sex cords accompanied by cortical expression in ovary that continues through to adult differentiation (Shoemaker et al., 2007b) (see Table I). Similarly, in the American alligator (*Alligator mississippiensis), FoxL2* is expressed before the sensitive period in both FPT and MPT animals. Then, the expression becomes sexually dimorphic in FPT individuals (Janes et al., 2013; Yatsu et al., 2016) (see Table I). Such data would argue for higher *Foxl2* expression being a secondary consequence of FPT exposure. In that respect, the temperature would restrictively influence Foxl2 expression, suggesting sequential control between the environment, gene expression, and the female fate respectively.

In Birds, FOXL2 has been studied only in chicken where its expression is low and not dimorphic between male and female gonads at (Embryonic days E4.7) (Govoroun et al., 2004). Higher activation of chicken Foxl2 expression is then observed in female left and right between E4.7 and E5.7 before reaching a plateau at E6.7. Then by E10.7, a decrease of Foxl2 expression is apparent in the right gonads of females, reflecting the asymmetric gonad development in female chicken. Always in the male gonads, Foxl2 expression, although detectable, is 10 to 250 times less abundant than in observed female gonads. Interestingly, immunohistochemistry revealed that Foxl2 protein is localized in the medullar somatic cells of the ovary as well as the aromatase protein while both being

absent from the cortical part. Foxl2 expressing cells surrounding the oocytes correspond to the granulosa cells of primordial and primary follicles (Figure 3 and Table I). It was also shown that foxl2 is additionally highly expressed in maturing and ovulated oocytes, suggesting a possible additional late role in oocyte maturation. Although significantly lower, expression of foxl2 was also detected in theca cells (Figure 3). Interestingly, regardless of the cell types or sub-populations, the gonadal expressions of the avian Foxl2 transcripts and proteins are clearly female-biased and apparently very dynamically regulated toward oocyte maturation.

In Amphibians, *Foxl2* has not been extensively studied (Table I). In the frog, *Rana rugosa, Foxl2* transcripts were detected before the onset of sex differentiation in both sexes following by female dimorphic expression during the sex differentiation period in tadpoles. Foxl2 protein is localized in somatic cells surrounding the oocytes in the ovary prior metamorphosis. In adults, *Foxl2* mRNA is highly expressed in female frogs, but clear expression is also detected in males (Oshima et al., 2008). In *Xenopus laevis*, *Foxl2* expression is detected in ZW female individual gonads during the period of first expression of the sex-determining gene DM-W. Then, *Foxl2* is expressed in both ZZ and ZW gonads with 5-fold higher expression in ZW individuals. This increasing expression is correlated to ovarian formation (Okada et al., 2009). Similarly in the adult wood frog, *L. sylvaticus*, Foxl2 is expressed in both gonads although a clear dimorphic expression in the ovaries is observed (Navarro-Martin et al., 2012). Hence in amphibians Foxl2 is also linked to gonad feminization. It is worth to note that *Foxl2* is nevertheless detectable in male adult testis. Its function in this tissue remains elusive.

In many **Teleost fish species**, *foxl2* displays a clear sexually dimorphic expression in the differentiating and adult gonads with higher expressions in ovaries compared to testes (Baron et al., 2005a; Baron et al., 2004; Nakamoto et al., 2006b; Vizziano et al., 2007; Wang et al., 2004a) (see also

in Table 1). *foxl2* expression is predominantly in somatic cells of female developing gonads ((Nakamoto et al., 2006b; Nakamoto et al., 2009; Wang et al., 2007a) and Table 1). At adult stages, the Foxl2 protein is mainly present in follicular ovarian cells, i.e., granulosa cells and theca cells, surrounding the oocytes (Figure 3). In male salmonids and seabass, a low but significant level of *foxl2* expression is detected by sensitive quantitative methods (RT-qPCR) in the adult testis throughout sexual development (Baron et al., 2004; Crespo et al., 2013; von Schalburg et al., 2010; von Schalburg et al., 2011). In zebrafish, Foxl2 expression has been localized to Leydig and germ cells by immunolocalization (Caulier et al., 2015a) (Table I). Medaka *foxl2* is clearly expressed in a dimorphic fashion with no testicular expression (Nakamoto et al., 2006b). To investigate the possible role of Foxl2 during medaka ovarian differentiation, the distribution of Foxl2 protein was analyzed by immunohistochemistry (Herpin et al., 2013). Throughout the transition of germ line stem cells to oocytes Foxl2 protein is first present in the germ line stem cells of the cradle and maintained during meiosis until oogenesis (Nakamoto et al., 2006b). During these early stages of oocyte formation no Foxl2 protein is detected in the interwoven threadlike ovarian cord cells where the supporting follicular cells reside. In the following steps of oogenesis, Foxl2 protein is progressively localized in the surrounding cells accompanying oocyte development (Herpin et al., 2013). In the rest of the ovary, Foxl2 is detected within the follicular cells of the pre-vitellogenic and vitellogenic follicles and then gradually lost while maturation proceeds (Herpin et al., 2013; Nakamoto et al., 2006b). Particularly, Foxl2 is localized in the nuclei of all granulosa cells. Unexpectedly, and in contrast to mammals, a minority of theca cells also express Foxl2 in medaka (Herpin et al., 2013) (Figure 3). Reminiscent of the observations made in birds, the teleost expression data, showing *foxl2* female-biased early somatic expression, being maintained throughout the transition of germ line stem cells to oocytes, as well as in sub-populations of theca cells, would imply conserved functions among the ovarian determining and maintenance pathways in non-mammal vertebrates (Figure 5).

In Protostomes, *foxl2/3* has also been characterized, for instance in insects (De Loof et al., 2010), but overall expression data remains scarce. In the pea aphid, *Acyrthosiphon pisum*, which displays a very unusual reproductive pattern alternating between parthenogenesis and sexual morphs (Figure 2), RNA-seq data from parthenogenetic (asexual) females, oviparous (sexual) females and males revealed that the pea aphid *foxl2/3* transcript is specifically expressed when females switch to a sexual reproduction mode (Figure 2). In molluscs, analysis of *foxl2/3* expression in the Pacific oyster *Crassostrea gigas*, Hong Kong oyster *Crassostrea hongkongensis*, and Black-lip pearl oyster *Pinctada margaritifera* revealed predominant expression of *foxl2/3* in female gonads (Teaniniuraitemoana et al., 2015; Teaniniuraitemoana et al., 2014; Tong et al., 2015; Zhang et al., 2014). In *C. gigas*, *in situ* hybridization specifically localized expression of *foxl2/3* transcripts in the oogonia and early-developed oocytes of the female ovary as well as in male germ cells from spermatogonia until spermatids of the testis (Naimi et al., 2009). Somatic gonadal expression has not been clearly observed, in contrast with the follicular somatic cell expression reported in most species (Table I). The expression pattern of the oyster nevertheless clearly indicates a potential function for *foxl2/3* during male gonadal maturation. Similar to *C. gigas*, in another mollusc, i.e., *Chlamys farreri*, *foxl2/3* is also expressed in both gonads, but in a dimorphic fashion, with higher expression in the ovary (Liu et al., 2012). Being mainly expressed at the proliferative stage of developing ovaries, *foxl2/3* transcripts are present in follicle cells as well as in germ cells (Liu et al., 2012). Of note, mRNA localization is also observed in male germ cells of the testis, except spermatozoa (Liu et al., 2012) (Table I).

Similar to molluscs, the Chinese mitten crab exhibits a higher expression of *foxl2/3* in the nucleus of the oogonia and vitellogenic oocytes during ovarian development and in the nucleus of follicular cells in adults. It is worth noting that *foxl2/3* mRNA and Foxl2/3 proteins are constantly expressed in the developing and adult testis reinforcing a possible role of *foxl2/3* in this tissue (Liu et al., 2015) (Table I).

Interestingly, the presence of *foxl2/3* has also been reported in the sponge *Suberites domuncula* (Adell and Muller, 2004). In that species, a polyclonal antibody raised against Foxl2/3 (Sd-Foxl2) revealed the ubiquitous presence of the protein in the nuclei of all tissues in the sponge as well as in the primmorphs (dissociated *in-vitro* sponge cultured cells) without any sexual dimorphism (Adell and Muller, 2004) (Table I and Figure 5). These results suggest that despite a high degree of identity between the DNA-binding domains of Foxl2 proteins from mammals and Foxl2/3 protein in sponges, sequence homology obviously does not necessarily imply functional homology and conservation of the downstream gene regulatory networks.

Indeed, being ubiquitously present in basal protostomes or expressed in the "whole" gonads (somatic and germ line) of mollusks and insects already indicate that the functional evolution of Foxl2/3 proteins might have paralleled the acquisition of their gonadal cell-specific pattern of expression (Figures 1 and 5). Whether this evolution was gradual or sequential and always concerted between function and patterns of expression will be discussed in the following paragraphs.

Expression and function of Foxl3, another key player of gonadal sex differentiation.

The first expression data for *foxl3* were reported in rainbow trout, in which *foxl3* is expressed in the differentiating female gonad peaking transiently just before and during the first oocyte meiosis; its expression remains undetectable in males (Baron et al., 2004). In Atlantic salmon and European sea bass adult stages, the expression pattern is somehow different with *foxl3* transcripts predominantly expressed in the testis compared to ovaries (Crespo et al., 2013; von Schalburg et al., 2010; von Schalburg et al., 2011). The onset of *foxl3* expression was also observed in immature testes, then decreasing while spermatogenesis proceeds and reaching a minimum when germ cells entered

meiosis. In the ovaries of sea bass, both *foxl2* and *foxl3* are expressed although the expression of *foxl3* is much lower (Crespo et al., 2013). Highest expression was observed in previtellogenic ovaries while steadily decreasing thereafter. Accordingly, it has been suggested that *foxl3* could be possibly involved in the onset of oocyte meiosis in females as well as for regulating male testis development or maturation (Baron et al., 2004; Crespo et al., 2013). Apart from its expression in gonads, *foxl3* is also highly expressed in male gills compared to a slight expression in females, and a weak expression is also observed in male spleen and in female hypothalamus examined in European sea bass (Crespo et al., 2013). Though *foxl2* and *foxl3* paralogs both encode for transcription factors that likely recognize similar target DNA sequences due to highly similar DNA binding domains, they also display distinct expression patterns in teleosts, pointing out likely non-redundant functions. Interestingly, the poorly conserved C-terminal domain of Foxl3 when compared to Foxl2 might also indicate possible different transactivating properties between the two paralogs (Crespo et al., 2013). Although specific targets subjected to Foxl3 transactivation have not yet been identified, similar to Foxl2, over expression of Foxl3 in sea bass ovarian follicular cells notably induced high expression of Lhr (Crespo et al., 2013). Likewise, expression of other foxl2-regulated genes was similarly modulated after foxl3 up-regulation, although at much lower levels (Crespo et al., 2013). Otherwise the intrinsic function of foxl3 remains elusive.

In medaka during the sex differentiation period, *foxl3* is expressed both in female and male gonads (Nishimura et al., 2015). Transcripts and proteins are first detectable in germ cells of both XX and XY embryos during the onset of gonadal sex determination (stage 35) (Nishimura et al., 2015), where *foxl3*/Foxl3 were localized specifically in a subset of mitotically active germ cells. Whereas *foxl3*/Foxl3 expressions remain detectable throughout gonadal development in XX female fish, expressions were not observable anymore in germ cells of XY fish by ten days after hatching (Nishimura et al., 2015). Interestingly, *foxl3*$^{-/-}$ XX gonads did not develop any oocytes but were rather filled with cystic and meiotic germ cells as well as spermatid-like cells, expressing the sperm-specific marker protamine, at the periphery of the gonads (Nishimura et al., 2015). On the other hand adult *foxl3*$^{-/-}$ XY gonads

developed as morphologically and functionally normal testes, indicating that *foxl3* is dispensable for male gonadal development and maintenance. Together, these findings indicate that Foxl3 is a germline-intrinsic factor involved in sperm-egg fate decision (Nishimura et al., 2015; Nishimura and Tanaka, 2016).

The data from the sponge, namely that Foxl2/3 is ubiquitously and not dimorphically expressed in the nucleus of all cell types studied (Figure 5) and neither is regulated in cell culture or in the resting gemmules of that organism suggest that the primary, ancestral function(s) of this protein are related to basic cellular processes. What that processes is remain to be elucidated. Hence, a handful of studies already stressed the fact that Fox proteins in general might not only act as canonical transcription factors but might also have a broader function in regulating basic cellular functions.

From ancestral function of Foxl2/3 to new functions for Foxl2 and Foxl3.

Rising the question of the ancestral function(s) of fox genes, it has been observed that many *forkhead domain* genes from yeast (FKH1 and FKH2) to mammals (*FOXO* and *FOXM1*) are not only ubiquitously expressed but are specifically committed to cell cycle regulation and growth (Alvarez et al., 2001; Burgering and Kops, 2002) and therefore might not solely act as “pure” transcription factors. Into that direction in yeast, FKH1 and 2, after associating with the coding regions of certain genes, coordinate early transcription elongation and pre-mRNA processing by regulating the elongation activity of RNA polymerase II (Morillon et al., 2003). For instance FoxA is able, after binding to its target sites, to open compacted chromatin and allow other transcription factors to bind and regulate gene expression (Cirillo et al., 2002). It is then clear that, like suggested by Carlsson and Mahlapuu, the function of the first Fox genes, in unicellular or simple multicellular organisms, was

fundamental in the cell metabolism (Carlsson and Mahlapuu, 2002). Then the metazoan Fox genes have undergone a more recent expansion, coinciding with the evolving anatomical complexity of animal body plans (Carlsson and Mahlapuu, 2002) (Figure 5). Identically one could reasonably imagine that the diversification of the *Foxl2* genes recently observed in vertebrates (Figures 1 and 5) likely permitted specialization of this sub-family toward –female- gonadal functions.

Another variation when looking at the common theme of Foxl2 expression among metazoans is whether their sexually dimorphic expression was acquired in a stepwise and sequential fashion during the course of evolution (Figure 5). While ovarian somatic expression of *foxl2*/Foxl2 is always predominant in vertebrates, testicular mRNA expression is nevertheless detected in many species, including some mammals, birds and fish but in these species the Foxl2 protein is often not detectable in testis (Cocquet et al., 2005; Crisponi et al., 2001; Govoroun et al., 2004; Herpin et al., 2013; Loffler et al., 2003; Pannetier et al., 2006). Such a discrepancy between the testicular expression of the *foxl2* gene and the presence of the Foxl2 protein suggest post-transcriptional or translational regulation mechanisms that could be mediated by antisense RNAs. Natural antisense *foxl2* transcripts (NAT-*Foxl2*) have been described in mouse, scallop and oyster (Cocquet et al., 2005; Liu et al., 2012; Naimi et al., 2009), and could specifically mediate sense transcript silencing after forming RNA-RNA duplexes. Although antisense *Foxl2* RNA expression levels were shown to be similar to that of the sense RNA in the gonads of mice and scallop (Cocquet et al., 2005; Liu et al., 2012), data gathered in the oyster suggests that such antisense RNA might indeed be involved in the regulation of Foxl2 (Santerre et al., 2012). Oyster NAT-*foxl2* is significantly more expressed than *Foxl2* in 2-month-old oyster larvae as well as in mature males (Santerre et al., 2012). Whether this evolutionary conserved mechanism of Foxl2 protein expression modulation could account for sequential foxl2/3 sub-functionalization and dimorphic functions remains to be investigated.

A role outside of the granulosa cells?

One of the major roles of Foxl2 during gonadal differentiation and maintenance has emerged *via* the mutual antagonistic relationship of Foxl2 and Dmrt1, inhibiting each other's transcription (see for review (Herpin and Schartl, 2008, 2015)). Additionally, the strict co-expression of Foxl2 and Aromatase (Cyp19) in the mammalian ovary led to the demonstration that Foxl2 is involved in the regulation of estrogen synthesis *via* direct transcriptional up-regulation of ovarian-type Aromatase (see for review (Pannetier et al., 2006)). Interestingly, while Foxl2 has been stated to be a strong inducer of the steroidogenic activity of granulosa cells (Guiguen et al., 2010; Hudson et al., 2005; Wang et al., 2007a), unexpectedly, and in contrast to mammals, theca cell expression of Foxl2 has also been reported in chicken (Govoroun et al., 2004) and medaka (Herpin et al., 2013). Hence, the canonical view of *foxl2* being the major inducer of *aromatase* expression is now seriously challenged (see Figure 3).

In medaka the presence of two types of cyp19a1a-positive theca cells, which are either Foxl2 positive or Foxl2 negative were detected (Herpin et al., 2013) (Figure 3). In contrast to mammals where ovarian-type aromatase is produced only by granulosa cells, the biological significance of cells expressing both *Cyp19a1* and *foxl2* within the thecal layer remains unclear. Whether these indeed represent two separated subpopulation of theca cells or just different steps of theca cell differentiation remains to be clarified.

In a similar way, Foxl2 transcripts and protein were both detected in the theca cell layer of the adult chicken ovary (Govoroun et al., 2004) (Figure 3). In chicken, aromatase transcripts are present only in theca cells of the developing follicles (Oreal et al., 2002) as well as in the external theca layer of maturing follicles (Kato et al., 1995). Foxl2 has never been detected in chicken granulosa cells, although these cells are known for being a site of specific aromatase expression in mammals. Consequently, and reminiscent of the observations made in medaka, it seems that the area of *foxl2* expression is much broader than that of aromatase. This conclusion not only suggests that aromatase

expression is regulated by additional factors besides Foxl2 and that by implication, Foxl2 function is not restricted to the control of aromatase, at least in the ovaries of medaka and chicken (Figure 3).

In that perspective it is interesting to note that birds also have multiple populations of theca cells, some of which are also steroidogenic (Nitta et al., 1991). In contrast to the main consensus, the discordance of spatial expression patterns of Foxl2 and ovarian-type aromatase (Cyp19a1a) calls into question an exclusive transcriptional regulation of *cyp19a1* by Foxl2 in the ovary of medaka and chicken. This conclusion indicates that Foxl2 is not always required for the maintenance of aromatase expression. On the other hand, several other factors (e.g., testosterone, TGF-β1, TNF-β, and glucocorticoids) have been shown to direct the expression of the aromatase gene in Sertoli, Leydig, and germ cells of rat testis (see for review (Bourguiba et al., 2003)).

Conclusions

Based on the recent literature "*foxl2*" evolution appears much more complex than initially though with multiple genes i.e., *foxl2/3*, *foxl2*, *foxl2a*, *foxl2b*, *foxl2b1*, *foxl2b2*, and *foxl3*, resulting from different whole genome duplications (VGD1 and/or VGD2, TGD and SaGD) followed by multiple and independent lineage-specific losses. Such gene complexity goes along with an increasing complexity of expression patterns, probably reflecting a functional evolution and specialization of theses genes. However, a few trends emerge from that apparent complexity with for instance the idea that (i) the initial ubiquitous expression of Foxl2/3 in sponges was followed by a progressive acquisition of an important role in gonadal development (like for instance in protostomes) and (ii) with an additional specialization of jawed vertebrates Foxl2 and Foxl3. In line with these ideas we proposed a gonadal sub-functionalization scenario for Foxl2 and Foxl3 after the vertebrate duplication of foxl2/3; Foxl2 being predominantly expressed in ovarian somatic cells and Foxl3 in male germ cells. Foxl2 and Foxl3 would then have complementary roles during gonadal differentiation. However many questions

remain to be solved to be able to link the evolution and the function(s) of all these "*foxl2*" genes. Their precise gene evolution is for instance not well understood, like the presence / absence of *Foxl3* in some tetrapod lineages that is still puzzling, or the paralogy relationships of *foxl2a* and *foxl2b* in teleosts. What are the molecular targets of Foxl3? and do Foxl2 and Foxl3 regulate the same genes or at least the same network of genes? So far the role of Foxl3 has only been demonstrated in fish, but its expression and function should now be explored in some birds, reptiles or marsupials. Answering these questions may shed new light on Foxl2 and Foxl3 evolution after duplication and on whether they remained major evolutionary conserved players required for respectively somatic and germinal differentiation of gonadal sex.

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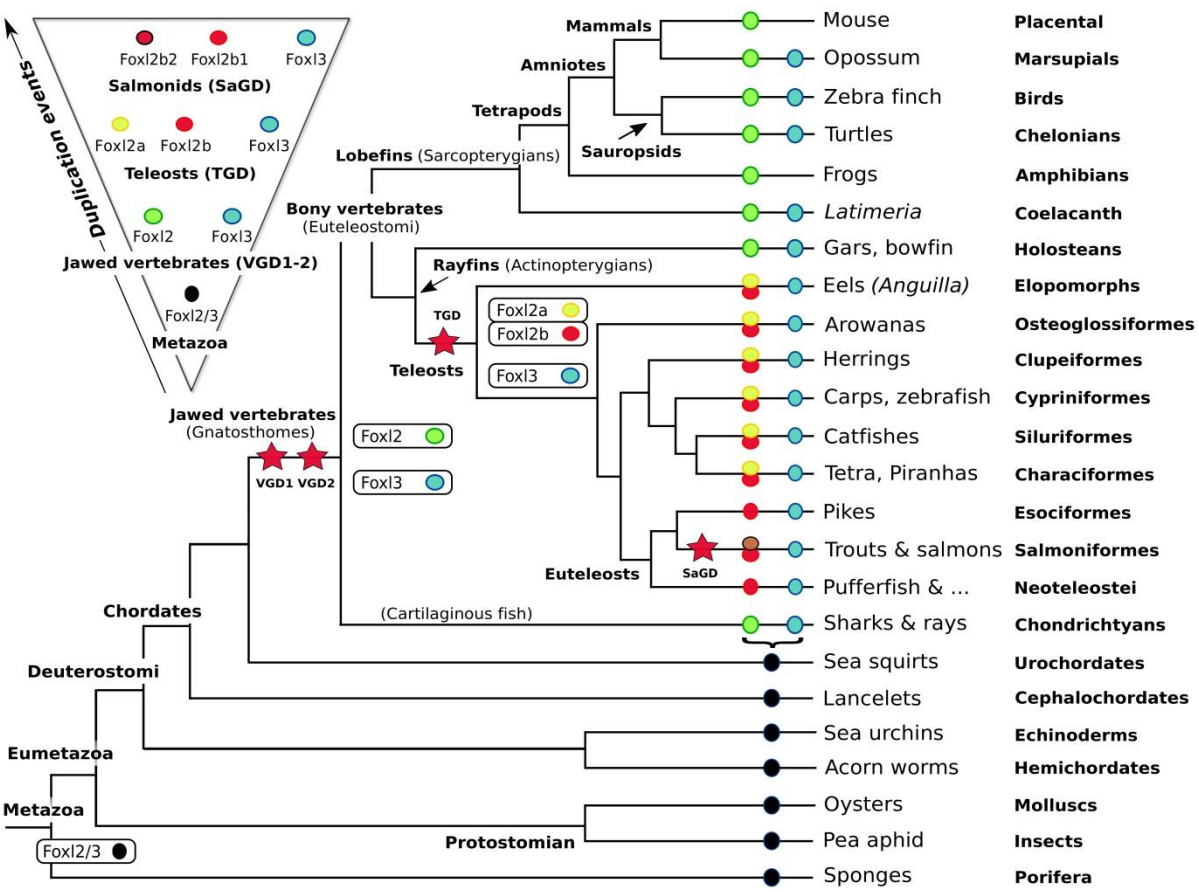

Figure 1. Evolution of "Foxl2" genes in Metazoans. Schematic cladogram representing the evolution of Foxl2, Foxl3, and its ancestor Foxl2/3. See text for details. VGD1, VGD2, TGD and SaGD (red stars) represent respectively the two rounds (VGD1 and VGD2) of whole genome duplications (WGDs) that occurred at the root of the vertebrate lineage, the teleost-specific whole duplication (TGD) that occurred at the base of the teleost radiation and the salmonid-specific whole genome duplication (SaGD). A few examples of species are given on the right of this cladogram within each taxon (in bold type on the right). Neoteleostei is a very large clade of teleost fish including species such as cods, medakas, guppies, mollies, sticklebacks, perches, tunas, flatfishes, wrasses, pufferfishes and pipefishes. The evolution of foxl2 in teleosts after TGD shown in this figure is based on the hypothesis of a single duplication of *foxl2* after TGD leading to two ohnologs (*foxl2a* and *foxl2b*) followed by a secondary loss of *foxl2a* in the Euteleost lineage (see text for details).

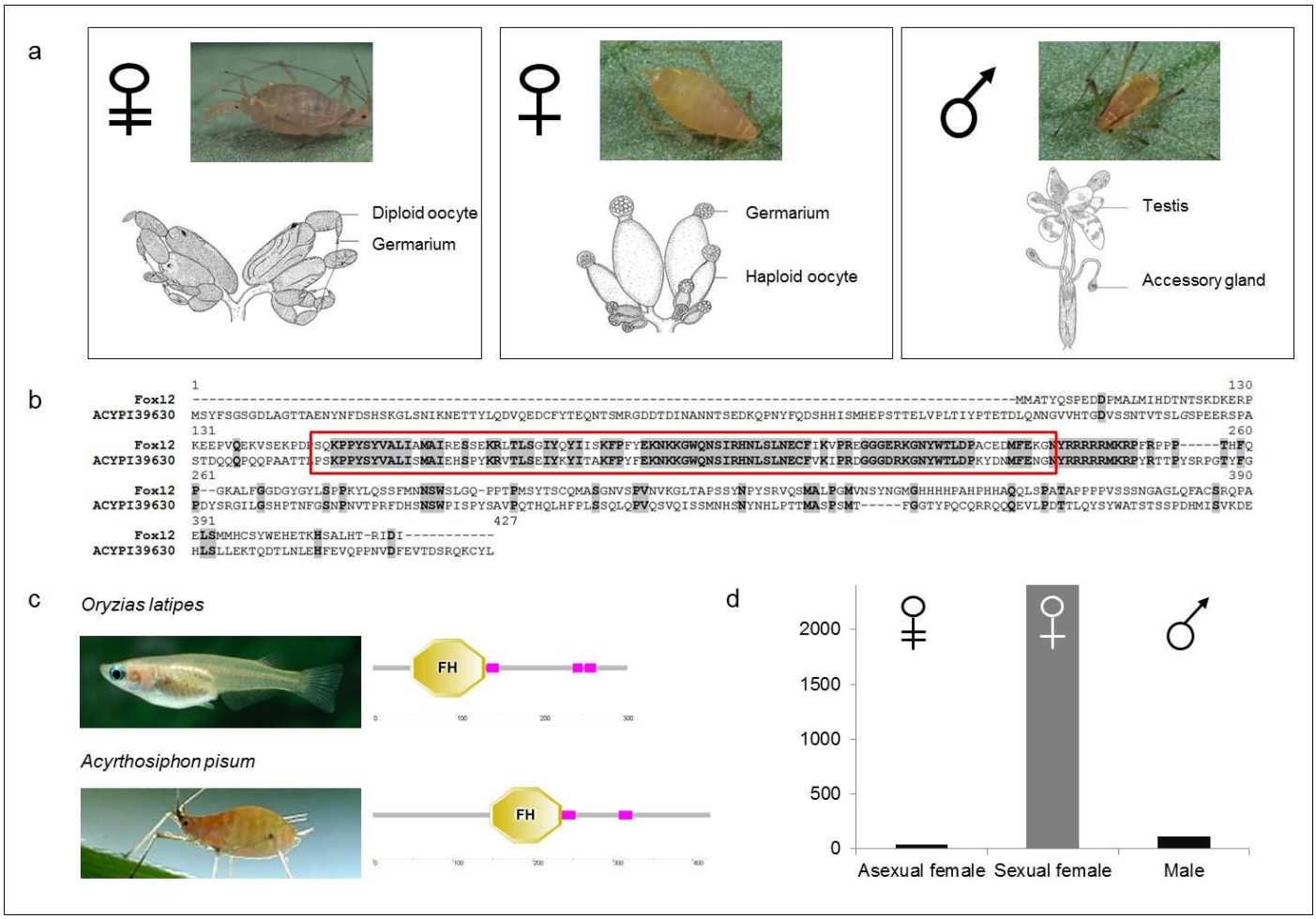

Figure 2. Foxl2 characterization and expression in the pea aphid, *Acyrthosiphon pisum*. Aphids are among the major pests of a wide range of crops and display an unusual reproductive pattern. **(A)** They reproduce by parthenogenesis (asexual reproduction) during spring and summer, but then switch their reproductive mode in autumn when the photoperiod shortens to produce truly sexual morphs that mate and lay cold-resistant eggs that overwinter. As a result, three distinct morphs **(A)** occur during the aphid life cycle: asexual females in which the germarium contains diploid oocytes that will viviparously give rise to diploid embryos; sexual females that contain true haploid oocytes; and males that contain testis that harbor haploid spermatozoa. **(B)** Sequence alignment of medaka (*Oryzias latipes*) Foxl2 protein and the aphid Foxl2-3 homologue (ACYPI39630). **(C)** Domain analysis revealed a strong amino-acid conservation of the typical Forkhead domain (framed in red), reflected by protein domain prediction (carried out using the SMART algorithm (Letunic et al., 2006)) of these two sequences. **(D)** RNA-seq data from asexual females, sexual females and males revealed that the aphid *foxl2-3* transcript is specifically expressed in sexual females.

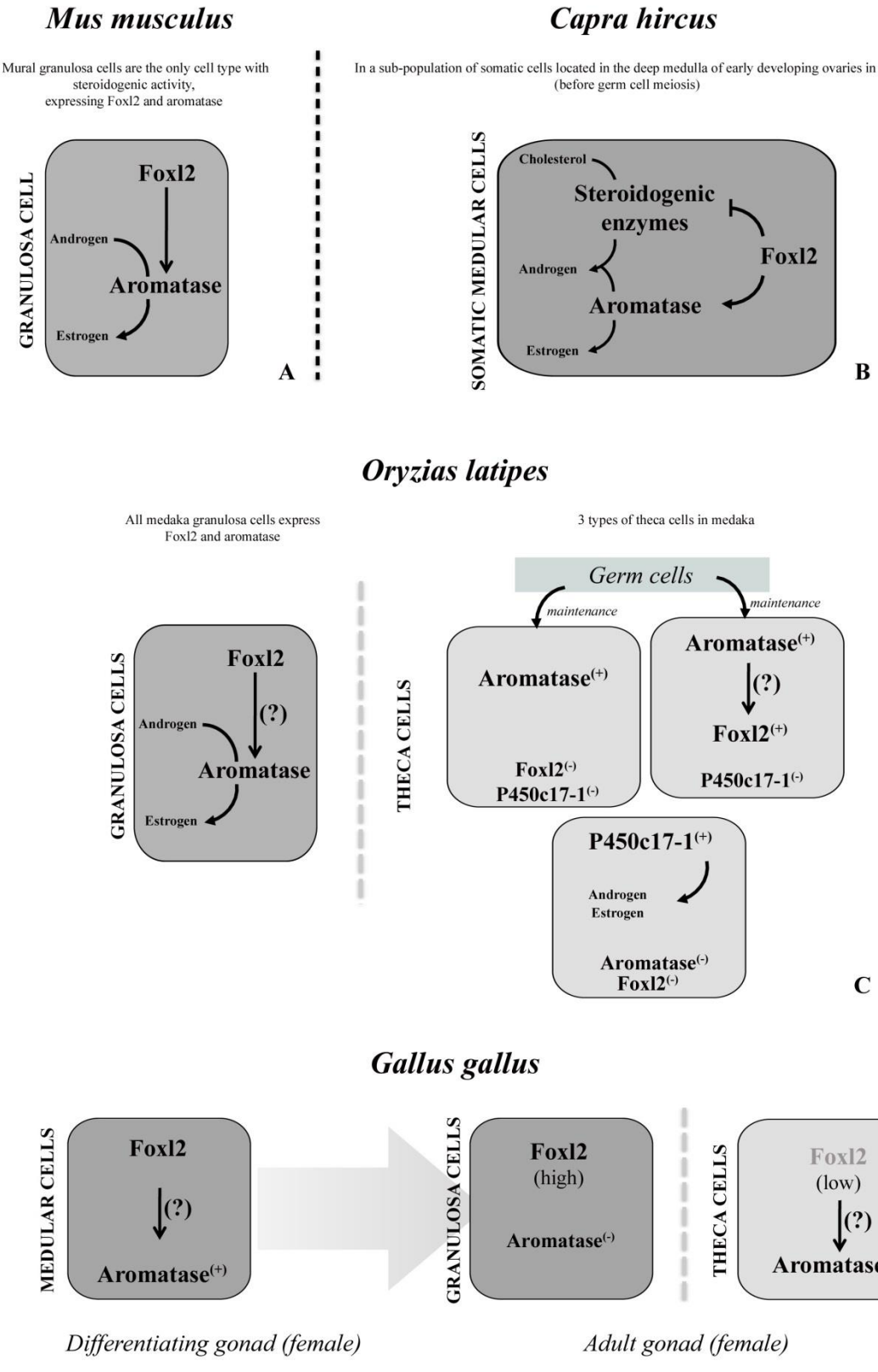
Mus musculus
Mural granulosa cells are the only cell type with steroidogenic activity, expressing Foxl2 and aromatase
GRANULOSA CELL
Foxl2
Androgen
Aromatase
Estrogen
A
Capra hircus
In a sub-population of somatic cells located in the deep medulla of early developing ovaries in goat (before germ cell meiosis)
SOMATIC MEDULAR CELLS
Cholesterol
Steroidogenic enzymes
Foxl2
Androgen
Aromatase
Estrogen
B
Oryzias latipes
All medaka granulosa cells express Foxl2 and aromatase
GRANULOSA CELLS
Foxl2
(?)
Androgen
Aromatase
Estrogen
3 types of theca cells in medaka
THECA CELLS
Germ cells
maintenance
maintenance
Aromatase(+)
Foxl2(-)
P450c17-1(-)
Aromatase(+)
(?)
Foxl2(+)
P450c17-1(-)
P450c17-1(+)
Androgen
Estrogen
Aromatase(-)
Foxl2(-)
C
Gallus gallus
MEDULAR CELLS
Foxl2
(?)
Aromatase(+)
GRANULOSA CELLS
Foxl2
(high)
Aromatase(-)
THECA CELLS
Foxl2
(low)
(?)
Aromatase(+)
Differentiating gonad (female)
Adult gonad (female)
D

Figure 3. Schematic representation of expression, regulation and physiology of Foxl2 in mouse (A), goat (B), medaka (C) and chicken (D). (A) In mice (*Mus musculus*), mural granulosa cells are the only cell type with steroidogenic activity expressing both Foxl2 and aromatase. Aromatase expression is directly induced by *foxl2*. **(B)** Analogously in goat (*Capra hircus*), foxl2 is expressed in a sub-population of somatic cells located in the deep medulla of early developing ovaries before the onset of germ cell meiosis. In these cells, Foxl2 controls the production of both androgen and estrogen through either negative regulation of steroidogenic enzymes or positive regulation of aromatase transcription respectively. **(C)** In medaka, as in mouse, granulosa cells express both Foxl2 and aromatase. Challenging the view of *foxl2* being the major inducer of *aromatase* expression, in medaka, a sub-population of theca cells expressing *aromatase* but not *foxl2* has also been described. Examination of Foxl2 distribution in the medaka ovary revealed a new sub-population of theca cells expressing both Foxl2 and aromatase. This situation is similar to what has been observed in birds **(D)**. **(C)** In that perspective, it is interesting to note that birds also have multiple populations of theca cells some of which are also steroidogenic but not correlated with *foxl2* expression **(D)**.

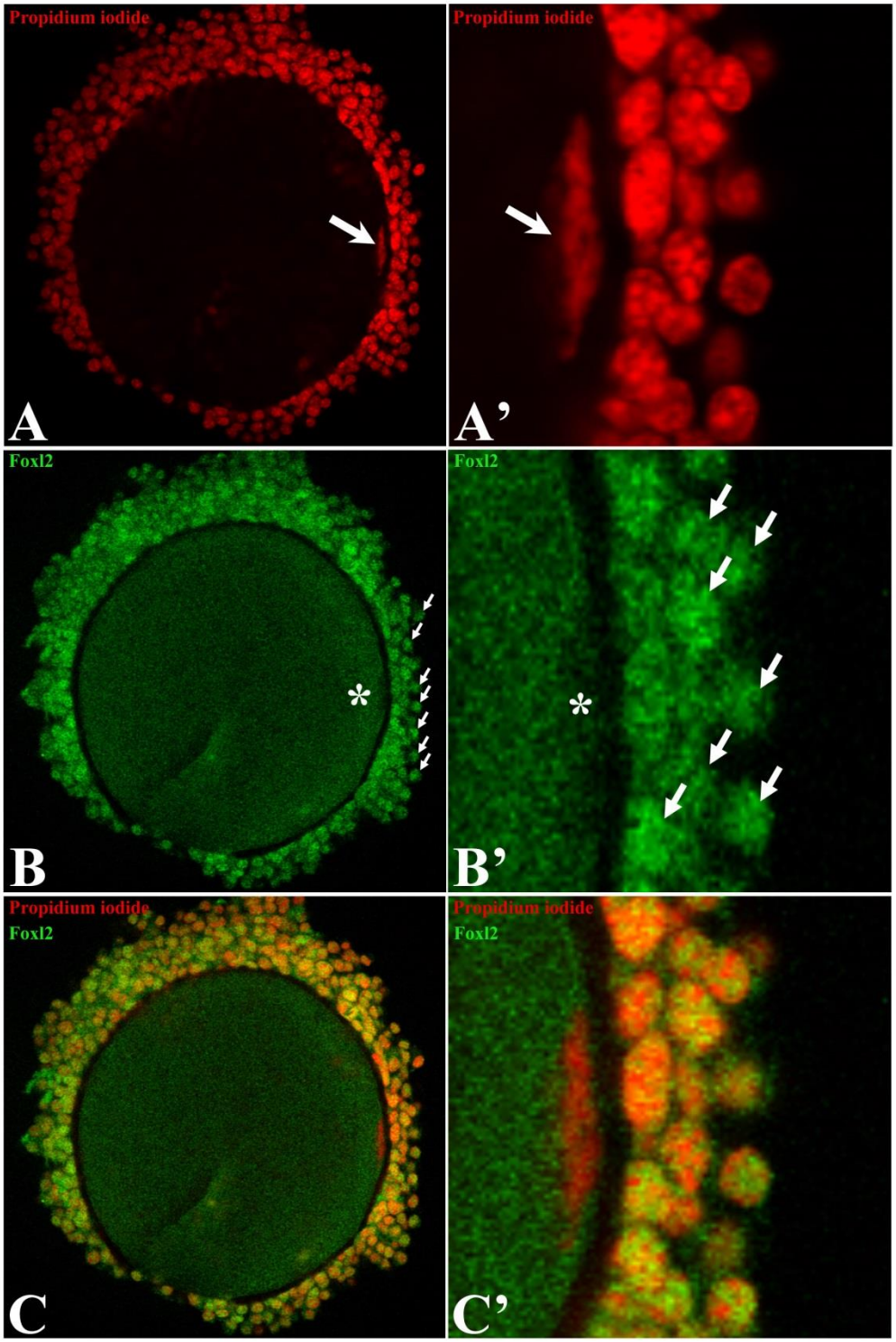
Propidium iodide
A
Propidium iodide
A'
Foxl2
B
Foxl2
B'
Propidium iodide
Foxl2
C
Propidium iodide
Foxl2
C'

Figure 4. FOXL2 protein localization in the goat ovary.

In goat (*Capra hircus*) FOXL2 proteins are not detected in the nuclei of the oocytes (**A and A'** compared to **B and B'**). FOXL2 proteins are nevertheless clearly localized in the nuclei of the granulosa cells supporting the large follicles (**B' and C'**). **(A and A')** Nuclear staining of the nuclei of the follicle, including oocyte and granulosa cells; the arrow indicates the nucleus of the oocyte. **(B to C')** Immunolocalization of the FOXL2 protein, being specifically localized in the nuclei of the granulosa cells. The asterisk (*) indicates the absence of FOXL2 signal in the nucleus of the oocyte. Arrows indicate the nuclear localization of FOXL2 signal in the granulosa cells of the follicle.

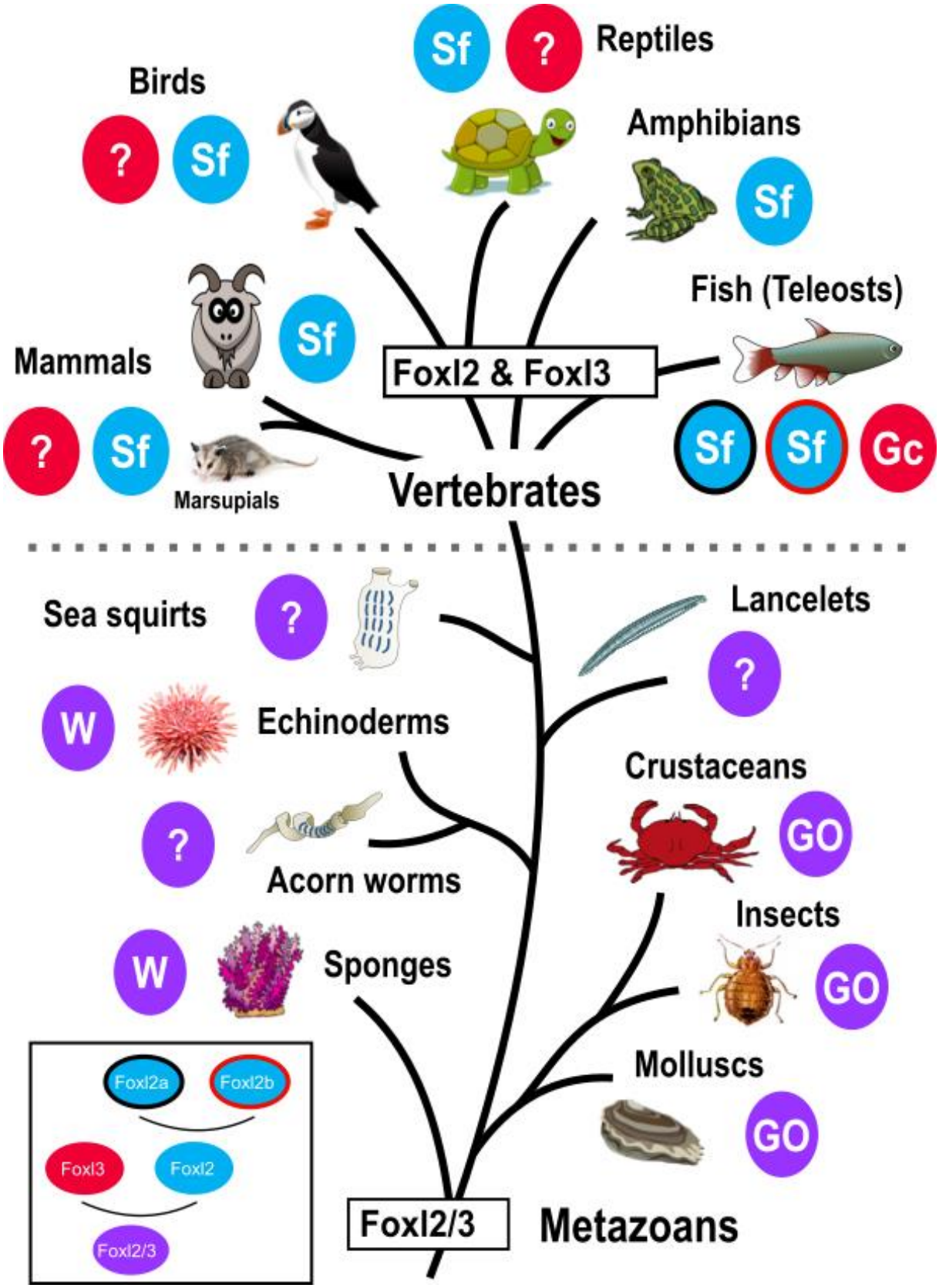
Sf
?
Reptiles
Birds
?
Sf
Amphibians
Sf
Fish (Teleosts)
Mammals
Sf
Foxl2 & Foxl3
?
Sf
Sf
Sf
Gc
Marsupials
Vertebrates
Sea squirts
?
Lancelets
?
W
Echinoderms
Crustaceans
GO
?
Acorn worms
Insects
W
Sponges
GO
Foxl2a
Foxl2b
Molluscs
Foxl3
Foxl2
GO
Foxl2/3
Foxl2/3
Metazoans

Figure 5. Schematic representation of the evolution of gonadal expression of Foxl2 and its relatives (Foxl2/3 and Foxl3) in Metazoa.

Foxl2/3 is represented in blue, Foxl2 in purple and Foxl3 in red. Following the teleost fish genome duplication (TGD), some species retained two foxl2 genes, Foxl2a (purple with a black circle) and Foxl2b (purple with a red circle). "W": Whole animal (no restricted expression), "GO": expression in gonads, "Sf": predominant gonadal expression in female somatic tissue, "Gc": predominant gonadal expression in germ cells, "?": expression pattern not described.

Table 1. *Foxl2* and its relatives (*Foxl3* and *Foxl2/3*) expression pattern in Metazoa.

Abbreviations and symbols: (A) adult stage, (E) embryonic stage, EST (Expressed sequence tag), ISH (in situ hybridization), IHC (Immunohistochemistry), (L) larvae, mRNA (messenger RNA), NB (Northern blot), RT-qPCR (Reverse transcription-quantitative Polymerase chain reaction), TSP (Temperature-dependent sex determination), WB (Western Blot). Identified means that *foxl2/3* gene is found and publicly available. The term expression is relative to its description in the original article. Each gene is delimited by a line. Each clade is separated by a dashed line.