Candidate protein interactors of sexually dimorphic on the Y chromosome (SdY) in Atlantic salmon

by

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Abstract

Atlantic salmon depend on genetic cues to determine whether an individual is male or female. A novel sex-determining gene, sexually dimorphic on the Y chromosome (*sdY*), is found exclusively in all salmonids. Unlike other sex-determining genes, *sdY* lacks a DNA-binding domain. Instead, it is a divergent, truncated form of interferon regulatory factor 9. As a recently discovered gene, little is known about *sdY*; how it is involved in sex-determination and what proteins interact with it. Identification of protein interactors was done through a variety of techniques including yeast two-hybridization, co-immunoprecipitation and histidine-tagged pull down assays. These assays identified several proteins: SdY itself, 40S ribosomal protein S16 and SA, isocitrate dehydrogenase, heat shock protein HSP 90-beta, and ras GTPase-activating-like protein IQGAP1, as well as creatine kinase, GDP-mannose-4,6-dehydratase, sodium/potassium-transporting ATPase subunit alpha-1, AP-1 complex subunit beta-1, and hydroxysteroid dehydrogenase (17-beta) 4. The yeast two-hybrid assay also identified 3' UTR of annexin A7-like and transmembrane protein 91-like, most likely false positives. This broad range of candidates has led me to believe that SdY is involved either in the biosynthesis of testosterone or in the testosterone signalling pathway.

Keywords: SdY, Atlantic salmon, sex-determination, yeast two-hybridization

This project is dedicated to my parents and my sister, Asia, for their unwavering support and confidence in me.

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List of Acronyms

Rps16	40S ribosomal protein S16
AD	GAL4 activation domain
Anxa7	Annexin A7
Co-IP	Co-immunoprecipitation
DNA-BD	DNA-binding domain
ESD	Environmental sex-determination
GSD	Genetic sex-determination
Irf9	Interferon regulatory factor 9
Ni-NTA	Nickel-nitrilotriacetic acid
SdY	Sexually dimorphic on the Y chromosome
Tmem91	Transmembrane protein 91

Glossary

ADE2	A gene that encodes for phosphoribosylaminoimidazole carboxylase that is involved in the IMP biosynthesis pathway via *de novo* pathway, which is a part of purine metabolism. Presence of this gene allows yeasts to grow in the absence of adenine (P21264 (PUR6_YEAST)).
AUR1-C	Dominant mutant form of the AUR1-C gene which is expressed in the Y2HGold yeast strain which causes colonies to be resistant to the Aureobasidin A drug during a mating between the GAL4 binding and activating domains (Clontech).
Aureobasidin A	An antibiotic that is highly toxic to yeast as it inhibits inositol phosphorylceramide synthase an important step in sphingolipid biosynthesis in yeast (Clontech, P36107 (AUR1_YEAST)).
DDO	Double dropout medium that lacks leucine and tryptophan (Clontech).
DDO/X/A	Double dropout medium that lacks leucine and tryptophan and is supplemented with X-α-galactosidase and Aureobasidin A (Clontech).
HIS3	A gene that encodes for imidazoleglycerol-phosphate dehydratase that is involved in the biosynthesis of histidine (P06633 (HIS7_YEAST)).
MEL1	A gene that encodes for α-galactosidase, which when expressed results in blue colonies in the presence of the chromagenic substrate X-α-Gal (Clontech).
QDO	Quadruple dropout medium that lacks adenine, histidine, leucine, and tryptophan (Clontech).
QDO/X/A	Quadruple dropout medium that lacks adenine, histidine, leucine, and tryptophan and is supplemented with X-α-galactosidase and Aureobasidin A (Clontech).
SD/leu	Single dropout minimal medium that is comprised of a nitrogen base and a carbon source and it lacks leucine (Clontech).
SD/trp	Single dropout minimal medium that is comprised of a nitrogen base and a carbon source and it lacks tryptophan (Clontech).
Sex-determination	A developmental event involving genetic, environmental, behavioural, and physiological factors that establish the sex of an organism (Devlin and Nagahama 2002).
X-α-galactosidase	A chromogenic substrate for yeast galactosidase used for detecting GAL4-based yeast two-hybrid interactions directly on agar, yielding blue colonies (Clontech).

YPDA	A medium comprised of yeast extracts, peptone, and dextrose optimal for growth of yeast strains and is additionally supplemented with adenine hemisulfate (Clontech).

Chapter 1.

Introduction

Sexual reproduction is a costly venture with more risks than benefits to the participants. Not only do individuals risk coming into contact with predators, diseases, or harm, but they also only transmit fifty percent of their genes to the offspring (Otto 2009). With such detrimental costs, it is assumed that sexual reproduction would be a rare occurrence and yet majority of metazoans, in particular all vertebrates, use this method to reproduce (Otto 2009). The persistence of sex may be attributed to the reintroduction of variation in a population. Sexual reproduction enables recombination to occur which is beneficial when genetic associations are no longer favorable (Otto 2009). Strong evolutionary pressures have been applied on vertebrates to develop diverse sex-determination pathways with essentially the same outcome: the production of males and females and in some cases hermaphrodites (Beukeboom and Perrin 2014).

1.1. Sex-Determination in Vertebrates

The sex of an organism is never visualized at the moment of conception (Beukeboom and Perrin 2014). A series of factors is triggered after fertilization to initiate the determination of sex. For the purpose of this thesis, sex-determination is a developmental event involving factors that establish the sex of an organism, while the further development of testes or ovaries is referred to as sex-differentiation (Cutting et al. 2013; Devlin and Nagahama 2002).

Sex-determination pathways are complex and elaborate systems, relying extensively on cues either genetic or environmental or a mixture of both to commit a vertebrate to its sexual fate (Bachtrog et al. 2014). Some vertebrates, like mammals and birds, have only one type of system, while others like reptiles, amphibians, and fish have

a mixture of systems used to determine sex (Figure 1.1). In genetic sex-determination (GSD), males and females arise through chromosomal determination. To date, there are only two known chromosomal determination systems: XY and ZW (Ezaz et al. 2006). The genes responsible for GSD are inherited at the moment of fertilization, but will not be activated until Sertoli or granulosa cells have been developed (Beukeboom and Perrin 2014). In environmental sex-determination (ESD), external factors like temperature, pH, and salinity dictate the development of an organism's gender, however ESD in vertebrates is mostly due to temperature changes (Kobayashi et al. 2013; Trukhina et al. 2013). Within organisms, the system of sex-determination is not always set as both systems can influence the sex of an individual. Even though an organism may have a GSD system, environmental factors can still alter the sex (Bachtrog et al. 2014). The preference for an ESD system depends on the environment, particularly when it is more beneficial for one sex (Bachtrog et al. 2014). In unpredictable environments, GSD systems are favoured which prevent biased sex ratios (Bachtrog et al. 2014).

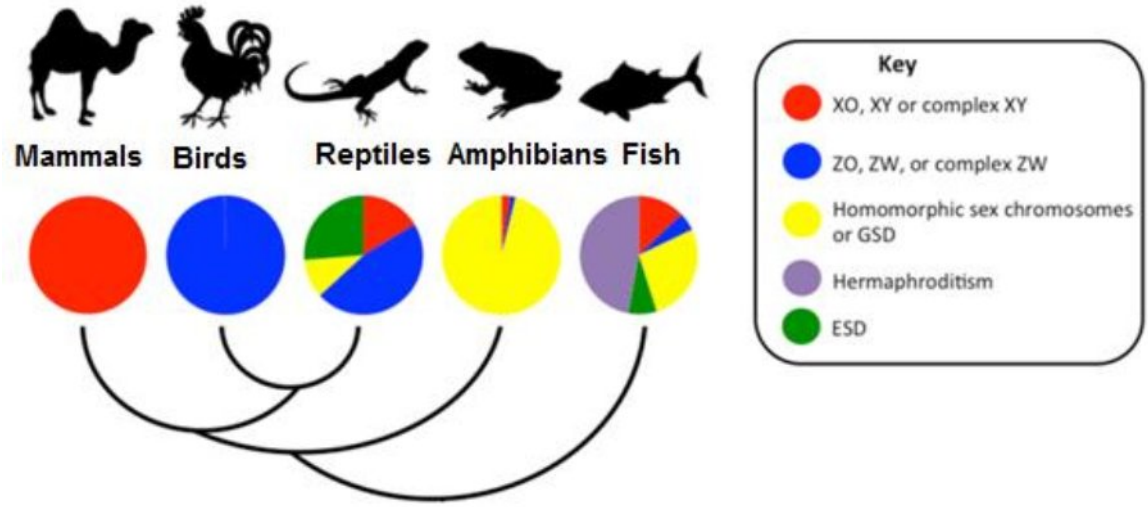


Figure 1.1 The various sex-determination systems present in vertebrates

Vertebrates are comprised of five classes: mammals which have an XX/XY system, birds which have a ZZ/ZW system, reptiles which have XX/XY, ZZ/ZW, GSD, and ESD systems, amphibians which have a predominantly GSD system, and fish which have XX/XY, ZZ/ZW, hermaphroditism, GSD, and ESD systems. Modified figure from Bachtrog et al. 2014 which is under the Creative Commons Attribution License.

Sex-determination is an ancient process where the same set of sex-determining genes remains conserved across all vertebrates, with only the master switch varying (Cutting et al. 2013; Bachtrog et al. 2014). In general to become a male, SOX9 (sry-box 9), FGF9 (fibroblast growth factor 9), and DMRT1 (doublesex and mab-3 related transcription factor 1) play a role in inhibiting the female pathway by upregulating *amh* (anti-Müllerian hormone) to stimulate testes development (Beukeboom and Perrin 2014). Along the female pathway, a feedback loop comprised of WNT4 (Wingless-type MMTV integration site family, member 4), RSPO1 (R-spondin-1), and β-catenin is involved in the development of ovaries along with foxl2 (forkhead box L2), which upregulates aromatase (Beukeboom and Perrin 2014). These genes remain present in all vertebrates regardless of whether it is a GSD or ESD system, but may play different roles in determining the sex.

The genes of the sex-determining pathway form a cascade with a master sex-determining gene at the top. It has been originally thought that all genes involved in sex-determination are transcription factors with either Zinc fingers (Sinclair et al. 1990) or DM domains (Matsuda et al. 2002), but as more master sex-determining genes are being discovered, it is evident that other gene families outside of transcription factors have evolved into key components in the sex-determination cascade. Further elaboration on these genes is explored in the later sections.

There are two possible origins of a master sex-determining gene. The gene could have already been part of the sex-determining pathway and took on a greater role in the pathway, which led to it becoming the main switch (Cutting et al. 2013). Another possibility is that the gene was originally part of a different pathway and adopted a new function related to sex-determination (Cutting et al. 2013).

1.1.1. Evolution of Sex Chromosomes

Sex chromosomes are essential for GSD. It is generally believed that sex chromosomes have evolved from identical autosomes that have stopped recombining (Bachtrog et al. 2014; Betrán et al. 2012). An ancestral autosome becomes a proto-Y chromosome by acquiring a male-determining gene, which leads to sexually antagonistic

variation, resulting in the accumulation of male-specific genes (Figure 1.2; Graves 2006; Landeen and Presgraves 2013, Betrán et al. 2012). Autosomes may also obtain mutations such as a gain-of-function that leads to sex-determination (Devlin and Nagahama 2002). These mutations may result in elevated activity of the gene or in a completely new function (Devlin and Nagahama 2002). As the accumulation of mutations and male-specific genes continues, recombination gets repressed as a result of modification from these genes, leaving only a small pseudoautosomal region between the two chromosomes (Graves 2006; Landeen and Presgraves 2013; Bergero and Charlesworth 2009). With no more recombination, the Y chromosome degrades. As the Y chromosome is degraded, only genes that have a male advantage remain active while the rest are lost. In extreme cases, the Y chromosome degrades completely resulting in an XO system (Bachtrog et al. 2014).

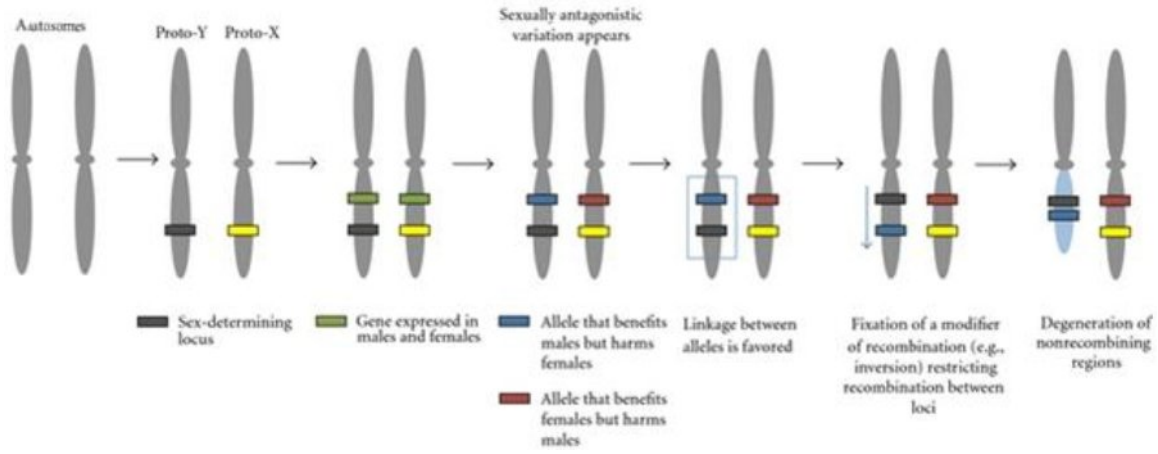


Figure 1.2 The process of autosomes becoming heteromorphic sex chromosomes

Originally, two autosomes are recombining until one of the autosomes acquires a sex-determining locus (shown in black). Genes continue to be expressed on both chromosomes until sexually antagonistic variation appears. Linkage between the male-specific genes and the sex-determining locus forms repressing recombination. This leads to the degradation of the Y chromosome, with active genes getting lost on the Y chromosome where only genes with a male advantage remain active. Modified image taken from Betrán et al. 2012 which is under the Creative Commons Attribution License.

Within the ZW system, the same process of evolution of sex chromosomes occurs as with the XY system, with the accumulation of male-specific genes occurring on the Z chromosome. Much like the Y chromosome, the W chromosome undergoes degradation (Namekawa and Lee 2009), but to date, no vertebrates have been found to

have a ZO system. Oddly enough, even though the ZW system follows the same pattern in evolving its sex chromosomes as the XY system, there is no homology between the two systems, indicating that they evolved from different autosomal regions (Ezaz et al. 2006). Nevertheless, transitions between these two systems have occurred numerous times across taxa (Ezaz et al. 2006).

Regardless of whether an organism has an XY or a ZW system, the master sex-determining gene is male specific, located on either the Y or the Z chromosome. In the case of the ZW system, both males and females have the sex gene, but the dosage triggers the sex-determining pathway (Nanda et al. 2008).

1.1.2. Evolution of Sex-determining Genes

Ancestral sex-determining genes may have been associated with DM domain genes, which encode transcription factors similar to *doublesex*, a sex-regulator gene first identified in *Drosophila* (Volff et al. 2002), as all vertebrates have these highly conserved genes play a role in sex-determination (Cutting et al. 2013). DM domain genes have been conserved for hundreds of millions of years having arisen to regulate sexual development in metazoans (Matson and Zarkowe 2012). However, DM domain genes are not the only genes involved in regulating sex-determination.

A key driver of the evolution of sex-determining genes is genome duplication. Vertebrates have undergone a whole genome duplication which resulted in many redundant copies of genes (Dehal and Boore 2005). Subsequently, many of these genes became novel sex-determining genes through neofunctionalization, where one gene retains its original function while another obtains a new function (Mawaribuchi et al. 2012). Across vertebrates, several sex-determining genes have arisen from DM domain genes, while others came from other gene families.

Another reason for the evolution of sex-determining genes is due to sex chromosome undifferentiation (Mawaribuchi et al. 2012). In organisms that have not yet differentiated their sex chromosomes, sex-determining genes are not stable and can be altered. This allows for a novel gene to take over the sex-determining pathway in a neofunctionalization-like manner (Mawaribuchi et al. 2012). Once the sex chromosomes

been established, the genes stabilize on the chromosomes, resulting in them becoming sex-determining genes (Mawaribuchi et al. 2012).

1.2. Sex-Determination in Mammals

All mammals have an XX/XY female/male GSD sex system (Figure 1.1, Graves 2015) where their chromosomes are highly dimorphic (Graves 2006). Distinguishing between males and females begins with the development of sex-specific gonads which is triggered by *Sry* (*sex-determining region of the Y chromosome*) (Kashimada and Koopman 2010). With the exception of monotremes, *Sry* is conserved in all therians and not present in any other vertebrate (Wallis et al. 2007; Cutting et al. 2013).

Sry is an evolved form of *Sox3* (*sry-box 3*), an X-linked gene involved in gonadal development but not sex-determination, which has an HMG (high mobility group)-box motif that enables it to bind to DNA and assist in transcription (Marshall Graves and Peichel 2010). Having gained the sex-determining function through neofunctionalization (Cutting et al. 2013; Weiss et al. 2003; Mawaribuchi et al. 2012), *Sry* is the master sex-determining gene located at the top of the sex-determination pathway, where with SF1 (splicing factor 1) it activates *Sox9* expression (Cutting et al. 2013). In its activated form, SOX9 inactivates *Sry* expression and then proceeds to activate *Fgf9*, *Pgds* (*prostaglandin d synthase*), and *Amh* for testes development (Figure 1.3; Cutting et al. 2013). The upregulation of *Dmrt1* by FGF9 leads to the inhibition of FOXL2 and the RSPO1/β-catenin/WNT4 pathway, while possibly also activating *Amh* (Beukeboom and Perrin 2014). The role of AMH is to degrade the Müllerian ducts which would later develop into fallopian tubes (Cutting et al. 2013).

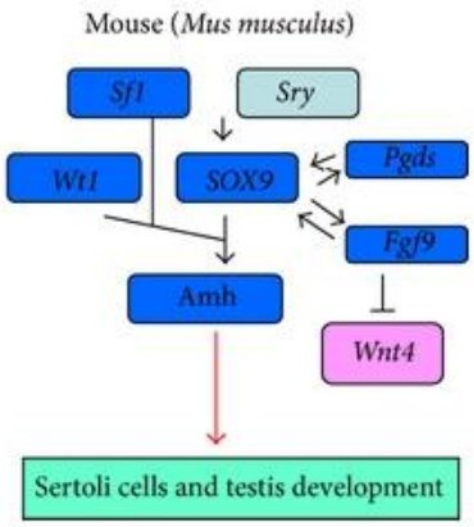


Figure 1.3 An overview of the sex-determining pathway in mammals

Using mouse as a model, *Sry* activates *Sox9* which proceeds to activate *Fgf9*, *Pgds*, and *Amh* for testes development. Activation of *Amh* requires not only SOX9 but also SF1 and WT1. Inhibition of the female pathway is done by FGF9. Light blue represents the master sex-determining gene, blue represents the male pathway, and pink represents the female pathway. Modified image taken from Trukhina et al. 2013 which is under the Creative Commons Attribution License .

In the absence of *Sry*, FOXL2 and the WNT4/RSPO1/β-catenin pathway are no longer inhibited and are able to activate aromatase and the development of ovaries (Beukeboom and Perrin 2014). Even though FOXL2 and the WNT4/RSPO1/β-catenin pathway are required for ovarian development, they are independent of each other (Cutting et al. 2013).

There are always unusual exceptions present in nature that do not conform to the norm. The mole vole (*Ellobius lutescens*) and the spiny rat (*Tokudaia muenninki*) lack both the *Sry* gene and the Y chromosome, but still have males and females (Just et al. 2007; Graves 2015). The platypus (*Ornithorhynchus anatinus*) has 5X and 5Y chromosomes and lacks the *Sry* gene (Rens et al. 2004). Within these 5X chromosomes, some have typical mammalian genes, while others have the *Dmrt1* gene (Graves 2006). In both cases the mechanism for sex-determination is unknown.

1.3. Sex-Determination in Birds

Birds determine sex strictly through GSD using a ZZ/ZW female heterogametic system as seen in Figure 1.1 (Trukhina et al. 2013). With the exception of the ratites (ostrich, emu, and cassowary), all birds have distinct Z and W chromosomes (Schartl 2004), where unlike in the XX/XY system, the Z chromosome does not undergo inactivation (Smith et al. 1999).

The process of sex-determination in birds is not well understood with two models proposed to describe this event. The determination of sex may either rely on sex-determining genes on the W chromosome or through dosage dependency on the Z chromosome (Cutting et al. 2013). Both models are supported by evidence, but neither has enough to refute the other model.

Regarding the W-linked gene hypothesis, several candidate genes have been found to support this hypothesis; these include *WPKCI* (W-linked PKC inhibitor/interacting protein), a gene similar in function to a protein kinase C inhibitor (Schartl 2004), *FET1* (female-expressed transcript 1), a gene involved in the female urogenital system (Schartl 2004), and *ASW* (avian sex-specific W-linked), a gene related to the histidine triad family (O'Neil et al. 2000). All three genes are located on the W chromosome and are involved in ovary development; however their exact functions still remain uncertain (Hori et al. 2000; Schartl 2004; O'Neil et al. 2000).

Another key model for sex-determination is through a dosage-dependent manner involving *DMRT1*. First discovered in chicken (*Gallus gallus*), *DMRT1* is located on the Z chromosome where both males and females have it, but a double dose is required for testes development (Smith et al. 2009; Cutting et al. 2013). Comprised of a zinc finger-like DM domain, *DMRT1* binds to DNA where it regulates transcription (Marshall Graves and Peichel 2010). It is not entirely clear how *DMRT1* functions in sex-determination, but a double dose of *DMRT1* activates *SOX9* which will either repress FOXL2 and the WNT4/RSPO1/β-catenin pathway (Beukeboom and Perrin 2014) or regulate expression of *PGDS* for testes development (Figure 1.4; Cutting et al. 2013). Rather than SOX9, DMRT1 may repress FOXL2 and the ovarian pathway (Figure 1.4; Cutting et al. 2013). Another key player in the sex-determining pathway is *AMH*, which is activated by

DMRT1 to possibly upregulate *SOX9* and inhibit *aromatase* expression (Cutting et al. 2013).

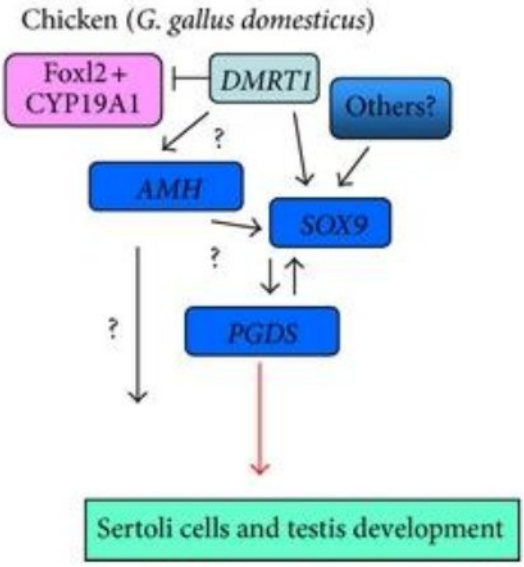


Figure 1.4 An overview of the sex-determining pathway in birds

Using chicken as a model, *DMRT1* activates *SOX9* and inhibits FOXL2 and CYP19A1. *SOX9* activates *PGDS* which leads to testes development. *DMRT1* may also activate *AMH* for testes development, as there are still plenty of unknowns regarding the sex-determination pathway. Light blue represents the master sex-determining gene, blue represents the male pathway, and pink represents the female pathway. Modified image taken from Trukhina et al. 2013 which is under the Creative Commons Attribution License.

Along the ovarian pathway, a single dose of *DMRT1* may either be not enough to inhibit FOXL2 and the WNT4/RSPO1/β-catenin pathway or it may be suppressed by an unknown gene or mechanism (Beukeboom and Perrin 2014). In the absence of suppression, FOXL2 and the WNT4/RSPO1/β-catenin pathway are active to stimulate estrogen production and ovary development (Beukeboom and Perrin 2014; Cutting et al. 2013).

1.4. Sex-Determination in Reptiles

Reptiles are split between GSD and ESD with either male or female heterogametic systems (Figure 1.1). For snakes, many lizards, and some turtles, sex is

genetically determined by either a male (in lizards and turtles) or female (only in snakes) heterogametic system (Pieau et al. 1999). For the rest of the reptiles, including all crocodiles, sex is determined environmentally, specifically by temperature during egg incubation (Pieau et al. 1999). The mechanism behind ESD is not well understood but evidence indicates that thermosensitive genes may be involved.

It is known that the determination of sex occurs during the most optimal time for the gonads to respond to changes in ambient temperature, which takes place during embryogenesis (Schroeder et al. 2016). During this temperature sensitive period, several genes are differentially expressed in the developing gonads. The expression of these genes varies between the masculinizing and feminizing temperatures, prompting them to be candidate sex-determining genes. Some of the possible candidates are CIRBP (cold-inducible RNA-binding protein) in the common snapping turtle (*Chelydra serpentine*) (Schroeder et al. 2016), and *wt1* (*Wilm's tumor-associated gene*) in turtles (*Chrysemys picta*) (Valenzuela 2008), both of which are thermosensitive, but their functions in sex-determination still remain unclear. It is known that estrogen is very important for ovary development in ESD and that *aromatase* and *foxl2* are both expressed at higher levels at feminizing temperatures, while *dmrt1* and *sox9* are expressed at masculinizing temperatures (Rhen and Schroeder 2010). Regarding WT1, it is proposed that WT1 may act to activate SF1 which would then either activate *sox9* and *amh* at masculinizing temperatures or *aromatase* at femininizing temperatures (Pieau et al. 1999). Aromatase would stimulate the production of estrogen and the development of ovaries while potentially inhibiting *sox9* and *amh* (Pieau et al. 1999; Crews et al. 2001).

Regarding GSD, with the exception of snakes which only have a ZZ/ZW system, all other GSD reptiles have both XX/XY and ZZ/ZW heterogametic systems (Pieau et al. 1999). Many GSD reptiles still retain some thermosensitive genes, primarily those whose ancestors had an ESD system (Valenzuela 2008). In the case of turtles, GSD was derived from an ESD ancestor and has retained some of the thermosensitive genes. These genes are predominantly ineffective during sex-determination, but a few have retained some thermal sensitivity, enough to affect gonadal development (Valenzuela 2008). For instance, *wt1* is differentially expressed in both GSD and ESD turtles and has

retained its thermosensitivity (Valenzuela 2008). Other sex-determining genes that have retained thermosensitivity are *sf1*, *dax1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), and *sox9* (Trukhina et al. 2013). Whether a reptile has a GSD or ESD system, no known master sex-determining gene has been identified, however many candidates have been proposed.

In general, reptiles reproduce sexually using either GSD or ESD systems; however, squamate (scaled) reptiles, which are the snakes and lizards, are also capable of reproducing asexually, through facultative parthenogenesis (Booth et al. 2012). However, such a method of reproduction predominantly occurs in captivity, but it is possible that it can also occur in the wild.

1.5. Sex-Determination in Amphibians

Sex-determination in amphibians is predominantly genetic (Figure 1.1). Unlike in birds or mammals where there is a fixed heterogametic sex system, amphibians have both XY and ZW systems indicating that evolution of sex chromosomes has occurred many times (Nakamura 2009). Not only can amphibians be either XY or ZW, but both systems can be present within one species as is the case with the wrinkled frog (*Rana rugosa*) (Nakamura 2009). It is important to note that even though amphibians have XY and ZW systems, their sex chromosomes are indistinguishable from autosomes and that it is through back-crosses of sex-reversed and normal individuals that the systems have been determined (Yoshimoto and Ito 2011).

Although amphibians have a GSD system, environmental factors are capable of affecting sex-determination. At ambient temperatures, sex is determined genetically with a 1:1 ratio of males to females being produced (Nakamura 2009). However exposure to extreme temperatures, hot or cold, will favour one sex over the other. Generally higher temperatures favour males while lower temperatures favour females (Nakamura 2009). It is worth noting that such temperatures are not commonly encountered by amphibians.

Many genes have been shown to be involved in sex-determination including *sox9*, *wnt4*, *dax1, wt1*, and *fgf9*, but none are the master sex-determining gene as their

expression does not vary between males or females (Nakamura 2009). Instead of a master sex-determining gene, it is probable that steroid hormones determine the sex of amphibians. The steroidogenic enzymes, Cyp19 (P450 aromatase) and Cyp17 (P450 17alpha-hydroxylase/C17-20 lyase), are present in frogs and are expressed in undifferentiated gonads during sex-determination, with expression of *cyp19* in females and *cyp17* in males (Nakamura 2009). Regulation of these enzymes is done by *foxl2* and *sox3* where f*oxl2* is upregulated in the gonads of female tadpoles to promote expression of *cyp19* (Nakamura 2009).

Due to the variation in sex systems within this taxon, there is currently only one master sex-determining gene identified in frogs. In the African clawed frog (*Xenopus laevis*), which has a ZZ/ZW system, a paralog of *dmrt1* (*dm-w*) has been identified as the sex-determining gene (Yoshimoto et al. 2008). *Dm-w* is localized on the W chromosome, making it a female sex-determining gene, required for ovarian development (Yoshimoto et al. 2008). As a paralog of *dmrt1*, *dm-w* shares high sequence similarity with *dmrt1*, particularly in the DNA-binding domain region, however the C-terminal region shares no similarity (Yoshimoto et al. 2008). The C-terminal region of *dmrt1* has a transactivation domain which is lacking in *dm-w* (Yoshimoto et al. 2010). Oddly enough, both *dmrt1* and *dm-w* are found on the chromosomes, with *dmrt1* localized on the Z chromosome, while *dm-w* is on the W chromosome, suggesting that these two genes compete with one another for the DNA-binding site (Yoshimoto et al. 2008; 2010). During sex-determination, *dm-w* forms either a homodimer or a heterodimer with *dmrt1*, where it inhibits the gene cascade for testes formation, allowing for ovarian development to take place (Yoshimoto et al. 2010).

1.6. Sex-Determination in Fish

With a broad diversity in fish species and their aquatic habitats, a diverse range of sex-determination mechanisms has evolved ranging from hermaphroditism, male or female heterogametic, GSD or ESD systems (Figure 1.1). Sexuality of fish falls under three categories: gonochorism, hermaphroditism, and unisexuality. Mentioned below are the various forms of sex-determination that have evolved in fish.

1.6.1. Gonochorism

Gonochorism determines sex through either genetic or environmental or a combination of both cues. Gonochoristic individuals develop only as either male or female and maintain that gender throughout their lifetime (Devlin and Nagahama 2009). Under genetic cues, fish either have an XX/XY or a ZW/ZZ system as seen in Figure 1.1. An exception to this rule is seen in the platyfish (*Xiphophorus maculatus*) which has three sex chromosomes: X, Y, and W (Volff and Schartl 2001). Male heterogamety has been speculated to favour male size advantage while female heterogamety favours female size advantage (Devlin and Nagahama 2009). Subsequently, it is of no surprise that majority of the gonochoristic fish have an XY male heterogametic system.

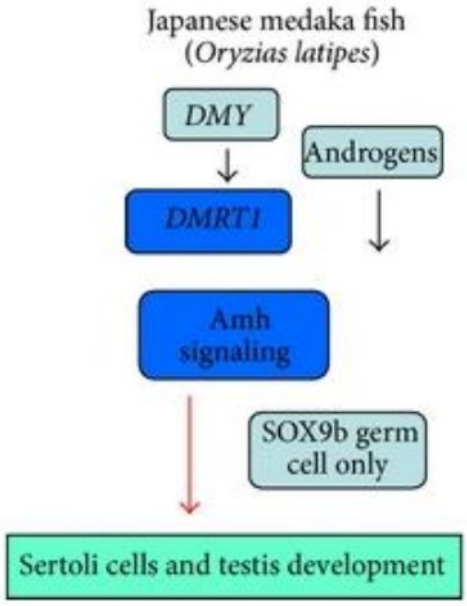


Figure 1.5 An overview of the sex-determining pathway in gonochoristic fish

In the Japanese medaka fish, the sex-determination pathway is still unclear. *Dmy* is the master sex-determining gene and the Amh signalling pathway is important for testes development with *sox9* involved in the control of testes, but how they all relate to each other is still unknown. Light blue represents the master sex-determining gene, blue represents the male pathway, and pink represents the female pathway. Modified image taken from Trukhina et al. 2013 which is under the Creative Commons Attribution License.

The second sex-determination gene discovered in vertebrates and first in fish is *dmy* (*Y-specific DM-domain gene*) found in the Japanese medaka (*Oryzias latipes*)

(Matsuda et al. 2002; Kobayashi et al. 2013). *Dmy* is specific to the medaka species with it being the master sex-determining gene for *O. latipes*. How it function in *O. latipes*, which has an XX/XY system (Schartl 2004) is not entirely clear, possibly leading to activation of *amh* for testes development (Figure 1.5). In other medaka species, like in *O. luzonensis*, *gsdf* (gonadal somatic cell derived factor) is the master sex-determining gene (Myosho et al. 2012). The variety in sex-determining genes is not surprising due to the wide diversity in fish. To date, known sex-determining genes include *amhr2* (*anti-Müllerian receptor type II*) in tiger pufferfish (*Takifugu rubripes*) (Kamiya et al. 2012), *amhy (Y-linked anti-Müllerian hormone duplication)* in Patagonian pejerrey (*Odontesthes hatchery*) (Hattori et al. 2012), and *sdY* (*sexually dimorphic on the Y chromosome*) in rainbow trout (Oncorhynchus *mykiss*) (Yano et al. 2012).

In the case of platyfish which has three chromosomes, how sex-determination works still remains a mystery. Male platyfish are either YY or XY, while females are XX, XW, or YW (Volff and Schartl 2001; Böhne et al. 2009). These potential combinations suggest that the sex-determining genes are present on all three chromosomes, where only those on the Y chromosome are active, while the W chromosome has a suppressor specific for the Y chromosome, allowing for YW females (Volff and Schartl 2001). Another suggestion is that sex-determination with the three chromosomes is dosage dependent (Böhne et al. 2009). Each chromosome would carry different copies of the master sex-determining gene; Y would have two, X one, and W zero (Böhne et al. 2009). The amount of copies present would dictate whether testes or ovaries would develop. If an individual has three or more copies than it would be destined to be male, while two copies or less would result in females (Böhne et al. 2009).Regardless of where the sex-determining genes are located, the actual genes involved are still unknown. All that is known is that sex-determination in platyfish does not correspond to DM-domain genes (Böhne et al. 2009).

1.6.2. Hermaphroditism

Hermaphroditism refers to the presence of both male and female sexes within one individual. In fish, there is no order that only has hermaphrodites (Avise and Mank 2009). Instead, it is a mixture of hermaphrodites and gonochoristic species, suggesting

that hermaphroditism evolved from gonochorism through convergent evolution many times throughout the taxon (Avise and Mank 2009). The switch from gonochorism to hermaphroditism is not well understood, but it has been theorized that the need to switch evolved due to natural or sexual selection to provide individuals with the maximum production of offspring (Avise and Mank 2009; Devlin and Nagahama 2009). Further selection was applied on hermaphrodite species resulting in two types of hermaphroditism: sequential or simultaneous.

The more common of the two types, sequential hermaphroditism deals with a change in sex in an individual during its lifetime. This change can occur in multiple different ways and is governed by social cues and hormonal changes. Protogynous fish, commonly seen in reef fish, begin as females and at some point in life, change into males (Avise and Mank 2009). Rarely do males switch to females as it is more costly to produce eggs than sperm. Anemonefish (*Amphiprion ocellaris*) are one of the few examples of protoandrous species that switch from male to female in the absence of females in a population (Avise and Mank 2009). An even more unusual hermaphroditism is the bidirectional sex change where individuals not only change sex many times in the course of their lifetime, but in both directions as seen in goby (*Trimma*) species (Avise and Mank 2009). In all three forms of sequential hermaphroditism, the cause of sex change is stimulated by mate monopolization.

Simultaneous or synchronous hermaphroditism has the simultaneous production of both male and female gametes in one individual (Avise and Mank 2009; Devlin and Nagahama 2009). Such hermaphrodites can either be outcrossing or self-fertilized. Although both male and female gametes are present, outcrossing hermaphrodites need to encounter another individual in order to mate and reproduce. In such situations, a sex-role decision must be made between the two individuals (Avise and Mank 2009). Most of the simultaneous hermaphrodites are of the outcrossing nature, but the mangrove killifish (*Kryptolebias marmoratus*) is the only known example of a self-fertilizing hermaphrodite which does not require a partner to mate (Avise and Mank 2009). In both situations, simultaneous hermaphrodites have arisen due to sparse populations (Avise and Mank 2009).

1.6.3. Unisexuality

Unisexuality in fish has arisen through interspecific hybridization (Neaves and Baumann 2011). In unisexual species like the Amazon molly (*Poecilia formosa*) all individuals are female and rely on hybrid mating with a male of a closely related species in order to maintain the population (Lampert and Schartl 2008). This dependency on hybrid mating has allowed the Amazon molly to maintain some form of genetic variability through paternal introgression. Paternal DNA gets incorporated into the genome by either small parts of the paternal genome remaining in the oocyte or the whole genome fertilizes the egg, producing polyploid offspring (Lampert and Schartl 2008). However, paternal introgression has no effect on the sex of unisexual fish, as they are always female.

1.7. Sex-Determination in Salmonidae

The family Salmonidae is comprised of salmon, trout, char, freshwater whitefish, and grayling (Nelson, Grande, and Wilson 2006) whose common ancestor underwent whole genome duplication 88-103 million years ago (Macqueen and Johnston 2014). After a whole genome duplication event, there are duplicate chromosomes and genes present (Force et al. 1999). To avoid redundancy, chromosomes obtain null mutations in regulatory regions which can then lead to either non-functional gene copies (nonfunctionalization), subdivision of gene function (subfunctionalization), or new functions allowing for both copies to exist (neofunctionalization) (Figure 1.6; Force et al. 1999). This is crucial for sex-determination as sex-determining genes must either delete one copy of the duplicate or recruit a novel sex-determining master gene (Davidson et al. 2009). In the case of salmonids, a novel sex-determining gene emerged: *sdY*.

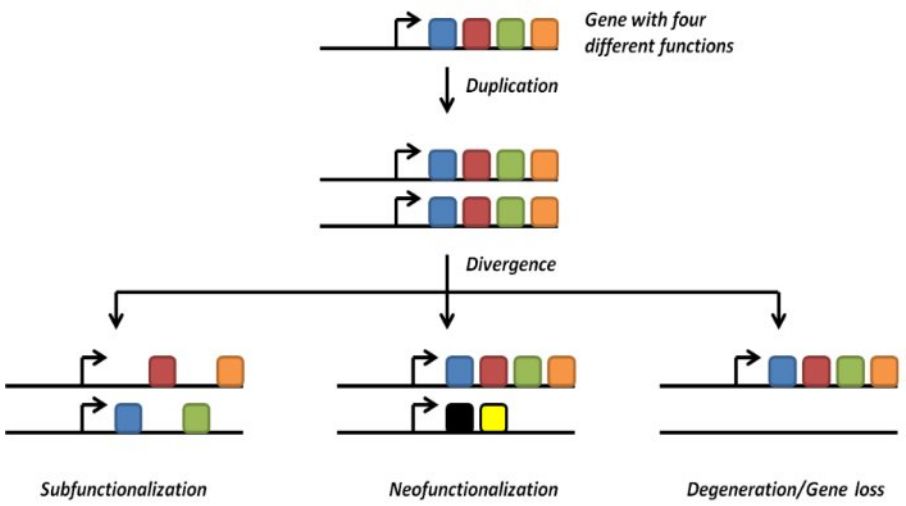


Figure 1.6 Consequences of whole genome duplication

Whole genome duplication results in two copies of the entire genome. Over the course of time, one copy acquires null mutations which will lead to three possible fates for the duplicated genes. Null mutations may result in subfunctionalization where the gene function is partitioned between the two copies, neofunctionalization where the mutation results in a new function on one of the copies and allowing both copies to exist, or nonfunctionalization also known as degeneration or gene loss where one gene copy is lost. From "Evolutionary fate of duplicate genes" by Veryhuman, 2012, https://en.wikipedia.org/wiki/Gene_duplication. Copyright CC BY-SA 3.0.

Sex-determination in salmonids is generally viewed as being a male heterogametic sex-determining system (Davidson et al. 2009). This is predominantly based on the extensive research done on salmon, trout, and char while limited knowledge is present regarding whitefish and grayling (Davidson et al. 2009). Furthermore, there is a lack of synteny of the phenotypic sex locus (SEX) within the salmonids due to the localization being on various different chromosomes, sometimes even within one species (Woram et al. 2003). In Tasmanian Atlantic salmon (*Salmo salar*), the SEX locus is present on chromosomes 2, 3, and 6 (Eisbrenner et al. 2014). Such variability in the location of SEX may be a result of the transposition of the sex-determining gene between chromosomes or that there are different SEX loci where each represents unique sex-determining genes (Woram et al. 2003; Davidson et al. 2009). Regardless of the different locations of the SEX locus, *sdY* has been found to be highly conserved in all salmonid species (Yano et al. 2013).

The exact pathway of sex-determination in salmonids is not known, but many genes have been implicated. Differential expression of *sdY*, *gsdf, amh*, and *cyp19a* is

observed in males and females during development (Lubieniecki et al. 2015a). During testes development, *amh,* either alone or with *gsdf*, inhibits ovarian development through the inhibition of *cyp19a* (von Schalburg et al. 2011; Lubieniecki et al. 2015a). A key element in the pathway is regulating *cyp19a*. *Sf1* and *dax1* are consistently high in both testes and ovaries where they mediate activation of steroidogenesis (von Schalburg et al. 2010; 2011). The levels of *dax1* dictate the role of *sf1* in mediating regulation of *cyp19a*. At low levels of *dax1*, *sf1* is repressed while at high levels *sf1* is co-activated (von Schalburg et al. 2010). *Dax1* and *sf1* associate with *foxl2* which leads to the transactivation of *cyp19a* (von Schalburg et al. 2011). Upregulation of *cyp19a* increases levels of estradiol E2, thus promoting ovarian development (Lubieniecki et al. 2015a).

The role *sdY* plays in sex-determination remains uncertain. Speculations have been made that *sdY* may be involved in the upregulation of *gsdf* and *amh* particularly since it is differentially expressed before them, starting at 58 days post fertilization (Lubieniecki et al. 2015a). Also, in the absence of *sdY*, repression of *cyp19a* is not seen (Lubieniecki et al. 2015a). However, no concrete evidence has been shown to indicate the role of *sdY*. To further complicate matters, *sdY* is unlike any other sex-determining gene discovered as it has no homology to any well-known factor already identified in sex-determination.

1.7.1. Origins of *sdY*

First discovered in rainbow trout, *sdY* is a gene encoding a putative protein of 192 amino acids that shares sequence similarity with the carboxy-terminal domain of interferon regulatory factor 9 (Irf9), a transcription factor involved in triggering immune responses (Yano et al. 2012; Tamura et al. 2008). There is no similarity present with the N-terminal binding domain of Irf9 as SdY lacks this, suggesting that SdY is a truncated, divergent form of Irf9 (Yano et al. 2012). The C-terminal domain shared by Irf9 and SdY contains an IRF association domain which enables Irf9 to form a complex with Stat (Signal transducer and activator of transcription) 1 and 2 to elicit type I interferon signalling (Takoaka and Yanai 2006).

No known involvement of the interferon signalling pathway during testicular differentiation in vertebrates has been reported, but this does not immediately exclude *sdY* as the sex-determining gene in salmonids. Since SdY is a divergent, truncated form of Irf9, the sex-determination function may have evolved through neofunctionalization to compensate for the whole genome duplication, making *sdY* a paralog of *Irf9* (Yano et al. 2012). However, salmonids have two copies of *irf9*, making those paralogs of the ancestral *irf9* rather than *sdY* (Yano et al. 2012).

Transposition of *sdY* is evident in Atlantic salmon (Lubieniecki et al. 2015b). Coupled with the discovery of the SEX locus being located on three chromosomes (Eisbrenner et al. 2014), it is possible that *sdY* may have transposed onto a chromosome which altered its function, gaining a new role as a sex-determining gene. Many models have been proposed as to how *sdY*, an immune-related gene, became a sex-determining gene, but the exact origins of *sdY* still remain a mystery.

1.8. Project Aims

Although *sdY* has been classified as the sex-determining gene in rainbow trout and found to be male-specific in the majority of the salmonid species (Yano et al. 2013), the function of SdY still remains a mystery. Therefore, the aim for this project is to determine how SdY functions by identifying its interactors. With ten genera and over 200 species of salmonids (Nelson, Grande, and Wilson 2006), particular emphasis will be on Atlantic salmon as it is the main salmon species of the world (Gilbert 2002). I intend to determine interactors of SdY using yeast two-hybridization, co-immunoprecipitation, and his-tag pull down assays. By the end of this project, a better understanding of how SdY functions in salmon should be formed.

Chapter 2.

Methods

2.1. Yeast Two-Hybridization

Yeast two-hybridization experiments were done using the Matchmaker® Gold Yeast Two-Hybrid System from Clontech which is based on the methodology established by Fields and Song. Within this system, a bait protein is fused to the GAL4 DNA-binding domain (DNA-BD) of yeast, while libraries of prey proteins are fused to the GAL4 activation domain (AD). When the bait and prey fusion proteins interact, the two domains are in close proximity to each other resulting in the activation of transcription of four independent reporter genes (*AUR1-C*, *ADE2*, *HIS3*, and *MEL1*). These reporter genes allow for the yeast strains to be resistant to Aureobasidin A, to grow in the absence of adenine and histidine, and to express α-galactosidase which results in blue colonies in the presence of X-alpha-gal. Isolating positive interactors are selected on stringent plates that promote the activation of all four reporter genes. A simplified procedure of the yeast two-hybridization assay can be seen in Figure 2.1, where each step is elaborated in the following sections. All the yeast strains and plasmids used in this assay are summarized in Table 2.1.

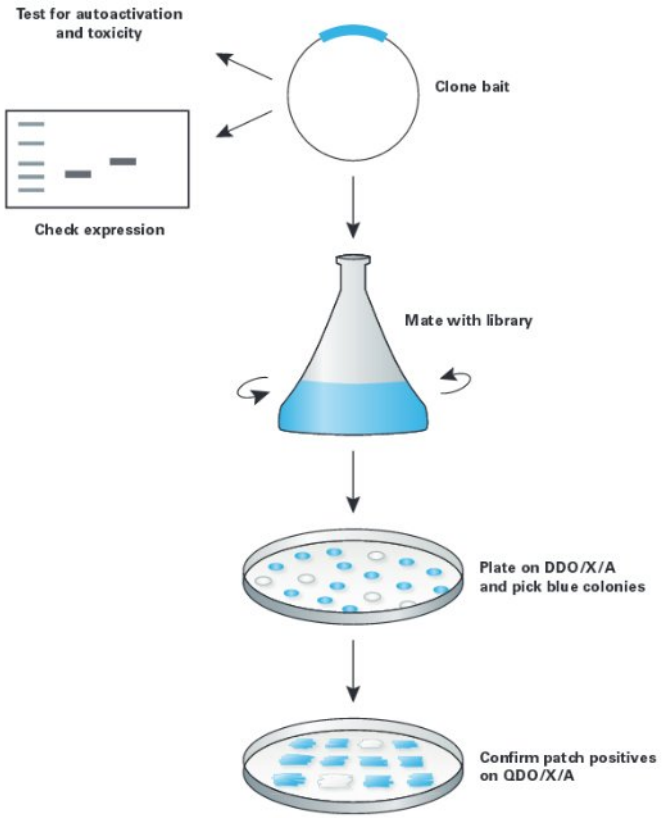


Figure 2.1 Yeast two-hybrid screening

The bait protein is cloned into a pGBKT7 vector and transformed into the Y2HGold yeast strain. Tests for autoactivation and toxicity are performed followed by mating with the cDNA library cloned into a pGADT7 vector and transformed into the Y187 yeast strain. Overnight mating results in diploids that contain the four reporter genes. Diploids are plated onto DDO/X/A followed by QDO/X/A plates to activate the reporter genes. Image taken from Matchmaker® Gold Yeast Two-Hybrid System User Manual from Clontech.

Table 2.1 Yeast strains and plasmids

Yeast strain	Genotype	Reporter Genes	Selectable Markers (yeast)	Reference
Y2HGold	*MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, GAL4Δ, gal80Δ, LYS2 : : GAL1UAS–Gal1TATA–His3, GAL2UAS–Gal2TATA–Ade2 URA3 : : MEL1UAS–Mel1TATA AUR1-C MEL1*	*AUR1-C, HIS3, ADE2, MEL1*	*trp1, leu2*	Matchmaker™ Gold Yeast Two-Hybrid System User Manual
Y187	*MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, GAL4Δ, gal80Δ, met–, URA3 : : GAL1UAS–Gal1TATA–LacZ, MEL1*	*MEL1, LacZ*	*trp1, leu2*	Harper et al. 1993
Plasmid	Information	Selectable Genes (bacteria, yeast)	Corresponding Yeast strain	Reference
pGBKT7-BD	A plasmid that expresses proteins fused to the GAL4 DNA-BD	*Kan^r, trp1*	Y2HGold	Louret et al. 1997
pGBKT7-53	A positive control plasmid that encodes a fusion of the murine p53 protein and the GAL4 DNA-BD	*Kan^r, trp1*	Y2HGold	Iwabuchi et al. 1993
pGBKT7-lam	A negative control plasmid that encodes a fusion of the human lamin C protein and the GAL4 DNA-BD	*Kan^r, trp1*	Y2HGold	Bartel et al. 1993
pGADT7-AD	A plasmid that expresses a protein fused to the GAL4 AD	*Amp^r, leu2*	Y187	Chien et al. 1997
pGADT7-rec	A plasmid that has the GAL4 AD and a SmaI restriction site used to generate a prey library	*Amp^r, leu2*	Y187	Make Your Own "Mate & Plate™" Library System User Manual
pGADT7-T	A positive control plasmid that encodes a fusion of the SV40 large T antigen and the GAL4 AD	*Amp^r, leu2*	Y187	Li and Fields, 1993

* All yeast strain information was taken from Matchmaker™ Gold Yeast Two-Hybrid System User Manual. All vector information was taken from Clontech.

2.1.1. Cloning of Bait

The GAL4 DNA-BD of the pGBKT7 vector was fused in frame with *sdY*, my gene of interest, through PCR cloning. The pGBKT7 vector was linearized through double digestion with BamHI (Thermo Scientific) and EcoRI (Thermo Scientific).

sdY was amplified by PCR using the following primers:

Forward: Y2H SDY F: 5'-CATGGAGGCCGAATTCATGGTTGACA-3'
Reverse: Y2H SDY R: 5'-CAGGGGAGGAGGATCCGTCGACCTGC-3'

PCR reactions were performed on T1 thermocyclers under the following conditions: an initial denaturation at 95^{o}C (4min) followed by 35 cycles of denaturation at 98^{o}C (20s), annealing at 60^{o}C (30s), and extension at 72^{o}C (30s). A final extension at 72^{o}C for 5min followed before completion of the PCR. Each 10 µl reaction contained 6.4 µl PCR grade water (Clontech), 2 µl 2X Kappa HiFi HotStart ReadyMix (KAPA Biosystems) 0.3 µl of 0.3 µM forward primer (Y2H SDY F), 0.3 µl of 0.3 µM reverse primer (Y2H SDY R), 0.5 µl of 5% DMSO (New England Bio Labs), and 0.5 µl of *sdY* (15 ng/µl). The PCR reaction was then purified by NucleoSpin® Gel and PCR Clean-Up kit.

In-fusion cloning of the linearized pGBKT7 vector and my bait (*sdY*) was performed using In-fusion® Advantage PCR cloning kit from Clontech. 10-200ng of the purified PCR fragment (*sdY*, 68.9 ng/µl) was combined with 2 µl 5X In-fusion HD enzyme premix, 50-200ng of the linearized vector (pGBKT7), and to 10 µl of deionized water. The reaction was incubated at 50^{o}C for 15min and briefly on ice before being transformed into Stellar^{TM} Competent Cells purchased from Clontech. No more than 5 ng of DNA was mixed with 50µl of competent cells in a 1.5mL microcentrifuge tube. The mixture was incubated on ice for 30min followed by heat shock at 42^{o}C for 1min and another incubation on ice for 1-2 min. SOC medium (Clontech) was added to a final volume of 500 µl followed by incubation for 1 hour at 37^{o}C, shaking at 250rpm. Afterwards, 200 µl were plated onto LB agar with either 50 µg/mL kanamycin (Sigma) or 100µg/mL ampicillin (Sigma) and were incubated overnight at 37^{o}C.

Plasmids were isolated from the cells using QIAprep® Miniprep from Qiagen. The isolated plasmid DNA was verified through restriction digestion using BamHI (Thermo Scientific) and EcoRI (Thermo Scientific).

2.1.2. Generation of cDNA Two-Hybrid Library

My testis cDNA library was generated using the Make Your Own "Mate & Plate™" Library System from Clontech where all components, unless specified otherwise, came from Clontech. Primers used to generate the cDNA library include:

CDS III Primer: 5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30VN-3'
SMART III Oligo: 5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTATGGCCGGG-3'
5' PCR Primer: 5'-TTCCACCCAAGCAGTGGTATCAACGCAGAGTGG-3'
3' PCR Primer: 5'-GTATCGATGCCCACCCTCTAGAGGCCGAGGCGGCCGACA-3'

The cDNA was generated from total RNA from testes following the protocol provided with the Make Your Own "Mate & Plate™" Library System from Clontech. 1 µl of the RNA (2079.5 ng/µl) was used to generate the cDNA.

Amplification of the cDNA was achieved through long distance PCR. Each PCR reaction included 2 µl of the first-strand generated cDNA, 70 µl deionized water, 10 µl 10X Advantage® 2 PCR buffer, 2 µl 50X dNTPs, 2 µl 5' PCR primer, 2 µl 3' PCR primer, 10 µl 10X Melting solution, and 2 µl 50X Advantage 2 polymerase mix. The PCR reactions were run on T1 thermocyclers using the following parameters: a denaturation at 95^{o}C for 30s followed by 20 cycles of annealing at 95^{o}C for 10s and extension at 68^{o}C for 6 min, where the extension time was extended by 5s each cycle. After a final extension of 68^{o}C for 5min, double stranded (ds)-cDNA was generated.

The ds-cDNA was purified using the CHROMA SPIN™ +TE-400 columns from Clontech following the provided protocol. After purification, the ds-cDNA was used for library construction. The two-hybrid library was constructed via a library-scale transformation using the Yeastmaker Yeast Transformation System 2 from Clontech. Aliquots of 1 ml and 50ml were stored at -80^{o}C.

2.1.3. Yeast Two-Hybrid Mating

Yeast mating was achieved using Matchmaker® Gold Yeast Two-Hybrid System from Clontech. The mated culture was plated onto DDO/X/A plates. After the appearance of colonies on DDO/X/A plates, blue colonies were transferred onto QDO/X/A plates and grown for another 3-5 days at 30^{o}C.

Prior to the mating between my bait (SdY) and my prey library, control mating, using the Yeastmaker Yeast Transformation System 2 supplied by Clontech, was performed to verify that the experiments were working properly. Plasmid pGBKT7-Lam encodes the GAL4 DNA-BD fused with lamin and mating with this plasmid serves as a negative control when mated with plasmid pGADT7-T. Plasmid pGBKT7-53 encodes the GAL4 DNA-BD fused with a murine p53 and mating with this plasmid serves as a positive control when mated with the pGADT7-T plasmid. Control mating was done according to the provided protocols from Clontech.

Plasmid transformation of yeast colonies was achieved following a modified protocol from Elble 1992. 10 µl of carrier DNA (salmon sperm), was combined with 5 µl of plasmid, one colony of Y2HGold or Y187 yeast, and 500 µl of PLATE (50% PEG, 1M LiAC, and 10X TE) solution. The mixture was incubated at room temperature for overnight to 4 days before being pelleted for 10s at 8000-10,000rpm. The supernatant was removed and the cells were resuspended in 200 µl of distilled water and spread onto appropriate plates. Colonies were grown at 30^{o}C for 3 days. After colonies have grown, one colony from Y2HGold strain and one colony from the Y187 strain were picked and combined in a 1.5 mL microcentrifuge tube with 500 µl of 2X YPDA followed by an overnight incubation at 30^{o}C with shaking at 200rpm. The mated cultures were then diluted (1/10, 1/100, and 1/1000) and incubated at 30^{o}C for 3-5 days on SD/trp, SD/Leu, DDO, and DDO/X/A plates.

The number of clones screened was calculated using the following formula:

$$\text{Number of Clones screened} = \frac{(\#\text{ of colonies})(\text{resuspension volume})}{(\text{plating volume})(\text{dilution factor})} \quad (1)$$

where the viability is calculated using the formula:

$$\text{Viability} = \frac{(\#\text{ of colonies})}{(\text{plating volume})(\text{dilution factor})} \quad (2)$$

The mating efficiency of the yeast two-hybridization using Clontech ranges from 2-5%, which is determined using the following equation:

$$\text{Mating efficiency} = \frac{\text{Viability of diploid}}{\text{Viability of limiting partner}} \times 100\% \quad (3)$$

To test whether my bait autonomously autoactivates or is toxic, an empty pGBKT7-BD vector and *sdY* cloned into pGBKT7 were transformed into Y2HGold using the same protocol as mentioned above. Diluted (1/10 and 1/100) samples were plated onto SD/Trp, SD/Trp/X, and SD/Trp/X/A plates and grown for 3-5 days at 30^{o}C.

2.1.4. Rescue and Isolation of Library Plasmid

To identify the protein(s) responsible for the positive interaction(s), blue colonies on QDO/X/A plats were streaked onto QDO/X plates (grown for 3-5 days at 30^{o}C) and rescued using the Easy Yeast Plasmid Isolation kit from Clontech. The purified DNA was transformed into Stellar^{TM} Competent Cells following the protocol mentioned earlier in section 2.1.1. Colonies were picked and isolated using QIAprep® Miniprep from Qiagen with visualization of the DNA inserts done through restriction digestion using HindIII (Invitrogen). Upon verifying that inserts are present, the DNA was sequenced using either the 377 DNA Sequencer or the 310 Genetic Analyzer from ABI PRISM®. Preparation of the DNA for sequencing was done using the DYEnamic ET Terminators Cycle Sequencing kit from GE Healthcare Life Sciences. The DNA was mixed with 0.5 µl primer, 1 µl sequencing mix and 1 µl sequencing buffer from the kit, and to 5 µl distilled water. The sample was then run in a thermocycler (either T1 or T personal) under the following conditions: 39 cycles of denaturation at 95^{o}C for 20s, annealing at 50^{o}C for 15s, and extension at 60^{o}C for 2min, followed by a final extension at 60^{o}C for 10min.

Sequence reactions were then cleaned up by adding 2 µl sodium acetate/EDTA buffer and 80 µl 95% EtOH. After centrifuging at 13000rpm for 20min, 200 µl 70% EtOH was added and centrifuged at 13000rpm for 10min. Loading dye was added, either 2 µl of formamide dye or 15 µl of MegaBACE^{TM} loading solution (GE Healthcare Life Sciences) and the samples were loaded onto the sequencer. For the 377 DNA Sequencer, a gel had to be made, comprised of urea, water, 50% Long Ranger^{TM} acrylamide, 10X TBE (1M Tris-Base, 1M Boric Acid, 0.5M Na_2EDTA), 10% APS, and TEMED which was poured over sequencing plates.

Sequencing involved the following primers:

T7 Sequencing Primer: 5'-TAATACGACTCACTATAGGG-3'
3' AD LD Insert 5'-GTGAACTTGCGGGGTTTTTCAGTATCTAC-3' (Clontech)

For further elongation of the sequences, additional primers were used:

Forward:

Y2H-A1: 5'-ACACTTGATTGAATCTATTTTCGCT-3'
Y2H-F7: 5'-ATAAGTCATATCAGAGTTTAGAGA-3'
F7-Y2H- F7E: 5'-GCCTTCCACGCTACGGATTGAATC-3'
E2B11-F: 5'-GAGTGTGTAGTTGATGGAAATAGAG-3'
40RPS-F: 5'-CAGAAGTCTTACCGTTAACCTCTC-3'

Reverse:

40RPS-R: 5'-TCGGATGTCAACTCCAGCAAAACG-3'
T90A-FR: 5'-TTATGATATATCCCACAGGGCACA-3'

2.1.5. Reciprocal Transfer of Proteins

To verify that the interactions are positive, a reciprocal transfer of proteins was done by switching DNA binding to activating domain fusions. In the case of SdY, it was cloned into the pGADT7 vector rather than the pGBKT7 vector, while the interactors were

cloned into pGBKT7 vectors. The same protocols were used as described in section 2.1.3 with a few minor differences as described below.

The interactors were amplified by PCR using the following primers:

Forward: Y2H 40RPS-F: 5'-CATGGAGGCCGAATTCGGGTGGCCTCAT-3'
Reverse: Y2H 40RPS-R: 5'-GCAGGTCGACGGATCCGCTTTCTCCCTCAAC-3'

and

Forward: Y2H T90A-F: 5'-CATGGAGGCCGAATTCGGGGACACACTG-3'
Reverse: Y2H T90A-R: 5'-GCAGGTCGACGGATCCAGAGGTCAGTGTGTGATT-3'

PCR reactions were performed on T1 thermocyclers under the following conditions: 33 cycles of denaturation at 98^{o}C (10s), annealing at 55^{o}C (15s), and extension at 72^{o}C (5s). Each 10 µl reaction contained 3.4 µl PCR grade water (Clontech), 5 µl CloneAmp HiFi PCR premix, 0.3 µl of 0.3 µM forward primer, 0.3 µl of 0.3 µM reverse primer, and 1 µl of DNA. The PCR reactions were run on a 1.6% agarose gel with the bands of interest being extracted using the NucleoSpin® Gel and PCR Clean-Up kit.

The rest of the experiments involved with yeast two-hybrid mating, including in-fusion cloning, transformations, controls, two-hybrid library screening, and rescue and isolation of interactors was done according to the protocols mentioned in sections 2.1.1 to 2.1.3.

2.2. Protein Assays

Tissues of approximately 1 year old Atlantic salmon used in the protein assays were generously donated by Fisheries and Oceans Canada from West Vancouver. The antibodies used in this section were polyclonal antibodies raised against SdY in rabbits which were generated by ImmunoPrecise in Victoria, BC. Prior to being used, the antibodies were purified by diluting in a 1:1 ratio with Protein A IgG binding buffer (Thermo Scientific) for proper ionic strength and pH. The diluted antibodies were applied onto a protein A agarose column (GenScript) and flown through. Following a wash of 15 ml binding buffer (same as mentioned above) and an elution with 5 ml IgG elution buffer

(Thermo Scientific), 2 ml fractions were collected in tubes with 200 µl Neutralization buffer (1M Tris-HCl, pH of 7.5-9). Fractions with the highest absorbance were pooled together before being ready to use.

The recombinant SdY used in this section has a 6X histidine tagged N-terminus (generated by W. Eisbrenner) which was purified from *Escherichia coli* on a nickel-nitrilotriacetic acid (Ni-NTA) column. The purified SdY was eluted with 1X TBS (200mM Tris, 100mM NaCl, pH 8) and various concentrations of imidazole ranging from 100mM to 1M.

To obtain total cell lysates, approximately 0.125g of tissue was grounded up into powder in liquid nitrogen. The powdered tissues were placed into a 1.5 ml tube with cold RIPA lysis buffer (10 ml per 1 g, Sigma) and 50 µl 25X stock solution of cOmplete protease inhibitor (Roche). Testes and ovaries were lysed using either RIPA lysis buffer or a non-denaturing buffer (20mM Tris-HCl pH 8, 137mM NaCl, 10% glycerol, 1% Triton X-100, 2mM EDTA). All other tissues were lysed using only RIPA buffer. Samples were vortexed for 1 min followed by incubation on ice for 45 min with vortexing 4-6 times and centrifugation for 20 min at 14000g.

Protein concentrations from tissues were determined using the BCA protein assay kit from Thermo Scientific. Based on the diluted BSA standards, a linear regression line was generated which was used to calculate the concentrations of the proteins.

2.2.1. Affinity Chromatography

Two forms of affinity chromatography were performed on the testes lysates: co-immunoprecipitation (co-IP) assay and the histidine-tagged pull down assay on a Ni-NTA column.

For the co-IP assay, 5 ml of testis lysate (10.5 mg/ml) were incubated with 1 ml of rabbit anti-SdY antibodies for 2 hours before being placed into a column with protein A agarose beads supplied by GenScript. The antibodies bind to the beads and the samples were washed with 30 ml Protein A IgG binding buffer and eluted with 10-15 ml IgG elution

buffer. The eluate fractions were then neutralized by the Neutralization buffer. All three buffers are the same as those mentioned in section 2.2.

For the Ni-NTA assay, 1 ml of testis lysate (10.5 mg/ml) was incubated with 6X histidine-tagged SdY for 2 hours before being placed into a column with nickel-agarose beads supplied by Thermo Scientific and QIAGEN. The histidines bind to the beads and the samples were washed with 1X TBS and 5mM imidazole and eluted with 1X TBS and 250mM imidazole.

Eluates from both assays were run on 15% (for co-IP assay) and 12% (for Ni-NTA assay) SDS-PAGE gels using the molecular weight broad range from Bio-Rad as ladder. The bands indicating protein interactions with SdY were excised and placed in tubes with 50% MeOH before being shipped to UVic Genome BC Proteomics Centre for In-Gel Digest LC-MS/MS analysis with the proteins being digested with trypsin. Peptide sequences were generated based on the molecular weights.

2.2.2. Comparative Analysis of Proteins

Identity of the peptide sequences was determined through BLAST against the Atlantic salmon database (http://blast.ncbi.nlm.nih.gov/, tBlastn, default settings). For sequences that did not give a result, they were BLASTed again, but without masking low complexity regions. A list of contaminants was provided with the results by the UVic Genome BC Proteomics Centre and those sequences corresponding to the contaminants were removed from the list. The rest of the sequences were organized on Excel spreadsheets. Full length sequences of the corresponding proteins were obtained from NCBI (http://www.ncbi.nlm.nih.gov/protein/) which were then used in aligning the peptide sequences to determine the coverage of the peptides using the Geneious software.

2.2.3. Western Blotting

Proteins obtained from cell lysates were run on 15% SDS-PAGE gels at a voltage of 150-200V, using MagicMark + SeeBlue pre-stained ladder from Thermo Scientific. Bands were electrophoretically transferred onto a nitrocellulose membrane that has been soaked in MeOH for 10s, water for 5 min, and transfer buffer (25 mM Tris, 192 mM

glycine, 10% MeOH) for 10 min. Electroblotting was done at a voltage of 100V for 1 hour at 4^{o}C. Membranes were then washed 5-6 times in water and then blocked for 1 hour at 4^{o}C using Blotto a mixture of instant skim milk powder buffered with 1X TBST (20mM Tris, 100mM NaCl, 0.05% Tween-20). The presence of interactors was assessed using primary rabbit anti-SdY antibodies (1:1000 unless specified otherwise) suspended in Blotto with the membranes incubated at 4^{o}C overnight. Following incubation, the membranes were washed 3 times with 1X PBS (BioWhittaker) and once with 1X TBST, for 10 min intervals. After these washes, the membranes were exposed to horseradish peroxidase conjugated goat anti-rabbit secondary antibody (1:5000 dilution, Sigma) in Blotto for 1 hour at 4^{o}C. Exposure was followed by two washes with 1X PBS, again for 10 min intervals. Bands were detected using enhanced chemiluminescence reagents, 2 mL of each reagent from the Clarity™ Western ECL Blotting Substrate kit (Bio-Rad) for 5 min, followed by drying and then wrapping of the membrane in a plastic wrap. Visualization of the bands was done by Fujifilm LAS4000 luminescent imager with a 100s increment exposure for 200s.

Chapter 3.

Results

3.1. Identifying interactors of SdY using yeast two-hybridization

Since *sdY* was recently discovered, there is an absence in knowledge in how SdY functions during sex-determination. As such, I intend to identify the interactors using a yeast two-hybridization assay using SdY as my bait and a testis cDNA library as my prey. Necessary controls were performed to verify that the yeast two-hybridization assay is working properly. These controls include performing positive and negative control mating (Figure A1) and testing that there is no bait autoactivation or toxicity (Figure A2). No issues were observed. Prior to generating a cDNA library, I tested whether SdY interacts with itself by transforming *sdY* into bait (pGBKT7-BD) and prey (pGADT7-AD) vectors. Mating between the two resulted in diploids with 1.36 million clones screened. Colonies are seen on DDO and DDO/X/A plates (Figure 3.1), indicating that SdY putatively interacts with itself. However, upon mating SdY with the testis cDNA library, no such results were seen. Mating between SdY and the testis cDNA library resulted in growth of colonies on both DDO/X/A and QDO/X/A plates. Having screened 9.36×10^5 clones, SdY was shown to interact with the 3' untranslated regions of annexin A7-like (Anxa7-like, Figure 3.2a) and transmembrane protein 91-like (Tmem91-like, Figure 3.2b), as well as with 40S ribosomal protein S16 (Rps16, Figure 3.3).

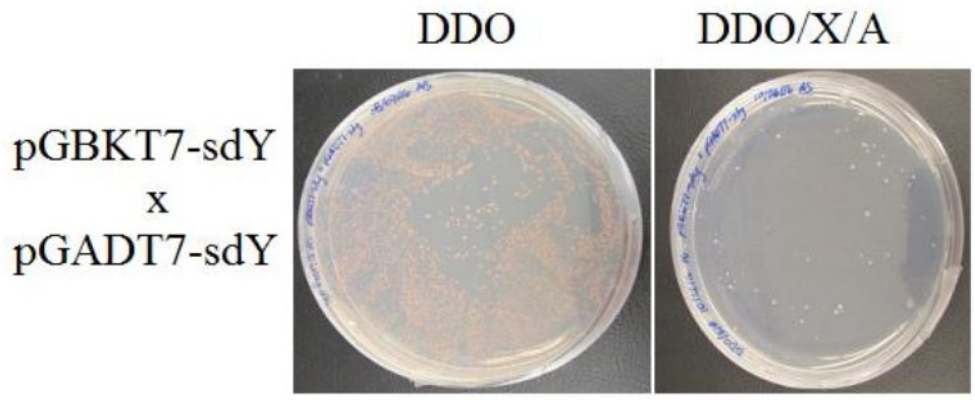


Figure 3.1 Mating between SdY and itself

The *sdY* gene was cloned into both pGBKT7-BD and pGADT7-AD vectors and transformed into Y2HGold and Y187 yeast strains. Diploids appear on both DDO and DDO/X/A plates, with blue colonies appearing in the latter.

Anxa7-like and Tmem91-like are highly similar in sequence to each other (Figure 3.2c), resulting in the sequence of the interactor of SdY giving a BLAST hit to the 3' UTR of both of these proteins. It is unusual that the yeast two-hybrid assay is detecting 3' UTR as this assay is designed to only detect protein-protein interactions and not protein-RNA interactions. As such, this result is likely to be a false positive rather than a true interaction. It should be noted that from the sequence obtained from the yeast two-hybridization, only approximately a fourth of the sequence aligned to Anxa7-like and approximately half to the Tmem91-like, in both cases just to the 3' UTR. The rest of the sequence aligned to no known sequence in the Atlantic salmon database.

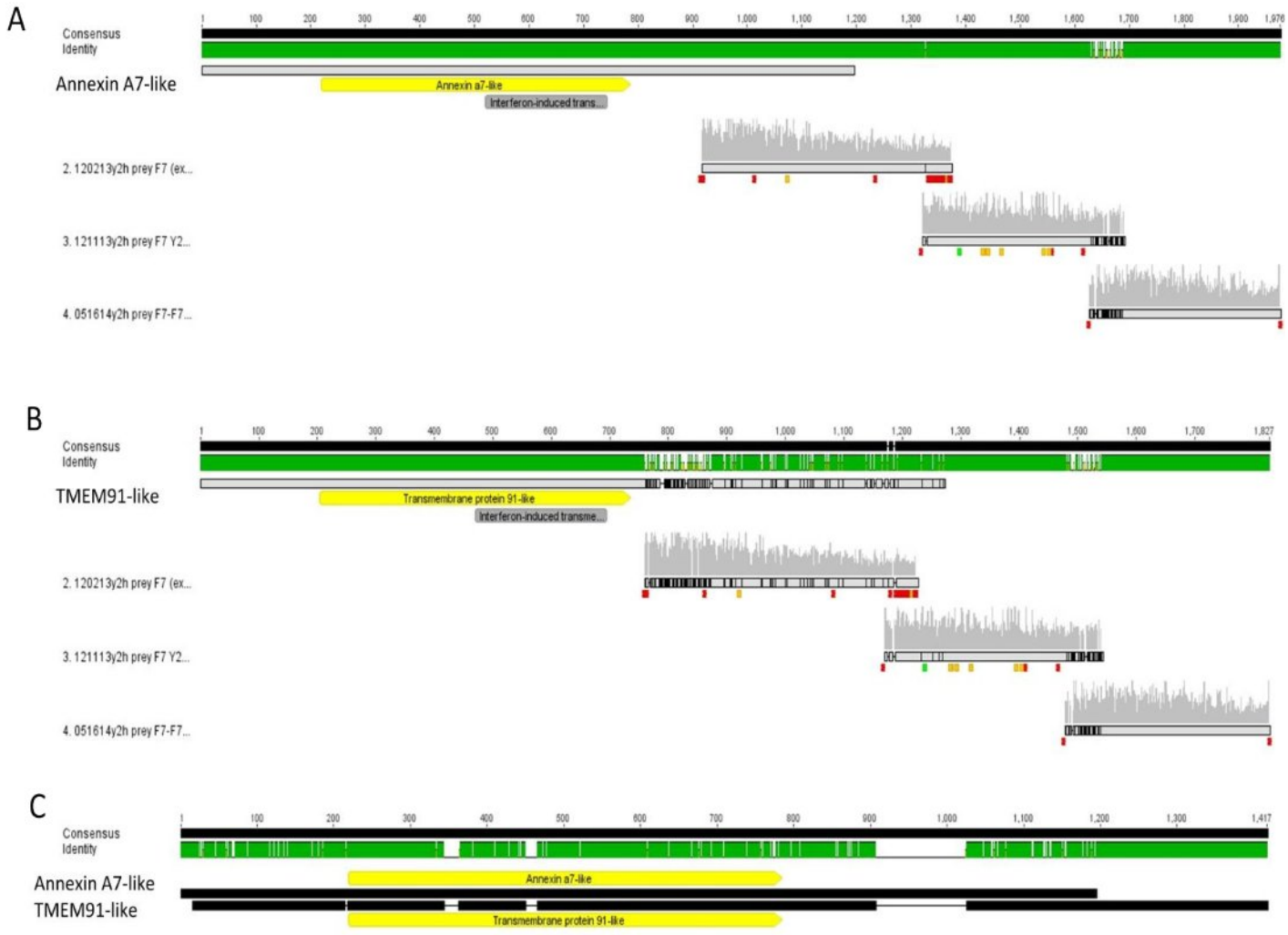

A
Consensus
Identity
Annexin A7-like
Annexin a7-like
Interferon-induced trans...
2. 120213y2h prey F7 (ex...
3. 121113y2h prey F7 Y2...
4. 051614y2h prey F7-F7...
B
Consensus
Identity
TMEM91-like
Transmembrane protein 91-like
Interferon-induced transme...
2. 120213y2h prey F7 (ex...
3. 121113y2h prey F7 Y2...
4. 051614y2h prey F7-F7...
C
Consensus
Identity
Annexin A7-like
TMEM91-like
Annexin a7-like
Transmembrane protein 91-like

Figure 3.2 Consensus sequence of Annexin A7-like and Transmembrane protein 91-like

Sequence obtained from yeast two-hybridization aligned to A) annexin A7-like with 100% identity to the 3' UTR and B) transmembrane protein 91-like with 80.2% identity to the 3' UTR. Numbers 2-4 in A and B correspond to a portion of the sequence of the interactor obtained from the hybridization using a particular primer. A total of three primers were used to obtain the full sequence of the interactor. C) Comparison of annexin A7-like and transmembrane protein 91-like, with an 81% sequence similarity to each other. Yellow bar represents the location of the coding region, while gray bar denotes the location of domains. Percentages obtained from Geneious.

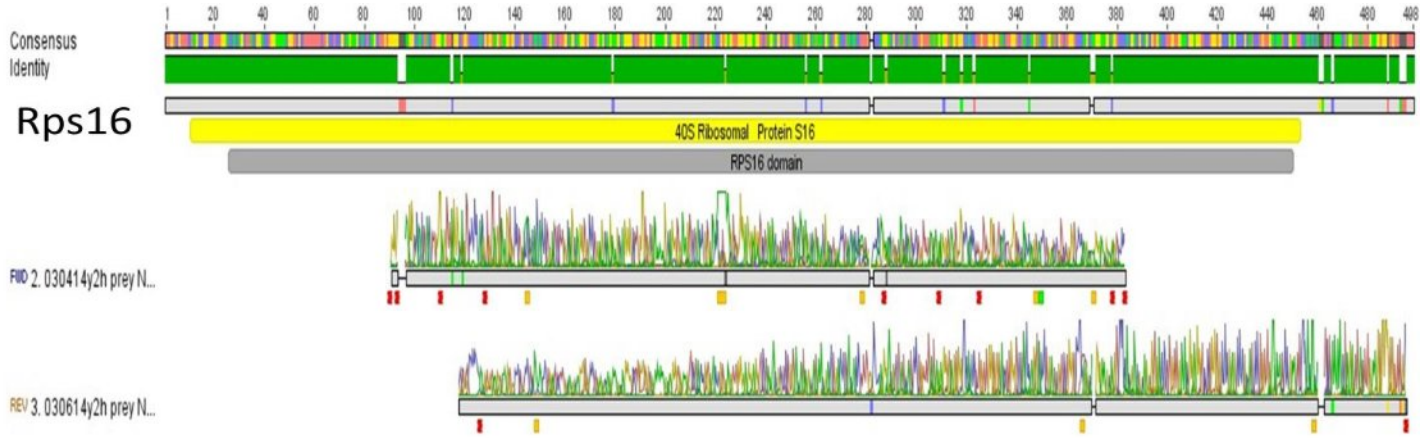


Figure 3.3 Consensus sequence of 40S ribosomal protein S16

Sequence obtained from yeast two-hybridization aligned to 40S ribosomal protein S16 with a 93.8% identity. Numbers 2-3 correspond to a portion of the sequence of the interactor obtained from the hybridization using a particular primer. A total of two primers were used to obtain the full sequence of the interactor. Yellow bar represents the location of the coding region, while gray bar denotes the location of domains. Percentage obtained from Geneious.

Unlike Anxa7-like or Tmem91-like, Rps16 is a small protein comprised of 146 amino acids (NP_001134097.1). As such, most of the coding region and the 3' UTR of the protein interact with sdY (Figure 3.3). Rps16 has one domain, the 40S ribosomal protein S16 domain (NP_001134097.1), where most of it interacts with SdY.

After obtaining the interactors of SdY, a reciprocal transfer of the proteins occurred to verify the interactions, especially the interaction with the 3' UTR. The sequences that were obtained from the yeast two-hybrid assay were cloned into pGBKT7-BD vectors and transformed into the Y2HGold yeast strain to serve as baits, while SdY was cloned into pGADT7-AD vector and transformed into the Y187 yeast strain to serve as the prey. Mating between the baits and the prey resulted in colonies growing on both DDO/X/A and QDO/X/A plates. Screening 1.15 million (Rps16) and 1.2 million (Anxa7/Tmem91-like) clones resulted in the same outcome: SdY as the interactor of both Rps16 and the 3' UTR of Anxa7-like/Tmem91-like (Figure 3.4). However, there were inconsistencies in reproducing the results for the mating between Rps16 and SdY and 3' UTR of Anxa7-like/Tmem91-like with SdY. Many times, there was either no evidence of colonies or the colonies were tiny. These inconsistencies provide doubt as to whether these are true interactors of SdY. For full sequence of each interactor see Table B1. Results from yeast two-hybridization assays always require further analysis using other methods to verify the results. As such, I have chosen to use protein assays to further identify the interactors of SdY.

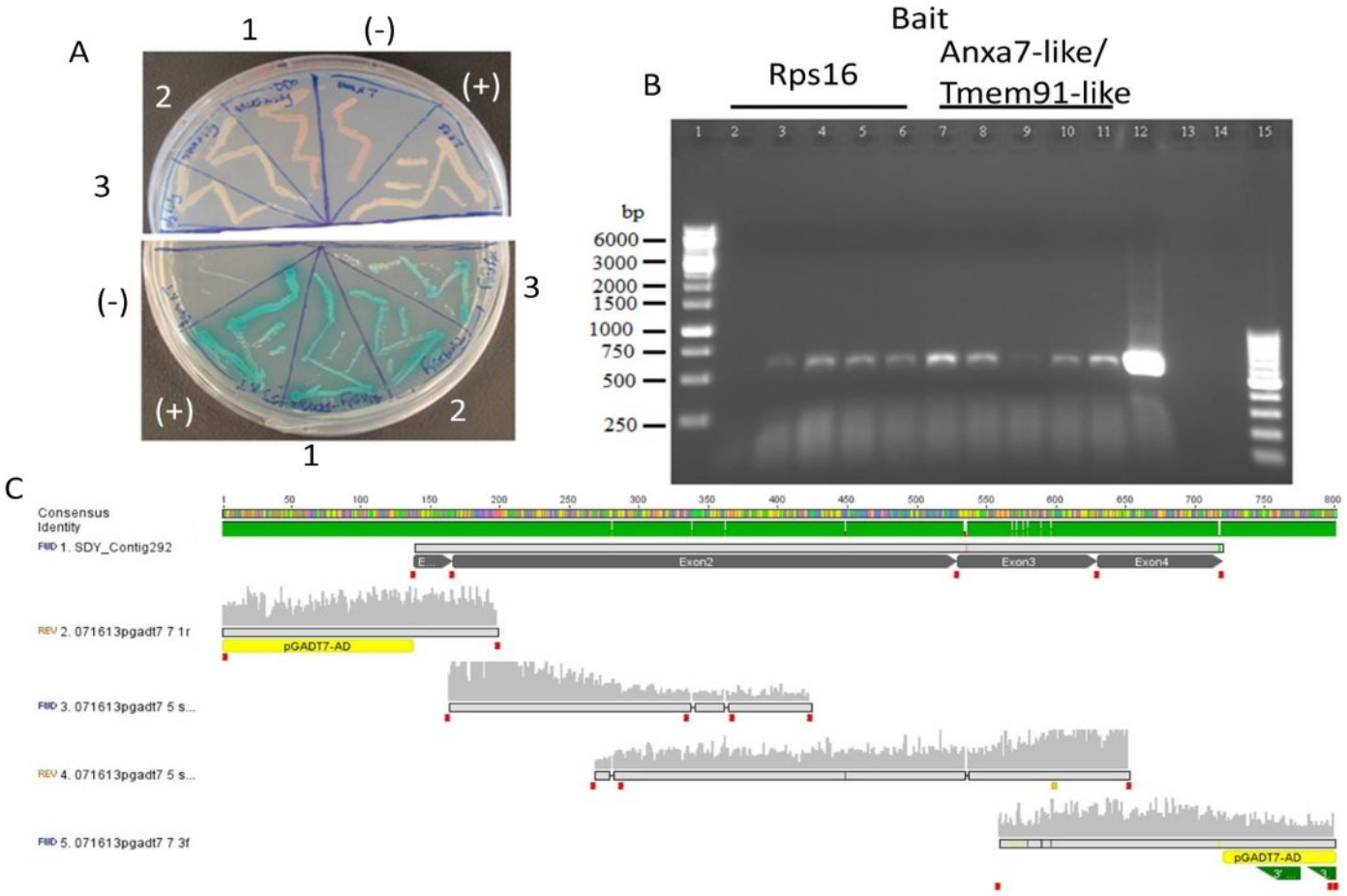


Figure 3.4 Interactors from reciprocal transfer mating between Rps16 and sdY and between Anxa7-like/ Tmem91-like and sdY

A) All positive interactions on DDO (on top) and DDO/X/A (on bottom) plates obtained from reciprocal transfer mating along with the positive and negative control mating. Mating: (+): pGBKT7-53 x pGADT7-T, (-): pGBKT7-lam x pGADT7-T, 1: pGBKT7-Rps16 x pGADT7-SdY, 2: pGBKT7-Anxa7-like/Tmem91-like x pGADT7-SdY, 3: pGBKT7-SdY x pGADT7-SdY. B) 1.3% agarose gel electrophoresis of interactors from reciprocal transfer mating between pGBKT7-Rps16 x pGADT7-SdY, and pGBKT7-Anxa7-like/Tmem91-like x pGADT7-sdY. Lane 1: 1kb ladder, Lanes 2-11: *sdY*, Lane 12: Positive Control (SdY), Lane 13: empty, Lane 14: Negative Control, Lane 15: 100bp ladder. C) Consensus sequence of SdY. Sequence was obtained from yeast two-hybridization through reciprocal transfer with the sequence aligned to SdY. Full SdY sequence is interacted with 97.8% identity to the full gene. Numbers 2-5 correspond to a portion of the sequence of the interactor obtained from the hybridization using a particular primer. A total of four primers were used to obtain the full sequence of the interactor. Yellow bar represents the vector of pGADT7-AD. Percentages were obtained from Geneious.

3.2. Preparation for affinity assays

To identify the protein interactors of SdY, a purified SdY protein needed to be prepared. Using a recombinant SdY protein with a 6X histidine tagged N-terminus (previously made by W. Eisbrenner), SdY was purified on a nickel column and eluted with various concentrations of imidazole (Figure 3.5). SdY is shown to be present at 30kDa where there are additional two bands of 50 and 14kDa present in the elution with 250mM imidazole.

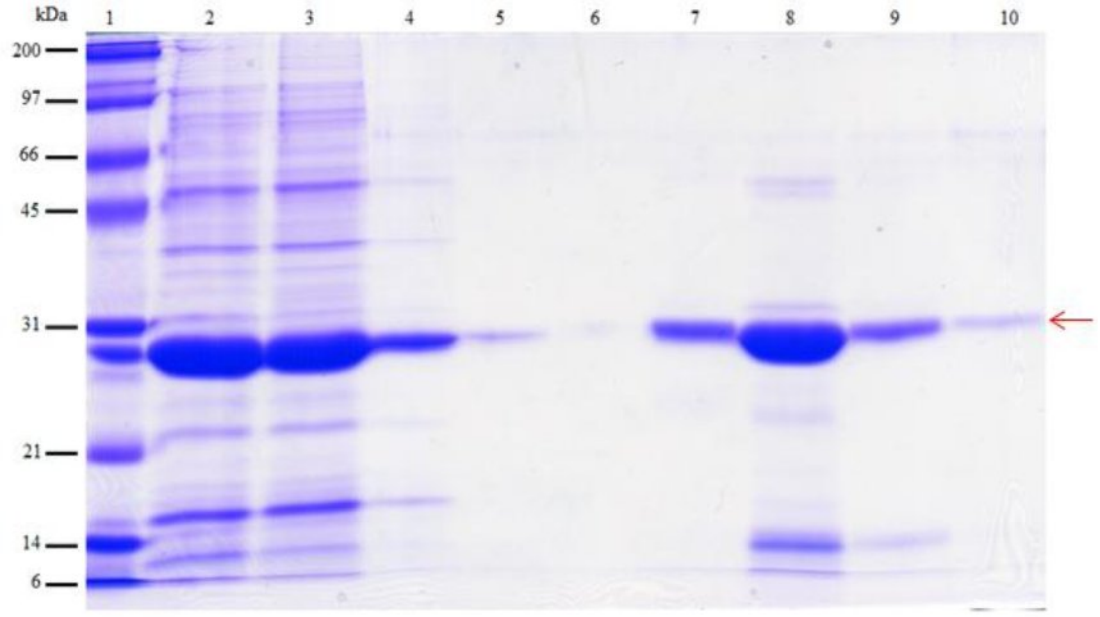


Figure 3.5 Purification of recombinant SdY

15% SDS-PAGE gel electrophoresis of recombinant sdY purified from e.coli on Ni-NTA agarose column. Lane 1: ladder, Lane 2: SdY in 8M urea, Lane 3: third flow-through from the column, Lane 4: flow-through after 8 M urea, 5mM imidazole wash, Lane5: Flow-through 25% 1xTBS 75% 8M urea wash, Lane 6: flow-through 100% 1xTBS wash, Lane 7: elution 1xTBS 100mM imidazole, Lane 8: elution 1xTBS 250mM imidazole, Lane 9: elution 1xTBS 550mM imidazole, Lane 10: Elution 1xTBS 1M imidazole. Red arrow indicates SdY.

To verify the specificity of the antibodies, I tested the antibodies against the selected target, SdY, on a Western blot where a single band was observed at the expected size of 30kDa (Figure 3.6). There were also present two additional bands at approximately 50kDa and 25kDa in lanes 2 and 3 and faintly seen in lane 4, but by lane 5 those bands have disappeared. These bands may be a result of non-specific binding. The 50kDa band may be a result of dimerization.

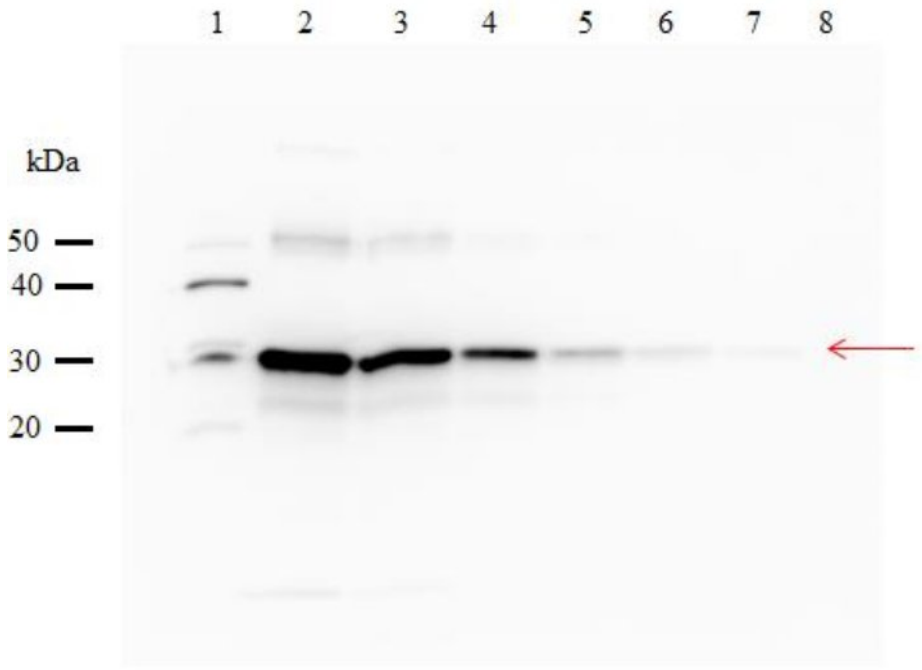


Figure 3.6 Specificity of polyclonal antibodies against SdY

A Western blot of serial dilutions of SdY using polyclonal antibodies (1:5000) against SdY. Recombinant SdY was separated on a 15% SDS-PAGE and analysed by Western blot. Lane 1: ladder, Lanes 2-8: serial dilutions of SdY. SdY protein was purified on a Ni-NTA column and eluted with 100mM imidazole. A total of 10µl of each sample was loaded onto each well. Purified SdY was serially diluted (1:3) to the following amounts: 10µl (lane 2), 3µl (lane 3), 1µl (lane 4), 0.3µl (lane 5), 0.1µl (lane 6), 0.03µl (lane 7), 0.01µl (lane 8). Membrane was exposed for 60s. Red arrow indicates SdY.

3.3. SdY is localized in the testes

Since Atlantic salmon have a male-heterogametic sex system with *sdY* a master sex-determining gene in rainbow trout (Yano et al. 2012), it should be located in testes and not in ovaries. Validation of the polyclonal antibodies was done by Western blot against the tissues of an Atlantic salmon, where ovaries act as a negative control. Several tissues were analyzed by SDS-PAGE gel electrophoresis and Western blotting (Figure 3.7a-b). As expected, SdY is located in the testis and not in ovaries additionally not present in the head kidney, posterior kidney, intestine, and pyloric caeca as well (Figure 3.7a-b). The two bands of 25 and 50kDa seen in Figure 3.6 are strongly evident in sdY that was eluted in 250mM imidazole and dialysed. Other bands are also present in lane 1, which are most likely a result of non-specific binding. An additional band of approximately 14kDa is present in the liver, kidney, heart, spleen, stomach, eye, swim bladder, gill, and native testis (Figure 3.7b). This could correspond to post-translational modifications of SdY. There are three bands present in the ovaries which may be a result of non-specific binding.

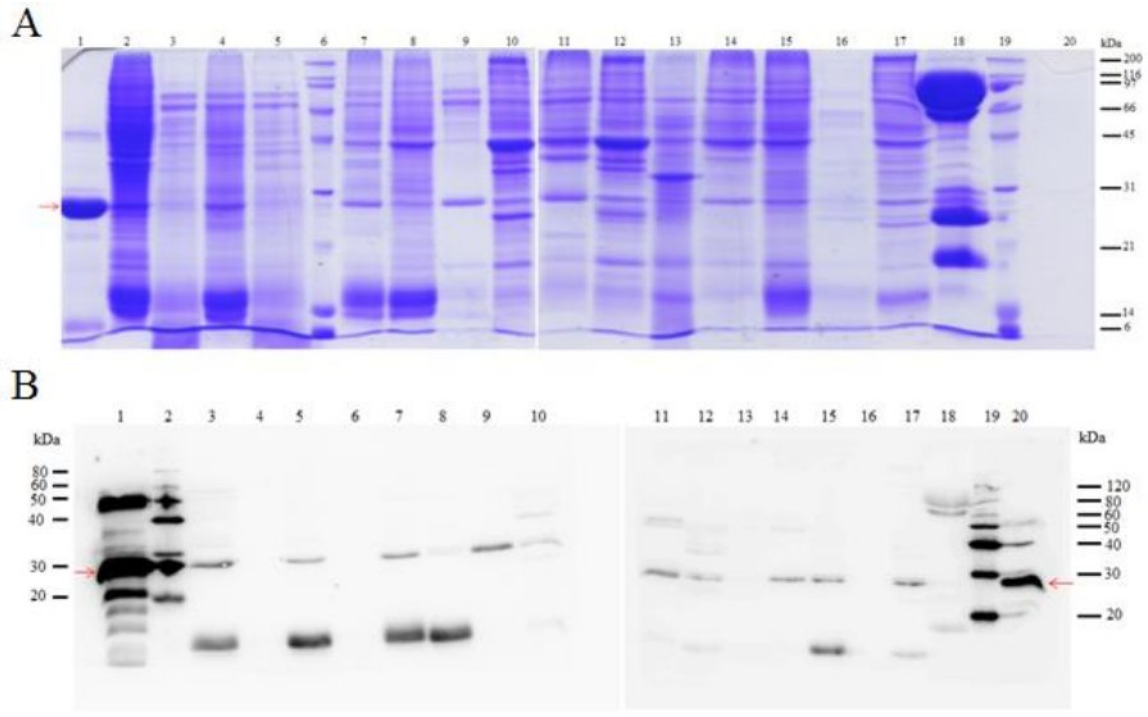


Figure 3.7 Location of SdY in tissues

Location of SdY in various tissues using **A)** 15% SDS-PAGE gel and **B)** Western blot analysis using polyclonal antibodies against SdY. Approximately 25µg of protein extract was loaded into each lane, separated on a 15% SDS-PAGE gel and then analyzed by Western blot. **A)** Lane 1: SdY pure protein purified on a Ni-NTA column and eluted with 250mM imidazole and dialysed, Lane 2-5 tissues (liver, head kidney, kidney, posterior kidney), Lane 6and 19: ladder, Lane 7-18: tissues (heart, spleen, brain, stomach , eye, swim bladder, intestine, skin, gill, pyloric caeca, native testis, and native ovaries), Lane 20: SdY pure protein purified on a Ni-NTA column and eluted with 100mM imidazole. **B)** Lane 1: SdY pure protein purified on a Ni-NTA column and eluted with 250mM imidazole and dialysed, Lane 2 and 19: ladder, Lanes 3-18: tissues (liver, head kidney, kidney, posterior kidney, heart, spleen, brain, stomach, eye, swim bladder, intestine, skin, gill, pyloric caeca, native testis, and native ovaries), Lane 20: SdY pure protein purified on a Ni-NTA column and eluted with 100mM imidazole. Red arrows indicate SdY.

Since an additional band was detected in the native testis, is it also present in testes under denaturing conditions? To test this, I compared testis lysates that were treated with RIPA buffer or non-denaturing buffer on a 15% SDS-PAGE gel and Western blot (Figure 3.7a-b). More bands were observed in the native testis (Figure 3.8a) with SdY being detected in both lysed and native testes (Figure 3.8b). Only native testes had the additional band of 14kDa present; the band was not seen in testis lysed in RIPA buffer.

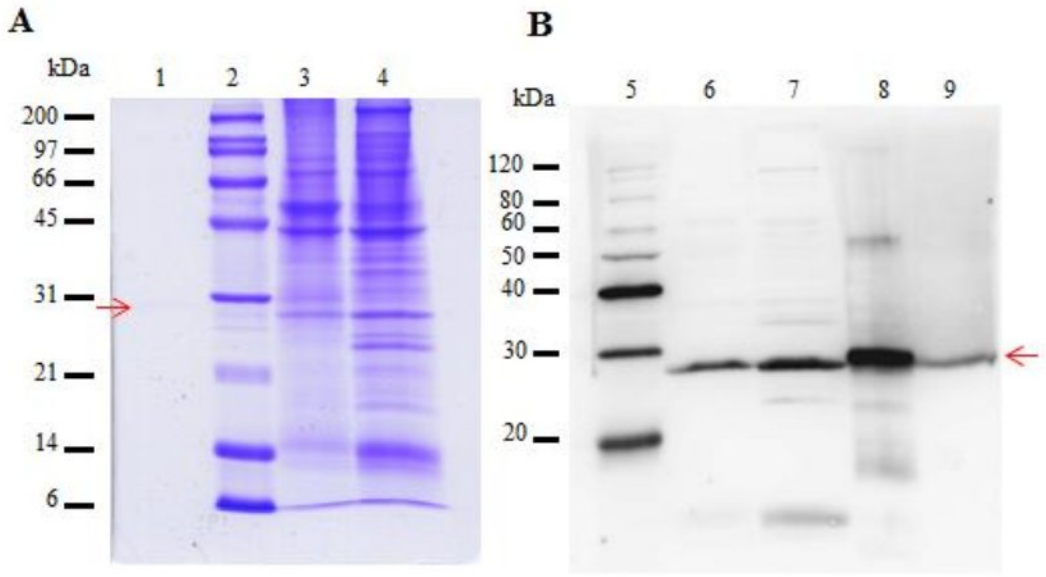


Figure 3.8 Analysis of testis lysates for SdY

Analysis of testis lysates for SdY was performed using **A)** 15% SDS-PAGE gel electrophoresis and **B)** Western blot using the polyclonal antibodies raised against SdY. Approximately 25µg of protein extract was loaded into each well. Lanes 1 and 8: SdY pure protein purified on a Ni-NTA column and eluted with 100mM imidazole, Lanes 2 and 5: ladder, Lanes 3 and 6: testis lysed in RIPA buffer, Lanes 4 and 7: native testis in non-denaturing buffer, and Lane 9: spillover from lane 8. Red arrow indicates SdY.

3.4. Co-immunoprecipitation of SdY to identify interacting proteins

With the generation of polyclonal antibodies against SdY that are specific for SdY (Figure 3.6), a co-IP assay was performed to identify interacting proteins of SdY. Eluate samples from the co-IP assay were collected and ran on a 15% SDS-PAGE gel. Based on those results, appearance of any interactions were evident starting with eluate fraction #10 (Figure 3.9). As such, eluate fraction #11 was chosen for further experiments. As a negative control, I used a mixture of antibody and testis lysate rather than the pre-immune serum. The bands present in lane 4 were a result of non-specific binding with the antibody (Figure 3.9).

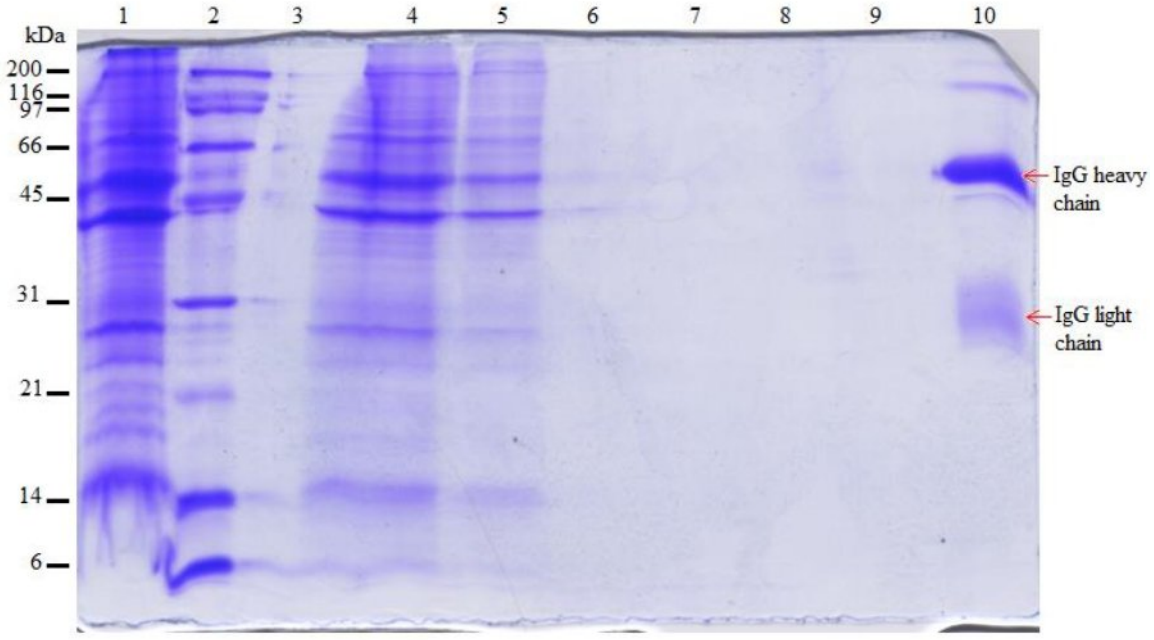


Figure 3.9 Fractions collected from co-IP assay

15% SDS-PAGE gel electrophoresis of testis lysate from co-IP assay. Lane 1: native testis lysate, Lane 2: ladder, Lane 3: SdY pure protein purified on a Ni-NTA column and eluted with 100mM imidazole, Lane 4: SdY polyclonal antibodies mixed with testis lysate, Lane 5: wash fraction # 1, Lane 6: wash fraction #19, Lane 7: elution fraction #2, Lane 8: elution fraction #4, Lane 9: elution fraction # 6, Lane 10: elution fraction #10.

To identify interactors of SdY, eluate fraction #11 was loaded onto 9 wells and ran on a 15% SDS-PAGE gel, resulting in six distinct bands (Figure 3.10) where bands C and E correspond to the heavy and light chains of the IgG antibody (50 and 23kDa). The rest of the bands (A, B, D, and F) were of 128, 119, 45, and 21kDa in size. These were excised out of the gel and sent off for mass spectrometry analysis. Even though lanes two to ten were loaded with the same sample in equal amounts, lane four produced two additional bands (128 and 21kDa), while the rest of the lanes showed no difference between each other. These bands may be a result of contamination, but nevertheless these were excised and sent for mass spectrometry analysis.

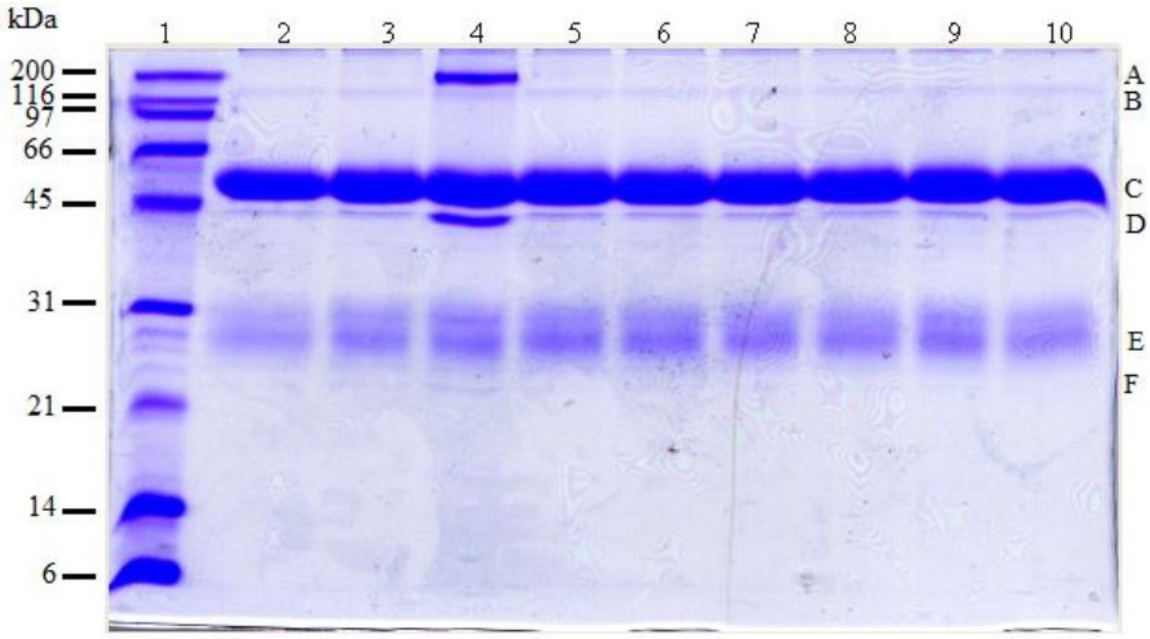


Figure 3.10 Interactors of SdY using co-IP assay

15% SDS-PAGE gel of co-IP Assay of native testes lysate under reducing conditions. Lane 1: ladder, Lane 2-10: eluate fraction #11. Letters A-F indicate the bands produced.

3.5. His-tag pull down of SdY

The recombinant his-tagged SdY was incubated with testes and placed over a nickel-column where it was eluted out with various concentrations of imidazole. Of the concentrations, the first elution with TBS and 250mM imidazole provided strongest intensity of the interactors of SdY (Figure 3.11a) and therefore was chosen for further experiments. As a negative control, the recombinant his-tagged SdY was incubated with ovaries and was eluted out with various concentrations of imidazole (Figure 3.11b). In ovaries, SdY does not appear to be present. Running testes sample eluted with TBS and 250mM imidazole on a 12% SDS-PAGE gel resulted in five distinct bands (Figure 3.12). Band A was greater than the protein ladder and as such was discarded from further analysis as the rate of migration may have been affected. The rest of the bands (H, I, J, and K) were of 97, 63, 45, and 30kDa in size. These bands were excised and sent off for mass spectrometry analysis to identify the interactors.

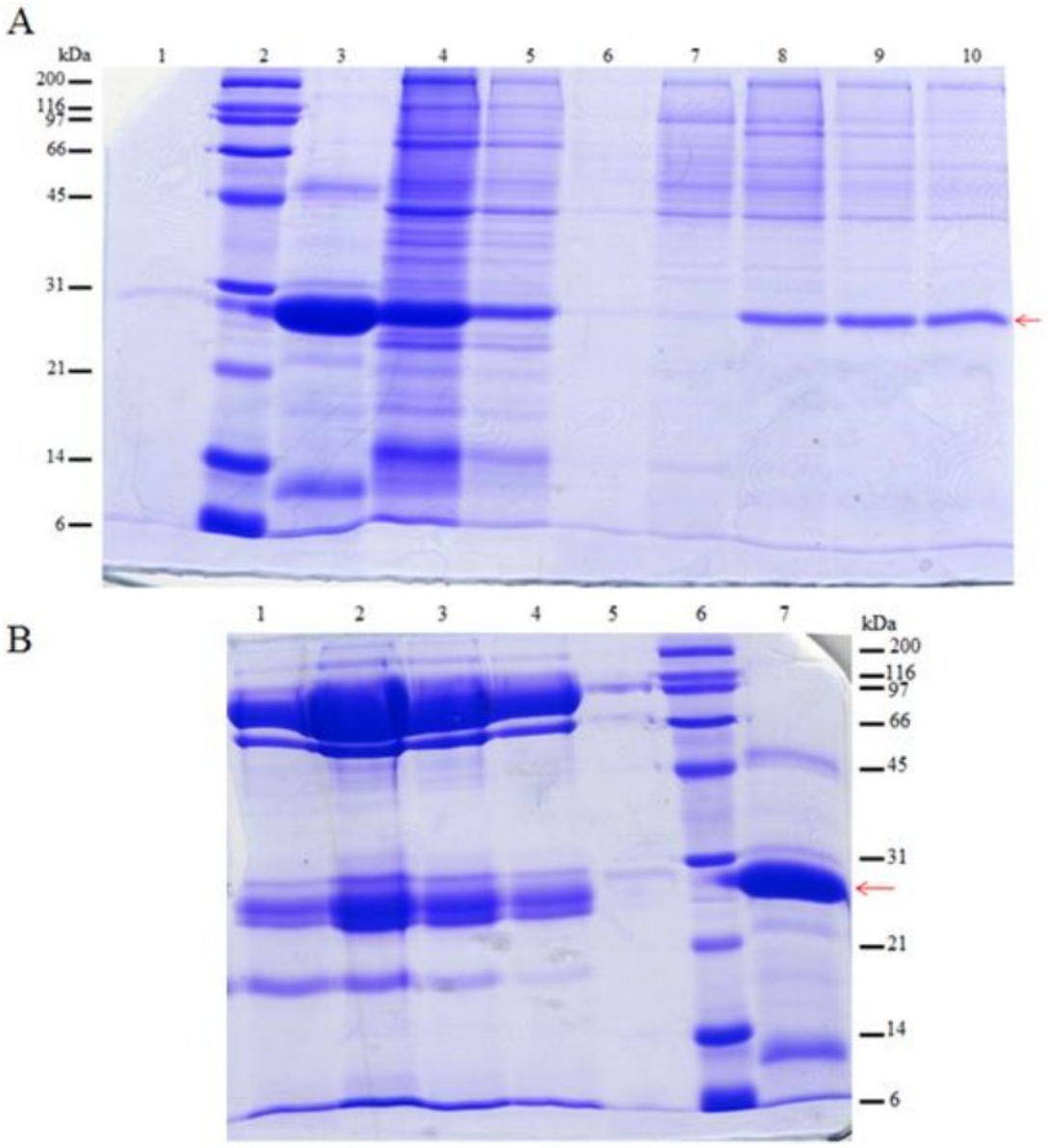

A
kDa
1
2
3
4
5
6
7
8
9
10
200
116
97
66
45
31
21
14
6
B
1
2
3
4
5
6
7
kDa
200
116
97
66
45
31
21
14
6

Figure 3.11 Fractions collected from Ni-NTA using various imidazole concentrations

15% SDS-PAGE gel electrophoresis of **A)** testis/SdY mixture and **B)** ovaries/SdY mixture. For testes: Lane 1: SdY pure protein purified on a Ni-NTA column and eluted with 100mM imiadzole, Lane 2: ladder, Lane 3: SdY pure protein purified on a Ni-NTA column and eluted with 250mM and dialysed, Lane 4: testis/SdY beads mix, Lane 5: testis/SdY wash with TBS, Lane 6: testis/SdY wash with TBS and 5mM imidazole, Lane 7: testis/SdY elution with TBS and 100mM imidazole, Lane 8: testis/SdY elution fraction #1 with TBS and 250mM imidazole, Lane 9: testis/SdY elution fraction #2 with TBS and 250mM imidazole, Lane 10: testis/SdY elution fraction #3 with TBS and 250mM imidazole. For ovaries: Lane 1: ovaries/SdY elution with TBS and 100mM, Lane 2: ovaries/SdY elution fraction #1 with TBS and 250mM imidazole, Lane 3: ovaries/SdY elution fraction #2 with TBS and 250mM imidazole Lane 4: ovaries/SdY elution fraction #3 with TBS and 250mM imidazole, Lane 5: SdY pure protein purified on a Ni-NTA column and eluted with 100mM imiadzole, Lane 6: ladder, Lane 7: SdY pure protein purified on a Ni-NTA column and eluted with 250mM and dialysed. Red arrow indicates SdY.

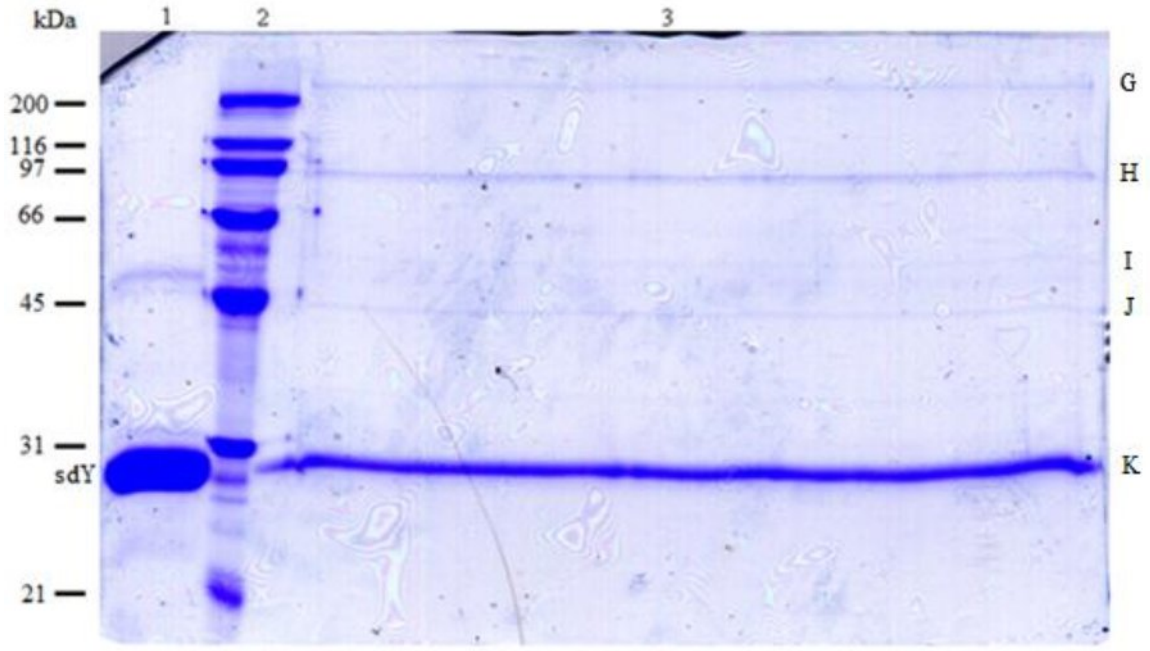


Figure 3.12 Interactors of SdY using Ni-NTA assay

12% SDS-PAGE gel of Ni-NTA Assay of testes lysate under reducing conditions. Lane 1: SdY pure protein purified on a Ni-NTA column and eluted with 250mM imidazole and dialysed, Lane 2: ladder, Lane 3: testis/SdY elution with TBS and 250mM imidazole. Letters G-K indicate the bands produced.

3.6. Mass Spectrometry Analyses

A total of eight bands were sent for mass spectrometry (four co-IP and four Ni-NTA) where peptide sequences were generated based on molecular weights and sent back to me. Through tBLASTn, identity of these sequences was revealed. In order for a protein to be classified as a candidate interactor of SdY, they must be localized in the cytoplasm as sdY is believed to reside in the cytoplasm (von Schalburg, personal communication). As such, the top candidate interactors of SdY from each band are isocitrate dehydrogenase, SdY, heat shock protein HSP 90-beta, and ras GTPase-activating-like protein IQGAP1 (Table 3.1). The sequences of these four proteins obtained from the affinity assays covered most of the full length sequence of the proteins (Figure 3.13). Other potential interactors of interest are creatine kinase, GDP-mannose-4,6-dehydratase, sodium/potassium-transporting ATPase subunit alpha-1, 40S ribosomal protein SA, and hydroxysteroid dehydrogenase (17-beta) 4 as they have large protein sequence coverage (data not shown).

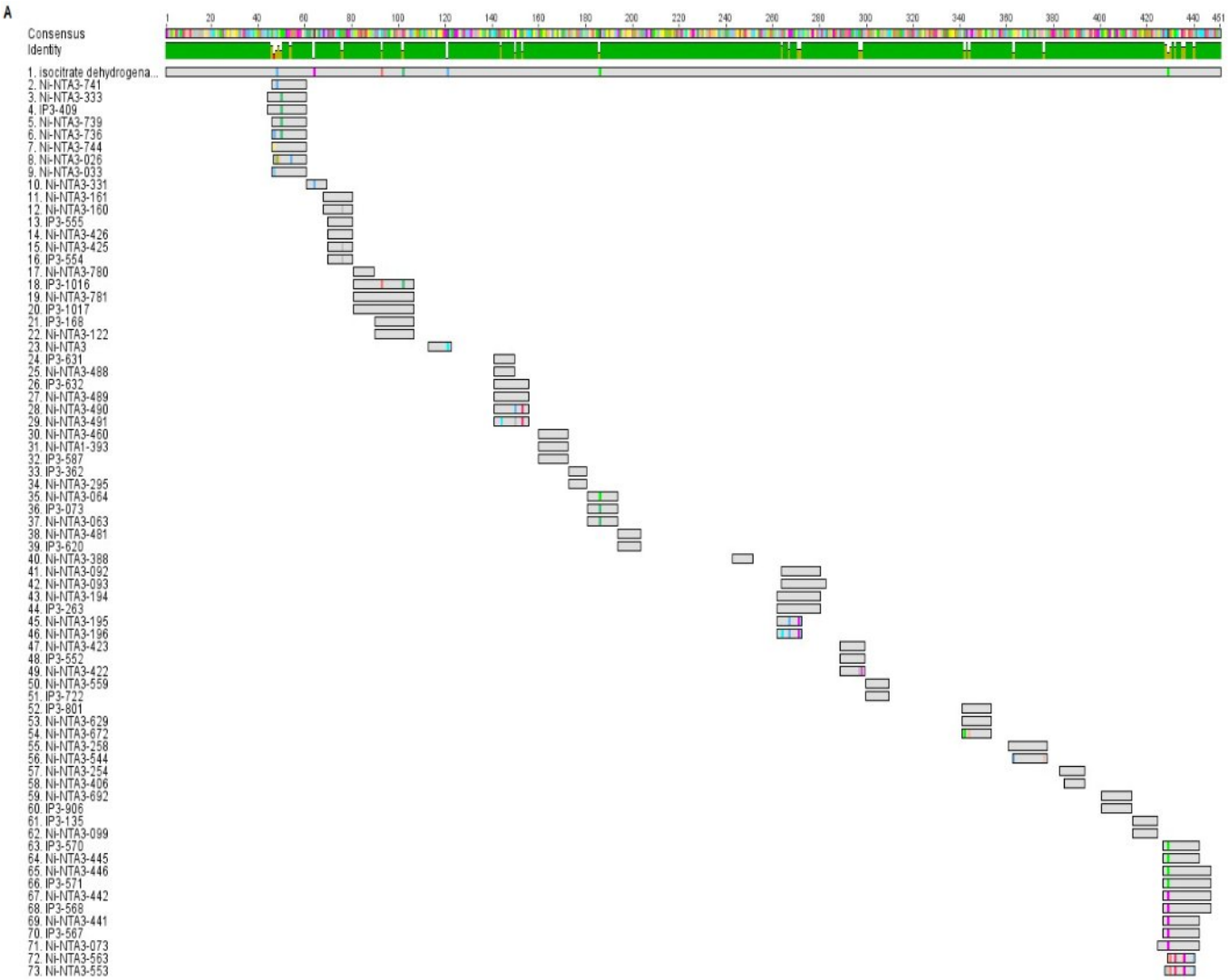
Overall, the candidates are exclusively found in a distinct band. The exception to this is SdY which has been detected in all the bands except band B (Table 3.1). Based on its molecular weight, SdY should only be found in the lowest bands (F from co-IP; Figure 3.10, and K from Ni-NTA; Figure 3.12). The presence of sdY in numerous bands suggests that it is a ubiquitous contaminant. Interestingly enough, 40S ribosomal protein SA has been identified as a potential interactor of SdY, where a different protein fold of the 40S subunit (S16) has previously been detected in the yeast two-hybridization assay. However, no sequences corresponding to Anxa7-like or Tmem91-like were detected in the two affinity assays.

Within each protein, there is no full sequence coverage of the protein as there are gaps with missing peptide sequences (Figure 3.13a-d). The lack of peptide sequences may be due to the protein not being fully digested or the peptide sequence was too small to be analyzed. No inferences can be made by the lack of certain peptides.

Table 3.1 Candidate interacting proteins obtained from protein assays

Protein	Molecular Weight (kDa)	Location in the cell	# of Peptides	Ni-NTA bands				Co-IP bands			
				H	I	J	K	A	B	D	F
Isocitrate dehydrogenase	50.56	Cytoplasm, mitochondria	72	1		49				22	
SdY	21.78	Cytoplasm*	47	7	7	7	13	6		3	4
Heat shock protein HSP 90-beta	83.33	Cytoplasm	44		44						
Ras GTPase-activating-like protein IQGAP1	188.16	Intracellular	25	20				5			
Creatine kinase brain/testis isozymes	42.75	Cytoplasm	30			14				16	
GDP mannose 4,6 dehydratase	41.7	Cytoplasm	28			19				9	
Sodium/potassium-transporting ATPase subunit alpha-1	113.17	Membrane	28	16				12			
40S ribosomal protein SA	34.90	Cytoplasm, nucleus, membrane	18			17				1	
AP-1 complex subunit beta-1	112.55	Cytoplasm	18						18		
Hydroxysteroid dehydrogenase (17-beta) 4	79.18	Membrane, mitochondria**	9			9					

*Location of SdY is based on preliminary histochemistry results done by von Schalburg, personal communication. **Location in the cell of humans.


A
1 20 40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 451
Consensus
Identity
1. isocitrate dehydrogena...
2. Ni-NTA3-741
3. Ni-NTA3-333
4. IP3-409
5. Ni-NTA3-739
6. Ni-NTA3-736
7. Ni-NTA3-744
8. Ni-NTA3-026
9. Ni-NTA3-033
10. Ni-NTA3-331
11. Ni-NTA3-161
12. Ni-NTA3-160
13. IP3-555
14. Ni-NTA3-426
15. Ni-NTA3-425
16. IP3-554
17. Ni-NTA3-780
18. IP3-1016
19. Ni-NTA3-781
20. IP3-1017
21. IP3-168
22. Ni-NTA3-122
23. Ni-NTA3
24. IP3-631
25. Ni-NTA3-488
26. IP3-632
27. Ni-NTA3-489
28. Ni-NTA3-490
29. Ni-NTA3-491
30. Ni-NTA3-460
31. Ni-NTA1-393
32. IP3-587
33. IP3-362
34. Ni-NTA3-295
35. Ni-NTA3-064
36. IP3-073
37. Ni-NTA3-063
38. Ni-NTA3-481
39. IP3-620
40. Ni-NTA3-388
41. Ni-NTA3-092
42. Ni-NTA3-093
43. Ni-NTA3-194
44. IP3-263
45. Ni-NTA3-195
46. Ni-NTA3-196
47. Ni-NTA3-423
48. IP3-552
49. Ni-NTA3-422
50. Ni-NTA3-559
51. IP3-722
52. IP3-801
53. Ni-NTA3-629
54. Ni-NTA3-672
55. Ni-NTA3-258
56. Ni-NTA3-544
57. Ni-NTA3-254
58. Ni-NTA3-406
59. Ni-NTA3-692
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61. IP3-135
62. Ni-NTA3-099
63. IP3-570
64. Ni-NTA3-445
65. Ni-NTA3-446
66. IP3-571
67. Ni-NTA3-442
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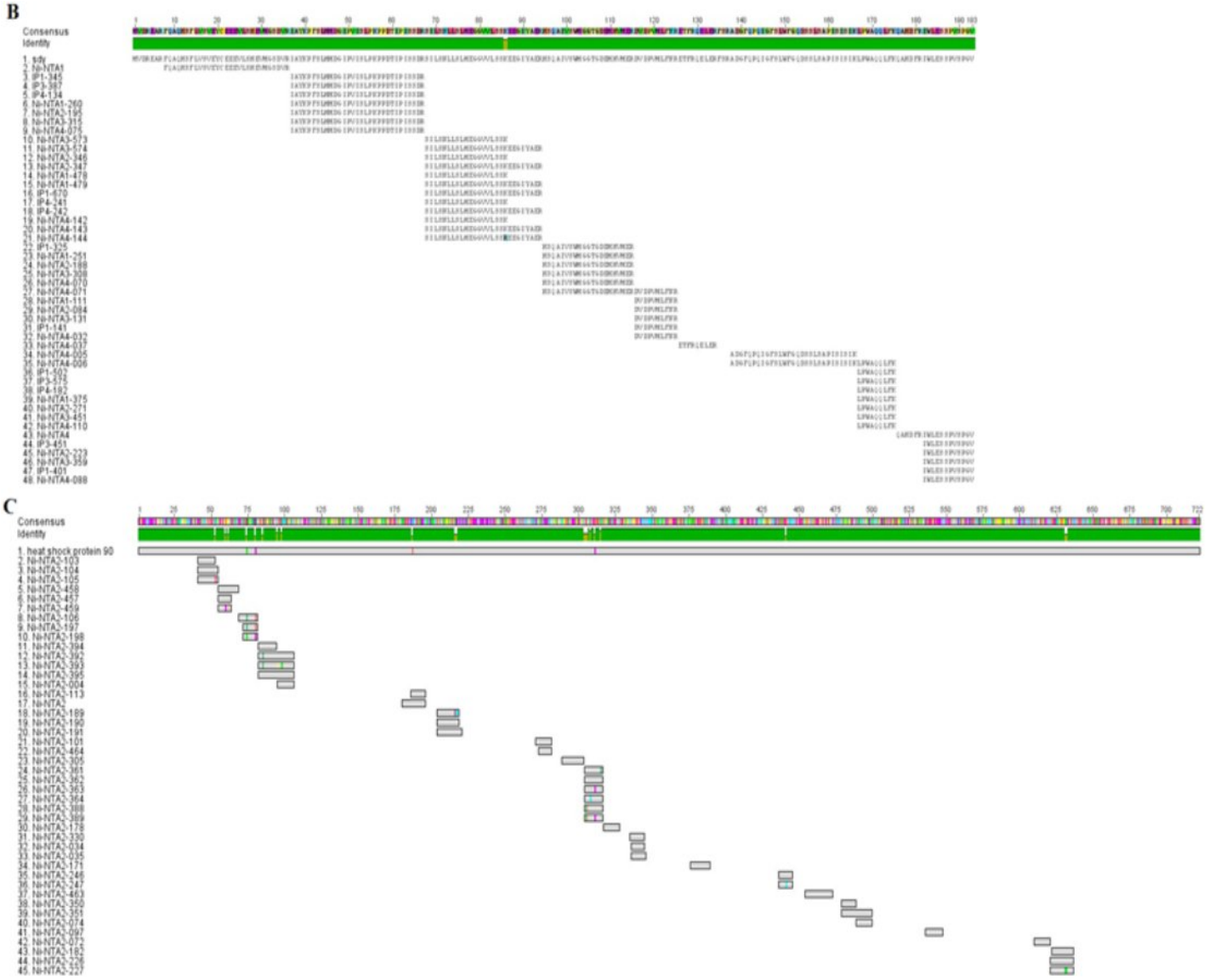
71. Ni-NTA3-073
72. Ni-NTA3-563
73. Ni-NTA3-553


B
Consensus
Identity
C
Consensus
Identity
1. heat shock protein 90

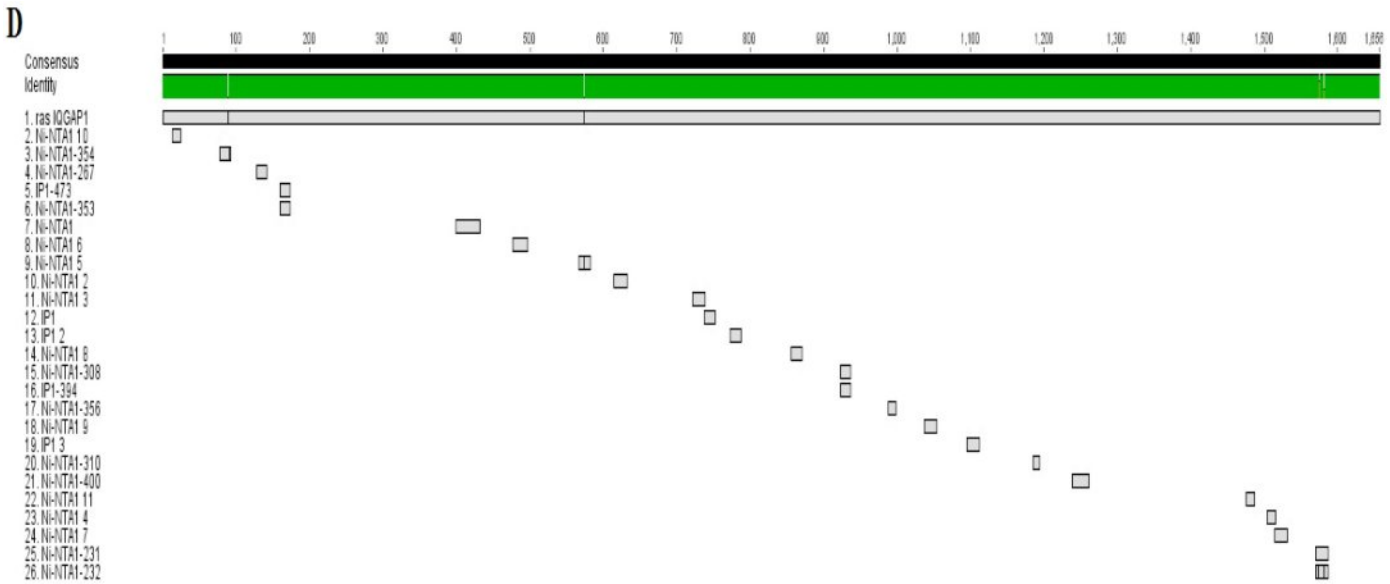


Figure 3.13 Alignment of peptide sequences to proteins

Peptide sequences aligned to proteins: A) isocitrate dehydrogenase, B) SdY, C) heat shock protein HSP 90-beta, and D) ras GTPase-activating-like protein IQGAP1.

Table 3.2 Domains of the top 4 candidate interacting proteins

Protein	RefSeq Accession	Domain(s)	Location in sequence (amino acid)	Domain(s) covered by protein sequence
Isocitrate dehydrogenase	NP_001133197.1	PTZ00435 (isocitrate dehydrogenase)	39-451	Yes
SdY	AKP41008.1	IRF-3	12-178	Yes
Heat shock protein HSP 90-beta	NP_001117004.1	PTZ00272, hatpase-c, HSP90	13-722, 34-169, 190-712	Yes Yes Yes
Ras GTPase-activating-like protein IQGAP1	XP_013983053.1	CH, BAR, ras-GAP, ras-GAP_IQGAP1, ras-GAP_C	43-157, 696-893, 991-1342, 1002-1381, 1453-1579	No, Yes, No, No, No

All four of the top candidate protein interactors have domains which are covered by the peptide sequences, except in the case of the ras GTPase-activating-like protein IQGAP1 (Table 3.2). No inferences can be made as to whether SdY interacts specifically with these domains as my affinity assays were not designed to distinguish the domains SdY interacts with, but rather identifying the proteins as a whole.

All the sequences obtained from the mass spectrometry analysis can be found in Appendix C. The proteins corresponding to the sequences were identified through tBLASTn against the Atlantic salmon database. There are a number of sequences that did not provide any hits. Some of them were later identified by the UVic Genome BC Proteomics Centre which also ran a BLAST on the sequences against the Atlantic salmon. Those sequences are distinguished by [Salmo salar] at the end of the protein name. A small percentage of the peptide sequences still provided no hits. These unknown peptide sequences may constitute the missing gaps of the proteins. The BLAST done by the centre also resulted in a longer list of sequences than originally provided to me and subsequently those sequences are distinguished by a red font colour. Further information on the peptide sequences regarding their location in the cell, molecular weight, and domains is found in Appendix D. Proteins in red correspond to the top four candidates while those in green are other potential candidates.

Chapter 4.

Discussion

4.1. False positives

Several proteins have been identified as candidate interactors of SdY, where there is no uniformity among them. With such a range of candidates, some may indeed be false positives.

Within the yeast two-hybrid assay, SdY has been shown to interact with the 3' UTR of Anxa7-like and Tmem91-like and with the Rps16 protein. The yeast two-hybrid assay I used is designed to identify protein-protein interactions and not protein-RNA interactions. As such, it is highly probable that the candidates: Anxa7-like and Tmem91-like are false positives especially since no open reading frames were detected and there were inconsistencies in growth on plates during the reciprocal transfer. How this assay identified the 3' UTR in the first place is unknown, but it may be that the sequence corresponds to a protein that has not yet been annotated in the Atlantic salmon, which has sequence similarity to the 3' UTR of Anxa7-like and Tmem91-like. This is plausible as a part from the hit to the 3' UTR, there is a portion of the sequence that aligns to no known protein.

Traditionally, ribosomal proteins are classified as false positives in yeast two-hybridization assays (Hengen 1997), which makes the candidate Rps16 a potential false positive. However, ribosomal proteins have been identified as interactors of SRY in nuclear speckles using yeast two-hybridization assays (Sato et al. 2011). Furthermore, certain ribosomal proteins, like 40S ribosomal protein S4 Y isoform 2, are believed to be involved with spermatogenesis (Lopes et al. 2010). Due to these discoveries, it is possible that ribosomal proteins, in particular Rps16, can also interact with SdY through

some unknown mechanism. There were inconsistencies in growth of colonies after a reciprocal transfer, but this may be due to cloning only the sequence obtained from the yeast two-hybrid assay and not the full sequence of the *rps16* gene into the pGBKT7 vector. Rps16 is a cytoplasmic protein involved in protein translation, belonging to the 40S subunit of the ribosome (Figure 4.1; Gene ID: 6217), as such SdY may interact with Rps16 to control translation of sex-determining genes. 40S ribosomal proteins were not only identified in the yeast two-hybridization assay but also in the histidine-tagged pulldown assay, making this protein more likely a potential interactor than a false positive.

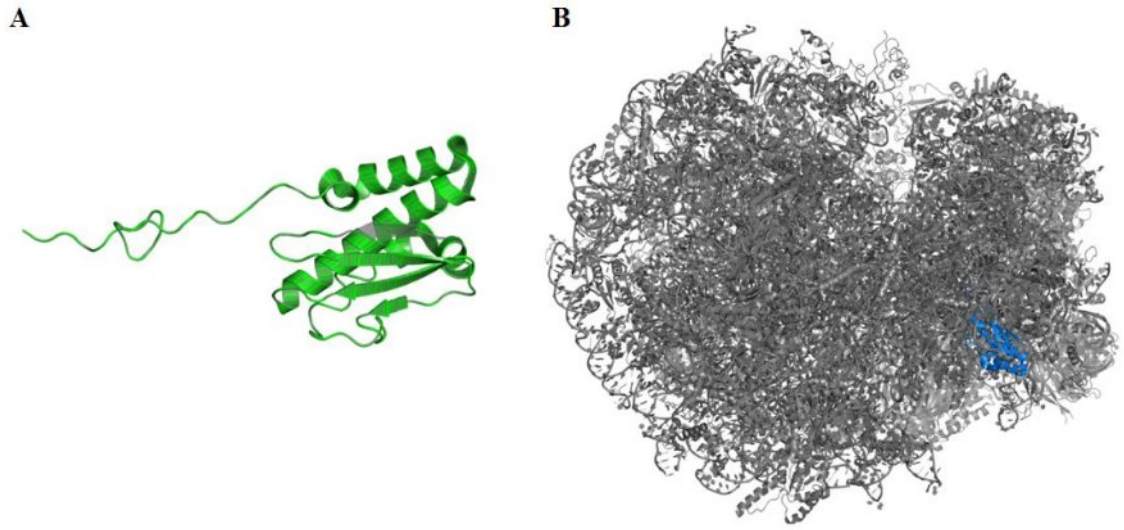


Figure 4.1 Structure of 40S ribosomal protein S16

A) The structure of 40S ribosomal protein S16. **B)** The location of 40S ribosomal protein S16 (shown in blue) in the 40S ribosome subunit. From "5aj0>40S ribosomal protein S16" by the Protein data bank in Europe, 2012, https://www.ebi.ac.uk/pdbe/entry/pdb/5aj0/protein/66. Copyright 2012 EMBL-EBI.

Considering how the actual location of SdY within a cell is unknown, it is difficult to eliminate candidates based on their location in a cell. Preliminary studies on testes indicate that SdY is not in the nucleus and most likely found in the cytoplasm (von Schalburg, personal communication). Within the affinity assays, proteins that resulted in few hits or matched to one particular sequence were ignored as these are most likely a result of non-specific interaction to antibodies or beads. Ideally, eliminating non-specific proteins would be achieved by sending bands from the lane with the antibody mixed with

testis extract for mass spectrometry analysis. The protein sequences obtained from such an analysis would indicate non-specific binding to the antibody and thus eliminate any false positives. However, since it is costly to do, this was omitted. An additional negative control to further reduce false positives would be to have run and analyzed the pre-immune serum.

The omission of band G (Figure 3.12) from further analysis was done due to the belief that the rate of migration may have been affected as it was above the protein ladder. However, in hindsight, this band may be an interactor of SdY and should not have been ignored.

4.2. SdY may be a dimer

Through yeast two-hybridization assays, SdY appears to interact with itself (Figure 3.1) suggesting that it may function as a dimer. This is further supported by the presence of a band approximately twice the size of SdY in the purified SdY lane of SDS-PAGE gels and Western blots (Figure 3.8a-b).

In order for Irf9 to bind to DNA, it needs to form a dimer with itself (Tang et al. 2007). With SdY being a divergent form of Irf9, it may have retained some features of Irf9. Much like Irf9, SdY may also dimerize in order to become active. Although SdY lacks a DNA-binding domain, the dimerization function may have still been retained. It is possible that SdY becomes activated through dimerization as there have been female Atlantic salmon found that have part of the *sdY* gene present (Eisbrenner 2014, Lubieniecki 2015b). A similar situation has been reported in Chinook salmon where a small portion of females also has *sdY* present (Cavileer et al. 2015). In most cases, the females lack exon 1 although a few have been reported to have all four exons present (Cavileer et al. 2015). The reason for having the *sdY* gene but remaining female may be because the SdY did not dimerize and subsequently did not become active.

It is not unique for SdY to be a dimer, as other proteins involved in sex-determination have been identified as dimers. Amh is a dimerized glycoprotein required for the silencing of the female pathway through the destruction of Müllerian ducts (Heule

et al. 2014; Kumar et al. 2010). Another case is SOX9 which functions as both a monomer and a dimer in mammals (Bernard et al. 2003). As a dimer, SOX9 is involved in the development of cartilages, while as a monomer SOX9 plays an important role in sex-determination (Bernard et al. 2003). It is possible that SdY may also have a dual role as both a monomer and dimer where its interaction to proteins depends on its structure, one of which is involved with sex-determination especially since SdY is not exclusively found in testes but also in many other tissues (Figure 3.7).

Although SdY was identified as an interactor of itself when mating between the two was done, it is interesting to note that when mating between SdY and the testis cDNA library was performed, SdY was not among the interactors. Upon generating the testis cDNA library, it was not tested whether it was large enough and had enough independently derived clones as the process was too timely and costly. As such, there is a chance that the *sdY* cDNA was not well represented in the library, resulting in it not being identified as an interactor.

4.3. SdY potentially associates with testosterone

Identifying the top candidates from the co-IP and Ni-NTA assays (isocitrate dehydrogenase, heat shock protein HSP 90-beta, and ras GTPase-activating-like protein IQGAP1) has led me to believe that SdY is associated with testosterone. Two possible scenarios as to how SdY may interact with testosterone are: 1) SdY plays a role in the synthesis of testosterone or 2) SdY is involved with the testosterone signalling pathway.

4.3.1. Scenario 1: SdY and the synthesis of testosterone

Testosterone is an androgen synthesized in the smooth endoplasmic reticulum (ER) of Leydig cells (Kim et al. 2016). Biosynthesis of testosterone as with any steroid hormone synthesis begins with cholesterol either synthesized or dietary (Nelson and Cox 2013). Cholesterol moves into the mitochondria where it gets converted into pregnenolone which will then be transported into the smooth ER (Kim et al. 2016). Once in the ER, a series of reactions occur which will lead to the generation of testosterone. The final step is the conversion of androstenedione to testosterone mediated by

hydroxysteroid dehydrogenase (17-beta), where isozymes 2 and 4 are involved in the inactivation of estradiol (Mindnich et al. 2004).

Several of the proteins interacting with SdY are associated with the testosterone synthesis pathway, leading me to believe that SdY plays a role in generating testosterone. One of the proposed candidate interactor of SdY is isocitrate dehydrogenase which is best known for converting isocitrate to alpha-ketoglutarate in the Krebs cycle in the mitochondrial matrix. There is also a cytoplasmic isoform of isocitrate dehydrogenase that is involved in generating NADPH for the biosynthesis of steroid hormones in adrenal glands (Frederiks et al. 2007). This cytoplasmic isocitrate dehydrogenase is also found in testes where it maintains high NADPH/NADP+ gradient in the cytoplasm (Sherbet and Auchus 2007). SdY may interact with isocitrate dehydrogenase in the cytoplasm to promote the generation of NADPH needed for testosterone biosynthesis. Furthermore, both isozymes (2 and 4) of 17β-hydroxysteroid dehydrogenase were identified in my protein assays, which are key enzymes involved in synthesizing testosterone.

The production of testosterone is stimulated by luteinizing hormone (LH) in Leydig cells and follicle-stimulating hormone (FSH) in Sertoli cells (Sanderson 2006). In *O. mykiss*, several of the genes found to be upregulated by LH (Sambroni et al. 2012) are similar to the ones identified in my protein assays as interactors of SdY. For instance, cytochrome c oxidase subunit 3, coatomer subunit alpha, and COP9 signalosome complex subunit 5 are upregulated by LH while protein cytochrome c oxidase subunit 2, coatomer subunit alpha, and COP9 signalosome complex subunit 3, 4, and 8 were identified in my protein assays. With these genes being upregulated by LH, they may be involved in steroidogenesis which could also be the case for the identified proteins from my assays. However, only coatomer protein complex alpha is found in the cytoplasm while the other proteins reside in the mitochondria or nucleus. Assuming that SdY is in the cytoplasm, it is unlikely that they interact with SdY.

Furthermore, interferons are known to inhibit steroidogenesis (Diemer et al. 2003). Since SdY is a truncated, divergent form of Irf9, it is possible that SdY retained this function, but through the loss of the N-terminus of Irf9 is now promoting

steroidogenesis, particularly the generation of testosterone. The notion that SdY is involved with steroidogenesis is plausible as steroids are important inducers of fish gonadal differentiation (Devlin and Nagahama 2002; Jalabert et al. 2000).

4.3.2. Scenario 2: SdY and the testosterone signalling pathway

Testosterone is a lipid hormone, capable of entering and leaving the plasma membrane, where it is part of either a classical or non-classical signalling pathway (Figure 4.2; Walker 2011). In the classical pathway, it binds to androgen receptors, releasing them from heat shock proteins which allows the receptors to travel into the nucleus to stimulate gene transcription for spermatogenesis (Walker 2011).The non-classical pathway involves testosterone either activating a kinase cascade or increasing the levels of Ca^{+2} in Sertoli cells (Walker 2011; Lyng et al. 2000).

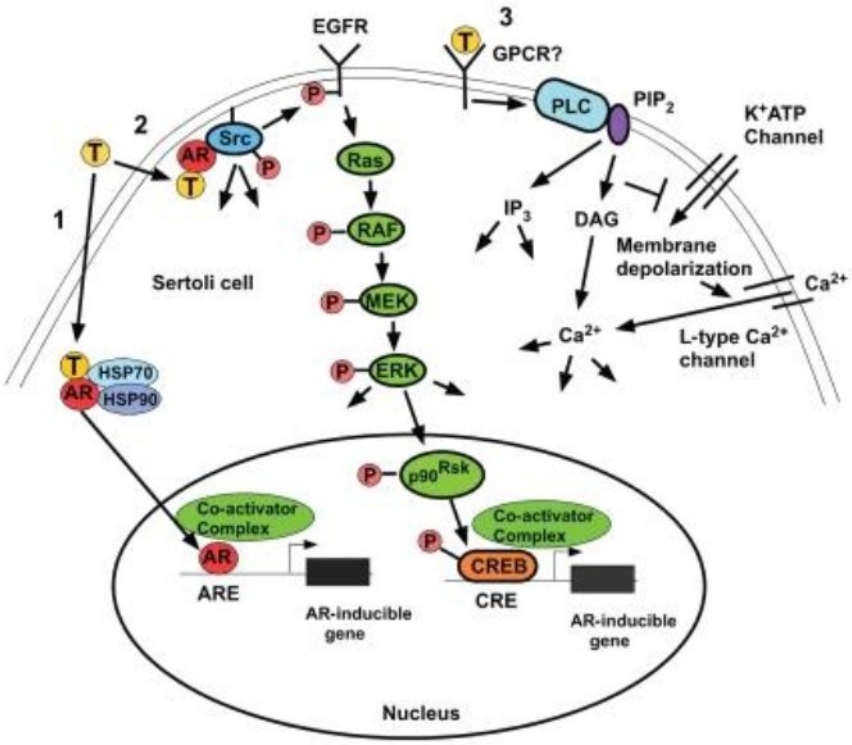


Figure 4.2 Testosterone signalling pathway

There are two types of testosterone signalling pathway: 1) classical pathway: testosterone (T) enters the plasma membrane and binds to an androgen receptor (AR) where it is released from heat shock proteins and enters the nucleus to activate transcription. 2) Non-classical pathway: binding to AR can also result in the activation of Src which will activate EGF receptors and a cascade of MAP kinases that will eventually lead to gene expression. 3) Non-classical pathway: T binds to a receptor which will lead to inhibition of K^+ATP channels and increase of Ca^{+2} into the cell through channels. Taken from Walker 2011 which is under the Creative Commons Attribution-NonCommercial 3.0 Unported License.

Among the top four candidates are heat shock protein HSP 90-beta, a chaperone protein involved in regulating proteins through conformational change (P08238(HS90B_HUMAN)) and ras GTPase-activating-like protein IQGAP1, a scaffold protein that binds to cell division control protein 42 (Cdc42) and is associated with calmodulin (P46940 (IQGA1_HUMAN)). Heat shock proteins 70 and 90 are bound to the androgen receptors and released once testosterone binds to the receptors (Figure 4.2). SdY may bind to heat shock proteins to assist them in binding to the androgen receptors in the absence of testosterone.

Ras proteins have been identified as part of the testosterone non-classical pathway (Figure 4.2). Apart from binding to calmodulin and Cdc42, ras GTPase-activating-like protein IQGAP1 can also bind to β-catenin, Rac1, and Erk1, acting as a regulator of the kinase signalling pathway (Joyal et al. 1997; Roy et al. 2004). The kinase signalling pathway is triggered once testosterone binds to androgen receptors and activating src (Figure 4.2). Another portion of the non-classical pathway involves testosterone binding to a receptor other than the androgen receptor leading to the inhibition of K+ATP channels causing membrane depolarization through the increase of Ca^{+2} into the cell (Figure 4.2; Walker 2011). As mentioned earlier, ras GTPase-activating-like protein IQGAP1 binds to calmodulin which is a Ca^{+2}-binding protein (B5DGN6 (B5DGN6_SALSA)).As Ca^{+2} enters the cell during membrane depolarization, calmodulin would bind to it, thus enabling it to bind to ras GTPase-activating-like protein IQGAP1. The potential relationship between SdY and ras GTPase-activating-like protein IQGAP1 may involve SdY altering the IQ motifs of the GTPase to allow it to bind to calmodulin.

Both candidates play a role in the testosterone signalling pathway. Heat shock protein HSP 90-beta bind to androgen receptors, keeping them inactive until testosterone binds to them, while ras proteins like ras GTPase-activating-like protein IQGAP1 cause a MAP kinase cascade once androgen receptors activate Src in the presence of testosterone (Figure 4.2). Ras GTPase-activating-like protein IQGAP1 may also be involved with Ca^{+2} which enter the cell in the presence of testosterone. As such, it has led me to believe that SdY is involved with the testosterone signalling pathway. Overall, the components of the non-classical pathway have not been well studied with new molecules constantly being discovered. As such, SdY can be a part of this pathway, as a link between the components of the pathway and testosterone as several of the candidate interactors of SdY are involved with testosterone.

4.4. Other potential candidates

The top four candidates were chosen as they were present in the cytoplasm and covered most of the full sequence of the protein with significant matches to more than one distinct peptide sequence. Many more proteins were identified in the mass spectrometry of the bands from the affinity assays, which were excluded as they were either not located in the cytoplasm or their peptide sequences provided a smaller coverage of the protein. A main criterion for the candidates is that they need to be located in the cytoplasm as preliminary studies suggest that SdY is localized there. If however SdY is shown to be located not only in the cytoplasm but in other compartments of the cell, other candidates which were excluded earlier may be the interactors of SdY.

Other potential candidates include sodium/potassium-transporting ATPase subunit alpha-1, AP-1 complex subunit beta-1, creatine kinase, and GDP-mannose 4,6-dehydratase. ATPase subunit alpha-1 is a membrane bound protein involved in the hydrolysis of ATP while exchanging sodium and potassium ion across the plasma membrane (P05023(AT1A1_HUMAN)). ATPases are not known to be involved in sex-determination. There are K+ATP channels in the testosterone signalling pathway that become inhibited in the presence of testosterone to allow for Ca^{+2} to enter into the cell. Interaction between SdY and ATPase subunit alpha-1 may involve regulating the

movement of K+ and Na+ into and out of the cell which will affect the membrane potential. However, since the ATPase is a membrane-bound protein, if SdY indeed interacts with it, then SdY would have limited binding regions to the protein as most of it would be imbedded in the membrane. AP-1 complex subunit beta-1 is part of the clathrin-associated adaptor protein complex 1 located on the cytoplasmic face of vesicles from the Golgi where it is involved with recruiting clathrin to the membrane as well as recognizing the sorting signals of transmembrane receptors (Gene ID 162). SdY may interact with the AP-1 complex to help mediate endocytosis.

Regarding creatine kinase, there are two isozymes that were identified in my assays: the testis and the brain. In salmonids, there is a testis isozyme of creatine kinase which attributes to the high level of creatine kinase activity in the spermatozoa (Saudrais et al. 1996), where the isozyme is specific to germ cells in trout (Kaldis et al. 1997). Creatine kinase, in particular the testis isozyme, is involved in energy transduction in the testes and the spermatozoa (P24722 (KCRT_ONCMY)). It is unusual to have the brain isozyme identified in my assay as I only looked at interactions of SdY in testes. SdY has been found to be present in the brain (Figure 3.7) so there is a chance that it can interact with the creatine kinase brain isozyme. In roosters, the brain isozyme is also present in the spermatozoa to provide additional sources of energy for the sperm (Kaldis et al. 1997). No reports of the brain isozyme being present in testes have been identified in salmonids, but it could function similarly as in roosters. Furthermore, *sdY* is classified as a sex-determining gene and as such would function early in development. Production of sperm is important for males, but it occurs once the male pathway has already been determined.

GDP-mannose 4,6-dehydratase is another potential candidate where its peptide sequences provided significant matches to most of the protein. The GDP-fucose pathway is comprised of three reactions where GDP-mannose 4,6-dehydratase is one of the enzymes of the pathway (Becker and Lowe 2003). It is uncertain how SdY would interact with GDP-mannose 4,6-dehydratase as the GDP-fucose pathway does not have any known role in sex-determination. GDP-mannose 4,6-dehydratase belong to the short-chain dehydrogenase/reductase (SDR) superfamily which is involved in NAD(P)H-dependent oxidation-reduction reactions (Kallberg, Oppermann, and Persson 2010).

Within this family belong the hydroxysteroid dehydrogenases which are involved in generating testosterone and as such GDP-mannose 4,6-dehydratase may contribute to the generation of NADPH for testosterone biosynthesis.

4.5. Further validation of interactors

Of all the potential candidate interactors identified through all three protein assays, no candidate was identified that was known to be involved in sex-determination. This may either suggest that SdY is not involved in sex-determination or since adult testes were used, the interactions with SdY during early development are not occurring. The latter is more plausible as the *sdY* gene has been shown to be involved in sex-determination in salmonids (Yano et al. 2012). Testes from adult Atlantic salmon were used in the affinity assays as well as to generate the cDNA library; therefore the interactors identified in my protein assays may indicate the role of SdY after sex-determination since levels of *sdY* remain constant after 63 days post fertilization (Lubieniecki et al. 2015a). Using testes from different developing stages of the Atlantic salmon would be beneficial in validating these interactors. However, the first sign of testes occurs shortly before hatching (Laird et al. 1978), therefore such studies would be done on the entire embryo which is why adult testes were used instead. Looking at the interactions of SdY in the developing testes would help establish the role of SdY in sex-determination.

Validation of the interactors needs to be done to determine which of these candidates are indeed interacting with SdY while simultaneously eliminating any false positives. One way is to transform the candidates obtained from the co-IP and his-tag pull down assays into yeast to validate their interaction with SdY. Additionally, the interactors identified in the yeast two-hybrid assays could be verified through a co-IP assay. In both cases, there are risks of obtaining false positives and as such, it would be beneficial to use other methods to validate the interactors. Apart from yeast two-hybrid and co-IP assays, validation of the interactors can be done through bimolecular fluorescence complementation (BiFC).This assay involves fusing the proteins of interest to two non-fluorescent fragments of a fluorescent protein such as the yellow fluorescent protein (YFP) where the interactions between the two proteins results in YFP fluorescing

in a living cell (Kerppola 2008).This method would not only verify the interactors but also confirm the location of SdY within a cell.

Chapter 5.

Conclusion

Salmonids are an interesting group of teleost fish as they have undergone whole genome duplication 88-103 million years ago. Consequently, they are still dealing with the redundancy of the duplicate genes, particularly those involved with sex-determination. The recently discovered sex-determining gene, *sdY*, is a truncated divergent form of *irf9*, which is speculated to have arisen as a result of the whole genome duplication. *sdY* is currently exclusively found in salmonids and shares no homology to any known sex-determining factor. Since *sdY* was recently discovered, very little information is known about this gene, particularly its role in sex-determination. My project is focused on elucidating the function of SdY protein based on its interactions with other proteins with emphasis being placed on Atlantic salmon as they are the main salmon species of the world.

Within my project, I have used yeast two-hybridization, co-IP, and his-tag pull down assays to identify the possible interactors of SdY. To prepare for these assays, a testis cDNA library, polyclonal antibodies against SdY, and his-tag recombinant SdY were generated. The first portion of my project dealt with identifying interactors through a yeast two-hybridization assay. Mating was performed between SdY and a testis cDNA library as there was no previous information on any interactors of SdY. Also, SdY was mated with itself which resulted in growth of colonies, indicating a positive interaction. However, mating between SdY and the testis cDNA library did not reveal SdY as an interactor. From the yeast two-hybridization assay, three interactors were determined: Rps16 and the 3' UTR of Anxa7-like and Tmem91-like, where Anxa7-like and Tmem91-like are most likely false positives as my yeast two-hybrid assay cannot detect protein-RNA interactions. Reciprocal transfer of the interactors was performed to verify the positive interactions where there were inconsistencies in the growth of diploids, providing

doubt whether SdY indeed interacts with them. Yeast two-hybridization assays are known to result in false negative and positive results and require further validation methods to provide confidence in the results. Among common false positives are ribosomal proteins, however since ribosomal proteins have been linked to sex-determination in mammals, they remain as candidate interactors of SdY.

The second portion of my project dealt with identifying interactors of SdY using co-IP and his-tag pull down assays where the identity was determined based on mass spectrometry analysis. From these assays, four proteins were classified as candidate interactors: isocitrate dehydrogenase, SdY, heat shock protein HSP 90-beta, and ras GTPase-activating-like protein IQGAP1. These four proteins were chosen as potential candidates as they provided the most peptide sequences, providing significant matches to multiple distinct peptide sequences, and are located in the cytoplasm, where SdY is believed to reside. Other potential candidates include creatine kinase, GDP-mannose-4,6-dehydratase, sodium/potassium-transporting ATPase subunit alpha-1, 40S ribosomal protein SA, and hydroxysteroid dehydrogenase (17-beta) 4. A wide variety of interactors were identified, all of which have no known role in sex-determination. These interactions have led me to believe that SdY is involved in either the biosynthesis of testosterone or with its signalling pathway.

The exact function of SdY still remains to be determined and further validation studies need to be performed to test these candidate interactors. These studies include, but are not limited to, performing additional yeast two-hybridization assays on the interactors identified in the co-IP and his-tag pull down assays to test whether they do interact with SdY, or performing the co-IP assay on the candidates obtained from the yeast two-hybridization (SdY, Anxa7-like/Tmem91-like and Rps16), particularly on Rps16 to test if they are indeed interactors of SdY. Another way to validate these interactions is through bimolecular fluorescence complementation (BiFC) which visualizes protein interactions in living cells.

Other future directions may include sending band G (Figure 3.12) for mass spectrometry analysis as well as bands produced from a mixture of antibody and testis lysate to test for any non-specific binding which would assist in identifying any false

positives. Since adult testes were used for this project, it would be useful to test testes in developing individuals to check if SdY is interacting differently. With a wide variety of candidate interactors being identified in my protein assays, looking at their differential expressions may help in determining whether they are involved in the sex-determining pathway of salmonids. Nevertheless, this work brings us one step closer in understanding how SdY functions in Atlantic salmon.

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Appendix A.

Controls of Yeast Two-Hybridization

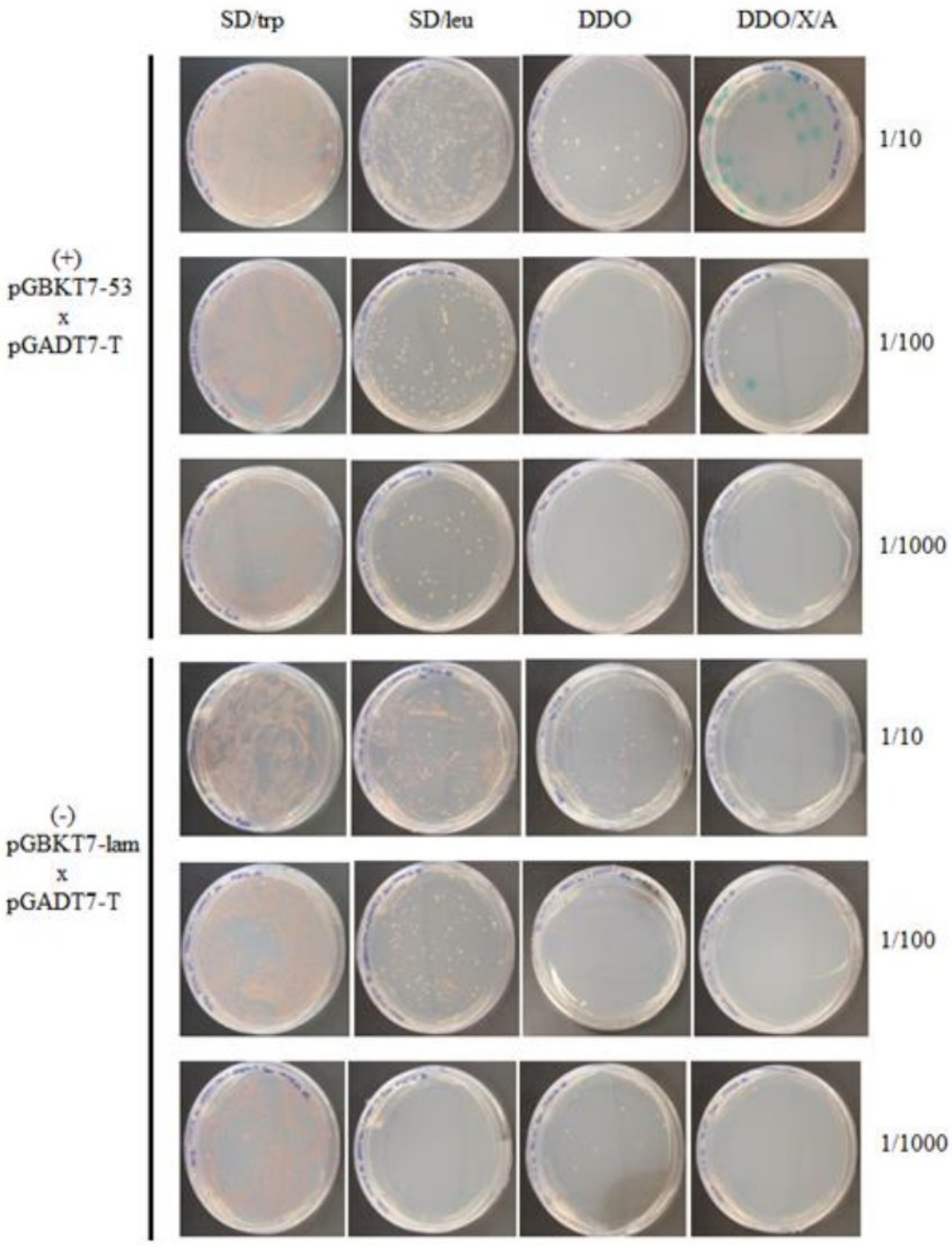

Figure A1. Positive and negative control mating

Positive control mating was between Y2HGold pGBKT7-53 and Y187 pGADT7-T while negative control mating was between Y2HGold pGBKT7-lam and Y187 pGADT7-T. Bait (pGBKT7) colonies grew on SD/trp, prey (pGADT7-T) colonies grew on SD/leu and diploids grew on DDO and DDO/X/A. For positive control, approximately equal numbers of colonies have grown on both DDO and DDO/X/A plates where colonies are blue on DDO/X/A. For the negative control mating, colonies have grown on DDO, but not on DDO/X/A.

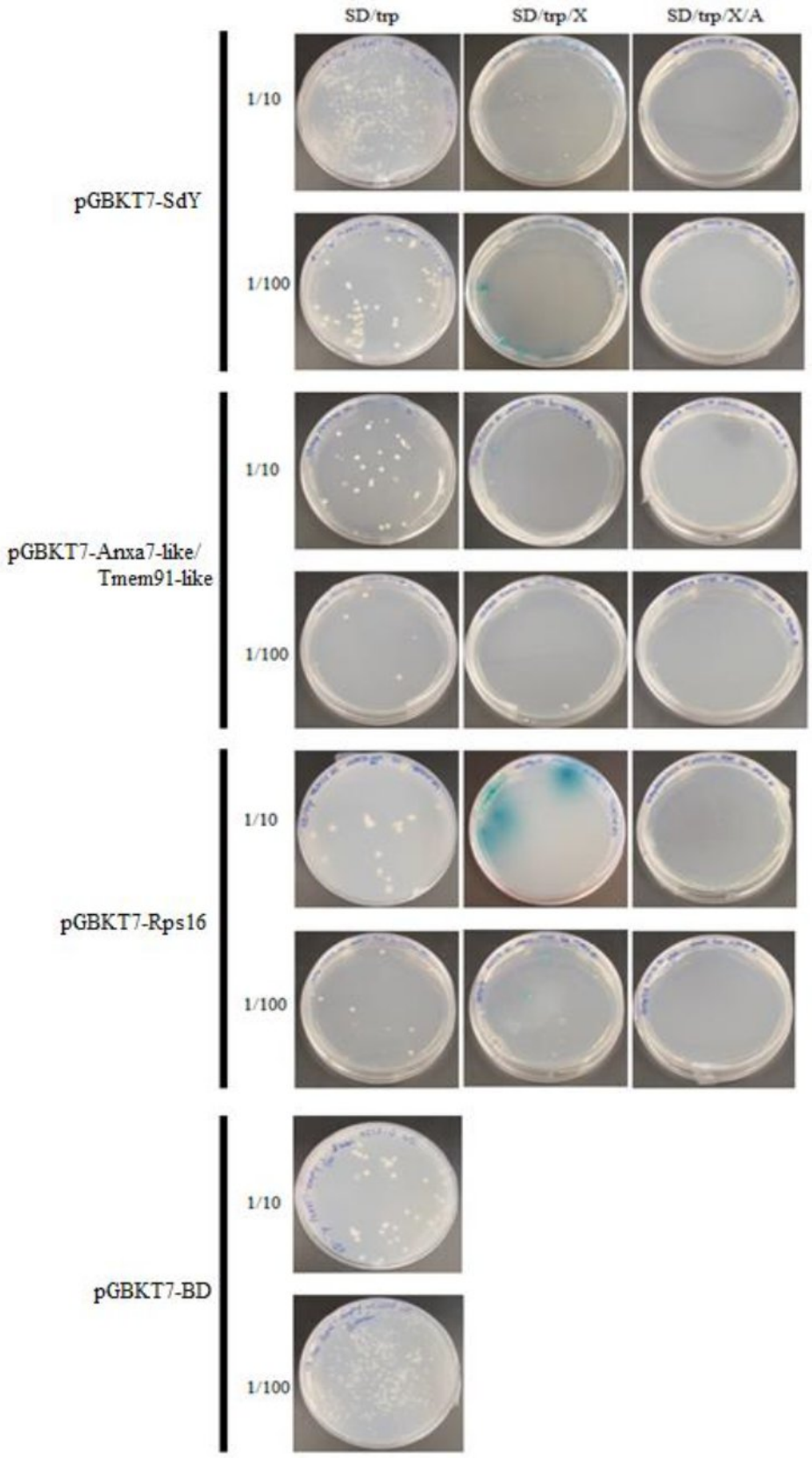
SD/trp
SD/trp/X
SD/trp/X/A
pGBKT7-SdY
1/10
1/100
pGBKT7-Anxa7-like/
Tmem91-like
1/10
1/100
pGBKT7-Rps16
1/10
1/100
pGBKT7-BD
1/10
1/100

Figure A2. Bait Autoactivation and Toxicity Test

Testing for autoactivation and toxicity on three baits: pGBKT7-SdY, pGBKT7-Anxa7-like/Tmem91-like, and pGBKT7-Rps16.No autoactivation occurred as the three baits have distinct colonies on SD/trp and SD/trp/X plates with blue or white colonies on the latter, while no growth on SD/trp/X/A plates. Colonies are not toxic as there is a similar size between the baits and the empty vector (pGBKT7-BD).

Appendix B.

Sequences of the interactors

Table B1 Sequences of interactors obtained from yeast two-hybridization

Interactor	Sequence
Anxa7-like/ Tmem91-like	GGGGACACACTGGTTGAATCAACGTTGTTTCCACGTCGTTTCAATGAAATG GCGTTGAACCAATGTGGAATAGACGTTGAATTGACACCTGTGCCCTGTGG GATATATCATAATGCTGTTTATTCATCAATGTTTATGACACACATTTTGAAAA ACTATACATCTAATGACTTACTTTTTTAAATACCTGCAGAACAGATAGTTTA AATCATTCATTATTTTGCCTGTTTAATTGTATATTTAATATACATAAATAAAC ACCAAATTATGGACACTAATTGTATTTTGGAATATTTCTAATACATTTCTGAA GGATAGTGTATGTATATTCAGTGTGTATGGATAAGTCATATCAGAGTTTAG AGACAGATTTTGACAATTGTTATTGAGAATGCAGCAGAAAAGAGACNTATT CTTAATCTGAGAGGACATGTATTACTGGTTGTCATTACACAGAGTGCCAGT GAGGTTACCACTCACAAGAGTAAACATTTTACAACTCGATGTCAAGTATTTA CTTATTGTTACACAAATGTATCAATTTGCTCAATTCAGTGTTGTGGGCTGTC AGCAATGACACAAGCAAGACTCAACTCAACTTATTTTGGTCAACTGTGGAG ACTAAGGGCTAGATTCAATTTAAAGGTAATTTAAAGGTAATTTCCATTTGAG TCGACATATGCAGCGTTTACCAGTGAACGCAGTCTCTCCGCTGTCCAGTTT GGCCTTACAGTTGATTCATTTGGTAAAAATGTCCACACTATCAATGTCATTT AGAGTCACATATTGCCAGGATTATGTATAACACTGTATATCGTTAGCGTATA TATTTTATTTGACTATGTATTCAGTGTTGCTCAGCTCTTCTATTTACCAAGG GGTTGTACTTTATCCTTCTCTGAGTGTGTAGTTNATGGAAATAGTGTTTACC TTGCTATCATGTCTGTTTGTTTGTGAAAACACAAAGGAAGAGACCATTATCA NAATGCAGATATGTAAACATAATTNGTNTTAAAATAACGTNCAGTGTAAATA AAAATCACACACTGACCTCTA
Rps16	GGGTGGCCTCATCAAGGTGAATGGCAGACCCCTCGAGATGATTGAGCCA GCCACTCTCCAGTACAAGCTGCTGGAGCCAGTGCTGTTGCTGGGCAAGG AGCGTTTTGCTGGAGTTGACATCCGAGTCCGAGTGAAGGGTGGTGGACAT GTCGCACAGATCTATGCTATTCGTCAGTCCATCTCCAAAGCCCCTGGTCG CATACTACCAGAAATATGTGGATGAGGCCTCCAAGAAGGAGATCAAGGAC ATCCTGATCCAGTACGACAGGACCCTGCTGGTTGCTGACCCTCGTCGCTG CGAGTCCAAGAAGTTCGGTGGACCAGGAGCCCGTGCCCGCTACCAGAAG TCTTACCGTTAACCTCTCTACATTTTCATGTAATAAAGTTGAGGGAGAAAGC
SdY* *sequence was a result of reciprocal transfer	ATGGTTGACAGAGAGGCCAGATTCCAAGCCCAGCACTCTTTTCTTGTCTCAGTGGA GTACTGCGAAGAGGAGGTGCTTAGTCATGAGGTTATGGGGAGTGATGTCAGAATT GCCTACAAGCCCTTCTCCCTGATGATGGATGNGATCCCCGTCATCTCTCTCCCAAA GCCCCCCGACACCATTCCCATCTCCTCTGACCGTNCAATCCTCTCCAACCTGCTTT CCNTCATGGAGGGTGGAGTGGTTTTAAGCTCTAAGGAGGAAGGCATCTATGCTGA ACGGCATAGCCAAGCCATAGTCTCCTGGATGGNCGGCACGGGGGATGAGATGCA CGTGATGGAGCGTGATGTGGATCCTGTGATGCTCTTCAACAGGGAGACCTTCAGA CAGGAGTTGNACGCTTCAGCAGAGCAGATGGCTTCCAACCGCAAATTGGGTTCAG CCTATGGTTCGGACAAGACTCATCACTCAGTGCACCAATCTCTATATCGATTAAATT GCCATGGGCTCAGCAGCTATTCAAGCAAGCTCACGACTTCAGGATCTGGCTTGAG TCCTCCCCTGTCTCTCCTGGAGTCGGA

Appendix C.

Complete list of all sequences from mass spectrometry

The attached worksheets file (Appendix_C.xlsx) is a part of this thesis. The file contains two tabs: tables C1 and C2. Table C1 shows a list of all the sequences of proteins that were generated through mass spectrometry. Table C2 shows a list of sequences corresponding to the contaminants.

Appendix D.

Detailed inventory of proteins generated from mass spectrometry

The attached worksheets file (Appendix_D.xlsx) is a part of this thesis. The file contains three tabs: table D1, D2, and D3. Table D1 shows a list of all the proteins whose sequences were generated through mass spectrometry and were not classified as contaminants. Proteins that are highlighted in red are the top 4 candidates. Table D2 shows a list of the proteins which have 7 or more unique sequences and their distribution across the two protein assays. The top 4 candidates are shown first, above the red box. Table D3 shows a list of proteins that have 15 or more peptides with their domains and whether they are covered by the peptide sequences generated from the protein assays.