EXPRESSION AND FUNCTION OF WILLIAMS SYNDROME TRANSCRIPTION FACTOR (WSTF) IN THE NEURAL DEVELOPMENT OF *XENOPUS LAEVIS*

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Abstract

Imitation Switch (ISWI) is a member of the Switch2/Sugar Non-Fermenting2 (SWI2/SNF2) superfamily of ATP (adenosine triphosphate) - dependent chromatin remodelers. Twenty different ISWI complexes have been identified so far in yeast, *Drosophila*, *Xenopus* and mammals. Three ISWI-containing complexes, WSTF-ISWI chromatin remodeling complex (WICH), ATP-dependent chromatin assembly and remodeling factor (ACF) and chromatin accessibility complex (CHRAC), have been characterized in *Xenopus*. Loss of ISWI function in *Xenopus* embryos results in severe defects in neural and eye development, including loss of retinal differentiation and formation of cataracts. We have begun to dissect the contributions of individual ISWI-dependent complexes to development, by using *in situ* hybridization and antisense morpholino knockdowns against subunits unique to different ISWI-containing complexes. Here I have investigated the WICH complex in *Xenopus* and have targeted the WSTF subunit. Whole mount *in situ* hybridization shows WSTF localized in the neural tissue including eye, brain, branchial arches and neural tube/ spinal cord. Injection of antisense morpholino oligonucleotides into fertilized eggs leads to misregulation and aberrant expression of genes involved in neural patterning and development, such as Bone morphogenetic protein 4 (BMP4) and Sonic hedgehog (Shh). Whole mount *in situ* hybridization shows mislocalization of BMP4 in the probable deformed neural tube of WSTF knockdowns. All these data suggest that WSTF is critical for neural development.

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Chapter 1

Introduction

1.1: Chromatin structure and remodeling

In eukaryotic cells, DNA, the genetic material, is housed within the nucleus. For a cell to survive and function, the DNA must be transcribed into RNA, which in turn must be translated into functional protein molecules. The resulting proteins have countless critical roles in the body, including structural and enzymatic functions.

The nucleus of a mammalian cell is typically 5-50μm in diameter while the total DNA is approximately over 2 meters in length. Despite this length, the nucleus is able to house the DNA, because the DNA is highly condensed into a complex structure called "chromatin", which is a complex of DNA and proteins. The basic structural unit of chromatin is the nucleosome (Figure 1.1).

Each nucleosome consists of 147bp of DNA coiled around a histone octamer leading to the first order of compaction. The histone octamer consists of two copies each of four core histones: H2A, H2B, H3 and H4. All histones have N-terminal tails that extend beyond the DNA in the nucleosome, while histone H2A has both N- and C-terminal tails (note that these tails are not visible in the ribbon diagram constructed from the crystal structure shown in Figure 1.1). Nucleosomes are rich in basic amino acids (lysine and arginine) and the histone core domains are among the most highly conserved protein domains in eukaryotes.

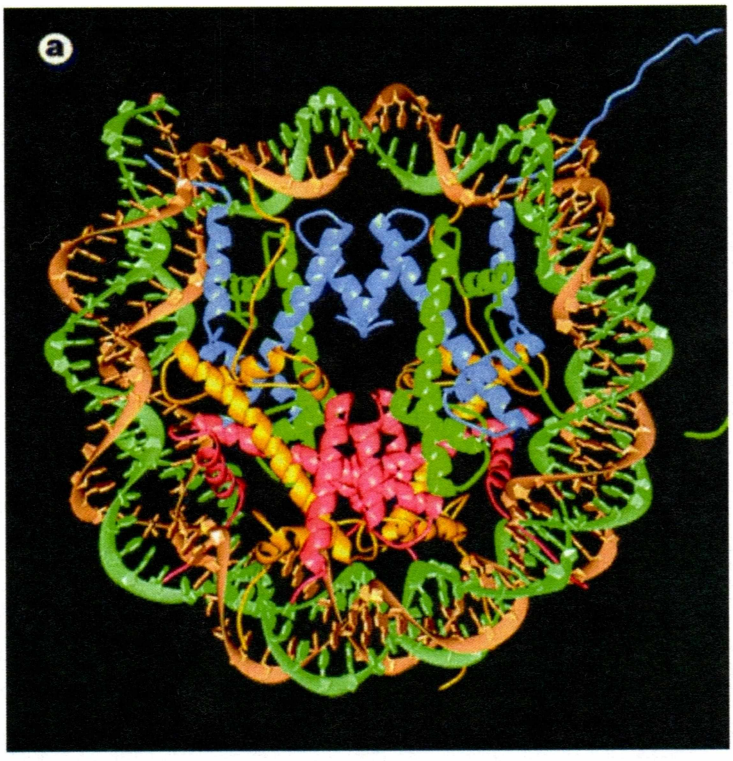


Figure 1.1. Nucleosome core particle: Ribbon diagram for the 147-bp DNA phosphodiester backbones (brown and light green). The eight histone protein core domains are shown in blue: H3; green: H4; yellow: H2A; red: H2B (adapted from Luger et al., 1997).

Nucleosomes are connected by variable lengths of linker DNA. This nucleosomal organization is repeated throughout the length of the DNA forming a "beads on string" arrangement that forms a DNA fiber 11 nm in diameter. Besides the core histones there are linker histones like H1 that interacts with nucleosomal core as well as linker DNA. Linker histones bind the entry and exit site of the nucleosome. The basic amino acids on histones tails also interact with the negatively charged DNA on other nucleosomes through electrostatic interactions. All these interactions facilitate the formation of the second order of compaction, which is a chromatin fiber 30 nm in diameter. Beyond this, the chromatin folds into higher order chromatin structures, which include loops and superhelical structures that are poorly understood, ultimately leading to the highly compacted metaphase chromosome that is 1400 nm in diameter (Figure 1.2).

This high level of compaction of the DNA in eukaryotic nucleus renders it inaccessible to the general transcription machinery. Thus transcriptional regulation is not only dependent upon the activation of the transcription factors but also on the complex chromatin structure. There are also specific histone modifications on histone tails that lay down the 'histone code' that also contribute towards the transcriptional profile of a particular cell type in a specific developmental stage.

Thus, chromatin remodeling is essential for altering chromatin compaction so that cells can function. This remodeling is achieved by two general mechanisms by two groups of chromatin remodeling enzymes: histone modifying enzymes and ATP-dependent chromatin remodeling complexes.

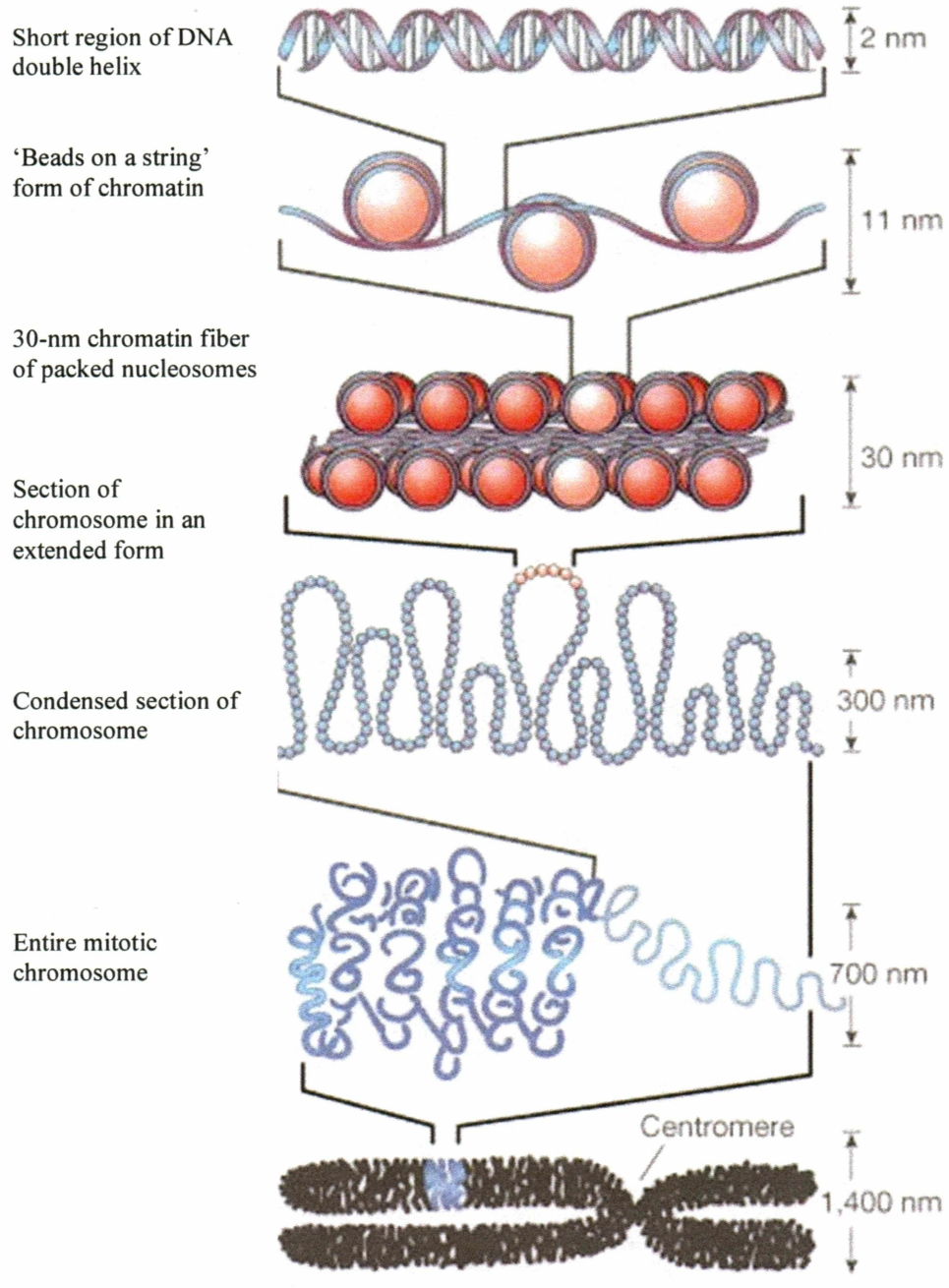


Figure 1.2. Condensation of DNA: DNA is wound around the octamer of core histones forming the nucleosomes, which further condense ultimately forming the 1400 nm mitotic chromosome, so that the DNA can fit inside the eukaryotic nucleus. Higher order structures are hypothetical (Felsenfeld and Groudine, 2003).

Histone modifying enzymes covalently modify particular amino acid residues on histone tails. Many histone modifications have been described, but the best characterized are acetylation, phosphorylation, methylation and ubiquitylation. These covalent modifications alter the interactions between DNA and histones and produce new binding surfaces for other factors to bind (Imhof, 2006). Some of these modifications are correlated with activation of certain genes while some of them lead to repression.

Figure 1.3 illustrates two examples of how histone modifications work. The unmodified histones have a high degree of positive charge, mainly from lysine and arginine residues, that allows the negatively charged DNA to associate tightly with the histones in a closed configuration. This DNA-histone contact is altered through changes in electrostatic interaction. For example, lysine 16 (K16) residue on H4 tail has a positive charge when unmodified. This residue can be acetylated by KATs (lysine acetyltransferases, previously known as HATs, or histone acetyltransferases). This leads to neutralization of the positive charge leading to the formation of a more open chromatin structure making the chromatin environment conducive to gene activation (reviewed in Wang et al., 2007a).

On the other hand, some modifications can form binding surfaces for specific non-histone proteins. These proteins have domains that recognize only specific modifications on histone tails. For example, the 'bromodomains' recognize only acetylated lysine (Zeng and Zhou, 2002) while 'chromodomains' bind to methylated lysine (Bannister et al., 2001). Bromodomains are involved in gene activation.

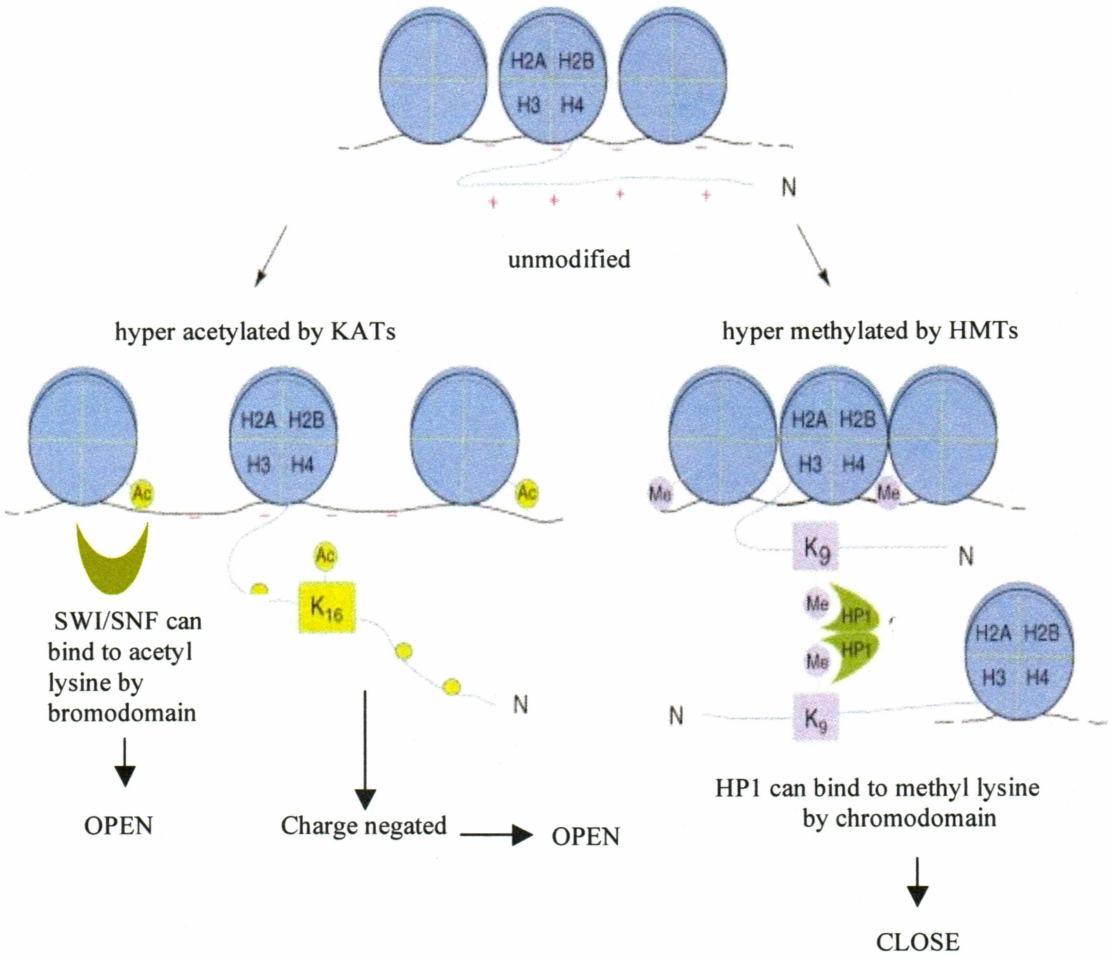


Figure 1.3. Histone covalent modifications: Mechanisms of covalent modifications of histone tails (adapted from Wang et al., 2007a). SWI/SNF subfamily proteins contain bromodomains. Me: methylated; Ac: acetylated.

For example, Rsc4, a component of yeast chromatin remodeling complex RSC, contains two bromodomains that bind acetylated lysine 14 (K14) on H3 (H3K14ac), resulting in gene activation (Kasten et al., 2004). Recognition of histone acetylation by bromodomains is also selective in nature. For example, Gcn5 binds to H4K16ac (Owen et al., 2000) while Brd2 protein recognizes H4K12ac (Kanno et al., 2004). Chromodomains, on the other hand, are involved in either gene activation or repression depending on which lysine has been methylated. An example of a chromodomain containing protein is HP1 that recognizes methylated lysine (K9) on histone H3 (H3K9me3), facilitating the formation of a closed conformation leading to gene repression or heterochromatin formation (reviewed in Wang et al., 2007a). On the other hand, the bromodomain of Chd1 protein interacts with H3K4me which is associated with active transcription (Pray-Grant et al., 2005).

The second group of chromatin modifiers, the ATP-dependent chromatin remodelers, works through two distinct mechanisms (Figure 1.4). They utilize the energy of ATP hydrolysis to alter the DNA-histone interactions. Firstly, they can slide and displace the nucleosome along the DNA. Secondly, they can also exchange one nucleosomal subunit (like H2A-H2B dimers with H2A variants etc, which can be unmodified or pre-modified) for another. These activities expose or conceal specific sites on the DNA to cellular machinery, or alter the basic chromatin composition by exchanging one histone variant for another (Cairns, 2005; Johnson et al., 2005).

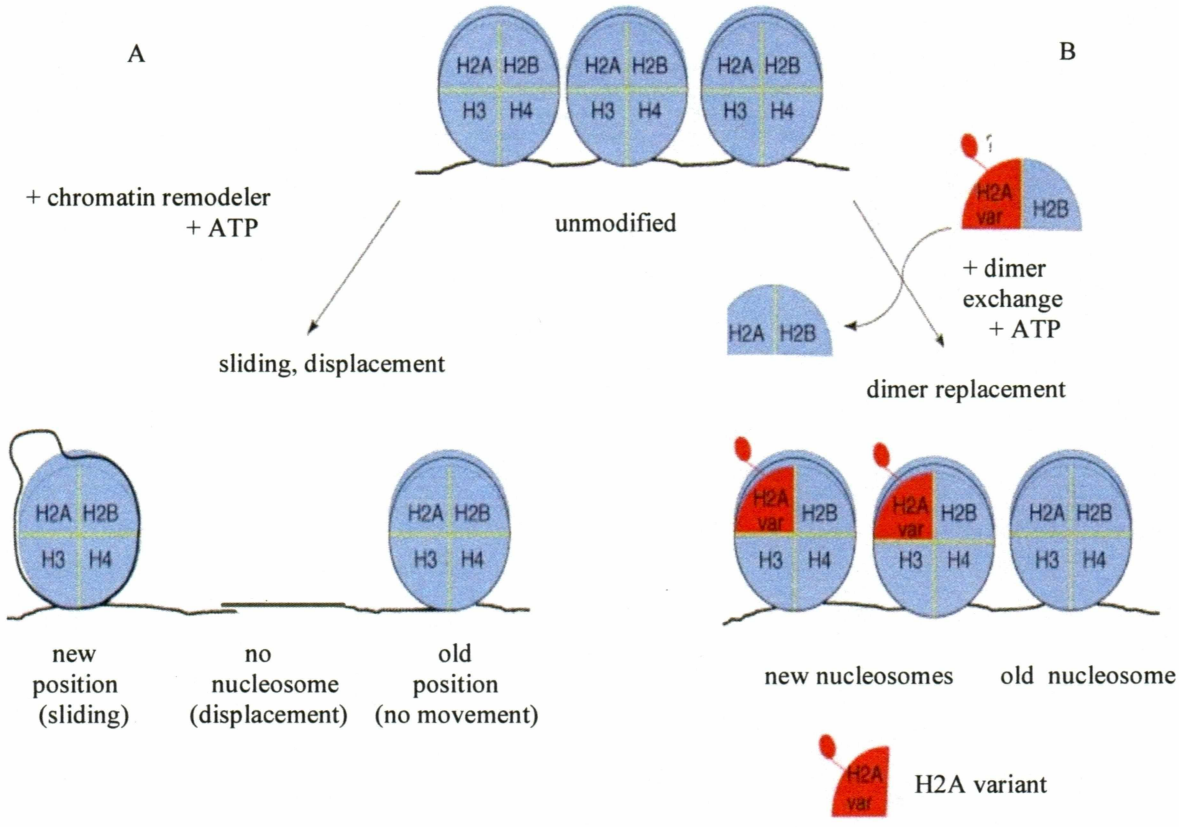


Figure 1.4. ATP-dependent chromatin remodeling: Schematic showing the two general mechanisms (A and B) of ATP-dependent chromatin remodeling as described in text. The red mark indicates some histone modification (adapted from Wang et al., 2007b).

All of these ATP-dependent chromatin remodelers belong to the SW12/SNF2 superfamily, which has at least 7 major subfamilies. These subfamilies include SNF2, CHD1, INO80, CSB, RAD54, DDM1 and ISWI. Many of these have been identified in several organisms and their structures and functions characterized. All contain a catalytic subunit belonging to the SWI2/SNF2 superfamily of proteins (Eisen et al., 1995). The structure and function of these enzymes are highly conserved in eukaryotes from yeast to humans. Subfamilies are defined by the degree of similarity between their ATPase domains and the presence of other characteristic domains. Members of the SNF2 subfamily contain a bromodomain (Marmorstein and Berger, 2001). Members of the CHD (Chromodomain Helicase DNA binding) protein family contain two tandem chromodomains and may also contain PHD fingers; both of which interact with methylated histone tails (Li et al., 2006a; Pena et al., 2006; Shi et al., 2006; Woodage et al., 1997; Wysocka et al., 2006). All INO80 homologs found in humans, *Drosophila*, yeast and plants contain a conserved domain known as DBINO (DNA binding INO) (Bakshi et al., 2004); this domain of human INO80 has been shown to bind DNA non-specifically (Bakshi et al., 2006). The CSB subfamily members enable transcription-coupled repair (Laposa et al., 2007). Members of the ISWI (Imitation Switch) subfamily contain a HAND-SANT domain in the carboxy-terminal half of the protein (Boyer et al., 2002) which is linked to a SLIDE domain by an alpha-helical spacer (Grune et al., 2003); the SLIDE domain interacts with nucleosomal DNA (reviewed in Dirscherl and Krebs, 2004; Mellor, 2006).

There is evidence of cross talk between the two kinds of remodelers. For example, Gcn5 (a KAT), acetylates lysine 753 on ISWI and lysine 14 on H3 both *in vitro* and *in vivo*, suggesting a direct cross-talk between two different remodeling mechanisms (Ferreira et al., 2007). Also, in *Drosophila*, NURF (an ISWI complex) is suggested to recruit ADAC (a KAT) to enable H4-K12 acetylation in order to regulate global chromosome structure (Carre et al., 2007). Thus the distinct affinity of a chromatin remodeler for one or more specific histone modifications may serve to target it to a point in the chromatin that has been specifically marked by the gene regulatory apparatus. This may impart to it distinct roles in developmental processes (reviewed in de la Cruz et al., 2005; de la Serna et al., 2006). All of the SWI2/SNF2 ATPases function as subunits of larger protein complexes. While the ATPase subunit serves as the motor that hydrolyses ATP and translocates histone cores along the DNA, the non-ATPase subunits of remodeling complexes may interact with tissue-specific transcription factors to target remodeling activity to specific genes, or may alter other structural features of the complex. The targeting of remodeling complexes both by specific histone marks and by tissue-specific transcription factors can exquisitely regulate remodeling activities to play a variety of roles in development (Cairns, 2005; Saha et al., 2006).

This thesis describes the expression patterns and developmental function of WSTF, which is a subunit of WICH - an ISWI-dependent complex in *Xenopus*. Therefore in the next section, I will discuss in detail the role of Imitation Switch (ISWI) subfamily in development of different organisms known to date.

1.2: The role of ISWI subfamily in development

The ISWI family is the largest and most diverse subfamily of ATP-dependent remodelers characterized thus far. In addition to the SWI2/SNF2 superfamily ATPase domain, members of the ISWI family are distinguished by the SANT-SLIDE domains in the C-terminal half of the protein. The ISWI protein was first identified in *Drosophila,* in which it is found in three different chromatin remodeling complexes: NURF (nucleosome remodeling factor), ACF (ATP-dependent chromatin assembly and remodeling factor), and CHRAC (chromatin accessibility complex) (Becker et al., 1994; Ito et al., 1997; Tsukiyama et al., 1994; Tsukiyama et al., 1995; Tsukiyama and Wu, 1995). Subsequently, ISWI-containing complexes have been identified in yeast, *Xenopus*, *Arabidopsis* and mammals. There are two ISWI homologs in budding yeast, Isw1and Isw2 (Tsukiyama et al., 1995), that are present in the 1sw1a, Isw1b, and Isw2/yCHRAC complexes (Iida and Araki, 2004; Tsukiyama et al., 1999; Vary et al., 2003). In *Xenopus* three ISWI-containing complexes have been characterized: ACF, CHRAC and WICH (Bozhenok et al., 2002; Guschin et al., 2000). Mammals have two ISWI homologs, SNF2L and SNF2H, which show tissue-specific expression patterns (Barak and Shiekhattar, 2004). SNF2H is present in at least 7 different complexes, including RSF (remodeling and spacing factor) (LeRoy et al., 1998; Loyola et al., 2003), hACF/WCRF (WSTF–related chromatin-remodeling factor) (Bochar et al., 2000; LeRoy et al., 2000), hCHRAC (Poot et al., 2000), hWICH (Bozhenok et al., 2002), hB-WICH (Cavellan et al., 2006), and NoRC (nucleolar remodeling complex) (Strohner et al., 2001). SNF2H has also been found to be associated in a large complex containing cohesin and subunits of the NuRD complex (nucleosome remodeling and histone deacetylase complex)

that also contains the Mi-2 ATPase (a member of the CHD subfamily) (Hakimi et al., 2002). SNF2L is the catalytic subunit of the hNURF complex (Barak et al., 2003) and CERF (CECR2 containing remodeling factor) complex (Banting et al., 2005). Recently, a *Caenorabditis elegans* ISWI homolog (isw-1) was identified, which appears to be present in a *C. elegans* NURF complex along with a nematode ortholog of NURF301 called NURF-1 (Andersen et al., 2006). The next section will concentrate on the developmental roles of these ISWI complexes in multicellular organisms.

1.2.1: Developmental roles of the ISWI ATPase

Because ISWI is present in so many different complexes, studies of *in vivo* roles for ISWI are complicated by the need to dissect the role of ISWI in the context of these different complexes. Two general strategies are generally taken: interference with the function of ISWI itself, which is assumed to impact all ISWI-dependent complexes, and inhibition of specific subunits within individual ISWI-containing complexes. I will first discuss the developmental roles of ISWI itself, and then I will discuss data that address the roles of specific ISWI complexes in development. The known developmental roles of these ISWI complexes have also been summarized in Tables 1.1 and 1.2.

In *Drosophila,* null mutations in *ISWI* are lethal, resulting in death at the late larval/early pupal stages (Deuring et al., 2000). In order to study the role of this essential gene, these researchers used somatic clonal analysis (in which patches of *ISWI* mutant tissue are generated in viable heterozygous animals) and dominant-negative *ISWI* mutants to study the effects of loss of ISWI in different tissues during development.

Table 1.1. Developmental roles of ISWI subfamily members across species: The known expression patterns and developmental functions of ISWI subfamily members are listed for several metazoan species. In many cases these proteins are essential for early development or for viability of individual cells; therefore some functions listed reflect data utilizing partial loss-of-function strategies. d; *Drosophila melanogaster,* x; *Xenopus laevis*, m; mammals (mouse or human).

PROTEINS	EXPRESSION PATTERNS	FUNCTIONS	REFERENCES
dISWI	- Restricted to CNS and gonads after germ band retraction	- Essential for late larval /early pupal development - Self renewal of GSCs	Elfring et al., 1994; Deuring et al.,2000
xISWI	- Brain, neural tube, eye	- Essential for normal neural and eye development	Dirscherl et al., 2005
mISWI	- (mouse) SNF2H is ubiquitously expressed but SNF2L is restricted to brain and gonads - (human) SNF2L and SNF2H are ubiquitously expressed	- Normal differentiation and survival of embryo - Corpus luteum formation - Blood cell formation - engrailed genes expression	Stopka and Skoultchi 2003; Lazzaro et al., 2006; Barak et al., 2004
dNURF301[a]	- Not Done except wing expression	- Essential for late larval/early pupal metamorphosis	Badenhorst et al., 2002; Badenhorst et al., 2005; Deuring et al., 2000

[a] subunit of NURF complex; [b] subunit of NURF complex

Table 1.2. Developmental roles of ISWI subfamily members across species: The known expression patterns and developmental functions of ISWI subfamily members are listed for several metazoan species. In many cases these proteins are essential for early development or for viability of individual cells; therefore some functions listed reflect data utilizing partial loss-of-function strategies and therefore cannot be considered an exhaustive list of functions. d; *Drosophila melanogaster,* x; *Xenopus laevis*, m; mammals (mouse or human). Hippocampus is a part of the brain involved in memory and spatial navigation; CNS, Central Nervous System; GSC, Germline Stem Cell.

PROTEINS	EXPRESSION PATTERNS	FUNCTIONS	REFERENCES
xBPTF[b]	- Not Done	- Essential for normal body axis, gut development	Wysocka et al., 2006
mBPTF	- Hippocampus and cerebellum of adult mouse brain	- Required for normal expression of engrailed genes involved in mid-brain development	Barak et al., 2003
dTau[c]	- Dorsal most thoracic region, wing imaginal disc, wing pouch	- Essential for sensory organ development (Dorso-Central bristle)	Vanolst et al., 2005
xWSTF[e]	- Eye, brain, neural crest cells	- Essential for normal eye and CNS development	Cus et al., 2006; S. Malakar, J. Henry, J. Krebs, unpublished results
mCECR2[d]	- Throughout nervous tissue	- Essential for neurulation	Banting et al., 2005

[c] TIP-5 related protein; [d] subunit of CERF complex; [e] subunit of WICH complex

In fact, any tissue expressing dominant-negative ISWI results in subsequent loss of corresponding adult structures derived from that tissue, indicating that ISWI is globally required for either cell viability or division. Before death at early pupal stages, *iswi* mutants also show defects in transcription of the segmentation gene *engrailed* and the homeotic gene *Ultrabithorax.* In addition, the structure of polytene chromosomes is altered in *iswi* mutants, particularly the male X chromosome, which is much shorter and broader than wild type. This could reflect a defect in replication or chromatin assembly in these mutant larvae.

Drosophila ISWI is also required for the maintenance of the self-renewal activity of germline stem cells (GSC) in the ovary (Xi and Xie, 2005). A FLP-mediated recombination method was used to eliminate ISWI function in GSCs. 99% of the homozygous *iswi* mutant germline stem cells are lost within a two-week period after elimination of *ISWI*, compared to 35% loss of wild type GSCs. The GSC division rates in *iswi* mutants are also reduced compared to wild type, suggesting that ISWI is required to stimulate division of GSCs.

In *Xenopus,* ISWI is essential for survival during early development, particularly neurulation, and is also critical for later stages of neural development and retinal differentiation (Dirscherl et al., 2005). Inhibition of ISWI *in vivo* with anti-ISWI morpholinos or a dominant negative ISWI mutant leads to defects in gastrulation and neural fold closure, aberrant eye development, and formation of cataracts. It also leads to misregulation of a number of genes required for neural patterning and development, such as Sonic hedgehog (Shh) and Bone Morphogenetic Protein 4 (BMP4).

The two ISWI homologs in mammals, SNF2H and SNF2L, perform different functions *in vivo*. While both of these genes are expressed in nervous tissue and gonads in mice, they are expressed at different times or in different subpopulations within these tissues (Lazzaro and Picketts, 2001). SNF2H is transiently up regulated in proliferating neural cell populations during embryogenesis and early post-natal development, while SNF2L expression is increased in terminally differentiated neurons after birth and in adult animals. Similarly, SNF2H is also expressed in proliferating cells within the ovary and testis, while SNF2L is prevalent in differentiated cells in these tissues.

The expression patterns of SNF2H and SNF2L differ somewhat between mouse and human. In adult mice, SNF2H is expressed ubiquitously and SNF2L is restricted to the brain and gonad, while in humans, SNF2H and SNF2L are both ubiquitously expressed (Barak et al., 2004). However, in humans, a splice variant of SNF2L called SNF2L+13 is highly expressed in non-neuronal tissue. SNF2L+13 lacks chromatin-remodeling activity; therefore, functional SNF2L dominates in the nervous system, while in other tissues the inactive isoform is the predominant source of SNF2L. This limits the major activity of SNF2L to the nervous system, as in mice. This differential pattern of expression probably suggests different developmental functions of these two homologs.

Consistent with the ubiquitous expression of SNF2H, and its upregulation in highly proliferative cells, *snf2h* homozygous mutant mice embryos die at the peri-implantation stage (Stopka and Skoultchi, 2003). Outgrowth of blastocysts *in vitro* is also impaired in these mutant mice due to growth arrest, loss of normal differentiation of the trophoectoderm and inner mass cells, and ultimately cell death within 3-6 days of

culture. These researchers also inhibited SNF2H in human primary hematopoietic progenitors, which then failed to differentiate into mature erythroid cells upon cytokine induction, indicating roles for SNF2H in both embryonic and adult differentiation programs.

Recent studies indicate that SNF2L may play a key role in the development of the corpus luteum in mammalian cells (Lazzaro et al., 2006), consistent with the restriction of mouse SNF2L expression to gonad and brain. While SNF2H is strongly expressed during growth of preovulatory follicles, SNF2L expression peaks during the process of luteinization, which represents the final stage of differentiation of the ovarian follicle. SNF2L interacts directly with Progesterone Receptor A, which is essential for activation of genes required for ovulation. Gonadotropin stimulation, which initiates luteinization, leads to binding of SNF2L to the proximal promoter of the StAR (Steroidogenic acute regulatory protein) gene, which is essential for steroidogenesis. Elimination of SNF2L results in a failure to activate StAR, interfering with a key stage in the luteinization process.

All the findings described above indicate that ISWI proteins play a wide and crucial role in development, including fundamental roles in cell viability, as well as more specific functions in embryogenesis, development of normal reproductive organs, and development of neural tissues. In the following section, we will dissect the developmental roles of individual ISWI complexes, where the functions of individual complexes have been addressed.

1.2.2: Developmental roles of individual ISWI complexes

In this section we will focus on the NURF, NoRC, CERF, WICH, and CHRAC complexes. The WICH and CHRAC complexes have some functional links, in that both may be involved in preventing the spread of heterochromatin and aiding in the movement of the replication fork through heterochromatin (Bozhenok et al., 2002; Collins et al., 2002). On the other hand NURF, NoRC, and WICH/B-WICH complexes have all been shown to have roles in transcriptional regulation. The NURF complex in humans is known to be involved in transcriptional activation (Barak et al., 2003), while other ISWI complexes appear to be primarily involved in transcriptional repression. NoRC is involved in repression of Pol I transcription (Zhou et al., 2002), and the yeast ISWI complexes repress a wide variety of genes (Fazzio et al., 2001; Goldmark et al., 2000; Kent et al., 2001; Ruiz et al., 2003; Vary et al., 2003). The conservation of different ISWI complexes may also reflect similar developmental roles of these complexes in different species. Most is known about the NURF complex; therefore, I will begin by illustrating its developmental role in different species.

NURF complex

The NURF complex was first identified in *Drosophila.* It consists of four subunits: ISWI, NURF38 (inorganic pyrophosphatase), NURF 301, and NURF55 (Gdula et al., 1998; Martinez-Balbas et al., 1998). *In vivo* studies show that null mutations of *nurf301* result in embryonic lethality during late larval/early pupal stages (Badenhorst et al., 2002). Like the *iswi* mutants, *nurf301* mutations result in impaired transcription of *Ultrabithorax* (*Ubx*) and *engrailed* (*en*). In homozygous *nurf301* mutants, expression of

ubx is undetectable in haltere and third leg discs of third instar larvae. Loss of UBX protein leads to homeotic transformation where the third thoracic segment (which normally includes the vestigial haltere and no sensory bristles) transforms into the second thoracic segment, resulting in increased size and sensory bristle development, and transformation of the haltere towards the wing fate. Also, normal expression of EN in the posterior compartment of the haltere and the leg discs in these mutants is reduced. In *nurf301* mutants the females are sterile and the males have highly aberrant X chromosome that is reduced in length and breadth, again consistent with the effect of an *iswi* mutant, suggesting that the major developmental phenotypes observed in *iswi* mutants are primarily due to loss of the NURF complex (Badenhorst et al., 2002; Deuring et al., 2000).

Comparison of genome-wide expression profiles of wild type and *nurf301* flies reveals that NURF regulates a large number of ecdysone-responsive genes (Badenhorst et al., 2005). Upon ecdysone binding, the ecdysone receptor activates numerous genes during larval-pupal development in wild type flies; however, these transcriptional changes are absent in *nurf301* mutants. Purified NURF complex physically associates with ecdysone receptor. The data indicate that the *Drosophila* NURF complex is required for ecdysteroid signaling and metamorphosis.

Human NURF, containing the SNF2L ATPase, has been implicated in transcriptional activation of genes involved in neuronal development in the mid-hindbrain (Barak et al., 2003). Depletion of *snf2l* by RNAi results in downregulation of the human engrailed genes *en-1* and *en-2* (regulators of midbrain development), which are homologs

of the *Drosophila en* gene that also requires NURF for its proper expression (described above). Likewise, depletion of the human NURF301 homolog, BPTF (Bromodomain and PHD finger Transcription Factor) results in reduced expression of *en-1* and probably *en-2*. Transfection of a mouse neuroblastoma cell line with wild type SNF2L results in significant potentiation of neurite outgrowth, also consistent with the role of NURF in promoting neural development in mammals.

Recent work has uncovered a developmental role for a *C.elegans* NURF complex, containing ISW-1 and a NURF301 homolog NURF-1 (Andersen et al., 2006). This study implicated worm NURF in promoting vulval cell fates, in opposition to several negative regulators of vulval development, such as the worm homolog of the NuRD complex.

Recent *in vitro* and *in vivo* studies in mammals and *in vitro* studies in *Drosophila* suggest that BPTF in humans and NURF301 in *Drosophila,* through their PHD zinc finger domains, specifically associate with trimethylated lysine 4 of histone H3 (H3K4) (Wysocka et al., 2006). Trimethylated H3K4 marks the transcription start site for almost all active genes (Ruthenburg et al., 2007). Depletion of trimethylated H3K4 results in dissociation of BPTF and SNF2L from the *HOXC8* promoter, which results in a compromised pattern of expression of this gene during development. In *Xenopus,* depletion of BPTF mRNA by anti-BPTF morpholino injections leads to axial deformities, gut mis-patterning, and blood defects. *Xenopus* BPTF depletion also causes deregulation of *HOXC8* expression, leading to posteriorization of Hox expression by several somite lengths (Wysocka et al., 2006). Thus the axial deformities and posteriorization of Hox expression in BPTF-depleted *Xenopus* embryos and homeotic transformation in *nurf301*

mutant flies (as mentioned earlier) might indicate a general role of NURF complex in proper patterning of cells leading to a normal morphology during development.

NoRC complex

The mammalian NoRC complex consists of a heterodimer of SNF2H and TIP5. It is responsible for transcriptional repression of Pol I genes, and acts by recruiting co-repressors to the rDNA promoters and by positioning nucleosomes to silence transcription (Li et al., 2006b; Zhou et al., 2002); recruitment of NoRC appears to require intergenic transcription from the rDNA intergenic spacers (Mayer et al., 2006). While a role for NoRC in mammalian development has not been investigated, in *Drosophila* the TIP5-related Tou (Toutatis) protein is necessary for sensory bristle development in association with Pnr (Pannier, a transcription factor that binds dorsocentral enhancer) and its co-factor Chip (Vanolst et al., 2005). Tou interacts directly with Iswi in both yeast and Cos cells, and Iswi also positively regulates Pnr/Chip function. This suggests that Tou and ISWI may act as subunits of the same multiprotein complex influencing sensory organ development. It is not yet known whether a *Drosophila* NoRC complex also represses Pol I transcription, or whether the mammalian NoRC complex has additional roles in regulation of Pol II genes.

CERF complex

CERF (CECR-2 containing remodeling factor) is a heterodimeric chromatin remodeling complex identified in mouse, which consists of CECR-2 (cat eye syndrome chromosome region candidate-2) and SNF2L (Banting et al., 2005). CECR-2 is mostly concentrated in nervous tissue. Homozygous mutant mice, generated by a Cecr2 gene-

trap-induced mutation, exhibit exencephaly, a neural tube defect which is similar to human anencephaly and arises due to failure of neural tube closure in the midbrain. This is reminiscent of the neural tube closure defects observed in ISWI knockdowns in *Xenopus* (Dirscherl et al., 2005). There is also a lack of cranium formation and lack of eyelids in exencephalic *cerc-2*$^{-/-}$ mice. As discussed above, murine SNF2L has previously been proposed to have a role in neural development, particularly in later stages of differentiation; however, there is not a *snf2l* knockout mouse available for study. The identification and characterization of this SNF2L-containing CERF complex provides direct evidence for a role of SNF2L in normal neurogenesis.

WICH complex

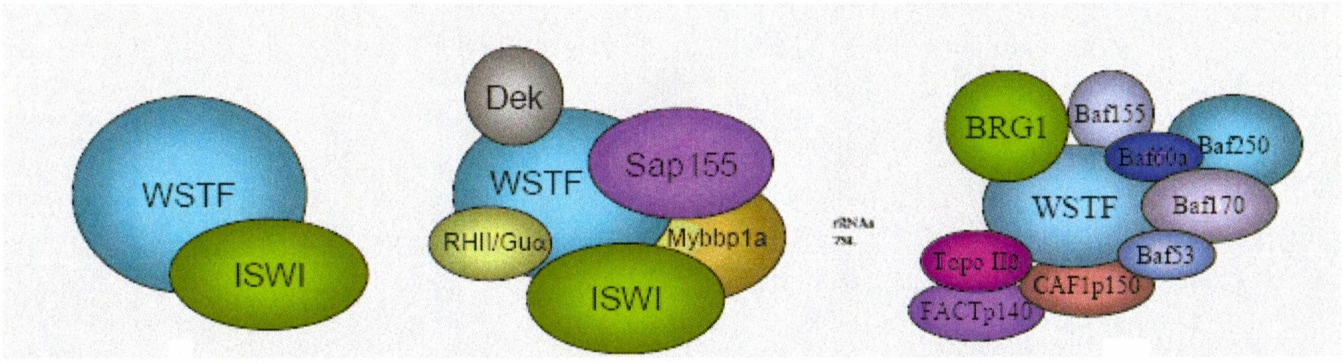
The WICH complex has been identified in both mammals and *Xenopus*, and consists of WSTF (Williams Syndrome Transcription Factor) and ISWI/SNF2H. The main focus of this thesis is the expression and function of WSTF subunit of the WICH (WSTF-ISWI chromatin remodeling complex) complex. Therefore, I will discuss WSTF in detail in the following section.

1.3: Williams syndrome transcription factor

Williams syndrome transcription factor (WSTF) was first identified in search for genes deleted in Williams syndrome (WS), which is an autosomal dominant hereditary disorder characterized by mental retardation, growth deficiency, elfin face, congenital vascular lesions, and other deficits (Lu et al., 1998). These developmental defects cannot necessarily all be attributed to lack of WICH function, as other genes are also deleted in Williams syndrome patients (Ewart et al., 1993).

WSTF exists in at least three different complexes: the WICH complex in *Xenopus* and mammals and the B-WICH and WINAC (WSTF including nucleosome assembly complex) complexes in mammals (Figure 1.5).

The WICH complex consists of WSTF and ISWI/SNF2H. In B-WICH, the WICH interacts with several nuclear proteins to form a 3 MDa complex. The B-WICH complex consists of core proteins WSTF, SNF2H and NM1 (nuclear myosin1). Nuclear proteins involved in transcription and processing of rRNA, such as RNA helicase II/Guα, the myb-binding protein 1a, CSB and the proto-oncogene Dek are also found in B-WICH. Besides, during active transcription, the core becomes associated with 45Sr RNA, 5s rRNA, 7SLRNA and U2 small nuclear RNA indicating its possible role in PolI and PolIII transcription (Cavellan et al., 2006). The WINAC complex on the other hand does not contain SNF2H as its catalytic subunit. Instead, it has the ATPase subunits, BRG1, which are components of the SWI2/SNF2 subfamily and not ISWI. It consists of



WICH **B-WICH** **WINAC**

Figure 1.5. Subunit composition of WSTF-containing complexes: WICH has been characterized in both *Xenopus* and mammals, while the B-WICH and WINAC complexes have only been identified in mammals thus far. WICH (WSTF-ISWI chromatin remodeling complex); WSTF (Williams syndrome transcription factor); SNF2H (sugar non-fermenting 2H); RHII/Guα (RNA helicaseII/Guα); Mybbp1a (myb-binding protein 1a); Sap155(splisosome associated protein 155); Dek (proto-oncogene)WINAC (WSTF including nucleosome assembly complex); BRG1(brahma related gene-1); Baf 53(BRG1 associated factor 53); Baf 60a (BRG1 associated factor 60a); Baf 70 (BRG1 associated factor 70); Baf 250 (BRG1 associated factor 250); TopoIIβ (topoisomerase IIβ); FACTp140 (chromatin specific transcription elongation factor); CAF(CREBBP associated factor).

at least 13 subunits. Two of these subunits, Topollβ and CAF-1p150, are DNA replication factors. Another subunit called FACTp140 is involved in transcriptional elongation. None of the above three subunits has been identified in any other known chromatin remodeling complexes. Besides, there are several BAFs (Baf53, Baf60a, Baf70, Baf155 and Baf250) which are chromatin remodelers consisting of BRG1/hBrm as their ATPase subunit (Kitagawa et al., 2003). Baf60 is a common subunit found in all BAF complexes and therefore found in WINAC as well. This subunit is known to interact with nuclear receptors and form a bridge between them and other BAF complexes (Debril et al., 2004; Hsiao et al., 2003). Recently it was found to play a role in development of heart and formation of right-left axis symmetry in mouse (Lickert et al., 2004; Takeuchi et al., 2007).

WSTF, the common subunit in all of these complexes, is a large protein of 1442 amino acids in length (Lu et al., 1998). WSTF contains three conserved domains – the DDT domain, the PHD domain and the Bromo domain (Lu et al., 1998 and Figure 1.6A). Alignment of the predicted domains of *Xenopus* WSTF proteins show that all these domains are well conserved among major model organisms (Cus et al., 2006).

In WSTF, only the structure of the PHD domain has been determined by Wright laboratory as a conserved interdigitated zinc finger motif with two variable loops (Pascual et al., 2000) and Figure 1.6.B. The variable loops vary in both length and sequence in all proteins containing PHDs, and might mediate a wide variety of functions. In WSTF, the variable loops form a deep cavity on the surface of the molecule. This architecture may indicate formation of a binding site for specific physiological partners.

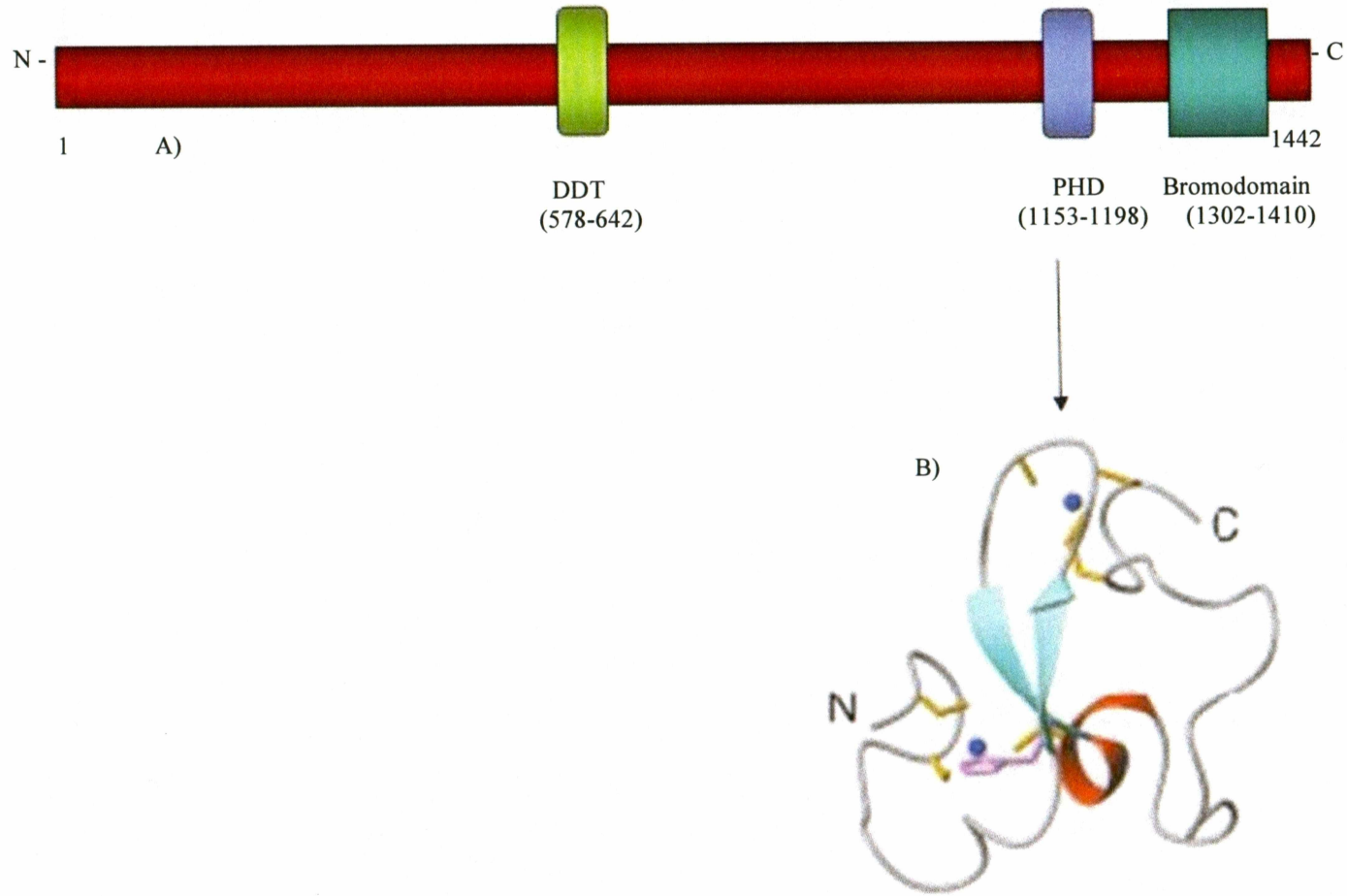


Figure 1.6. Schematic representation of the WSTF protein: WSTF is 1442 a.a in length. (A) The conserved domains of WSTF are indicated. DDT (yellow) PHD (purple) and bromodomain (teal). (B) Ribbon diagram of the PHD domain from human WSTF showing zinc ions in blue (adapted from Pascual et al., 2000).

No data is currently available on its function or binding specificity. However, some details on PHD domains in other proteins have been identified which might throw some light on the possible function of PHD finger of WSTF as well.

The PHD of human BPTF, the largest subunit of the NURF complex in humans, binds trimethylated lysine 4 at H3 (H3K4me3) (Li et al., 2006a). H3K4me3 is associated with the transcription start site of almost all active genes (Bernstein et al., 2005; Pokholok et al., 2005; Schneider et al., 2004). Both *in vitro* and *in vivo* assays show that human or mouse BPTF bind specifically to H3K4me3 peptides and nucleosomes and not to its di-/mono-methylated forms. Mutation of the PHD finger in human BPTF abolishes recognition of this mark. In *Xenopus*, BPTF knockdown compromises expression of the Hoxc8 gene and produces axial, blood and gut defects in the developing embryo (Wysocka et al., 2006). Blood defects are also observed in patients with Omenn syndrome where the PHD finger of a protein called RAG2 (involved in maturation of antigen receptors) is severely mutated (Sobacchi et al., 2006). The PHD finger of RAG2 binds both lysine and arginine at H3K4me3 and H3R2me2 respectively (Ramon-Maiques et al., 2007). In mouse, the PHD domain of ING2, a tumor suppressor, also targets the trimethylated lysine3 mark. *In vivo*, substitution of the histone binding residues of PHD finger in ING2 prevents its ability to induce apoptosis (Pena et al., 2006). ING2 is also a part of the repressor complex mSin3a-HDAC1 (Doyon et al., 2006). As a DNA damage response, ING2 binds to H3K4me3 through its PHD domain and stabilizes the repressor complex at the promoters of proliferating genes, thus playing a role in gene repression as

well (Shi et al., 2006). This is the first evidence of H3K4me3 involved in active gene repression.

On the other hand, Bromodomains bind acetylated lysines on histone tails, and like PHD domains, they show specificity for particular modified lysines in specific histone tails. *In vitro* experiments show that in the context of the WINAC complex, WSTF interacts physically with acetylated histones through its bromodomain in order to recruit vitamin-D receptors (VDR) to the promoter regions of vitamin D-regulated genes (Fujiki et al., 2005).

The third domain, DDT, is a conserved structures present in numerous PHD-containing proteins, and is considered a putative DNA binding motifs, although this has not been confirmed experimentally (Doerks et al., 2001).

The expression pattern of WSTF mRNA has been characterized in both humans and *Xenopus.* In humans, WSTF is expressed in almost all tissues of the body including brain, heart, skeletal muscle, lung, liver, spleen, thymus, prostrate, testis, ovary, small intestine, colon, placenta, kidney and pancreas. Northern blot analysis reveals stronger expression in the brain than in the lung, liver or kidney in adult tissues, while there were similar levels of expression in all these tissues in fetuses (Lu et al., 1998). In *Xenopus* embryos *in situ* hybridization reveals that WSTF is differentially expressed in neural tissue, especially in the eye, brain and neural crest cells (Cus et al., 2006), and our unpublished results). Defects resulting from WSTF depletion in *Xenopus* embryos are discussed in detail in the results section of this thesis.

Several possible roles of WSTF in transcription have been identified. Studies have shown that the B-WICH complex may be involved in both Pol1 and PolIII transcription. A ChIP (chromatin immunoprecipitation) using human autoimmune serum that recognizes the largest subunit of Pol1, show that the core of B-WICH that includes WSTF-SNF2H-NM1, is physically associated with RNA Pol I. Inhibition of WSTF proteins *in vivo* by anti-WSTF antibody prevents PolI transcription on pre-assembled chromatin templates but not on naked DNA suggesting that SNF2H of the B-WICH is involved in remodeling during PolI transcription. A ChIP assay using NM1 antibody revealed that WSTF, SNF2H and NM1 also occupy the rDNA promoter and coding regions. In addition, WSTF silencing by RNAi in HeLa cells results in a 39% reduction of 45s pre-rRNA transcript (Percipalle and Farrants, 2006; Percipalle et al., 2006). The transcription of genes associated with PolIII, like 5SrRNA and 7SLRNA, is also markedly reduced in cells depleted of WSTF, pointing towards the involvement of B-WICH in PolIII transcription (Cavellan et al., 2006).

Unlike B-WICH that supports active transcription, WINAC complex acts as a repressor of vitamin-D receptor (VDR) associated genes. VDRs belong to the nuclear receptor (NR) gene family and are ligand-inducible transcription factors. ChIP reveals that WINAC is associated with ligand binding domain (LBD) of unliganded VDR. VDR recruitment to the target promoters is impaired in patients with WS. Over expression of WSTF in the fibroblast cells of Williams syndrome patients rescues recruitment of VDRs to their targets, indicating that WINAC helps in the recruitment of unliganded VDR to the VDR response elements on target gene promoters (Kato et al., 2004; Kitagawa et al.,

2003). *In vitro* histone binding experiments with purified histone octamers from HeLa cells show that WSTF interacts with histone octamers through its bromodomain. This may suggest that besides interacting with VDR before recruitment to the DNA, WSTF also interacts with acetylated lysines on the promoter through its bromodomain. Supporting this, a mutant WSTF lacking its bromodomain acts as dominant-negative and prevents normal repression of 1-alpha hydroxylase gene which is a Vitamin-D regulated gene (Fujiki et al., 2005).

Interestingly, WSTF has also been implicated in replication. Immunofluorescence experiments in HeLa cells indicate that WSTF accumulates at pericentric heterochromatin during replication and is stably associated with mitotic chromosomes, suggesting a role of WSTF in the replication of heterochromatin (Bozhenok et al., 2002). WSTF interacts directly with the DNA clamp, PCNA, at the replication foci and recruits SNF2H to the replication site. Depletion of WSTF or SNF2H by RNAi leads to aberrant compaction of newly replicated heterochromatin and increased amounts of heterochromatin markers *in vivo*. These studies suggest that WSTF is important in maintaining the proper chromatin structure during and immediately after DNA replication. A model has been proposed in which WSTF (WICH complex) keeps the chromatin structure open immediately after replication, thus allowing the reassociation of DNA-binding factors so that the proper epigenetic state of the chromatin is maintained in the newly replicated DNA (Poot et al., 2005, 2004). Recently, chromatographic and spectrometric studies also showed that WSTF is associated with

Cdt1, a protein central to DNA replication and cell cycle, reconfirming the role of WSTF in replication (Sugimoto et al., 2007).

WINAC is also involved in replication. *In vitro* assays determined that WINAC could facilitate formation of chromatin structure on newly replicated DNA by nucleosomal arrangement (Kitagawa et al., 2003). WINAC is also detected throughout all stages of cell cycle. In addition, RNAi mediated depletion of WSTF or BRG1/Brm ATPases results in reduced DNA synthesis in cultured human cells (Kitagawa et al., 2003; Sugimoto et al., 2007).

In brief, WSTF resides in several complexes and is involved in both transcription activation and repression, as well as in replication, where it may be important for the proper epigenetic inheritance of the newly replicated DNA. In addition, the presence of splicing factor Sf3b/SAP155 and DNA repair factor CSB in the B-WICH complex (Cavellan et al., 2006) might indicate its involvement in splicing and DNA repair as well. All these essential and intriguing functions demand further investigation of the WSTF protein.

Previous work in our lab showed that in *Xenopus*, ISWI is essential for early embryonic development, and is also critical for later stages of neural development and retinal differentiation (Dirscherl et al., 2005). *In vivo*, inhibition of ISWI led to defects in neural fold closure, aberrant eye development, formation of cataracts and misregulation of certain genes required for neural patterning and development, such as Shh and BMP4. I hypothesize that the WICH complex, being an ISWI-dependent complex, might play a critical role in the normal neural development of the *Xenopus* embryos as well.

Inhibition of this complex might lead to abnormalities that would represent a subset of defects (morphological, histological and transcriptional) that we observe in the ISWI knockdown *Xenopus laevis* embryos. These abnormalities might include neural defects involving the eye (especially the retina and the lens), brain and the spinal cord as well as perturbation of transcription of certain neuro-developmental genes.

So the overall goal of this research is to understand the role of the WICH complex in the neural development of *Xenopus*, particularly the eye, and how this WICH complex functions to contribute to the global role of all the ISWI complexes. As a first step in this evaluation of WICH function, I performed whole mount in situ hybridization to detect the expression pattern of the WSTF subunit that is unique to the WICH complex that would distinguish it from the other ISWI complexes characterized in *Xenopus*. Next I analyzed the phenotypes of embryos in which WSTF has been knocked down via anti-WSTF morpholino injections. I also performed Western analysis of protein extracts obtained from embryos injected with morpholinos in order to confirm that the defects observed in the WSTF knockdowns were actually due to reduced levels of endogenous WSTF protein. To further characterize the defects that I observed, I also obtained histological sections of the eye of the deformed embryos from our collaborator Dr Jonathan Henry (University of Illinois Urbana). I also measured the effects of WSTF knockdown on transcription and of Shh and BMP4 through rtRT-PCR. These genes are critical for eye development and were also found to be perturbed in the ISWI knockdowns. I also performed whole mount in situ hybridization to detect the changes in the spatial localization of BMP4 in WSTF knockdowns.

Chapter 2

Materials and Methods

2.1: Morpholinos:

A 25-mer antisense morpholino was designed against the 5' UTR of *Xenopus WSTF* (5'- GCTTCTCGTGGGATGATAGTCCCGC-3') adjacent to the translation start site (Gene Tools, LLC, Philomath, OR). A 25-mer inverse-WSTF morpholino (5'-CGCCCTGATAGTAGGGTGCTCTTCG-3') was used as a control. As recommended by Gene Tools, these morpholinos were resuspended in sterile nanopure water at 1mM and injected into embryos to give a final concentration in the range of 250 to 500μM.

2.2: Microinjection:

Adult *Xenopus laevis* were purchased from Xenopus Express (Plant City, Florida). Eggs were collected from female frogs (induced by injection of 0.8 ml of chorionic gonadotropin at a potency of 14000 IU/ mg) and fertilized *in vitro* using surgically isolated testes. Microinjections were performed at the one-cell stage using standard protocols (Sive et al., 2000). Morpholinos were injected at 500 μM and 250 μM concentrations (equivalent to 4.2 ng/nl and 2.1 ng/nl). Each embryo received 10 nl injection volume. The injected embryos were incubated at 16^0C for 24-48 hrs and staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967).

2.3: Protein isolation and immunoblotting:

Total proteins were collected at stages 37/38, 41 and 45 from both normal and WSTF knockdown embryos, using the protocol of (Merzdorf and Goodenough, 1997) with the following modifications: chymostatin, DIFP and Trasylol were omitted from the

protein isolation buffer and a final concentration of 1μM PMSF was added to the buffer. SDS-gel electrophoresis was performed with these samples and transferred using standard methods (Sambrook and Gething, 1989). Dr. Paul Wade (NIEHS) generously provided us with WSTF and ISWI antibodies. E-Cadherin antibody (Developmental Studies Hybridoma Bank, University of Iowa) was used as a loading control.

2.4: Whole mount in situ hybridization:

After *in vitro* fertilization, embryos were collected and fixed in MEMFA and stained (Sive et al., 2000), with the following changes: A preantibody incubation step was added with 2% BMB (10% w/v BMB in MAB) blocking reagent and 20% MAB (100 mM maleic acid, 150 mM NaCl; pH 7.5) at room temperature for 1 hour. The RNase treatment step was omitted. AP buffer without levamisol (100mM Tris; pH 9.5, 50mM $MgCl_2$, 100mM NaCl, 0.1% Tween20) was used as a detection buffer. BM purple (Roche Diagnostics, Mannheim, Germany) was used for the detection method. *WSTF* and BMP4 sense RNA was used as negative controls respectively for *WSTF* and BMP4 *in situs*. Digoxygenin (DIG)-labelled RNA antisense probe was prepared for both WSTF (446 bp) and BMP4 (400bp). A fragment (corresponding to nucleotide 1790 – 2236) was amplified from complete WSTF cDNA (kindly provided by Dr. Paul Wade, NIEHS) and inserted into pGEM vector (Promega) and *in vitro* transcribed into WSTF *in situ* probe. For BMP4 probe, all the procedures were exactly the same, except the fragment (2102 to 2502) inserted into pGEM was amplified from genomic DNA. All antisense and sense probes for both the genes were used at a concentration of 0.5μg/ml.

2.5: rtRT-PCR:

Total RNA was isolated from *Xenopus* embryos at several stages with RNA WIZ™ (Ambion). Real time reverse transcriptase PCR was performed on a Cepheid Smartcycler using Lightcycler ™ reagents. Each reaction contained 1μg total RNA and 2.5 pmoles of primers and subjected to 40 cycles. Annealing temperatures were optimized for each primer set. Products were detected with Sybr Green. A standard curve was prepared for each gene of interest. For making this standard curve several dilutions of total RNA was made. RT-PCR reactions were performed (40 cycles) for each primer set (each gene of interest) and the amounts of DNA produced were detected by a fluorescent signal (using Sybr Green). The intensity of the signal is proportional to the amount of double-stranded DNA formed in the reaction. This standard curve was then used as a reference to compare and quantitate the total amount of double-stranded DNA produced in the experimental samples. The quantitation was performed using the Cepheid SmartCycler® 3.0 software loaded on the rtPCR machine (Smartcycler). For each reaction, a growth curve was plotted as the fluorescence intensity of the product increased. The cycle threshold for the sample was then quantitated relative to the cycle threshold values for the standard curve generated with the same primers. Primers were obtained from Sigma Genosys (St. Louis, MO). Sequences for the primers used are as follows:

EF1alpha: 5-CAGATTGGTGCTGGATATGC-3 + 5'-

ACTGCCTTGATGACTCCTAG-3'

BMP4*:* 5’- GGAGCTTCCATCACGAGGAGCAT-3' + 5’-

CACGCTGGACCATGGTCTATTTGTT-3'

Shh*:* 5’-GTCTTTGCCAGCAACATCCAACCA-3'+ 5’-

TTAGAGGCGCATAAGCTCCAGTGT-3'

Chapter 3

Results

3.1: Xenopus WSTF is expressed primarily in anterior neural tissue

As a first step towards understanding the contribution of WSTF in *Xenopus laevis* development, I performed *in situ* hybridization to determine the expression pattern of WSTF. Figure 3.1 shows *in situ* hybridization for WSTF mRNA expression in *Xenopus* embryos at different stages of development. The sense controls are shown in the Appendix. In the embryos probed with the antisense probe, I observed very strong expression of WSTF in the anterior neural structures such as the brain, eye, branchial arches and much weaker expression at the posterior part of the embryo. This is consistent with results from the Kühl laboratory, which have also recently shown similar patterns of expression for WSTF at a later stage, as well as expression in the migrating neural crest cells in some earlier stages of development (Cus et al., 2006). Strong WSTF expression is also seen in the fetal and adult brain tissues of humans (Lu et al., 1998). WSTF expression data show high overlap with ISWI expression in the brain, eye and branchial arches (Dirscherl et al., 2005). Overlap of expression might indicate overlap of functions - both ISWI and WSTF knockdown embryos have neural defects, which I discuss next.

3.2: Xenopus WSTF is critical for normal development of the eye and brain

Previous study in our lab found that complete ISWI knockdown embryos in *Xenopus* had failure of neural tube closure and a lethal phenotype at an early stage while partial ISWI knockdowns developed congenital cataract in the eyes (Dirscherl et al., 2005).

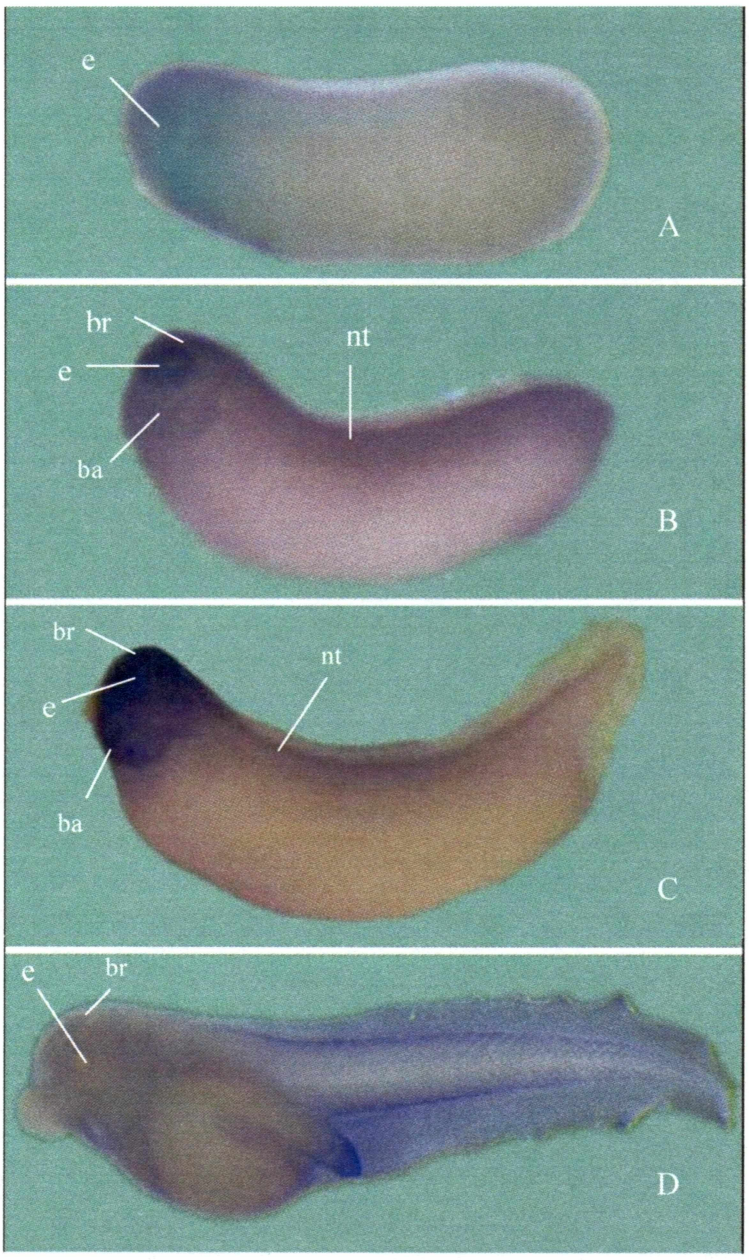


Figure 3.1. WSTF expression in *Xenopus* embryos: A-D: Whole mount *in situ* hybridization of *Xenopus* embryos using a 468 base WSTF digoxigenin-labeled probe. Left lateral views of stage 24 (A), 26 (B), 33 (C), and 45 (D) embryos showing expression of WSTF mRNA in neural tube, brain and eye. All embryos are oriented with the anterior end to the left side of the figure, and dorsal to the top. Eye (e) designates the optic vesicle at stage 24; the primary eye vesicle at stage 26; the optic cup at stage 33 and optic ventricles at stage 41. e: eye; nt: neural tube; br: brain; ba: branchial arches.

Since I observed overlap of expression between WSTF and ISWI, I wanted to determine the contribution of WSTF (or WICH complex) in the neural development of *Xenopus*. I hypothesized that WSTF knockdown embryos might have neural abnormalities that might indicate a subset of defects seen in ISWI knockdown embryos.

In order to determine WSTF function, I injected 42 and 21ng of anti-WSTF morpholino (MO) into fertilized eggs to inhibit the translation of endogenous WSTF mRNA. I also injected a lower concentration (10 ng) without any obvious phenotype. For negative controls, I used identical concentrations of inverse morpholino. After injection, I incubated the embryos at 16°C.

Representative injected embryos are shown in Figure 3.2. The WSTF knockdowns (Figure 3.2 B, D, F, H, J) have extremely reduced or absent eye development and their brains are highly deformed compared to the wild type (Figure 3.2 A, C, E, G, I), as revealed by the undersized and misformed heads of the knockdowns. The pattern of head deformation suggests that these embryos have severely defective forebrain and midbrain structures; this could be confirmed by *in situ* hybridization to detect markers of forebrain and midbrain development.

The embryos first start showing defects at stage 37/38 (53.5 hours post-fertilization); this stage in eye development marks the beginning of arrangement of the three retinal layers and differentiation of visual cells. Defects become more pronounced in the later stages (like stage 41 and stage 45), during which more mature brain and optic structures (like optic ventricles, rods and cones in the retina, cerebral hemispheres) are

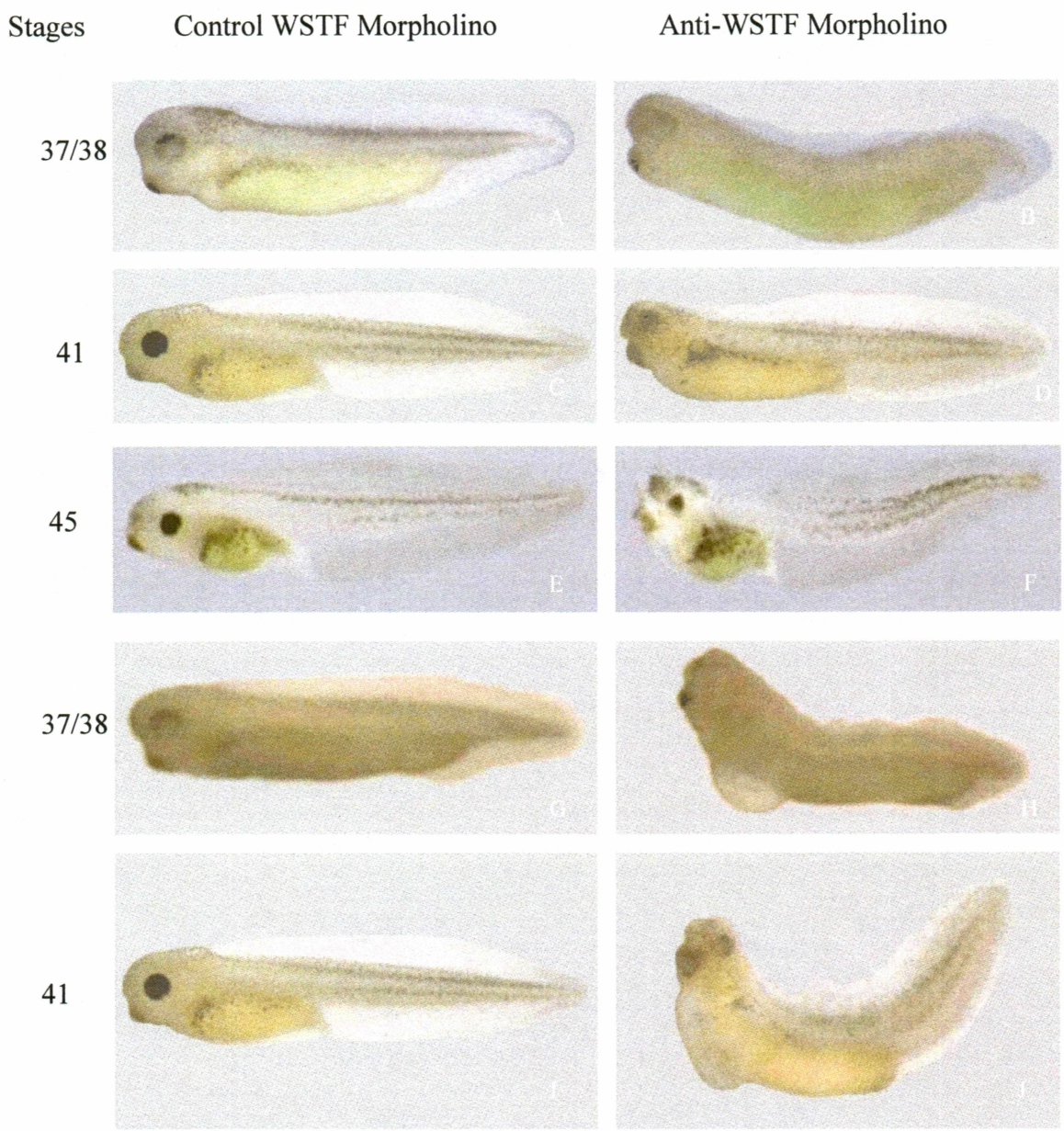


Figure 3.2. Phenotypes of embryos lacking WSTF: *Xenopus laevis* embryos were injected with control morpholino (A,C,E,G,I) or anti-WSTF morpholino (B,D,F,H,J). The embryos were either injected with 2.1 ng/nl (A-F) or 4.2 ng/nl (G-J) of morpholino. **A-B, G-H:** Embryos photographed when control embryos reached approximately stage 37/38. **C-D, I-J:** Embryos photographed when control embryos reached stage 41. **E-F:** Embryos photographed when control injected embryos reached stage 45. Embryos injected with 4.2ng/nl of anti-WSTF morpholino did not survive to stage 45.

formed. All WSTF knockdowns injected with 42 ng of morpholino died shortly after stage 41, and very few of the 21 ng-injected embryos survived until stage 45, and even these died shortly thereafter. The early lethality seen in higher dosage injections might be due to complete knockdown of all WICH complexes. Besides, like mammals, *Xenopus* might have other WSTF containing complexes, other than WICH, that are important for neural development, and the knockdown of all those complexes might contribute to the lethality.

To quantitate the effects of anti-WSTF morpholino injections, for every experiment I also counted the number of defects that present in both the knockdowns and the controls. Table 3.1 shows the summarized data for multiple injection experiments. Out of 441 embryos injected with 41 ng of anti-WSTF morpholino that survived until stage 37/38, 80% exhibited defects in neural development. Embryos injected with 21 ng exhibited neural defects in 57% of survivors. In contrast, injection of the control morpholino resulted in only 12-13% defective embryos, and these included a variety of non-specific defects (compared to the consistent phenotypes of the WSTF knockdowns), which is a typical outcome of any injection.

There is no significant qualitative phenotypic difference between the embryos injected with the two different morpholino concentrations. These abnormalities are consistent with the strong expression of WSTF in neural tissue at later stages of development. Although, complete knockdown of ISWI leads to neural defects and lethality early on, no observable defects are seen in the earlier stages of development for

Table 3.1. Neural phenotypes in injected embryos: [a]Includes brain, eye and spinal deformities. Total number of embryos was counted at stage 37/38 where the embryos first start showing defects. The numbers are a total from at least three different injections. MO stands for morpholino. There was 52% death in the embryos injected with 4.2ng/nl of anti-WSTF MO before it reached stage 37/38. This percentage was substantially higher than 19% death observed in the control injections. No such substantial difference was observed in the lower concentration injections.

	Concentration (x 10nl injected embryos)	Total # of embryos	Total # of defective embryos[a]	% of defective embryos
Uninjected		1764	33	2%
Control MO	2.1ng/nl	774	103	13%
	4.2 ng/nl	643	82	12%
Anti-WSTF MO	2.1ng/nl	839	480	57%
	4.2ng/nl	441	351	80%

WSTF knockdowns, in spite of the fact that Cus et al. (2006) observed expression of WSTF in the migrating neural crest cells. This might indicate that the WICH complex is redundant in the earlier stages or that later defects are a downstream effect of early depletion of WSTF.

3.3: WSTF is specifically inhibited in the WSTF knockdowns

Next, I wanted to confirm that the abnormalities seen in the knockdowns were actually due to inhibition of endogenous WSTF and not due to any non-specific interactions in the embryo. So, I performed Western analysis to compare the level of expression of WSTF proteins in the WSTF knockdowns with those of the controls. Total protein was isolated from embryos injected with either 42 ng or 21 ng of morpholino. The collected samples were run on an SDS-page gel and probed with anti-WSTF antibody, kindly provided by Dr. Paul Wade (NIEHS).

Western analysis revealed that WSTF protein is significantly reduced in WSTF knockdowns throughout several stages of development as indicated in the blot itself (Figure 3.3 A) as well as the bar chart depicting its quantitation (Figure 3.3 B). I used E-Cadherin as a loading control, which showed similar levels of expression in all the samples. I also detected ISWI levels in these samples. All the samples showed similar levels of ISWI indicating that the ISWI levels remain unperturbed in the WSTF knockdowns.

Taken together, these results indicate the specificity of the anti-WSTF morpholino, and confirm that the abnormalities observed in the WSTF knockdowns are due to reduced levels of endogenous WSTF protein.

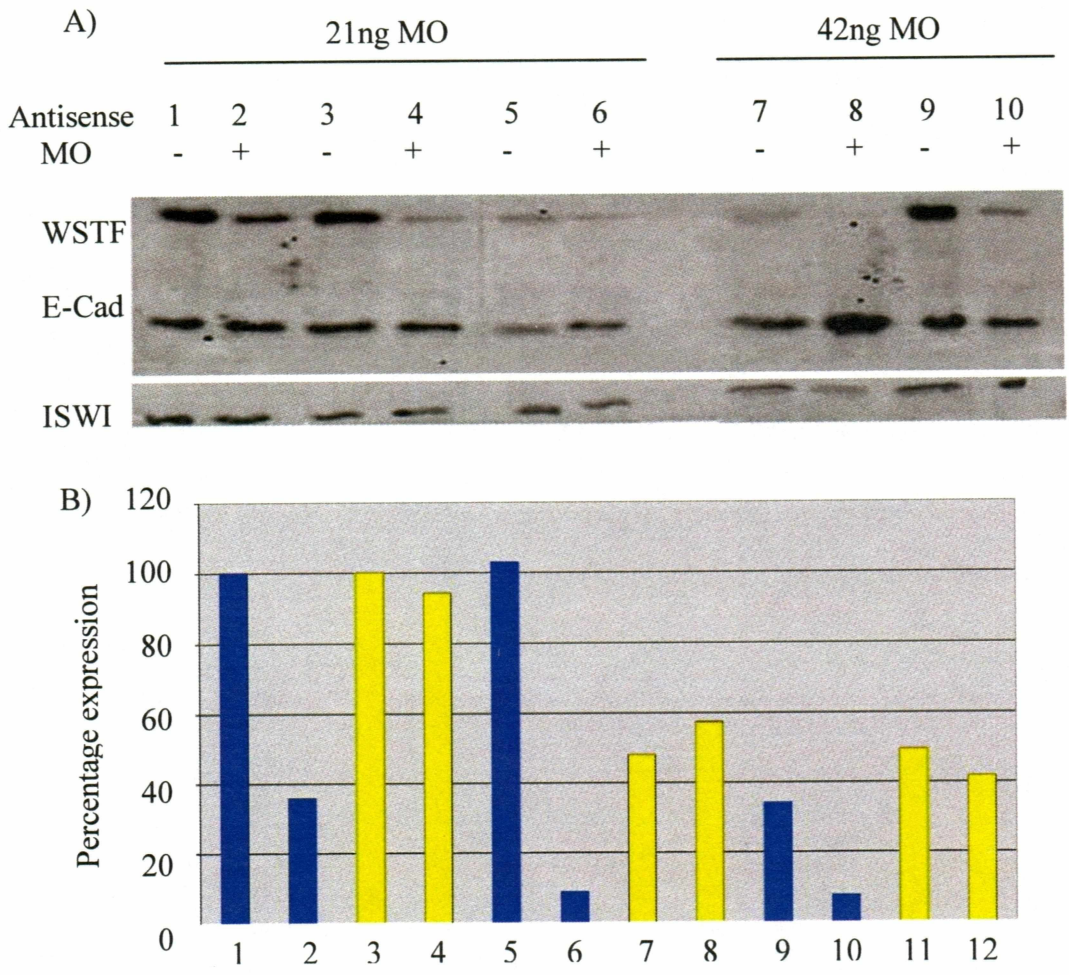


Figure 3.3: WSTF protein levels in injected embryos. (A) Western blot showing decreased levels of WSTF protein in embryos injected with 21 ng or 42 ng of antisense WSTF MO. '-' indicates samples collected from embryos injected with control MO. '+' indicates samples collected from embryos injected with antisense MO. Lanes 1, 2, 7, 8: stage 37/38; lanes 3, 4, 9, 10: stage 41; lanes 5,6: stage 45. E-Cadherin is a loading control. (B) Quantitation of Western blot (21 ng MO) where blue bars represent WSTF protein levels in controls (bars 1, 5 and 9) and in WSTF knock-down embryos (bars 2, 6 and 10), yellow bars represent ISWI protein levels in controls (bars 3, 7 and 11) and in ISWI knock-down embryos (bars 4, 8 and 12). Bars 1, 2, 3 and 4: stages 37/38; bars 5, 6, 7 and 8: stage 41; bars 9, 10, 11 and 12: stage 45. Samples normalized to E-Cadherin. Vertical axis represents percentage expression.

3.4: WSTF knockdowns have severely impaired eye development

Morphologically, the ISWI deficient embryos show cataract in their eyes and histological sections show posterior subscapular cataract, small lens and defects in retinal differentiation (Dirscherl et al., 2005). In contrast, the eyes in WSTF knockdowns are extremely reduced or absent. So I wanted to further characterize these abnormalities in the WSTF deficient embryos, and therefore, fixed and shipped the embryos to our collaborator Dr. Jonathon Henry (University of Illinois-Urbana) for histological sectioning.

Sections of some representative stages are presented in Figure 3.4. The eye development in these WSTF knockdowns is delayed and highly abnormal. Retinal differentiation is almost absent, resulting in a solid mass of undifferentiated cells, especially the sensory retinal epithelium, which is highly disorganized. The rest of the undifferentiated retinal tissue is dissociated from the pigmented epithelium.

In some embryos injected with 21 ng of morpholino, some primary lens fibers are formed but there is complete absence of secondary lens fibers (Figure 3.4B). In embryos injected with 42 ng of morphlino, the furthest development observed is the presence of a lens placode (a thickening of the overlying ectoderm), but there is no differentiation of any lens fiber (Figure 3.4D). These deformed lens placodes are intimately associated with the surface ectoderm and are herniated outward through the surrounding epithelium away from the optic vesicle (red star in Figure 3.4D). Corresponding defects were also seen in the central nervous system, which reveals a disorganized brain and spinal cord (data not shown),

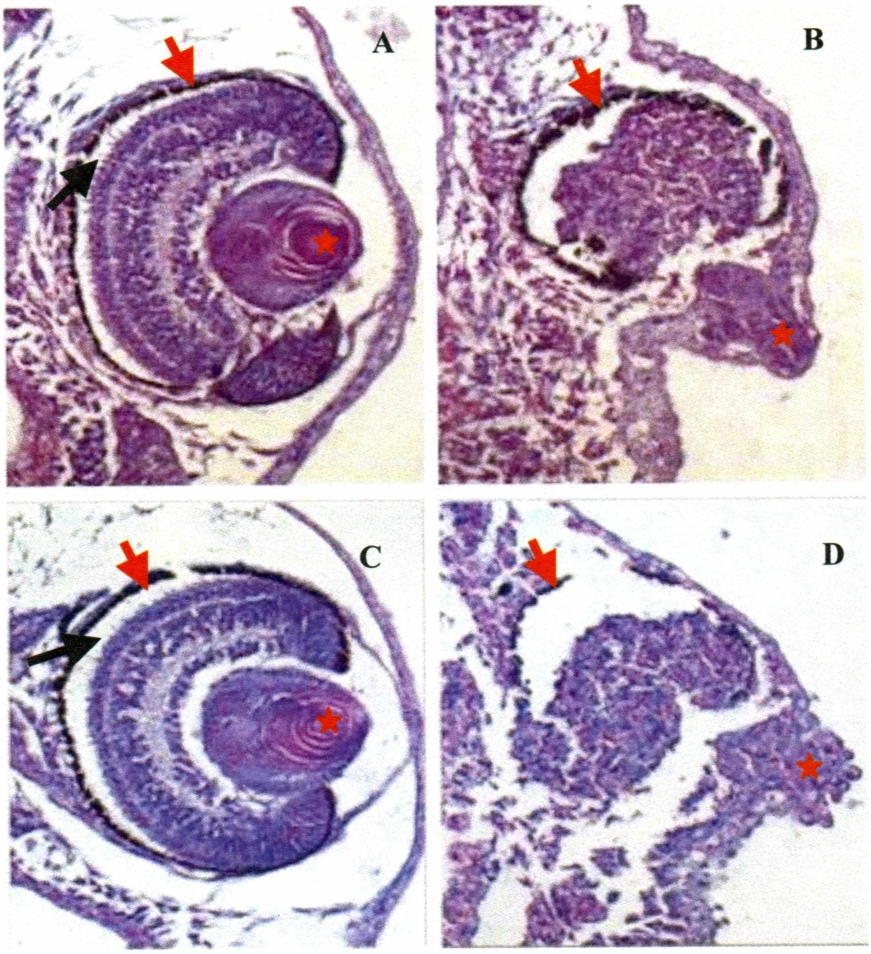


Figure 3.4: Impaired eye development in WSTF knockdowns. **A-B.** Eye sections of embryos injected with 21ng of control (A) and anti-WSTF morpholino (B). **C-D.** Eye sections of embryos injected with 42 ng of control (C) and anti-WSTF morpholino (D). All embryos are at stage 41. Note that normal eyes (A, C) at this stage consist of a well-defined lens, and a retina differentiated into several retinal layers. Red arrows: pigmented retinal epithelium; Black arrows: sensory retinal epithelium; Red stars: lens/ presumptive lens. Sectioning kindly performed by Dr. Jonathon Henry (University of Illinois-Urbana).

Therefore, in compliance with the more deformed eye morphology seen in the WSTF knockdowns as compared to the ISWI knockdowns, the histological sections also show severe abnormalities. This might indicate that the WICH complex is more critical for eye development or WSTF might exist in other complexes, other than WICH, that play important role in neural development of *Xenopus*.

3.5: WSTF is required for normal expression of genes involved in neural patterning and development

ATP-dependent chromatin remodelers are known to be involved in the regulation of transcription of several genes. Previous work in our lab showed that inhibition of ISWI leads to aberrant expression of several genes critical for neural development and differentiation, such as BMP4 (bone morphogenetic protein 4) and Shh (sonic hedgehog) (Dirscherl et al., 2005). In ISWI knockdowns, BMP4 mRNA is upregulated, while Shh mRNA levels decrease, consistent with the loss of neural structures in ISWI knockdowns. Since WSTF is complexed with ISWI in the WICH complex and there is an overlap of expression of these two proteins in *Xenopus,* I wanted to determine if these two neural regulatory genes are also influenced by WSTF depletion.

I performed real time reverse transcriptase PCR (rtRT-PCR) on total RNA samples collected from anti-WSTF morpholino-injected embryos. Figure 3.5 shows quantitation of *rt*RT-PCR products. Expression levels were normalized to the mRNA levels of the ubiquitously expressed EF1α.

I looked at Shh expression at the neural fold stage (stage 15). Shh is an early notochord marker first expressed at gastrulation, which gives a neural fate to ectodermal

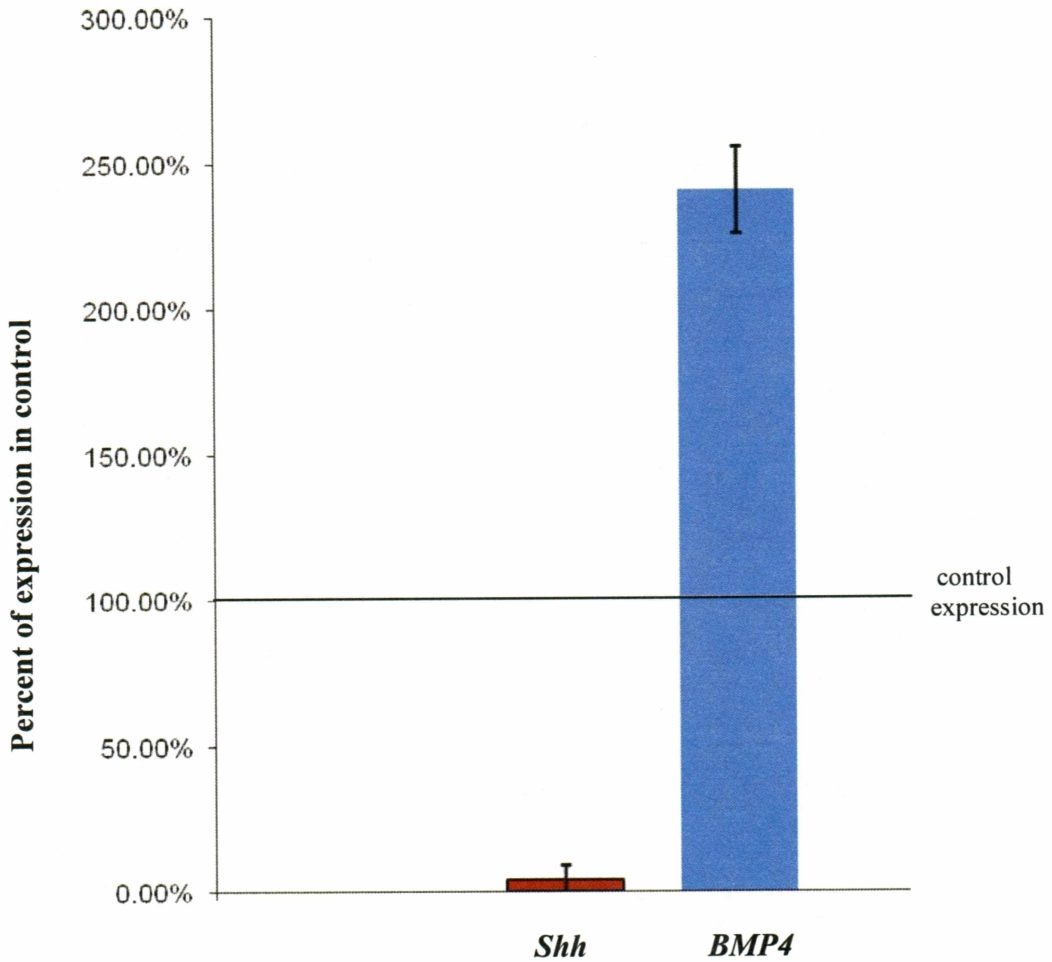


Figure 3.5: Shh and BMP4 are misexpressed in WSTF knockdowns. Real time-RT-PCR for Shh and BMP4 in anti-WSTF morpholino injected embryos. The bar graph shows levels of expression of each Shh and BMP4 expressed as a percentage of the expression levels in the control-injected embryos. Both Shh and BMP4 are normalized to EF1α levels. Data is the average from three injections. Shh was measured at stage 15 while BMP4 was measured at stage 37/38.

cells. In early developmental period, Shh is required for neural fold patterning and ventral specification of the central nervous system by influencing the fate of neural precursors (Di Marcotullio et al., 2006; Palma and Ruiz i Altaba, 2004; Ruiz i Altaba et al., 2002). At stage 15, the neural folds become distinct and the initial sharp inner ridges on the neural folds become prominent. This is also the stage when Shh is first expressed in the dorsal-midline area of the neural fold (Rorick et al., 2007).

I observed that, at this stage, similar to the phenotype of ISWI knockdowns, the level of expression of Shh is decreased, to about 4 percent of normal levels in the WSTF knockdowns. This indicates that WSTF acts directly or indirectly as an activator of Shh, although apparently neural fold patterning is not affected. This might also indicate that the defects seen at the later stages may be due to an effect of early depletion of Shh.

In contrast to Shh function, BMP4 is an inhibitor of neural tissue formation. Prior studies on BMP4 expression focused primarily on early stages of development, and indicated that BMP4 is crucial for early eye patterning and growth. However, recently the McFarlane lab observed expression of BMP4 and its receptors in later stages of eye development in *Xenopus* and found that it is also expressed in the eye between stages 26 and 35/36 (Hocking and McFarlane, 2007). This finding, as well as the fact that we saw neural and eye phenotypes at these later stages of development, prompted us to look at the expression of BMP4 in the WSTF knockdowns at late stages.

I observed that BMP4 expression increases significantly to about 250% of wild type in the WSTF knockdowns at stage 37/38 (stage at which the defects were first detected), indicating that WSTF is formally acting as a repressor of BMP4 at least in this

stage. However, we do not know for sure if WSTF is a direct repressor of BMP4 or not. ChIP data on ISWI indicates that ISWI binds directly to BMP4 promoter and the pattern of BMP4 expression in the ISWI knockdowns are similar to WSTF knockdown embryos (Dirscherl et al., 2005). Therefore, there is a strong possibility that WSTF (at least in WICH) may be involved in direct repression of BMP4 as well.

3.6: BMP4 is spatially misregulated in WSTF knockdowns

Development of an organism not only depends on the proper levels of expression of developmental genes, but also on the precise temporal and spatial localization. As my *rt*RT-PCR data showed misexpression (250% more than normal) of BMP4 in WSTF knockdowns, I hypothesized that the expression pattern of BMP4 may also be affected in the knockdown embryos. I wanted to confirm if the over expression of BMP4 seen in the WSTF knockdowns was within the same domains of normal BMP4 expression or in domains where normally BMP4 is not expressed.

Therefore, I performed, whole mount *in situ* hybridization to detect the spatial expression of BMP4 mRNA. BMP4 was detected in the head and eye of the normal *Xenopus* embryo (Figure 3.6). Previous studies have also detected expression of BMP4 in the retina of late-stage embryos (Hocking and McFarlane, 2007). In chicken, BMP4 is expressed in the dorsal neural retina and lens placode at later stages of development. In the dorsal optic cup, it is responsible for creating a balance between cell proliferation and programmed cell death to allow proper eye development (Trousse et al., 2001). As hypothesized, the WSTF knockdowns showed an aberrant pattern of BMP4 expression

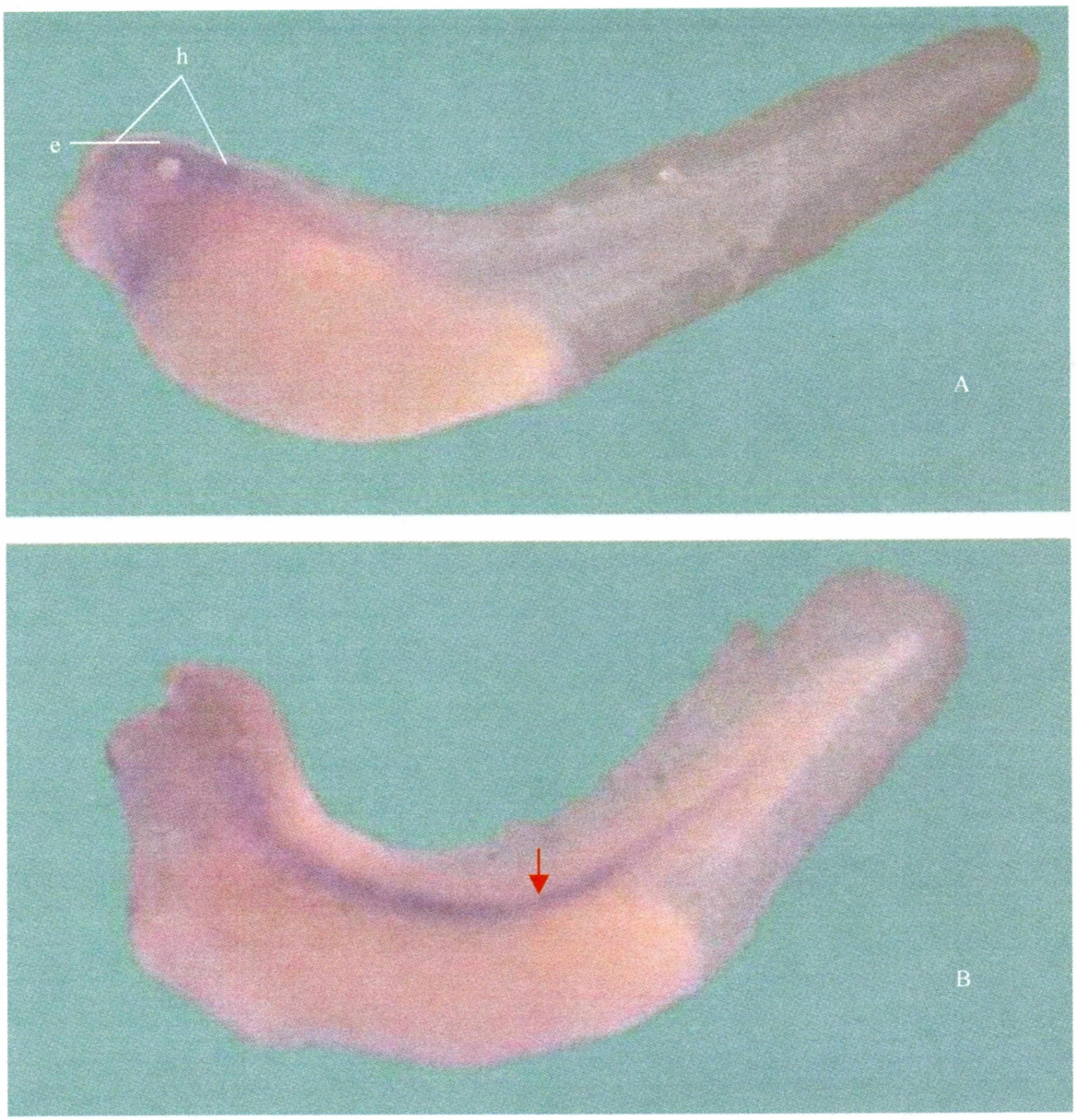


Figure 3.6: Aberrant expression of BMP4 in WSTF knockdown. Whole mount *in situ* hybridization of *Xenopus* embryos with a 400 base BMP4 digoxigenin labeled probe. **A**. Control embryo showing expression of BMP4 mRNA in the head and heart. **B.** WSTF knockdown embryo showing expression of BMP4 in a structure resembling a deformed neural tube. Both embryos are oriented with the anterior end to the left side of the figure. Both embryos are at stage 41. h: head; e: eye; red arrow: deformed neural tube.

(Figure 3.6). No expression was detected in the anterior region of the body like the control embryos. This result is surprising. Given, the anterior neural abnormalities detected in the WSTF knockdowns, one would expect very strong expression in the head region. Instead, strong expression was detected in a structure running through the dorsal region of the embryo, which resembles a deformed neural tube. This indicates that WSTF acts as a direct or indirect repressor of BMP4 in the neural tube in the later stages of development.

Chapter 4

Discussion

I have shown that in *Xenopus* WSTF mRNA is expressed in the neural tissue especially in the eye, brain, branchial arches and the spinal cord. This expression overlaps with the expression of ISWI (Dirscherl et al., 2005). However, WSTF shows lower expression in the spinal cord compared to ISWI.

The WSTF knockdowns also have extremely defective eye and brain development, obvious in both the whole mount and sectioned embryos shown in Figures 3.1 and 3.4. These defects correspond well to the strong localization of WSTF mRNA in the anterior neural tissue. WSTF knockdowns have underdeveloped, disorganized retinas and failure of lens formation in the later stages of development, while the late survivors of ISWI knockdowns, that survive due to partial inhibition of ISWI, have congenital cataracts. The complete ISWI knockdown embryos have a lethal phenotype due to neurulation failure (Dirscherl et al., 2005). These differences in expression patterns as well as the greater severity in eye and brain development of WSTF knockdowns compared to ISWI knockdowns suggests two alternative models: (1) that the WICH complex is central to eye development, and that more severe phenotypes result from the more complete (and specific) knockdown of the WICH complex, or (2) that WSTF exists in other complexes other than WICH and functions independently of ISWI in the development of the nervous system.

As discussed in the Introduction, three ISWI-containing complexes have been biochemically characterized so far in *Xenopus* (Guschin et al., 2000) and three WSTF-

containing complexes have been identified in mammals (Bozhenok et al., 2002; Cavellan et al., 2006; Kato et al., 2004). In *Xenopus,* BPTF, a subunit of the ISWI-dependent NURF complex, has been identified, although a xNURF complex has not yet been characterized (Wysocka et al., 2006). However, it will not be surprising if other ISWI-dependent chromatin remodeling complexes are present in *Xenopus*, or that WSTF will be shown to be present in other complexes in *Xenopus* as well.

I have observed that mRNA levels of the neurogenic gene Shh is greatly reduced in the WSTF knockdowns during the beginning of neural crest formation at stage 15. Shh is expressed in the dorsal midline at this stage (Rorick et al., 2007) and is responsible for the induction of two symmetrical retinal primordia (Chow and Lang, 2001). Depletion of Shh is associated with cyclopia or holoprosencephaly in *Xenopus*, chick, mice and humans (Chiang et al., 1996; Li et al., 1997; Roessler et al., 1996; Wallis and Muenke, 2000). Cyclopia has been observed in some ISWI knockdowns and ISWI knockdowns show reduced Shh levels (Dirscherl et al., 2005). However, I did not observe any morphological abnormalities in the WSTF knockdowns at early stages, nor did I observe cyclopia at later stages. The knockdown phenotype seen in the later stages of development may be partially due to an effect of depletion of Shh (and probably other neural specific genes) early on. The level of expression of Shh in the WSTF knockdowns was one third of that of the ISWI knockdowns. This might indicate that there was more complete knockdown of WSTF in the WSTF knockdowns than there was for ISWI in the ISWI knockdowns, which led to more decrease in expression of Shh in the WSTF depleted embryos as compared to ISWI deficient ones.

Shh is expressed in the mouse retina and is required for retinal progenitor cell (RPC) proliferation (Jensen and Wallace, 1997; Levine et al., 1997; Wang et al., 2005, 2002). It also plays an important role in the retinal cell fate determination including specification of ganglion, photoceceptor and Müller glial cells (Jensen and Wallace, 1997; Levine et al., 1997; Stenkamp et al., 2000). It induces the formation of ciliary marginal zone (CMZ) in chick eye and retinal margin in the eye of rodents (Moshiri et al., 2005; Moshiri and Reh, 2004). The CMZ and the retinal margin harbors undifferentiated progenitor cells (stem cells) which is important for specification of post embryonic neurons and glia (Goodrich and Scott, 1998). Given all these functions that Shh has in retinal development, the undifferentiated, malformed retina observed in the WSTF knockdowns may be wholly or partially attributed to loss of Shh signaling.

I have also observed that the WSTF knockdown embryos show BMP4 over-expression and *in situs* for BMP4 show aberrant expression throughout a structure that appears to be a deformed neural tube. This strongly suggests that WSTF acts as a repressor of BMP4 in the neural tube. Therefore, WSTF may facilitate the formation of neural tissue by repressing genes that prevent neural tissue formation like BMP4. However, we do not know for sure if BMP4 is a direct target for WSTF or whether it is acting further downstream in a WSTF-dependent pathway. A ChIP analysis would help answer this question. ChIP with ISWI antibodies indicate that ISWI binds directly to BMP4 gene (Dirscherl et al., 2005). As WSTF is a specific ISWI complex and the BMP4 over expression in the ISWI and WSTF knockdown embryos are similar, therefore there is a strong possibility that WSTF may directly bind to BMP4 as well.

In chicken, BMP4 is expressed in the overlying optic vesicle (Muller et al., 2007) and in mice it is expressed in the presumptive lens ectoderm (Furuta et al., 1997). In mice BMP4 is responsible for proper patterning of gene expression in the ectoderm overlying the optic cup from where lens is formed (Furuta and Hogan, 1998; Sasagawa et al., 2002). Optic vesicle explants cultured from Bmp4 null mice only forms lens when treated with exogenous BMP4 indicating that BMP4 is essential for lens formation (Furuta and Hogan, 1998). In Bmp4 $^{+/-}$ mice, the optic cup is formed, but the eyes are smaller in size (micropthalmia) (Chang et al., 2001). BMP4 over expression in chick and *Xenopus* embryos induces ectopic *Tbx5* (dorsal optic cup marker) expression in the retina (Koshiba-Takeuchi et al., 2000; Sasagawa et al., 2002). Increasing BMP4 signaling by addition of exogenous BMP4 alters the patterns of gene expression and results in a reduced retinal volume, primarily due to reduced cell proliferation and alterations in the axial length of the optic cup, indicating that correct levels of BMP signaling are important for the growth and the shape of the embryonic eye (Behesti et al., 2006). Besides cell proliferation, BMP4 is also implicated in programmed cell death in the dorsal optic cup in chick retina and in the proper distribution and localization of retinal ganglion cells (Koshiba-Takeuchi et al., 2000; Trousse et al., 2001).

In the nervous system, BMPs have important roles to play in inhibition of neural induction, cell fate determination, proliferation and apoptosis (Mehler et al., 1997). Thus, lack of normal neural development, especially the lack of proliferation and proper differentiation in the lens tissue in the WSTF knockdowns may be associated with misexpression of BMP4.

This study has focused on the role of WSTF in *Xenopus.* Ultimately we hope to determine if there are any functional differences between the PHD, bromo and DDT domains of WSTF, by mutation and deletion studies.

In WS, the whole WSTF gene is deleted. But we do not know the effect of only PHD domain impairment. This would be interesting to see because many mutations in the PHD motifs of several proteins are associated with a variety of developmental disorders. Mostly, mutations in the first Zn binding site and the nearby putative interaction surfaces are related to human diseases. For example, in XLMR syndrome (X-linked mental retardation which has characteristic facial dysmorphic features), a point mutation in the ATRX gene is found (Gibbons et al., 2000). This mutation also corresponds to the same amino acid residue that is present in the predicted functional region of the WSTF-PHD.

The other domain in WSTF, the bromodomain, interacts with acetylated histone tails and helps in the recruitment of VDR to its targets. Impaired recruitment of VDR is found in WS patients. In cells isolated from WS patients this condition is rescued by over expression of WSTF (Fujiki et al., 2005). The structure of bromodomain was first revealed from a human transcriptional co activator PCAF (Dhalluin et al., 1999). Eventually, structures of bromodomains in several proteins including Brg1 and BPTF have been identified (Li et al., 2006a; Shen et al., 2007). All of these consist of 4 conserved helices with two inter-helical loops with variable length and sequences. These loops are known to stabilize the protein and interact with acetyl lysine. Since the amino acid residues in bromodomains involved in acetyl lysine recognition are highly conserved

in the large bromodomain family of proteins, mutation of the corresponding residues in the bromodomain of WSTF protein may throw some light on its function.

The third domain in WSTF protein is the DDT domain. These are putative DNA binding motifs (Doerks et al., 2001) but their binding specificity and functional significance is not known. So it will also be interesting to see if the deletion of this domain in WSTF protein has any functional role during development.

Besides, the mutation and deletion analysis, determination of transcriptional profiles of some lens, retina and brain specific markers in the WSTF knockdowns will help us understand which developmental genes are under the control of WSTF protein. Some of these markers include *Pax-6*, *Six-3*, *Sox-2*, *Sox-3*, *XlNLRR-6, Slug*, *Snail* and *HoxB9*. *Pax-6* is a central player in eye development and can induce ectopic eye formation in vertebrates (Chow et al., 1999; Pichaud and Desplan, 2002). *Six3, Sox2 and Sox3* are homeodomain transcription factors required for normal lens development (Kamachi et al., 2001; Zhou et al., 2000; Zuber et al., 2003; Zygar et al., 1998). *XlNLRR-6* is a leucine-rich repeat protein essential for retina, lens and cornea development, and is necessary for differentiation of cell layers in the retina (Wolfe and Henry, 2006). Since previous study in our lab has also shown reduced expression of the neural crest genes like *Slug* and *Snail*, and the general CNS/spinal cord marker *HoxB9* in the ISWI knockdowns it will be useful to evaluate their expression in the WSTF knockdowns as well (Dirscherl et al., 2005).

WSTF knockdowns also show severe disorganization of anterior neural structures. So it will also be useful to measure expression of genes specific for different structures

within the CNS like *Xaxin* (anterior midbrain marker), *Krox20* (hindbrain marker), and *Xbcan* (notochord and hindbrain marker) (Hedgepeth et al., 1999; Sander et al., 2001).

Real time RT-PCR will determine any perturbation in the level of expression of these genes in the WSTF knockdowns while *in situs* will decipher any difference in their spatial expression. It will also assist in deciphering the specific defects in the neural development in WSTF knockdowns. We also hope to find some genes that are directly activated or repressed by WSTF through chromatin immunoprecipitation (ChIP) assays. This will help us understand the specific interaction of the WICH complex with its targets during neural development in *Xenopus*.

References:

Andersen, E. C., Lu, X. and Horvitz, H. R. (2006). C. elegans ISWI and NURF301 antagonize an Rb-like pathway in the determination of multiple cell fates. *Development* **133,** 2695-704.

Badenhorst, P., Voas, M., Rebay, I. and Wu, C. (2002). Biological functions of the ISWI chromatin remodeling complex NURF. *Genes Dev* **16,** 3186-98.

Badenhorst, P., Xiao, H., Cherbas, L., Kwon, S. Y., Voas, M., Rebay, I., Cherbas, P. and Wu, C. (2005). The Drosophila nucleosome remodeling factor NURF is required for Ecdysteroid signaling and metamorphosis. *Genes Dev* **19,** 2540-5.

Bakshi, R., Mehta, A. K., Sharma, R., Maiti, S., Pasha, S. and Brahmachari, V. (2006). Characterization of a human SWI2/SNF2 like protein hINO80: demonstration of catalytic and DNA binding activity. *Biochem Biophys Res Commun* **339,** 313-20.

Bakshi, R., Prakash, T., Dash, D. and Brahmachari, V. (2004). In silico characterization of the INO80 subfamily of SWI2/SNF2 chromatin remodeling proteins. *Biochem Biophys Res Commun* **320,** 197-204.

Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C. and Kouzarides, T. (2001). Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410,** 120-4.

Banting, G. S., Barak, O., Ames, T. M., Burnham, A. C., Kardel, M. D., Cooch, N. S., Davidson, C. E., Godbout, R., McDermid, H. E. and Shiekhattar, R. (2005). CECR2, a protein involved in neurulation, forms a novel chromatin remodeling complex with SNF2L. *Hum Mol Genet* **14,** 513-24.

Barak, O., Lazzaro, M. A., Cooch, N. S., Picketts, D. J. and Shiekhattar, R. (2004). A tissue-specific, naturally occurring human SNF2L variant inactivates chromatin remodeling. *J Biol Chem* **279,** 45130-8.

Barak, O., Lazzaro, M. A., Lane, W. S., Speicher, D. W., Picketts, D. J. and Shiekhattar, R. (2003). Isolation of human NURF: a regulator of Engrailed gene expression. *Embo J* **22,** 6089-100.

Barak, O. G. and Shiekhattar, R. (2004). Preparation and assays for mammalian ISWI complexes. *Methods Enzymol* **377,** 389-401.

Becker, P. B., Tsukiyama, T. and Wu, C. (1994). Chromatin assembly extracts from Drosophila embryos. *Methods Cell Biol* **44,** 207-23.

Behesti, H., Holt, J. K. and Sowden, J. C. (2006). The level of BMP4 signaling is critical for the regulation of distinct T-box gene expression domains and growth along the dorso-ventral axis of the optic cup. *BMC Dev Biol* **6,** 62.

Bernstein, B. E., Kamal, M., Lindblad-Toh, K., Bekiranov, S., Bailey, D. K., Huebert, D. J., McMahon, S., Karlsson, E. K., Kulbokas, E. J., 3rd, Gingeras, T. R., Schreiber, S. L. and Lander, E. S. (2005). Genomic maps and comparative analysis of histone modifications in human and mouse. *Cell* **120,** 169-81.

Bochar, D. A., Savard, J., Wang, W., Lafleur, D. W., Moore, P., Cote, J. and Shiekhattar, R. (2000). A family of chromatin remodeling factors related to Williams syndrome transcription factor. *Proc Natl Acad Sci U S A* **97,** 1038-43.

Boyer, L. A., Langer, M. R., Crowley, K. A., Tan, S., Denu, J. M. and Peterson, C. L. (2002). Essential role for the SANT domain in the functioning of multiple chromatin remodeling enzymes. *Mol Cell* **10,** 935-42.

Bozhenok, L., Wade, P. A. and Varga-Weisz, P. (2002). WSTF-ISWI chromatin remodeling complex targets heterochromatic replication foci. *EMBO J* **21,** 2231-41.

Cairns, B. R. (2005). Chromatin remodeling complexes: strength in diversity, precision through specialization. *Curr Opin Genet Dev* **15,** 185-90.

Carre, C., Ciurciu, A., Komonyi, O., Jacquier, C., Fagegaltier, D., Pidoux, J., Tricoire, H., Tora, L., Boros, I. M. and Antoniewski, C. (2007). The Drosophila NURF remodelling and the ATAC histone acetylase complexes functionally interact and are required for global chromosome organization. *EMBO Rep.*

Cavellan, E., Asp, P., Percipalle, P. and Farrants, A. K. (2006). The WSTF-SNF2h chromatin remodeling complex interacts with several nuclear proteins in transcription. *J Biol Chem* **281,** 16264-71.

Chang, B., Smith, R. S., Peters, M., Savinova, O. V., Hawes, N. L., Zabaleta, A., Nusinowitz, S., Martin, J. E., Davisson, M. L., Cepko, C. L., Hogan, B. L. and John, S. W. (2001). Haploinsufficient BMP4 ocular phenotypes include anterior segment dysgenesis with elevated intraocular pressure. *BMC Genet* **2,** 18.

Chiang, C., Litingtung, Y., Lee, E., Young, K. E., Corden, J. L., Westphal, H. and Beachy, P. A. (1996). Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383,** 407-13.

Chow, R. L., Altmann, C. R., Lang, R. A. and Hemmati-Brivanlou, A. (1999). Pax6 induces ectopic eyes in a vertebrate. *Development* **126,** 4213-22.

Chow, R. L. and Lang, R. A. (2001). Early eye development in vertebrates. *Annu Rev Cell Dev Biol* **17,** 255-96.

Collins, N., Poot, R. A., Kukimoto, I., Garcia-Jimenez, C., Dellaire, G. and Varga-Weisz, P. D. (2002). An ACF1-ISWI chromatin-remodeling complex is required for DNA replication through heterochromatin. *Nat Genet* **32,** 627-32.

Cus, R., Maurus, D. and Kuhl, M. (2006). Cloning and developmental expression of WSTF during *Xenopus laevis* embryogenesis. *Gene Expr Patterns* **6,** 340-6.

Debril, M. B., Gelman, L., Fayard, E., Annicotte, J. S., Rocchi, S. and Auwerx, J. (2004). Transcription factors and nuclear receptors interact with the SWI/SNF complex through the BAF60c subunit. *J Biol Chem* **279,** 16677-86.

de la Cruz, X., Lois, S., Sanchez-Molina, S. and Martinez-Balbas, M. A. (2005). Do protein motifs read the histone code? *Bioessays* **27,** 164-75.

de la Serna, I. L., Ohkawa, Y. and Imbalzano, A. N. (2006). Chromatin remodelling in mammalian differentiation: lessons from ATP-dependent remodellers. *Nat Rev Genet* **7,** 461-73.

Deuring, R., Fanti, L., Armstrong, J. A., Sarte, M., Papoulas, O., Prestel, M., Daubresse, G., Verardo, M., Moseley, S. L., Berloco, M., Tsukiyama, T., Wu, C., Pimpinelli, S. and Tamkun, J. W. (2000). The ISWI chromatin-remodeling protein is required for gene expression and the maintenance of higher order chromatin structure in vivo. *Mol Cell* **5,** 355-65.

Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K. and Zhou, M. M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. *Nature* **399,** 491-6.

Di Marcotullio, L., Ferretti, E., De Smaele, E., Screpanti, I. and Gulino, A. (2006). Suppressors of hedgehog signaling: Linking aberrant development of neural progenitors and tumorigenesis. *Mol Neurobiol* **34,** 193-204.

Dirscherl, S. S., Henry, J. J. and Krebs, J. E. (2005). Neural and eye-specific defects associated with loss of the Imitation Switch (ISWI) chromatin remodeler in *Xenopus laevis*. *Mech Dev* **122,** 1157-70.

Dirscherl, S. S. and Krebs, J. E. (2004). Functional diversity of ISWI complexes. *Biochem Cell Biol* **82,** 482-9.

Doerks, T., Copley, R. and Bork, P. (2001). DDT -- a novel domain in different transcription and chromosome remodeling factors. *Trends Biochem Sci* **26,** 145-6.

Doyon, Y., Cayrou, C., Ullah, M., Landry, A. J., Cote, V., Selleck, W., Lane, W. S., Tan, S., Yang, X. J. and Cote, J. (2006). ING tumor suppressor proteins are critical regulators of chromatin acetylation required for genome expression and perpetuation. *Mol Cell* **21,** 51-64.

Eisen, J. A., Sweder, K. S. and Hanawalt, P. C. (1995). Evolution of the SNF2 family of proteins: subfamilies with distinct sequences and functions. *Nucleic Acids Res* **23,** 2715-23.

Elfring, L.K., Deuring, R., McCallum, C.M., Peterson, C.L and Tamkun, J.W. (1994). Identification and charecterization of Drosophila relatives of yeast transcriptional activator SNF2/SWI2. *Mol.Cell.Biol* 14, 2225-2234.

Ewart, A. K., Morris, C. A., Atkinson, D., Jin, W., Sternes, K., Spallone, P., Stock, A. D., Leppert, M. and Keating, M. T. (1993). Hemizygosity at the elastin locus in a developmental disorder, Williams syndrome. *Nat Genet* **5,** 11-6.

Fazzio, T. G., Kooperberg, C., Goldmark, J. P., Neal, C., Basom, R., Delrow, J. and Tsukiyama, T. (2001). Widespread collaboration of Isw2 and Sin3-Rpd3 chromatin remodeling complexes in transcriptional repression. *Mol Cell Biol* **21,** 6450-60.

Felsenfeld, G. and Groudine, M. (2003). Controlling the double helix. *Nature* **421,** 448-53.

Ferreira, H., Flaus, A. and Owen-Hughes, T. (2007). Histone modifications influence the action of Snf2 family remodelling enzymes by different mechanisms. *J Mol Biol* **374,** 563-79.

Fujiki, R., Kim, M. S., Sasaki, Y., Yoshimura, K., Kitagawa, H. and Kato, S. (2005). Ligand-induced transrepression by VDR through association of WSTF with acetylated histones. *EMBO J* **24,** 3881-94.

Furuta, Y. and Hogan, B. L. (1998). BMP4 is essential for lens induction in the mouse embryo. *Genes Dev* **12,** 3764-75.

Furuta, Y., Piston, D. W. and Hogan, B. L. (1997). Bone morphogenetic proteins (BMPs) as regulators of dorsal forebrain development. *Development* **124,** 2203-12.

Gdula, D. A., Sandaltzopoulos, R., Tsukiyama, T., Ossipow, V. and Wu, C. (1998). Inorganic pyrophosphatase is a component of the Drosophila nucleosome remodeling factor complex. *Genes Dev* **12,** 3206-16.

Gibbons, R. J., McDowell, T. L., Raman, S., O'Rourke, D. M., Garrick, D., Ayyub, H. and Higgs, D. R. (2000). Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. *Nat Genet* **24,** 368-71.

Goldmark, J. P., Fazzio, T. G., Estep, P. W., Church, G. M. and Tsukiyama, T. (2000). The Isw2 chromatin remodeling complex represses early meiotic genes upon recruitment by Ume6p. *Cell* **103,** 423-33.

Goodrich, L. V. and Scott, M. P. (1998). Hedgehog and patched in neural development and disease. *Neuron* **21,** 1243-57.

Grune, T., Brzeski, J., Eberharter, A., Clapier, C. R., Corona, D. F., Becker, P. B. and Muller, C. W. (2003). Crystal structure and functional analysis of a nucleosome recognition module of the remodeling factor ISWI. *Mol Cell* **12,** 449-60.

Guschin, D., Geiman, T. M., Kikyo, N., Tremethick, D. J., Wolffe, A. P. and Wade, P. A. (2000). Multiple ISWI ATPase complexes from *xenopus laevis*. Functional conservation of an ACF/CHRAC homolog. *J Biol Chem* **275,** 35248-55.

Hakimi, M. A., Bochar, D. A., Schmiesing, J. A., Dong, Y., Barak, O. G., Speicher, D. W., Yokomori, K. and Shiekhattar, R. (2002). A chromatin remodelling complex that loads cohesin onto human chromosomes. *Nature* **418,** 994-8.

Hedgepeth, C. M., Deardorff, M. A. and Klein, P. S. (1999). *Xenopus* axin interacts with glycogen synthase kinase-3 beta and is expressed in the anterior midbrain. *Mech Dev* **80,** 147-51.

Hocking, J. C. and McFarlane, S. (2007). Expression of Bmp ligands and receptors in the developing Xenopus retina. *Int J Dev Biol* **51,** 161-5.

Hsiao, P. W., Fryer, C. J., Trotter, K. W., Wang, W. and Archer, T. K. (2003). BAF60a mediates critical interactions between nuclear receptors and the BRG1 chromatin-remodeling complex for transactivation. *Mol Cell Biol* **23,** 6210-20.

Iida, T. and Araki, H. (2004). Noncompetitive counteractions of DNA polymerase epsilon and ISW2/yCHRAC for epigenetic inheritance of telomere position effect in Saccharomyces cerevisiae. *Mol Cell Biol* **24,** 217-27.

Imhof, A. (2006). Epigenetic regulators and histone modification. *Brief Funct Genomic Proteomic* **5,** 222-7.

Ito, T., Bulger, M., Pazin, M. J., Kobayashi, R. and Kadonaga, J. T. (1997). ACF, an ISWI-containing and ATP-utilizing chromatin assembly and remodeling factor. *Cell* **90,** 145-55.

Jensen, A. M. and Wallace, V. A. (1997). Expression of Sonic hedgehog and its putative role as a precursor cell mitogen in the developing mouse retina. *Development* **124,** 363-71.

Johnson, C. N., Adkins, N. L. and Georgel, P. (2005). Chromatin remodeling complexes: ATP-dependent machines in action. *Biochem Cell Biol* **83,** 405-17.

Kamachi, Y., Uchikawa, M., Tanouchi, A., Sekido, R. and Kondoh, H. (2001). Pax6 and SOX2 form a co-DNA-binding partner complex that regulates initiation of lens development. *Genes Dev* **15,** 1272-86.

Kanno, T., Kanno, Y., Siegel, R. M., Jang, M. K., Lenardo, M. J. and Ozato, K. (2004). Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. *Mol Cell* **13,** 33-43.

Kasten, M., Szerlong, H., Erdjument-Bromage, H., Tempst, P., Werner, M. and Cairns, B. R. (2004). Tandem bromodomains in the chromatin remodeler RSC recognize acetylated histone H3 Lys14. *EMBO J* **23,** 1348-59.

Kato, S., Fujiki, R. and Kitagawa, H. (2004). Vitamin D receptor (VDR) promoter targeting through a novel chromatin remodeling complex. *J Steroid Biochem Mol Biol* **89-90,** 173-8.

Kent, N. A., Karabetsou, N., Politis, P. K. and Mellor, J. (2001). *In vivo* chromatin remodeling by yeast ISWI homologs Isw1p and Isw2p. *Genes Dev* **15,** 619-26.

Kitagawa, H., Fujiki, R., Yoshimura, K., Mezaki, Y., Uematsu, Y., Matsui, D., Ogawa, S., Unno, K., Okubo, M., Tokita, A., Nakagawa, T., Ito, T., Ishimi, Y., Nagasawa, H., Matsumoto, T., Yanagisawa, J. and Kato, S. (2003). The chromatin-remodeling complex WINAC targets a nuclear receptor to promoters and is impaired in Williams syndrome. *Cell* **113,** 905-17.

Koshiba-Takeuchi, K., Takeuchi, J. K., Matsumoto, K., Momose, T., Uno, K., Hoepker, V., Ogura, K., Takahashi, N., Nakamura, H., Yasuda, K. and Ogura, T. (2000). Tbx5 and the retinotectum projection. *Science* **287,** 134-7.

Laposa, R. R., Huang, E. J. and Cleaver, J. E. (2007). Increased apoptosis, p53 up-regulation, and cerebellar neuronal degeneration in repair-deficient Cockayne syndrome mice. *Proc Natl Acad Sci U S A* **104,** 1389-94.

Lazzaro, M. A., Pepin, D., Pescador, N., Murphy, B. D., Vanderhyden, B. C. and Picketts, D. J. (2006). The imitation switch protein SNF2L regulates steroidogenic acute regulatory protein expression during terminal differentiation of ovarian granulosa cells. *Mol Endocrinol* **20,** 2406-17.

Lazzaro, M. A. and Picketts, D. J. (2001). Cloning and characterization of the murine Imitation Switch (ISWI) genes: differential expression patterns suggest distinct developmental roles for Snf2h and Snf2l. *J Neurochem* **77,** 1145-56.

LeRoy, G., Loyola, A., Lane, W. S. and Reinberg, D. (2000). Purification and characterization of a human factor that assembles and remodels chromatin. *J Biol Chem* **275,** 14787-90.

LeRoy, G., Orphanides, G., Lane, W. S. and Reinberg, D. (1998). Requirement of RSF and FACT for transcription of chromatin templates *in vitro*. *Science* **282,** 1900-4.

Levine, E. M., Roelink, H., Turner, J. and Reh, T. A. (1997). Sonic hedgehog promotes rod photoreceptor differentiation in mammalian retinal cells *in vitro*. *J Neurosci* **17,** 6277-88.

Li, H., Ilin, S., Wang, W., Duncan, E. M., Wysocka, J., Allis, C. D. and Patel, D. J. (2006a). Molecular basis for site-specific read-out of histone H3K4me3 by the BPTF PHD finger of NURF. *Nature* **442,** 91-5.

Li, H., Tierney, C., Wen, L., Wu, J. Y. and Rao, Y. (1997). A single morphogenetic field gives rise to two retina primordia under the influence of the prechordal plate. *Development* **124,** 603-15.

Li, J., Langst, G. and Grummt, I. (2006b). NoRC-dependent nucleosome positioning silences rRNA genes. *Embo J* **25,** 5735-41.

Lickert, H., Takeuchi, J. K., Von Both, I., Walls, J. R., McAuliffe, F., Adamson, S. L., Henkelman, R. M., Wrana, J. L., Rossant, J. and Bruneau, B. G. (2004). Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. *Nature* **432,** 107-12.

Loyola, A., Huang, J. Y., LeRoy, G., Hu, S., Wang, Y. H., Donnelly, R. J., Lane, W. S., Lee, S. C. and Reinberg, D. (2003). Functional analysis of the subunits of the chromatin assembly factor RSF. *Mol Cell Biol* **23,** 6759-68.

Lu, X., Meng, X., Morris, C. A. and Keating, M. T. (1998). A novel human gene, WSTF, is deleted in Williams syndrome. *Genomics* **54,** 241-9.

Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F. and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 A resolution. *Nature* **389,** 251-60.

Marmorstein, R. and Berger, S. L. (2001). Structure and function of bromodomains in chromatin-regulating complexes. *Gene* **272,** 1-9.

Martinez-Balbas, M. A., Tsukiyama, T., Gdula, D. and Wu, C. (1998). Drosophila NURF-55, a WD repeat protein involved in histone metabolism. *Proc Natl Acad Sci U S A* **95,** 132-7.

Mayer, C., Schmitz, K. M., Li, J., Grummt, I. and Santoro, R. (2006). Intergenic transcripts regulate the epigenetic state of rRNA genes. *Mol Cell* **22,** 351-61.

Mehler, M. F., Mabie, P. C., Zhang, D. and Kessler, J. A. (1997). Bone morphogenetic proteins in the nervous system. *Trends Neurosci* **20,** 309-17.

Mellor, J. (2006). Imitation switch complexes. *Ernst Schering Res Found Workshop,* 61-87.

Merzdorf, C. S. and Goodenough, D. A. (1997). Localization of a novel 210 kDa protein in Xenopus tight junctions. *J Cell Sci* **110 (Pt 8),** 1005-12.

Moshiri, A., McGuire, C. R. and Reh, T. A. (2005). Sonic hedgehog regulates proliferation of the retinal ciliary marginal zone in posthatch chicks. *Dev Dyn* **233,** 66-75.

Moshiri, A. and Reh, T. A. (2004). Persistent progenitors at the retinal margin of ptc+/- mice. *J Neurosci* **24,** 229-37.

Muller, F., Rohrer, H. and Vogel-Hopker, A. (2007). Bone morphogenetic proteins specify the retinal pigment epithelium in the chick embryo. *Development* **134,** 3483-93.

Nieuwkoop, P.D. and Faber, J. (1967). "Normal Table of *Xenopus laevis*." Daudine: North-Holland, Amsterdam.

Owen, D. J., Ornaghi, P., Yang, J. C., Lowe, N., Evans, P. R., Ballario, P., Neuhaus, D., Filetici, P. and Travers, A. A. (2000). The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. *EMBO J* **19,** 6141-9.

Palma, V. and Ruiz i Altaba, A. (2004). Hedgehog-GLI signaling regulates the behavior of cells with stem cell properties in the developing neocortex. *Development* **131,** 337-45.

Pascual, J., Martinez-Yamout, M., Dyson, H. J. and Wright, P. E. (2000). Structure of the PHD zinc finger from human Williams-Beuren syndrome transcription factor. *J Mol Biol* **304,** 723-9.

Pena, P. V., Davrazou, F., Shi, X., Walter, K. L., Verkhusha, V. V., Gozani, O., Zhao, R. and Kutateladze, T. G. (2006). Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature* **442,** 100-3.

Percipalle, P. and Farrants, A. K. (2006). Chromatin remodelling and transcription: be-WICHed by nuclear myosin 1. *Curr Opin Cell Biol* **18,** 267-74.

Percipalle, P., Fomproix, N., Cavellan, E., Voit, R., Reimer, G., Kruger, T., Thyberg, J., Scheer, U., Grummt, I. and Farrants, A. K. (2006). The chromatin remodelling complex WSTF-SNF2h interacts with nuclear myosin 1 and has a role in RNA polymerase I transcription. *EMBO Rep* **7,** 525-30.

Pichaud, F. and Desplan, C. (2002). Pax genes and eye organogenesis. *Curr Opin Genet Dev* **12,** 430-4.

Pokholok, D. K., Harbison, C. T., Levine, S., Cole, M., Hannett, N. M., Lee, T. I., Bell, G. W., Walker, K., Rolfe, P. A., Herbolsheimer, E., Zeitlinger, J., Lewitter, F., Gifford, D. K. and Young, R. A. (2005). Genome-wide map of nucleosome acetylation and methylation in yeast. *Cell* **122,** 517-27.

Poot, R. A., Bozhenok, L., van den Berg, D. L., Hawkes, N. and Varga-Weisz, P. D. (2005). Chromatin remodeling by WSTF-ISWI at the replication site: opening a window of opportunity for epigenetic inheritance? *Cell Cycle* **4,** 543-6.

Poot, R. A., Bozhenok, L., van den Berg, D. L., Steffensen, S., Ferreira, F., Grimaldi, M., Gilbert, N., Ferreira, J. and Varga-Weisz, P. D. (2004). The Williams syndrome transcription factor interacts with PCNA to target chromatin remodelling by ISWI to replication foci. *Nat Cell Biol* **6,** 1236-44.

Poot, R. A., Dellaire, G., Hulsmann, B. B., Grimaldi, M. A., Corona, D. F., Becker, P. B., Bickmore, W. A. and Varga-Weisz, P. D. (2000). HuCHRAC, a human ISWI chromatin remodelling complex contains hACF1 and two novel histone-fold proteins. *EMBO J* **19,** 3377-87.

Pray-Grant, M. G., Daniel, J. A., Schieltz, D., Yates, J. R., 3rd and Grant, P. A. (2005). Chd1 chromodomain links histone H3 methylation with SAGA- and SLIK-dependent acetylation. *Nature* **433,** 434-8.

Ramon-Maiques, S., Kuo, A. J., Carney, D., Matthews, A. G., Oettinger, M. A., Gozani, O. and Yang, W. (2007). The plant homeodomain finger of RAG2 recognizes histone H3 methylated at both lysine-4 and arginine-2. *Proc Natl Acad Sci U S A* **104,** 18993-8.

Roessler, E., Belloni, E., Gaudenz, K., Jay, P., Berta, P., Scherer, S. W., Tsui, L. C. and Muenke, M. (1996). Mutations in the human Sonic Hedgehog gene cause holoprosencephaly. *Nat Genet* **14,** 357-60.

Rorick, A. M., Mei, W., Liette, N. L., Phiel, C., El-Hodiri, H. M. and Yang, J. (2007). PP2A:B56epsilon is required for eye induction and eye field separation. *Dev Biol* **302,** 477-93.

Ruiz, C., Escribano, V., Morgado, E., Molina, M. and Mazon, M. J. (2003). Cell-type-dependent repression of yeast a-specific genes requires Itc1p, a subunit of the Isw2p-Itc1p chromatin remodelling complex. *Microbiology* **149,** 341-51.

Ruiz i Altaba, A., Sanchez, P. and Dahmane, N. (2002). Gli and hedgehog in cancer: tumours, embryos and stem cells. *Nat Rev Cancer* **2,** 361-72.

Ruthenburg, A. J., Allis, C. D. and Wysocka, J. (2007). Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol Cell* **25,** 15-30.

Saha, A., Wittmeyer, J. and Cairns, B. R. (2006). Mechanisms for nucleosome movement by ATP-dependent chromatin remodeling complexes. *Results Probl Cell Differ* **41,** 127-48.

Sambrook, J. and Gething, M. J. (1989). Protein structure. Chaperones, paperones. *Nature* **342,** 224-5.

Sander, V., Mullegger, J. and Lepperdinger, G. (2001). Xenopus brevican is expressed in the notochord and the brain during early embryogenesis. *Mech Dev* **102,** 251-3.

Sasagawa, S., Takabatake, T., Takabatake, Y., Muramatsu, T. and Takeshima, K. (2002). Axes establishment during eye morphogenesis in Xenopus by coordinate and antagonistic actions of BMP4, Shh, and RA. *Genesis* **33,** 86-96.

Schneider, R., Bannister, A. J., Myers, F. A., Thorne, A. W., Crane-Robinson, C. and Kouzarides, T. (2004). Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. *Nat Cell Biol* **6,** 73-7.

Shen, W., Xu, C., Huang, W., Zhang, J., Carlson, J. E., Tu, X., Wu, J. and Shi, Y. (2007). Solution structure of human Brg1 bromodomain and its specific binding to acetylated histone tails. *Biochemistry* **46,** 2100-10.

Shi, X., Hong, T., Walter, K. L., Ewalt, M., Michishita, E., Hung, T., Carney, D., Pena, P., Lan, F., Kaadige, M. R., Lacoste, N., Cayrou, C., Davrazou, F., Saha, A., Cairns, B. R., Ayer, D. E., Kutateladze, T. G., Shi, Y., Cote, J., Chua, K. F. and

Gozani, O. (2006). ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. *Nature* **442,** 96-9.

Sive, H.L., Grainger, R.M., Harland, R.M. (2000). "Early Development of Xenopus laevis." New York, Cold Spring Harbor Laboratory Press.

Sobacchi, C., Marrella, V., Rucci, F., Vezzoni, P. and Villa, A. (2006). RAG-dependent primary immunodeficiencies. *Hum Mutat* **27,** 1174-84.

Stenkamp, D. L., Frey, R. A., Prabhudesai, S. N. and Raymond, P. A. (2000). Function for Hedgehog genes in zebrafish retinal development. *Dev Biol* **220,** 238-52.

Stopka, T. and Skoultchi, A. I. (2003). The ISWI ATPase Snf2h is required for early mouse development. *Proc Natl Acad Sci U S A* **100,** 14097-102.

Strohner, R., Nemeth, A., Jansa, P., Hofmann-Rohrer, U., Santoro, R., Langst, G. and Grummt, I. (2001). NoRC--a novel member of mammalian ISWI-containing chromatin remodeling machines. *Embo J* **20,** 4892-900.

Sugimoto, N., Kitabayashi, I., Osano, S., Tatsumi, Y., Yugawa, T., Narisawa-Saito, M., Matsukage, A., Kiyono, T. and Fujita, M. (2007). Identification of Novel Human Cdt1-Binding Proteins by a Proteomics Approach: Proteolytic Regulation by APC/CCdh1. *Mol Biol Cell.*

Takeuchi, J. K., Lickert, H., Bisgrove, B. W., Sun, X., Yamamoto, M., Chawengsaksophak, K., Hamada, H., Yost, H. J., Rossant, J. and Bruneau, B. G. (2007). Baf60c is a nuclear Notch signaling component required for the establishment of left-right asymmetry. *Proc Natl Acad Sci U S A* **104,** 846-51.

Trousse, F., Esteve, P. and Bovolenta, P. (2001). BMP4 mediates apoptotic cell death in the developing chick eye. *J Neurosci* **21,** 1292-301.

Tsukiyama, T., Becker, P. B. and Wu, C. (1994). ATP-dependent nucleosome disruption at a heat-shock promoter mediated by binding of GAGA transcription factor. *Nature* **367,** 525-32.

Tsukiyama, T., Daniel, C., Tamkun, J. and Wu, C. (1995). ISWI, a member of the SWI2/SNF2 ATPase family, encodes the 140 kDa subunit of the nucleosome remodeling factor. *Cell* **83,** 1021-6.

Tsukiyama, T., Palmer, J., Landel, C. C., Shiloach, J. and Wu, C. (1999). Characterization of the imitation switch subfamily of ATP-dependent chromatin-remodeling factors in *Saccharomyces cerevisiae*. *Genes Dev* **13,** 686-97.

Tsukiyama, T. and Wu, C. (1995). Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell* **83,** 1011-20.

Vanolst, L., Fromental-Ramain, C. and Ramain, P. (2005). Toutatis, a TIP5-related protein, positively regulates Pannier function during Drosophila neural development. *Development* **132,** 4327-38.

Vary, J. C., Jr., Gangaraju, V. K., Qin, J., Landel, C. C., Kooperberg, C., Bartholomew, B. and Tsukiyama, T. (2003). Yeast Isw1p forms two separable complexes *in vivo*. *Mol Cell Biol* **23,** 80-91.

Wallis, D. and Muenke, M. (2000). Mutations in holoprosencephaly. *Hum Mutat* **16,** 99-108.

Wang, G. G., Allis, C. D. and Chi, P. (2007a). Chromatin remodeling and cancer, Part I: Covalent histone modifications. *Trends Mol Med* **13,** 363-72.

Wang, G. G., Allis, C. D. and Chi, P. (2007b). Chromatin remodeling and cancer, Part II: ATP-dependent chromatin remodeling. *Trends Mol Med* **13,** 373-80.

Wang, Y. P., Dakubo, G., Howley, P., Campsall, K. D., Mazarolle, C. J., Shiga, S. A., Lewis, P. M., McMahon, A. P. and Wallace, V. A. (2002). Development of normal retinal organization depends on Sonic hedgehog signaling from ganglion cells. *Nat Neurosci* **5,** 831-2.

Wang, Y., Dakubo, G. D., Thurig, S., Mazerolle, C. J. and Wallace, V. A. (2005). Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. *Development* **132,** 5103-13.

Wolfe, A. D. and Henry, J. J. (2006). Neuronal leucine-rich repeat 6 (XlNLRR-6) is required for late lens and retina development in *Xenopus laevis*. *Dev Dyn* **235,** 1027-41.

Woodage, T., Basrai, M. A., Baxevanis, A. D., Hieter, P. and Collins, F. S. (1997). Characterization of the CHD family of proteins. *Proc Natl Acad Sci U S A* **94,** 11472-7.

Wysocka, J., Swigut, T., Xiao, H., Milne, T. A., Kwon, S. Y., Landry, J., Kauer, M., Tackett, A. J., Chait, B. T., Badenhorst, P., Wu, C. and Allis, C. D. (2006). A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* **442,** 86-90.

Xi, R. and Xie, T. (2005). Stem cell self-renewal controlled by chromatin remodeling factors. *Science* **310,** 1487-9.

Zeng, L. and Zhou, M. M. (2002). Bromodomain: an acetyl-lysine binding domain. *FEBS Lett* **513,** 124-8.

Zhou, X., Hollemann, T., Pieler, T. and Gruss, P. (2000). Cloning and expression of xSix3, the Xenopus homologue of murine Six3. *Mech Dev* **91,** 327-30.

Zhou, Y., Santoro, R. and Grummt, I. (2002). The chromatin remodeling complex NoRC targets HDAC1 to the ribosomal gene promoter and represses RNA polymerase I transcription. *Embo J* **21,** 4632-40.

Zuber, M. E., Gestri, G., Viczian, A. S., Barsacchi, G. and Harris, W. A. (2003). Specification of the vertebrate eye by a network of eye field transcription factors. *Development* **130,** 5155-67.

Zygar, C. A., Cook, T. L. and Grainger, R. M., Jr. (1998). Gene activation during early stages of lens induction in Xenopus. *Development* **125,** 3509-19.

Appendix

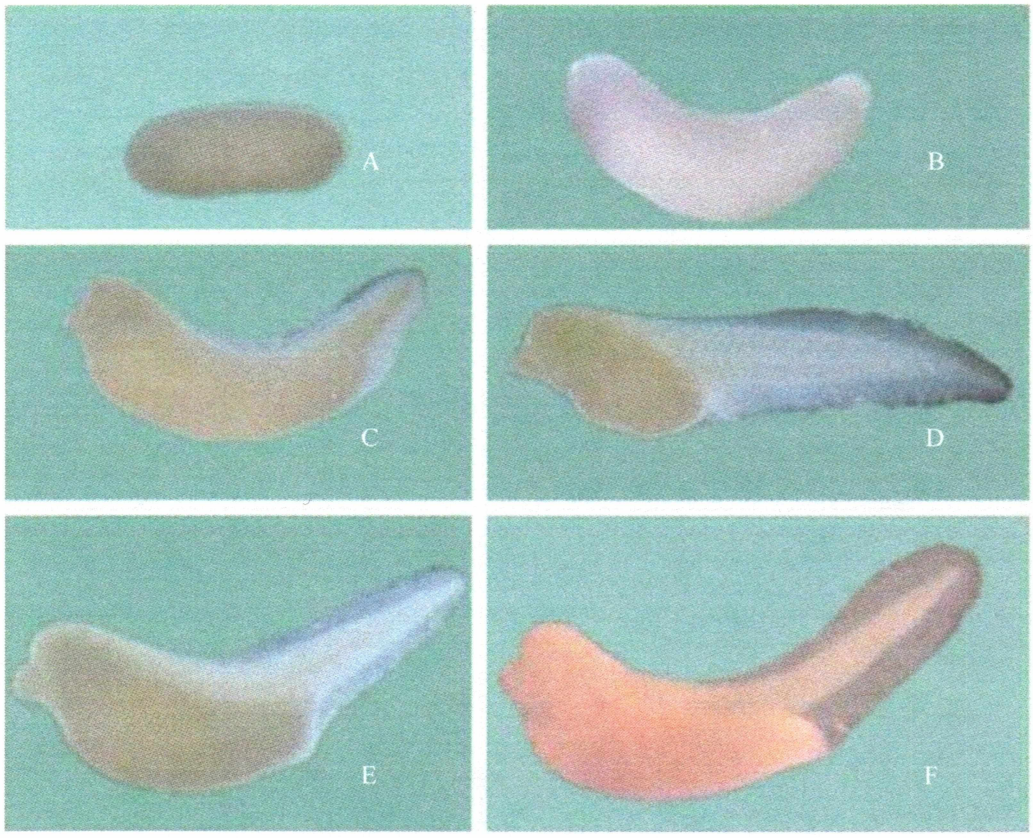


Figure A.1: Representative negative controls for *in situ* hybridization. A-C. Represents embryos incubated with WSTF sense strand digoxygenin labeled probe. **E-F**. Represents embryos incubated with BMP4 sense strand digoxygenin labeled probe. A:stage 24; B: stage 28; C: stage 33/34; D: stage 45; E: stage 41(normal); F: stage 41 (WSTF knockdown).