The regulation of Sox3 function in zebrafish embryonic development

By

Chi Man Lam, BSc

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Abstract

Embryogenesis in vertebrates is regulated by highly complicated signaling networks, which involve various signaling pathways and factors. Sox3 is known to have critical roles during the whole of embryonic development in vertebrates. In zebrafish, it has been shown that Sox3 restricts organizer formation as well as inhibiting Fgf signaling, which is required for the expression of organizer genes. On the other hand, SUMOylation has been suggested to be a regulator of the transcriptional activity of Sox3. The SUMOylaton has previously been demonstrated on mouse Sox3 and chick Sox3.

In this study, I examined the role and regulation of Sox3 in early embryogenesis in zebrafish. In the first part of the study, I inspected the detailed expression of *gsc* and *chd*, in comparison to *foxd3* expression. It was demonstrated that Sox3 and Fgf signaling could repress both *gsc* and *chd* independently from each other. Sox3 was also found to be able to directly act on the promoter region of *gsc*. In the second part of the study, it was shown that the SUMOylation of Sox3 appeared to occur in zebrafish embryos. The SUMOylation of Sox3 was also shown to correlate with the chromatin fraction, although there was a very small fraction of Sox3 SUMOylated. The biological effect of SUMOylation of Sox3 has also been analysed. It was shown that SUMOylation of Sox3 could enhance the transcriptional repressor activity of Sox3. The result of luciferase reporter assay on the promoter region of *boz* also suggested that SUMOylation eliminated the transcriptional activator activity of Sox3. These data raise the possibility that SUMOylation of Sox3 might act as the switch from a transcriptional activator to a repressor.

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Chapter 1 Introduction

Pre-Introduction

This project investigated the role of Sox3 in zebrafish embryogenesis. In the first part of the study, I looked into the intricate signaling network by which Sox3 and Fgf signaling pathways regulate *goosecoid* and *chordin* expression at organizer formation stage. Since Sox3 is a multi-functional transcription factor in zebrafish development, it is interesting to inspect how Sox3 is regulated to play diverse roles in different situations. Therefore, in the second part of my study, I examined one of the post-translational modifications, SUMOylation, of Sox3 in zebrafish.

1.1 The development of zebrafish

1.1.1 zebrafish as a research animal model in developmental biology

The field of developmental biology has been studied throughout modern history. However, since the 1950s when molecular biology was born, the questions surrounding genetic regulation were introduced into the field of developmental biology. At that time, many model organisms of developmental genetics were established, including *Drosophila*, *Xenopus*, *Gallus Gallus* (chicken) and *Mus musculus* (mouse). In the 1970s, there were two other animals, *Caenorhabditis elegans* and *Danio rerio* established as new model organisms in developmental biolgoy.

Danio rerio, discovered and classified in 1822 by Francis Hamilton, commonly known as the zebrafish, is a teleost fish belonging to the family *Cyprinidae* in the class *Actinopterygii*. The zebrafish is a model system in developmental biology, environmental biology, neurobiology, toxicology and evolutionary biology (Powers, 1989). Due to their genetic amenability, transgenic fish have also been used for drug

screening and as a model for specific human diseases, including Alzheimer's disease, heart disease and cancers (Langenau et al., 2003, Lee et al., 2002).

In developmental biology, there are prominent advantages in using zebrafish as a model system. The fertilization and embryogenesis of zebrafish embryos are external. Their embryonic development is rapid, and it takes only 72 hours for a fertilized egg to proceed through the major processes during embryonic development. Moreover, the embryos are large and completely transparent in the first 24 hours after fertilization. This allows the easy manipulation and observation of the embryos. All these factors make the zebrafish a good animal model in studying developmental events. The zebrafish system was first developed and exploited by George Streisinger at University of Oregon in Eugene. Since then, the group widely developed the zebrafish system and isolated the first developmental mutants, *cyclops* and *spadetail*. Afterwards, the zebrafish research community rapidly increased in number and this animal model system became more well-established. To date, the zebrafish Genome Project, started by the Sanger Institute in 2001, is now at the maintenance stage, and the zebrafish Mutation Project is still ongoing.

1.1.2 The early development of zebrafish

Embryonic development initiates subsequent to fertilization (Figure 1.1). During early development of the zebrafish, there are several points for critical developmental events. At the tenth cell cycle after fertilization, about 512-cell to 1000-cell stage, the mid-blastula transition (MBT) begins. That coincides with the zygotic genome activation, when zygotic gene transcription starts (Lee et al., 2014). At sphere stage, some signals start to be expressed in the dorsal mesoderm, and that marks the formation of organizer and the onset of epiboly (De Robertis et al., 2000). There are

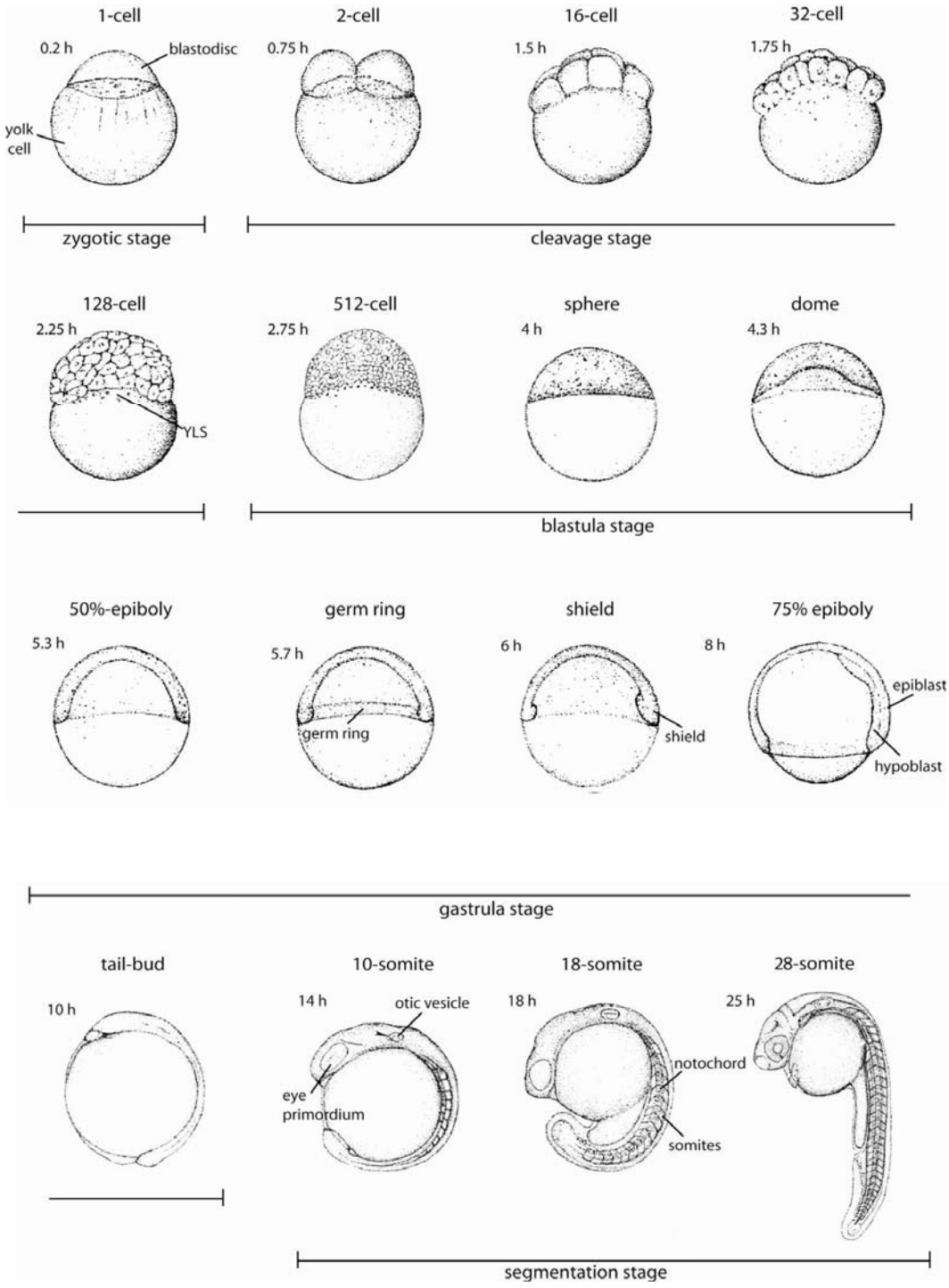


Figure 1.1 The early development of zebrafish
The morphology of the zebrafish embryogenesis is represented in the schematic pictures. Stages of zygotic, cleavage, blastula, gastrula and segmentation are depicted. The hours post fertilization indicated is under the standard condition that the zebrafish embryos are incubated at 28°C. (pictures adapted from Kimmel *et al*., 1995)

three typical types of cell movements during the epiboly stage; intercalation from late blastula to 50% epiboly stage, involution from 50% epiboly which marks gastrulation, and convergent extension occurring simultaneously with epiboly intercalation and involution (Figure 1.2) (Warga and Kimmel, 1990). During late gastrulation, neural development arises at the inception of neural induction.

The staging of the embryonic development of zebrafish is according to Kimmel's study in 1995 as a standard (Kimmel et al., 1995). Embryogenesis in zebrafish is divided into seven periods; Zygote, Cleavage, Blastula, Gastrula, Segmentation, Pharyngula and Hatching. My study is mainly focussed on the early stages, from the blastula period to gastrula period, marking the organizer formation and neural induction.

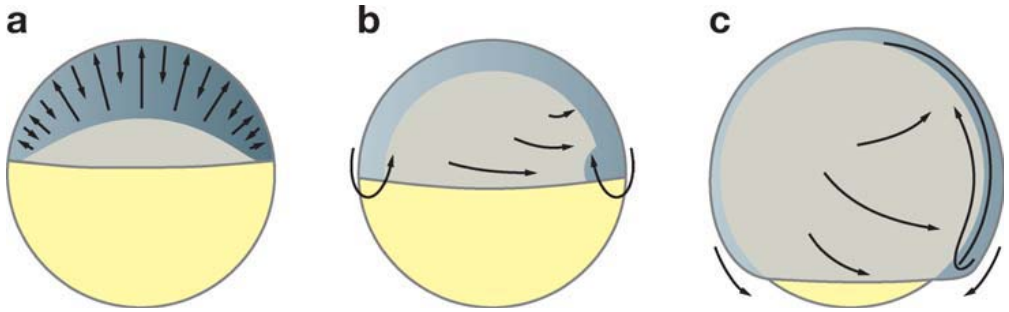


Figure 1.2 Cell movements during gastrulation
There are three types of cell movement (indicated by arrows) during gastrulation. (a) Cell intercalation starting at dome stage. (b) Cell involution at shield stage. Cells migrate dorsally and internally towards animal pole. (c) Involution and convergent extension happens during the whole epiboly stage.

1.1.3 The formation of the organizer

The concept of the organizer was first described in 1924. Hans Spemann and his student Hilde Proescholdt Mangold first demonstrated, using transplantation, that a group of cells from the dorsal lip of amphibian blastula embryos has the ability to form dorsal mesodermal structures autonomously. This triggered the neighbouring host cells to become a secondary body. They named that specific area of cells the "organizer". Since then, thousands of studies have focused on the formation, regulation and function of organizer. In 1973, the Nieuwkoop centre, a region preceeding the Spemann-Mangold organizer, was discovered by Pieter Nieuwkoop in the amphibian embryo (Nieuwkoop, 1973). In his study, the organizer was suggested to be induced by the dorsal vegetal cells just beneath the organizer. Later transplantation studies performed in *Xenopus* also supported this idea (Gurdon, 1960b, Gurdon, 1960a).

There are several factors that critically contribute to organizer induction. The two best characterized signal pathways are the Wnt signaling pathway and the TGF-β signaling pathway (De Robertis et al., 2000). Following the discovery of the organizer and the Nieuwkoop centre in *Xenopus*, later studies implicated the maternal gene, *β-catenin*, in determining Nieuwkoop centre formation. It was suggested that β-catenin, which was originally known as a cell-cell adhesion protein, was required and sufficient for the development of dorsal structures by acting downstream of the Wnt signaling pathway (Guger and Gumbiner, 1995, Heasman et al., 1994). It was then discovered that β-catenin could act as a transcriptional co-factor, as well as a cadherin-mediated adhesion protein. β-catenin translocates to the nucleus where it co-operates with other transcription factors, such as LEF/TCF. In the absence of β-catenin, LEF/TCF acts as a repressor of downstream genes. Interaction with β-

catenin converts LEF/TCF to an activator. Thereupon, the targeted genes will be turned on in the presence of β-catenin (Figure 1.3) (Behrens et al., 1996, Molenaar et al., 1996, Schneider et al., 1996).

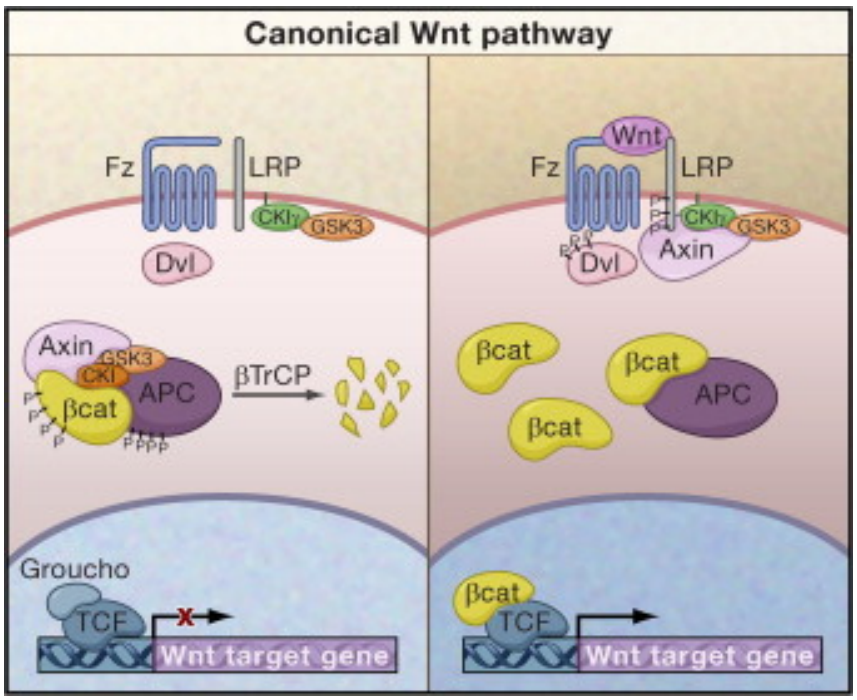


Figure 1.3 The Wnt signaling pathway
In the absence of Wnt protein, β-catenin is subjected to degradation. When the Wnt protein binds to its receptor, Dsh is activated and thereby Gsk3 is inactivated. Consequently, β-catenin is prevented from degradation. β-catenin is then translocated into the nucleus and cooperated with TCF to activate the transcription of target genes.

Although β-catenin is expressed throughout the whole embryo, it is only translocated into nuclei at the dorsal side of the blastula. Therefore, there must be other factors participating in determining β-catenin's activity in distinct areas. An explanation for this phenomenon is that Glycogen synthase kinase 3 (GSK-3), acting in the Wnt signaling pathway, targets β-catenin for degradation by phosphorylation in the absence of Disheveled and GSK3 binding protein (GBP) (Dominguez et al., 1995, Fagotto et al., 1997, He et al., 1995). When an oocyte is fertilized, Disheveled and

GBP are transported to the proposed dorsal mesoderm through microtubules during cortical rotation in *Xenopus*. GSK3 is consequently inactivated and β-catenin is preserved (Figure 1.3) (Miller et al., 1999, Weaver et al., 2003).

Apart from β-catenin, the TGF-β signaling pathway also plays an important role in organizer formation. It was also found that there is communication between Wnt/β-catenin signaling and TGF-β signaling through the Smad proteins (Figure 1.4) (Letamendia et al., 2001). It is known that the marginal cells from the mesendoderm are induced by vegetal signals to become endoderm. Signals from the endoderm then induce mesoderm formation. In *Xenopus*, it has been suggested that mesoderm induction essentially depends on the maternal mRNA, *veg T*. Veg T, which is a transcription factor, triggers the expression of zygotic Fgfs and members of the TGF-β family, including Xnrs, Derriere and Activin, which instruct mesoderm formation

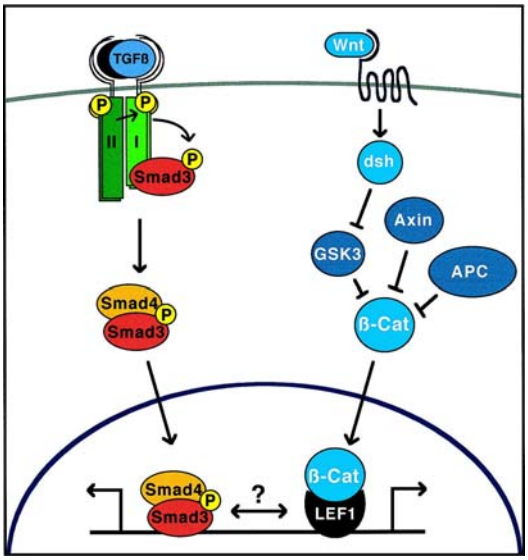


Figure 1.4 TGF-β signaling pathway
The TGF-β ligand binds to the TGF-β receptor II, which initiates the signlling cascade. The receptor I activates a Smad protein, which interact with another Smad protein and regulate the expression of target gene. TGF-β signaling pathway has crosstalk with Wnt/β-catenin signaling.

(Kofron et al., 1999). As well as *Veg T*, another maternal mRNA, *vg1* which is also a member of TGF-β family, plays an essential role in mesoderm induction and organizer formation (Birsoy et al., 2006).

However, as cells receiving those mesoderm inducing signals and being specified to be mesoderm, what cell types they would give rise to mainly depends on the concentration of Nodal signals (Gritsman et al., 2000). It has been suggested that there is a Nodal gradient, regulated by Veg T, Vg1 and β-catenin, along the equator of the embryo. The Nodal gradient, along with β-catenin and BMP signals, determines the dorsal-ventral axis in the mesoderm (Agius et al., 2000). The area receiving the highest levels of Nodal, acting synergistically with β-catenin, activates *siamois* and *twin*, which in turn activates the downstream genes, such as *goosecoid*, that characterize the organizer (Figure 1.5) (Yamanaka et al., 1998, Fekany et al., 1999, Kimelman, 2006).

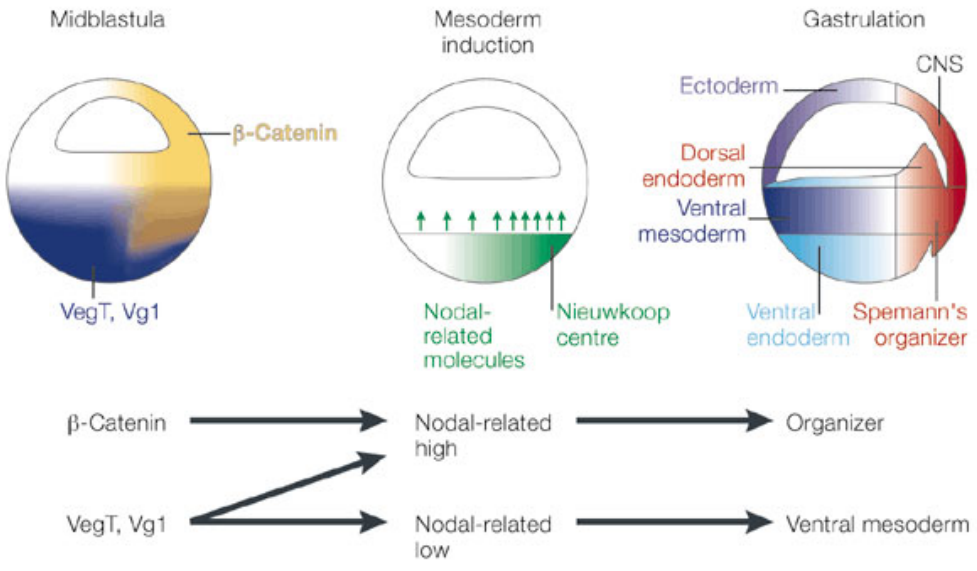


Figure 1.5 The signaling network during organizer formation in *Xenopus*
Maternal mRNA VegT and Vg1 were distributed in vegetal pole. They triggered the TGF-β family proteins which then activated the TGF-β signaling and finally instructed organizer and mesoderm formation. The combined signaling pathways contribute to a network regulating the gastrulation process.

As mentioned above, *siamois* is activated by the Wnt/β-catenin signaling pathway. It was discovered in *Xenopus* (Brannon and Kimelman, 1996, Lemaire et al., 1995) that Siamois is a marker of Nieuwkoop centre. Siamois cooperates with other cofactors, such as Smad2/4, and activates the expression of the organizer genes. *goosecoid*, which encodes a transcription factor, was the first organizer gene to be discovered (Cho et al., 1991), followed by Noggin and Chordin, both BMP antagonists (Sasai et al., 1994, Smith and Harland, 1992).

Since both the Spemann-Mangold organizer and Nieuwkoop centre were initially defined in amphibian embryos, later studies aimed to identify homologs in other species, such as *Danio rerio*. β-catenin in the Wnt signaling cascade was first demonstrated to play a role in axis formation in zebrafish. Overexpression of β-catenin was shown to induce ectopic expression of *gsc* and axis duplication (Kelly et al., 1995). Another study using the microsugical and cell labeling experiments to characterize the shield in zebrafish also reported that the shield region was sufficient to induce a secondary axis (Shih and Fraser, 1996). The gene *bozozok (boz)* was later identified as the earliest organizer gene to be expressed in zebrafish. It has similar function to *siamois*, and therefore *boz* is suggested to be the equivalent marker of Nieuwkoop centre in zebrafish. *boz* expression was also found to require the Wnt/β-catenin signaling pathway. It was proposed that the accumulation of β-catenin at dorsal mesoderm cells and yolk syntical layer (YSL) induced the expression of *boz*. On the other hand, it was also found that singals from vegetal yolk cell in zebrafish specified dorsal organizer formation (Ober and Schulte-Merker, 1999). These vegetal signals include the Nodal-related gene, *squint* (*sqt*), which is also induced by the accumulation of β-catenin in dorsal mesoderm and YSL. Overepxpression of *boz* induced ectopic expression of two other organizer genes, *goosecoid* (*gsc)* and *chordin*

(*chd)*. While *boz* and *sqt* are expressed in dorsal mesoderm and YSL, they are both required for organizer formation in zebrafish. Yet *boz* was suggested to act parallelly to the Nodal signaling. It was shown that Boz could partially induce *chd* but not *gsc* in the absence of Nodal siganling (Shimizu et al., 2000, Sirotkin et al., 2000). *chd* and *gsc* are expressed in organizer in zebrafish following the initial expression of *boz* and *sqt*. As mentioned, both *gsc* and *chd* expression are induced by *boz* and *sqt*. The combined signals from dorsal organizer restrict the BMP signaling and the ventral genes, such as *ved*, *vox* and *vent*, and vice versa. For instance, Boz was demonstrated to directly act on *bmp2b* and *ved* (Leung et al., 2003, Shimizu et al., 2002). Later on, the BMP antagonists *chd* and *nog* also come into function. After MBT stage, the combined signaling network establishes a BMP gradient, which works together with other signaling pathways and contributes to the dorsal-ventral axis formation. (Figure 1.6)

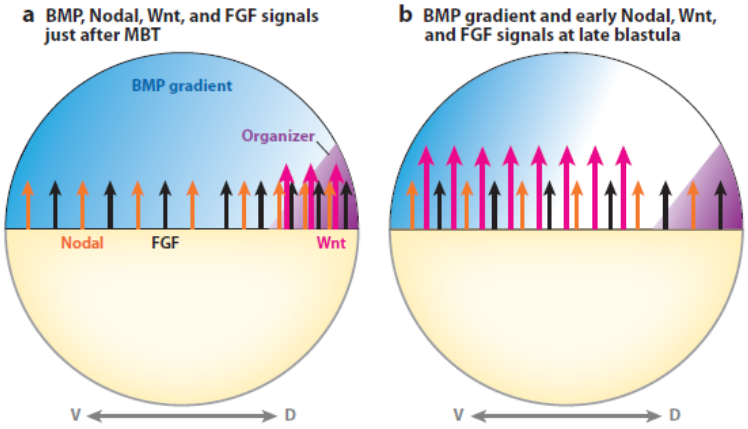


Figure 1.6 The signaling network of organizer and axis formation in zebrafish
The BMP, Nodal, Wnt and Fgf signals are expressed in different ways before and after MBT stage in zebrafish embryos. Nodal and Fgf signals are expressed in the margin. The gradient of BMP signals contributes to dorsal-ventral determination. The Wnt signals expressed in the area of dorsal organizer cooperates with other signaling pathway and contribute to organizer formation.

1.1.4 Neural induction

Following organizer formation, as gastrulation proceeds, neural induction is triggered by a combination of signals. First, Wnt/β-catenin signaling induces the expression of organizer genes such as *boz* and *chd*, which directly represses Bmp signaling or encodes a Bmp antagonist respectively. Thus, the dorsal organizer is the initial signaling centre for neural induction. Transplantation experiments also demonstrated that the shield tissue from the zebrafish embryo induced the nervous system at the ventral side of the embryo (Shih and Fraser, 1996). Secondly, Bmp signaling inhibition is required for neural induction. That is accomplished by both the functions of Bmp antagonists, including Chd, Nog and Follistatin, and Fgf signaling. Knockdown of Bmp antagonists showed the reduction of neural plate in zebrafish. In contrast, knockdown of Bmp signals leads to expansion of neural plate. Fgf signaling functions to restrict Bmp signaling prior to gastrulation and continues its function during neural induction. Apart from Bmp signaling inhibition, Fgf signaling induces neural cell fate in a Bmp-inhibition-independent pathway.

On the other hand, Sox3 is another key factor contributing to neural induction. Overexpression of Sox3 induced the expansion of prospective neural ectoderm markers and restricted the non-nerual marker. Thus, the prospective neural cells receiving the combination of induction by Fgf signaling, elimination of Bmp signaling, as well as Sox3 function, adopt the neural cell fate. Afterwards, Sox3 promotes the proliferation of neural progenitor cells and prevents neuronal differentiation. Thus, Sox3, together with other signaling pathways, contributes to cell fate determination in axis formation during zebrafish embryogensis (Figure 1.7).

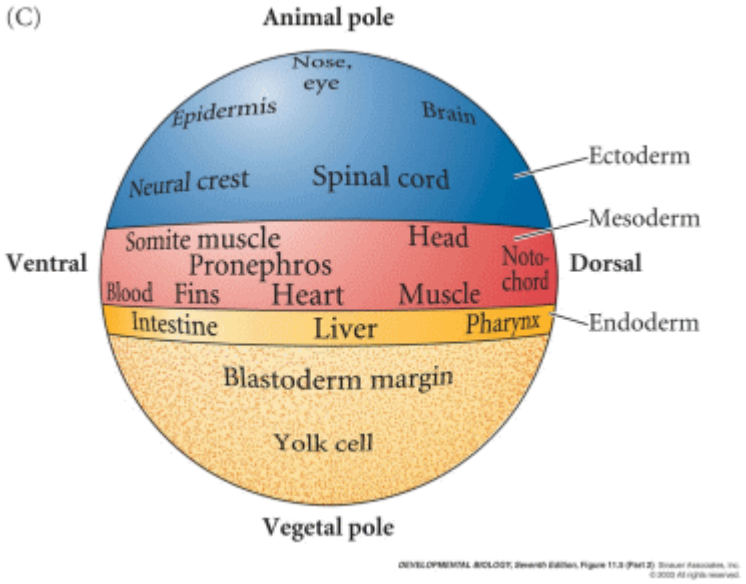


Figure 1.7 Cell fate of the zebrafish embryo at organizer formation and neural induction stage

The dorsal mesoderm area is the organizer. The endoderm (yellow) and mesoderm (red) cells are usually mixed and named as mesendoderm. The ectoderm (blue) consists of prospective neural cells and epidermis cells. The dorsal half of ectoderm gives rise to nervous system and the ventral half of it gives rise to epidermis and neural crest cells. The cells at the animal pole are the prospective dorsal anterior cells.

1.2 The regulatory network in axis formation in zebrafish development

The dorsalanterior and ventralposterior axis in zebrafish embryogenesis is regulated by a complicated signaling network. This includes several major signaling pathways, the Wnt signaling pathway, the Fgf signaling pathway, the Nodal signaling pathway and the Bmp signaling pathway. The dorsal organizer formation is induced by the first three signaling pathways, which are further introduced in this section, whereas the Bmp signaling pathway acts to restrict organizer formation (Figure 1.6). The combination of these signaling pathways precisely regulates the axis formation in zebrafish, giving rise to cell fate determination at organizer and neural induction stage (Figure 1.7).

1.2.1 The Wnt signaling pathway

The name Wnt is derived from the Drosophila gene *wingless* and its human homolog *int1*. The first Wnt signaling pathway identified was the canonical Wnt/β-catenin pathway (Figure 1.3). This is initiated when Wnt proteins bind to the Frizzled-LRP5/6 receptors. The signal is transduced by Disheveled (Dsh), which inactivates GSK3 and prevents β-catenin from degradation. β-catenin then enters the nucleus and, along with transcription factors such as LEF/TCF, activates its target genes (Hatsell et al., 2003, Zeng et al., 2008, Mao et al., 2001). In non-canonical pathways, the planar cell polarity pathway or the Wnt/Ca^{+} pathway, LRP5/6 does not participate. Frizzled receptor is activated and Dsh triggers other signal transduction cascades.

The Wnt/β-catenin pathway is the canonical Wnt pathway. It plays important roles in diverse developmental events such as the regulation of dorsal-ventral patterning in early development. It was first discovered in *Xenopus* that the accumulation of Dsh and other proteins in the prospective dorsal side of the embryos stabilize β-catenin. A

mutant form of Dsh blocked the Wnt signaling in *Xenopus* (Sokol, 1996). In *Xenopus*, the specification of the dorsal side is then activated by the initiating factor Wnt11 working along with Frl1 (Tao et al., 2005). The β-catenin/TCF protein complex induces expression of *twin* and *siamois* in *Xenopus*, or *boz* and *sqt* in zebrafish, and thereby initiates organizer formation. Dorsally expressed Wnt8 in *Xenopus* was also found to repress *bmp4* and induce neural development (Baker et al., 1999). The Wnt/β-catenin pathway also has a pivotal role in anterior-posterior patterning. During late gastrulation, Wnt inhibitors such as secreted Frizzled related protein (sFRP), Cerberus, and Wnt inhibitor factor (WIF) are released and bind to Wnt proteins, subsequently preventing the interaction between Wnt proteins and Fz receptors, resulting in the degradation of β-catenin in the anterior region (Kawano and Kypta, 2003). The gradient of β-catenin levels determines the anterior-posterior axis, as well as the forebrain structure and heart formation (Yamaguchi, 2001). On the other hand, the Wnt/β-catenin pathway has also been shown to mediate self-renewal in stem cells and cancer cells.

1.2.2 The Fgf signaling pathway

The fibroblast growth factor (Fgf) family comprises polypeptides functioning in diverse developmental processes. It is named after its first member being identified as growth factor of fibroblasts. In zebrafish, there are twenty seven members in the Fgf family (Itoh and Konishi, 2007). Six of them are paralog genes acquired from whole genome or local gene duplication during evolution. Most of Fgfs are secreted polypetides, such as Fgf8, Fgf9, Fgf18, and Fgf20, while a few of them are non-secreted, such as Fgf11- Fgf14. Fgfs can act in a paracrine, autocrine or an endocrine manner (Goldfarb, 2005, Revest et al., 2000, Miyakawa et al., 1999). Fgf signaling is

most often initiated by the binding of Fgfs to a particular set of receptor tyrosine kinases (RTKs), the fibroblast growth factor receptors (Fgfrs). The Fgfr is activated by its dimerization followed by auto-phosphorylation (McKeehan et al., 1998). Fgf signaling can be transduced in four pathways (Figure 1.8). The Ras/Mapk pathway, in which the mitogen-activated protein kinase (Mapk) is activated through the Ras G protein cascades, results in Mapk entering the nucleus and activating its target transcription factor by phosphorylation (Kouhara et al., 1997). The phospholipase C gamma (PLC-γ) pathway, in which Raf is activated by Protein kinase C-δ through PLC-γ activation, leads to Ca^{+} release and the Ras-independent Mapk pathway (Mohammadi et al., 1992, Peters et al., 1992). The Pl3K/Akt pathway, in which phosphatidylinositol 3-kinase is activated, directly regulates the target protein Akt (Carballada et al., 2001). The Jak/Stat pathway, in which Stats are phosphorylated by Jak, homo- or hetero-dimer of Stats is formed and target transcription is activated.

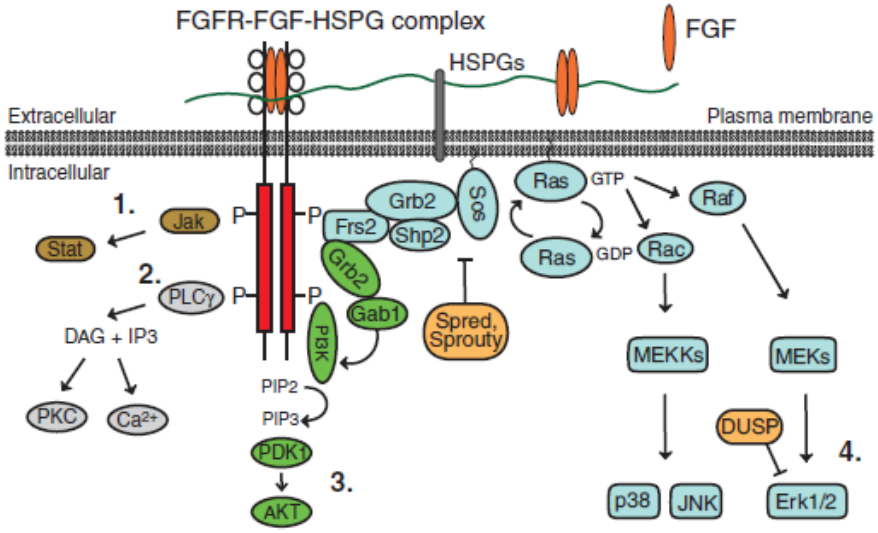


Figure 1.8 The Fgf signaling pathways
The four Fgf signaling pathways are activated downstream of the Fgf receptor. The (1) Jak/Stat pathway (brown), (2) PLC-γ pathway (grey), (3) Pl3K/Akt pathway (green) and (4) Ras/Mapk pathway (blue) are regulated at different levels.

During early development, Fgfs are expressed as early as early blastula stages at the dorsal margin. They have essential roles in dorsal-ventral patterning in zebrafish. Fgfs participates in organizer formation and subsequent developmental events by inducing organizer genes. For example, Fgf8 can expand the dorsal lateral genes by inhibition of Bmp expression. Therefore Fgfs are believed to act together with Bmp antagonists in dorsal-ventral modelling (Furthauer et al., 2004). Together with Nodal signaling, Fgfs regulate organizer and mesoderm formation, by Bmp-inhibition dependent and independent pathways. Fgf2 was the first mesoderm inducer discovered. Fgfs participate also in the induction of mesoderm formation and maintenance of ventral-posterior mesoderm by regulating T-box transcription factors such as Ntl, Tbx6, and Spt (Griffin et al., 1995, Griffin et al., 1998). Fgfs regulate cell movement during gastrulation, in concert with their dynamic expressions over the time. In addition, Fgfs take part in neural induction and anterior-posterior patterning. At later stages, Fgf3 and Fgf8 induce otic and epibranchial placodes development (Sun et al., 2007).

1.2.3 The Nodal signaling pathway

Nodal was first discovered in mouse. Nodal belongs to the transforming growth factor β (TGF-β) superfamily, and activates the canonical TGF-β pathway (Figure 1.4). It is initiated when a Nodal protein binds to an Activin receptor and an EGF-CFC co-receptor, resulting in stimulation of the phosphorylation of Smad2 or Smad3, which then associates with Smad4 (Figure 1.9) (Schier, 2003). This complex interacts with transcription factors, such as FoxH1 and Mixer, and activates the transcription of target genes. On the other hand, Nodal signaling is regulated by the Nodal

antagonists such as Lefty and Cerberus. Nodal signaling can act within a small region as well as at a long distance as a morphogen.

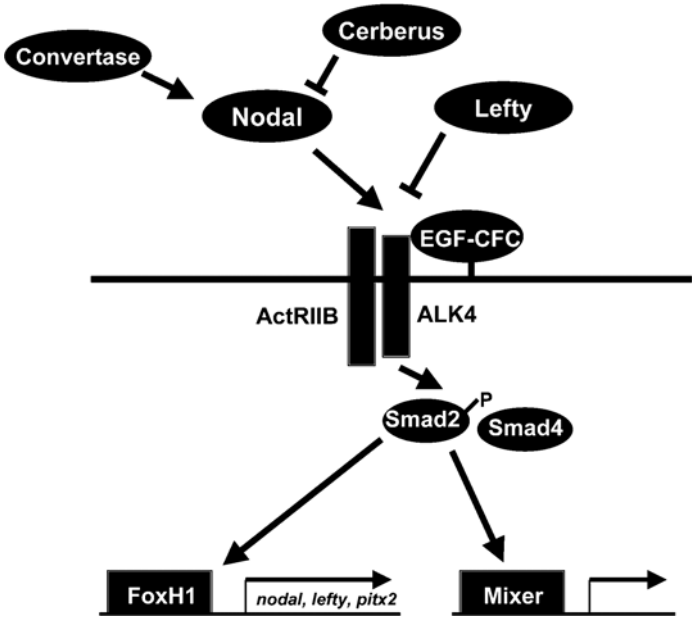


Figure 1.9 The Nodal signaling pathway
Nodal Signaling is activated with Nodal proteins bind to the Activin receptor. It triggers the downstream phosphorylation of Smad2 and its association with Smad4, which eventually activates the transcription factors for target genes. Nodal proteins are competed by the antagonists, Lefty or Cerberus families of proteins. (Schier, 2003)

Nodal signaling functions in organizer formation, mesendoderm induction and left-right axis determination. In the zebrafish organizer, the Nodal-related signal, Sqt, activates organizer genes *gsc* and *chd* (Chen and Schier, 2001). The *squint*;*cyclops* double mutant lacks expression of *gsc* and *flh* during gastrulation (Schier and Talbot, 2001, Dougan et al., 2003). Together with other signals such as Wnt and Fgfs, Nodal contributes to organizer formation. In zebrafish, Nodal-related genes, such as *sqt*, one of the organizer markers, are expressed before gastrulation occurs in the mesoderm and endoderm progenitors. In zebrafish, Nodal signaling is required for the formation of dorsal mesendoderm structures including the notochord, heart, blood, kidney,

pancreas and liver (Schier and Talbot, 2001, Feldman et al., 1998). Ectopic expression of Nodal in the presumptive ectoderm region results in the transformation into mesendoderm cell fates. Loss-of-function of Nodal signaling results in the presumptive notochord and prechordal plate becoming hindbrain and midbrain (Thisse et al., 2000). Nodal signaling also has a critical role in left-right axis determination (Hirokawa et al., 2006, Levin, 2005). Nodal genes are expressed in the left side of lateral plate of mesoderm of chordate. Overexpression of Nodal in the right side leads to misexpression of left-side specific genes. When Nodal signaling is absent or expressed symmetrically, the asymmetry of the body is randomized (Concha et al., 2000). Nodal signaling is tightly regulated during neural patterning. At the stage of neural induction, Nodal expression is excluded from prospective neural cells and restricted by Nodal antagonists. However, Nodal is later required for some cell specification in the ventral neural system.

1.2.4 The SoxB1 family

The Sry-related HMG box (Sox) family is a subfamily of transcription factors belonging to the high mobility group (HMG) superfamily. Members of this superfamily contain an HMG box domain that binds to the potential Sox binding DNA sequence 5'- (A/T) (A/T) CAA (A/T) G -3'and alters the DNA conformation by bending it (Figure 1.10) (van de Wetering and Clevers, 1992, Harley et al., 1994). The defining member, Sry, was first identified in the Y chromosome in mouse as a sex determining gene. There are more than 20 sox genes in mice and humans. They are divided into groups A-H according to their HMG sequence and biological functions. All the Sox proteins share at least 50% identity of amino acid sequence in their HMG sequence with Sry. They are usually highly conserved between

orthologues and members of the same group (Figure 1.11). Sox proteins within the same group often exhibit some functional redundancy (Kamachi and Kondoh, 2013, Lefebvre et al., 2007). Apart from DNA binding and bending, the Sox proteins accomplish their biological functions through interacting with other proteins from various families, including homeodomain proteins, helix-loop-helix leucine zipper proteins, and zinc finger proteins.

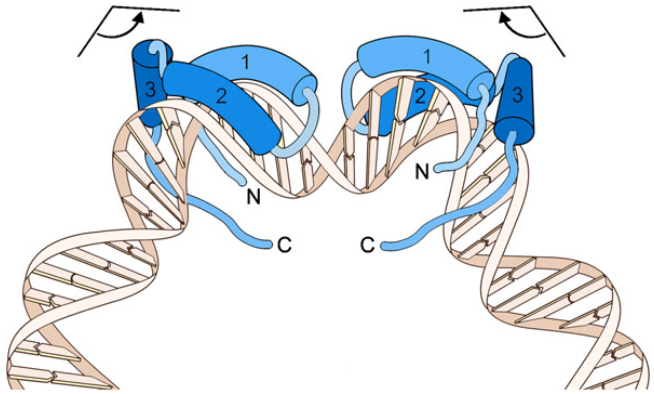


Figure 1.10 The Sox HMG box domain bending the DNA
The Sox HMG box domain (blue) binds to the double helix DNA at minor groove. It bends the DNA at 30° to 110°. The cylinders 1, 2, 3 are the alpha helix structure.

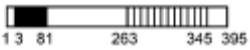
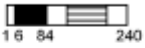
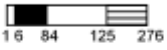
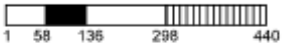
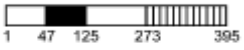
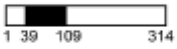
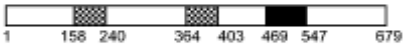
Group	Gene	Locus	Schematic
A	*Sry*	YC3	
B1	*Sox1*	8 A1-A2	
	Sox2	3 A2-B	
	Sox3	X A7.3-B	
B2	*Sox14*	9 E3.3	
	Sox21	14 E4	
C	*Sox4*	13 A3-A5	
	Sox11	12 A3	
	Sox12	2 G3	
D	*Sox5*	6 G3	
	L-Sox5	6 G3	
	Sox6	7 F1	
	Sox13	1 E4	
E	*Sox8*	17 A3	
	Sox9	11 E2	
	Sox10	15 E1	
F	*Sox7*	14 C3	
	Sox17	1 A1	
	Sox18	2 H4	
G	*Sox15*	11 B3	
H	*Sox30*	11 B1.1	

Figure 1.11 The classification and structural organization of Sox proteins
The structure of Sox proteins are shown in the boxes. The HMG box domain is represented by the black internal box. The transactivation domain is represented by vertical stripes. The transrepression domain is represented by horizontal stripes. The dimerization domain is represented by checkers.

The SoxB1 subfamily consists of Sox1, Sox2, Sox3 and, exclusively in zebrafish, Sox19a and Sox19b (Figure 1.12). They share 90% identity in their HMG box. SoxB1 family genes are expressed in early embryonic development. They contribute to complicated signaling networks with other signaling pathways such as Wnt, Bmp and Fgf signaling pathways. They regulate gene expression in a cell-specific manner (Oosterveen et al., 2013, Okuda et al., 2010). For instance, Sox2 cooperates with Oct3/4 and activates stem cell-specific genes such as *fgf4* and *utf1* in embryonic stem cells. Sox2 also forms a heterodimer with Pax6 and activates *δ-crystallin* in lens cells (Kamachi et al., 2001). There are redundant yet distinct functions between SoxB1 proteins. The expression of Sox1, Sox2 and Sox3 overlap in central nervous system (CNS). In mouse and human fibroblast cells, Sox1 or Sox3 can compensate the loss of Sox2 in inducing pluripotent cells (Nakagawa et al., 2008). However, since they have functional redundancy on each other, the malfunction of a SoxB1 gene led to defects in distinct regions in CNS. The knockout of sox1 in mice resulted in a complete loss of γ-crystallin and caused microphlthalmia and cataract, indicating that Sox1 is required for lens development (Nishiguchi et al., 1998). Another study also reported that $Sox1^{-/-}$ mice demonstrated abnormility in ventral forebrain development (Malas et al., 2003).

The SoxB1 family is involved in zebrafish development as early as maternal-zygotic transition stage. The SoxB1 family together with Nanog and Pou5f1 triggers the zygotic genome activation (ZGA) in zebrafish (Lee et al., 2013). Sox19b is one of the most highly translated transcription factors at pre-ZGA stage. The loss of SoxB1 genes resulted in gastrulation arrest and inactivation of zygotic gene transcription (Lee et al., 2013). It has been suggested that the SoxB1 family, especially Sox3, also participates in other signaling pathways and directly acts on organizer genes during

organizer formation (Shih et al., 2010). SoxB1 genes are also highly expressed in neural progenitor cells in the central nervous system. In chick it has been shown that the SoxB1 proteins act as transcriptional activators and they are required for the maintenance of neural progenitor cells. As neuronal specification occurs, SoxB1 genes are downregulated (Bylund et al., 2003). However, at a later stage, SoxB1 proteins have been shown to contribute to the differentiation of oligodendrocytes. In mice, Sox2 and Sox3 are expressed in oligodendrocytes and continue to be expressed during oligodendrocyte differentiation, while they are down-regulated during other neuronal specification, suggesting that Sox2 and Sox3 promote oligodendrocyte differentiation. It has also been shown that Sox2 acts directly on the myelin gene, *mbp*, and activates its expression (Hoffmann et al., 2014).

1.2.5 Sox3 in zebrafish development

In zebrafish, Sox3 is the earliest expressed member in the SoxB1 family. It can be detected from the 32-cell-stage by RT-PCR, indicating that there is maternal *sox3* mRNA playing a role before zygotic gene expression (Kuo, 2012). By 3.5 hpf, *sox3* expression is strongly detected by *in situ* hybridization. At the beginning of gastrulation, the expression of *sox3* covers the whole animal hemisphere. It continues to be expressed throughout embryonic development, but its expression is later restricted to the prospective neural ectoderm during early gastrulation (Kuo, 2012). Later its expression is mainly confined in dorsal anterior which eventually give rise to forebrain, midbrain, hindbrain and spinal cord.

During early stages, Sox3 together with other signaling pathways forms a complicated regulation network controlling a series of developmental events. Sox3 is restricted by

Bmp signaling pathway via the effector Vent. In addition, it is induced by Bmp antagonists. But then, Sox3 represses Bmp signaling to promote neural induction.

On the other hand, Sox3 expression is promoted and maintained by Fgf signaling pathway. However, in organizer formation, Sox3 and Fgf signaling play opposite roles. It has been suggested that Sox3 suppresses the Wnt/β-catenin pathway and directly represses organizer genes and therefore restricts organizer formation, whereas Fgf signaling, particularly Fgf3 and Fgf8, is required for the expression of organizer genes.

Later, Sox3 is a critical regulator for specification of germ layers. It promotes the expression of Nodal signaling repressors, such as Oct, Ectodermin, Coco and Xema. It directly represses Nodal-related genes as well, and therefore restricts mesoderm induction. During late gastrulation, the expression of Sox3 becomes restricted to neural ectoderm in the developing CNS. Sox3 is required for the determination of neural cell fate as well as maintenance of neural progenitor cells (Dee et al., 2008). However, while it has long been believed that Sox3 prevents neuronal differentiation in the CNS, a recent study demonstrated that it plays a part in differentiation of oligodendrocytes, as mentioned above (Kudoh et al., 2004, Shih et al., 2010, Rogers et al., 2008).

In addition, Sox3 takes part in gonadal development and gametogenesis. Although mutation of Sox3 does not affect sex determination in mouse, Sox3 null mice showed defects in gonadal development and oocytes or sperms production (Weiss et al., 2003).

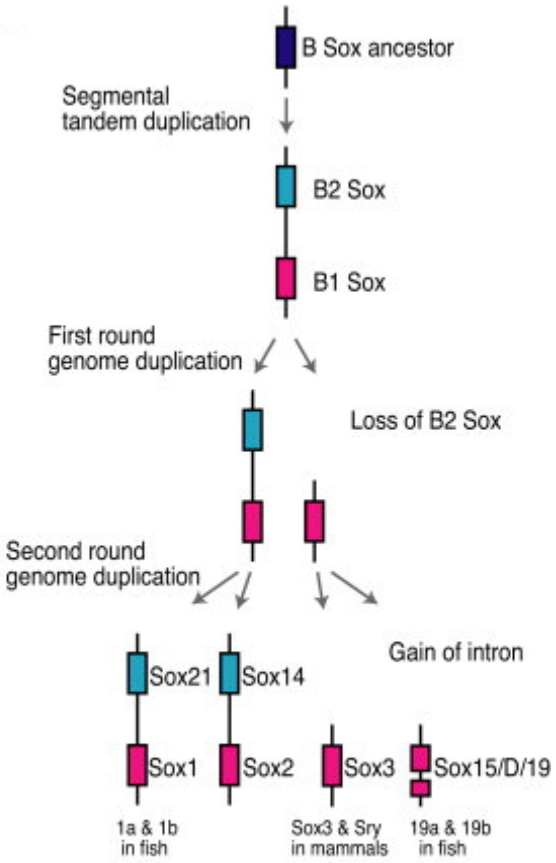


Figure 1.12 The evolution of SoxB family genes
During the evolution process, *soxb1* (pink) segment and *soxb2* (blue) segment are derived from the *soxb* ancestor gene through the process of segmental tandem duplication. After the first round of genome duplication, one of them lost a B2 segment. Afterwards, the chromosomes underwent a second round of genome duplication. The *soxb1* gene on one chromosome diversified into *sox1* and *sox2*, while the *soxb2* gene on the same chromosome diversified into *sox21* and *sox14*. The *soxb1* gene on the other chromosome which was loss of *soxb2* eventually diversified into *sox3, Sry* and other *soxb1* genes after second round of genome duplication. *sox19a* and *sox19b* are derived through the gain of intron process.

1.3 SUMOylation in developmental biology

1.3.1 The identification of SUMOylation, SUMO peptides and SUMOylation consensus

The Small-Ubiquitin-like-modifier (SUMO) was first identified in 1996 in humans (Shen et al., 1996). SUMO modification on protein was first discovered on RanGAP1 to target its re-localization to nuclear pore complex (Mahajan et al., 1997, Matunis et al., 1996). Soon after, the yeast gene *smt3* was also identified as encoding a SUMO peptite (Johnson et al., 1997, Takahashi et al., 1999). Since then, SUMOs have been found conjugated with various substrates and have roles in diverse cellular activities in different organisms (Tanaka et al., 1999, Kamitani et al., 1998, Mahajan et al., 1997, Matunis et al., 1996, Boddy et al., 1996, Kamitani et al., 1997, Johnson et al., 1997). There is only one SUMO gene in invertebrates, whereas there are usually three SUMO genes, SUMO1, SUMO2 and SUMO3 in vertebrates. SUMO2 and SUMO3 share about 95% identity in amino acid sequence in mammals, whereas they have only about 50% similarity with SUMO1. In 2004, a novel SUMO gene SUMO4 was discovered in human and found to be related to Diabetes type I (Bohren et al., 2004). Subsequently, many studies revolved around the association of SUMO4 polymorphism and diseases, including Diabetes Type I and Type II, Behcet's disease and Vogt-Koyanagi-Harada (VKH) syndrome in various populations (Kamoun et al., 2010, Hou et al., 2008, Tang et al., 2012). SUMO4 shares 86% amino acid identity with SUMO2. However, SUMO4 is exclusively discovered in human. In zebrafish, there are only three SUMOs identified.

SUMO conjugation requires target proteins to have a consensus motif ΨKX(D/E), in which Ψ represents a large hydrophobic residue, K is the lysine to be conjugated with SUMO, X is any amino acid, D is Aspartic acid and E is Glutamic acid (Rodriguez et

al., 2001, Sampson et al., 2001). The ΨKX(D/E) motif is recognized and bound by the Ubc9 which catalyses a critical conjugating step of SUMOylation (Sampson et al., 2001). However, subsequent studies have shown that SUMOylation can occur at a lysine residue outside of this motif in some rare cases (Xu et al., 2008). It is noteworthy that SUMO2 and SUMO3, but not SUMO1, have a consensus motif at their N-terminals, which allow the formation of poly-SUMO chains (Tatham and Hay, 2009, Matic et al., 2008).

1.3.2 The SUMOylation cycle

After the SUMO peptides have been synthesized, they are cleaved by a Sentrin/SUMO-specific protease (SENP) to reveal a C-terminal di-glycine residues which can be conjugated with the lysine residue on the target substrate protein. SUMOylation undergoes three steps: activation, conjugation and ligation (Figure 1.13). The C-terminal glycine residue is first activated by an E1 activating enzyme, such as the SAE1/SAE2 heterodimer, to form a thioester bond with the cysteine residue on the E1 enzyme (Hay, 2007). Following this, the flanking sequence of a lysine residue, mostly the consensus motif mentioned above, on the target protein is bound by the E2 conjugating enzyme, Ubc9. Ubc9 then transfers the SUMO peptide from E1 activating enzyme to the cysteine residue on itself, carrying the SUMO peptide to the target SUMOylation site. Finally, the ligation is facilitated by an E3 ligase, such as Siz in *S. cerevisiae* or PIAS in human, allowing the formation of a covalent bond between di-glycine residue on SUMO peptide and the lysine residue on the target protein.

SUMOylation is a reversible event modulated by de-SUMOylating SENP proteins (Yeh, 2009). There are six SENPs identified in human. They have different cellular

localization and substrate specificity. Accordingly, they are divided into three subfamilies. They are responsible for the cleavage of SUMO precursors as well as deconjugation of SUMO peptides from proteins or editing the SUMO chains.

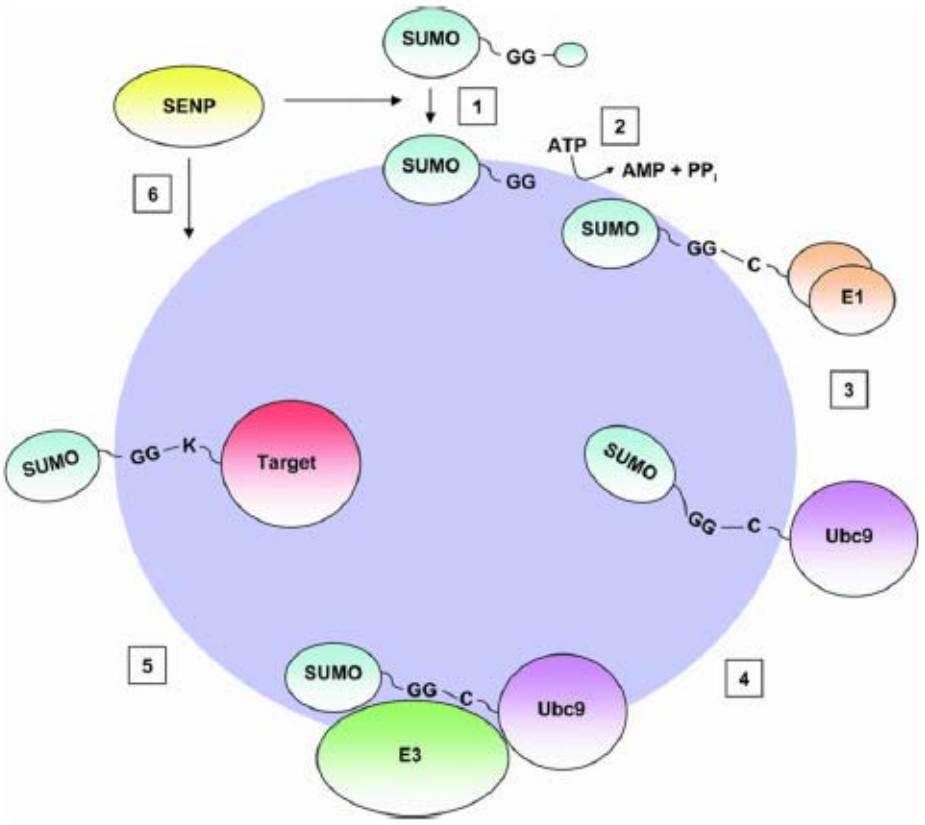


Figure 1.13 The SUMOylation cycle
(1) The C-terminal of SUMO peptide is cleaved by SENP protein to expose a di-glycine motif prior to SUMO conjugation to the target protein. (2) The SUMO peptide is then conjugated to the cysteine residue of an E1 activating enzyme. (3) The activated SUMO peptide is then transferred to an E2 conjugating enzyme, Ubc9. (4) Ubc9 recruits the E3 ligase. (5) Ubc9 catalyses the conjugation of SUMO peptide to a lysine residue on the target substrate. (6) de-SUMOylation can be catalysed by SNEP protein.
(Picture from (Wilkinson and Henley, 2010)

1.3.3 Non-covalent SUMO binding

In addition to covalent SUMO modification, the non-covalent binding of SUMO with SUMO-interacting motifs (SIMs) on other proteins is important in many developmental situations. The SIM is a consensus sequence with some variants with preferences for different SUMO peptides or SUMO chains. They usually contain a hydrophobic core and amino acids with charges, which contributes to different affinities and functionality of the SIM-SUMO associations (Song et al., 2004, Minty et al., 2000). This association is a strategy for recruiting the SUMOylated proteins to the SIM containing proteins to accomplish various events, including DNA replication, DNA repair, transcriptional regulation and protein localization (Ouyang et al., 2009, Pfander et al., 2005, Raffa et al., 2006, Lin et al., 2006). Some proteins having roles in SUMOylation cycle including the E3 ligases and the SUMO-activating enzymes also contain SIMs (Tatham et al., 2005, Truong et al., 2011).

1.3.4 The interplay between SUMOylation and other post-translational modifications

In addition to ubiquitination, SUMOylation also has interplay with other post-translational modifications, such as acetylation and phosphorylation. It is well known that in some cases SUMOylation and acetylation have competing roles. Some proteins can be conjugated by an acetyl group or SUMO peptides at the same lysine residues (Stankovic-Valentin et al., 2007). A SUMO-acetyl switch motif has been described as having a C-terminal proline residue in addition to the standard SUMOylation consensus (Stankovic-Valentin et al., 2007). Moreover, some histone deacetylases (HDACs) can act as an SUMO ligases in SUMOylation (Gregoire and Yang, 2005). As it is known that histone acetylation usually leads to transcriptional activation, the participation of HDACs in the interplay between SUMOylation and

acetylation demonstrated a tight relationship between acetylation and SUMOylation in transcriptional regulation (Cheng et al., 2004, Kirsh et al., 2002, Gregoire and Yang, 2005).

On the other hand, phosphorylation can have roles in inducing or preventing SUMOylation. It was first demonstrated that phosphorylation prevented SUMOylation of some transcription factors and tumor suppressors. It has also been reported that phosphorylation is required for SUMOylation in some proteins (Hietakangas et al., 2003, Yang et al., 2003). The phosphorylation-dependent-SUMOylation motif is described as ΨKXExxSP, the standard SUMOylation consensus followed by any two amino acids, and then a serine and proline (Yang and Gregoire, 2006). As the proline directs phosphorylation on the serine residue, that provides a negative-charged environment which benefits the SUMOylation on the lysine residue. Subsequently, an alternative negative-charged-dependent SUMOylation motif (NDSM) was also reported (Yang et al., 2006, Hietakangas et al., 2006). In that case, the negative-charged environment was provided by the C-terminal flanking acidic residues instead of the phosphorylated serine. Additionally, there is intricate reciprocal regulation between SUMOylation and phosphorylation. SUMOylation can occur on components of the phosphorylation machinery, of which kinases and phosphatases can be modified by SUMO peptides to modulate the dynamic state of phosphorylation (Eun Jeoung et al., 2008, Dadke et al., 2007). Phosphorylation can also affect SUMOylation via phosphorylation of components of the SUMOylation machinery such as E3 ligases (ref). For example, phosphorylation can serve as a switch of E3 ligase preference for ubiquitionation or SUMOylation. Phosphorylation also plays roles in non-covalent SUMO binding to the SIMs on some

proteins (Stehmeier and Muller, 2009). Overall, phosphorylation and SUMOylation can tightly regulate each other in order to accomplish adequate cellular processes.

1.3.5 The functions of SUMO in transcriptional regulation

SUMOylation has various roles in diverse biological events. It can change subcellular localization, protein-protein interaction, protein stability, transcriptional activities and other post-translational regulation. SUMOylation contributes to cell cycle, apoptosis, cell division, proliferation and differentiation by regulating nuclear transport, gene expression, genomic stability and chromosomal integrity.

In terms of transcriptional activity, SUMOylation favours repressing activity in most cases. It usually alters transcriptional activity by sequestration of the target proteins or direct interaction with the transcription complex and the promoter. SUMOylation of transcription factor Sp3 provokes its re-localization to the nuclear speckles and therefore reduces its transcriptional activity (Ross et al., 2002). In some cases, SUMOylation regulates transcription only when there is a transcriptional synergy effect. The transcription factor CCAAT/enhancer-binding protein α (C/EBPα) has a synergy control motif, which is a SUMO modification consensus, and can be modified by SUMO1 or SUMO3. When there is more than one C/EBPα binding on a promoter, a synergy control factor is recruited to the conjugated SUMO proteins and represses their transcriptional activity (Figure 1.14) (Subramanian et al., 2003).

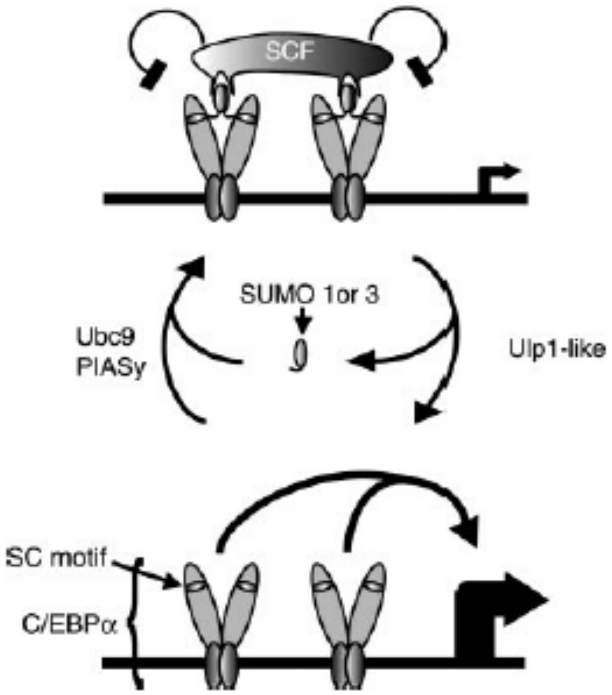


Figure 1.14 The model of SUMO synergy control motif
The binding of SUMO peptides to the SUMO synergy control motif of C/EBPα recruits SCF and thereby inhibits transcription.

However, SUMOylation does contribute to activation of transcriptional regulations in some cases. For example, it can activate a transcriptional activator by changing its DNA binding ability. Modification of heat shock transcription factor 1 (HSF1) and heat shock transcription factor 2 (HSF2) by SUMO1 under stress coverts them to an active DNA binding form and therefore induces their transcriptional activation ability (Goodson et al., 2001, Hong et al., 2001).

In addition, SUMOylation mediates transcription through transcriptional co-factors, such as HDAC1, HDAC4, p300 and SRC-1, and other post-translational modification. For example, in the case of p300, the CRD1 domain modified by SUMO1 in p300 recruits HDAC6 and histone deacetylase and finally results in DNA silencing (Girdwood et al., 2003).

Furthermore, it is important to note that PML and SP100, the components of promyelocytic leukaemia (PML) nuclear bodies (NBs) are both subject to SUMOylation while PML NBs play roles in transcriptional regulation. PML has been shown to be a co-activator or co-repressor while being able to interact with a number of transcription activators or repressors including AP-1, p53, RARα, pRB, Sp1 and Daxx (Zhong et al., 1999, Li et al., 2000, Vallian et al., 1998, Vallian et al., 1997, Alcalay et al., 1998). On the other hand, many transcription factors and co-factors, such as pRB and p53, have been found in PML nuclear bodies. This suggests that PML nuclear bodies could serve as a transient storage place for those factors in order to tune the degree of transcription activities. Moreover, regulators of SUMOylation and acetylation are also accumulated in the nuclear bodies, suggesting that transcription factors could be modulated by SUMOylation in nuclear bodies prior to their actions.

It is noteworthy that some SUMOylation regulators can also modulates transcriptional activity of the target protein in a non-SUMOylation way. For instance, the SUMO E3 ligase PIASy sequesters Lef-1 to the PML nuclear bodies and reduces its transcriptional activity (Sachdev et al., 2001). SUMOylation of Lef-1 is not required for this process (Sachdev et al., 2001). Besides, the conjugating enzyme Ubc9 can interact androgen receptor (AR) and modulates its transcriptional activity independent of SUMOylation (Poukka et al., 1999).

1.3.6 SUMOylation on developmental events

SUMOylation participates in diverse developmental events by different mechanisms. Thus, how the SUMOylation pathway is regulated is an important aspect to investigate. There is only one E2 conjugating enzyme Ubc9 in the SUMOylation

pathway; Ubc9 is the critical point in regulating global SUMOylation. In *Xenopus* egg extracts, SUMOylation of Topoisomerase-II by SUMO2 is crucial in cell mitosis (Azuma et al., 2003). A dominant negative form of Ubc9 blocked the dissociation of sister chromatids at metaphase-anaphase transition (Azuma et al., 2003). In zebrafish, a dominant negative form of Ubc9 led to cell apoptosis while antisense blockage of zygotic ubc9 led to more specific late developmental defects, such as cranial cartilage and brain structures formation (Nowak and Hammerschmidt, 2006).

Apart from Ubc9, which usually controls the global SUMOylation status, E3 ligases are another target for the regulatory mechanism of local SUMOylation status. Many E3 ligases can undergo autoSUMOylation (ref). E3 ligases have different preferences on substrate proteins and SUMO peptides. The best described E3 ligases for SUMOylation are members of the PIAS family, which contains a subset of proteins having non-redundant functions and distinct gene expression patterns in development. The large variety of E3 ligases contribute to the speicificity of SUMOylation in different substrates with different SUMO peptides.

In early zebrafish development, knockdown of all three SUMO peptides caused p53-dependent apoptosis, or brain and eye formation defects, similar to those seen in Ubc9 deficient phenotypes (Yuan et al., 2010). However, knockdown of an individual SUMO peptide can be compatible with normal development (Yuan et al., 2010). This showed that there is functional redundancy between SUMO peptides in zebrafish. Nevertheless, functional redundancy between SUMO peptides does not happen in all cases. Knockdown of SUMO1 at the translational level in *Xenopus* gave rise to abnormal axis formation (Yukita et al., 2007). SUMO1 in *Xenopus* is suggested to participate in Activin/Nodal signaling pathway and contribute to mesoderm induction (Yukita et al., 2007).

In *C. elegans*, knockout of zygotic *smo-1*, encoding the only SUMO protein in *C. elegans*, causes aberrant somatic gonad, germ line and vulval (Broday et al., 2004). LIN-11 was identified as the target of smo-1. SUMOylation of LIN-11 was suggested to promote the transcriptional repression function of LIN-11 and therefore inhibits the genes inducing vulval cell fates. The lack of SUMOylation on LIN-11 caused the loss of uterus-vulva connection in C. elegans. Thus, SUMOylation of LIN-11 is important for the postembryonic reproductive development and gametogenesis (Broday et al., 2004).

SENP proteins control deSUMOylation and consequently modulate the SUMOylation level of specific substrate proteins. Loss-of-function experiments on mouse revealed that SENP1 targets the conjugation of SUMO1 but not SUMO2/3 on a number of substrates and as a consequence causes embryonic development defects. Overexpression of SENP2 is also shown to be lethal in mice. Transgenic mice with cardiac-specific expression of SENP2 caused cardiac dysfunction, suggesting the SUMOylation is required for heart development in mice (Kim et al., 2012).

There are some prominent genes found to be regulated by SUMOylation during development. The p32 isoform of Pax6 can be SUMOylated by SUMO1. The SUMOylated p32 Pax6 is enhanced in DNA-binding activity and transcriptional activation. The target genes of SUMOylated p32 Pax6 promote embryonic brain and eye development (Yan et al., 2010). SUMOylation of Homeodomain-interacting protein kinase 2 (HIPK2) by SUMO1 is a dynamic modulator of its role in c-Jun NH2-terminal kinase (JNK) pathway and p53-independent anti-proliferation (Hofmann et al., 2003, Hofmann et al., 2005).

In zebrafish, dNp63, a member of p53 family is a target of Bmp signaling. It is a transcriptional repressor which suppresses neural development of the ectoderm

(Rentzsch et al., 2004). Overexpression of dNp63 promotes the expansion of non-neural markers and inhibits the neural markers during gastrulation (Rentzsch et al., 2004). It has been demonstrated that apart from ubiquitination, SUMOylation of dNp63 is another mechanism that contributes to the instability of dNp63 at the dorsal side of zebrafish embryo, where ubiquitination and SUMOylation associated genes *nedd4* and *ubc9* are restrictedly expressed (Bakkers et al., 2005). Thus neural development proceeds as dNp63 is destabilized. It has also been shown that SUMOylation of human p63 is essential for limb development (Huang et al., 2004).

The effects of SUMOylation can also be controlled by signaling pathways. The Erk/Mapk pathway has been revealed to control the SUMOylation status of transcription factors or cofactors in different circumstances. For example, the Erk/Mapk pathway regulates SUMOylation on Elk-1 through the PIAS protein, which can act separate to the role of an E3 ligase (Yang and Sharrocks, 2005). The PIAS xα promotes the de-SUMOylation and phosphorylation of Elk-1 and thereby switches on the transcriptionally active form of Elk-1 (Yang and Sharrocks, 2005). Mapk signaling can also up-regulate SUMOylation of PEA3 and switch it to be transcriptionally active (Guo and Sharrocks, 2009, Guo et al., 2011).

Since the sole E2 conjugating enzyme Ubc9 controls the global regulation of SUMOylation, the E3 ligase proteins are usually subject to delicate modulation of SUMOylation dynamics. The E3 ligase Pc2 is phosphorylated and activated by HIPK2 upon DNA damage. Pc2 then promotes the SUMOylation on HIPK2 which then acts as a transcriptional repressor (Roscic et al., 2006). Hence, the substrate of SUMOylation can sometimes establish a autoregulatory loop with the component of the SUMOylation machinery.

1.3.7 SUMOylation on Sox proteins

Sox proteins, like other transcription factors, are usually regulated by a number of post-translational modifications in order to play different roles in diverse developmental processes in different situations. Although the precise mechanisms of how SUMOylation affects the functions of target proteins remain largely unclear, it has been demonstrated that some of them are modified by SUMOylation and that is associated with diverse developmental events. SUMOylation can change their transcriptional activities in different ways, altering subcellular localization, DNA-binding activity, or switching between activator and repressor.

One of the substrates of SUMOylation is Sox6, of which the transcriptional activity is inhibited by altering its sub-nuclear distribution, although SUMOylation is not required for its nuclear localization. A study showed that SUMOylation of Sox6 occured *in vivo* and subsequently induced its recruitment in PML bodies, and it is likely that by this way Sox6 was sequestered from DNA binding (Fernandez-Lloris et al., 2006). It was also suggested that SUMOylation repressed the synergic effect of Sox6 and Sox9 on a chondrocyte-specific enhancer, while interestingly both Sox6 and Sox9 had been found to be regulated by SUMOylation (Hattori et al., 2006). Thus, SUMOylation of Sox6 is suggested to take part in chondrocyte differentiation (Fernandez-Lloris et al., 2006).

SUMOylation also has a determinant role in the SoxE subgroups regulating developmental processes. The SoxE family consists of Sox8, Sox9 and Sox10. Their roles in neural crest differentiation and inner ear development have been comprehensively studied in *Xenopus* and mouse (Honore et al., 2003, Aoki et al., 2003, Spokony et al., 2002, Watanabe et al., 2000, Saint-Germain et al., 2004, Mak et al., 2009). Extensive study of Sox9 and Sox10 demonstrated that SUMOylation

changes transcriptional activity but not DNA binding ability or subcellular localization of Sox9 and Sox10 (Taylor and Labonne, 2005). In this way, constitutively SUMOylated Sox9 promotes expression of the inner ear markers and enlarges the otic placode, whereas non-SUMOylatable Sox9 up-regulated the markers of neural crest cells and its derivatives (Taylor and Labonne, 2005).

The SoxB1 family are substrates of SUMOylation also. Sox2 is well known for its cooperation with Oct3/4, another transcription factor, in transcriptional activity during early embryogenesis. A study of a reporter assay of *fgf4* enhancer revealed that SUMO1 and Ubc9 overexpression inhibited the activation effect driven by Sox2 and Oct3/4 (Tsuruzoe et al., 2006). Moreover, constitutively SUMOylated Sox2 diminished the Oct3/4 mediated transcription activity. It was also demonstrated that the SUMOylated form of Sox2 interfered with its DNA binding ability on the *fgf4* enhancer sequence as well as the formation of the Sox2-Oct3/4-DNA ternary complex (Tsuruzoe et al., 2006).

Another study on the Drosophila gene SoxNeuro (SoxN) presented that SoxN can be SUMOylated by SUMO1 or SUMO2 and that SUMOylation represses the transcriptional activity of SoxN (Savare et al., 2005). The phenotypic experiments showed that SUMOylation of SoxN is crucial for embryogenesis in Drosophila. Disregulated SUMOylation of SoxN caused severe defects in CNS development (Savare et al., 2005). In addition, their results showed that conservative SUMOylation also occured in human Sox3, one of the homologous proteins of SoxN, and that SUMOylation of Sox3 was also shown to favour transcription repression (Savare et al., 2005). Nevertheless, the change of transcriptional activity of SoxN and Sox3 by SUMOylation is not associated with subcellular re-localization (Savare et al., 2005).

1.4 research objectives

Sox3 has been demonstrated to suppress organizer formation by directly repressing the downstream genes of Wnt/b-catenin signaling. Fgf signaling is required for the expression of these organizer genes. In my study, I aimed to investigate how Sox3 fits in the whole signaling network, focusing on the Fgf signaling pathway, and the detailed regulation of the organizer genes *gsc* and *chd*. On the other hand, it has been shown that Sox3 can act as a transcription activator or repressor. It is interesting to investigate how the function of Sox3 is regulated in order to perform various roles during embryogenesis. I propose that post-translational SUMOylation is a strategy by which Sox3 can accomplish different roles in different situation. In this study, I set out to investigate the role of SUMOylation of Sox3 in zebrafish development.

Chapter 2 Materials and Methods

2.1 Molecular cloning and analysis

2.1.1 Preparation of genomic DNA and messenger RNA for zebrafish embryos

Preparation of genomic DNA

Embryos were collected and dechorionated at 24 hpf. They were washed with PBST for 5 min x 3 times. They were then lysed with Lysis buffer (10mM Tris pH8.2, 10mM EDTA, 200mM NaCl, 0.5% SDS and 200 μg/ml Proteinase K) incubating at 50°C for 3 hours with mixing occasionally. Subsequently, 2 extraction volumes of 100% ethanol was added and chilled on ice for 30 min. The samples were then centrifuged at 10000 g for 10 min. The supernatant was removed. 200 μl 70% ethanol was added. After centrifuging for 2 min, ethanol was removed. The genomic DNA pellet was air dried and resuspended in water. Genomic DNA was stored in -20°C.

Preparation of messenger RNA

Embryos were collected and dechorionated at 24 hpf. They were then washed with PBST for 5 min x 3 times. 1ml Tri Reagent (Sigma-Aldrich) was added and embryos were homogenized by passing through a needle (25G BD Microlance ^{tm} 3). The liquid was then centrifuged for 10 min at 10000 g at 4°C. The supernatant was transferred to a new tube. 200μl Chloroform was added and mixed well with the sample. It was then centrifuged for 15 min at 10000 g at 4°C. Supernatant was collected and mixed with 500 μl isopropanol, followed by centrifuging for 10 min at 10000 g at 4°C. The liquid was discarded. The pellet was washed with 70% ethoanol by centrifuging for 5 min at 12000 rpm at 4°C. The RNA pellet was dissolved in DEPC water (Sigma-Aldrich).

2.1.2 Reverse transcription and Polymerase chain reaction

Reverse transcription was carried out with the SuperScript III Reverse Transcriptase kit (Life Technologies). RNA was first treated with DnaseI (New England Biolabs) with 10x DNase buffer in order to get rid of remaining DNA. The Digestion was stopped by incubating in 65°C for 10 min. The total product was proceeded to reverse transcriptase with the kit. Oligo dT primer was used. The reaction was set up according to the kit protocol and incubated at 25°C for 5 min, followed by incubation at 45°C for 60 min. The reaction was terminated by heating at 70°C for 5 min.

PCR reaction was set up with 0.1 – 0.2 μg DNA as template in a 50 μl reaction. Specific primers were added to a final concentration of 0.25 μM. dNTP was added to 0.5 mM. Taq polymerase (New England Biolabs) was added last. The DNA teamplate was denatured at 95°C for 30 sec, following by annealing step at certain temperature according to specific primers for 45 sec. The intended product was elongated at 72°C for certain time according to the length of the product. After running for 35-38 cycles, the final elongation was held for 10 min at 72°C. The product was then held at 4°C.

2.1.3 Agarose gel electrophoresis

50x Tris-Acetate-EDTA (TAE) buffer was made by dissolving 242 g Tris base, 57.1 g glacial acid and 100 ml 0.5 M EDTA into 1 L deionized water. The pH was adjusted to 8.5. To set a TAE gel, agarose powder was dissolved into 1x TAE buffer to make 1% TAE gel for general DNA electrophoresis purpose. Ethidium bromide (Sigma-Aldrich) or Safe Red (ABC Scientific) was added into the gel to make visible of the DNA.

2.1.4 DNA extraction from agarose gel

The DNA sample was run and separated on a TAE gel. The target DNA was identified under the UV light. The gel containing the target DNA was cut out. DNA extraction was applied with QIAquick Gel Extraction Kit (Qiagen). The DNA was finally eluted with DEPC water.

2.1.5 Endonuclease enzyme digestion

Single or double endonuclease enzyme (New England Biolabs) digestion was set up with corresponding buffer in DEPC water. The reaction was incubated at certain temperature for overnight. The product was either cleaned with phenol-chloroform extraction or isolated by agarose gel extraction.

2.1.6 DNA ligation

The ligation of pJET vector and the PCR product was set up according to the CloneJET PCR Cloning Kit (Thermo Scientific). The ligation of other DNA vector and the specific insert fragment were mixed at ratio of 1:3 or 1:5. T4 ligase (New England Biolabs) and the corresponding 10x ligation buffer were used. The ligation reaction was placed on ice water in room termperature for overnight, in order to let the temperature of the reaction gradually increased to room termperature.

2.1.7 Transformation

Competent cells (Bioline) were thawed on ice. The ligation product was mixed gently with competent cells. The mixture stood on ice for 5 min. Heat shock at 42°C was applied for 90 sec. The sample was cooled down on ice for another 10 min. The

competent cells were added with super optimal broth with Catabolite repression (SOC) medium and incubated in 37°C for 1 hour for recovery. It was then spread on a Luria broth (LB) plate containing specific antibiotics.

2.1.8 DNA plasmid preparation and verification

After transformation, a single colony on the plate was selected for inoculation. The colony was inoculated into LB medium which contained specific antibiotics. It was then incubated at 37°C with shaking for 16-20 hours. The following day, the bacterial was harvested by centrifuging the liquid culture. DNA plasmid was extracted by using GenElute^{TM} Plasmid Mini Kit (Sigma-Aldrich) or Qiagen Plasmid Maxi Kit (Qiagen). The concentration of plasmid DNA was measured with Nanodrop (Thermo Scientific). After the DNA purification, the plamid was proceeded with DNA sequencing process. 200ng DNA plasmid as template and 10pmols of primers were used for sequencing PCR using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies). A total volum of 5µl reaction was set up. The sequencing PCR started with hot start at 95°C for 5 min. It was then run for 99 cycles of denaturing at 95°C for 30s, 50°C for 20s and 60°C for 4 min. Sample was then cleaned up with the addition of 95µl H_2O, 10µl 3M sodium acetate, 1µl glycogen and 200µl 100% ethanol, followed by incubation at -20°C for 15 min. It was then precipitated and washed with 100µl 75% ethanol. The DNA pellect was finally collected and sent for sequencing analysis (GeneService)

2.1.9 Site-directed mutagenesis

A pair of complementary primers containing the intended mutation was designed according to the instructions in the QuikChange II Site-Directed Mutagenesis Kit

(Agilent Technologies). Site-directed mutagenesis was performed using the same kit. The PCR reaction was prepared by mixing the primers, plasmid DNA template, dNTP mix, 10x reaction buffer and PfuUltr HF DNA polymerase. The PCR started with 1 cycle at 95°C for 30 sec. Then it went through 95°C for denaturing, 55°C for annealing and 68°C for elongation for total 18 cycles. The PCR amplication product was then chilled on ice for 2 min. It was then subjected to restriction enzyme digestion with DpnI at 37°C for 1 hour to remove the parental plasmid DNA. The digestion product was then ready for transformation.

2.1.10 *in vitro* transcription

Template DNA plasmid was first linearized with specific restriction enzyme digestion. The digestion was then cleaned with phenol-chlorofrom extraction. Phenol-chloroform was added at the same volume as the reaction volume. It was mixed using vortex mixer for 30 sec, followed by centrifuge at 10000 g for 3 min. Then the bottom layer was removed and discarded. This phenol-chloroform extraction procedure was repeated once more. Then 1 volume of chloroform was added, mixed by vortex for 30 sec, followed by centrifuging at 10000 g for 3 min. The bottom layer was removed and discarded. This chloroform extraction procedure was repeated once again. Afterwards, 1/10 volume of 3M sodium acetate and 2 ½ volume of 100% ethanol were added in. The sample containing the DNA was then cooled down to -20°C for 30 min to allow it to precipitate. It was centrifuged at 10000 g for 30 min at 4°C. The liquid was discarded and the pellet was rinsed with pre-cooled 70% ethanol. Ethanol was removed after centrifuging. The DNA pellet was air dried and re-dissolved in DEPC water (Sigma-Aldrich).

In vitro transcription reaction was set up by according to the mMessage mMachine High Yield Capped RNA transcription kit with Sp6, T7 or T3 RNA polymerase (Life Technologies). The linear DNA digested above was used as template. The reaction was incubated in 37°C for 5 hours. Afterwards, 1μl Dnase I was added. The template DNA was digested at 37°C for 20 min. The reaction was stopped by cooling down on ice and adding 15 μl ammonium acetate and 115 μl DEPC water (Sigma-Aldrich). Then, synthesized RNA was extracted by phenol-chloroform extraction once followed by chloroform extraction once. To precipitate the RNA, 1 volume of isopropanol was added. It was then put in -20°C for 30 min or more, followed by centrifuging at 10000 g for 15 min at 4°C. Supernatant was discarded. The pellet was air dried and resuspended in DEPC water. The concentration of RNA was measured with Nanodrop (Thermo Scientific). RNA was stored in -80°C.

2.2 Biological analysis

2.2.1 Maintenance of zebrafish and embryos harvest

Zebrafish were maintained at 28°C in an aquarium. The day before harvesting the embryos, a female fish and a male fish were selected for each pair and transferred to a tank which contained a device separating the female fish from the male fish. The following day, the partition was taken out to allow mating. The embryos were harvested after 15 min and subjected for microinjection. Embryos were incubated in water containing methylene blue at 28°C

2.2.2 Microinjection

Microinjection was carried out with glass needles at 1-4 cell stage. Injection was performed with the Picospritzer III Intracellular Microinjection Dispense System

(Parker). The injecting volume did not exceed 1/3 of the total cell volume of the embryos.

2.2.3 SU5402 treatment

3-[3-(2-Carboxyethyl)-4-methylpyrrol-2-methylidenyl]-2-indolinone (Calbiochem), SU5402 in short, is a FGF signaling inhibitor. It was diluted in DMSO and stored in -20°C. Embryos were incubated at 28°C until pre-MBT stage. They were then treated with SU5402, which was added in the methylene blue containing fish water to give a final concentration of 84μM. The SU5402 treatment was applied on embryos before MBT stage. The same amount of DMSO was applied on the control group for every experiment with SU5402 treatment.

2.2.4 Whole mount *in situ* hybridization

Probe synthesis

Plamid DNA was linearized with an appropriate restriction enzyme. The linear DNA was isolated by gel extraction and used as template for probe synthesis. The probe was synthesized with Sp6, T3 or T7 polymerase (Promega) with corresponding buffer. In a total volume of 20 μl reaction, 2 μl DTT, 0.5 μl RNase inhibitor and 10x Dig or Flu labelled NTP mix were added. The reaction was incubated at 37°C for 5 hours. Afterwards, the synthesized probe was diluted with 25 μl DEPC water. It was then cleaned by running through the Microspin G50 columns (GE Life Sciences). The probe was diluted in tRNA/Heparin containing pre-hybridization (pre-Hybe) buffer (50% formamide, 5x SSC, 0.92% 1M Citric Acid, 0.1% Tween 20, tRNA in final concentration 0.5mg/ml and Heparin in final concentration 0.05mg/ml in H2O) and stored in -80°C.

in situ hybridization

Embryos are collected at desired time point, fixed with 4% paraformaldehyde at 4°C overnight. The next day, embryos are washed with PBST, dehydrated with gradient PBST/methanol (75%/25%, 50%/50%, 25%/75% and 0%/100%) washes, then they were stored at -20°C. On the first day performing *in situ* hybridization, embryos were rehydrated with gradient methanol/PBST (75%/25%, 50%/50%, 25%/75%, 0%/100%), washed thoroughly with extra PBST washes. The embryos were then incubated in pre-Hybe buffer at 67°C for 3 hours, followed by probe hybridization in pre-Hybe buffer at 67^{0}C overnight. The following day, probe-containing pre-Hybe buffer was removed. The embryos were washed at 67°C with gradient Hybe buffers (50% formamide, 5x SSC, 0.92% 1M Citric Acid and 0.1% Tween 20 in H_2O) and 2x SSC (100%/0%, 75%/25%, 50%/50%, 25%/75% and 0%/100%). They are then washed with 0.2x SSC for 15 min x 4 times with shaking in room temperature. Finally, they were washed with gradient 0.2x SSC/MABT (75%/25%, 50%/50%, 25%/75% and 0%/100%) with shaking at room temperate. Embryos were blocked with 2% blocking reagent (Boehringer) in Maleic Acid Buffer (MAB) (0.1M Maleic Acid, 0.15M NaCl, pH adjusted to 7.5 by NaOH) for 2-3 hours. Afterwards, antibodies were applied with the 2% blocking solution at 4^{0}C overnight.

On the third day, antibodies were removed and embryos were washed with MABT (MAB containing 0.1% Tween 20) for 15 min x 8 times. Embryos were then equilibrated with BCL buffer (0.05M Mg2Cl, 0.1M NaCl, 0.1M Tris-HCl, 0.001% Tween 20). The signals were developed with BM purple (Roche) in BCL buffer III (100mM Tris-HCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$, 0.1% Tween 20), or fast red (Sigma-Aldrich). Reactions were stopped by washing with PBST for 5 min x 4 times.

After whole mount *in situ* hybridization was performed, embryos could be re-fixed with 4% paraformaldehyde for 30 min in room temperature, washed with PBST for 10 min x 4 times and kept in 90% glycerol in 4°C for long period of storage.

Double *in situ* hybridization

Double in situ hybridization can be performed following the first ISH signal development. Embryos were re-fixed with 4% paraformaldehyde for 30 min in room temperature. The embryos were then washed with PBST for 10 min x 4 times, followed by incubation in pre-Hybe buffer at 67°C for 3 hours. Another *in situ* hybridization can be performed following the same procedures described above. In double *in situ* hybridization, the first ISH signal was developed with Fast red and the second ISH signal was developed with BM purple or BCIP.

2.2.5 Whole mount immunohistochemistry

Prior to performing immunostaining, embryos were dehydrated with gradient PBST/methanol washes if from -20°C storage, or re-fixed with 4% paraformaldehyde for 30 min at room temperature if after *in situ* hybridization analysis. Embryos were washed with PBST for 15 min x 6 times. Then embryos were blocked with blocking solution (5% Bovine Serum, 1% DMSO and 0.1% Tween 20) for 2 hours at room temperature. Primary antibody diluted in blocking solution was applied. They were incubated with shaking at 4°C overnight. The next day, embryos were washed with PBST for 15 min x 4 times. Subsequently, embryos were incubated with secondary antibody with shaking for 3 hours at room temperature. The signal was detected by DAB substrate (Vector Laboratories). The reaction stopped by washing with PBST for 5 min x 4 times. Embryos were then re-fixed with 4% paraformaldehyde for 1

hour in room temperature. They were washed again with PBST for 10 min x 4 times. Embryos were finally stored in 90% glycerol at 4°C for long.

2.3 Biochemical analysis

2.3.1 Cell fractionation

The cell fractionation protocol was adapted from previous literature (Link et al 2006, *BMC Dev Biol* **6**, 1). Embryos were collected, dechorionated and deyolked in deyolking buffer (55mM NaCl, 1.8mM KCl, 1.25mM $NaHCO_3$) They were then centrifuged at 200g for 5 min. The supernatant was discarded. The pellet was collected and resuspended in buffer A (10mM HEPES, pH7.9, 10mM KCl, 1.5mM $MgCl_2$, 0.34M Sucrose, 10% glycerol, 1mM DDT, 1x Protease Inhibitor Cocktail (company)) with Triton-X added to 0.1% final concentration. Samples were then incubated on ice for 8 min and centrifuged at 1300g for 5 min. The supernatant (S1) was collected from the pellet (P1). The S1 fraction was centrifuged again at 20,000g for 5min to gain the final supernatant (S2), which is the cytosol fraction. P1 was then washed again with buffer A, lysed in buffer B (3mM EDTA, 0.2mM EGTA, 1mM DDT and 1x Protease Inhibitor Cocktail) by incubation on ice for 30 min. Afterwards, it was centrifuged at 1700g for 5 min to separate the supernatant (S3) which is the soluble nuclear fraction from the pellet (P3) which is the chromatin fraction. The P3 chromatin fraction was washed again with buffer B. It was then lysed in 2x SDS loading buffer and stored in -80°C.

2.3.2 Immunoprecipitation (IP)

IP with Sepharose beads

Embryos were collected and deyolked in deyolking buffer, centrifuged at 200g for 5 min (the pellet can be stored in -80°C). Prior to use, samples were allowed to thaw on ice for 10 min. The samples were then lysed in 3:1 Lysis/RIPA buffer (Lysis buffer: 20mM Tris pH 8.0, 137mM NaCl, 10% glycerol, 1% NP-40, 2mM EDTA. RIPA buffer: 50mM Tris pH 8.0, 150mM NaCl, 1% NP-40 or Triton X-100, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulphate), Protease Inhibitor Cocktail (Clontech) was added to 1x final concentration. The samples were then incubated with antibody overnight at 4°C. The next day, Sepharose beads (Sigma-Aldrich) were washed with 3:1 Lysis/RIPA buffer for 3 times, by centrifuging at 5000 g for 30 seconds prior to use. The samples and antibody mixture were then mixed with the Sepharose beads and allowed to bind by shaking at 4°C overnight.

The following day, the sample/bead mix was centrifuged and the unbound fraction was removed. The beads were then washed with PBST containing 1x Protease Inhibitor Cocktail and 0.5% NP-40 for 3 times. 2x Sodium Dodecyl Sulphate (SDS) loading buffer was added to the beads. The samples were then ready for Western blot or could be stored in -80°C.

IP with anti-HA beads or SUMO- Qapture-T^{tm} Kit

The procedure mainly followed IP with Sepharose beads as describe above. Instead of let bind of antibody and sample protein, the sample was directly incubated with anti-HA beads. The IP applied with the SUMO-Qapture- T^{tm} Kit followed the SUMO-Qapture protocol. SUMO-Qapture-T matrix provided from the kit was used instead of Sepharose or anti-HA beads.

2.3.3 Western Blot

Preparation of protein samples

Embryos were collected, dechorionated and transferred to an eppendorf. 200µl deyolking buffer was added following the removal of water. It was then homogenized by pipetting. The pellet was collected and supernatant was discarded after centrifuging at 100 g for 5 min. The protein sample was denatured by addition of 2x SDS sample buffer (company). 1µl SDS sample buffer was used per embryo. Samples could be stored in -80°C. Before use, protein samples were thawed on ice. They were then denatured at 100°C for 10 min and chilled on ice for another 10 min. The protein samples were centrifuged prior to loading in SDS gel.

Sodium Dodecyl Sulfate Polyacrylamid Gel Eletrophoresis (SDS-PAGE)

SDS gels were set up by two steps. The SDS resolving gel (Acrylamine-Bis-Acrylamine 29:1 (Bio-rad), Solution B (1.57M Tris-HCl pH8.8, 0.4% SDS), H_2O, 10% Ammonium persulfate and TEMED (Sigma-Aldrich)) was set at the bottom, followed by addition of 70% ethanol. When the running gel was polymerized and set, the ethanol was discarded. The SDS stacking gel (Acrylamine-Bis-Acrylamine 29:1 (Bio-rad), Solution C (500mM Tris-HCl pH 6.8, 0.4% SDS), H_2O, 10% Ammonium persulfate (Sigma-Aldrich) and TEMED (Sigma-Aldrich)) was set above. The protein samples were loaded into the gel and run in a low voltage (35V - 45V) in the stacking gel and high voltage 80V – 95V in the running gel in SDS-PAGE running buffer (0.303% Tris, 1.88% Glycine and 0.1% SDS). The protein ladder SeeBlue Plus2 Pre-Stained Standard (invitrogen) was used.

Western Blot

After the protein was separated on the SDS gel, it was then transferred to a Nitrocellulose membrane with Transfer buffer (0.303% Tris, 1.44% Glycine and 20%

Methanol) in cool temperature. Then, the membrane was blocked with 5% Marvel Milk (Marvel) in PBS for 1-3 hour, followed by the application of first antibody diluted in 2% milk containing PBS for overnight. Membrane was then washed with PBST (PBS containing 0.1% tween 20) for 5 min x 3 times before applied for secondary antibody for 2 or more hours. Signal was detected with Licor Odyssey system (Licor Biosciences) or Binary Cooperative Ligands (BCL) system (Life Technologies).

Antibody	Dilution	Host
Anti-hSox3	1:5000	Mouse
Anti-HA	1:4000	Rabbit
Anti-Myc	1:1000	Mouse
Anti-SUMO2	1:250	Rabbit
Anti-H3	1:1000	Rabbit
Anti-Actin	1:4000	Rabbit
Anti-Mouse (Licor)	1:4000	Goat
Anti-Rabbit (Licor)	1:4000	Goat
Anti-Mouse-HRP	1:2000	Goat
Anti-Rabbit-HRP	1:2000	Donkey

2.3.4 Luciferase assay

Embryos were collected and dechorionated at specific stage. 5x Passive Lysis buffer (Promega) was diluted with DEPC water (Sigma-Aldrich) to 1x final concentration. 5µl 1x Lysis buffer was used per embryo. After added with 1x lysis buffer, embryos were lysed and mixed well by vortex. The sample could be stored in -80°C at this stage or proceed to following steps. The sample was then centrifuged at 10000 g for 5 min. The supernatant was transferred to a 96-well white plate. The luciferase activity was analyzed with the Dual-Luciferase Reporter Assay System (Promega). The

LARII buffer was prepared by resuspending the Luciferase Assay Substrate into Luciferase Assay Buffer II. The Stop & Glo Reagent was prepared by diluting the Stop & Glo Substrate into Stop & Glo Reagent. The protein lysate samples were subjected to measurement of luciferase activity with LARII and STOP & Glo reagent using the luminometer machine.

2.3.5 Mass spectrometry

Embryos were collected, dechorionated and lysed at certain stage. The interested protein samples were isolated through of IP. The samples after IP application were analysed by western blot for confirmation of isolation of the protein. The samples were then sent out for Mass spectroscopy analysis.

Chapter 3 The regulation of *gsc* and *chd* expression by Sox3

3.1 Introduction

During the early embryogenesis of zebrafish, organizer formation is a notable event patterning the dorsal-ventral axis and the later central nervous system. The formation and functions of the organizer are regulated by a complicated network of signals including Wnt/β-catenin signaling, BMP signaling, Nodal Signaling, and organizer genes such as *boz*, *gsc*, *chd*, *sqt* and *nog* (Shih et al., 2010). According to previous studies, Sox3 has been found to play an important role in inhibiting multiple steps during organizer formation. Among them, it represses organizer gene expression including *boz*, *gsc*, *chd* and *sqt* (Figure 3.1) (Shih et al., 2010).

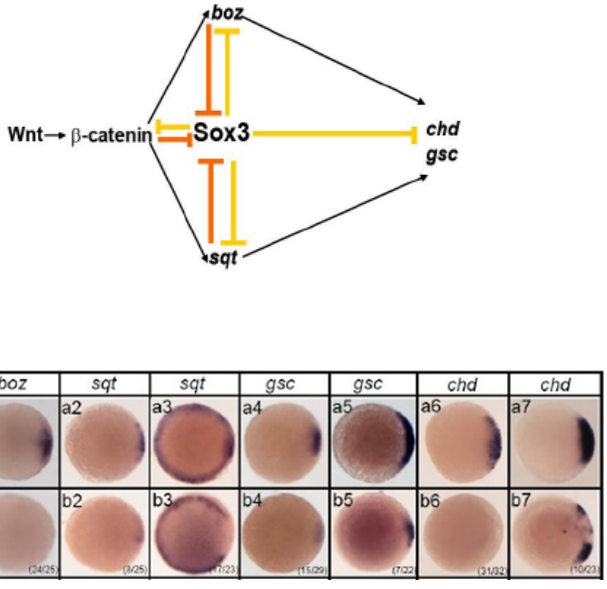


Figure 3.1 Sox3 represses the downstream targets of Wnt/β-catenin Signaling
(A) Model of the function of Sox3 on Wnt/β-catenin signaling and its downstream targets. Orange arrows indicate activation and yellow T-bars indicate inhibition of expression. (B) Embryos were uninjected(a1-a7) or injected with 50pg *sox3* mRNA(b1-b7) at 1-4 cell stage and collected at 4.5 hpf. The expression of *boz*, *sqt*, *gsc* and *chd* were analyzed. It showed that overexpression of Sox3 restricted the expression of organizer genes.
(diagram and data from Shih *et al*., 2010)

Although it has been shown that organizer genes are suppressed by Sox3, the mechanism of how the organizer genes interact with each other and how that fits into the regulatory network still required exploring. *boz* encodes Dharma in zebrafish. It is functionally equivalent to Spemann organizer marker Siamois/Twin in Xenopus. The regulation of *boz* by Sox3 has been studied in details by Kuo (Kuo, 2012). Previous studies showed that Boz cooperated with a Nodal-related factor, Sqt, to induce the expression of other organizer genes, including *gsc* and *chd,* and formation of dorsal mesoderm. Here, I focussed on the regulation of two of the organizer genes, *gsc* and *chd* by Sox3 and other related signals at the stage of organizer formation.

goosecoid (gsc), named after the Drosophila genes *gooseberry* and *bicoid*, is a homeodomain-containing transcription factor first identified in *Xenopus Laevis* as the most abundant organizer molecule (Blumberg et al., 1991). Later on, it was found that *gsc* was responsible for the "organizer phenomena", namely, it gave rise to another body axis when being overexpressed in ventral cells. Since then, studies in *Xenopus* unveiled the diverse functions of *gsc* on organizer formation, gastrulation and later anterior development (Niehrs and De Robertis, 1991, Cho et al., 1991).

On the other hand, soon after *gsc* was identified, many signals participating in organizer formation were discovered. Among these, *chordin (chd),* coding for a secreted protein, was first found to be activated by *gsc* in *Xenopus* and its expression was initiated immediately after the expression of *gsc*. It was also demonstrated that *chd* induced a secondary axis (Sasai et al., 1994). *chd* was later shown to be involved in many signaling models. For example, it was found to be an antagonist of BMP-4 and inhibiting BMP signaling (Piccolo et al., 1996). Together with FGF signaling, *chd* has a role in neural induction also (Sasai et al., 1996). However, the precise relationship between the early expression patterns of *gsc* and *chd* in zebrafish have

not been defined in detail. Therefore, prior to investigating the detailed signaling network among them, I explored the expression of *gsc* and *chd* at early stages of zebrafish development by *in situ* hybridization.

Moreover, another transcription factor Foxd3 has been shown to promote organizer genes (ref). I therefore compared the expression *gsc* or *chd* with *foxd3*. In zebrafish, *foxd3* was first described in 1998 (Odenthal and Nusslein-Volhard, 1998). As a member of the class V sub-family, it was originally suggested to be a transcriptional activator (Schuddekopf et al., 1996). However, later studies suggested that it can also act as a transcriptional repressor. *foxd3* is initially expressed in involuting dorsal-mesodermal cells, neural crest cells and ventral-posterior cells at later stages (Odenthal and Nusslein-Volhard, 1998). Since Foxd3 has also been described to induce organizer formation, I investigated its effects on *sox3* and other SoxB1 genes, *sox19a* and *sox19b*.

After determining the expression of *gsc* and *chd* at early stages, I set out to look into the signaling network in which Sox3 restricts their expressions. Recent study has suggested that Fgf signaling promotes organizer formation (Maegawa et al., 2006), however, it remains unclear how these factors playing opposite roles fit in the signaling network of organizer formation. I therefore examined the intricate mechanism of Sox3 and Fgf signaling in regulating *gsc* and *chd*.

Furthermore, as it has been suggested that Sox3 represses organizer genes directly (Shih et al., 2010), I inspected the putative promoter region of *gsc* in an attempt to establish if Sox3 acts on these upstream sequences.

3.2 Organizer gene expression

3.2.1 The early expression pattern of *goosecoid* and *chordin*

In order to look at the early expression pattern of *gsc* and *chd* in zebrafish embryos, wildtype uninjected embryos were collected and fixed at stages between 3.5 hpf to 70% epiboly. Whole mount *in situ* hybridization was performed. *gsc* and *chd* were both initially detectable by *in situ* hybridization in the dorsal mesoderm as early as at high stage (3.5 hpf) (Figure 3.2 aa', ii'). The *gsc* expression remained weak at 4 hpf and 4.5 hpf stages (Figure 3.2 bb'-cc'). Until 30% epiboly, the *gsc* expression level dramatically increased in addition to its expression pattern expanding more laterally along the animal-vegetal margin. (Figure 3.2 dd') From 50% epiboly onwards, its expression became more intense but the shape of its domain of expression remained similar (Figure 3.2 ee'). At shield stage, the expanded pattern of *gsc* was contracted as it intensely accumulated at the dorsal marginal area (Figure 3.2 gg'). At 70% epiboly, it extends towards the animal pole, forming a stick-shaped pattern (Figure 3.2 hh'). On the other hand, *chd* was expressed weakly at 3.5 hpf (Figure 3.2 ii'). It gradually became more dense and expanded laterally along the margin since initial expression (Figure 3.2 jj'-kk'). At 30% epiboly, its expression level largely increased (Figure 3.2 ll'). Until 50% epiboly, its pattern appeared like a quarter of a ring around the margin of the embryos (Figure 3.2 mm'). Later it continued to accumulate and become denser at germ ring stage (Figure 3.2 nn'). From shield stage to 70% epiboly, the expression of *chd* remained around the margin and extended upwards to the animal pole as well (Figure 3.2 oo'- pp').

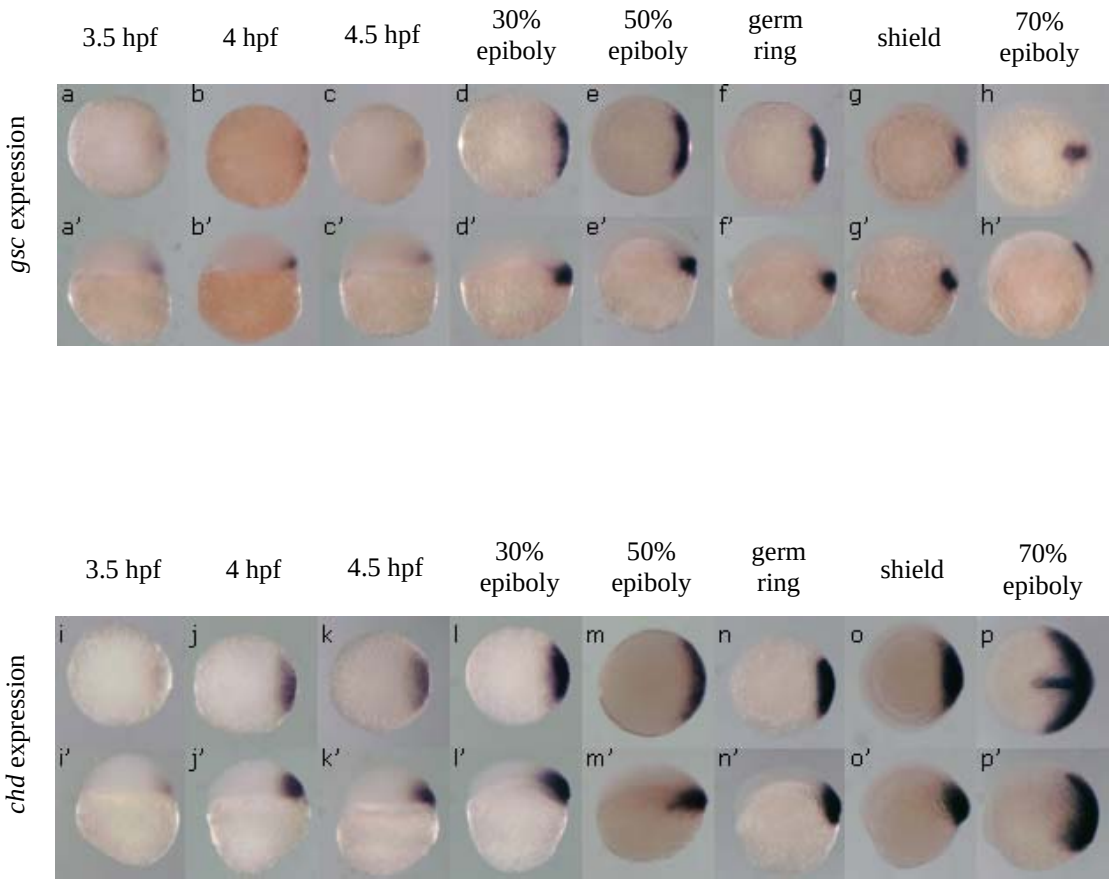


Figure 3.2 Early expression pattern of *gsc* and *chd*
gsc and *chd* start expression at 3.5 hpf and continue their expression throughout the early development. Embryos were collected at different stages. The expression of *gsc* and *chd* were assayed by *in situ* hybridization. (a-p) animal pole view, dorsal to the right. (a'-p') lateral view, dorsal to the right.

3.2.2 The early expression pattern of *foxd3*

Embryos were collected and fixed at high, sphere, 30% epiboly and shield stages. Whole mount *in situ* hybridization was performed with a *foxd3* probe. *foxd3* was undetectable at high stage (Figure 3.3 a). It was initially detected at sphere stage in the dorsal side (Figure 3.3 b-d). Its expression at this stage was relatively weak. However, at later stages it gradually became more intense and its expression pattern extended laterally, in the similar way as *gsc* and *chd*. *foxd3* slightly extended laterally and its edges of its domain of expression became more defined at 30% epiboly (Figure 3.3 e-g). At shield stage, *foxd3* further lengthened to about half way around the embryo (Figure 3.3 h-j). Interestingly, its pattern appeared as an arch-shaped at the dorsal-most area at this stage (Figure 3.3 j). This left a small region at the dorsal/vegetal region devoid of *foxd3* expression. That also raises the question as to whether the region devoid of *foxd3* expression is where organizer genes are expressed.

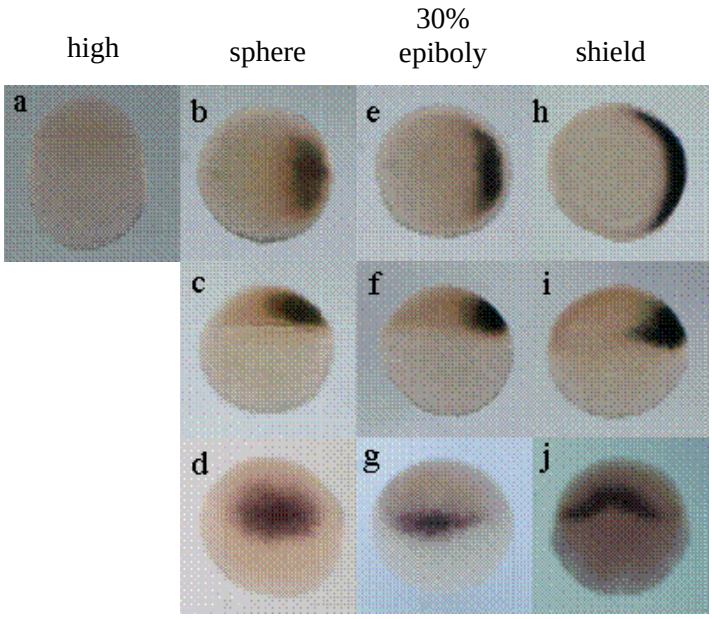


Figure 3.3 Early expression pattern of *foxd3*
Embryos were collected at high, sphere, 30% epiboly and shield stage. *In situ* hybridization was performed with *foxd3* probe. (a, b, e, h) animal pole view, dorsal to the right. (c, f, i) lateral view, dorsal to the right. (d, g, j) dorsal lateral view.

3.2.3 Double *in situ* hybridization of *gsc*/*chd* and *foxd3*

To unveil the detailed relative areas expressing *gsc*, *chd* and *foxd3* during organizer formation, double *in situ* hybridization of *gsc*/*chd* and *foxd3* was performed on uninjected embryos at dome, 50% epiboly and 70% epibolgy stages. As mentioned above, *foxd3*, *gsc* and *chd* were already expressed by the dome stage. At this stage, *foxd3* was expressed just above *gsc* and *chd* at the dorsal mesoderm region (Figure 3.4 a-a'', d-d''). At 50% epiboly, as their expression pattern gradually beame more intense and extended laterally, *foxd3* appeared to expand downwards and became overlapped with *gsc* (Figure 3.4 b-b'). *chd* was expressed below *foxd3* and partially overlapped with *foxd3* at this stage (Figure 3.4 e-e'). At 70% epiboly stage, as the *foxd3* pattern appeared as the arch-shaped at the shield area as shown in Figure 3.3 j, *chd* mostly overlapped with *foxd3* at the shield region as well as at the lateral margin (Figure 3.4 f-f''). However, as *gsc* extended towards the animal pole and formed the stick-shaped pattern as shown in Figure 3.2 hh', it overlapped with *foxd3* only at the shield region (Figure 3.4 c-c''). Interestingly, *chd* but not *gsc* was expressed at the area below the arch-shaped *foxd3* pattern, as *gsc* elongated as a line and migrated towards animal pole.

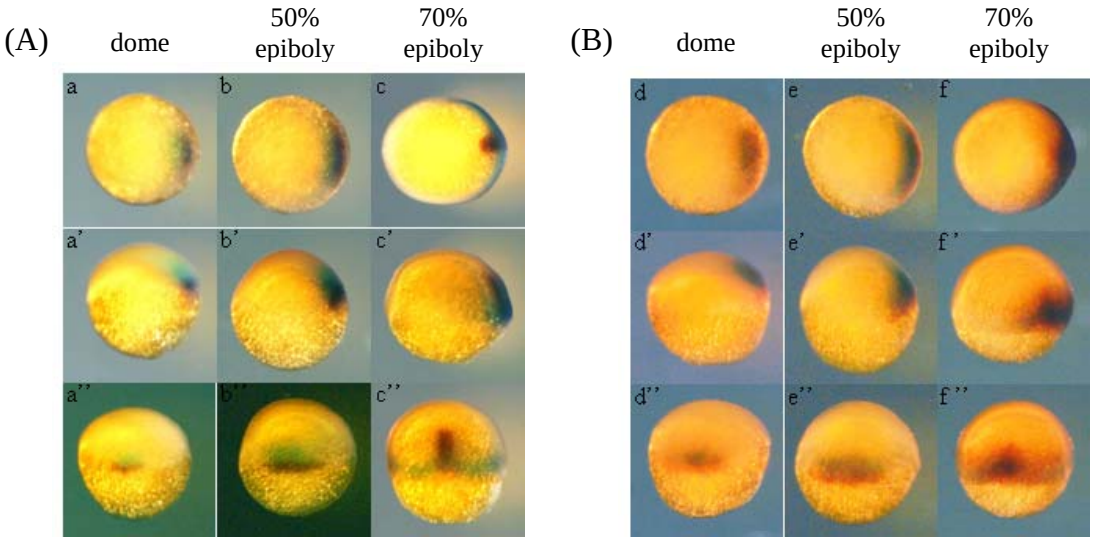


Figure 3.4 The expression of *gsc* and *chd* partially overlapped with *foxd3*
(A) Double *in situ* hybridization of *gsc* and *foxd3* was applied in three different stages, dome (a-a''), 50% epiboly (b-b'') and 70% epiboly (c-c''). *gsc* signal was developed with fast red and appeared in red. *foxd3* signal was developed with BCIP and appeared in blue.
(B) Double *in situ* hybridization of *chd* and *foxd3* was applied in three different stages, dome (d-d''), 50% epiboly (e-e'') and 70% epiboly (f-f''). *chd* signal was developed with fast red and appeared in red. *foxd3* signal was developed with BCIP and appeared in blue.

3.3 The interaction between *foxd3* and SoxB1 genes

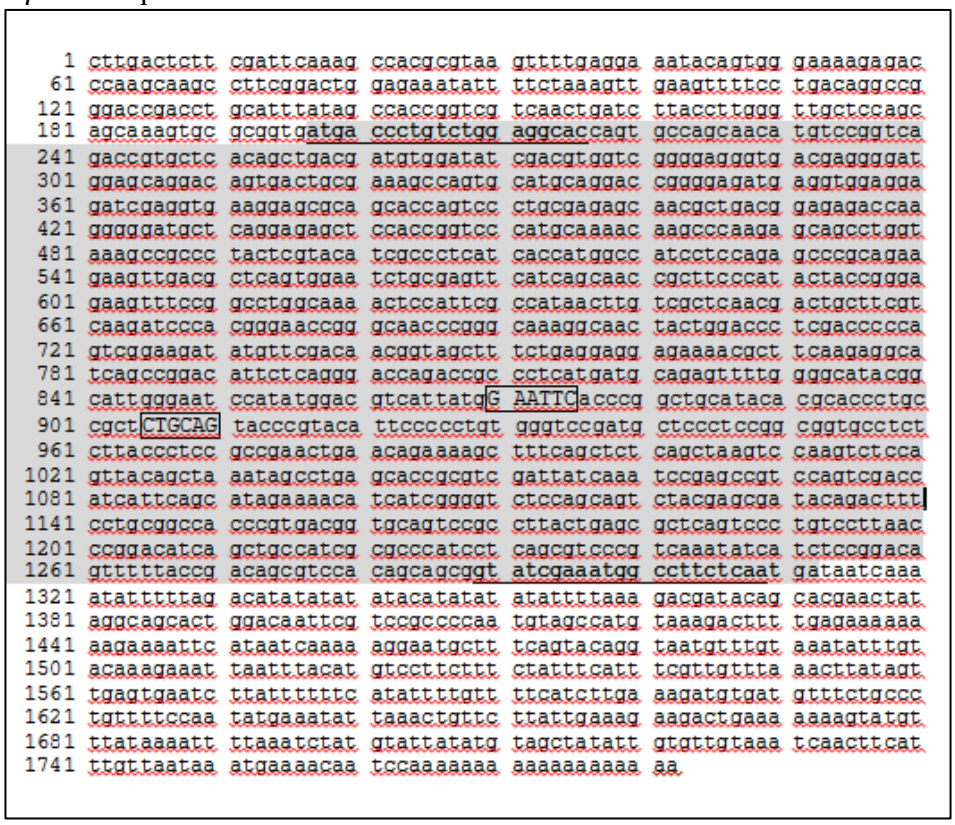
Results in previous sections showed that *foxd3, gsc* and *chd* were expressed in the area of dorsal mesoderm during organizer formation. Previous studies have also demonstrated that Foxd3 induced organizer genes, while Sox3 repressed them. The fact that gsc and chd are both regulated by Foxd3 and Sox3 raised the question of whether Foxd3 promoted organizer and mesoderm genes by repressing SoxB1 proteins. In this section, I inspected the relationship between Foxd3 and Sox3, as well as the effect of Foxd3 on other *soxb1* gene expression.

3.3.1 The generation of pβut2-*foxd3*-HA construct

Gain-of-function experiments are a good strategy to further study the function of *foxd3*, therefore I generated a construct for synthesis of *foxd3* mRNA. The pβut2 vector was used because it contains the 5' and 3' untranslated region of Hemoglobin, which can stabilize the synthesized mRNA. However, the only restriction sites for linearizing this vector for *in vitro* mRNA synthesis, *PstI* and *EcoRI*, are both present in the *foxd3* sequence (Figure 3.5 A). I therefore modified this pβut2 vector by inserting a multiple cloning site which contains three extra restriction sites, *SbfI*, *SpeI* and *NotI* between the *PstI* and *EcoRI* sites of the original vector. *SbfI*, *SpeI* or *NotI* is not present in the *foxd3* sequence and therefore can be used to linearize the vector for *in vitro* mRNA synthesis. The strategy was to design two complementary oligos containing those three restriction sites sequences overhangs complementary to *PstI* or *EcoRI* sites at 5'ends, which allows ligation with the linearized pβut vector. The oligos were annealed to form a complimentary polylinker before ligating with the linearized original pBut vector (Figure 3.5 B).

(A)

foxd3 sequence



Forward primer: **TCTAGA**ATGACCCTGTCTGGAGGCAC
Reverse primer: **CTCGAG**ATTGAGAAGGCCATTTCGATAC

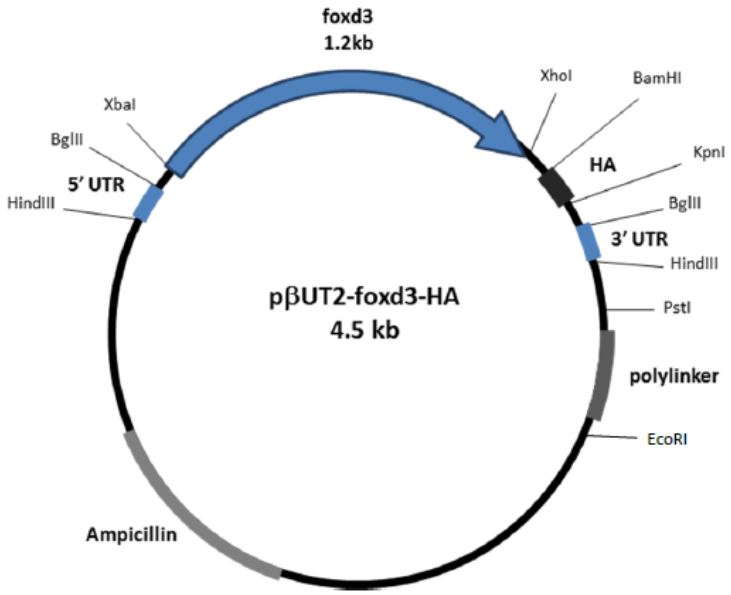
Figure 3.5 A Generation of pBut-*foxd3*-HA construct
(A) Sequence of full-length foxd3 transcript. The coding sequence is in grey shade. The sequence underlined is complementary with the primers. The restriction sites *PstI* and *EcoRI* are in squares. The sequence in bold in two primers are *XbaI* and *XhoI* sites.

Meanwhile, wildtype uninjected embryos were collected at shield stage, when *foxd3* is comparatively highly expressed. The embryos were lysed and mRNA was extracted. Reverse transcription was performed to gain the cDNA which was used as template in PCR amplifying the *foxd3* gene. Primers were designed to have restriction sites *XbaI* or *XhoI*, which are the subcloning sites on pbut2 vector, at the 5'end. The PCR product was ligated to the linear pJET vector, with which the competent cells transformed. At the next day, a colony was selected for inoculation in liquid medium. After 18 hrs incubation, the plasmid DNA was extracted and verified by DNA sequencing. The *foxd3* fragment in pJET vector was then subcloned into pβut2 by double digestion with *XbaI* and *XhoI* followed by ligation. Competent cells were transformed with the ligation product. A colony was selected for inoculation followed by plasmid DNA extraction. The clone with pβut2-*foxd3*-HA was again verified by DNA sequencing (Figure 3.5 B).

3.3.2 Reciprocal repression between *sox3* and *foxd3*

As the results showed in Figure 3.4, *foxd3* is expressed at the area around where *gsc* and *chd* are expressed. Foxd3 was also previously suggested to activate organizer and mesoderm genes such as *gsc*, *chd* and *ntl* (Chang and Kessler, 2010). That gives rise to the question of whether Foxd3 activates organizer and mesoderm genes by way of repressing *sox3*. Therefore, I looked into whether Foxd3 overexpression resulted in inhibition of *sox3*. Embryos were injected with 50pg, 100pg or 200pg of *foxd3-HA* mRNA at 1-2 cell stage and collected at sphere stage. Expression of *sox3* was examined by *in situ* hybridization and was compared to uninjected embryos or embryos injected with 50pg *gfp* mRNA as controls.

(B)



Polylinker:

	SbfI		SpeI		NotI	
AATTCATC	CCTGCAGG	ATG	ACTAGT	AAC	GCGGCCGC	CGCA
GTAG	GGACGTCC	TAC	TGATCA	TTG	CGCCGGCG	GCGTTTAA

Figure 3.5 B Generation of pBut-*foxd3*-HA construct
(B) The diagram of the modified pBut-*foxd3*-HA contruct. The sequences of two oligos containing *SbfI*, *SpeI* and *NotI* are stated.

The results showed that Foxd3 repressed *sox3* in a dose dependent manner (Figure 3.6 A). As *foxd3* is regarded as a mesoderm gene and the fact that Sox3 represses organizer as well as mesoderm genes, I also inspected the effect of Sox3 on *foxd3* expression. Embryos were injected with *sox3-HA* mRNA at 1-2 cell stage and the expression of *foxd3* was examined. This showed that overexpression of Sox3 resulted in substantial suppression of *foxd3* expression (Figure 3.6 B). Interestingly, overexpression of Sox3 in a relative small amount led to notable suppression of *foxd3*, while Foxd3 repressed *sox3* to a high degree only when it was overexpressed at quite high levels.

To confirm the protein expression of exogenous mRNA in injected embryos, Western blot analysis was performed. Uninjected, *sox3-HA* injected or *foxd3-HA* injected embryos were deyolked for protein extraction, followed by SDS-PAGE and Western blot analysis. Anti-HA was applied to detect the protein expression of Sox3-HA or Foxd3-HA, while anti-Actin was applied to detect Actin expression as control.

As shown in Figure 3.6 C, actin was detected at about 47 kDa in both injected and uninjected samples. Anti-HA detected the Sox3-HA at about 50 kDa in injected samples (Figure 3.6 C). Compared to this, the weaker band appeared in uninjected sample was likely to be the non-specific signal. Foxd3-HA was also detected by anti-HA at about 42 kDa in injected sample but not in uninjected ones (Figure 3.6 D). This result confirmed that exogeneous Sox3-HA and Foxd3-HA were successfully expressed in the embryos.

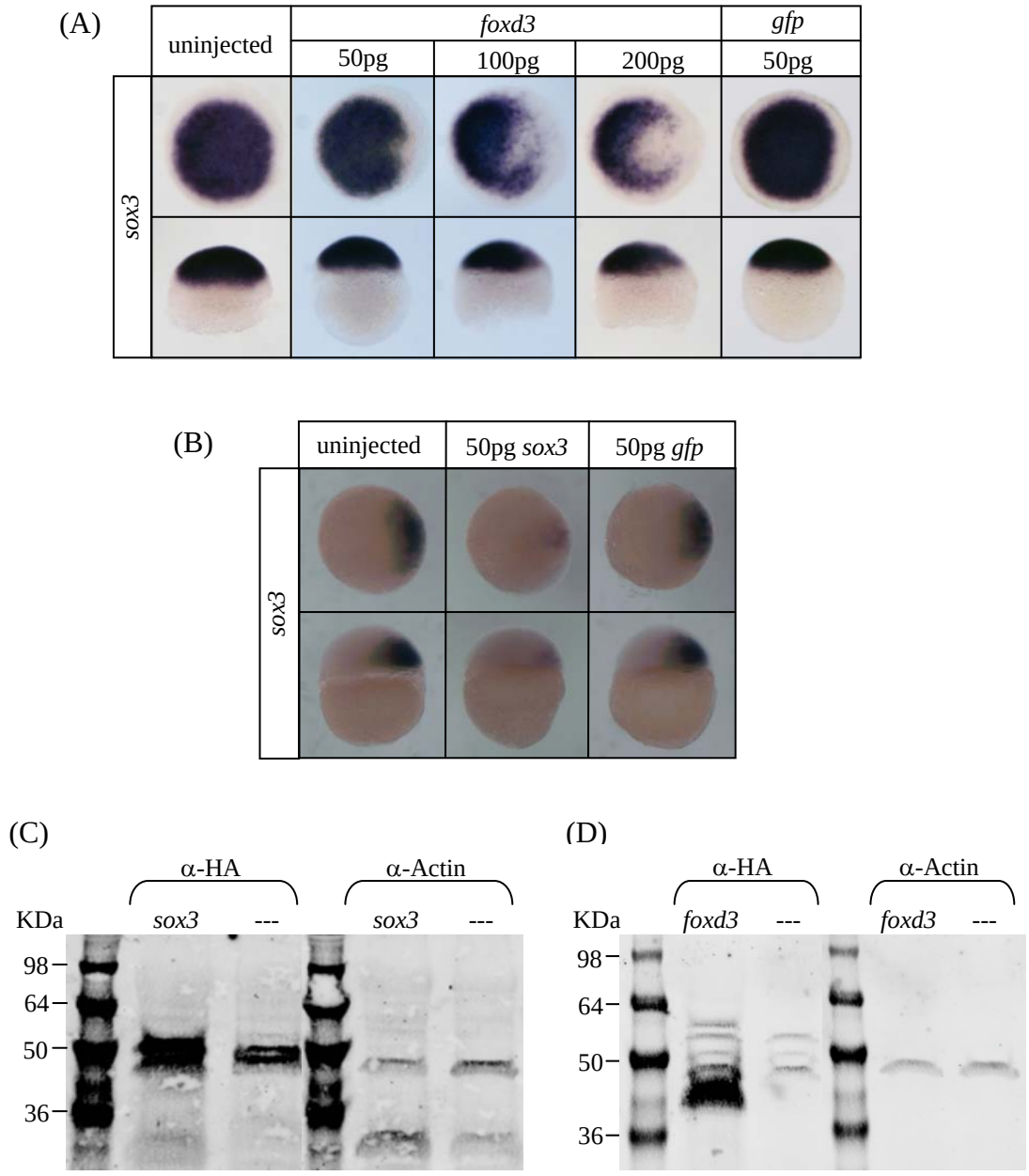


Figure 3.6 Sox3 and Foxd3 represses each other
(A) Uninjected embryos or embryos injected with various amounts of *foxd3* mRNA or *gfp* mRNA were collected at sphere stage. The expression of *sox3* was examined by *in situ* hybridization. (B) Uninjected embryos and embryos injected with 50pg of *sox3* or *gfp* mRNA were collected at sphere stage. The expression of *foxd3* was examined by *in situ* hybridization. (C, D) Uninjected embryos and embryos injected with *foxd3-HA* or *sox3-HA* were lysed at sphere stage. Protein was extracted and analysed by western blot. Expression of Foxd3-HA or Sox3-HA was detected by anti-HA and Actin was detected by anti-Actin as controls.

3.3.3 *foxd3* induces *sox19a* and *sox19b*

It has been suggested that there is functional redundancy between the SoxB1 family genes. Two of the SoxB1 genes, *sox19a* and *sox19b* have been found to have similar activities in repressing organizer formation (Kuo, 2012). Hence, in addition to *sox3*, I explored whether Foxd3 affected *sox19a* and *sox19b* expression. *foxd3* was overexpressed by injection of 50pg of mRNA at 1-2 cell stage. The expression of *sox19a* and *sox19b* were examined at sphere, 50% epiboly, and 85% epiboly stages. The results were compared to uninjected and 50pg *gfp* injected controls (Figure 3.7). As early as sphere stage, both *sox19a* and *sox19b* are expressed throughout the whole animal pole in uninjected embryos (Figure 3.7 a, j).

As shown in Figure 3.7 a, b, the expression of *sox19a* was enhanced by overexpression of *foxd3* at sphere stage. At 50% epiboly, *sox19a* expression was all over the animal pole in uninjected embryos (Figure 3.7 d). *foxd3* caused more intense expression of *sox19a* as at earlier stage. At 85% epiboly, when *sox19a* is almost restricted to presumptive neuroectoderm, *foxd3* overexpression caused expansion of *sox19a* expression (Figure 3.7 g, h). *foxd3* has similar influences on *sox19b* as on *sox19a*. The expression of *sox19b* became prominently intense when *foxd3* was overexpressed at sphere stage (Figure 3.7 j, k). Even though the *sox19b* expression in uninjected embryos decreases at 50% epiboly stage, *foxd3* overexpression up-regulated *sox19b* at this stage (Figure 3.7 m, n). At 85% epiboly, the expression level of *sox19b* in uninjected embryos becomes largely reduced and is restricted at the very dorsal side within the presumptive neuroectoderm (Figure 3.7 p). However, overexpression of *foxd3* caused the expansion of *sox19b* expression (Figure 3.7 q).

It was originally expected to demonstrate the repression of *sox19a* and *sox19b* by Foxd3. However, it turned out that while Foxd3 induces *sox19a* and *sox19b*

expressions while it repressed *sox3* expression. Previous studies also found that Foxd3 promoted organizer genes whereas Sox19a and Sox19b repressed them (Chang and Kessler, 2010, Kuo, 2012). Taken together, the results on Foxd3 are not consistent with the model of the regulation on organizer formation by SoxB1 family, and therefore it is not very meaningful to carry out further studies.

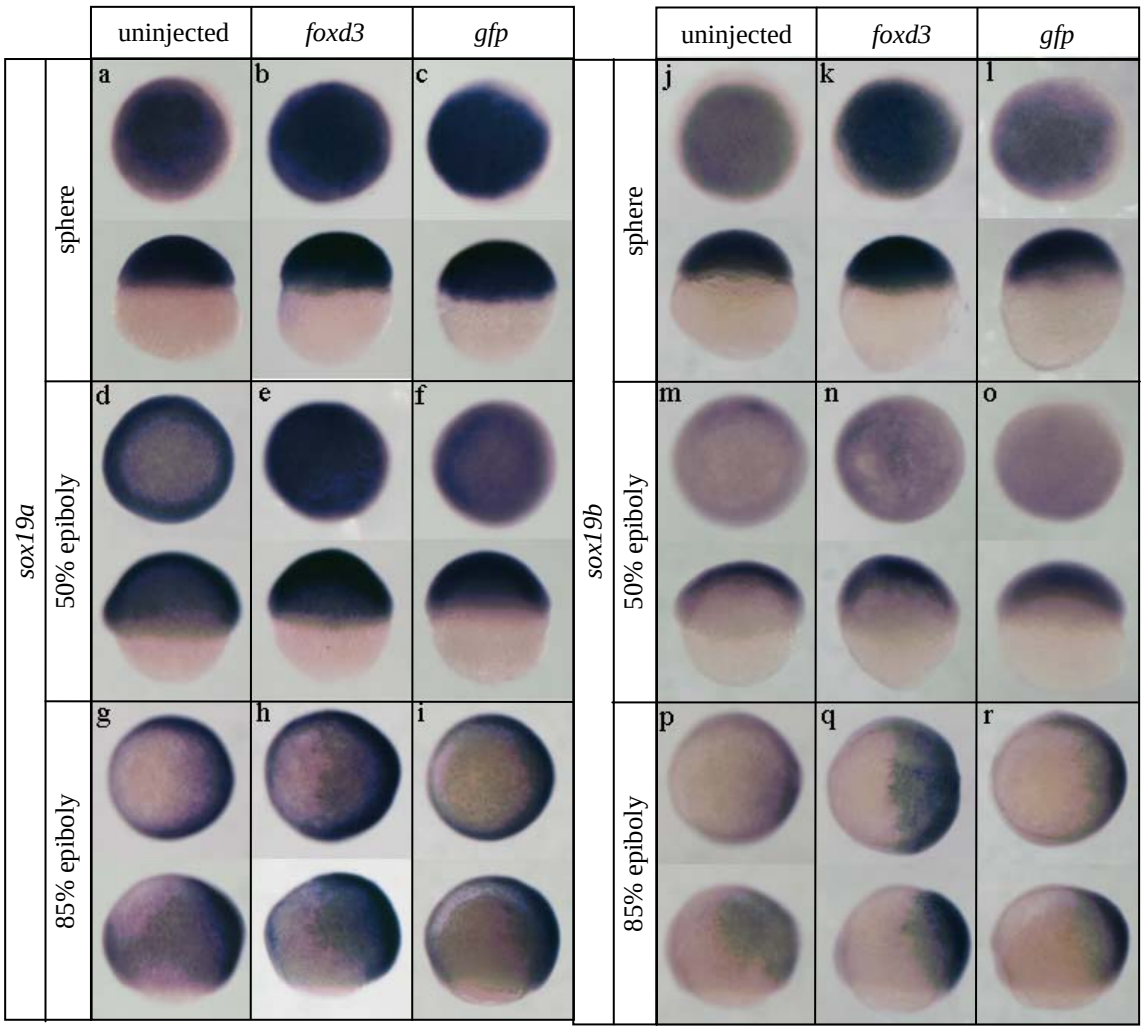


Figure 3.7 Foxd3 up-regulates *sox19a* and *sox19b*
Uninjected embryos, embryos injected with 50pg *foxd3* mRNA or 50pg *gfp* mRNA at 1-2 cell stage were collected at sphere, 50% or 85% epiboly stage. *In situ* hybridization was performed to analyse the expression of *sox19a* and *sox19b*

3.4 The regulation of *gsc* and *chd* by Sox3 and Fgf signaling

The regulation of organizer genes is controlled by an intricate signaling network in which each involving factor may have effects one another. As mentioned earlier, the organizer genes, *gsc* and *chd*, are restricted by Sox3 and promoted by Fgf signaling (Maegawa et al., 2006, Shih et al., 2010). On the other hand, Gsc can activate the expression of *chd* (Sasai et al., 1994). Sox3 has also been found to repress Fgf signaling (Kuo, 2012). It is interesting to investiage whether Sox3 or Fgf signaling regulates the expression of *chd* via *gsc*. It is also worth investiaging whether Sox3 restricts the expression of *gsc* and *chd* via repressing Fgf signaling. The strategy to look into these problems was to test whether overexpression of *gsc* could rescue the effects casued by *sox3* overexpression or Fgf signaling blockade on *chd* expression (Figure 3.8). Moreover, it was also tested whether Sox3 was able to inhibit the expansion of *chd* expression caused by Fgf signaling (Figure 3.8).

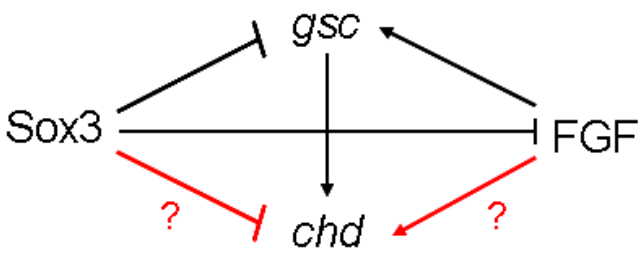


Figure 3.8 The signaling network of Sox3, Fgf and organizer genes
Model of signaling network of between Sox3, Fgf and the organizer genes, gsc and chd. Arrows indicate activation and T-bar indicate repression. The relationship indicated in red is to be investigated in following section.

3.4.1 The generation of pβut2-*gsc*-HA

The overexpression of Sox3 or blockage of Fgf signaling would be accomplished by using the pβut2-*sox3*-HA construct or SU5402. The pβut2-*sox3*-HA construct has been generated in our lab. However, in order to overexpress *gsc* in embryos, a gsc expressing contruct was needed for in vitro synthesis of *gsc* mRNA. Wildtype uninjected embryos were collected at 24 hpf. The embryos were lysed and mRNA was extracted. Reverse transcription was performed to gain the cDNA which was used as template in PCR amplifying the *gsc* gene. Primers were designed to have restriction sites *XbaI* and *XhoI* at the 5'end. The PCR product was ligated to the linear pJET vector. The plasmid DNA was verified by DNA sequencing. The *gsc* fragment in pJET vector was then subcloned into pβut2 vector by double digestion with *XbaI* and *XhoI* followed by ligation. The clone with pβut2-*foxd3*-HA was again verified by DNA sequencing (Figure 3.9).

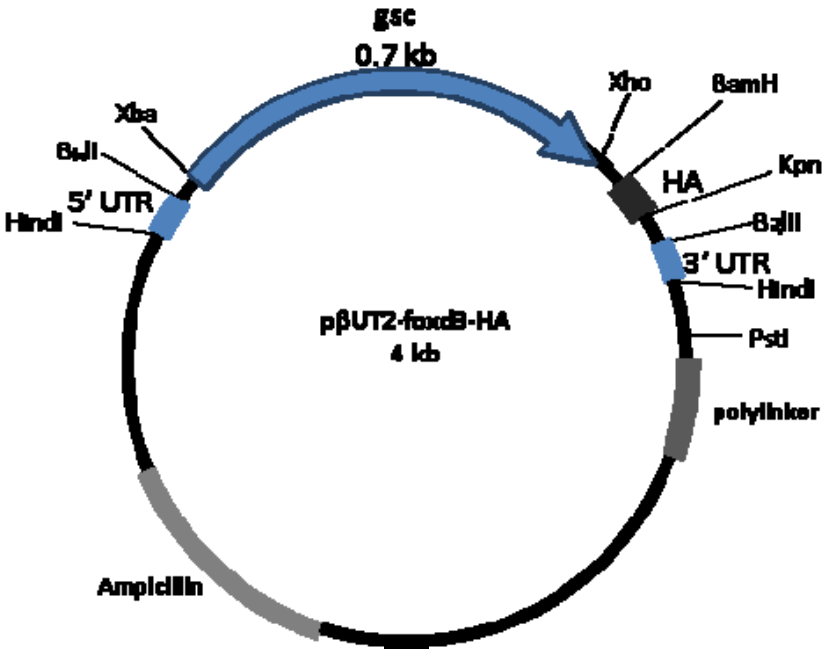
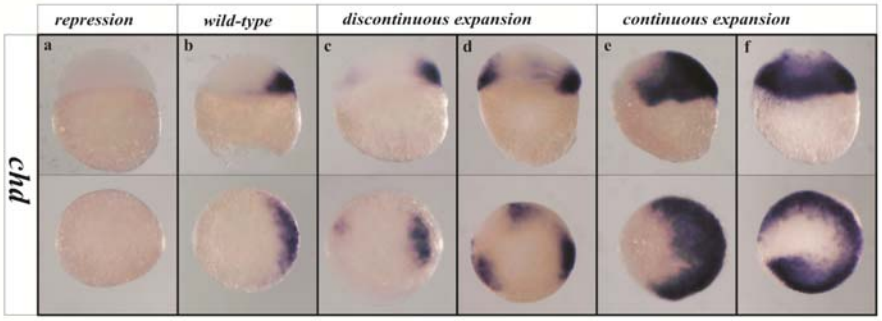


Figure 3.9 The generation of pBut-*gsc*-HA construct
(A) The diagram of pbut2-gsc-HA construct. The modified pbut2 vector which contain an extra polylinker was used as backbone. (B) The sequence of primers used in PCR amplification of *gsc* gene.

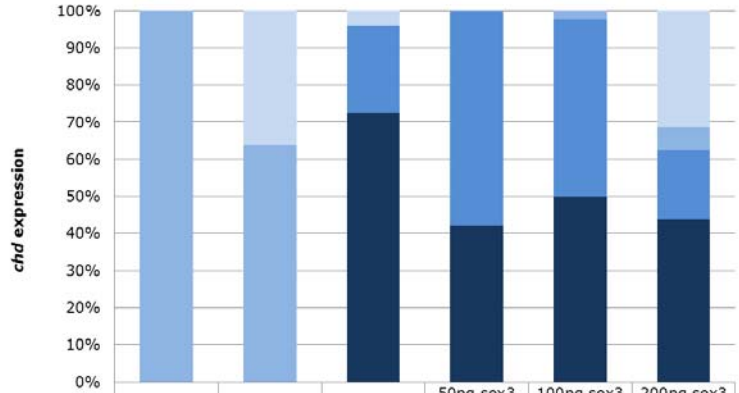
3.4.2 Sox3 regulates *chd* independently from regulation on *gsc*

Sox3 represses both *gsc* and *chd* expression (Shih et al., 2010). It has also been shown that *gsc* induces *chd* expression (Sasai et al., 1994), it was therefore possible that Sox3 repressed *chd* via repressing *gsc*. I therefore looked into the precise mechanisms by which Sox3 regulates the expression of *gsc* and *chd*. To determine whether Sox3 represses *chd* exclusively by its repression on *gsc* or Sox3 can directly act on both *gsc* and *chd*, experiments were designed to test whether Gsc could rescue the repression of *chd* caused by Sox3. Embryos were injected with *sox3* alone, *gsc* alone, or both *sox3* and *gsc* in various dosage combinations at 1-2 cell stage, collected and fixed at dome stage. The expression of *chd* was analysed by *in situ* hybridization. As anticipated, Sox3 had a slight repressing effect on *chd* while *gsc* led to a wide range expansion of *chd* expression (Figure 3.10 A, B). When *sox3* and *gsc* were co-overexpressed by injecting mRNA, ectopic *chd* expression was relatively moderate (Figure 3.10 A, B). In order to examine whether the area devoid of *chd* expression was where Sox3 was overexpressed, immunostaining of the exogeneous Sox3-HA protein was performed following the development of the *chd* ISH signal. Although increasing the amount of injected *sox3* mRNA amount did not result in a considerably great repression of *chd*, within the broad *chd* expansion area, however, there are patches in which *chd* expression is absent and Sox3 was expressed (Figure 3.10 C). Since the embryos were injected with the mixture of gsc and sox3 mRNAs, the immunostaining of Sox3-HA also indicated where *gsc* was expressed. As *chd* was expressed surrounding where *gsc* and *sox3* were injected, it is likely that *chd* was repressed by Sox3 cell-autonomously and regulated by Gsc in a non-cell-autonomous manner. This experiment also suggested that *gsc* was not able to rescue the repressing effect on

(A)



(B)



	uninjected	50pg sox3	50pg gsc	50pg sox3 + 50pg gsc	100pg sox3 + 50pg gsc	200pg sox3 + 50pg gsc
repression	0	13	2	0	0	10
wildtype	39	23	0	0	1	2
discontinuous expansion	0	0	12	22	21	6
continuous expansion	0	0	37	16	22	14

(C)



(D)

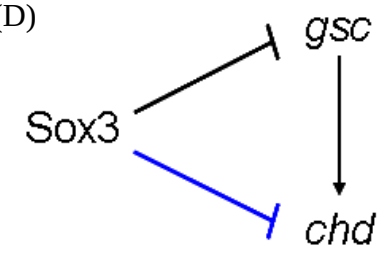


Figure 3.10 The repressing effect of Sox3 on *chd* expression cannot be rescued by *gsc*

Embryos were injected with various mixtures of *sox3* and *gsc* mRNA at 1-2 cell stage, collected and assayed at dome stage. (A) various range of *chd* expression. Upper panel: lateral view and dorsal to the right. Lower panel: animal pole view and dorsal to the right. (B) percentage of embryos with different range of *chd* expression after *sox3* and/or *gsc* overexpression. (C) Immunostainging showed the area of protein expression of injected *sox3* and *gsc*. *chd* expression was examined by ISH in dark purple. Whole mount immunostaining of Sox3-HA is shown in brown spots. (D) Sox3 can directly repress *chd* other than via its repression on *gsc*.

chd caused by Sox3, implying that Sox3 can directly repress *chd* other than via repressing *gsc* expression (Figure 3.10 D).

3.4.3 Fgf signaling regulates *chd* independently from regulation on *gsc*

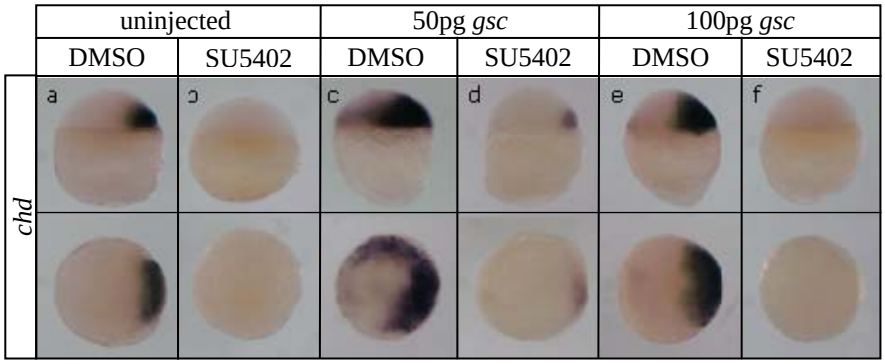
Fgf signaling is known to be necessary for organizer genes expression. When Fgf signaling is blocked, it leads to a loss of *gsc* and *chd* expression (Maegawa et al., 2006, Kuo, 2012). I therefore investigated whether the blockade of Fgf signaling caused the loss of *chd* expression due to the loss of *gsc*. To determine this, I blocked the Fgf signaling in the embryos with SU5402, a Fgf signal blocking reagent, and examined whether the loss of *chd* expression could be rescued by *gsc* overexpression. I overexpressed *gsc* by injecting the embryos with *gsc* mRNA at 50pg or 100pg at 1-2 cell stage. The embryos were then treated with DMSO as a control or SU5402 by MBT stage (~512-cell to 1000-cell stage). Embryos were collected and fixed at dome stage. The expression of *chd* was anaylsed by *in situ* hybridization.

The results showed the loss of *chd* expression on uninjected embryos treated with SU5402 (Figure 3.11 A a, b). On DMSO treated embryos, *gsc* injection caused expansion of *chd* expression (Figure 3.11 A c, e). However, injection of *gsc* mRNA at any dosage did not rescue the expression *chd* when Fgf signaling was blocked (Figure 3.11 A d, f). This result implies that Fgf signaling is necessary for *chd* expression and it promotes *chd* independently from its regulation on *gsc*, despite that Gsc can act on *chd* as well (Figure 3.11 C).

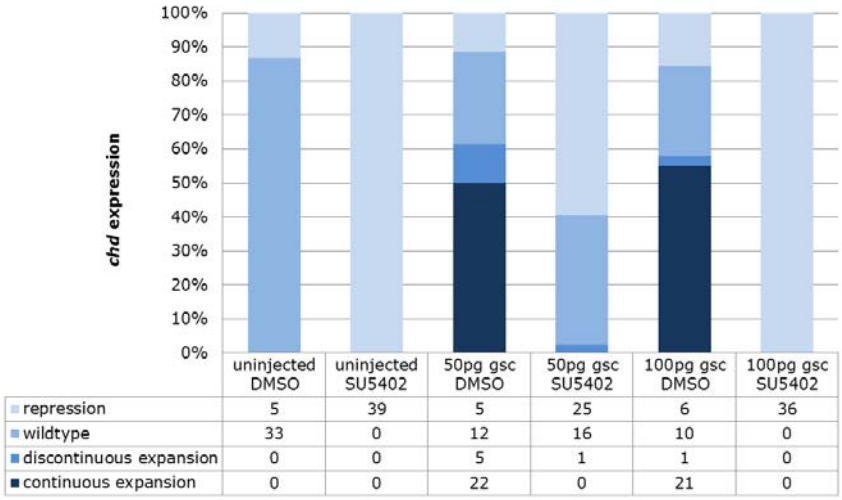
3.4.4 Sox3 can inhibit the expansion of *chd* expression caused by Fgf signaling

As previously mentioned, both Sox3 and Fgf signaling regulate the organizer gene *chd*. Given the fact that Sox3 inhibits the expression of organizer genes and inhibits

(A)



(B)



(C)

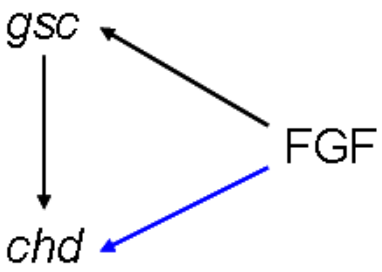


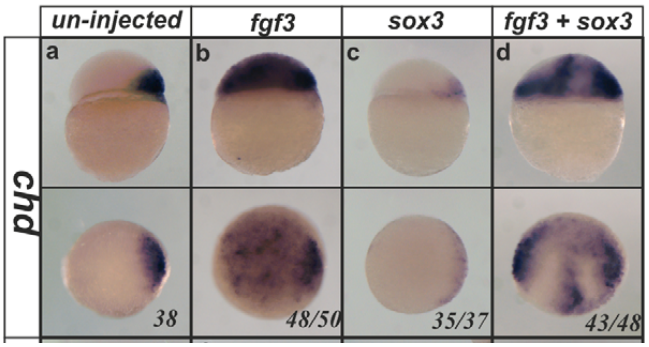
Figure 3.11 The repressing effect of SU5402 on *chd* expression cannot be rescued by *gsc*

Embryos were injected with various amount of *gsc* mRNA at 1-2 cell stage, treated with DMSO or SU5402 at MBT stage, collected and assayed at dome stage. (A) various range of *chd* expression. Upper panel: animal pole view and dorsal to the right. Lower panel: lateral view and dorsal to the right. (B) percentage of embryos with different range of *chd* expression. (C) FGF is necessary for *chd* expression independently from regulation on *gsc*.

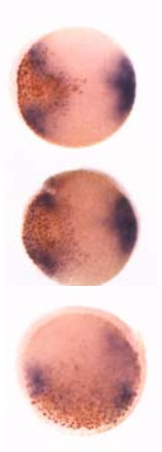
Fgf signaling, and that Fgf signaling is necessary for the expression of organizer genes, I set out to test if the inhibition of organizer genes by Sox3 is due to its repression of Fgf signaling. The strategy was to examine whether Sox3 can inhibit the expansion of *chd* expression caused by Fgf3 overexpression. Embryos were injected with *sox3*, *fgf3* or both at 1-2 cell stage. They were then collected and fixed at dome stage. The expression of *chd* was analysed by *in situ* hybridization.

Sox3 caused the loss of *chd* expression as expected. Fgf3 caused a large expansion of *chd* all over the animal hemisphere (Figure 3.12 A a, b, c). However, Sox3 was able to partially rescue the level of ectopic expression (Figure 3.12 A d). When Sox3 was co-overexpressed with Fgf3, the etopic expression pattern of *chd* mostly changes from continuous to discontinuous (Figure 3.12 B). As Sox3 appears to inhibit the *chd* expansion caused by Fgf signaling to some extent, it can be suggested that Sox3 also represses *chd* independently from inhibiting Fgf signaling. Since the co-overexpression of Sox and Fgf3 caused the discontinuous pattern of *chd* expression, I next examined the area where Sox3 and Fgf3 were overexpressed by immunostaining for Sox3-HA. The result showed that the area lacking in *chd* expression among the discontinuous pattern was where injected Sox3 was ectopically expressed (Figure 3.12 C). The immunostaining marked the cells in which both exogeneous Sox3 and Fgf3 were expressed. Considering Sox3 is a transcription factor and Fgf is a secreted molecule, Sox3 therefore repressed *chd* restrictedly in those cells, whereas Fgf3 was secreted and therefore induced etopic *chd* expression in surrounding cells. As Sox3 appeared to inhibit the *chd* expansion caused by Fgf signaling to some extent, it can be suggested that Sox3 also represses *chd* independently from inhibiting Fgf signaling (Figure 3.12 D).

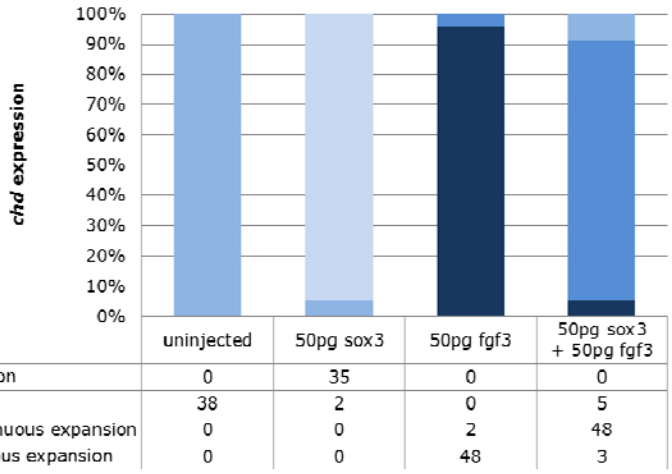
(A)



(C)



(B)



	uninjected	50pg sox3	50pg fgf3	50pg sox3 + 50pg fgf3
repression	0	35	0	0
wildtype	38	2	0	5
discontinuous expansion	0	0	2	48
continuous expansion	0	0	48	3

(D)

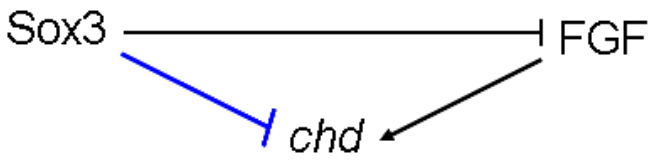


Figure 3.12 The expansion of *chd* caused by Fgf3 was inhibited by Sox3
Embryos were injected with *fgf3*, *sox3* or both at 1-2 cell stage, collected and assayed at dome stage. (A) *chd* expression analysed by *in situ* hybridization. Upper panel: lateral view and dorsal to the right. Lower panel: animal pole view and dorsal to the right. (B) percentage of embryos with different range of *chd* expression after *sox3* and/or *fgf3* overexpression. (C) Immunostaining showed the area of protein expression of injected *sox3* and *fgf3*. *chd* expression is in dark purple. Whole mount immunostaining of Sox3-HA is shown in brown spots.

3.5 The effect of Sox3 on the *gsc* promoter

A previous study from our lab has also looked into the potential promoter region of another organizer gene, *boz.* However, the result of luceferase assay showed that Sox3 activated the luciferase activity driven by *5'boz1.4* element, while the endogenous function of Sox3 represses *boz*. This result indicated that there is a distal regulatory region which is not included in *5'boz1.4* from the gene *boz*. Hence, I chose another organizer gene, *gsc*, and carried out luciferase reporter assay to inspect the regulation of Sox3 on organizer gene in a transcriptional level. The promoter region of gsc has been extensively studied in mouse and Xenopus. Distal and proximal elements responding to a set of factors involved in various signaling pathways had been identified (Genes and Dev 9:3038). Here I attempted to study the promoter region of gsc in zebrafish.

3.5.1 The generation of two *gsc* reporter constructs

In Xenopus, organizer genes, such as *cerberus(cer)* and *gsc*, have been demonstrated to be bound and activated by transcription factors expressed during organizer formation. The cis-regulatory modules of *gsc* has been identified in Xenopus. Their luciferase reporter assay result showed that *gsc* was regulated by VegT through the three T-box sites within -492bp of 5'region of *gsc* promoter. It has therefore been suggested that *gsc* is likely to be regulated by a set of other transcription factors, such as Sia, Otx2 and Lim1. In zebrafish, since it has been suggested that Sox3 directly represses *gsc*, it is possible that Sox3 acts on *gsc* promoter through the same domain as in the *gsc* promoter in *Xenopus* study.

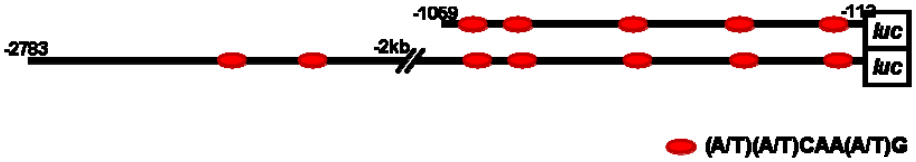
Therefore, in addition to revealing the detailed regulation of *gsc* at gene expression level, I also set out to investigate the putative promoter of *gsc* by luciferase reporter

assay. I aimed to test whether Sox3 can act directly on the upstream region of *gsc*. First of all, *gsc* reporter plasmids were designed and constructed. The upstream region of *gsc* was screened for potential Sox binding sites, (A/T)(A/T)CAA(A/T)G (Figure 3.13 A). Within the 2.6 kb region, six potential Sox binding sites were identified (Figure 3.13 A). Hence, two fragments, *5'gsc2.6* and *5'gsc0.9*, which contains 6 or 4 potential Sox binding sites respectively, were selected (Figure 3.13 B). Wildtype uninjected embryos were collected at 24 hpf, lysed and the genomic DNA was extracted. The genomic DNA was used as a template in PCR amplifying *5'gsc2.6* and *5'gsc0.9*. Specific primers were designed having restriction enzyme sites *NotI* and *SalI* at their 5'ends. After the amplification, those two fragments were ligated with pJET vector. They were then verified by DNA sequencing. Subsequently, the pJET-*5'gsc2.6* and pJET-*5'gsc0.9* were selected for digestion with *NotI* and *SalI*, which allows their subcloning into a luciferase expressing construct pFlash. The same selecting process was done to gain the constructs pFlash-*5'gsc2.6*-Luc and pFlash-*5'gsc0.9*-Luc (Figure 3.11 C).

(A)



(B)



(C)

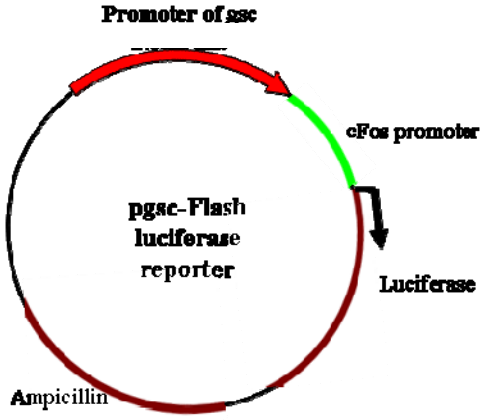


Figure 3.13 The generation of pFlash-5'gsc-luciferase contructs

(A) The upstream sequence of *goosecoid.* The sequences in red are complementary to primers. The six potential Sox binding sites are in square. The *5'gsc2.6* fragment is from -112 to -1059 whereas *5'gsc0.9* is from -112 to -2783. (B) Schematic diagram for the upstream region of *goosecoid*. 2.6 kb or 0.9 kb fragments *5'gsc2.6* or *5'gsc0.9* of upstream region of *gsc* coding sequence is cloned into luciferase expressing construct. The red ovals represent potential Sox binding sites. (C) The map of pFlash-5'gsc2.6-Luc or pFlash-5'gsc0.9-Luc.

3.5.2 Sox3 can regulate the *gsc* promoter directly

Biological analysis has shown that Sox3 represses *gsc* in vivo (Shih et al., 2010). I next set out to look into the regulation of *gsc* promoter by Sox3 at the transcriptional level. Luciferase reporter assays were carried out with pFlash-*5'gsc2.6*-Luc or pFlash-*5'gsc0.9*-Luc, luciferase expressing constructs. In this experiment, I aimed to test whether Sox3 and other factors including Boz, Sqt, Foxd3 and Fgf signaling, which are supposed to regulate *gsc* in vivo, can act on the putative *gsc* promoter region. An activation of luciferase activity would be expected in Boz, Sqt, Foxd3 and Fgf3 overexpressing embryos. Sox3 was expected to have negative effect on luciferase activation. On the other hand, luciferase acitivty was also measured under the condition of overexpression of combination of *sox3* and other factors. In section 3.4, Sox3 has been suggested to have a competitive role with Fgf signaling on organizer gene regulation. If Fgf signaling activates pFlash-*5'gsc2.6*-Luc or pFlash-*5'gsc0.9*-Luc, the combination of Fgf3 and Sox3 would be expected to reduce the luciferase activity.

Embryos were injected at the 1-2 cell stage with pFlash-5'gsc2.6 or pFlash-5.gsc0.9 constructs, *gfp* mRNA as a control or various mRNA as indicated. Embryos were then collected and lysed at dome stage, followed by protein extraction and measurement of luciferase activity.

Although Sox3 represses the expression of endogenous *gsc* during organizer formation, it was found to activate the luciferase reporters, pFlash-*5'gsc2.6*. Fgf3, a factor in the Fgf family and is expressed in the dorsal organizer in zebrafish, it is known to promote organizer gene expression. While the early expression of *gsc* and *chd* is Fgf signaling dependent, it was found that Sox3 could repress *gsc* and *chd* expressions independently from repression on Fgf genes (section 3.4). In this

luciferase assay, Fgf3 overexpression activated pFlash-*5'gsc2.6* also. Even so, there is no significant difference on luciferase acitivity between samples overexprssed with Sox3 alone and combination of Sox3 and Fgf3 (Figure 3.14). *5'gsc2.6* was then studied extensively with a set of other factors. As shown in Figure 3.14, Boz or Foxd3 overexpression caused no effect on pFlash-*5'gsc2.6*-Luc. On the other hand, Sqt activated pFlash-*5'gsc2.6*-Luc in great extent. Interestingly, while Boz or Foxd3 was overexpressed in combination with Sox3, the activation brought by Sox3 on pFlash-*5'gsc2.6*-Luc was eliminated. The activation caused by Sqt was reduced when Sox3 was co-overexpressed.

The effects of Fgf3, Sox3 or both on pFlash-*5'gsc0.9*-Luc has also been studied. The result showed that Sox3 activated the pFlash-*5'gsc0.9*-Luc also. When Sox3 was overexpressed in combination with Fgf3, the activation on pFlash-*5'gsc0.9*-Luc was still significant. Fgf3 alone, however, did not cause significant activation on pFlash-*5'gsc0.9*-Luc (data not shown).

Although Sox3 unexpectedly activated both pFlahs-*5'gsc2.6*-Luc and pFlash-*5'gsc0.9*-Luc, taken together with the unexpected results caused by Boz or Foxd3, it is very likely that the 2.6 kb upstream region of *gsc* gene was not sufficient to perform the *in vivo* biological activity.

Although Sox3 alone activates both *5'gsc2.6* and *5'gsc0.9* in the reporter assay, it seems to have a greater extent of activation on *5'gsc2.6* than *5'gsc0.9* (data not shown). This may indicate that Sox3 could bind and act on those two Sox binding sites between -2 kb to -2.7 kb on *5'gsc2.6*. This result suggested that Sox3, Sqt and Fgf signaling components can bind to and act directly on the putative promoter region of *gsc*, although there is likely distal cis-elements of *gsc* gene needed for the accurate regulation by Sox3. The fact that Sox3 reduced the activation on pFlahs-*5'gsc2.6*-Luc

caused by Sqt possibly implied the competing role of Sox3 and Sqt on regulating *gsc* promoter. When Sox3 was overexpressed, a proportion of Sqt was competed out by Sox3 and therefore the luciferase reporter was activated to a lesser extent. The activation on pFlahs-*5'gsc2.6*-Luc caused by Sox3 was also eliminated by Boz or Foxd3 (Figure 3.14). There is possibly a competing role between Sox3 and Boz or Foxd3 as well. This may also imply that the *5'gsc2.6* region was sufficient for Boz or Foxd3 binding, although it was not sufficient to perform the biological function of the promoter of *gsc*. In this case, when Boz or Foxd3 was overexpressed in combination with Sox3, Sox3 might be competed out and therefore no activation was shown on the pFlahs-*5'gsc2.6*-Luc.

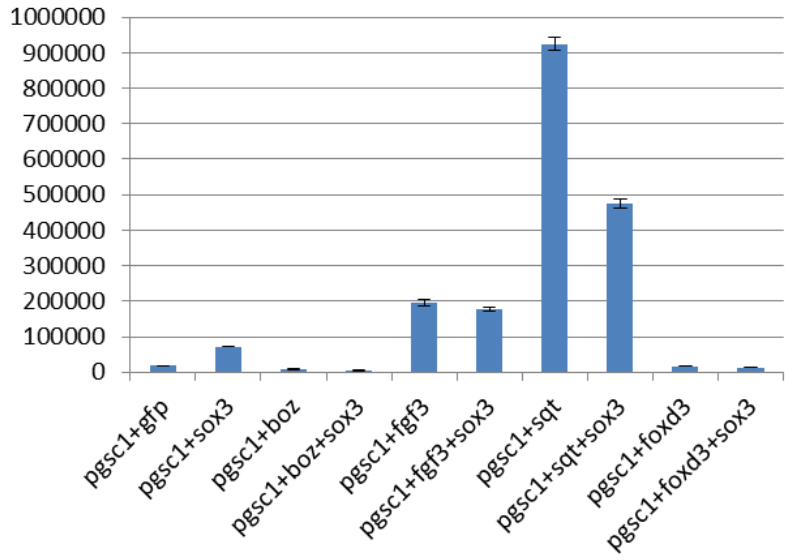


Figure 3.14 Sox3 and Fgf3 activates pFlash-*5'gsc2.6*-Luc
Embryos were injected at 1-2 cell stage with various mRNA and luciferase expressing vector pFlash-*5'gsc2.6*-Luc. Luciferase expressing contruct was injected at 5 pg and all the mRNA indicated was injected at 50 pg. Embryos were collected and lysed and at dome stage. Experiments were done in triplicates of which 10 embryos were used in each. Luciferase assay was carried out and the luciferase activities were measured using the Dual-Luciferase Reporter assay system and GlomaxTM Luminescence Detection.

3.5.3 The analysis of *gsc* promoter across species

In section 3.5, it was shown that Sox3 could directly act on regulatory elements of *gsc* gene. However, the unexpected activation of *5'gsc2.6*-Luc by Sox3 indicated that 2.6 kb upstream regulatory region of *gsc* in zebrafish was insufficient for endogenous biological regulation by Sox3. Therefore, I screened the sequence of introns and downstream region of *gsc* in zebrafish for potential Sox3 binding sites also. In order to speculate whether these potential Sox binding sites have conservative roles across species, the regulatory regions of *gsc* were also analysed in human, Xenopus, mouse and chick.

Figure 3.15 showed that potential Sox binding sites indeed exist in intron and downstream of the *gsc* gene in zebrafish. In human, there are three potential Sox binding sites within the 3 kb region upstream of *gsc* and a single one in intron. In Xenopus, mouse and chick, potential Sox binding sites distribute throughout upstream and downstream region of *gsc*. The existence of Sox binding sites downstream of *gsc* in zebrafish, Xenopus, mouse and chick indicate that it is likely that those potential Sox binding sites have conservative roles among these species.

Taken together with the result shown in Figure 3.14 and the existence of potential Sox binding sites in both upstream and downstream region of *gsc*, it can be suggested that Sox3 binding and bending of the DNA is required for execution of complete biological regulation on *gsc* gene by Sox3.

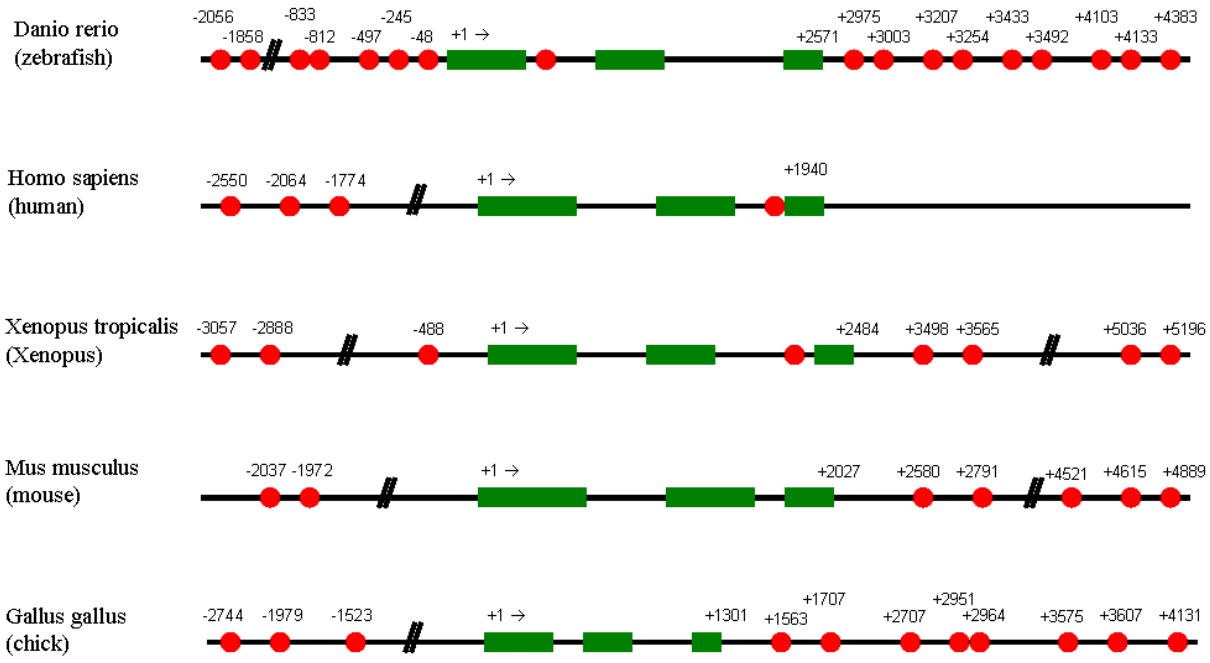


Figure 3.15 Sox binding sites in regulatory regions of *gsc* gene across species
Introns, 3kb upstream and 3kb downstream of *gsc* gene in zebrafish, human, Xenopus, mouse and chick were screened for potential Sox binding sites. Green boxes represent exons of *gsc* gene. Red dots represent potential Sox3 binding sites (A/T)(A/T)CAA(A/T)G.

3.6 Discussion

3.6.1 The expression pattern of *gsc* and *chd* and the signaling regulations on them

chordin was first identified in *Xenopus*, where its expression first appears in the dorsal marginal zone, then exclusively at the forming dorsal blastopore lip. Once gastrulation happens, involution and epiboly take place, *chd* becomes intense and is expressed in superficial and involuting cells of dorsal lip including the bottle cells. In zebrafish, I found that the expression of *chd* is initially detectable in the dorsal margin, the region of presumptive organizer at 3.5 hpf (Figure 3.2 ii'). *chd* expression gradually expands laterally and covered quarter of the margin (Figure 3.2 jj'-mm'), consistent with the convergent extension caused by intercalation during gastrulation. This is analogous to what was reported in *Xenopus* (ref). As gastrulation continues, involuting cells, led by the perspective pharyngeal endoderm and head mesoderm cells, move towards the animal cap. This is reflected by the trigeminal-like *chd* expression at 70% epiboly (Figure 3.2 p). The migration trend of *chd* expressing cells is consistent with the reports that pharyngeal and dorsal gut endoderm which derive from the bottle cells, prechordal plate which derived from anterior mesoderm, chordamesoderm giving rise to notochord, forebrain and tailbud are all expressing chordin at corresponding stages.

In Xenopus, *gsc* was found to be expressed in the organizer at early stage and expressed at dorsal anterior tissues including pharyngeal endoderm and prechordal plate at later stages. In zebrafish, I also found that as the expression results showed, *gsc* expressing cells from the dorsal marginal region migrated upwards to the animal pole as involution took place.

On the other hand, *foxd3* is known as a mesoderm gene (Odenthal and Nusslein-Volhard, 1998). It is initially expressed at the dorsal margin. *foxd3* expressing cells

do not migrate upwards until shield stage. The arch-shaped expression pattern of foxd3 formed at shield stage is possibly a result of the cell movement during involution. As cell involution started, the cells at the dorsal most area migrated inward and upward, leaving an area avoid of the foxd3 expression.

However, there are some limitations in these experiments. First of all, the precise expression level of the genes cannot be quantified by *in situ* hybridization. It cannot be estimated whether there was a difference in the expression level of *gsc* between stages in the *in situ* hybridization results. Secondly, there are difficulties in confirming the precise expression area of a gene at particular stage due to the biological variants and the possible deviation in staging embryos. Thirdly, the mRNA expression levels may not represent their protein expression levels, due to different translation level and the steady state of the proteins. Although the precise mRNA expression level at different stages could be compared by performing real-time PCR, as well as the precise expression area of *foxd3*, *gsc* or *chd* could also be determined by doing embryos sectioning, it was, however, not worth proceeding with further studies since the preliminary results of the effects of Foxd3 on SoxB1 genes was not encouraging for further investigation.

3.6.2 The interaction between SoxB1 family and Foxd3

The SoxB1 family has been shown to be required in various developmental events during early embryogenesis. For instance, Sox3 is found to regulate organizer formation by directly restricting the expressions of target genes downstream of Wnt signaling pathway. Sox2 is also found to be expressed at later stage and has a role in the initiation of neurogenesis and the maintenance of prospective neural ectoderm in chick and Xenopus. Among the SoxB1 family, *sox3* and *sox19b* are found to be

expressed earliest, with *sox19a* following. Gain-of-function experiments showed that Foxd3 and Sox3 could mutually repress each other expression at sphere stage. Since *foxd3* is more likely to be a transcriptional repressor, it can be proposed that Foxd3 induces organizer genes indirectly by repressing *sox3*. However, this hypothesis needs to be further tested. There is literature showing that foxd3 can also act as an activator, suggesting that it is possible that Foxd3 directly acts on *sox3* and organizer genes separately. On the other hand, Sox3 is believed to regulate organizer by acting directly on organizer genes. Here I found that Sox3 represses *foxd3*, which is therefore another way Sox3 might contribute to the repression of organizer gene. Interestingly, overexpression of *foxd3* does not repress the other *soxb1* genes, *sox19a* and *sox19b*. Instead, Foxd3 activates sox19a and sox19b at the stages of sphere, 50% epiboly and 85% epiboly which represent the stages of organizer formation, gastrulation and neurogenesis respectively. Foxd3 is known as a mesoderm gene that functions in initiation of mesoderm formation. It is also critical in neural crest determination and the maintenance of neural crest progenitors at later stages. *sox19a* and *sox19b* are expressed in prospective ectoderm. They are important to the neural development. This might be an explanation for that Foxd3 induces *sox19a* and *sox19b* at later stages. Since Foxd3 induces *chd*, which acts as a neural inducer at later stage to activate SoxD factor, it is also likely that *sox19a* and *sox19b* are also up-regulated in a similar situation. However, this explanation might not be consistent with the fact that Sox19a and Sox19b represses organizer in zebrafish. Hence, the up-regulation of *sox19a* and *sox19b* could be explained by the functional redundancy between SoxB1 family genes. As *sox3* is repressed by Foxd3, *sox19a* and *sox19b* might be triggered to be expressed in a higher level to overcome the developmental events as shown by Kamachi (Okuda et al., 2010). However, further investigation on

the effects of *foxd3* and *sox3* on each other at later stages is needed to make further conclusion.

3.6.3 Sox3 and Fgf signaling independently regulate both *goosecoid* and *chordin*

The organizer is a singalling centre regulated by a complicated network of signals. Among these, Sox3 and Fgf singalling are suggested to have opposing effects. On the other hand, it is important to study the regulation of *gsc* on *chd* very carefully since blockage of *chordin* expression inhibited the induction of a secondary central nervous system by an organizer graft (Oelgeschlager et al., 2003), and that implies that *chd* is a main character executing the organizer function on neural induction. Immunostaining results showing that *gsc* was not able the rescue the loss of expression of *chd* in cells expressing Sox3 indicate that Sox3 represses *chd* cell-autonomously and *gsc* induces *chd* in a non-cell-autonomous manner. Together with the fact that *gsc* could not rescue the loss of *chd* caused by inhibition of Fgf signaling, it can be postulated that both Sox3 and Fgf signaling act upon both *gsc* and *chd* via independent pathways, although sox3 and Fgf signaling can also both regulate each other. Previous studies have suggested that Sox3 directly represses organizer genes. Here that Sox3 inhibited *chd* expansion brought by Fgf3 also supports this idea as that indicates Sox3 acts cell-autonomously and inhibits *chd* by competing with Fgf signaling.

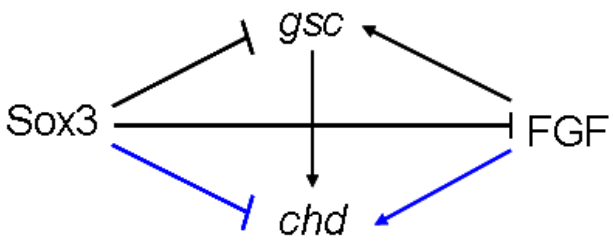


Figure 3.15 The model of regulation on gsc and chd by Sox3 and Fgf signaling
Gsc induces *chd* expression. Sox3 has been found to repress *chd* independently from repressing *gsc*. Fgf can also promote *chd* expression independently from inducing *gsc* expression. On the other hand, Sox3 directly regulates *gsc* and *chd* expression, apart from its repressing function on Fgf signaling.

3.6.4 Sox3 and Fgf3 can act on the promoter region of *goosecoid*

The regulatory elements of *gsc* by some early expressed transcription factor such as VegT, Siamois and Otx2 has been studied in *Xenopus*. Their results showed that a proximal regulatory module within 492 bp upstream of *gsc* is enough to strongly respond to VegT. In my experiments, the sequences analysed start from -112 upstream of *gsc*. There is no potential Sox binding site within the most proximal 112 bp from the transcription start site. The luciferase reporter result gives an unexpected activation of *5'gsc2.6* by Sox3, while Sox3 represses endogenous *gsc in vivo*. However, this result suggests that Sox3 can regulate *gsc* directly. The activation on *5'gsc2.6*-Luc by Sox3 reflected that this assay is insufficient to recapitulate the endogenous function of Sox3 to repress *gsc* expression. There are perhaps distal regulatory elements which are not contained in *5'gsc2.6*. It is also a possibility that there are other co-repressors needed for repression of *gsc* by Sox3. However, since the co-repressors could already be expressed there, more investiagation would be needed to test that. In fact, there is a previous study on the 1.4kb upstream region of

bozozok and that showed the similar result that Sox3 activated the luciferase activity driven by *5'boz1.4* (Kuo, 2012). However, the greater activation of luciferase activity driven by *5'gsc2.6* suggested that one or both of the two Sox potential binding sites excluded from *5'gsc0.9* are important for Sox3 activity.

The analysis of potential Sox binding sites across species showed that the downstream regulatory elements are possibly essential for endogenous regulation of *gsc* by Sox3. The fact that Sox3 is a architectural transcription factor, which can induce conformational change of DNA, also supports the idea that both upstream and downstream regulatory elements are required for recapitulation of endogenous function of Sox3.

On the other hand, Fgf3 gives strong activation on both *5'gsc0.9*-Luc and *5'gsc2.6*-Luc. It is likely that in addition to Sox3, this regulatory element responds to factor(s) activated by Fgf signaling. Morever, this 2.6 kb region is enough to respond to the activator(s).

Chapter 4 SUMOylation of Sox3 in zebrafish

4.1 Introduction

Previous literatures and the data from our group have shown that Sox3 can act as transcriptional activator or repressor in many developmental processes throughout embryogenesis (Dee et al., 2008, Shih et al., 2010, Oshima et al., 2009). For instance, a Xenopus study showed that Sox3 functions as an activator to induce *sox2* and *geminin* at the initiation of neurogenesis (Rogers et al., 2009). According to previous studies from our group, Sox3 caused expansion of neural plate and neural tissue. It has also been demonstrated to induce neural fate markers, such as *sox2* and *sox19b*, and inhibit non-neural fate markers, such as *gata2* during early neural induction in zebrafish (Dee et al., 2008). At the stage of organizer formation in zebrafish, Sox3 can also restrict organizer and mesoderm genes (Shih et al., 2010). Their Sox3HMG-EnR and Sox3HMG-VP16 overexpression results suggested that Sox3 functioned as a transcription repressor on organizer genes (Shih et al., 2010). However, it is not yet known how Sox3 is regulated in order to participate into diverse developmental events with these two opposite roles.

Post-translational modification including phosphorylation, ubiquitination, acetylation and SUMOylation is an important way by which transcription factors are regulated to accomplish diverse roles, both spatially and temporally in different cells. It has been found that SUMOylation has roles in various transcription factors including Sox proteins. Some of the Sox proteins, including Sox2, Sox3, Sox6, Sox9 and Sox10, have been shown to be affected by SUMOylation (Tsuruzoe et al., 2006, Savare et al., 2005, Fernandez-Lloris et al., 2006, Lee et al., 2012, Taylor and Labonne, 2005). SUMOylation changes their transcriptional activities by altering DNA binding affinity, subcellular localisation or protein-protein interaction. However, while Sox3

in zebrafish has been shown to act as transcriptional activator or repressor, SUMOylation of Sox3 has not been studied.

SUMOylation has been reported to contribute to transcriptional repression activity of a set of transcription factors by sequestering them to the nuclear bodies or recruiting other transcriptional co-repressor to form the repressor complexes. SUMOylation usually works in combination with other modification pathways to accomplish its regulation on transcriptional repression. The transcription factor Elk-1 had been reported to be SUMOylated (Salinas et al., 2004) It was also shown that this SUMOylation resulted in the recruitment of histone deacetylase 2 (HDAC-2). The histones were then deacetylated and therefore the target genes of Elk-1 would be silenced.

A previous study from our group demonstrated that chicken Sox3 and mouse Sox3 can be SUMOylated (Figure 4.1). In these species only a very small amount of Sox3 appeared to be endogenously SUMOylated (Laghari, 2010). Previous literature also reported that in most cases there is only a small fraction of protein subjected to SUMOylation (Johnson, 2004). Moreover, the SUMOylated Sox3 was exclusively located in the chromatin fraction (Laghari, 2010). It was also suggested that SUMOylation of Sox3 caused a decrease in its transcriptional activation activity (Laghari, 2010).

In this chapter, I investigated various aspects of the SUMOylation of Sox3 in zebrafish embryos. Considering the fact that SUMOylation negatively modulates transcription in most cases, plus the results from the previous study of SUMOylation on chicken Sox3 and mouse Sox3, my hypothesis was that SUMOylation switches zebrafish Sox3 from a transcription activator to repressor. First of all, I demonstrated the expression level as well as subcellular distribution of endogenous

zfSox3 at various stages. Secondly, I screened the amino acid sequence of Sox3 and identified the potential SUMOylation sites. Then I set out to determine the critical SUMOylation site on zfSox3. I also attempted to examine the SUMOylation of Sox3 *in vivo*. Finally I analysed the biological effects of SUMOylation of Sox3. I also analysed the functional difference between wtSox3, Sox3K230R and Sox3K230R-SUMO proteins at transcriptional level by looking into their effects on promoter region of *boz* and *gsc* and the expression of marker genes of several major developmental events during zebrafish embryogenesis.

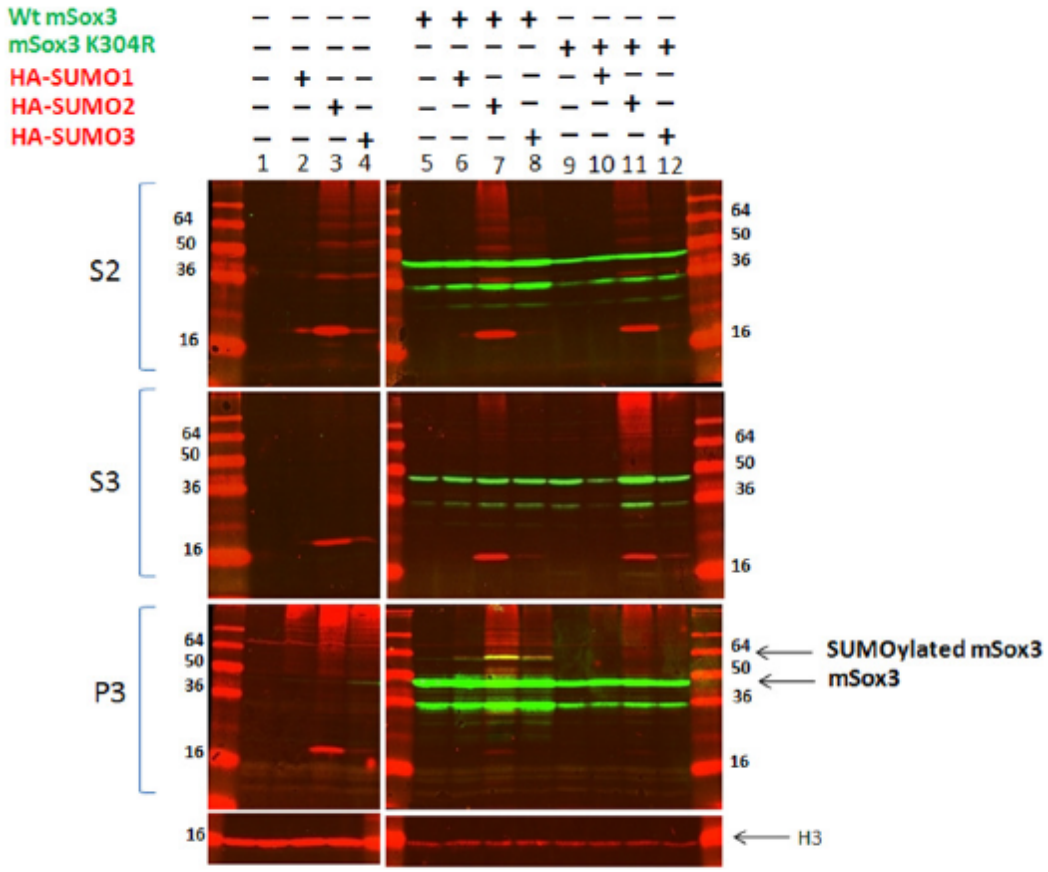


Figure 4.1 SUMOylated mouse Sox3 was exclusively in chromatin fraction. Subcellular fractionation demonstrated the distribution of mouse Sox3 in cytosol (S2), soluble nuclear (S3) and chromatin (P3) fraction. A large proportion of Sox3 was expressed in the chromatin fraction. SUMOylated mouse Sox3 (yellow band) was detected exclusively in chromatin fraction. (adapted from Laghari, 2010)
The red signals represented the HA-SUMO expression. The green signals represented the Myc-Sox3 expression.

4.2 The expression of Sox3 in zebrafish embryos

4.2.1 The subcellular distribution of Sox3

The subcellular distribution of mouse and chicken Sox3 had previously been studied in cell culture (Laghari, 2010). The results showed that most Sox3 was in the cytosol and chromatin fractions. SUMOylated Sox3 was exclusively found in the chromatin fraction. Here I looked at the subcellular distribution of Sox3 in zebrafish. Wildtype or mutant zfSox3 was overexpressed by injection of HA-tagged *wtsox3* or *sox3K230R* mRNA at 1-2 cell stage. Embryos were then collected, dechorionated and lysed at 6.5 hpf. Subcellular fractionation was then carried out followed by western blot analysis. The blot was probed using anti-HA which indicated the expression of exogenous wtSox3 or Sox3K230R. Histone 3 was detected exclusively in the chromatin fractions of uninjected, wtSox3 overexpressed, and Sox3K230R overexpressed embryos (Figure 4.2), indicating that the fractionation had successfully isolated the chromatin fractions.

The results demonstrated that there was no detectable Sox3 in the soluble nuclear fraction (S3). In the cytosol fraction (S2), however, there is a small amount of injected wtSox3 and a tiny amount of Sox3K230R. Injected HA-tagged Sox3 was mostly found in the chromatin fraction (P3) at about 40 kDa. Uninjected embryos were used as a negative control in all fractions. Interestingly, there is a faint band at about 58 kDa in wtSox3 overexpressing embryos whereas it did not appear in Sox3K230R overexpressed embryos (Figure 4.2, blue arrow), suggesting that this modification of Sox3 was prevented by the K230R mutation. The SUMOylated form of mSox3 and cSox3 suggested in previous studies were at about 61 kDa and 55 kDa respectively, whereas mSox3 and cSox3 without SUMO modification were detected at about 46 kDa and 40 kDa respectively (Figure 4.1) (Laghari, 2010). The SUMO

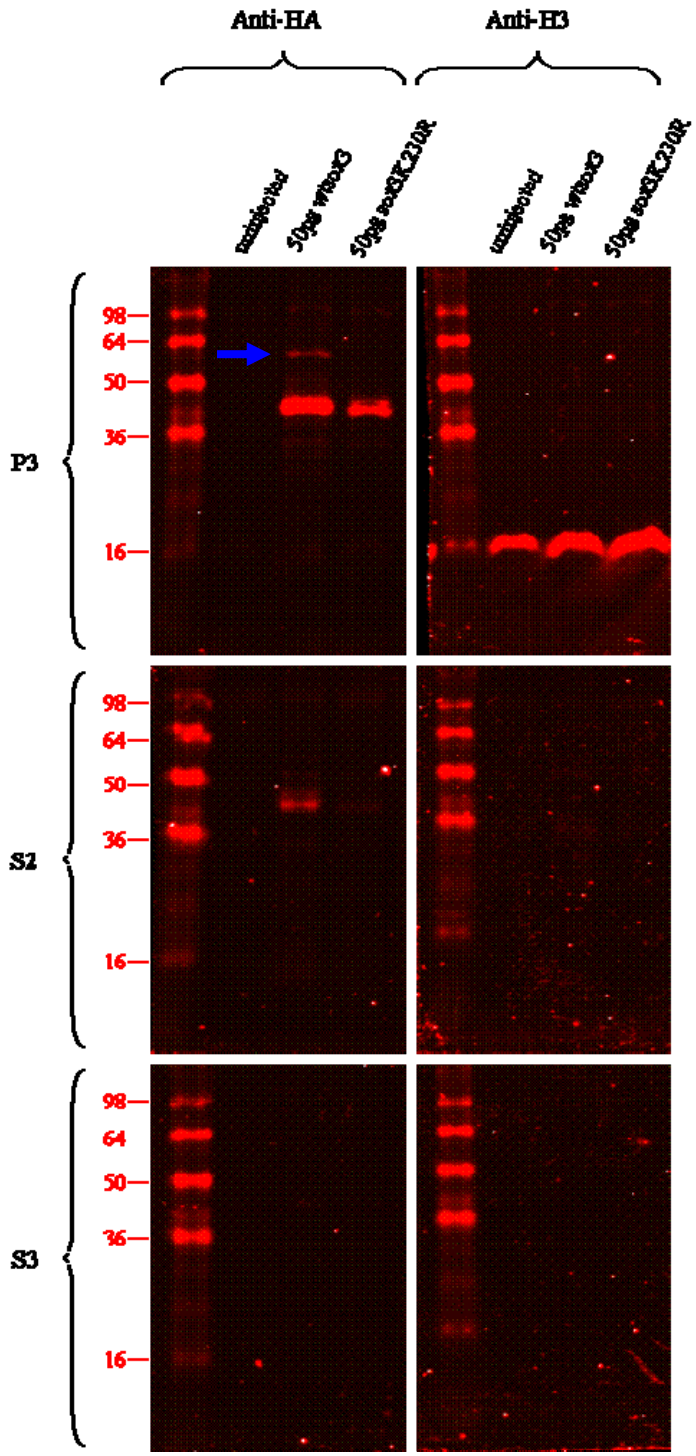


Figure 4.2 Subcellular distribution of overexpressed Sox3
Uninjected embryos and embryos injected with HA-tagged *wtsox3* or *sox3K230R* were collected at 6.5 hpf. Following the subcellular fractionation, protein was extracted for western blot analysis. Overexpressed wtSox3 or Sox3K230R were detected by anti-HA first antibody followed by anti-rabbit secondary antibody. Signal was developed with the Licor system. The blots were then detected with anti-H3 first antibody followed by anti-rabbit. S2 represented the cytosol fraction. S3 represented the soluble nuclear fraction. P3 represented the chromatin fraction.

modification of mSox3 and cSox3 gave rise to a 15 kDa shift when running on the the SDS gel. It is therefore this 58 kDa band could suggest that it was possibly the SUMOylation form of zfSox3, while zfSox3 is at about 40 kDa without SUMO modification.

4.2.2 The expression of Sox3 during the first 24 hours after fertilization

In advance of looking in further detail at SUMOylation of Sox3, I studied the expression level and modification pattern of endogenous Sox3 at different stages within 24 hours after fertilization in order to select the optimal stages for further experiments. As the results shown above, Sox3 is mainly expressed in chromatin fraction. In addition, SUMOylated Sox3 is believed to be expressed exclusively in chromatin fraction. Here I collected wildtype uninjected embryos at various stages within 24 hours after fertilization. Embryos were then dechorionated and lysed. Subcellular fractionation was performed and the protein was extracted. The chromatin fractions (P3) were prepared for western blot analysis. Expression of endogenous Sox3 was detected by anti-hSox3.

As the result showed (Figure 4.3 A), Sox3 was barely detectable at sphere stage. There was an impressive increase at shield stage. It is also noteworthy that at this stage Sox3 was detected as double bands at about 36 - 38 kDa. At the 70% epibloy stage, there was another remarkable increase in expression level accompanied by the appearance of additional faint bands between 50 kDa to 64 kDa. The expression level of Sox3 and the pattern of larger bands remained similar at bud stage and 5-somite stage. However, at 20 hpf, there is a striking change in the Sox3 bands pattern. The level of Sox3 in size of 36 - 38 kDa dramatically decreased while the level of larger bands between 50 kDa to 64 kDa increased. At the 24 hpf stage, the ratio of 36 - 38

kDa Sox3 and larger Sox3 bands remained similar to that of 20 hpf, yet the total protein level largely reduced.

However, the double bands of Sox3 at 36-38 kDa could not be clearly seen at later stages. In addition, there was an interesting sudden change of the level of the larger Sox3 bands from 5-somite stage to 20 hpf stage, more detailed inspection was needed to find out Sox3 expression between these stages. Therefore I collected samples every two hours between 5-somite stage (~12 hpf) to 24 hpf stage. Embryos were then lysed and the protein was extracted for western blot analysis.

Although the overall level of Sox3 was much lower in this experiment, it showed that the 36-38 kDa Sox3 double bands were present at later stages as well. The expression level of Sox3 larger bands gradually increased a little over time, but there was a dramatic shift between 18 hpf and 20 hpf. After that, as the total Sox3 protein level dramatically decreased, the larger Sox3 bands were less remarkable at 22 hpf and 24 hpf (Figure 4.3 B).

It is not clear whether the larger Sox3 bands at around 58 kDa were the SUMOylated Sox3. However, together with the results demonstrated in Figure 4.2, it supports the idea that the larger bands shown here were SUMOylated Sox3. On the other hand, it can also be suggested that a large proportion of Sox3 protein has been subjected to various post-translational modifications at about 20 hpf.

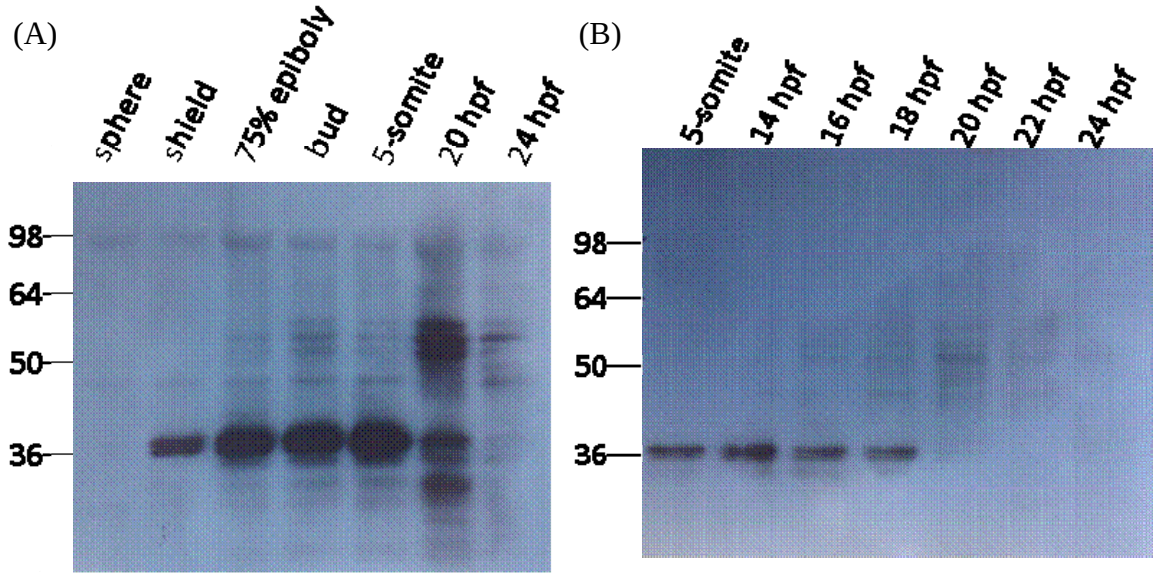


Figure 4.3 The protein expression of Sox3 within the first 24 hpf
Wildtype uninjected embryos were collected at specific stages. They are dechorionated and deyolked followed by subcellular fractionation. The chromatin (P3) fractions were collected. Protein samples were subjected for Western blot analysis with mouse anti-hSox3 (Abcam ab55795) as first antibody and anti-mouse as secondary antibody. (A) Protein samples from sphere, shield, 75% epiboly, bud, 5-somite, 20 hpf and 24 hpf stages. (B) Protein samples from 5-somite/12 hpf, 14 hpf, 16 hpf, 18 hpf, 20 hpf, 22 hpf and 24 hpf stages.

4.3 The SUMOylation of Sox3

4.3.1 Potential SUMOylation sites in zfSox3

According to the previous literature, SUMOylation occurs at lysine residues within a specific consensus sequence ΨKXE, whereas Ψ is a large hydrophobic residue, K is a Lysine residue to be conjugated with SUMO protein(s), X is any amino acid and E is Glutamic acid (Rodriguez et al., 2001, Sampson et al., 2001). To investigate the SUMOylation of Sox3, I generated Sox3 constructs with mutation(s) at potential SUMOylation sites. First of all, I identified two potential SUMOylation sites, K115 and K230 in protein sequence of zfSox3 (Figure 4.4 B). Amino acid sequence comparison between different species also showed that these are conserved potential SUMOylation motifs aross species (Figure 4.4 A).

According to the previous studies on mouse Sox3 from our lab, K304 on mSox3 is the critical SUMOylation site. Protein sequence alignment results showed that K230 on zfSox3 is homologous to K304 on mSox3 (Figure 4.4 A). It is likely that K230 is the only SUMOylation site in zfSox3. However, to assure whether K230 is the exclusive SUMOylation site in zfSox3 since this was not explicitly tested in mouse, I also studied the other potential SUMOylation site K115 in zfSox3. Hence, I set out to generate expressing constructs of another muatant Sox3, Sox3K115R, and the double mutant, Sox3K115RK230R (Figure 4.4 B).

HA-tagged Sox3K230R expressing construct was previous generated in our lab. It includes an AAG to AGG mutation at the nucleotide position 688-690 that turns the Lysine 230 residue to Arginine (Figure 4.4). Hence, I carried out site-directed mutagenesis on pβut2-wtSox3-HA or pbut2-Sox3K230R-HA to create pβut2-Sox3K115R-HA and pβut2-Sox3K115RK230R-HA respectively (Figure 4.5). A forward primer and its complementary reverse primer were designed (Figure 4.5 A)

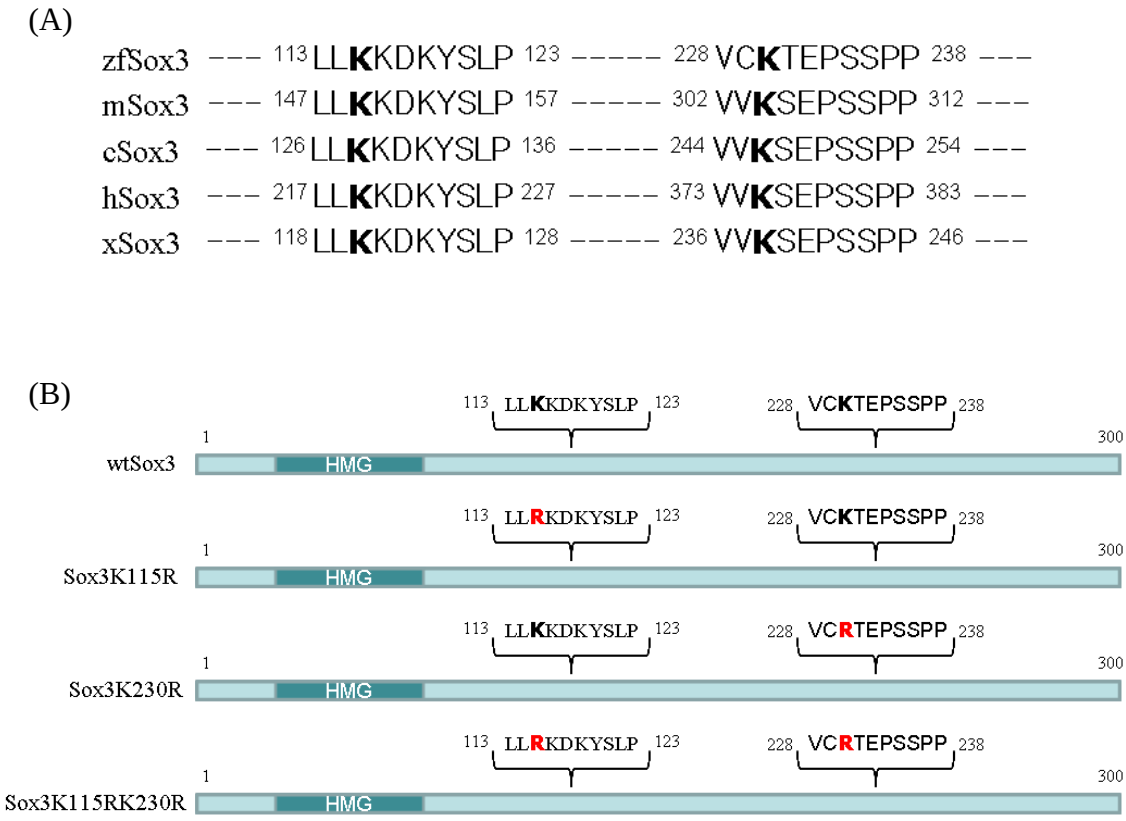


Figure 4.4 The conserved SUMOylation motifs on Sox3
(A) The sequences of the conserved SUMOylation motifs on Sox3 across zebrafish, mouse, chick, human and xenopus. (B) The four wildtype or mutant forms of Sox3 to be investigated. The amino acid mutation is marked in red. Sox3K115R and SoxK115RK230R expressing constructs were generated from pβut2-*wtsox3*-HA or pβut2-*sox3K230R*-HA respectively.

(A)

```
  1 atgtataaca tgatggaaac cgagattaaa agccccattc cgcagtccaa cacgggctcg
 61 gtgacgggcg gcaaaaacaa cagtgccaac gaccaggacc gggtgaagcg gcctatgaat
121 gctttcatgg tgtggtctcg cgggcagcgg aggaagatgg ctcaggagaa tcctaaaatg
181 cacaactcgg agatcagcaa gcgcctcggt gctgactgga aacttttgac tgacgccgag
241 aagagaccct tcattgacga ggccaagcgg ttacgagcca tgcacatgaa ggagcacccg
301 gattacaaat accgtccccg caggaagacc aagaccctgc tgaagaaaga caagtattct
361 ttgccagggg gactcctggc gcccggtgcc aacgctgtca acaacgcggt gtcggtgggc
421 cagcggatgg actacacgca catgaacgga tggacgaaca gcgcatactc cctcatgcag
481 gaccagctgg cctaccctca acatcccagt atgaacagcc cccagatcca gcagatgcac
541 cggtacgaca tggcgggact tcagtaccca atgatgtcca cggctcagac ctacatgaac
601 gccgcgtcca cgtacagcag catgtcacca gcatacacgc aacaaacttc cagtgcaatg
661 ggtttgggct ccatggcttc ggtgtgcaag acggagccca gctcccctcc tccggccata
721 acctctcact ctcagcgtgc ttgtttggga gacctgagag atatgataag catgtacctg
781 ccgcccggtg gagacagcgc cgaccactcc agtctacaga ccagtcggtt acacagcgtt
841 catccgcact atcaaagcgc agggacaggc gtgaacggaa cgctaccct aacccacatt
901 tga
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5'- GAAGACCAAGACCCTGCTGAGGAAAGACAAGTATTCTTTG – 3'
5'- CAAAGAATACTTGTCTTTCCTCAGCAGGGTCTTGGTCTTC – 3'

(B)

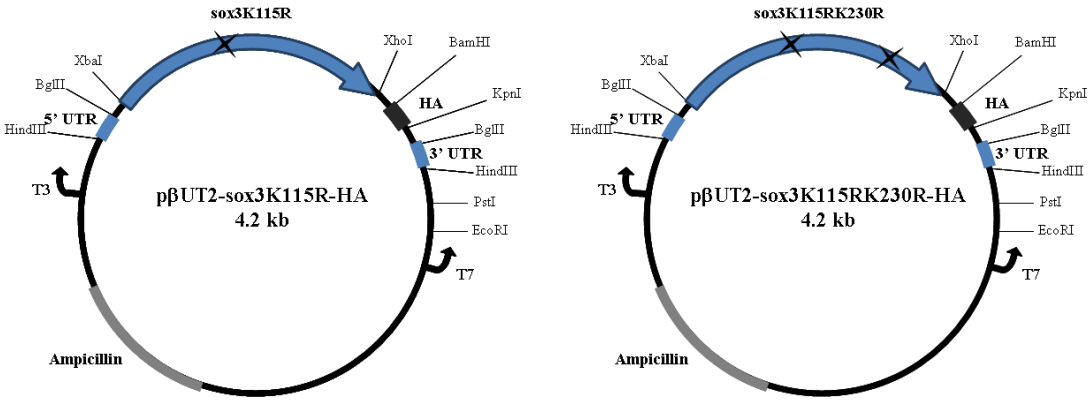


Figure 4.5 Generation of Sox3K115R and Sox3K115RK230R expressing constructs

(A) Primers were designed for site-directed mutagenesis which gave rise to an A→G change at position 344 on *wtsox3* or *sox3K230R*, giving arise to *sox3K115R* or *sox3K115RK230R* respectively. (B) The construct maps of pbut2-*sox3K115R*-HA and pbut2-*sox3K115RK230R*-HA. The crosses indicated the lysine to arginine mutations.

for site-directed mutagenesis. This changed the nucleotide A to G at position 344 which gave rise to K115R mutation (Figure 4.5 B). pβut2-*wtsox3*-HA or pβut2-*sox3K230R*-HA were used as template for PCR amplification. Following the *DpnI* enzyme digestion to remove the template plasmids, competent cells then transformed with the PCR product. Several colonies were selected for DNA sequencing. The constructs pβut2-*sox3K115R-HA* and pβut2-*sox3K115RK230R*-HA were gained after DNA plasmind extraction and confirmed by DNA sequencing (Figure 4.5 B). I next set out to determine the critical SUMOlation site(s) by looking into their abilities in being SUMOylated.

4.3.2 The generation of pGloMyc-SUMO2 construct

To investigate the SUMOylation of Sox3 in zebrafish embryos, I intended to overexpress wildtype or mutant Sox3 with or without co-expressed SUMO peptides. I generated a Myc-tagged SUMO2 expressing constructs by subcloning the *sumo2* sequence from a pcDNA3 vector to a pGlomyc vector which contains a myc tag (Figure 4.6). First of all, a linear pGlomyc vector was prepared by restriction enzyme digestion on pGlomyc-*mGrg5*. The linear pGlomyc vector was gained from gel extraction. Meanwhile, primers having the restriction sites *NotI* or *XhoI* at 5'end were designed for PCR amplification of human *sumo2*. pcDNA3-HA-*sumo2* was used as the template. The PCR product was run on a TAE gel and the *sumo2* fragment was isolated by gel extraction. *sumo2* was then ligated to pJET vector and competent cells transformed. A colony containing the pJET-*sumo2* construct was identified after DNA sequencing. That colony was selected for further amplification. The *sumo2* sequence was then subcloned from pJET-sumo2 to the linear pGlomyc vector by double digestion of *NotI* and *XhoI* followed by ligation. A colony containing the

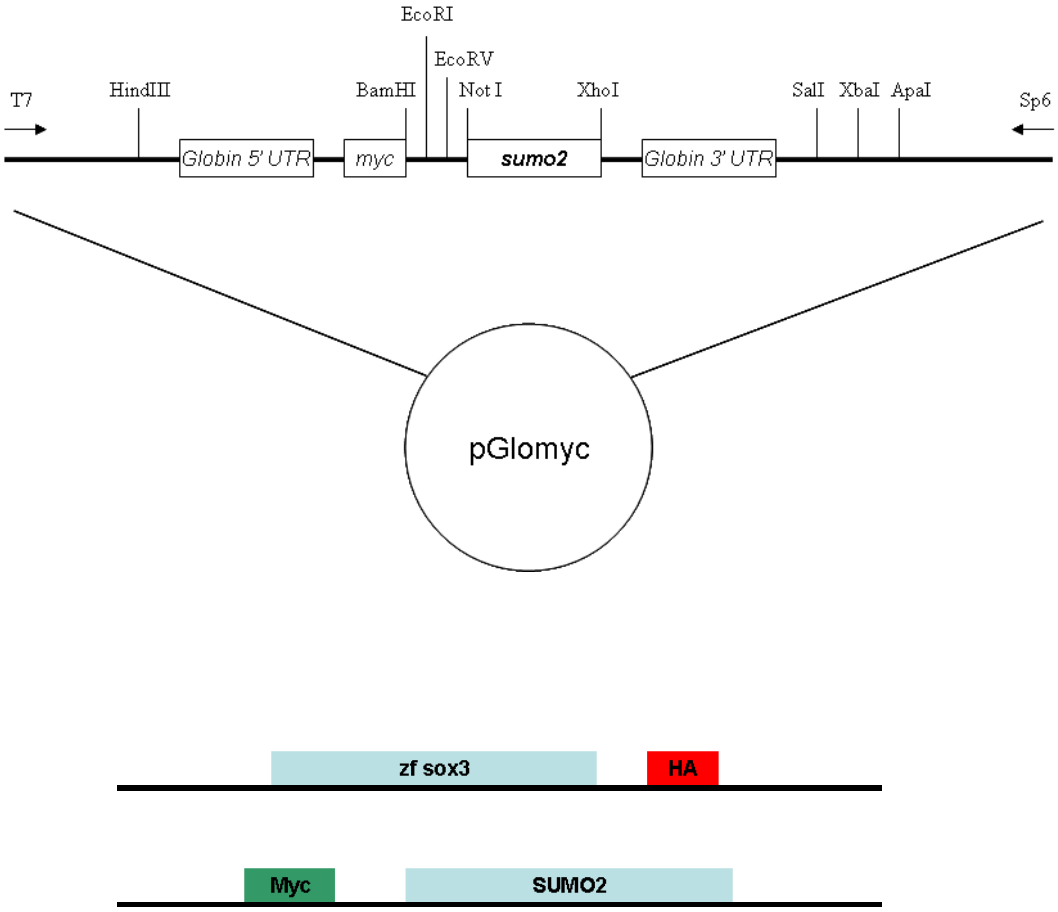


Figure 4.6 Generation of pGlomyc-SUMO2
The map showed the pGlomyc vector expressing Myc-tagged SUMO2 protein. The *sumo2* sequence was subcloned from pcDNA-*sumo2*. The diagram below showed that various forms of HA-tagged Sox3 with or without Myc-tagged SUMO2 would be overexpressed in embryos in the following experiments.

pGlomyc-*sumo2* was identified and selected for plasmid DNA extraction and verified by DNA sequencing.

In the following experiment, various forms of HA-tagged Sox3 with or without Myc-tagged *sumo2* would be overexpressed in embryos, followed by Western blot analysis for the protein expression.

4.3.3 Determination of the critical SUMOylation site(s)

To test whether SUMOylation can happen on Sox3 in zebrafish embryos, embryos were injected with *wtsox3-HA*, *sox3K115R-HA*, *sox3K230R-HA* or *sox3K115RK230R-HA* with or without *Myc-sumo2*. Embryos were then collected and dechorionated at 6.5 hpf or 16 hpf. Embryos were lysed and protein was extracted for western blot analysis. The protein expression of wtSox3-HA, Sox3K115R-HA, Sox3K230R-HA or Sox3K115RK23oR-HA was detected by anti-HA and Myc-SUMO2 was detected by anti-Myc.

At 6.5 hpf, all of the samples showed a bright red band at about 40 kDa, which is the size of HA-tagged Sox3 (Figure 4.7). In *wtsox3-HA* or *sox3K115R-HA* injected samples, there was clear double red bands at about 60 – 62 kDa. However, these double red bands were not apparent in the samples injected with *sox3K230R-HA* or *sox3K115RK230R-HA*. As expected, the Myc-tagged SUMO2 was expressed only in the samples co-injected with *myc-sumo2*. Myc-SUMO positive proteins appeared as a smear with some apparent bands. Interestingly, there was a slight yellow band at about 64 kDa on *wtsox3-HA* with *myc-sumo2* or *sox3K115R-HA* with *myc-sumo2* injected sample, while it did not appear in *sox3K230R-HA* with *myc-sumo2* or *sox3K115RK230R-HA* with *myc-sumo2* injected sample. The yellow band indicated immunoreactivity to both anti-HA and anti-Myc, suggesting that is was the

SUMOylated Sox3. The absence of yellow band in *sox3K230R* or *sox3K115RK230R* injected sample suggested that K230R mutation was likely to prevent Sox3 from being SUMOylated. The K115R mutation, however, did not prevent SUMOylation on Sox3. In the *sox3K230R* or *sox3K115RK230R* injected sample, the absence of double bands at 60 – 62 kDa, which appeared in wtsox3 or sox3K115R injected samples, indicated the possibility that K230 could be subjected to other post-translational modification(s) other than SUMOylation. It is also possible that they are the Sox3 SUMOylated with endogeneous SUMO peptides. Interestingly, these double bands appeared as a single band at about 60 kDa while *sumo2* was co-overexpressed and the yellow band appeared in wtSox3 or Sox3K115R expressing samples. The same pattern was also shown in Sox3K115R mutant. It can be speculated that the 62 kDa band could possibly be the exogeneous Sox3 modified by the endogeneous SUMO peptides. While exogeneous SUMO2 was overexpressed, it substituted the endogeneous SUMO peptides and modified the exogeneous wtSox3 or Sox3K115R, giving rise to the appearance of the yellow band at 64 kDa.

At 16 hpf, there was no apparent yellow band indicating the possible SUMOylation of Sox3 (Figure 4.7). Other modifications, however, did occur on Sox3 as indicated by the multiple red bands between 40 – 60 kDa.

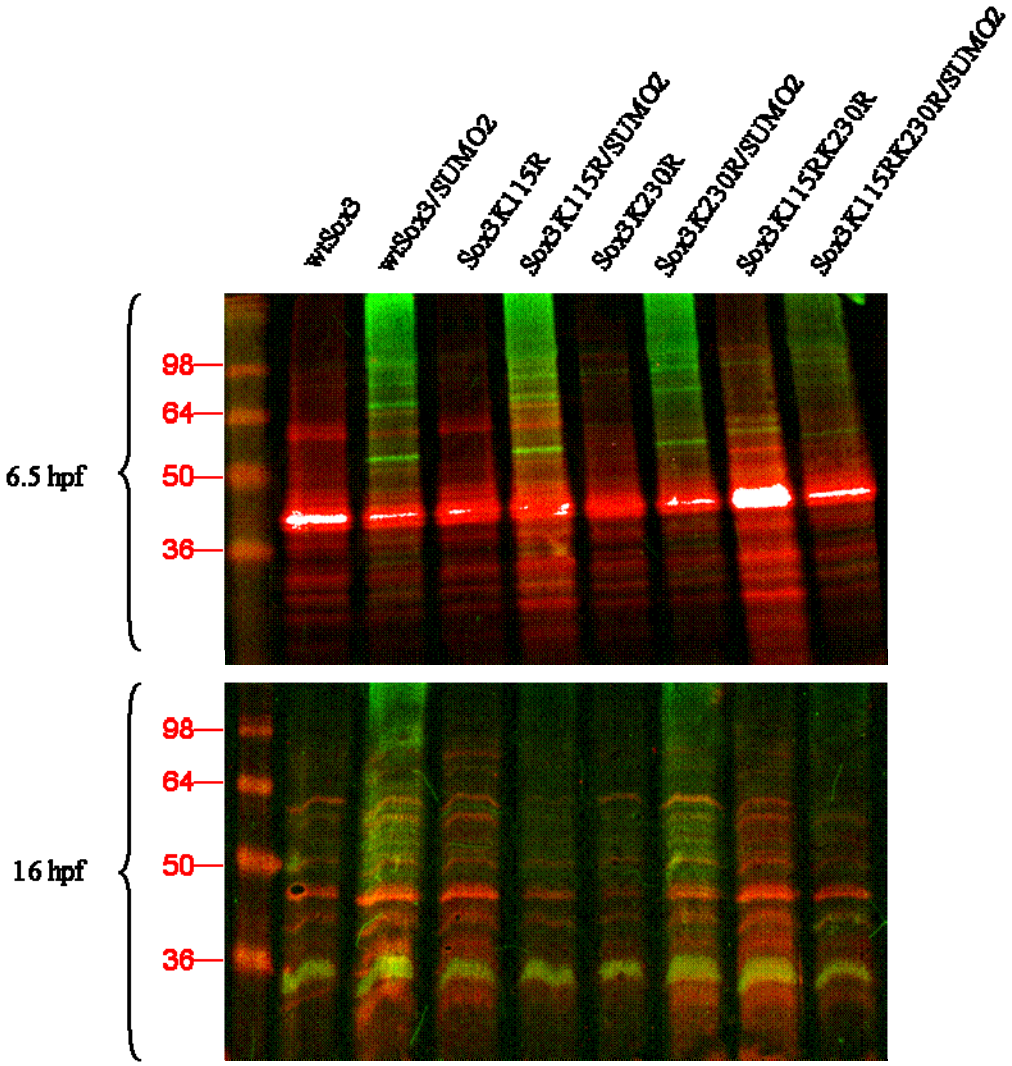


Figure. 4.7 The expression of HA-tagged Sox3 and Myc-tagged SUMO2 at 6.5 hpf and 16 hpf

Embryos were injected with mRNAs of *wtsox3-HA*, *sox3K115R-HA*, *sox3K230R-HA*, *sox3K115RK230R-HA* with or without *Myc-sumo2*. They were collected at 6.5 hpf or 16 hpf. Embryos were deyolked and lysed in SDS sample buffer, followed by western blot analysis. The protein expression of wtSox3, Sox3K115R, Sox3K230R and Sox3K115RK230R were detected with anti-HA as first antibody, anti-rabbit as secondary antibody and appeared in red. The SUMO2 was detected with anti-Myc as first antibody, anti-mouse as secondary antibody and appeared in green.

4.3.4 The SUMOylation of Sox3

In order to confirm SUMOylation of Sox3 occurs *in vivo,* I attempted to demonstrate this by performing immunoprecipitation and mass spectroscopy. The strategy for immunoprecipitation analysis was to overexpress HA-tagged wtSox3 and Myc-tagged SUMO2 in zebrafish embryos, followed by immunoprecipitation using HA reactive agarose beads and examined whether Myc-tagged SUMO2 could be detected.

In this experiment, embryos were injected with *wtsox3* with or without *sumo2*. They were collected and dechorionated at 6.5 hpf. They were then lysed with Lysis-RIPA buffer and mixed with anti-HA beads, followed by shaking overnight in 4°C. After washing with PBS, the lysate was washed with HA peptides overnight. After performing IP, the supernatant was then collected for western blot. The wtSox3 overexpressing sample was analysed with anti-Sox3 or anti-HA. SUMO2 was detected with anti-Myc. Unfortunately, the results of the immunoprecipitation experiments did not show the SUMOylation of Sox3. I tried to isolate the HA-tagged proteins in several attempts but did not succeed in isolating the Sox3-HA, or Sox3-HA was detected but with no Myc-SUMO signal.

On the other hand, I also set out to do the mass spectroscopy analysis. Embryos were injected with 100pg *wtsox3* or 100pg *sox3K230R*. 150 embryos each were collected and lysed at 6.5 hpf. Immunoprecipitation was then carried out to isolate the injected HA-tagged wtSox3 or Sox3K230R. Afterwards, one third amount of each sample was used in SDS-PAGE followed by Western blot assay (Figure 4.8). The Western blot result showed that both wtSox3 and Sox3K230R were detected on Western blot, suggesting that the isolation of proteins in immunoprecipitation was successful. The rest of the samples were then sent for mass spectroscopy analysis. I intended to look into the modification difference between wtSox3 and Sox3K230R. However, the

mass spectrometry result did not show the appearance of SUMO-modified Sox3 (data not shown). Due to technical difficulties, such as isolation of target protein and detection of it on Western blot, this experiment failed to demonstrate the SUMOylation of Sox3.

However, the 58 kDa band shown in Figure 4.2 and the yellow band shown in Figure 4.7 suggested that SUMOylation on Sox3 is likely to have occured. The fact that the loss of SUMO mutant Sox3K230R did not show the putative SUMOylation form also supports this idea. Taken together with the previous results on chick Sox3 and mouse Sox3, on balance, it appears very likely that Sox3 can also be SUMOylated in zebrafish. I therefore set out to inspect the biological effects of SUMOylation of Sox3 using the same mutants described above.

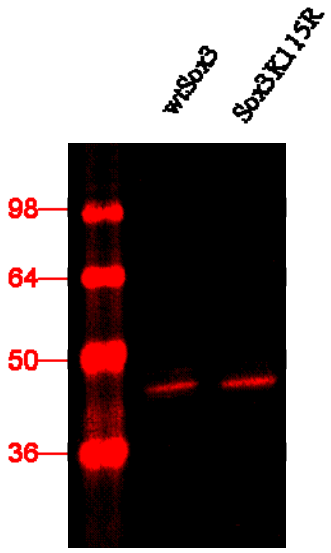


Figure. 4.8 The expression of wtSox3 and Sox3K115R
Embryos were injected with *wtsox3* of *sox3K115R*. Immunoprecipitation was performed. Samples prepared for mass spectrometry analysis were detected with anti-HA on western blot.

4.4 The biological effects of SUMOylation of Sox3

From the previous sections, although SUMOylation of Sox3 has not been categorically demonstrated in zebrafish, on the basis of the evidence it seems likely that SUMOylation of Sox3 occurs in zebrafish and that the K230R mutant will cause loss of that SUMOylation (Figure 4.7) (Laghari, 2010). Here I set out to investigate the biological effects of Sox3 SUMOylation using the wtSox3, Sox3K230R and a series of Sox3K230R-SUMO fusions on zebrafish embryos. Sox3K230R was used as a non-SUMOylatable form of Sox3 as K230 was suggested to be the exclusive SUMOylation site in zfSox3 according to former results (Figure 4.7). Sox3K230R-SUMO1/2/3 fusion proteins are believed to mimic the SUMOylated Sox3. It has been shown the SUMO fusion proteins of Sox9 and Sox10 mimic the constitutive SUMOylation. It has been claimed that the di-glycine epitope can link to a epsilon amino group of lycine residue, and thereby form the protein aggregates (Xu and Jaffrey, 2009). Since the di-glycine motif is present in the Sox3-SUMO fusion proteins, SoxK230R instead of wtSox3 was used to generate the Sox3K230R-SUMO proteins in order to prevent protein aggregation caused by diglycine-lysine modification if there is any. The Sox3K230R-SUMO1/2/3 expressing constructs were previously generated in our lab. They have a SUMO1/2/3 peptide fused to the C-terminus of the Sox3K230R expressing protein. In the following study, wtSox3, Sox3K230R and Sox3K230R-SUMO1/2/3 were overexpressed in zebrafish embryos, expression of various marker genes at certain stages were analysed afterwards. On the other hand, the morphology and mortality of embryos after overexpression of different forms of Sox3 were also observed. Finally I looked into their effects at transcription level by inspecting the *boz* and *gsc* promoters.

4.4.1 The effects of SUMOylation of Sox3 on its ability to repress the expression of organizer genes

As introduced earlier, *gsc* and *chd* are markers of the organizer. *boz* is the earliest marker of the organizer in zebrafish. It encodes a homeodomain protein bozozok/dharma. *boz* is one of the earliest zygotic genes expressed. It is activated by Wnt/β-catenin pathway (Fekany et al., 1999). In the early blastula, bozozok functions as a transcriptional repressor and negatively regulates *bmp2b*, contributing in the dorsal-ventral asymmetry expression of Bmp signaling (Leung et al., 2003). *sqt* encodes a Nodal-related protein, a member of TGF-β superfamily. It is a maternal gene expressed at the dorsal blastoderm and dorsal yolk syncytial layer (YSL) at early blastula stage. Its initial expression is dependent on the maternal Wnt signal (Feldman et al., 1998). Together with *boz*, *sqt* initiates organizer formation and triggers the expression of other organizer genes such as *gsc* and *chd* (Shimizu et al., 2000). Yet *boz* and *sqt* act parallelly to contribute to organizer formation and mesoderm induction (Sirotkin et al., 2000). In this section, I analyzed the expression of *boz*, *gsc*, *chd* and *sqt* under the overexpression of various forms of Sox3.

To investigate the effect of SUMOylation of Sox3 on these organizer genes, embryos were injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at the 1-2 cell stage. Embryos were collected and fixed at dome stage (~5.5 hpf). *in situ* hybridization was performed. The expression of these genes was detected using digoxigenin-labelled *boz*, *gsc* or *sqt* probes or flu-labelled *chd* probe.

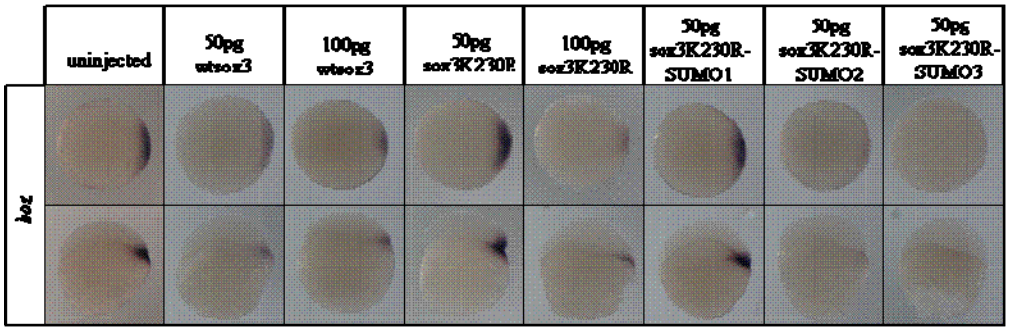
boz was expressed in dorsal mesoderm at dome stage as shown in Figure 4.9. wtSox3 inhibited *boz* in 18% or 32% of embryos at low (50pg) or high (100pg) dosage. Sox3K230R did not inhibit *boz* at all at low dosage, whereas at high dosage, it

repressed *boz* in 26% of embryos. Sox3K230R-SUMO1 did not have any effect on *boz* expression, while Sox3K230R-SUMO2 and Sox3K230R-SUMO3 repressed *boz* expression in 30% or 63% of embryos. Thus Sox3K230R-SUMO2/3 overexpression resulted in an increased extent of repression level on *boz*, compared to those embryos injected with wtSox3 or Sox3K230R. Sox3K230R-SUMO3 caused almost complete loss of *boz* expression (Figure 4.9).

sqt was expressed as a ring-shaped around the embryos throughout the presumptive mesoderm. wtSox3 did not inhibit *sqt* expression at low dosage and inhibited *sqt* expression in 19% of the embryos at high dosage, as shown by the gap on the circular expression pattern of *sqt* (Figure 4.10). Sox3K230R and Sox3K230R-SUMO1 did not exhibit any effect on the embryos at all. Sox3K230R-SUMO2 and Sox3K230R-SUMO3, however, gave rise to striking repression on *sqt* expression. They cause slight repression of *sqt* in 58% or 5% of embryos respectively, and strong repression of sqt in 42% or 95% of embryos respectively.

The results above showed that the constitutively expressed SUMO-modified Sox3 repressed *boz* and *sqt* in a greaer extent than wtSox3 did. In addition, Sox3K230R gave very slight or no effect on their expression. These results indicated that SUMOylation of Sox3 by SUMO2 or SUMO3, but not SUMO1, enhanced the repressor function of Sox3 in regulating *boz* and *sqt.* While SUMOylation was prevented by K230R, Sox3K230R lost its repression ability on *boz* and *sqt* expression.

(A)



(B)

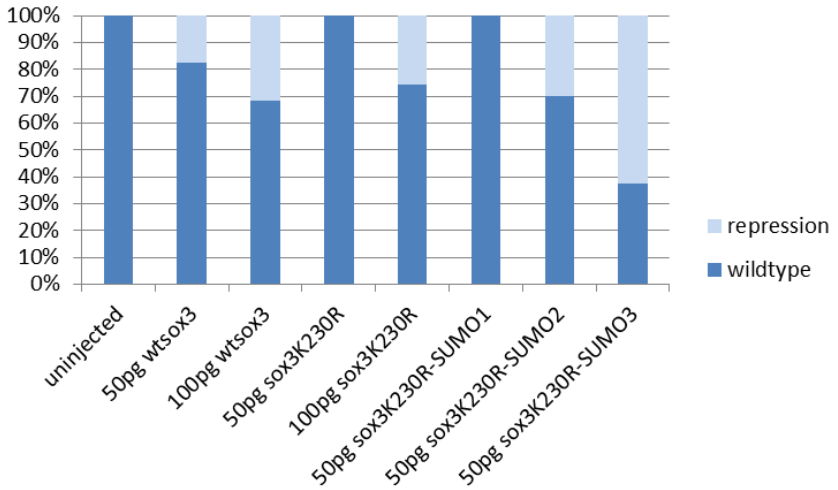
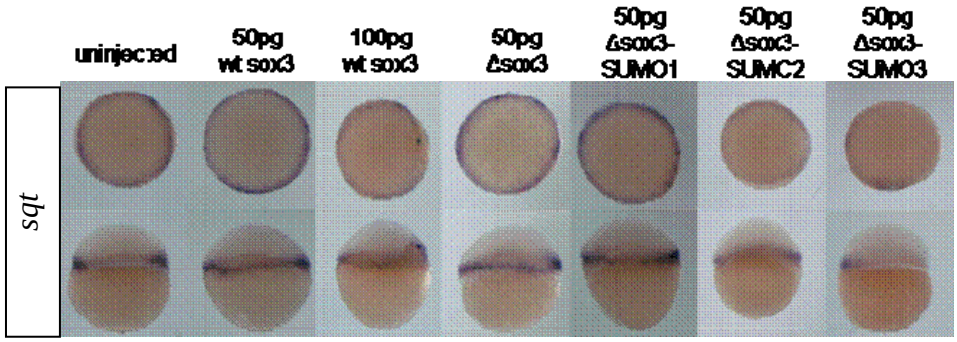


Figure 4.9. The effect of SUMOylation of Sox3 on *boz* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at dome stage (~5.5 hpf). The expression of organizer gene *boz* was examined. (A) *in situ* hybridization results showed the *boz* expression on embryos with different injections. (B) precentage of embryos with different range of *boz* expression.

(A)



(B)

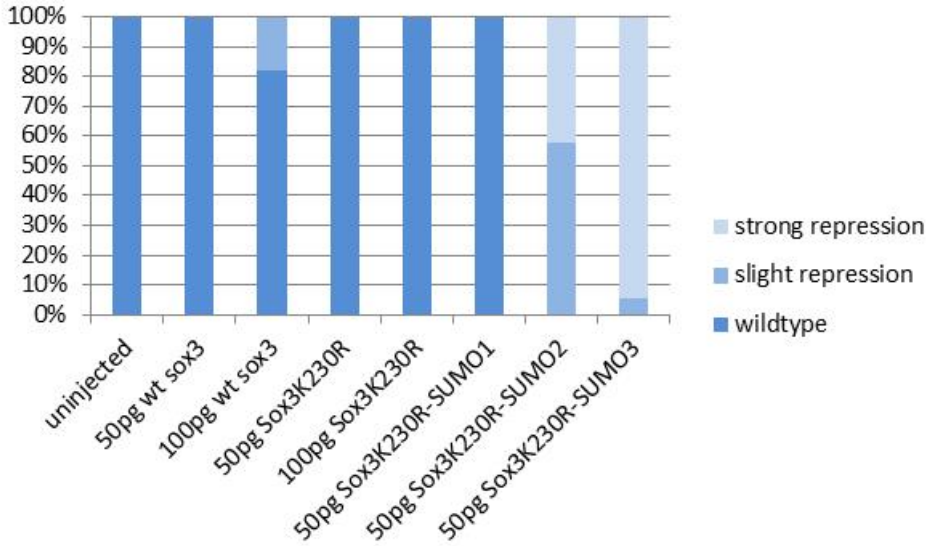
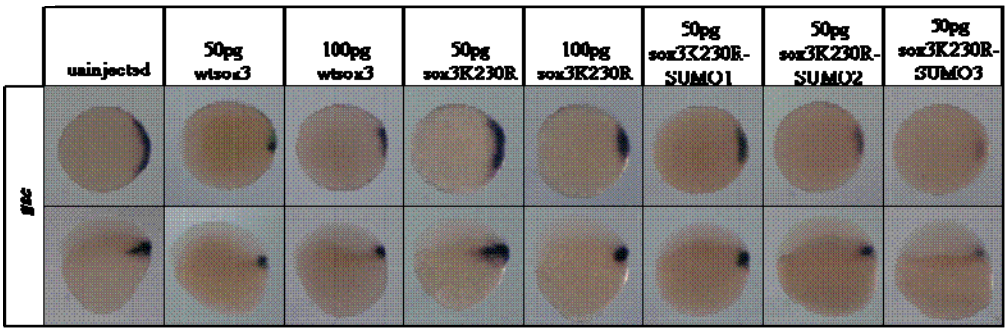


Figure 4.10. The effect of SUMOylation of Sox3 on *sqt* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-2 cell stage. They were collected at dome stage (~5.5 hpf). The expression of organizer gene *sqt* was examined. (A) *in situ* hybridization results showed the *sqt* expression on embryos with different injections. (B) precentage of embryos with different range of *sqt* expression.

gsc is also expressed in dorsal mesoderm, appearing like a quarter of a ring at dome stage. Injection of different forms of Sox3 caused repression on *gsc* to different extents. Repression of *gsc* was classified as repression, slight repression or wildtype (Figure 4.11). wtSox3 at low dosage caused slight repression on gsc in 21% of embryos and repression in 3% of embryos. At high dosage, wtSox3 gave rise to repression of *gsc* expression in 35% of embryos. Sox3K230R at low dosage slightly repressed *gsc* in only 10% of embryos, while at high dosage it caused slight repression on *gsc* in 22% of embryos and strong repression in 6% of embryos. Overexpression of Sox3K230R-SUMO1 resulted in slight inhibition of *gsc* in 38% of embryos and strong inhibition in 27% of embryos. Sox3K230R-SUMO2 led to slight repression of *gsc* in 35% of embryos and strong repression in 47% of embryos. All of the embryos exhibited *gsc* suppression in Sox3K230R-SUMO3 overexpressing embryos. 78% of them showed severe *gsc* repression and the rest of them showed mild repression (Figure 4.11).

chd is expressed in dorsal mesoderm as shown before. Neither wtSox3 nor Sox3K230R at low dosage had a notable repressing effect on *chd* (Figure 4.12). However, at high dosage, wtSox3 repressed *chd* in 23% of embryos while Sox3K230R repressed *chd* in 18% of embryos. *chd* expression was restricted at the dorsal most area under this repression. *sox3K230R-sumo1* injection had no effect on *chd* expression. Sox3K230R-SUMO2 and Sox3K230R-SUMO3 both repressed *chd* to a great extent. *chd* expression was almost entirely inhibited in 60% or 70% of embryos respectively (Figure 4.12).

(A)



(B)

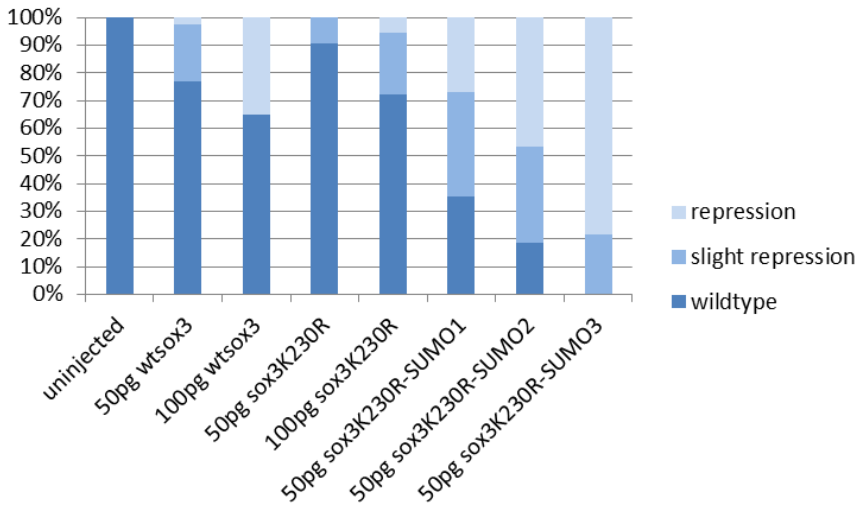
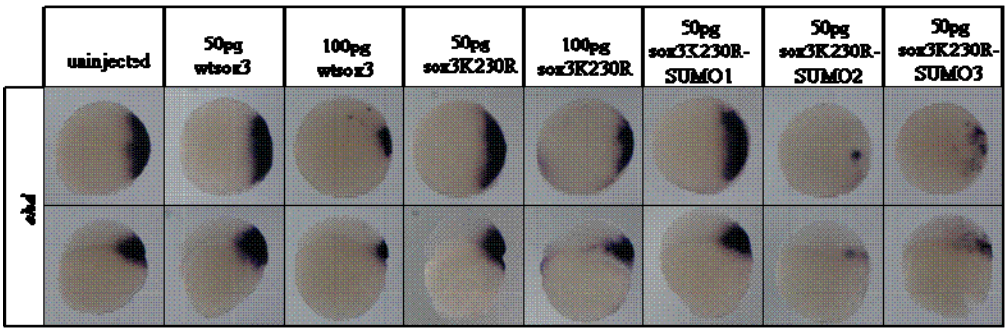


Figure 4.11. The effect of SUMOylation of Sox3 on *gsc* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at dome stage (~5.5 hpf). The expression of organizer gene *gsc* was examined. (A) *in situ* hybridization results showed the *gsc* expression on embryos with different injections. (B) precentage of embryos with different range of *gsc* expression.

(A)



(B)

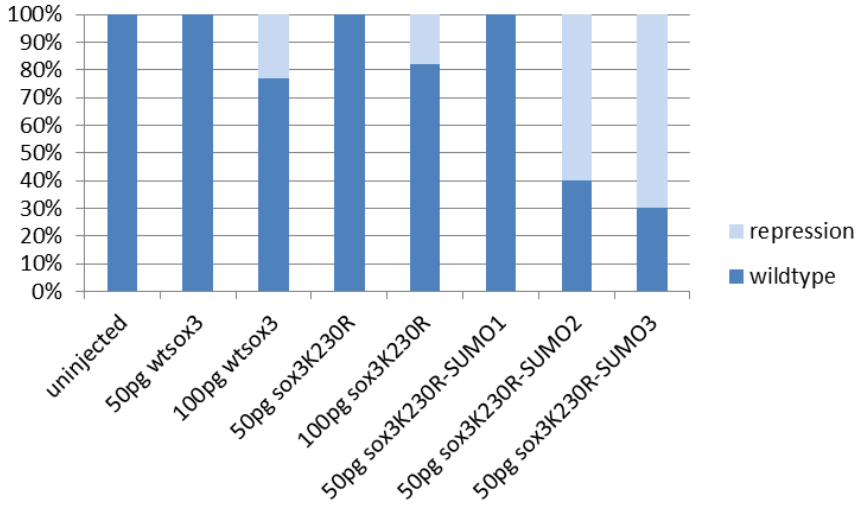


Figure 4.12. The effect of SUMOylation of Sox3 on *chd* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at dome stage (~5.5 hpf). The expression of organizer gene *chd* was examined. (A) *in situ* hybridization results showed the *chd* expression on embryos with different injections. (B) precentage of embryos with different range of *chd* expression.

4.4.2 The effects of SUMOylation of Sox3 on its ability to repress the expression of mesoderm genes

Apart from the dorsal organizer genes, Sox3 can also repress the expression of mesoderm genes (Shih et al., 2010). Therefore, I set out to investigate the influence of SUMOylation of Sox3 on mesoderm development. In the following experiments, I explored the expression of three mesoderm markers, *sp5* transcription factor-like (*sp5l*), no tail (*ntl*) and floating head (*flh*) following the overexpression of wtSox3, Sox3K230R or Sox3K230R-SUMO1/2/3.

sp5l encodes for a sp5-like, zinc-finger transcription factor. It is expressed in the hypoblast and epiblast during early gastrulation. It is then expressed in mesodermal and neural tissue at later stages. Sp5l acts downstream of Fgf signaling and has an early and important role in mesoderm induction. It can induce the expression of *ntl* (Zhao et al., 2003). It is also a direct target of Wnt signaling in mesoderm and neuroectoderm patterning (Weidinger et al., 2005).

To examine whether SUMOylation of Sox3 has a role in *sp5l* expression, gain-of-function experiment on zebrafish embryos was carried out. Embryos were injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-2 cell stage. Embryos were then collected and fixed at 50% epiboly stage (~6.5hpf). *sp5l* expression was analyzed using digoxigenin labelled *sp5l* probe. As shown in Figure 4.13, the expression pattern of *sp5l* in uninjected embryos is very similar to *ntl*. It was expressed in mesoderm around the embryo, appearing as a ring-like pattern. *wtsox3* injection caused repression of *sp5l* in 43% of embryos at low dosage and 76% of embryos at high dosage. Sox3K230R led to repression on *sp5l* in only 7% or 33% of embryos at low or high dosage respectively. Embryos overexpressing Sox3K230R-SUMO1 mostly displayed the wildtype uninjected

phenotype. Only 2% of them exhibit repression of *sp5l*. On the other hand, Sox3K230R-SUMO2 overexpression caused 67% of embryos to show repression of *sp5l*. Among those injected with *sox3K230R-sumo3*, 97% has repression on *sp5l*.

ntl encodes a T-box transcription factor. It is a homologue of the mouse brachyury gene (Schulte-Merker et al., 1994). *ntl* is a early response to mesoderm induction. It is critical for mesoderm patterning, especially the development of medial floor plate, trunk and tail mesoderm. It also directs the expression of *flh*, another mesoderm gene. Ntl cooperates with Flh in regulating the development of notochord. Previous study suggested that Sox3 restricted *ntl* expression as well as organizer formation (Shih et al., 2010). Here I looked into the effect of SUMOylation of Sox3 over the expression of ntl.

Embryos were injected with mRNAs of *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-2 cell stage. Embryos were then collected and fixed at 50% epiboly stage (~6.5hpf). The expression of *ntl* was examined by *in situ* hybridization with fluorescence-labelled *ntl* probe. *ntl* is expressed in the germ ring cells at this stage in wildtype uninjected embryos. wtSox3 overexpression inhibited normal *ntl* expression (Figure 4.14). In wildtype uninjected embryos, *ntl* was expressed presumtive mesoderm, appearing a ring-shaped pattern. Inhibition of *ntl* by Sox3 caused a gap on the *ntl* expressing ring, forming a C-shaped pattern. At low dosage, wtSox3 caused slight repression in 34% of embryos and strong repression in 3% of embryos. At high dosage, *ntl* was slightly inhibited in 48% of embryos and largely inhibited in 20% of embryos. Sox3K230R also inhibited *ntl* expression. 38% of embryos exhibited slight repression at both dosages. 8% or 13 % of embryos exhibited strong repression at when Sox3K230R was overexpressed at low or high dosage. All of the *sox3K230R-sumo1* injected embryos expressed normal

ntl expression pattern. However, Sox3K230R-SUMO2 caused 56% of embryos to exhibit slight repression and 18% with strong repression. *sox3K230R-sumo3* injection resulted in repression on *ntl* to very large extent. 18% embryos exhibited slight repression and the rest of them exhibited strong repression on *ntl*.

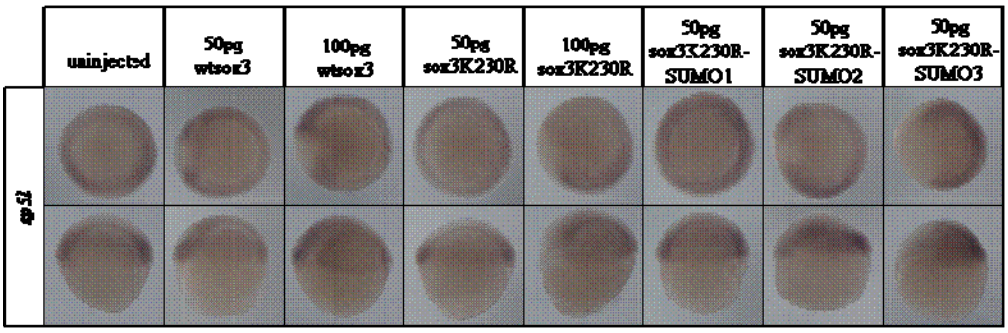
flh is a homeobox gene encoding for a transcription factor. It is responsible for the maintenance of notochord forming during axial mesoderm development (Halpern et al., 1995). On the other hand, Flh takes part in epiphysical neurogenesis in zebrafish by targeting *ash1a* and *neurogenin1*, which encode for transcription factors essential for generation of neurons in epiphysis (Cau and Wilson, 2003).

Embryos were injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-2 cell stage. They were collected and fixed at 50% epiboly stage (~6.5hpf). The expression of flh was analyzed by in situ hybridization with Digoxigenin-labelled *flh* probe. In wildtype uninjected embryos, *flh* was weakly expressed at dorsal mesoderm at this stage (Figrue 4.15). Overexpression of wtSox3 at low or high dosage caused slight repression on *flh* expression in 18% or 19% of embryos. Sox3K230R at low dosage caused repression of flh in 7% of embryos, while at higher dosage it caused repression in 24% of embryos. *sox3K230R-sumo2* and *sox3K230R-sumo3*, but not *sox3K230R-sumo1*, gave rise to strong repression on *flh*. *sox3K230R-sumo1* injected embryos showed exactly the same phenotype as uninjected embryos. In contrast, 24% showed slight repression and 11% showed strong repression of *flh* in *sox3K230R-sumo2* injected embryos. For the embryos injected with *sox3K230R-sumo3*, 18% displayed slight repression and 33% displayed strong repression on *flh*.

From the gene expression analysis of *ntl*, *sp5l* and *flh* under the overexpression of various forms of Sox3, it can be suggested that SUMOylation of Sox3 enhanced the

ability of Sox3 in restricting the mesoderm development, in addition with its repressing function on organizer.

(A)



(B)

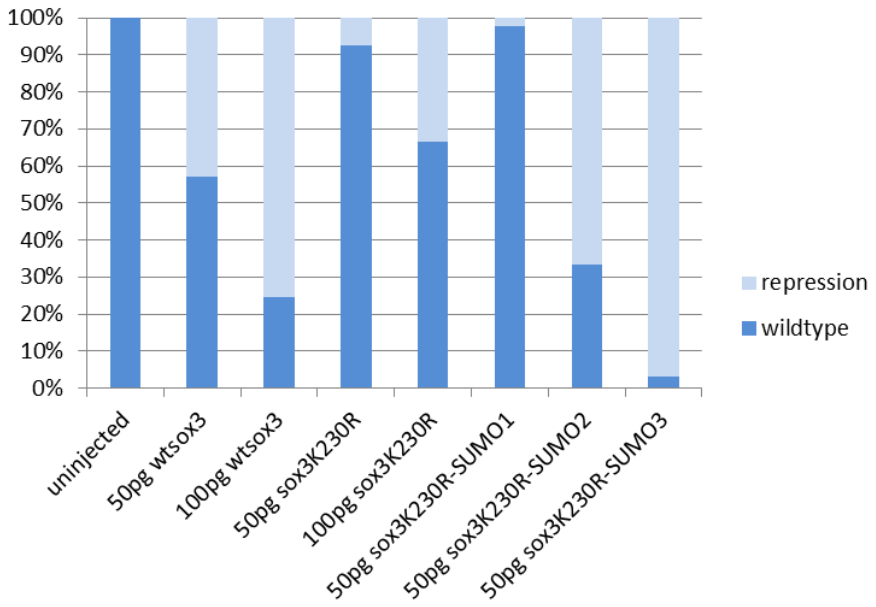
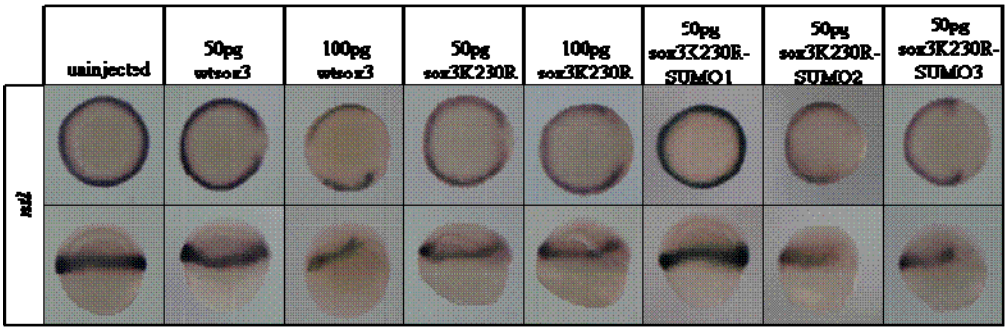


Figure 4.13 The effect of SUMOylation of Sox3 on *sp5l* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at 50% epiboly stage (~6.5 hpf). The expression of organizer gene *sp5l* was examined. (A) in situ hybridization results showed the *sp5l* expression on embryos with different injections. (B) precentage of embryos with different range of *sp5l* expression.

(A)



(B)

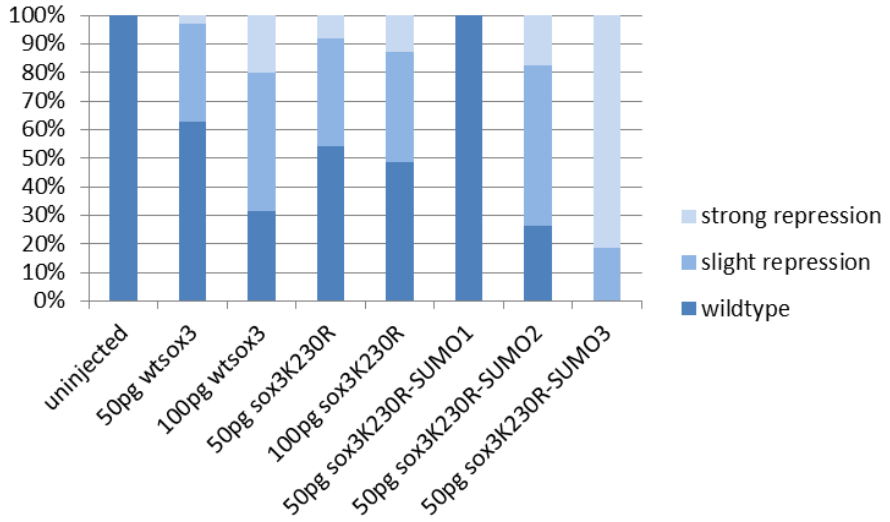
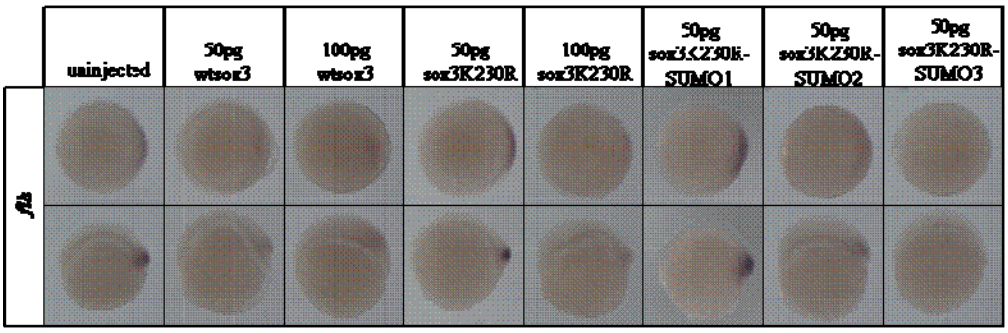


Figure 4.14. The effect of SUMOylation of Sox3 on *ntl* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at 50% epiboly stage (~6.5 hpf). The expression of organizer gene *ntl* was examined. (A) *in situ* hybridization results showed the *ntl* expression on embryos with different injections. (B) precentage of embryos with different range of *ntl* expression.

(A)



(B)

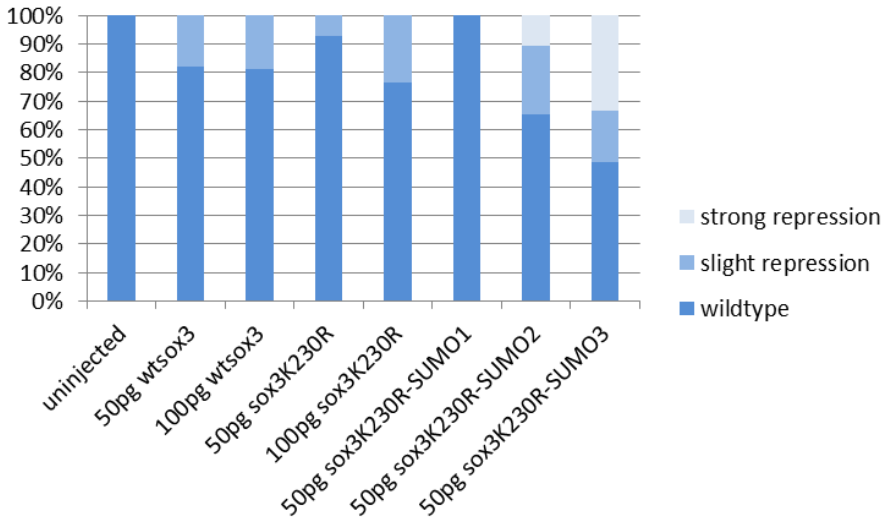


Figure 4.15. The effect of SUMOylation of Sox3 on *flh* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at 50% epiboly stage (~6.5 hpf). The expression of organizer gene *flh* was examined. (A) *in situ* hybridization results showed the *flh* expression on embryos with different injections. (B) precentage of embryos with different range of *flh* expression.

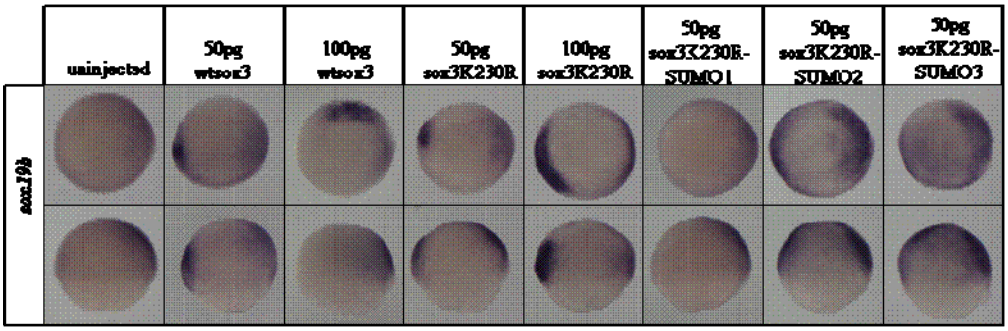
4.4.3 The effects of SUMOylation of Sox3 on neural induction

Previous study from our group has demonstrated that Sox3 also has an important role in neural induction and maintenance of neural progenitor cells (Dee et al., 2008). In early ectoderm development, Sox3 overexpression led to expansion of neural fate markers and inhibition of non-neural fate marker (Dee et al., 2008). At later stages, Sox3 injection caused neural plate expansion as well as duplication of the CNS. In this section, I investigated the effect of SUMOylation of Sox3 on neural fate determination by looking into the expression of a neural fate marker *sox19b*, and the non-neural fate marker *gata2*.

Embryos were injected with mRNA of *wtsox3, sox3K230R, sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-2 cell stage. They were then collected and fixed at 70% epiboly stage (~8 hpf). *In situ* hybridization was performed. The mRNA expression of *sox19b* and *gata2* were detected by using the digoxigenin-labelled *sox19b* or *gata2* probes.

sox19b is expressed in presumptive neural ectoderm (Figure 4.16). Injection of *wtsox3* induced etopic expression of *sox19b*. When wtSox3 was overexpressed at low dosage, *sox19b* repression slightly increased in 46% of embryos and largely increased in 51% of embryos. When wtSox3 was at high dosage, 97% of embryos exhibited strong increase in *sox19b* expression. Sox3K230R at low dosage induced large increase of *sox19b* in 80% of embryos and slight increase in 20% of embryos. All of the *sox3K230R-sumo1* injected embryos exhibited normal *sox19b* expression as uninjected embryos. Both Sox3K230R-SUMO2 and Sox3K230R-SUMO3 caused *sox19b* expansion as well as ectopic expression in a great extent that all of the embryos displayed extensively strong increase of *sox19b* expression.

(A)



(B)

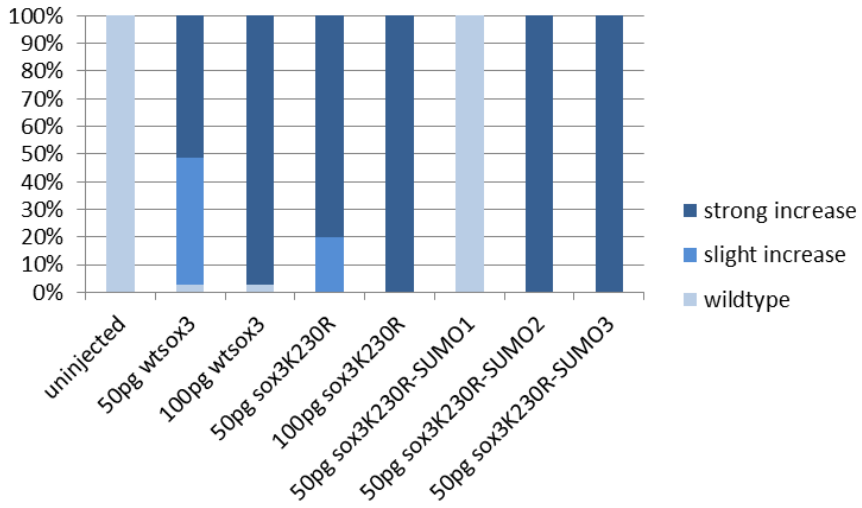
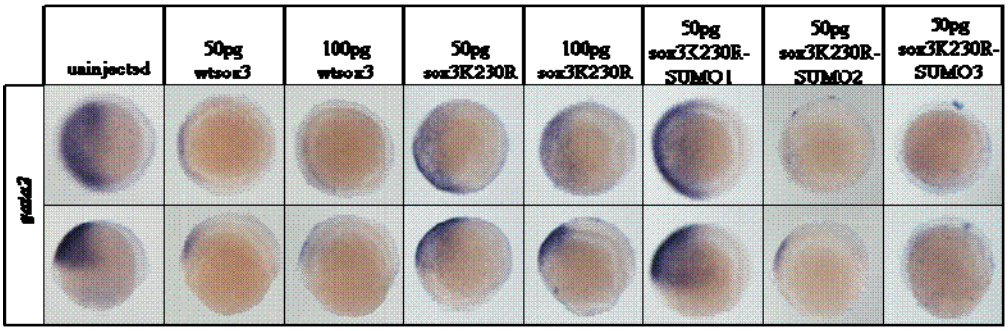


Figure 4.16 The effect of SUMOylation of Sox3 on *sox19b* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at 70% epiboly stage (~8 hpf). The expression of organizer gene *sox19b* was examined. (A) *in situ* hybridization results showed the *sox19b* expression on embryos with different injections. (B) precentage of embryos with different range of *sox19b* expression.

gata2 is expressed in prospective non-neural progenitor cells (Figure 4.17). Injection of *wtsox3* even at low dosage led to a strong suppression of *gata2* in 71% of embryos and slight suppression in 24% of embryos. At high dosage wtSox3 caused strong repression on *gata2* in 87% of embryos and the rest of them exhibited slight repression of *gata2*. Sox3K230R at low or high dosage caused slight repression of *gata2* in 72% or 61% of embryos and strong repression in 14% or 14% of embryos respectively. Sox3K230R-SUMO1 caused only slight repression on *gata2* in 27% of embryos. Sox3K230R-SUMO2 largely suppressed *gata2* in 77% of embryos and slightly suppressed it in 16% of embryos. Sox3K230R-SUMO3 largely suppressed *gata2* in 75% of embryos and slightly suppressed in 25% of embryos.

(A)



(B)

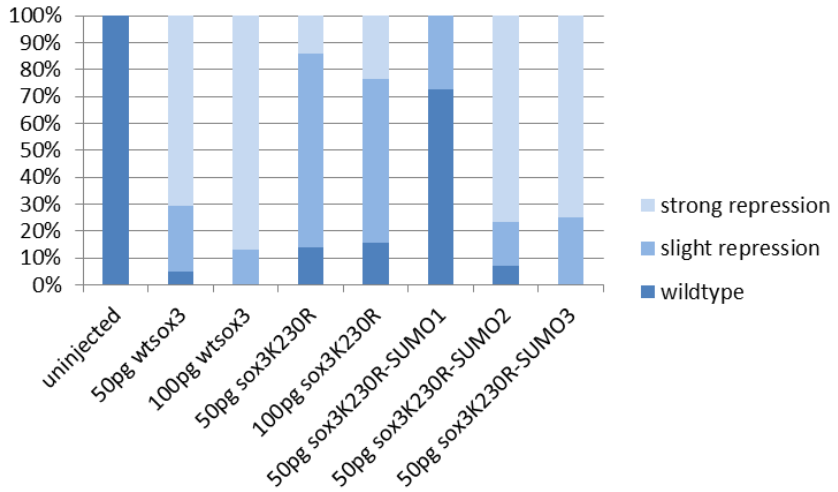


Figure 4.17 The effect of SUMOylation of Sox3 on *gata2* expression
Embryos were uninjected or injected with *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-4 cell stage. They were collected at 70% epiboly stage (~8 hpf). The expression of organizer gene *gata2* was examined. (A) *in situ* hybridization results showed the *gata2* expression on embryos with different injections. (B) precentage of embryos with different range of *gata2* expression.

4.4.4 The effectS of SUMOylation of Sox3 on its ability to disrupt morphology of embryos within 24 hpf

According to the results in earlier sections, Sox3K230R-SUMO1, among those Sox3K230R-SUMO proteins, does not enhance the repressing effect of Sox3, while Sox3K230R-SUMO2 and Sox3K230R-SUMO3 have dramatic repressing effects on a variety of developmental events which were examined by several organizer, mesoderm and neural fate markers. Here I chose sox3K230R-SUMO2 for further study. The effect of *wtsox3*, *sox3K230R* and *sox3K230R-sumo2* on the survival and morphology of embryos within 24 hours after fertilization were observed and compared to the uninjected ones.

At 4.5 hpf, there was no striking difference between injected and uninjected embryos. As shown in Figure 4.18, embryos injected with 50pg *wtsox3* mRNA have the same phenotype as the uninjected ones. When the dosage increased to 100pg, only 1% of embryos died. 5% of embryos overexpressing *sox3K230R* at 50pg died by 4.5 hpf. 3% of the embryos overexpressing 100pg *sox3K230R* died by 4.5 hpf. Embryos injected with 50pg *sox3K230R-sumo2* have 5% mortality rate, higher then 1% mortality rate in those injected with 100pg *sox3K230R-sumo2*. However, these statistic results do not seem to be significant. The mortality rate was possibly due to the artifacts caused by injection.

Embryos survived at 4.5 hpf were collected to proceed to later stages. I observed them at 6.5 hpf, around shield stage. At this stage, *wtsox3* caused obvious morphologic change on the embryos. 74% of 50pg *wtsox3* injected embryos and all of the 100pg *wtsox3* injected embryos had thickening of one side of the blastoderm (Figure 4.19). Embryos overexpressing *sox3K230R* showed the same phenotype. The blastoderm became thickened in 80% or 93% of embryos when *sox3K230R* was

overexpressed at 50pg or 100pg. Sox3K230R-SUMO2 overexpression not only caused the thickening of blastoderm in 85% or 92% of embryos, it also led to the mortality in 2% or 6% of embryos, at low or high dosage respectively.

The surviving embryos were observed again at 10 hpf, when the epiboly finished and the somite formation had started. *wtsox3* at 50pg caused almost no effect. Only 1% of embryo died at this dosage, while *wtsox3* at 100pg caused mortality in 8% embryos and delay in development in the rest of embryos (Figure 4.20). Sox3K230R had almost no effect on the surviving embryos. Only 1% of embryos died under low or high dosage of *sox3K230R*. Hence, about 10% or 78% of embryos injected with 50pg or 100pg *sox3K230R-sumo2* respectively did not survive through this stage. There were also 17% of embryos that had delay in development and 5% had abnormal appearance among the 100pg *sox3K230R-sumo2* injected embryos.

Finally, I looked at their survival and morphology at 24hpf. Among the embryos injected with 50pg *wtsox3* that had survival to 10 hpf, 9% died, 47% of them had abnormal phenotypes, including defects in head, tail and trunk (Figure 4.21). At 100pg, wtsox3 lead to 27% mortality and that the rest of embryos appeared more severe phenotypes. Among *sox3K230R* injected embryos, however, only 18% at low dosage and 42% at high dosage displayed head defects. 7% or 29% of embryos died while Sox3K230R-SUMO2 was overexpressed at low or high dosage. 51% or 71% of embryos exhibited severe phenotypes including head structure defects, incomplete trunk development, shortened or lack of tail under low or high dosage of Sox3K230R-SUMO2 overexpression (Figure 4.21).

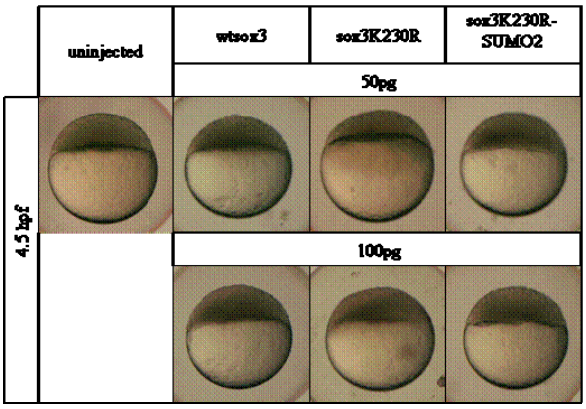


Figure 4.18 The effect of SUMOylation of Sox3 on survival and morphology of zebrafish embryos at 4.5 hpf

The morphology and mortality of the embryos injected with 50pg/100pg mRNAs of *wtsox3*, *sox3K230R* or *sox3K230R-sumo2* were compared with uninjected ones. Embryos with different injections showed the same phenotype at this stage. No significant mortality rate difference had been observed.

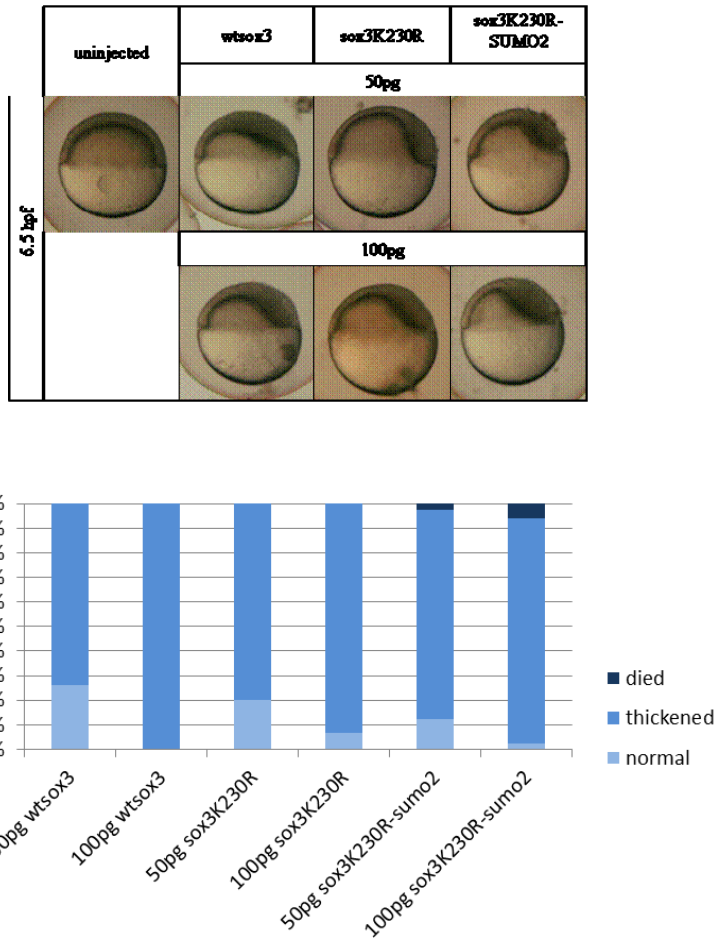
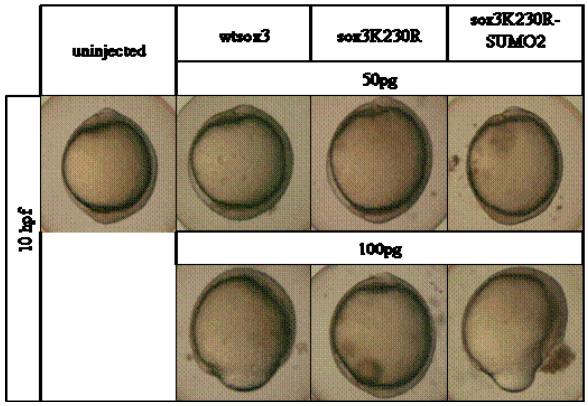


Figure 4.19 The effect of SUMOylation of Sox3 on survival and morphology of zebrafish embryos at 6.5 hpf

The morphology and mortality of the embryos injected with 50pg/100pg mRNAs of *wtsox3*, *sox3K230R* or *sox3K230R-sumo2* were compared with uninjected ones. The precentages of embryos with different phenotype were shown in the graph. The dead embryos from earlier stages are taken out from graph statistics of later stages.



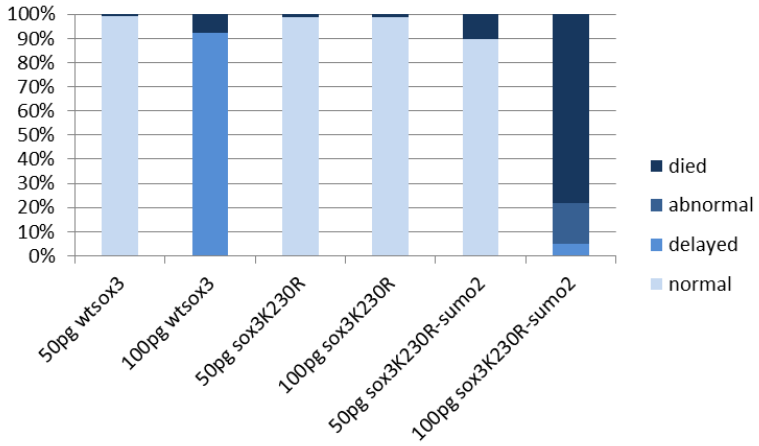
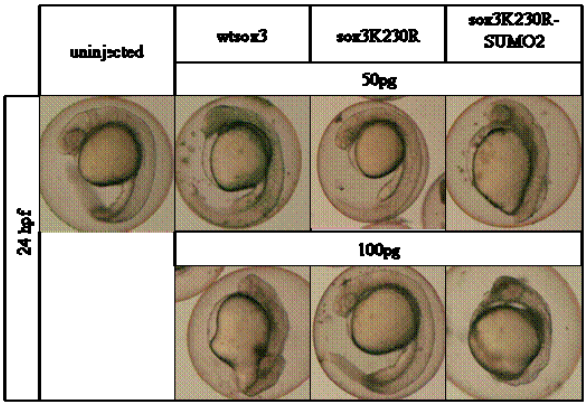


Figure 4.20 The effect of SUMOylation of Sox3 on survival and morphology of zebrafish embryos at 10 hpf

The morphology and mortality of the embryos injected with 50pg/100pg mRNAs of *wtsox3*, *sox3K230R* or *sox3K230R-sumo2* were compared with uninjected ones. The precentages of embryos with different phenotype were shown in the graph. The dead embryos from earlier stages are taken out from graph statistics of later stages.



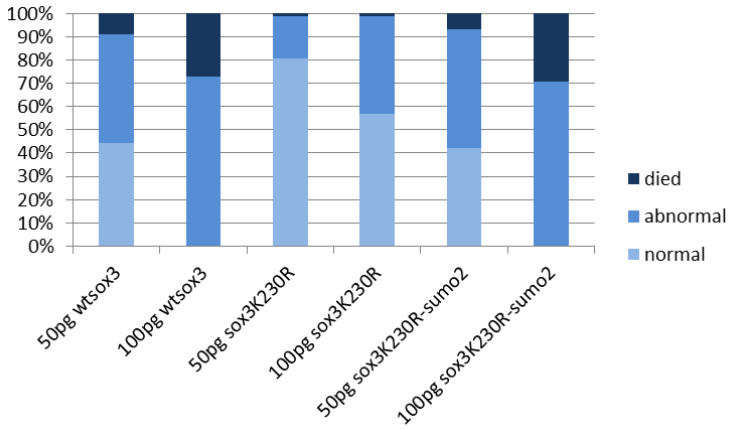


Figure 4.21 The effect of SUMOylation of Sox3 on survival and morphology of zebrafish embryos at 24 hpf

The morphology and mortality of the embryos injected with 50pg/100pg mRNAs of *wtsox3*, *sox3K230R* or *sox3K230R-sumo2* were compared with uninjected ones. The precentages of embryos with different phenotype were shown in the graph. The dead embryos from earlier stages are taken out from graph statistics of later stages.

4.4.5 The effects SUMOylation on transcriptional activator activity of Sox3 in a luciferase reporter assay

To investigate the effects of SUMOylation on the ability of Sox3 to directly act on the promoter region of its target genes, I inpected the activity of various Sox3 constructs on the promoter regions of *boz* and *gsc*. The pFlash-*5'boz1.4*-Luc luciferase reporter of the *5'boz1.4* region which contains six potential Sox binding sites was created and examined by Dr. Kuo (Kuo, 2012). It has been found that wtSox3 can activate the luciferase activity driven by pFlash-*5'boz1.4*-Luc reporter. Here I compared the activities of wtSox3, Sox3K230R, Sox3K230R-SUMO1, Sox3K230R-SUMO2 and Sox3K230R-SUMO3 on pFlash-*5'boz1.4*-Luc and pFlash-*5'gsc2.6*-Luc reporters. Although wtSox3 represses *boz* and *gsc in vivo,* it had been shown that wtSox3 drove activation on pFlash-*5'boz1.4*-Luc and pFlash-*5'gsc2.6*-Luc (Kuo, 2012) (Figure 3.14). According to the results in previous sections, it is believed that SUMOylation enhanced the repressing activity of Sox3. Since then, Sox3K230R is believed to diminish its repressing activity or enhance the activating activity, while Sox3K230R-SUMO fusions were believed to have opposite effects.

In this experiment, embryos were injected with pFlash-*5'boz1.4*-Luc or pFlash-*5'gsc2.6*-Luc construct and mRNA of *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, s*ox3K230R-sumo2* or *sox3K230R-sumo3*. Embryos were then collected and lysed at dome stage. Protein lysates were collected for luciferase assay.

Compared to the control in which only the luciferase expressing construct pFlash-*5'boz1.4*-Luc was injected, wtSox3 significantly activates pFlash-*5'boz1.4*-Luc at a low level. None of the Sox3K230R-SUMO fusions had an effect on the pFlash-*5'boz1.4*-Luc reporter, suggesting that SUMOylation of Sox3 eliminilated its

activation activity on pFlash-*5'boz1.4*-Luc. Moreover, Sox3K230R causes a dramatic activation which is 15 times that caused by wtSox3.

On the other hand, I attempted to analyse the effect of SUMOylation of Sox3 on pFlash-*5'gsc2.6*-Luc also. The result, however, did not show any significant effect caused by any of the *sox3* constructs. There were technical difficulties needed to be addressed in this experiment. Unfortunately, due to limit of time, this experiment has not been repeated.

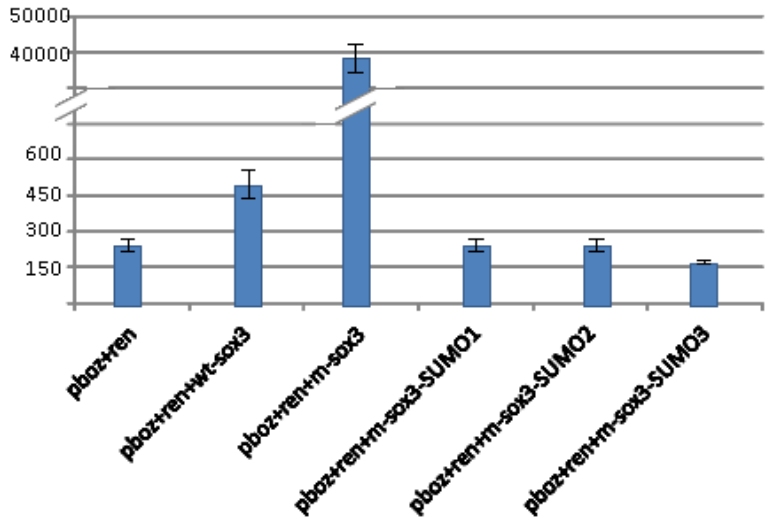


Figure 4.22 The effect of SUMOylation of Sox3 on *pFlash-5'boz1.4-Luc*
Embryos were injected with pFlash-*5'boz1.4*-Luc at 5 pg and indicated mRNAs at 50 pg. They were collected at dome stage, dechorionated and lysed with 5x lysis buffer. The protein lysate were collected by centrifuging and proceeded with luciferase activity analysis. The graph showed the results of triplicates which contained 10 embryos each. The error bars signify the standard deviation.

4.5 The protein expression level of of SUMO constructs

In section 4.4, it was shown that injection of sox3K230R-sumo2 or *sox3K230R-sumo3,* but not *sox3K230R-sumo1,* caused dramatic repression effects compared with *wtsox3* on expression of organizer and mesoderm genes. However, injection of *sox3K230R* caused the loss of function of wtSox3.

It is therefore of interest to investigate whether those phenotypes were due to different protein expression levels of injected mRNAs or caused by the function of proteins.

To examine the protein expression level of mRNAs used in gain-of-function experiments in section 4.4, embryos were injected with the same amount of *wtsox3, sox3K230R, sox3K230R-sumo1, sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-2 cell stage. Embryos were collected and dechorionated at 50% epiboly stage. They were deyolked with deyolking buffer and lysed in SDS sample buffer. The protein samples were then subjected for Western blot analysis.

The result, however, showed that the expression levels were dramatically different among wtSox3, mutant Sox3 and Sox3K230R-SUMO proteins. Sox3K230R was expressed much less than wtSox3. Expression of Sox3K230R-SUMO2 and Sox3K230R-SUMO3 were a lot more than wtSox3, while Sox3K230R-SUMO1 was not detected in this blot at all. This result coincides with the analysis of biological functions in section 4.4. Therefore, this result suggested that the biological effects demonstrated in section 4.4 were possibly largely due to the different protein expression levels of injected mRNAs.

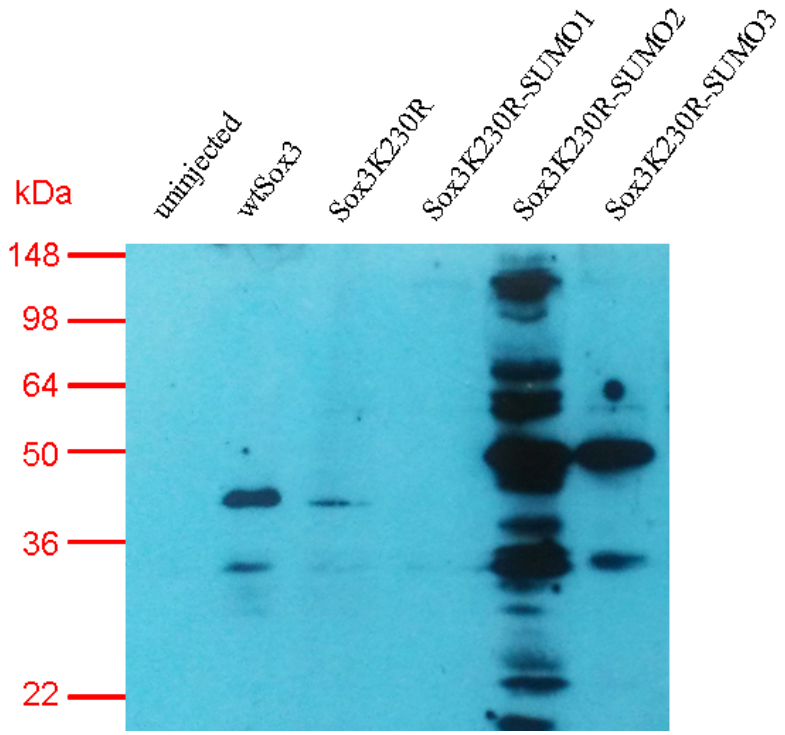


Figure 4.23 Expression level of wtSox3, mutant Sox3 and Sox3-SUMO conjugated proteins

Embryos were injected with 50 pg mRNAs of *wtsox3*, *sox3K230R*, *sox3K230R-sumo1*, *sox3K230R-sumo2* or *sox3K230R-sumo3* at 1-2 cell stage, deyolked and lysed at 50% epiboly stage. Protein samples were analysed by Western blot. 100 embryos were used in each sample. Protein was detected with anti-HA as first antibody and anti-Rabbit-HRP as secondary antibody.

4.6 Discussion

4.6.1 The expression and subcellular distribution of Sox3 in zebrafish embryo

Previous studies showed that Sox3 was expressed as early as 3 hpf (Shih, 2010, Kuo, 2012). There is a large increase in expression level of Sox3 between sphere stage and shield stage (Figure 4.3). This is consistent with the *in situ* hybridization results in previous study (Kuo, 2012). In zebrafish, after the mid-blastula transition at 3 hpf, the zygotic genome activation starts. This results in accumulation of large amount of transcripts followed by their translation. The large increase of Sox3 protein also implied that zygotic Sox3 expression starts at this stage. In fact, Sox3 has been shown to play a critical role in restricting organizer during gastrulation. From shield stage to 75% epiboly stage, there was another dramatic increase on Sox3 expression. During this period, the expression of Sox3 is restricted at the presumptive neural ectoderm. Sox3 has an important role in neural induction as well as maintenance of neural progenitor cells at this stage. In addition, there are multiple modified forms of Sox3 appearing from shield stage on and became more apparent at later stages. Interestingly, at 20 hpf, a substantial amount of Sox3 was detected as multiple upper bands while the amount of Sox3 at 36-38 kDa decreased. Sox3 is an essential transcription factor in early embryogenesis. It plays diverse roles in a series of developmental events. The sudden change of modification pattern of Sox3 at 20 hpf may also signify that Sox3 was subjected to more post-translational modifications, which might include ubiquitination resulted in proteolysis. There was only a small amount of Sox3 protein detected at 24 hpf (Figure 4.3). It is possible that a certain amount of Sox3 by this stage has been subjected to degradation, as its expression is restricted to neural stem cells (NSC) at later stages. Since there was the sudden change of modification pattern of Sox3 at 20 hpf, indicating Sox3 was subjected to

another subset of modifications, which might lead to its degradation. However, it is also possible that Sox3 became insoluble at this stage, and therefore could not be detected in Western blot. There was a white precipitation which was insoluble in the SDS sample buffer in the protein sample of 24 hpf,

Another striking feature was that endogenous wtSox3 was detected as double bands at 36-38 kDa from shield stage on (Figure 4.3). At later stages, the expression level of Sox3 dramatically increased. However, the double band forms were still clearly seen in Figure 4.3. It can be suggested that there was likely a small modification such as phosphorylation on Sox3.

On the other hand, the subcellular distribution of Sox3 revealed that Sox3 is mainly expressed in chromatin fraction. The putative SUMOylated Sox3 was also exclusively detected in chromatin fraction, suggesting that there is association between SUMOylation of Sox3 and its chromatin location.

4.6.2 Detection of SUMOylated Sox3 and other modifications on Sox3

As the result demonstrated in Figure 4.7, the yellow bands, which indicated the immunoreactivity of both HA-tagged Sox3 and Myc-tagged SUMO2, appeared at about 64 kDa in *wtsox3/sumo2* and *sox3K115R/sumo2* overexpressing samples is an evidence of *in vivo* SUMOylation of Sox3. However, this yellow band was absent in *sox3K230R/sumo2* or *sox3K115RK230R/sumo2* overexpressed samples. This suggested that sox3K230R and sox3K115RK230R resulted in lack of the ability to be SUMOylated, indicating that K230 was the only functional SUMOylation site on zfSox3. Previous study from our lab had also demonstrated the SUMOylation of mouse Sox3 and chick Sox3. K304 on mSox3 and K246 on cSox3 were found to be

the critical SUMOylation sites (Laghari, 2010). Sequence alignment analysis shows that K230 on zfSox3 is homologous to K304R on mSox3 and K246R on cSox3.

Phosphorylation-dependent SUMOylation usually occurs in transcriptional regulators. For example, Sox9 has been shown to be SUMOylated in chick embryos and its SUMOylation is phosphorylation dependent (Liu et al., 2013, Hietakangas et al., 2006). In my results, as mentioned above, Sox3 usually appeared as double bands on western blot at 36-38 kDa. There are several reasons giving double bands pattern of a protein on SDS-PAGE. There might be a small post-translational modification or proteolytic processes on the protein. It is also possible that the protein has alternative translation form. Nevertheless, there was only a single yellow band which signified the putative SUMOylated Sox3 shown in wtSox3 or Sox3K115R overexpressing samples (Figure 4.7). I therefore screened the Sox3 sequence again. It was found that at position 229 to 236 the amino acid sequence, CKTEPSSP, is consistent with the phosphorylation-dependent-SUMOylation motif ΨKX(D/E)xxSP, in which the lysine (K) residue is the SUMOylation site and the serine (S) residue is the proline (P) directly phosphorylation site (Hietakangas et al., 2006). Thus, it is likely that SUMOylation of Sox3 occurs on K230 in zebrafish and that it is phosphorylation dependent. However, further investigation is needed to confirm this.

4.6.3 Overexpression of SUMO-modified Sox3 disrupts gastrulation and segmentation during embryogenesis

From the observation on mortality and morphology change within 24 hpf, as shown in Figure 4.18 – Figure 4.21, neither wtSox3, Sox3K230R nor Sox3K230R-SUMO2 had apparent effect on morphology of the embryos at 4.5 hpf. Although there was 1% to 5% of embryo dead in injected groups, the morphology of embryos in these injected

groups showed no difference compared to uninjected embryos, and therefore it is likely that the death of embryos at this stage was due to the artifacts caused by injections. At 6.5 hpf, most of the injected embryos had thickened blastoderm at one side of the embryos. Only a few of the Sox3K230R-SUMO2 injected embryos died at this stage, suggesting that overexpression of Sox3K230R-SUMO2 was not toxic to the embryos at this stage and it could not prevent initiation of organizer formation or gastrulation, although SUMOylation of Sox3 did largely repress organizer genes. At 10 hpf, when segmentation period initiates in uninjected embryos, wtsox3 at high dosage cause delay in development in all the surviving embryos, while high dosage of Sox3K230R-SUMO2 cause nearly 80% embryos died and about 15% embryos having aberrant appearance. This suggested that although SUMOylation of Sox3 did not suppress the initiation of gastrulation, it prevented embryos from going through gastrulation stages. Interestingly, from 10hpf to 24 hpf, wtsox3 injected embryos and sox3K230R-sumo2 injected embryos had the similar phenotype and mortality rate. At 24 hpf, it can be clearly seen that the position of the head in wtsox3 or sox3K230R-sumo2 injected embryos were aberrant. There was incomplete and abnormal development in head, trunk and tail as well. It can be suggested that there was defect in morphogenetic movements as well as segmentation during this period.

Morever, the phenotypes appeared in the wtSox3 or Sox3K230R-SUMO2 overexpressing embryos were comparable to those knocked down with *soxb1* genes (Okuda et al., 2010). Knockdown of *soxb1* genes resulted in delay in epiboly, shortened anterior-posterior axis and defects in CNS development (Okuda et al., 2010). These are observed in wtSox3 or Sox3K230R-SUMO2 overexpressing embryos as well (Figure 4.21). Single knockdown of Sox3 was not enough to give severe phenotype mentioned above. Interestingly, the phenotype in wtSox3 or

Sox3K230R-sumo2 overexpressing embryos mimics those with quadruple knockdown of *sox2/3/19a/19b* (Okuda et al., 2010). Sox3 is a bi-functional transcription factor. To take multiple roles in diverse developmental events, Sox3 needs to be accurately regulated. Gain-of-function, loss-of-function, or constitutively expressed SUMO-modified Sox3, which is supposed to execute the repressing function of Sox3, all resulted in severe abnomalities in gastrualtion and neural development. In fact, it has been shown that overexpression of Sox3 distrupted gastrulation as well as causing the ectoic expression of neural tissues, while knockdown of Sox3 with a dominate-negative form of Sox3 resulted in axis duplication eventually (Shih, 2010).

4.6.4 SUMOylation of Sox3 changes its transcriptional activity

It has been suggested that Sox3 restricts organizer formation by acting directly on the organizer genes, such as *boz*, *gsc* and *chd* (Shih et al., 2010). The luciferase reporter analysis on putative promoter regions of *boz* and *gsc* also supported that Sox3 could directly act on *boz* and *gsc* (Figure 3.14) (Kuo, 2012). During mesoderm formation, Sox3 represses also mesoderm markers such as *ntl* and *nog1* (Shih et al., 2010). As shown in Figure 4.9 – Figure 4.15, SoxK230R-SUMO2 and Sox3K230R-SUMO3 enhanced the repressing function of Sox3 on dorsal organizer and mesoderm genes. One hypothesis is that SUMOylaion acts as a switch of Sox3 from an activator to a repressor. Sox3 is a bi-functional transcription factor. SUMOylation is likely to favour the repressing function of Sox3 based on these results. However, the SUMOylated form of Sox3 enhanced also the expansion of *sox19b* expression (Figure 4.16). This raises another hypothesis that SUMOylation does not favour neither activating nor repressing function of Sox3, yet it gives rise to the change of total Sox3

protein level, and therefore SUMOylated form of Sox3 exhibited enhanced functions compared to those of wtSox3. The analysis of protein expression level in Figure 4.23 supported this idea. However, while the protein levels of Sox3K230R-SUMO2 and Sox3K230R-SUMO3 dramatically higher than wtSox3, they did not give rise to activation of *pFlash-5'boz1.4-Luc* at all. This result determined that not only did the protein levels contributed to various levels of biological effects, but the function of proteins *per se* might have gave rise to different biological effects.

Nevertheless, the luciferase assay results showed that Sox3 activated both the pFlash-*5'boz1.4*-Luc and pFlash-*5'gsc2.6*-Luc reporters, suggesting that the *5'boz1*.4 or *5'gsc2.6* region does not cover the complete regulatory region of promoters of *boz* or *gsc* respectively. It is likely that there are other essential distal regulatory elements on *boz* and *gsc* promoters. Other transcription co-factors might also be required for bending the DNA and cooperating with Sox3 to execute the repressing activities on *boz* and *gsc* promoters. However, Sox3-SUMO did not significantly activate pFlash-*5'boz1.4*-Luc. This data suggested that SUMOylation of Sox3 eliminated its activation activity on pFlash-*5'boz1.4*-Luc. On the other hand, Sox3K230R activated pFlash-*5'boz1.4*-Luc in a much greater extent compared to that of wtSox3. This also supports that SUMOylation of Sox3 prevents its activating function on target genes.

Chapter 5 Discussion

Summary

In this study, the function of Sox3 in zebrafish development was investigated in two parts. In the first part, the regulation of organizer formation by Sox3 and the related signaling regulatory network were analyzed. Based on previous studies, I further looked into how Sox3 and Fgf signaling acted on the expression of *gsc* and *chd*. Together with the work of Cheng-Liang Kuo in our lab (Kuo, 2012), the roles of Sox3 and Fgf signaling on each other and organizer formation were analysed. In the second part of my study, the role of SUMOylation of Sox3 in early zebrafish development was investigated. Expression analysis of Sox3 throughout different periods of embryogenesis showed that there was a dramatic increase in Sox3 protein level at shield (6.5 hpf) stage. There was also striking change in modification pattern of Sox3 between 16 hpf and 20 hpf. Analysis of subcellular distribution of SUMOylation of Sox3 in zebrafish suggested that the SUMOylation of Sox3 correlates with the chromatin fraction. I therefore tried to analyse the endogenous SUMOylation of Sox3 at 6.5 hpf or 16 hpf using biochemical approaches. The results, however, were not sufficient to make a strong conclusion. Nevertheless, the biological effects of SUMOylation of Sox3 were studied by observing the morphological phenotypes, the expression of marker genes of certain critical developmental events, and the luciferase activity on a *boz* and *gsc* promoter reporter assay. Taken together, it seems probable that SUMOylation occurred on Sox3 endogenously in zebrafish embryos. Moreover, this SUMOylation appears to play a significant role in regulating the function of Sox3 and therefore affected the developmental regulation of zebrafish embryogenesis.

5.1 Sox3 and Fgf signaling in organizer formation

During the time that this project was completed, our lab demonstrated that the SoxB1 proteins, including Sox3, Sox19a and Sox19b, repress *fgf3* and *fgf8* expression (Kuo, 2012). It was also shown that Sox3 could bind to the *fgf3* promoter, suggesting that Sox3 directly repressed *fgf3* (Kuo, 2012). Previous studies found that Fgf signaling promoted organizer gene expression (Maegawa et al., 2006), while Sox3 has a repressing effect on organizer formation (Shih et al., 2010). Therefore, I focussed on investigating the complicated regulatory network of Sox3, Fgf signaling and organizer genes.

As mentioned, SoxB1 proteins repressed Fgf signaling. However, together with the results demonstrated in chapter 3, the regulation of *gsc* and *chd* by Sox3 and Fgf signaling were independent from each other. Moreover, although Gsc can induce the expression of *chd*, Sox3 and Fgf signaling can regulate *chd* expression independently from their regulation on *gsc*. The intricate signaling network has been extensively studied by Cheng-Liang Kuo (Kuo, 2012). It was also elucidated that the ectopic expression of organizer genes caused by inhibition of Sox3 was dependent on Fgf signaling (Kuo, 2012). While Sox3 and Fgf signaling have opposing functions on organizer gene expression, Sox3 could partially inhibit the expansion of *gsc* and *chd* caused by Fgf3 overexpressoion (Figure 3.12) (Kuo, 2012). Together with the fact that both Sox3 and Fgf signaling have been shown to act directly on the upstream region on *gsc* (Figure 3. 14), it can be suggested that Sox3 and Fgf3 have competitive roles in regulating *gsc* and *chd* expression.

This has allowed us to propose the following model for the involvement of Sox3 in organizer formation. Sox3 represses Wnt/β-catenin signaling as well as the organizer genes directly. Both Sox3 and Fgf signaling regulate *chd* expression independently

from their regulation on *gsc*, in spite of the fact that Gsc can induce *chd* expression. Sox3 has also been found to regulate on *gsc* and *chd* in a independent way of repressing Fgf signaling.

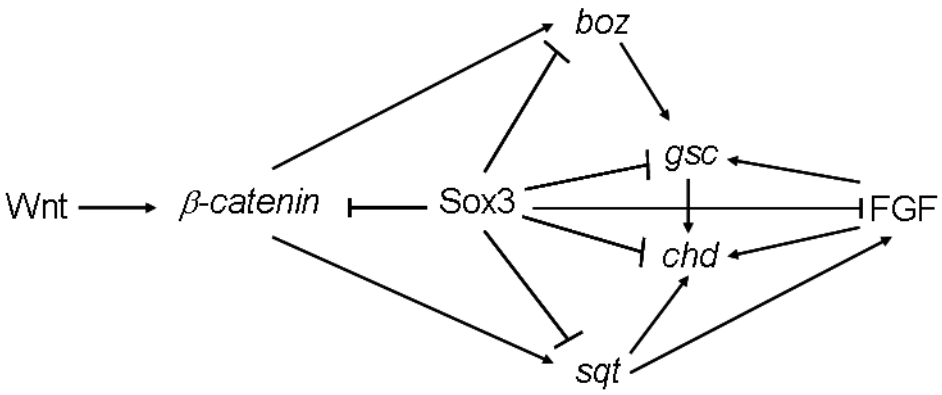


Figure 5.1 The signaling network of organizer formation
The signaling pathways and factor involved in organizer formation has been studied extensively. Arrows indicate activation of expression and T-bars indicate repression of expression. It has been previously found that Sox3 represses Wnt/β-catenin signaling as well as organizer genes and FGF signaling is required for expression of organizer genes. In this study, it has been found that Sox3 and Fgf signaling can act on both *gsc* and *chd* independently.

5.2 Modification of Sox3 changes as embryogenesis proceeds

Sox3 is early expressed during zebrafish embryogenesis. It is also believed that Sox3 is a maternal factor. It has different roles in different developmental events over the whole embryogenesis period. Sox3 protein is already detectable at the stage of organizer formation (Shih, 2010). As demonstrated in Figure 4.3, its expression dramatically increased prior to neural induction. However, at early stages, Sox3 was apparently detectable only at the size of 36-38 kDa. Multiple larger bands of Sox3 were distinctly shown only after 75% epiboly, indicating that multiple post-translational modified forms of Sox3 came into function. From the stage of 75% epiboly onwards, the amount of Sox3 protein seemed to increase, yet the modification

pattern of Sox3 did not change until 20hpf. At 20hpf, the ratio of larger bands of Sox3 dramatically increased. This change of Sox3 modification pattern implied that Sox3 might be subjected to very different functions at 20hpf. Interestingly, while there are multiple modified forms of Sox3 at later stages, the putative SUMOylated Sox3 was only detectable at shield stage (6.5 hpf) but not at 16hpf (Figure 4.7). Because of the technical difficulties in detecting SUMOylation of Sox3 due to the fact that only a small fraction of protein would be SUMOylated, it is possible that SUMOylation of Sox3 occurred at later stages as well but could not be detected. Alternatively, this may indicate that SUMOylation of Sox3 in zebrafish was essentially required only in earlier stages of embryogenesis. While Sox3K230R-SUMO2 overexpression usually caused stronger morphological changes on embryos than wtSox3 expression did, the mortality and abnormality caused by wtSox3 and Sox3K230R-SUMO2 at 24 hpf embryos were almost the same (Figure 4.21). The fact that similar phenotypes were shown at later stages regardless of whether wtSox3 or Sox3K230R-SUMO2 was overexpressed is probably due to the bi-functional property of Sox3 during the developmental processes. This may also imply that SUMOylation of Sox3 might not be required at later stages of embryogenesis. On the other hand, while Sox3K230R caused clearly apparent effects on embryos at 6.5 hpf, it led to only very slight abnormality on embryos at 10 hpf or 24 hpf. The embryos overexpressing Sox3K230R exhibited similar phenotype as the uninjected embryos. Therefore, Sox3K230R lost the function of wtSox3. However, if either wtSox3 or a SUMOylation form of Sox3 caused similar phenotypes at later stages, why would Sox3K230R overexpressing embryos exhibit a phenotype similar to uninjected embryos? One explanation is that K230 in zfSox3 may not be exclusively subjected for SUMO modification. K230R may also prevent Sox3 from other post-translational

modifications or protein-protein interactions, which would be essential for some of Sox3 activities, and therefore Sox3K230R overexpression had no or a very slight effect on embryos morphology at later stages compared to wtSox3 or Sox3K230R-SUMO fusions. This may also imply that the functioning Sox3 in embryos were post-translationally modified at K230. On the other hand, it is also possible that there might be other co-factors needed in order to allow SUMOylation of Sox3 to alter Sox3 function. In this case, if the presence of the co-factors of SUMOylated Sox3 might be restricted at certain stages, perhaps absent at later stages, then exogenous constitutively expressed SUMOylated Sox3 (Sox3K230R-SUMO2) would not have differ in its effect compared to wtSox3. However, more investigation is needed to further confirm whether SUMOylated Sox3 gives different effects comparing to wtSox3 at later stages.

5.3 Does SUMOylation switches Sox3 from a transcriptional activator to a repressor?

Sox3 is a bi-functional transcription factor having multiple roles in diverse developmental event in early embryogenesis. Yet little is known about how Sox3 is regulated in order to play different roles at different circumstances. The multiple bands of Sox3 shown in Figure 4.3 suggested that there are multiple modifications on Sox3. This indicates that Sox3 might be regulated by various post-translational modifications and these might change the functions of Sox3 at different stages. SUMOylation has been demonstrated to change the transcriptional activities in a variety of transcription factors, including some of the Sox proteins. The result in Figure 4.7 supported the hypothesis that Sox3 can be SUMOylated in zebrafish.

On the other hand, according to a previous study from our group, SUMOylation had been shown to occur on cSox3 and mSox3 (Laghari, 2010). In addition, SUMOylation of cSox3 and mSox3 has been demonstrated to reduce the transcriptional activation activity of cSox3 (Laghari, 2010). From the data presented in chapter 4, SUMOylation of Sox3 was shown to enhance its repressing functions at organizer and mesoderm formation stages. However, later at neural induction stages, Sox3 had been shown to induce neural fate marker *sox19b* and represses non-neural fate marker *gata2* (Dee et al., 2008). A SUMOylated form of Sox3 also promoted the expression of *sox19b* and repressed *gata2*. In Figure 4.16 – Figure 4.17, Sox3K230R-SUMO2 or Sox3K230R-SUMO3 up-regulated *sox19b* expression much more than wtSox3 did. If SUMOylation enhances the repressing function of Sox3, while that SUMOylation of Sox3 induced the expansion and ectopic expression of neural fate marker *sox19b*, it could be suggested that SUMOylation of Sox3 did not directly activate neural fate markers at this stage. However, it also raises the possibility that SUMOylation does not exclusively favour the repressing function of Sox3. SUMOylation might regulate the function of Sox3 through other processes, such as altering the DNA binding affinity or preference for other transcription co-factors. It is also possible that the enhanced functions of Sox3, either in up-regulating or down-regulation target genes, were due to the overexpression of the total Sox3 protein level. From the biological studies, Sox3 has been suggested to directly act on organizer genes such as *boz*, *gsc* and *chd*. According to the luciferase reporter assay on the *boz* promoter shown in Figure 4.22, wtSox3 acted as an activator in this assay on the *5'boz1.4* region. However, all the SUMOylation form of Sox3 activated luciferase reporter to a lesser extent then wtSox3 did, supporting that SUMOylation of Sox3 eliminated its activator activity. This is possibly due to the switch of Sox3 from an

activator to a repressor, or the inactivation of Sox3 by altering DNA binding affinity or protein-protein interactions with other co-activators. SUMOylated form of Sox3 therefore reduced the activation on pFlash-5'boz1.4-Luc compared to wtSox3. The striking activation driven by Sox3K230R suggested that the lack of SUMOylation on Sox3 favoured its activator activity. This also implied that there was certain modification(s), such as SUMOylation, occurred at K230 in endogeneous wtSox3 which diminished its activator activity on pFlash-5'boz1.4-Luc. It is therefore possible that while the wtSox3 naturally acts as an activator, SUMOylation can turn it into a repressor.

Taken together, it can be suggested that SUMOylation switched Sox3 to a transcriptional repressor. When SUMOylation was prevented, the repressing activity of Sox3 was reduced.

5.4 SUMOylation is required for the repressing activity of Sox3 in certain circumstances

It was also shown that only a small proportion of Sox3 was SUMOylated *in vivo*. Sox3K230R-SUMO2/3, which mimics constitutively SUMOylated Sox3, led to high mortality at later stages of the embryos, while it caused almost no effect on mortality at earlier stages. Considering that at organizer formation stage, Sox3 is believed to directly repress organizer genes as well as Fgf signals, however, there was only a very little amount of Sox3 SUMOylated at this stage. In addition, during embryogenesis, Sox3 has repressing activity at later stages as well. Yet the SUMOylated Sox3 was only detectable at 6.5 hpf but not at 16 hpf (Figure 4.7), suggesting that SUMOylation is not required for the repressing activity of Sox3 in all circumstances. It is likely that some of the Sox3 without the SUMO modification could also execute the repressing

activity. However, SUMOylation may limit the ways by which Sox3 executed its repression activity, for example, through the recruitment of other transcriptional co-factors.

5.5 SUMOylation may lead to the change of the total amount of Sox3 protein

From the results on gene expression analysis, survival and morphology observation and transcriptional activity study on overexpression of different forms of Sox3, it is clearly seen that Sox3K230R-SUMO1 had almost no effect on the embryos. It can be suggested that the functional SUMOylation on Sox3 was with SUMO2 or SUMO3. It is possible that SUMO1 modification on Sox3 does not occur during early zebrafish embryogenesis, and therefore the constitutively expressed SUMO1-modified Sox3 did not function to give biological effect on embryos. However, Figure 4.23 showed that there was no detectable Sox3K230R-SUMO1 after injection of *sox3K230R-sumo1* mRNA, suggesting that conjuction of SUMO1 led to protein degradation or that the mRNA of *sox3K230R-sumo1* was not translated into protein. Previous study on SUMOylation of cSox3 and mSox3 was also shown that the SUMO1-conjugated Sox3 was unstable and might be subjected to degradation (Laghari, 2010). It was shown that SUMO1 modification on mouse Sox3 dramatically down-regulated the total amount of Sox3 protein (Laghari, 2010). SUMO1 protein has been shown to exhibit the DNA binding activity (Eilebrecht et al., 2010). It can bind to dsDNA in a sequence-independent manner (Eilebrecht et al., 2010). This binding may interfere and compete with other protein-DNA binding (Smet-Nocca et al., 2011). Thus, Sox3 might be destabilized from the DNA binding because of the SUMO1 peptide. This may be an explanation of the fact that Sox3K230R-SUMO1 did not give any biological effects on gene expression or morphological phenotypes at various stages.

On the other hand, the high expression levels of Sox3K230R-SUMO2 and Sox3K230R-SUMO3 may also suggest a very high level of translation or that SUMO2 or SUMO3 conjugation stablilized the Sox3 protein. Sox3K230R-SUMO2 and Sox3K230R-SUMO3 led to dramatic differences in both gene expressions and morphology of embryos. Considering that Sox3K230R-SUMO2 and Sox3K230R-SUMO3 repressed the genes that are critical for organizer formation (Figure 4.9 – Figure 4.12), gastrulation and mesoderm formation (Figure 4.13 – Figure 4.15), but not neural induction (Figure 4.16, Figure 4.17), and the fact that expression levels of Sox3K230R-SUMO2 and Sox3K230R-SUMO3 were much higher than the level of wtSox3, it is very likely that exogenous SUMO-conjugated Sox3 actually enhanced their repressing function by up-regulating the total amount of Sox3 protein. In other words, overexpression of SUMO modified Sox3 enhanced either the repressing or activating activities of Sox3 because the total Sox3 level was up-regulated in those embryos. However, this hypothesis is not consistent with the luciferase result demonstrated in Figure 4.22. According to the luciferase assay analysis, SUMOylation eliminated the transcriptional activator activity of Sox3. Yet the K230R mutant enhanced its transcriptional activator activity. Overall, it can be suggested that SUMOylation favoured the transcriptional repressor activity of Sox3, while it could regulated the function of Sox3 by changing its protein level as well.

5.6 SUMOylation of zfSox3 correlates to its subcellular localization

As shown in Figure 4.2, Sox3 mostly exists in the chromatin fraction of the zebrafish embryos. There was also some Sox3 expressed in the cytosol fraction. The putative SUMOylation of Sox3 was demonstrated exclusively in the chromatin fraction. Previous studies showed that SUMOylation may alter the subcellular localization of

their target proteins. However, previous study on cSox3 showed that SUMOylation did not change its subcellular localization (Laghari, 2010). It is possible that the association of SUMOylated Sox3 and chromatin was because the SUMOylation machinery was recruited and functioned after Sox3 had bound to the DNA, and therefore SUMOylated Sox3 would always be associated with chromatin. Another explanation why SUMOylated Sox3 existed exclusively in chromatin is that SUMOlated Sox3 in other location would be subjected to degradation. It has been found that SUMOylation could be a signal for polyubiquitination and proteolytic degradation. It is possible that DNA bound SUMOylated Sox3 prevented it from proteolytic process. On the other hand, the localization of SUMOylated Sox3 can be explained in a functional aspect. Since Sox3 is a transcription factor, SUMOylation of Sox3 is likely to change its transcriptional activity. Hence, it makes energetic sense that SUMOylated Sox3 was located exclusively in chromatin.

5.7 The control of SUMOylation of Sox3

Although Sox3 could not been clearly seen to be SUMOylation from the immunoprecipitation and Western blot results, taken together with previous studies and the biological effect analysis in chapter 4, it seems likely that SUMOylation of Sox3 occurs in zebrafish. One explanation of the control of SUMOylation on Sox3 is the activator-repressor switch mechanism. When the Sox3 protein binds to the DNA, a certain proportion is SUMOylated, and therefore it is switched from an activator to a repressor. The SUMOylated Sox3 might cooperate with certain co-repressors to execute its repressing activity. It is also possible that certain co-factor(s) are needed for triggering the SUMOylation of Sox3 in chromatin. Thus, SUMOylation may control the co-factors that Sox3 works with. On the other hand, certain co-factors

expressed at specific stages may also control whether Sox3 would be SUMOylated and the amount of Sox3 being SUMOylated.

SUMOylation can also occur on Sox3 prior to its binding to the DNA. In this case, SUMOylation of Sox3 enhances its DNA binding activity and that SUMOylated Sox3 replaces some of the non-SUMOylated Sox3 in chromatin.

Conclusion

In this study, the relationship between Sox3, Fgf signaling and organizer genes has been studied in details. It has been found that Sox3 and Fgf3 can both regulate chd expression independently from their regulation on gsc. In addition, although Sox3 has inhibiting effect on Fgf signaling, Sox3 can regulate gsc and chd independently from its regulation on Fgf signaling. The regulation of Sox3 function by SUMOylation has also been investigated. Although the result from immunoprecipitation and mass spectroscopy failed to demonstrate the occurrence of SUMOylation of Sox3 in zebrafish, the western blot analysis suggested that SUMOylation of Sox3 occurred. In addition to this, the biological effects of SUMOylation of Sox3 promoted its transcriptional repressor activity. It can be suggested SUMOylation might act as a switch of Sox3 from activator to repressor. Luciferase reporter assay on pFlash-5'boz1.4-Luc supported SUMOylation diminished the activating function of Sox3 in zebrafish embryos.

Bibliography

AGIUS, E., OELGESCHLAGER, M., WESSELY, O., KEMP, C. & DE ROBERTIS, E. M. 2000. Endodermal Nodal-related signals and mesoderm induction in Xenopus. *Development,* 127**,** 1173-83.

ALCALAY, M., TOMASSONI, L., COLOMBO, E., STOLDT, S., GRIGNANI, F., FAGIOLI, M., SZEKELY, L., HELIN, K. & PELICCI, P. G. 1998. The promyelocytic leukemia gene product (PML) forms stable complexes with the retinoblastoma protein. *Mol Cell Biol,* 18**,** 1084-93.

AOKI, Y., SAINT-GERMAIN, N., GYDA, M., MAGNER-FINK, E., LEE, Y. H., CREDIDIO, C. & SAINT-JEANNET, J. P. 2003. Sox10 regulates the development of neural crest-derived melanocytes in Xenopus. *Dev Biol,* 259**,** 19-33.

AZUMA, Y., ARNAOUTOV, A. & DASSO, M. 2003. SUMO-2/3 regulates topoisomerase II in mitosis. *J Cell Biol,* 163**,** 477-87.

BAKER, J. C., BEDDINGTON, R. S. & HARLAND, R. M. 1999. Wnt signaling in Xenopus embryos inhibits bmp4 expression and activates neural development. *Genes Dev,* 13**,** 3149-59.

BAKKERS, J., CAMACHO-CARVAJAL, M., NOWAK, M., KRAMER, C., DANGER, B. & HAMMERSCHMIDT, M. 2005. Destabilization of DeltaNp63alpha by Nedd4-mediated ubiquitination and Ubc9-mediated sumoylation, and its implications on dorsoventral patterning of the zebrafish embryo. *Cell Cycle,* 4**,** 790-800.

BEHRENS, J., VON KRIES, J. P., KUHL, M., BRUHN, L., WEDLICH, D., GROSSCHEDL, R. & BIRCHMEIER, W. 1996. Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature,* 382**,** 638-42.

BIRSOY, B., KOFRON, M., SCHAIBLE, K., WYLIE, C. & HEASMAN, J. 2006. Vg 1 is an essential signaling molecule in Xenopus development. *Development,* 133**,** 15-20.

BLUMBERG, B., WRIGHT, C. V., DE ROBERTIS, E. M. & CHO, K. W. 1991. Organizer-specific homeobox genes in Xenopus laevis embryos. *Science,* 253**,** 194-6.

BODDY, M. N., HOWE, K., ETKIN, L. D., SOLOMON, E. & FREEMONT, P. S. 1996. PIC 1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukaemia. *Oncogene,* 13**,** 971-82.

BOHREN, K. M., NADKARNI, V., SONG, J. H., GABBAY, K. H. & OWERBACH, D. 2004. A M55V polymorphism in a novel SUMO gene (SUMO-4) differentially activates heat shock transcription factors and is associated with susceptibility to type I diabetes mellitus. *J Biol Chem,* 279**,** 27233-8.

BRANNON, M. & KIMELMAN, D. 1996. Activation of Siamois by the Wnt pathway. *Dev Biol,* 180**,** 344-7.

BRODAY, L., KOLOTUEV, I., DIDIER, C., BHOUMIK, A., GUPTA, B. P., STERNBERG, P. W., PODBILEWICZ, B. & RONAI, Z. 2004. The small ubiquitin-like modifier (SUMO) is required for gonadal and uterine-vulval morphogenesis in Caenorhabditis elegans. *Genes Dev,* 18**,** 2380-91.

BYLUND, M., ANDERSSON, E., NOVITCH, B. G. & MUHR, J. 2003. Vertebrate neurogenesis is counteracted by Sox1-3 activity. *Nat Neurosci,* 6**,** 1162-8.

CARBALLADA, R., YASUO, H. & LEMAIRE, P. 2001. Phosphatidylinositol-3 kinase acts in parallel to the ERK MAP kinase in the FGF pathway during Xenopus mesoderm induction. *Development,* 128**,** 35-44.

CAU, E. & WILSON, S. W. 2003. Ash1a and Neurogenin1 function downstream of Floating head to regulate epiphysial neurogenesis. *Development,* 130**,** 2455-66.

CHANG, L. L. & KESSLER, D. S. 2010. Foxd3 is an essential Nodal-dependent regulator of zebrafish dorsal mesoderm development. *Dev Biol,* 342**,** 39-50.

CHEN, Y. & SCHIER, A. F. 2001. The zebrafish Nodal signal Squint functions as a morphogen. *Nature,* 411**,** 607-10.

CHENG, J., WANG, D., WANG, Z. & YEH, E. T. 2004. SENP1 enhances androgen receptor-dependent transcription through desumoylation of histone deacetylase 1. *Mol Cell Biol,* 24**,** 6021-8.

CHO, K. W., BLUMBERG, B., STEINBEISSER, H. & DE ROBERTIS, E. M. 1991. Molecular nature of Spemann's organizer: the role of the Xenopus homeobox gene goosecoid. *Cell,* 67**,** 1111-20.

CONCHA, M. L., BURDINE, R. D., RUSSELL, C., SCHIER, A. F. & WILSON, S. W. 2000. A nodal signaling pathway regulates the laterality of neuroanatomical asymmetries in the zebrafish forebrain. *Neuron,* 28**,** 399-409.

DADKE, S., COTTERET, S., YIP, S. C., JAFFER, Z. M., HAJ, F., IVANOV, A., RAUSCHER, F., 3RD, SHUAI, K., NG, T., NEEL, B. G. & CHERNOFF, J. 2007. Regulation of protein tyrosine phosphatase 1B by sumoylation. *Nat Cell Biol,* 9**,** 80-5.

DE ROBERTIS, E. M., LARRAIN, J., OELGESCHLAGER, M. & WESSELY, O. 2000. The establishment of Spemann's organizer and patterning of the vertebrate embryo. *Nat Rev Genet,* 1**,** 171-81.

DEE, C. T., HIRST, C. S., SHIH, Y. H., TRIPATHI, V. B., PATIENT, R. K. & SCOTTING, P. J. 2008. Sox3 regulates both neural fate and differentiation in the zebrafish ectoderm. *Dev Biol,* 320**,** 289-301.

DOMINGUEZ, I., ITOH, K. & SOKOL, S. Y. 1995. Role of glycogen synthase kinase 3 beta as a negative regulator of dorsoventral axis formation in Xenopus embryos. *Proc Natl Acad Sci U S A,* 92**,** 8498-502.

DOUGAN, S. T., WARGA, R. M., KANE, D. A., SCHIER, A. F. & TALBOT, W. S. 2003. The role of the zebrafish nodal-related genes squint and cyclops in patterning of mesendoderm. *Development,* 130**,** 1837-51.

EILEBRECHT, S., SMET-NOCCA, C., WIERUSZESKI, J. M. & BENECKE, A. 2010. SUMO-1 possesses DNA binding activity. *BMC Res Notes,* 3**,** 146.

EUN JEOUNG, L., SUNG HEE, H., JAESUN, C., SUNG HWA, S., KWANG HUM, Y., MIN KYOUNG, K., TAE YOON, P. & SANG SUN, K. 2008. Regulation of glycogen synthase kinase 3beta functions by modification of the small ubiquitin-like modifier. *Open Biochem J,* 2**,** 67-76.

FAGOTTO, F., GUGER, K. & GUMBINER, B. M. 1997. Induction of the primary dorsalizing center in Xenopus by the Wnt/GSK/beta-catenin signaling pathway, but not by Vg1, Activin or Noggin. *Development,* 124**,** 453-60.

FEKANY, K., YAMANAKA, Y., LEUNG, T., SIROTKIN, H. I., TOPCZEWSKI, J., GATES, M. A., HIBI, M., RENUCCI, A., STEMPLE, D., RADBILL, A., SCHIER, A. F., DRIEVER, W., HIRANO, T., TALBOT, W. S. & SOLNICA-KREZEL, L. 1999. The zebrafish bozozok locus encodes Dharma, a homeodomain protein essential for induction of gastrula organizer and dorsoanterior embryonic structures. *Development,* 126**,** 1427-38.

FELDMAN, B., GATES, M. A., EGAN, E. S., DOUGAN, S. T., RENNEBECK, G., SIROTKIN, H. I., SCHIER, A. F. & TALBOT, W. S. 1998. Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature,* 395**,** 181-5.

FERNANDEZ-LLORIS, R., OSSES, N., JAFFRAY, E., SHEN, L. N., VAUGHAN, O. A., GIRWOOD, D., BARTRONS, R., ROSA, J. L., HAY, R. T. & VENTURA, F. 2006. Repression of SOX6 transcriptional activity by SUMO modification. *FEBS Lett,* 580**,** 1215-21.

FURTHAUER, M., VAN CELST, J., THISSE, C. & THISSE, B. 2004. Fgf signaling controls the dorsoventral patterning of the zebrafish embryo. *Development,* 131**,** 2853-64.

GIRDWOOD, D., BUMPASS, D., VAUGHAN, O. A., THAIN, A., ANDERSON, L. A., SNOWDEN, A. W., GARCIA-WILSON, E., PERKINS, N. D. & HAY, R. T. 2003. P300 transcriptional repression is mediated by SUMO modification. *Mol Cell,* 11**,** 1043-54.

GOLDFARB, M. 2005. Fibroblast growth factor homologous factors: evolution, structure, and function. *Cytokine Growth Factor Rev,* 16**,** 215-20.

GOODSON, M. L., HONG, Y., ROGERS, R., MATUNIS, M. J., PARK-SARGE, O. K. & SARGE, K. D. 2001. Sumo-1 modification regulates the DNA binding activity of heat shock transcription factor 2, a promyelocytic leukemia nuclear body associated transcription factor. *J Biol Chem,* 276**,** 18513-8.

GREGOIRE, S. & YANG, X. J. 2005. Association with class IIa histone deacetylases upregulates the sumoylation of MEF2 transcription factors. *Mol Cell Biol,* 25**,** 2273-87.

GRIFFIN, K., PATIENT, R. & HOLDER, N. 1995. Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. *Development,* 121**,** 2983-94.

GRIFFIN, K. J., AMACHER, S. L., KIMMEL, C. B. & KIMELMAN, D. 1998. Molecular identification of spadetail: regulation of zebrafish trunk and tail mesoderm formation by T-box genes. *Development,* 125**,** 3379-88.

GRITSMAN, K., TALBOT, W. S. & SCHIER, A. F. 2000. Nodal signaling patterns the organizer. *Development,* 127**,** 921-32.

GUGER, K. A. & GUMBINER, B. M. 1995. beta-Catenin has Wnt-like activity and mimics the Nieuwkoop signaling center in Xenopus dorsal-ventral patterning. *Dev Biol,* 172**,** 115-25.

GUO, B., PANAGIOTAKI, N., WARWOOD, S. & SHARROCKS, A. D. 2011. Dynamic modification of the ETS transcription factor PEA3 by sumoylation and p300-mediated acetylation. *Nucleic Acids Res,* 39**,** 6403-13.

GUO, B. & SHARROCKS, A. D. 2009. Extracellular signal-regulated kinase mitogen-activated protein kinase signaling initiates a dynamic interplay between sumoylation and ubiquitination to regulate the activity of the transcriptional activator PEA3. *Mol Cell Biol,* 29**,** 3204-18.

GURDON, J. B. 1960a. The developmental capacity of nuclei taken from differentiating endoderm cells of Xenopus laevis. *J Embryol Exp Morphol,* 8**,** 505-26.

GURDON, J. B. 1960b. Factors responsible for the abnormal development of embryos obtained by nuclear transplantation in Xenopus laevis. *J Embryol Exp Morphol,* 8**,** 327-40.

HALPERN, M. E., THISSE, C., HO, R. K., THISSE, B., RIGGLEMAN, B., TREVARROW, B., WEINBERG, E. S., POSTLETHWAIT, J. H. &

KIMMEL, C. B. 1995. Cell-autonomous shift from axial to paraxial mesodermal development in zebrafish floating head mutants. *Development,* 121**,** 4257-64.

HARLEY, V. R., LOVELL-BADGE, R. & GOODFELLOW, P. N. 1994. Definition of a consensus DNA binding site for SRY. *Nucleic Acids Res,* 22**,** 1500-1.

HATSELL, S., ROWLANDS, T., HIREMATH, M. & COWIN, P. 2003. Beta-catenin and Tcfs in mammary development and cancer. *J Mammary Gland Biol Neoplasia,* 8**,** 145-58.

HATTORI, T., EBERSPAECHER, H., LU, J., ZHANG, R., NISHIDA, T., KAHYO, T., YASUDA, H. & DE CROMBRUGGHE, B. 2006. Interactions between PIAS proteins and SOX9 result in an increase in the cellular concentrations of SOX9. *J Biol Chem,* 281**,** 14417-28.

HAY, R. T. 2007. SUMO-specific proteases: a twist in the tail. *Trends Cell Biol,* 17**,** 370-6.

HE, X., SAINT-JEANNET, J. P., WOODGETT, J. R., VARMUS, H. E. & DAWID, I. B. 1995. Glycogen synthase kinase-3 and dorsoventral patterning in Xenopus embryos. *Nature,* 374**,** 617-22.

HEASMAN, J., CRAWFORD, A., GOLDSTONE, K., GARNER-HAMRICK, P., GUMBINER, B., MCCREA, P., KINTNER, C., NORO, C. Y. & WYLIE, C. 1994. Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. *Cell,* 79**,** 791-803.

HIETAKANGAS, V., AHLSKOG, J. K., JAKOBSSON, A. M., HELLESUO, M., SAHLBERG, N. M., HOLMBERG, C. I., MIKHAILOV, A., PALVIMO, J. J., PIRKKALA, L. & SISTONEN, L. 2003. Phosphorylation of serine 303 is a prerequisite for the stress-inducible SUMO modification of heat shock factor 1. *Mol Cell Biol,* 23**,** 2953-68.

HIETAKANGAS, V., ANCKAR, J., BLOMSTER, H. A., FUJIMOTO, M., PALVIMO, J. J., NAKAI, A. & SISTONEN, L. 2006. PDSM, a motif for phosphorylation-dependent SUMO modification. *Proc Natl Acad Sci U S A,* 103**,** 45-50.

HIROKAWA, N., TANAKA, Y., OKADA, Y. & TAKEDA, S. 2006. Nodal flow and the generation of left-right asymmetry. *Cell,* 125**,** 33-45.

HOFFMANN, S. A., HOS, D., KUSPERT, M., LANG, R. A., LOVELL-BADGE, R., WEGNER, M. & REIPRICH, S. 2014. Stem cell factor Sox2 and its close relative Sox3 have differentiation functions in oligodendrocytes. *Development,* 141**,** 39-50.

HOFMANN, T. G., JAFFRAY, E., STOLLBERG, N., HAY, R. T. & WILL, H. 2005. Regulation of homeodomain-interacting protein kinase 2 (HIPK2) effector function through dynamic small ubiquitin-related modifier-1 (SUMO-1) modification. *J Biol Chem,* 280**,** 29224-32.

HOFMANN, T. G., STOLLBERG, N., SCHMITZ, M. L. & WILL, H. 2003. HIPK2 regulates transforming growth factor-beta-induced c-Jun NH(2)-terminal kinase activation and apoptosis in human hepatoma cells. *Cancer Res,* 63**,** 8271-7.

HONG, Y., ROGERS, R., MATUNIS, M. J., MAYHEW, C. N., GOODSON, M. L., PARK-SARGE, O. K. & SARGE, K. D. 2001. Regulation of heat shock transcription factor 1 by stress-induced SUMO-1 modification. *J Biol Chem,* 276**,** 40263-7.

HONORE, S. M., AYBAR, M. J. & MAYOR, R. 2003. Sox10 is required for the early development of the prospective neural crest in Xenopus embryos. *Dev Biol,* 260**,** 79-96.

HOU, S., YANG, P., DU, L., ZHOU, H., LIN, X., LIU, X. & KIJLSTRA, A. 2008. Small ubiquitin-like modifier 4 (SUMO4) polymorphisms and Vogt-Koyanagi-Harada (VKH) syndrome in the Chinese Han population. *Mol Vis,* 14**,** 2597-603.

HUANG, Y. P., WU, G., GUO, Z., OSADA, M., FOMENKOV, T., PARK, H. L., TRINK, B., SIDRANSKY, D., FOMENKOV, A. & RATOVITSKI, E. A. 2004. Altered sumoylation of p63alpha contributes to the split-hand/foot malformation phenotype. *Cell Cycle,* 3**,** 1587-96.

ITOH, N. & KONISHI, M. 2007. The zebrafish fgf family. *Zebrafish,* 4**,** 179-86.

JOHNSON, E. S. 2004. Protein modification by SUMO. *Annu Rev Biochem,* 73**,** 355-82.

JOHNSON, E. S., SCHWIENHORST, I., DOHMEN, R. J. & BLOBEL, G. 1997. The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *EMBO J,* 16**,** 5509-19.

KAMACHI, Y. & KONDOH, H. 2013. Sox proteins: regulators of cell fate specification and differentiation. *Development,* 140**,** 4129-44.

KAMACHI, Y., UCHIKAWA, M., TANOUCHI, A., SEKIDO, R. & KONDOH, H. 2001. Pax6 and SOX2 form a co-DNA-binding partner complex that regulates initiation of lens development. *Genes Dev,* 15**,** 1272-86.

KAMITANI, T., KITO, K., NGUYEN, H. P., FUKUDA-KAMITANI, T. & YEH, E. T. 1998. Characterization of a second member of the sentrin family of ubiquitin-like proteins. *J Biol Chem,* 273**,** 11349-53.

KAMITANI, T., NGUYEN, H. P. & YEH, E. T. 1997. Preferential modification of nuclear proteins by a novel ubiquitin-like molecule. *J Biol Chem,* 272**,** 14001-4.

KAMOUN, M., BEN DHIFALLAH, I., KARRAY, E., ZAKRAOUI, L. & HAMZAOUI, K. 2010. Association of small ubiquitin-like modifier 4 (SUMO4) polymorphisms in a Tunisian population with Behcet's disease. *Clin Exp Rheumatol,* 28**,** S45-9.

KAWANO, Y. & KYPTA, R. 2003. Secreted antagonists of the Wnt signaling pathway. *J Cell Sci,* 116**,** 2627-34.

KELLY, G. M., EREZYILMAZ, D. F. & MOON, R. T. 1995. Induction of a secondary embryonic axis in zebrafish occurs following the overexpression of beta-catenin. *Mech Dev,* 53**,** 261-73.

KIM, E. Y., CHEN, L., MA, Y., YU, W., CHANG, J., MOSKOWITZ, I. P. & WANG, J. 2012. Enhanced desumoylation in murine hearts by overexpressed SENP2 leads to congenital heart defects and cardiac dysfunction. *J Mol Cell Cardiol,* 52**,** 638-49.

KIMELMAN, D. 2006. Mesoderm induction: from caps to chips. *Nat Rev Genet,* 7**,** 360-72.

KIMMEL, C. B., BALLARD, W. W., KIMMEL, S. R., ULLMANN, B. & SCHILLING, T. F. 1995. Stages of embryonic development of the zebrafish. *Dev Dyn,* 203**,** 253-310.

KIRSH, O., SEELER, J. S., PICHLER, A., GAST, A., MULLER, S., MISKA, E., MATHIEU, M., HAREL-BELLAN, A., KOUZARIDES, T., MELCHIOR, F. & DEJEAN, A. 2002. The SUMO E3 ligase RanBP2 promotes modification of the HDAC4 deacetylase. *EMBO J,* 21**,** 2682-91.

KOFRON, M., DEMEL, T., XANTHOS, J., LOHR, J., SUN, B., SIVE, H., OSADA, S., WRIGHT, C., WYLIE, C. & HEASMAN, J. 1999. Mesoderm induction in Xenopus is a zygotic event regulated by maternal VegT via TGFbeta growth factors. *Development,* 126**,** 5759-70.

KOUHARA, H., HADARI, Y. R., SPIVAK-KROIZMAN, T., SCHILLING, J., BAR-SAGI, D., LAX, I. & SCHLESSINGER, J. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell,* 89**,** 693-702.

KUDOH, T., CONCHA, M. L., HOUART, C., DAWID, I. B. & WILSON, S. W. 2004. Combinatorial Fgf and Bmp signaling patterns the gastrula ectoderm into prospective neural and epidermal domains. *Development,* 131**,** 3581-92.

KUO, C. L. 2012. *The role of SoxB1 transcription factors in the formation of the embryonic organizer*. PhD Thesis, University of Nottingham.

LAGHARI, Z. A. 2010. *Sox3 as a transcriptional repressor*. PhD Thesis, University of Nottingham.

LANGENAU, D. M., TRAVER, D., FERRANDO, A. A., KUTOK, J. L., ASTER, J. C., KANKI, J. P., LIN, S., PROCHOWNIK, E., TREDE, N. S., ZON, L. I. & LOOK, A. T. 2003. Myc-induced T cell leukemia in transgenic zebrafish. *Science,* 299**,** 887-90.

LEE, K. Y., HUANG, H., JU, B., YANG, Z. & LIN, S. 2002. Cloned zebrafish by nuclear transfer from long-term-cultured cells. *Nat Biotechnol,* 20**,** 795-9.

LEE, M. T., BONNEAU, A. R. & GIRALDEZ, A. J. 2014. Zygotic genome activation during the maternal-to-zygotic transition. *Annu Rev Cell Dev Biol,* 30**,** 581-613.

LEE, M. T., BONNEAU, A. R., TAKACS, C. M., BAZZINI, A. A., DIVITO, K. R., FLEMING, E. S. & GIRALDEZ, A. J. 2013. Nanog, Pou5f1 and SoxB1 activate zygotic gene expression during the maternal-to-zygotic transition. *Nature,* 503**,** 360-4.

LEE, P. C., TAYLOR-JAFFE, K. M., NORDIN, K. M., PRASAD, M. S., LANDER, R. M. & LABONNE, C. 2012. SUMOylated SoxE factors recruit Grg4 and function as transcriptional repressors in the neural crest. *J Cell Biol,* 198**,** 799-813.

LEFEBVRE, V., DUMITRIU, B., PENZO-MENDEZ, A., HAN, Y. & PALLAVI, B. 2007. Control of cell fate and differentiation by Sry-related high-mobility-group box (Sox) transcription factors. *Int J Biochem Cell Biol,* 39**,** 2195-214.

LEMAIRE, P., GARRETT, N. & GURDON, J. B. 1995. Expression cloning of Siamois, a Xenopus homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell,* 81**,** 85-94.

LETAMENDIA, A., LABBE, E. & ATTISANO, L. 2001. Transcriptional regulation by Smads: crosstalk between the TGF-beta and Wnt pathways. *J Bone Joint Surg Am,* 83-A Suppl 1**,** S31-9.

LEUNG, T., BISCHOF, J., SOLL, I., NIESSING, D., ZHANG, D., MA, J., JACKLE, H. & DRIEVER, W. 2003. bozozok directly represses bmp2b transcription and mediates the earliest dorsoventral asymmetry of bmp2b expression in zebrafish. *Development,* 130**,** 3639-49.

LEVIN, M. 2005. Left-right asymmetry in embryonic development: a comprehensive review. *Mech Dev,* 122**,** 3-25.

LI, H., LEO, C., ZHU, J., WU, X., O'NEIL, J., PARK, E. J. & CHEN, J. D. 2000. Sequestration and inhibition of Daxx-mediated transcriptional repression by PML. *Mol Cell Biol,* 20**,** 1784-96.

LIN, D. Y., HUANG, Y. S., JENG, J. C., KUO, H. Y., CHANG, C. C., CHAO, T. T., HO, C. C., CHEN, Y. C., LIN, T. P., FANG, H. I., HUNG, C. C., SUEN, C. S., HWANG, M. J., CHANG, K. S., MAUL, G. G. & SHIH, H. M. 2006. Role of SUMO-interacting motif in Daxx SUMO modification, subnuclear localization, and repression of sumoylated transcription factors. *Mol Cell,* 24**,** 341-54.

LIU, J. A., WU, M. H., YAN, C. H., CHAU, B. K., SO, H., NG, A., CHAN, A., CHEAH, K. S., BRISCOE, J. & CHEUNG, M. 2013. Phosphorylation of Sox9 is required for neural crest delamination and is regulated downstream of BMP and canonical Wnt signaling. *Proc Natl Acad Sci U S A,* 110**,** 2882-7.

MAEGAWA, S., VARGA, M. & WEINBERG, E. S. 2006. FGF signaling is required for {beta}-catenin-mediated induction of the zebrafish organizer. *Development,* 133**,** 3265-76.

MAHAJAN, R., DELPHIN, C., GUAN, T., GERACE, L. & MELCHIOR, F. 1997. A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell,* 88**,** 97-107.

MAK, A. C., SZETO, I. Y., FRITZSCH, B. & CHEAH, K. S. 2009. Differential and overlapping expression pattern of SOX2 and SOX9 in inner ear development. *Gene Expr Patterns,* 9**,** 444-53.

MALAS, S., POSTLETHWAITE, M., EKONOMOU, A., WHALLEY, B., NISHIGUCHI, S., WOOD, H., MELDRUM, B., CONSTANTI, A. & EPISKOPOU, V. 2003. Sox1-deficient mice suffer from epilepsy associated with abnormal ventral forebrain development and olfactory cortex hyperexcitability. *Neuroscience,* 119**,** 421-32.

MAO, J., WANG, J., LIU, B., PAN, W., FARR, G. H., 3RD, FLYNN, C., YUAN, H., TAKADA, S., KIMELMAN, D., LI, L. & WU, D. 2001. Low-density lipoprotein receptor-related protein-5 binds to Axin and regulates the canonical Wnt signaling pathway. *Mol Cell,* 7**,** 801-9.

MATIC, I., VAN HAGEN, M., SCHIMMEL, J., MACEK, B., OGG, S. C., TATHAM, M. H., HAY, R. T., LAMOND, A. I., MANN, M. & VERTEGAAL, A. C. 2008. In vivo identification of human small ubiquitin-like modifier polymerization sites by high accuracy mass spectrometry and an in vitro to in vivo strategy. *Mol Cell Proteomics,* 7**,** 132-44.

MATUNIS, M. J., COUTAVAS, E. & BLOBEL, G. 1996. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol,* 135**,** 1457-70.

MCKEEHAN, W. L., WANG, F. & KAN, M. 1998. The heparan sulfate-fibroblast growth factor family: diversity of structure and function. *Prog Nucleic Acid Res Mol Biol,* 59**,** 135-76.

MILLER, J. R., ROWNING, B. A., LARABELL, C. A., YANG-SNYDER, J. A., BATES, R. L. & MOON, R. T. 1999. Establishment of the dorsal-ventral axis in Xenopus embryos coincides with the dorsal enrichment of dishevelled that is dependent on cortical rotation. *J Cell Biol,* 146**,** 427-37.

MINTY, A., DUMONT, X., KAGHAD, M. & CAPUT, D. 2000. Covalent modification of p73alpha by SUMO-1. Two-hybrid screening with p73 identifies novel SUMO-1-interacting proteins and a SUMO-1 interaction motif. *J Biol Chem,* 275**,** 36316-23.

MIYAKAWA, K., HATSUZAWA, K., KUROKAWA, T., ASADA, M., KUROIWA, T. & IMAMURA, T. 1999. A hydrophobic region locating at the center of

fibroblast growth factor-9 is crucial for its secretion. *J Biol Chem,* 274**,** 29352-7.

MOHAMMADI, M., DIONNE, C. A., LI, W., LI, N., SPIVAK, T., HONEGGER, A. M., JAYE, M. & SCHLESSINGER, J. 1992. Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. *Nature,* 358**,** 681-4.

MOLENAAR, M., VAN DE WETERING, M., OOSTERWEGEL, M., PETERSON-MADURO, J., GODSAVE, S., KORINEK, V., ROOSE, J., DESTREE, O. & CLEVERS, H. 1996. XTcf-3 transcription factor mediates beta-catenin-induced axis formation in Xenopus embryos. *Cell,* 86**,** 391-9.

NAKAGAWA, M., KOYANAGI, M., TANABE, K., TAKAHASHI, K., ICHISAKA, T., AOI, T., OKITA, K., MOCHIDUKI, Y., TAKIZAWA, N. & YAMANAKA, S. 2008. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. *Nat Biotechnol,* 26**,** 101-6.

NIEHRS, C. & DE ROBERTIS, E. M. 1991. Ectopic expression of a homeobox gene changes cell fate in Xenopus embryos in a position-specific manner. *EMBO J,* 10**,** 3621-9.

NIEUWKOOP, P. D. 1973. The organization center of the amphibian embryo: its origin, spatial organization, and morphogenetic action. *Adv Morphog,* 10**,** 1-39.

NISHIGUCHI, S., WOOD, H., KONDOH, H., LOVELL-BADGE, R. & EPISKOPOU, V. 1998. Sox1 directly regulates the gamma-crystallin genes and is essential for lens development in mice. *Genes Dev,* 12**,** 776-81.

NOWAK, M. & HAMMERSCHMIDT, M. 2006. Ubc9 regulates mitosis and cell survival during zebrafish development. *Mol Biol Cell,* 17**,** 5324-36.

OBER, E. A. & SCHULTE-MERKER, S. 1999. Signals from the yolk cell induce mesoderm, neuroectoderm, the trunk organizer, and the notochord in zebrafish. *Dev Biol,* 215**,** 167-81.

ODENTHAL, J. & NUSSLEIN-VOLHARD, C. 1998. fork head domain genes in zebrafish. *Dev Genes Evol,* 208**,** 245-58.

OELGESCHLAGER, M., KURODA, H., REVERSADE, B. & DE ROBERTIS, E. M. 2003. Chordin is required for the Spemann organizer transplantation phenomenon in Xenopus embryos. *Dev Cell,* 4**,** 219-30.

OKUDA, Y., OGURA, E., KONDOH, H. & KAMACHI, Y. 2010. B1 SOX coordinate cell specification with patterning and morphogenesis in the early zebrafish embryo. *PLoS Genet,* 6**,** e1000936.

OOSTERVEEN, T., KURDIJA, S., ENSTERO, M., UHDE, C. W., BERGSLAND, M., SANDBERG, M., SANDBERG, R., MUHR, J. & ERICSON, J. 2013. SoxB1-driven transcriptional network underlies neural-specific interpretation of morphogen signals. *Proc Natl Acad Sci U S A,* 110**,** 7330-5.

OSHIMA, Y., NARUSE, K., NAKAMURA, Y. & NAKAMURA, M. 2009. Sox3: a transcription factor for Cyp19 expression in the frog Rana rugosa. *Gene,* 445**,** 38-48.

OUYANG, J., SHI, Y., VALIN, A., XUAN, Y. & GILL, G. 2009. Direct binding of CoREST1 to SUMO-2/3 contributes to gene-specific repression by the LSD1/CoREST1/HDAC complex. *Mol Cell,* 34**,** 145-54.

PETERS, K. G., MARIE, J., WILSON, E., IVES, H. E., ESCOBEDO, J., DEL ROSARIO, M., MIRDA, D. & WILLIAMS, L. T. 1992. Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca2+ flux but not mitogenesis. *Nature,* 358**,** 678-81.

PFANDER, B., MOLDOVAN, G. L., SACHER, M., HOEGE, C. & JENTSCH, S. 2005. SUMO-modified PCNA recruits Srs2 to prevent recombination during S phase. *Nature,* 436**,** 428-33.

PICCOLO, S., SASAI, Y., LU, B. & DE ROBERTIS, E. M. 1996. Dorsoventral patterning in Xenopus: inhibition of ventral signals by direct binding of chordin to BMP-4. *Cell,* 86**,** 589-98.

POUKKA, H., AARNISALO, P., KARVONEN, U., PALVIMO, J. J. & JANNE, O. A. 1999. Ubc9 interacts with the androgen receptor and activates receptor-dependent transcription. *J Biol Chem,* 274**,** 19441-6.

POWERS, D. A. 1989. Fish as model systems. *Science,* 246**,** 352-8.

RAFFA, G. D., WOHLSCHLEGEL, J., YATES, J. R., 3RD & BODDY, M. N. 2006. SUMO-binding motifs mediate the Rad60-dependent response to replicative stress and self-association. *J Biol Chem,* 281**,** 27973-81.

RENTZSCH, F., BAKKERS, J., KRAMER, C. & HAMMERSCHMIDT, M. 2004. Fgf signaling induces posterior neuroectoderm independently of Bmp signaling inhibition. *Dev Dyn,* 231**,** 750-7.

REVEST, J. M., DEMOERLOOZE, L. & DICKSON, C. 2000. Fibroblast growth factor 9 secretion is mediated by a non-cleaved amino-terminal signal sequence. *J Biol Chem,* 275**,** 8083-90.

RODRIGUEZ, M. S., DARGEMONT, C. & HAY, R. T. 2001. SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem,* 276**,** 12654-9.

ROGERS, C. D., ARCHER, T. C., CUNNINGHAM, D. D., GRAMMER, T. C. & CASEY, E. M. 2008. Sox3 expression is maintained by FGF signaling and restricted to the neural plate by Vent proteins in the Xenopus embryo. *Dev Biol,* 313**,** 307-19.

ROGERS, C. D., HARAFUJI, N., ARCHER, T., CUNNINGHAM, D. D. & CASEY, E. S. 2009. Xenopus Sox3 activates sox2 and geminin and indirectly represses Xvent2 expression to induce neural progenitor formation at the expense of non-neural ectodermal derivatives. *Mech Dev,* 126**,** 42-55.

ROSCIC, A., MOLLER, A., CALZADO, M. A., RENNER, F., WIMMER, V. C., GRESKO, E., LUDI, K. S. & SCHMITZ, M. L. 2006. Phosphorylation-dependent control of Pc2 SUMO E3 ligase activity by its substrate protein HIPK2. *Mol Cell,* 24**,** 77-89.

ROSS, S., BEST, J. L., ZON, L. I. & GILL, G. 2002. SUMO-1 modification represses Sp3 transcriptional activation and modulates its subnuclear localization. *Mol Cell,* 10**,** 831-42.

SACHDEV, S., BRUHN, L., SIEBER, H., PICHLER, A., MELCHIOR, F. & GROSSCHEDL, R. 2001. PIASy, a nuclear matrix-associated SUMO E3 ligase, represses LEF1 activity by sequestration into nuclear bodies. *Genes Dev,* 15**,** 3088-103.

SAINT-GERMAIN, N., LEE, Y. H., ZHANG, Y., SARGENT, T. D. & SAINT-JEANNET, J. P. 2004. Specification of the otic placode depends on Sox9 function in Xenopus. *Development,* 131**,** 1755-63.

SALINAS, S., BRIANCON-MARJOLLET, A., BOSSIS, G., LOPEZ, M. A., PIECHACZYK, M., JARIEL-ENCONTRE, I., DEBANT, A. & HIPSKIND, R. A. 2004. SUMOylation regulates nucleo-cytoplasmic shuttling of Elk-1. *J Cell Biol,* 165**,** 767-73.

SAMPSON, D. A., WANG, M. & MATUNIS, M. J. 2001. The small ubiquitin-like modifier-1 (SUMO-1) consensus sequence mediates Ubc9 binding and is essential for SUMO-1 modification. *J Biol Chem,* 276**,** 21664-9.

SASAI, Y., LU, B., PICCOLO, S. & DE ROBERTIS, E. M. 1996. Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. *EMBO J,* 15**,** 4547-55.

SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L. K. & DE ROBERTIS, E. M. 1994. Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell,* 79**,** 779-90.

SAVARE, J., BONNEAUD, N. & GIRARD, F. 2005. SUMO represses transcriptional activity of the Drosophila SoxNeuro and human Sox3 central nervous system-specific transcription factors. *Mol Biol Cell,* 16**,** 2660-9.

SCHIER, A. F. 2003. Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol,* 19**,** 589-621.

SCHIER, A. F. & TALBOT, W. S. 2001. Nodal signaling and the zebrafish organizer. *Int J Dev Biol,* 45**,** 289-97.

SCHNEIDER, S., STEINBEISSER, H., WARGA, R. M. & HAUSEN, P. 1996. Beta-catenin translocation into nuclei demarcates the dorsalizing centers in frog and fish embryos. *Mech Dev,* 57**,** 191-8.

SCHUDDEKOPF, K., SCHORPP, M. & BOEHM, T. 1996. The whn transcription factor encoded by the nude locus contains an evolutionarily conserved and functionally indispensable activation domain. *Proc Natl Acad Sci U S A,* 93**,** 9661-4.

SCHULTE-MERKER, S., VAN EEDEN, F. J., HALPERN, M. E., KIMMEL, C. B. & NUSSLEIN-VOLHARD, C. 1994. no tail (ntl) is the zebrafish homologue of the mouse T (Brachyury) gene. *Development,* 120**,** 1009-15.

SHEN, Z., PARDINGTON-PURTYMUN, P. E., COMEAUX, J. C., MOYZIS, R. K. & CHEN, D. J. 1996. UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. *Genomics,* 36**,** 271-9.

SHIH, J. & FRASER, S. E. 1996. Characterizing the zebrafish organizer: microsurgical analysis at the early-shield stage. *Development,* 122**,** 1313-22.

SHIH, Y.-H. 2010. *The role of Sox3 in early zebrafish embryo development.* PhD Thesis, University of Nottingham.

SHIH, Y. H., KUO, C. L., HIRST, C. S., DEE, C. T., LIU, Y. R., LAGHARI, Z. A. & SCOTTING, P. J. 2010. SoxB1 transcription factors restrict organizer gene expression by repressing multiple events downstream of Wnt signaling. *Development,* 137**,** 2671-81.

SHIMIZU, T., YAMANAKA, Y., NOJIMA, H., YABE, T., HIBI, M. & HIRANO, T. 2002. A novel repressor-type homeobox gene, ved, is involved in dharma/bozozok-mediated dorsal organizer formation in zebrafish. *Mech Dev,* 118**,** 125-38.

SHIMIZU, T., YAMANAKA, Y., RYU, S. L., HASHIMOTO, H., YABE, T., HIRATA, T., BAE, Y. K., HIBI, M. & HIRANO, T. 2000. Cooperative roles of Bozozok/Dharma and Nodal-related proteins in the formation of the dorsal organizer in zebrafish. *Mech Dev,* 91**,** 293-303.

SIROTKIN, H. I., DOUGAN, S. T., SCHIER, A. F. & TALBOT, W. S. 2000. bozozok and squint act in parallel to specify dorsal mesoderm and anterior neuroectoderm in zebrafish. *Development,* 127**,** 2583-92.

SMET-NOCCA, C., WIERUSZESKI, J. M., LEGER, H., EILEBRECHT, S. & BENECKE, A. 2011. SUMO-1 regulates the conformational dynamics of

thymine-DNA Glycosylase regulatory domain and competes with its DNA binding activity. *BMC Biochem,* 12**,** 4.

SMITH, W. C. & HARLAND, R. M. 1992. Expression cloning of noggin, a new dorsalizing factor localized to the Spemann organizer in Xenopus embryos. *Cell,* 70**,** 829-40.

SOKOL, S. Y. 1996. Analysis of Dishevelled signaling pathways during Xenopus development. *Curr Biol,* 6**,** 1456-67.

SONG, J., DURRIN, L. K., WILKINSON, T. A., KRONTIRIS, T. G. & CHEN, Y. 2004. Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proc Natl Acad Sci U S A,* 101**,** 14373-8.

SPOKONY, R. F., AOKI, Y., SAINT-GERMAIN, N., MAGNER-FINK, E. & SAINT-JEANNET, J. P. 2002. The transcription factor Sox9 is required for cranial neural crest development in Xenopus. *Development,* 129**,** 421-32.

STANKOVIC-VALENTIN, N., DELTOUR, S., SEELER, J., PINTE, S., VERGOTEN, G., GUERARDEL, C., DEJEAN, A. & LEPRINCE, D. 2007. An acetylation/deacetylation-SUMOylation switch through a phylogenetically conserved psiKXEP motif in the tumor suppressor HIC1 regulates transcriptional repression activity. *Mol Cell Biol,* 27**,** 2661-75.

STEHMEIER, P. & MULLER, S. 2009. Phospho-regulated SUMO interaction modules connect the SUMO system to CK2 signaling. *Mol Cell,* 33**,** 400-9.

SUBRAMANIAN, L., BENSON, M. D. & INIGUEZ-LLUHI, J. A. 2003. A synergy control motif within the attenuator domain of CCAAT/enhancer-binding protein alpha inhibits transcriptional synergy through its PIASy-enhanced modification by SUMO-1 or SUMO-3. *J Biol Chem,* 278**,** 9134-41.

SUN, S. K., DEE, C. T., TRIPATHI, V. B., RENGIFO, A., HIRST, C. S. & SCOTTING, P. J. 2007. Epibranchial and otic placodes are induced by a common Fgf signal, but their subsequent development is independent. *Dev Biol,* 303**,** 675-86.

TAKAHASHI, Y., IWASE, M., KONISHI, M., TANAKA, M., TOH-E, A. & KIKUCHI, Y. 1999. Smt3, a SUMO-1 homolog, is conjugated to Cdc3, a component of septin rings at the mother-bud neck in budding yeast. *Biochem Biophys Res Commun,* 259**,** 582-7.

TANAKA, K., NISHIDE, J., OKAZAKI, K., KATO, H., NIWA, O., NAKAGAWA, T., MATSUDA, H., KAWAMUKAI, M. & MURAKAMI, Y. 1999. Characterization of a fission yeast SUMO-1 homologue, pmt3p, required for multiple nuclear events, including the control of telomere length and chromosome segregation. *Mol Cell Biol,* 19**,** 8660-72.

TANG, S. T., PENG, W. J., WANG, C. J., TANG, H. Q. & ZHANG, Q. 2012. Polymorphism M55V in gene encoding small ubiquitin-like modifier 4 (SUMO4) protein associates with susceptibility to type 1 (and type 2) diabetes. *Diabetes Metab Res Rev,* 28**,** 679-87.

TAO, Q., YOKOTA, C., PUCK, H., KOFRON, M., BIRSOY, B., YAN, D., ASASHIMA, M., WYLIE, C. C., LIN, X. & HEASMAN, J. 2005. Maternal wnt11 activates the canonical wnt signaling pathway required for axis formation in Xenopus embryos. *Cell,* 120**,** 857-71.

TATHAM, M. H. & HAY, R. T. 2009. FRET-based in vitro assays for the analysis of SUMO protease activities. *Methods Mol Biol,* 497**,** 253-68.

TATHAM, M. H., KIM, S., JAFFRAY, E., SONG, J., CHEN, Y. & HAY, R. T. 2005. Unique binding interactions among Ubc9, SUMO and RanBP2 reveal a mechanism for SUMO paralog selection. *Nat Struct Mol Biol,* 12**,** 67-74.

TAYLOR, K. M. & LABONNE, C. 2005. SoxE factors function equivalently during neural crest and inner ear development and their activity is regulated by SUMOylation. *Dev Cell,* 9**,** 593-603.

THISSE, B., WRIGHT, C. V. & THISSE, C. 2000. Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature,* 403**,** 425-8.

TRUONG, K., SU, Y., SONG, J. & CHEN, Y. 2011. Entropy-driven mechanism of an E3 ligase. *Biochemistry,* 50**,** 5757-66.

TSURUZOE, S., ISHIHARA, K., UCHIMURA, Y., WATANABE, S., SEKITA, Y., AOTO, T., SAITOH, H., YUASA, Y., NIWA, H., KAWASUJI, M., BABA, H. & NAKAO, M. 2006. Inhibition of DNA binding of Sox2 by the SUMO conjugation. *Biochem Biophys Res Commun,* 351**,** 920-6.

VALLIAN, S., CHIN, K. V. & CHANG, K. S. 1998. The promyelocytic leukemia protein interacts with Sp1 and inhibits its transactivation of the epidermal growth factor receptor promoter. *Mol Cell Biol,* 18**,** 7147-56.

VALLIAN, S., GAKEN, J. A., TRAYNER, I. D., GINGOLD, E. B., KOUZARIDES, T., CHANG, K. S. & FARZANEH, F. 1997. Transcriptional repression by the promyelocytic leukemia protein, PML. *Exp Cell Res,* 237**,** 371-82.

VAN DE WETERING, M. & CLEVERS, H. 1992. Sequence-specific interaction of the HMG box proteins TCF-1 and SRY occurs within the minor groove of a Watson-Crick double helix. *EMBO J,* 11**,** 3039-44.

WARGA, R. M. & KIMMEL, C. B. 1990. Cell movements during epiboly and gastrulation in zebrafish. *Development,* 108**,** 569-80.

WATANABE, K., TAKEDA, K., KATORI, Y., IKEDA, K., OSHIMA, T., YASUMOTO, K., SAITO, H., TAKASAKA, T. & SHIBAHARA, S. 2000. Expression of the Sox10 gene during mouse inner ear development. *Brain Res Mol Brain Res,* 84**,** 141-5.

WEAVER, C., FARR, G. H., 3RD, PAN, W., ROWNING, B. A., WANG, J., MAO, J., WU, D., LI, L., LARABELL, C. A. & KIMELMAN, D. 2003. GBP binds kinesin light chain and translocates during cortical rotation in Xenopus eggs. *Development,* 130**,** 5425-36.

WEIDINGER, G., THORPE, C. J., WUENNENBERG-STAPLETON, K., NGAI, J. & MOON, R. T. 2005. The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/beta-catenin signaling in mesoderm and neuroectoderm patterning. *Curr Biol,* 15**,** 489-500.

WEISS, J., MEEKS, J. J., HURLEY, L., RAVEROT, G., FRASSETTO, A. & JAMESON, J. L. 2003. Sox3 is required for gonadal function, but not sex determination, in males and females. *Mol Cell Biol,* 23**,** 8084-91.

WILKINSON, K. A. & HENLEY, J. M. 2010. Mechanisms, regulation and consequences of protein SUMOylation. *Biochem J,* 428**,** 133-45.

XU, G. & JAFFREY, S. R. 2009. Antibodies for ubiquitinated proteins. Google Patents.

XU, J., HE, Y., QIANG, B., YUAN, J., PENG, X. & PAN, X. M. 2008. A novel method for high accuracy sumoylation site prediction from protein sequences. *BMC Bioinformatics,* 9**,** 8.

YAMAGUCHI, T. P. 2001. Heads or tails: Wnts and anterior-posterior patterning. *Curr Biol,* 11**,** R713-24.

YAMANAKA, Y., MIZUNO, T., SASAI, Y., KISHI, M., TAKEDA, H., KIM, C. H., HIBI, M. & HIRANO, T. 1998. A novel homeobox gene, dharma, can induce the organizer in a non-cell-autonomous manner. *Genes Dev,* 12**,** 2345-53.

YAN, Q., GONG, L., DENG, M., ZHANG, L., SUN, S., LIU, J., MA, H., YUAN, D., CHEN, P. C., HU, X., QIN, J., XIAO, L., HUANG, X. Q., ZHANG, J. & LI, D. W. 2010. Sumoylation activates the transcriptional activity of Pax-6, an important transcription factor for eye and brain development. *Proc Natl Acad Sci U S A,* 107**,** 21034-9.

YANG, S. H., GALANIS, A., WITTY, J. & SHARROCKS, A. D. 2006. An extended consensus motif enhances the specificity of substrate modification by SUMO. *EMBO J,* 25**,** 5083-93.

YANG, S. H., JAFFRAY, E., SENTHINATHAN, B., HAY, R. T. & SHARROCKS, A. D. 2003. SUMO and transcriptional repression: dynamic interactions between the MAP kinase and SUMO pathways. *Cell Cycle,* 2**,** 528-30.

YANG, S. H. & SHARROCKS, A. D. 2005. PIASx acts as an Elk-1 coactivator by facilitating derepression. *EMBO J,* 24**,** 2161-71.

YANG, X. J. & GREGOIRE, S. 2006. A recurrent phospho-sumoyl switch in transcriptional repression and beyond. *Mol Cell,* 23**,** 779-86.

YEH, E. T. 2009. SUMOylation and De-SUMOylation: wrestling with life's processes. *J Biol Chem,* 284**,** 8223-7.

YUAN, H., ZHOU, J., DENG, M., LIU, X., LE BRAS, M., DE THE, H., CHEN, S. J., CHEN, Z., LIU, T. X. & ZHU, J. 2010. Small ubiquitin-related modifier paralogs are indispensable but functionally redundant during early development of zebrafish. *Cell Res,* 20**,** 185-96.

YUKITA, A., MICHIUE, T., DANNO, H. & ASASHIMA, M. 2007. XSUMO-1 is required for normal mesoderm induction and axis elongation during early Xenopus development. *Dev Dyn,* 236**,** 2757-66.

ZENG, X., HUANG, H., TAMAI, K., ZHANG, X., HARADA, Y., YOKOTA, C., ALMEIDA, K., WANG, J., DOBLE, B., WOODGETT, J., WYNSHAW-BORIS, A., HSIEH, J. C. & HE, X. 2008. Initiation of Wnt signaling: control of Wnt coreceptor Lrp6 phosphorylation/activation via frizzled, dishevelled and axin functions. *Development,* 135**,** 367-75.

ZHAO, J., CAO, Y., ZHAO, C., POSTLETHWAIT, J. & MENG, A. 2003. An SP1-like transcription factor Spr2 acts downstream of Fgf signaling to mediate mesoderm induction. *EMBO J,* 22**,** 6078-88.

ZHONG, S., DELVA, L., RACHEZ, C., CENCIARELLI, C., GANDINI, D., ZHANG, H., KALANTRY, S., FREEDMAN, L. P. & PANDOLFI, P. P. 1999. A RA-dependent, tumour-growth suppressive transcription complex is the target of the PML-RARalpha and T18 oncoproteins. *Nat Genet,* 23**,** 287-95.