# ATP-DEPENDENT CHROMATIN REMODELING COMPLEXES IN *XENOPUS* DEVELOPMENT

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**ATP-DEPENDENT CHROMATIN REMODELING COMPLEXES IN *XENOPUS* DEVELOPMENT**

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## Abstract

A central question in the study of vertebrate development is how to account for the exquisite interplay of genes within cells as they create the organs of the vertebrate embryo. Gene regulation by epigenetic processes adds a formerly unappreciated level of complexity to the regulatory network of development. One form of epigenetic gene regulation is embodied in ATP-dependent chromatin remodeling complexes. Chromatin remodeling complexes can both promote and repress expression of a gene at the appropriate time and place in vertebrate development. The list of their known roles in development is long and growing. Here I have studied the developmental role of CHRAC17, a subunit of the CHRAC and ATAC complexes, by visualizing its expression and by ablating CHRAC17 function in *Xenopus laevis* embryos. Whole mount *in situ* hybridization localized *CHRAC17* expression to the neural tube, cranial placodes, and myotomes. Loss of CHRAC17 function following injection of embryos with *CHRAC17*-specific morpholino oligonucleotides resulted in abnormal development in the neural tube, eyes, notochord, and pharyngeal pouches, underlining the critical importance of CHRAC17 function in *Xenopus* development. Similarly, ablating the function of CHD4, the ATPase motor of the NuRD chromatin remodeling complex, resulted in severe developmental abnormalities in early *Xenopus* development.

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# Chapter 1

## Introduction[1]


### Abstract

The development of a metazoan from a single-celled zygote to a complex multicellular organism requires elaborate and carefully regulated programs of gene expression. However, the tight packaging of genomic DNA into chromatin makes genes inaccessible to the cellular machinery and must be overcome by the processes of chromatin remodeling; in addition, chromatin remodeling can preferentially silence genes when their expression is not required. One class of chromatin remodelers, ATP-dependent chromatin remodeling enzymes, can slide nucleosomes along the DNA to make specific DNA sequences accessible or inaccessible to regulators at a particular stage of development. While all ATPases in the SWI2/SNF2 superfamily share the fundamental ability to alter DNA accessibility in chromatin, they do not act alone, but rather are subunits of a large assortment of protein complexes. Recent studies illuminate common themes by which the subunit compositions of chromatin remodeling complexes specify the developmental roles that chromatin remodelers play in specific tissues and at specific stages of development, in response to specific signaling pathways and transcription factors. In this review, we will discuss the known roles in metazoan development of three major subfamilies of chromatin remodeling complexes: the SNF2,


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ISWI and CHD subfamilies.

**Introduction: The SWI2/SNF2 superfamily of proteins**

At all stages of development of a single-celled zygote into a multicullular organism the genome must be maintained as densely packed chromatin, linear arrays of nucleosomes consisting of DNA wrapped around a core of histone proteins and further compacted into higher-order structures. The complex regulation of gene expression and other nuclear processes during development requires modifications to the chromatin to render the correct segment of DNA accessible to the nuclear machinery at the correct time. One mechanism for controlling access to DNA is the covalent modification of histones, which can alter the interactions between DNA and histones and produce new binding surfaces for other factors (Imhof, 2006). A second major mechanism depends on ATP-dependent chromatin remodeling complexes that translationally reposition or "slide" nucleosomes along the DNA to expose specific sites on the DNA to the cellular machinery (Johnson, 2005; Cairns, 2005).

Many ATP-dependent chromatin remodeling enzymes have been identified and their structures and functions characterized. All contain a catalytic subunit belonging to the SWI2/SNF2 superfamily of proteins (Eisen, 1995). The family is characterized by a distinctive ATPase domain that is the molecular motor driving nucleosome sliding. The structure and function of these enzymes are highly conserved in eukaryotes from yeast to human. 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 which is known to bind acetylated lysines of histones (Marmorstein, 2001). Members of the ISWI (Imitation Switch) subfamily contain the HAND-SANT domain in the carboxy-terminal half of the protein (Boyer, 2002) which is linked to a SLIDE domain by an alpha-helical spacer (Grune, 2003); the SLIDE domain interacts with nucleosomal DNA (reviewed in (Dirscherl, 2004; Mellor, 2006). Members of the CHD (Chromodomain Helicase DNA binding) protein family contain two tandem chromodomains and may also contain PHD fingers; these motifs have been shown to interact with methylated histone tails (Wysocka, 2006; Shi, 2006; Pena, 2006; Li, 2006a; Woodage, 1997).

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 Serna, 2006; de la Cruz, 2005). 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, 2006). In this review, we will summarize current data for the

differential expression patterns and developmental functions of SNF2-, ISWI-, and CHD-dependent chromatin remodeling complexes.

**The SWI2/SNF2 subfamily**

The yeast Swi2/Snf2 protein and proteins associated with it in the prototypical ySWI/SNF chromatin remodeling complex were identified in yeast deficient in mating type switching (SWItching mutants) and in sucrose fermentation (sucrose non-fermenters, SNF mutants). The ySWI/SNF complex is known to be necessary for the inducible transcription of a number of genes (Sudarsanam, 2000). Highly conserved homologs of SWI2/SNF2 are found in eukaryotes including *Arabidopsis, Drosophila*, zebrafish, *Xenopus*, chicken and mammals (Schofield, 1999; Randazzo, 1994; Brizuela, 1994; Gelius, 1999). All are subunits of SWI/SNF-related chromatin remodeling complexes that are also highly conserved in eukaryotes (Mohrmann, 2005). While the SWI2/SNF2 subunit alone is capable of limited ATP-dependent chromatin remodeling *in vitro*, other subunits may function to maintain the SWI/SNF protein complex's structure, alter its enzymatic activity, or to allow recruitment of the complex to target genes (Yudkovsky, 1999; Moshkin, 2007; Muchardt, 1995; Peterson, 2000).

The subunit composition of SWI/SNF complexes can be used to further subdivide them into two classes that are themselves highly conserved in eukaryotes. In yeast, the subclasses are represented by the ySWI/SNF and RSC chromatin remodeling complexes. They share two identical and at least four homologous subunits. ySWI/SNF contains

the ATPase Swi2/Snf2 and the Swi1 subunit. RSC on the other hand contains the ATPase Sth1, a paralog of Swi2/Snf2, and lacks Swi1, while it contains the subunits Rsc1, Rsc2 and Rsc4 not found in ySWI/SNF. The two types of chromatin remodeling complexes have distinct cellular functions in yeast (for reviews, see (Mohrmann et al., 2005; Martens, 2003) The relationships between the two yeast SWI/SNF protein complexes are conserved in eukaryotes. While *Drosophila* contains only one SWI2/SNF2 homolog, Brahma (BRM), it is found in two classes of chromatin remodeling complexes corresponding to ySWI/SNF (BRM-associated Proteins or BAP) and RSC (Polybromo-associated proteins or PBAP). BAP and PBAP contain orthologs of the yeast subunits in combinations similar to the yeast complexes, and they mediate distinct cellular functions (Moshkin et al., 2007). Similarly, the mammalian SWI2/SNF2 paralogs are mutually exclusive subunits of mammalian SWI/SNF chromatin remodeling complexes (Khavari, 1993). The corresponding human SWI/SNF complexes are differentiated by their use of BRG1 (brahma-related gene) or hBRM (human brahma) as the ATPase subunit.

In the following section, we will describe the known developmental roles of SWI/SNF complexes in *Drosophila,* zebrafish, *Xenopus*, and mammals. The differential expression and known developmental functions of these remodelers are also briefly summarized in Table I.

**Table 1** Developmental roles of SWI2/SNF2 subfamily members across species

| PROTEINS | EXPRESSION PATTERNS | FUNCTIONS | REFERENCES |
|---|---|---|---|
| **dBRM** | - Ubiquitous in earlier stages<br>- Restricted to neural tube in later stages | - Required for survival to early stages<br>- Peripheral nerve development<br>- Specification of anterior thorax, posterior head segments<br>- Required for normal wing development | Simon and Tamkun 2002; Brizuela and Elfring 1994; Elfring et al. 1998; Marenda et al. 2004 |
| **xBRM** | Widespread, absent in branchial arches and tailbud | - Not Done | Linder et al. 2004 |
| **mBRM** | - Not Done | - Adult liver-specific albumin expression | Inayoshi et al. 2006 |
| **zBRG1** | - Ubiquitous in early stages<br>- Confined to anterior region in later stages | )evelopment of neural tube<br>leural crest cell and retinal differentiation | Link et al. 2000; Gregg et al. 2003; Eroglu et al. 2006; Lewis et al. 2004 |
| **xBRG1** | - Widespread, absent in hindbrain, spinal cord, pronephros and somites. | - Required for neuronal differentiation | Linder et al. 2004; Seo et al. 2005 |
| **mBRG1** | - (Maternal transcript) oocyte<br>- Ubiquitous in early stages<br>- Restricted to neural tissues in later stages | - Zygotic genome activation<br>- Implantation<br>- Differentiation of glial cells, neurons<br>- Differentiation of myelocytes<br>- Differentiation of bone and muscle<br>- Fetal liver-specific albumin expression | Bultman et al. 2006; Bultman et al. 2000; Matsumoto et al. 2006; Seo et al. 2005; Bottardi et al. 2006; Kadam and Emerson 2003; Gebuhr and Kovalev 2003; Young et al. 2005;Roy et al. 2002, Ohkawa et al. 2006;Inayoshi et al. 2006 |
| **SNF5/ INI1**[a] | - Not Done | - Essential before blastocyst hatching<br>- Liver development | Klochendler-Yeiven and Fiette 2000<br>Gresh et al. 2005 |
| **Baf60c**[a] | - Early embryonic heart and somites<br>- Nodal-expressing cells | - Cardiac and skeletal muscle differentiation and heart morphogenesis<br>- Establishment of left-right asymmetry | Lickert et al. 2004<br>Takeuchi et al 2007 |

[a] Non-ATPase subunits of the SWI/SNF chromatin remodeling complex

The known expression patterns and developmental functions of SWI2/SNF2 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,* z: zebrafish, x: *Xenopus laevis,* m: mammals (mouse or human). Pronephros is primitive kidney, myelocytes are precursors to blood cells. Blastocyst hatching is the shedding of the early embryonic zona pellucida preparatory to implantation.

*The role of SWI/SNF in* Drosophila *development*

The SWI2/SNF2 homolog found in *Drosphila melanogaster* was named *brahma* after the Hindu god of fate, as it was originally identified as one of a group of genes (trithorax group) that determined cell fate. (Papoulas, 1998; Brizuela et al., 1994; Dingwall, 1995; Daubresse, 1999). The gene product BRM is similar enough to yeast Swi2/Snf2 that its ATPase domain is interchangeable with that of ySwi2, while that of Imitation Switch (ISWI, a member of a different SWI2/SNF2 subfamily) is not (Elfring, 1994). BRM is the ATPase subunit of a protein complex analogous to ySWI/SNF (Dingwall et al., 1995).

Both maternal and zygotic BRM are required for normal embryogenesis. Unfertilized eggs contain maternal *brm* transcripts and depleting *brm* transcripts in eggs results in developmental defects as early as the cellular blastoderm stage. *Brm* function is required for normal oogenesis and proper expression of the segmentation gene *engrailed (en)* (Brizuela et al., 1994). Embryos lacking *brm* function die in late embryogenesis. Embryos heterozygous for *brm* mutations exhibit a variety of developmental defects (Elfring, 1998). Loss of function studies show that *brm* function is required for normal development of the peripheral nervous system in *Drosophila.*

During *Drosophila* embryogenesis the identities of anterior thoracic and posterior head segments, including the primordium of the larval salivary gland, are determined by one of the *Antennapedia complex* genes, *sex combs reduced* (*Scr*). The

*Scr* expression domain is initially determined by segmentation genes and later by homeotic genes of the *Antennapedia* and *Bithorax complexes* (Kennison, 1998). Regulation of *Scr* expression is maintained in later development by two antagonistic groups of gene products: outside of its normal domain of expression it is repressed by genes of the *Polycomb group (Pc-G)*, while within its normal expression domain it is activated by those of the *trithorax group (trx-G)*, including *brm*.

Other proteins of the *trithorax* group have been found to be orthologous to yeast SWI/SNF subunits, and to physically interact with BRM (reviewed in (Simon, 2002). The *moira* gene, encoding the protein MOR, is homologous to yeast *SWI3*, a subunit of the ySWI/SNF complex, and coimmunoprecipitates with BRM in *Drosophila* embryo nuclear extracts (Crosby, 1999). *Moira* and *brm* have strong genetic interactions in *Drosophila* (Papoulas et al., 1998). Another *trx-G* gene, *osa,*was found to interact genetically with *brm* to regulate *Antennapedia* expression (Vazquez, 1999). OSA contains an ARID domain also present in the yeast Swi1 protein, another ySWI/SNF subunit. ARID domains usually confer non-sequence-specific DNA-binding function, with a general preference for AT-rich DNA binding; however, ySwi1 does not show significant DNA binding (Wilsker, 2004). Genetic analyses and loss of function studies have shown that SNR1, a homolog of the SWI/SNF subunit *SNF5*, interacts with BRM to regulate expression of genes involved in wing vein development (Marenda, 2004) and of ecdysone-responsive genes expressed at the larval-pupal transition (Zraly, 2006). Ecdysone is a steroid hormone required for the dramatic changes that occur during

insect metamorphosis. The specific roles of these non-ATPase subunits have not been elucidated, but one likely function is that they may help recruit the *Drosophila* SWI/SNF complex to promoters of target genes (Armstrong, 2002; Armstrong, 2005).

*Vertebrate SWI/SNF complexes*

In vertebrates the paralogous genes *brm* and *Brg1* function as alternative ATPase subunits of the SWI/SNF chromatin remodeling complex (Wang, 1996). It is clear that BRM and BRG1 proteins have diverged in function as they interact with different groups of transcription factors (Kadam, 2003). Studies in a variety of vertebrate model organisms point to distinct roles for BRG and BRM in development, some of which are conserved across vertebrate evolution.

*The roles of SWI/SNF in zebrafish development*

In zebrafish, Brg1 (encoded by the *smarca4* gene) is required for normal development of retina, brain, and neural crest cells, and loss of Brg1 function affects differentiation of the retina at a specific stage of development. *In situ* hybridization studies show that *Brg1* is expressed in early retinal development (Link, 2000; Gregg, 2003). *Brg1* mutant (also known as "young" or *yng*) embryos develop an abnormal retinal morphology that is phenocopied by *Brg1*-specific morpholino injection. The same abnormal morphology occurs in embryos mutant for *baf53*, a subunit of SWI/SNF complexes known to bind Brg1. To further characterize the role of Brg1 in retinal development, these researchers performed *in situ* hybridization in Brg1-deficient

embryos to detect markers of specified retinal cell types (*rx2 and vsx2*). They found that the retinas of mutant embryos undergo a normal process of specifying retinal cells, an early step in retinal development, but at later stages the retinal cells fail to develop the normal morphology of terminally differentiated retinal cells and don't express late-differentiation antigens (e.g., Zn-1 antigen for red and green photoreceptors, and ID1 antigen for rod photoreceptors). This indicates that Brg1 is required for terminal differentiation of the retina but does not play a role in the earlier step of retinal cell specification.

*In situ* hybridization of zebrafish embryos from the one-cell stage through the 24 hours post-fertilization (hpf) stage show that Brg1 mRNA is ubiquitous until 24hpf, when the pattern becomes restricted to the anterior region of the embryo (Eroglu, 2006). Expression is most pronounced in the brain. Injection of Brg1-specific morpholino into zebrafish embryos causes the expansion of the domain of *six3*, a forebrain marker, and reduction of the domains of midbrain boundary marker *engrailed2 (eng2)* and the hindbrain marker *krox20*. Overexpression (by injecting Brg1-specific mRNA) has the opposite effect on *six3*.

In addition to the defects in brain and retinal development described above, these experiments also revealed defects in development of neural crest cells. Neural crest cells derive from ectoderm and migrate laterally through the embryo to become skin pigment cells, peripheral neurons and glia, and form the cartilage and bones of

facial structures. Neural crest progenitor cells are induced at the gastrula stage in a process that requires function of the Wnt signaling pathway (Lewis, 2004). They later migrate away from neuronal cells and express neural crest specific genes.

Expression of neural crest specifiers is severely reduced in zebrafish embryos lacking Brg1 function; the embryos exhibit defects in neural crest-derived structures and fail to express neural crest markers *snail2, foxd3* and *tfap2a* (Eroglu et al., 2006). *Eng2* and *snail2* are both targets of Wnt signaling; Brg1 is known to bind the Wnt signaling pathway component β-catenin and is recruited to the T-cell transcription factor (TCF/LEF) binding site of target genes including *slug/snail2* (see (Gammill, 2003) for a review), so a role for Brg1 in specific Wnt-dependent pathways is not surprising. While these studies have revealed a key role for zebrafish Brg1 in the development of neural and neural crest-derived structures, little is known about corresponding roles of the zebrafish brm homolog (encoded by the *smarca2* gene).

*The roles of SWI/SNF in* Xenopus *development*

Western analysis of staged *Xenopus* embryos (Gelius et al., 1999) shows that, as in zebrafish, BRG1 is present at all stages of oogenesis and embryogenesis and is expressed ubiquitously in early development, later to be restricted to neural tissues. *In situ* studies of whole mount embryos demonstrate distinct expression patterns for the *Brg1* and *brm* paralogs. For instance, at the tailbud stage *brm* is expressed in the

hindbrain, spinal cord, pronephros and somites while *Brg1* is not, and *Brg1* is expressed in branchial arches and tailbud while *brm* is not (Linder, 2004).

Loss of BRG1 function in *Xenopus* prevents differentiation of neurons from proneural cells (Seo, 2005). The *Neuron-specific tubulin (N-tubulin)* gene is specifically expressed in differentiated neurons, dependent upon the proneural activities of the basic helix-loop-helix (bHLH) transcription factors Neurogenin-related-1 (Ngnr1) and NeuroD. Loss of Brg1 function results in both a reduction of *N-tubulin* expression and a failure of Ngnr1 and NeuroD to promote neuronal differentiation. Consistent with a direct role for Brg1 in neuronal differentiation, Brg1 coimmunoprecipitates with Ngnr1 and NeuroD. An analogous relationship between Brg1 and NeuroD2 was also demonstrated in a mammalian cell line that can be induced to differentiate into neurons by NeuroD.

*The roles of SWI/SNF complexes in mammalian development*

Considerable evidence shows that alternative mammalian SWI/SNF complexes containing either BRG1 or BRM perform different functions *in vivo,* despite the similarities between these complexes. GST pulldowns have demonstrated that BRG1 but not BRM binds to zinc finger transcription factors *in vivo,* while BRM but not BRG1 binds to ankyrin repeat proteins involved in the Notch signaling pathway. While overall the two proteins share 75% homology, BRG1 contains an N-terminal motif known to bind zinc finger proteins that is absent in the BRM protein (Kadam et al., 2003).

A number of studies of mouse development highlight the differences in BRG1 and BRM expression patterns and functions in development. *brg1*-null mouse embryos die around the time of implantation of the blastocyst while *brm* null mice exhibit only a mild phenotype (Bultman, 2000). As in zebrafish and *Xenopus,* BRG1 is expressed early in mouse development but progressively this expression becomes more restricted to neural tissue.

RT-PCR of *Brg1* and *brm* transcripts in mouse oocytes and embryos indicates that while both are abundant as maternally-derived products, only BRG1 is expressed at the start of zygotic transcription (LeGouy, 1998). Zygotic BRM expression begins later at the blastocyst stage when differentiation begins, and only in the inner cell mass. Similarly in *Rhesus* embryos monitored by RT-PCR, *Brg1* expression begins at the morula stage while *brm* zygotic expression begins later, at the hatched blastocyst stage (Zheng, 2004). In embryos conceived from conditional Brg1 mutant-derived eggs, Brg1 depletion leads to a zygotic genome activation failure that includes arrest at the two-cell stage and downregulation of about 30% of expressed genes (Bultman, 2006).

To visualize the expression of BRG1 and BRM in embryonic tissues, mouse embryo sections were immunostained with antibodies to BRG1 and BRM (Dauvillier, 2001). While BRG1 is expressed widely in embryos, BRM expression is restricted to mesodermal tissues involved in vasculogenesis, allantois (umbilical cord precursor), vitelline arteries, yolk sac and cardiogenic plate. As they are required early in

postimplanation development, these tissues are the first to be determined, coinciding with the onset of BRM expression.

In addition to its role in early embryonic viability, BRG1 has been implicated in a number of tissue-specific differentiation events, including differentiation in hematopoietic lineages. The zinc-finger protein Erythroid Kruppel-like factor (EKLF), required for tissue-specific expression of β-globin genes, associates with BRG1 *in vitro,* and *in vivo* is required for BRG1 recruitment to the β-globin Locus Control Region and promoter (Bottardi, 2006; Kadam, 2000). Mice with a partial loss-of-function mutation of *Brg1* exhibit a failure to switch from primitive yolk-sac-derived erythrocytes to definitive fetal-liver-derived erythrocytes, resulting in severe anemia and death at midgestation (Bultman, 2005). Paradoxically, other tissues develop normally in the mutant embryos, possibly because *brm* is expressed in those tissues and may compensate for the *brg1* partial loss of function, whereas *brm* expression is absent in erythrocyte precursors.

Brg1 loss of function also leads to a developmental block in myeloid differentiation to granulocytes at the promyelocyte/metamyelocyte precursor stage (Vradii, 2006). T lymphocyte-specific inactivation of *Brg1* in mice leads to CD4 derepression at the double negative (CD4-CD8-) stage of T cell development and a subsequent failure to develop to the next (CD4+ CD8+ double positive) stage of development (Gebuhr, 2003).

BRG1 is expressed in neural stem cells that give rise to both neurons and glial cell fates (astrocytes and oligodendrocytes) (Matsumoto, 2006). Targeted loss of BRG1 function in neural stem cells results in reduced expression of proteins required for stem cell maintenance, such as Pax6 and Sox1. Furthermore, BRG1 is required for gliogenesis, as *brg1*-null neural stem cells are unable to differentiate into glial cells and instead adopt neuronal fates. However, other studies have implicated BRG1 in neuronal differentiation as well. BRG1 is highly expressed in the mantle zone of the spinal cord in embryonic (day 12) mice; the mantle zone contains post-mitotic neurons whereas the underlying ventricular zone contains dividing neural stem cells and is the primary site of neural differentiation (Randazzo et al., 1994); this suggests a post-differentiation role for BRG1 as well. Also, as noted above, interference with BRG1 function prevents neuronal differentiation driven by NeuroD2 in a mouse cell line that can be induced to differentiate into neurons (Seo et al., 2005).

Indra and colleagues performed a set of experiments to specifically ablate BRG1 function in the surface ectoderm of developing mice, which gives rise to the dermal and epidermal layers of the skin (Indra, 2005). They constructed transgenic mice containing LoxP-flanked *Brg1* alleles and the Cre recombinase. The Cre recombinase was under the control of the K-14 promoter, which is active in surface ectoderm and the basal layer of the epidermis. While ablation of *Brg1* does not alter the early differentiation of keratinocytes, it does cause failure of the final stages of their differentiation, resulting in disruption of the skin permeability barrier. The loss of *Brg1* in developing limb

ectoderm results in profound hindlimb defects, indicating a role for BRG1 in limb patterning. Intriguingly, while BRM cannot substitute for BRG1 in limb formation, BRM does partially compensate for lack of BRG1 in terminal keratinocyte differentiation, revealing both redundant and non-redundant functions for BRM and BRG1.

Finally, BRG1 may also play an important role in bone and muscle differentiation. Young and colleagues demonstrated that BRG1 is expressed in the developing mouse skeleton, and showed that it is required for Bone Morphogenic Protein 2 (BMP2)-dependent induction of alkaline phosphatase (Young, 2005). Alkaline phosphatase is an early marker of osteoblast differentiation, dependent on the Runx2 transcription factor.

Studies of embryonic tissue and of cultured cells have revealed a requirement for BRG1 activation of genes required for muscle differentiation. In cultured fibroblasts inducibly expressing dominant negative BRM or BRG1, each of the basic helix-loop-helix myogenic regulatory factors MyoD, Myf5, Mrf4 require BRG1 or BRM to mediate expression of the myogenic markers myosin heavy chain and troponin T (Roy, 2002). Chromatin immunoprecipitation (ChIP) studies of differentiated embryonic muscle tissue demonstrate that myogenin binds at its own promoter and associates with BRG1 (Ohkawa, 2007). In cultured fibroblasts, BRG1 is required for MyoD-mediated myogenin expression, and that this is accompanied by chromatin remodeling at the promoter(de la Serna, 2001). These results suggest that BRG1 is required for both induction of myogenin expression by MyoD in early myogenesis, and subsequent maintenance of

expression by myogenin itself. These studies have also been extended into whole animals. In developing mouse embryos, RT-PCR and ChIP analyses demonstrate that myogenic late marker genes are expressed concomitant with the binding of BRG1, myogenin and Mef2D (a myogenic cofactor) to their promoters (Ohkawa, 2006).

*Roles of SWI/SNF complexes in human development*

The human homologs of SWI2/SNF2 and *Brahma* are designated human *brahma (hbrm)* and *Brahma related gene 1 (Brg1)* (Randazzo et al., 1994; Muchardt, 1993; Khavari et al., 1993; Chiba, 1994). Mammalian SWI/SNF complexes directly interact with regulatory proteins such as retinoblastoma protein, cyclin E and with a large number of transcription factors (Dunaief, 1994; McKenna, 1999; Glass, 2000).

Obviously, most work on the roles of BRG1 and BRM in development comes from studies in mice, as described in the previous section, or in work in cell culture models for different pathways of differentiation. However, some work has addressed how these results may translate to humans. For example, immunostaining of normal human tissue sections for BRG1 or BRM reveals different expression patterns for the paralogs. BRG1 is predominantly found in highly proliferative cell types (e.g., endodermal and ectodermal epithelium, B germinal centers of tonsils and spleen) while BRM is predominantly expressed in non-proliferating tissues such as brain and liver (Reisman, 2005). These different expression patterns are consistent with a number of the studies described above, in which BRG1 is commonly required for survival of proliferating cells

and early stages of differentiation, while BRM may play a more critical role in terminally differentiated, non-dividing cells.

*Non-ATPase subunits of SWI/SNF complexes*

It is well established that in interactions between SWI/SNF complexes and target genes, the ATPase subunit performs the same basic function, that of translocating nucleosomes along the DNA to facilitate regulation of the gene by other factors. The functions of the eight or more other subunits of SWI/SNF complexes have received relatively less attention from investigators.

Several studies have demonstrated that the SNF5/INI1 subunit, present in both BRG1- and BRM-containing SWI/SNF complexes, is also essential for mouse development (Klochendler-Yeivin, 2000; Guidi, 2001). While mice heterozygous for SNFR5/INI1 survive (albeit with an increased incidence of tumor formation), nullizygous embryos do not survive beyond the blastocyst stage. In culture, wild type blastocysts hatch from the zona pellucida and form a trophectoderm, but the nullizygous embryos fail to do so. These results, along with the results for *brg1*-null mice described above, make it clear that the SWI/SNF complex is essential for early development in mouse.

Conditional inactivation of the SNF5/INI1 subunit of SWI/SNF complexes in the developing mouse liver results in neonatal death accompanied by liver defects, including improper formation of hepatic epithelium and a failure to store glycogen (Gresh, 2005). Microarray analysis reveals that 70% of the genes normally upregulated during liver

development show reduced expression in SNF5/INI1-deficient mice. Interestingly, another study in hepatocytes revealed a requirement for BRG1 in expression of the liver-specific albumin gene in fetal hepatocytes, while expression of the same gene in hepatocytes from adult liver requires BRM (Inayoshi, 2006). These authors showed that BRG1 levels decrease and BRM levels increase during liver cell differentiation, consistent with other examples (discussed above) of roles for BRG1 and BRM in proliferating and post-mitotic cells, respectively

Another example of combinatorial assembly of SWI/SNF complexes is revealed by the alternative forms of Baf60: Baf60a, Baf60b and Baf60c, encoded by the *Smarcad1, Smarcad2* and *Smarcad3* genes, respectively. Baf60c is expressed specifically in the heart and somites of early mouse embryos (Lickert, 2004), suggesting that SWI/SNF complexes may have different subunit compositions in different tissues. In transgenic embryos, elimination of Baf60c by RNA interference disrupts normal cardiac and skeletal muscle differentiation and heart morphogenesis. In HeLa cells immunoprecipitation of BRG1 and epitope-tagged cardiac transcription factors shows that Baf60c is necessary for the interaction of BRG1 with cardiac transcription factors.

In zebrafish, Baf60c is expressed at late gastrulation in cells surrounding the forerunner of the ciliated organ of asymmetry, Kuppfer's vessicle (KV) analogous to the mouse node (Takeuchi, 2007). When left-right (LR) asymmetry arises during early somitogenesis, Baf60c is strongly expressed in notocord and around the KV, and later in eye, midbrain, forebrain and KV. In developing mice, Baf60c is expressed in the *Nodal-*

expressing cells at the periphery of the node. The normal breaking of bilateral symmetry requires the secretion of the *Nodal* protein in cells at the periphery of the node. Expression of *Nodal* requires both a functional Notch signaling pathway and functional Baf60c. Bac60c loss of function causes defective LR asymmetry such as abnormal looping of the heart. Expression of genes associated with the cascade of asymmetry establishment (e.g., *lefty1,2,3*) is also perturbed. Morpholino knockdown of zebrafish Baf60c causes *lefty1,2,3* and *southpaw* to be misexpressed or not expressed, demonstrating the conservation of functional relationships among these proteins in vertebrates.

In summary, the results discussed here indicate that BRG1- and BRM-containing SWI/SNF complexes have mostly non-redundant functions in vertebrate development. While their biochemical activities and certain other functions may overlap, their roles have diverged dramatically in the course of vertebrate evolution. Numerous examples support a division of labor in which BRG1-containing complexes are critical for the survival of dividing cells, maintenance of pluripotency, and early stages of differentiation, while BRM-containing complexes may have more restricted roles in terminal differentiation and transcriptional regulation in post-mitotic cell populations.

**The ISWI subfamily**

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) (Tsukiyama, 1995a; Becker, 1994; Tsukiyama, 1994; Tsukiyama, 1995b; Ito, 1997). 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., 1994) that are present in the 1sw1a, Isw1b, and Isw2/yCHRAC complexes (Tsukiyama, 1999; Vary, 2003; Iida, 2004). In *Xenopus* three ISWI-containing complexes have been characterized: ACF, CHRAC and WICH (Guschin, 2000; Bozhenok, 2002). Mammals have two ISWI homologs, SNF2L and SNF2H, which show tissue-specific expression patterns (Barak, 2004b). SNF2H is present in at least 7 different complexes, including RSF (remodeling and spacing factor) (LeRoy, 1998; Loyola, 2003), hACF/WCRF (WSTF–related chromatin-remodeling factor) (Bochar, 2000; LeRoy, 2000), hCHRAC (Poot, 2000), hWICH (Bozhenok et al., 2002), hB-WICH (Cavellan, 2006), and NoRC (nucleolar remodeling complex) (Strohner, 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 contains the Mi-2 ATPase (a member of the CHD subfamily (Hakimi, 2002). SNF2L is the catalytic subunit of the hNURF complex (Barak, 2003) and CERF (CECR2 containing remodeling factor) complex (Banting, 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, 2006). A detailed account of the subunit compositions of all the ISWI complexes and their homologies in different species is reviewed elsewhere (Dirscherl et al., 2004; Mellor, 2006), and Mellor and Morillon (Mellor, 2004) provide an excellent review of the functions of yeast ISWI complexes. Here we will concentrate on the developmental roles of these ISWI complexes in multicellular organisms.

*Developmental roles of the ISWI ATPase*

Because ISWI is present in so many different complexes, studies of the *in vivo* roles of 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. We will first discuss the developmental roles of ISWI itself, then we will discuss data that address the roles of specific ISWI complexes in development. The developmental roles of these ISWI complexes have also been summarized in Table 2.

In *Drosophila*, null mutations in *ISWI* are lethal, resulting in death at the late larval/early pupal stages (Deuring, 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. 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*. Additionally, 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, 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 also essential for survival during early development, particularly neurulation, and is also critical for later stages of neural development and retinal differentiation (Dirscherl, 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, 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. This is reminiscent of the separation of function between proliferating and post-mitotic cells observed for BRG and BRM, discussed earlier.

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, 2004a). 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

**Table 2** Developmental roles of ISWI subfamily members across species

| PROTEINS | EXPRESSION PATTERNS | FUNCTIONS | REFERENCES |
|---|---|---|---|
| **dISWI** | - Restricted to CNS and gonads after germ band retraction | - Essential for late larval /early pupal development<br>- 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 and Krebs 2005 |
| **mISWI** | - (mouse) SNF2H is ubiquitously expressed but SNF2L is restricted to brain and gonads<br>- (human) SNF2L and SNF2H are ubiquitously expressed | - Normal differentiation and survival of embryo<br>- Corpus luteum formation<br>- Blood cell formation<br>- 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., 2002b; Badenhorst et al. 2005; Deuring et al. 2000 |
| **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 | Vanolst et al. 2005 |
| **xWSTF**[e] | - Eye, brain, neural crest cells | - Essential for normal eye and CNS development | Cus et al. 2006; S.M., J. Henry, and J.E.K., unpublished results |
| **mCECR2**[d] | - Throughout nervous tissue | - Essential for neurulation | Banting et al. 2005 |

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

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; the sensory organ denotes the Dorso-Central bristle; CNS, Central Nervous System; GSC, Germline Stem Cell.

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, 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, 2006), in keeping 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.

*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, 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, 2002), and the yeast ISWI complexes repress a wide variety of genes (Fazzio, 2001; Goldmark, 2000; Kent, 2001; Ruiz, 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, we 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, 1998; Martinez-Balbas, 1998). *In vivo* studies show that, as for the *iswi* mutants described above, null mutations of *nurf301* result in embryonic lethality during late larval/early pupal stages (Badenhorst, 2002a). *nurf301* mutations result in impaired transcription of *Ultrabithorax* (*Ubx*) and *engrailed* (*en*), as was the case for *iswi* mutants, as well as the *hsp70* and *hsp26* heat shock genes. 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, 2002b; 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, 2005). Ecdysone is a steroid hormone required for the dramatic changes that occur during insect metamorphosis. 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 (see below).

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, 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 injection 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 (Zhou et al., 2002; Li, 2006b); recruitment of NoRC appears to require intergenic transcription from the rDNA intergenic spacers (Mayer, 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, 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 in *Xenopus*, and consists of WSTF (Williams Syndrome Transcription Factor) and ISWI/SNF2H. WSTF was first identified in a search for genes deleted in Williams syndrome, which is an autosomal dominant hereditary disorder characterized by mental retardation, growth deficiency, elfin face and congenital vascular lesions (Lu, 1998). These developmental defects cannot all be attributed to lack of WICH function, as other genes are also deleted in Williams syndrome patients. In *Xenopus* embryos WSTF is differentially expressed in neural tissue, especially in the eye, brain and neural crest cells (Cus, 2006) and our unpublished results). Our own work has revealed that inhibition of *Xenopus* WSTF results in severe defects in eye and central nervous system development (S.M., J.J.

Henry, and J.E.K., unpublished), indicating that a number of ISWI complexes play different roles in neural development in numerous species. Because WICH (and related B-WICH) complexes have been implicated in both transcriptional regulation and DNA replication, it will be interesting to see which of these functions is primarily responsible for the observed developmental defects.

**The CHD subfamily**

Numerous Chromodomain Helicase DNA-binding (CHD) proteins have been characterized in eukaryotes. As stated in the introduction, each contains two chromodomains that interact with methylated histone tails; some members of the CHD family also contain PHD domains, which have also been implicated in methyl-lysine recognition, while others have AT-rich DNA binding motifs (Wysocka et al., 2006; Shi et al., 2006; Pena et al., 2006; Li et al., 2006a; Woodage et al., 1997). Ruthenburg and colleagues have written an excellent recent review of methyl-lysine recognition by chromodomains and PHD fingers (Ruthenburg et al., 2007). Here we will describe the known roles of CHD family members in animal development, beginning with the best-characterized CHD proteins, CHD3/4 (also known as Mi-2α/β). Data for the CHD subfamily is also highlighted in Table III.

**Table 3** Developmental roles of CHD subfamily members across species

| PROTEINS | EXPRESSION PATTERNS | FUNCTIONS | REFERENCES |
|---|---|---|---|
| **cLET-418 CHD-3** | - Not Done | - LET-418 required for development to the first instar larva<br>- Antagonize vulval cell fate determination | Zelewsky et al. 2000 |
| **dMi-2**[a] | - Not Done | - Essential for development to the first or second instar larva<br>- Represses homeotic genes mediated by Hunchback and Polycomb<br>- Represses proneuronal gene expression | Kehle et al. 1998<br>Yamasaki and Nishida 2006 |
| **dp66**[a] | - Not Done | - Required for normal metamorphosis<br>- May be required for ecdysone-mediated gene Expression | Kon et al. 2005 |
| **xCHD2/ mCHD2**[b] | - Eyes and neural tube<br>- Branchial arches<br>- Otic vessicle (presumptive ear) | - Essential for survival to perinatal stage | Linder et al. 2004<br>Marfella et al. 2006 |
| **xCHD4/m CHD4/Mi-2β**[a,b] | - Eyes and neural tube<br>- Branchial arches<br>- Otic vessicle (presumptive ear)<br>- Somites | - Required for early stages of thymocyte differentiation<br>- Required for expression of CD4 surface marker<br>- Required for proliferation of mature T lymphocytes<br>- May function in nerve myelination | Linder et al. 2004<br>Williams et al. 2004<br>Srinivasan et al. 2006 |
| **xCHD5/ mCHD5**[b] | - Fetal and adult brain<br>- Otic vessicle (presumptive ear)<br>- Adrenal gland | - Possible role in development of nervous system | Thompson et al. 2003<br>Linder et al. 2004 |
| **mCHD7** | - Precursors of eye, ear, kidney, vascular system, olfactory epithelium | - Essential for survival to perinatal stage<br>- Functions in normal closure of optic fissure, inner ear, heart and genitourinary and inner ear morphogenesis | Sanlaville et al. 2006<br>Aramak et al. 2007(Bosman, 2005)<br>Aramaki et al. 2006 |

[a] Subunit of NuRD complex; [b] Expression studies were done for *Xenopus* only and functional studies for mammals only

The known expression patterns and developmental functions of CHD 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. c: *C. elegans,* d: *Drosophila melanogaster,* x: *Xenopus laevis*, m: mammals (mouse or human). Hunchback and Polycomb are transcription factors that repress HOX gene expression.

*CHD3/Mi2α and CHD4/Mi2β: NuRD complexes*

The CHD4 protein was initially identified by Seelig and colleagues in 1995 as the dermatomysitis-specific autoantigen Mi-2 (antigen recognized by patient Mitchell's autoimmune antibodies 2) (Seelig, 1995). Subsequently, several groups identified a related set of remodeling complexes containing either CHD3 or CHD4 as the ATPase subunit. These complexes include the *Xenopus* Mi-2 complex (Wade, 1998) and the human complexes NuRD/NURD/NRD (Zhang, 1998; Xue, 1998; Tong, 1998); we will refer to these generically as NuRD. NuRD complexes, which generally function as transcriptional repressors, also contain histone deacetylases, methyl DNA binding proteins (MBD2 or MBD3), members of the MTA (metastasis-associated) protein family, and Rb-associated proteins RbAp48/p46 (reviewed in (Bowen, 2004).

Two Mi2 homologs in *C. elegans*, CHD-3 and LET-418, play essential and non-identical roles in embryogenesis and vulval development (von Zelewsky, 2000). Null mutations in *let-418* are homozygous lethal at the L1 larval stage, while *chd-3* null animals are viable. However, combination of *chd-3* and *let-418* mutations results in early embryonic arrest, suggesting some redundant functions in early embryogenesis. These authors also showed that CHD-3 and LET-418 are negative regulators of vulval cell fate determination, and act by antagonizing the Ras signaling pathway required for

vulval induction—a role in vulval cell specification that opposes that described for the ISWI-containing NURF complex (Andersen et al., 2006), discussed above.

Work in *Drosophila* has also uncovered developmental roles for the *Drosophila* Mi2 homolog, dMi-2. Complete absence of dMi-2 is lethal; maternally deposited dMi-2 is sufficient for survival to the first or second larval instar (Kehle, 1998). Using heterozygous animals to alter the dosage of dMi-2, these authors showed that dMi-2 participates in the repression of homeotic genes mediated by Hunchback and Polycomb. An essential protein associated with *Drosophila* NuRD, p66 (a p66 homolog also copurifies with the *Xenopus* Mi2 complex), is required for normal metamorphosis and may be critical for ecdysone-regulated gene expression (Kon, 2005).

More recent work has uncovered a specific role for dMi-2 in sensory organ development in *Drosophila* (Yamasaki, 2006). While dMi-2 null mutants normally die during early larval stages, approximately 0.1% will actually survive to adulthood. Animals that escape embryonic lethality reveal ectopic development of sensory bristles, implicating dMi-2/NuRD in the repression of proneural gene expression. This is consistent with the known interaction between dMi-2 and Tramtrack69, a transcriptional repressor that regulates nervous system development (Murawsky, 2001; Badenhorst et al., 2002a).

Mammalian NuRD complexes have been implicated in cell differentiation. Recent work shows that CHD4/ Mi-2β is required for several steps in T cell development,

including early stages of thymocyte differentiation, CD4 expression, and proliferation of mature T cells (Williams, 2004). Other studies have also hinted at a role for CHD4 in another terminal differentiation event, nerve myelination, possibly via repression of *Rad*, a gene normally repressed in Schwann cells during peripheral nerve myelination (Srinivasan, 2006).

Human CHD5 is a poorly characterized member of the CHD family that is closely related to CHD3 and CHD4. It is preferentially expressed in fetal brain, adult brain, and the adrenal gland, suggesting that it too may play a role in the development of the neural system (Thompson, 2003).

*CHD2*

Unlike the other CHD family members, CHD1 and CHD2 lack the PHD domains found in CHD3, 4 and 5, and instead contain a unique DNA binding motif that preferentially binds AT-rich DNA (Stokes, 1995), though the role of this motif is not yet understood. A recent study from the Imbalzano laboratory reveals an essential role for *Chd2* in mouse development (Marfella, 2006). *Chd2* null mice exhibit perinatal lethality; i.e. they die shortly before or after birth and exhibit reduced body size compared to wild type littermates. Even heterozygous pups exhibit increased mortality, and present with multiple organ abnormalities. These studies have implicated *Chd2* in cell cycle progression.

*CHD7*

The CHD7 protein contains the diagnostic domains of the CHD subfamily, including the SWISNF2 ATPase domain and two chromodomains, and additionally contains a SANT domain and a BRK DNA-binding domain. In humans, mutations in the *Chd7* gene have been linked to CHARGE, a constellation of congenital abnormalities known by the acronym for the canonical symptoms: Coloboma (failure of optic or choroidal fissure to close), Heart septal defects, Atresia choanae (narrowing or blockage of nasal passages), Retardation of growth and/or development, Genitourinary anomalies, and Ear/olfactory/cranial nerve abnormalities (Williams, 2005). A patient diagnosed with Kallmann Syndrome (poor gonad development and impaired olfactory function) was also shown to carry a *Chd7* mutation (Ogata, 2006).

The link between CHD7 and CHARGE was uncovered by Vissers and colleagues, who used array comparative genomic hybridization to identify a translocation on chromosome 8 in an affected individual (Vissers, 2004). The translocated region contains the *Chd7* gene; when they sequenced the region in 17 affected individuals they identified 10 heterozygous mutations of the *Chd7* gene, suggesting that haploinsufficieny of the gene may be the cause of some or all cases of CHARGE. Genotyping of 23 patients presenting with CHARGE syndrome identified 17 *Chd7* heterozygous mutations (Aramaki, 2006). They exhibited varying levels of penetrance

for the major and minor characteristics of CHARGE. The remaining patients may represent mutations in regulatory regions of the gene or a clinically distinct group.

Bosman and colleagues identified a number of ENU-induced mutations in the murine *Chd7* homolog that result in behavioral defects attributable to inner ear malformations similar to those observed in CHARGE patients (Bosman, 2005). In normally developing mice, *Chd7* is expressed in the precursors of organs affected by CHARGE syndrome: eye, olfactory epithelium, ear, kidney and vascular system. In addition to the inner ear defects, mice heterozygous for *chd7* mutations also exhibit other defects similar to those found in CHARGE syndrome patients, such as heart and genitourinary defects. As in humans, all of these *Chd7* mutant mice are heterozygous and no homozygotes have been reported to survive past birth.

Expression patterns similar to those seen in mice are also found in normally developing human fetuses, including expression in tissues derived from the neural crest, as well as in cranial nerves, auditory and nasal tissues, and neural retina (Sanlaville, 2006). A recent study of the chicken CHD7 ortholog also reveals extensive neuronal expression of *Chd7* during early development, and expression in otic, optic and olfactory placodes, indicating a conserved function in development of specific organs across vertebrate species.

**Remodelers in development: vive la difference!**

In this review, we have discussed the known developmental expression patterns and functions of a diverse selection of SWI2/SNF2 chromatin remodeling enzymes. The cartoons shown in Figure 1 visually summarize the functions we have discussed. The studies discussed here have revealed an array of functions for these proteins, ranging from viability at the level of individual cells (often revealed by essential roles in early development), through roles in differentiation in specific tissues. We have highlighted both divisions of labor between pre- and post-differentiation stages of cell fate determination, as well as a striking preponderance of functions in neural development, particularly in vertebrates. The vast proliferation of the SWI2/SNF2 superfamily throughout evolution has resulted in an incredibly complex assortment of chromatin remodeling factors, which are able to serve in both unique and overlapping roles in the carefully orchestrated processes of metazoan development.

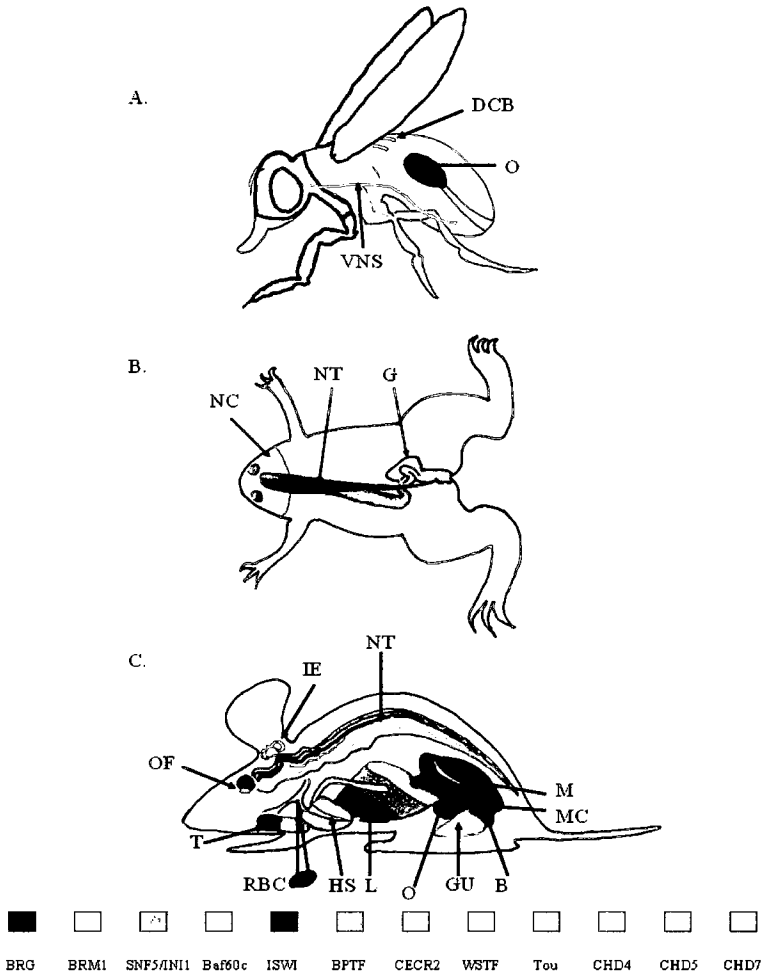


**Figure 1** Cartoon representation of developmental functions of chromatin remodeling enzymes in metazoan development. Shaded regions indicate tissues that require one or more remodelers for their normal development; different colors indicate the specific remodeling complex subunit as indicated in the color key (bottom). Multiple colors in a single tissue indicate contribution of multiple proteins; this is not meant to imply positional roles within tissues. For simplicity, the relevant adult tissues are indicated in the cartoons of *Drosophila* (A), *Xenopus* (B) or mouse (C), and therefore do not reveal the stage of development during which these activities are required. For further details see the text and tables. Structures are not to scale. O: ovary; DCB: Dorso-Central Bristles; VNS: ventral nervous system; NT: neural tube; NC: neural crest; G: gut; IE: inner ear; OF: optic fissure; T: Thymus; RBC: red blood cell; HS: heart septum; L: liver; GU: genitourinary system; MC: myelocyte; M: muscle; B: bone.

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# Chapter 2

## Materials and Methods

ATP-dependent chromatin remodeling is required for the carefully regulated expression of genes during development. The enzyme ISWI (Imitation Switch) is the ATPase motor of a number of protein complexes that exhibit chromatin remodeling activity. Studies have shown that while ISWI fills the same function in each complex, the unique complement of auxiliary proteins in each complex modifies the nature of the chromatin remodeling activity. This suggests that ISWI-containing complexes may have different roles to play in development.

While there are many obstacles to studying human development, the frog *Xenopus laevis* has proven to be well suited as an animal model of development in vertebrates. Three ISWI-containing chromatin remodeling complexes have been characterized in *Xenopus*: ACF, CHRAC and WICH (reviewed in [1]). Ablation of ISWI function results in mortality at early stages and neural and eye defects among the survivors. However, the individual roles of one ISWI-containing complex cannot be distinguished from another by ablating ISWI function, which inactivates the ATPase motor in all of them.

This thesis focuses on the developmental roles of the CHRAC complex and its impact on morphology in gene regulation in developing *Xenopus*. To help parse out the roles in development of individual ISWI-containing remodeling complexes I

hypothesized that the CHRAC complex's role in development would be distinct from that of all of the ISWI-containing complexes combined. Other chromatin remodeling complexes that share a common ATPase subunit have been shown to have distinct functions in development (reviewed in [2]), and the distinct complements of auxiliary proteins in ISWI-containing complexes suggest distinct roles in development.

CHD4 is an ATPase chromatin remodeling enzyme, the motor of a well-characterized protein complex called NuRD (Nucleosome Remodeling and histone Deacetylase) complex. The complex has been shown to be essential for early embryogenesis and for hematopoiesis. My collaborator, Dr. Jonathan Henry of the University of Illinois Urbana, identified it in a screen for genes upregulated in regenerated lens in xenopus. I hypothesized that CHD4 may also have a role in eye development, and to test this I ablated CHD4 function by morpholino injection.

My research embodies the Specific Aims that follow.

Specific Aim #1: To determine the expression pattern of *CHRAC17* and compare and contrast it with the pattern of total *ISWI* expression.

Specific Aim #2: To characterize the loss of function phenotype of CHRAC17.

Specific Aim #3: To characterize the CHRAC17 loss of function phenotype at the molecular level by measuring expression levels of putative target genes.

Specific Aim #4: To characterize the loss of function phenotype of CHD4.

I visualized *CHRAC17* expression by whole mount *in situ* hybridization. An expression pattern distinct from but with potential overlap with the pattern of *ISWI* would suggest that the ACF, CHRAC and WICH complexes may have unique roles in development and can function independently of each other. It is noteworthy that ISWI and CHRAC17 are both subunits of protein complexes other than the ISWI-containing chromatin remodeling complexes. Some of these are known to regulate developmental processes, and all act on chromatin.

The CHRAC17 protein is not found in the ACF or WICH complexes. Thus, ablation of CHRAC17 in developing embryos would shed light on the consequences of CHRAC17 loss as opposed to ISWI loss. I used morpholino oligonucleotides to ablate CHRAC17 function and observed the resulting gross morphology from stages 15 to 45. Phenotype analysis of embryonic structures (neural tube, eye, notochord, pharyngeal pouches) in finer detail was conducted by Dr. Jonathan Henry. Perturbation of gene regulation by CHRAC17 loss of function was measured by RT-qPCR and cDNA microarray analysis.

If there are no differences in morphology or target gene regulation between ISWI knockdown embryos and CHRAC17 knockdowns, it would suggest that the developmental role of the CHRAC complex is redundant to the other ISWI-containing complexes. But if there are differences between CHRAC17 knockdown embryos and ISWI knockdowns the hypothesis would be supported: the CHRAC complex's role in development is distinct. Again it is important to make such interpretations in light of

the fact that CHRAC17 and ISWI are both subunits of other protein complexes. As well, whether the effect of CHRAC17 knockdown is direct or indirect would require further research to determine.

I used morpholino oligonucleotides to ablate CHD4 function and observed the resulting gross morphology from stages 15 to 45. Phenotype analysis of embryonic structures (neural tube, eye, notochord, pharyngeal pouches) in finer detail was conducted by Dr. Jonathan Henry.

If CHD4 is involved in eye development I will expect to see abnormalities of the eyes on the gross morphological scale or histologically. Lack of perturbed development would indicate that CHD4 and the NuRD complex play no role in eye development or that they play redundant roles. Whether or not the effects are direct or indirect would require further research to determine.

**Generation of *Xenopus* embryos**

I induced ovulation in female Xenopus *laevis* by injecting human chorionic gonadotropin (Intervet, Millsboro, DE) into their dorsal lymph sac. I obtained testis by euthanizing a male frog in 0.06% benzocaine for 45 minutes and surgically excising the testes. Twelve to eighteen hours after injecting the females I mimicked amplexus to induce them to lay eggs and immediately exposed the eggs to *Xenopus* dissected testis in 0.1X MMR. After determining that fertilization was successful I dejellied the embryos by exposing them to L-cysteine for 3-5 minutes. I determined the developmental stage

of embryos according to Nieuwkoop and Faber [3]. All procedures were performed in compliance with approved University of Alaska Anchorage IACUC protocols.

***In situ* hybridization**

I generated digoxigenin labeled RNA probes complementary to mRNA of *CHRAC17.* Template DNA corresponding to the EST clone BJ623466 was generously provided by Dr. Paul Wade. A 474bp segment of it was amplified by PCR using primers

5'CGAAGATCTCAACTTGCCCAA3' and 5'TCCATCTTCTCATCTTCCTCC3'.

Using the Primer Blast tool of the National Center for Biotechnology Information (NCBI), I designed PCR primers to amplify probes for putative target genes of CHRAC17 knockdown from genomic DNA template. I used the BLAST tool of NCBI to rule out cross-reactions of probes with extraneous *Xenopus* coding sequences.

The primer sequences are as follows:

*BMP7*: 5'GTAGACTCAAGAACCATCTGGG3' and 5'CCCAGTGATTACCAGTTGC 3'

*CXCL12*: *5'*TCAATAGGGAGGGGCACAAG3' and 3'ATTGGCACAGGGGCTCTAAT-5'

*DAB2*: 5'AACATCCTCAGGTCCTGCTC3' and 5'TTTGTCTGCAGGTCTGTCCA3'

*FOXP1*: 5'ATCTACAACTGGTTCACACGG3' and 5'TCCATACTGCCCCTTTTACG3'

*FOX03*: 5' GGATTGGGTGGAATATCTGGG 3' and 5' TCGCTCTGGGTTTTAGTTGG 3'

*H1F0*: 5' CGTCCGGGAAGGTGCTG 3' and 3' AATTTGTGAGCAGAGCAGGC5'

*HOXA13*: 5' CAGCCTGGAGGAGATGAACA3' and 3' AGAAACCACGGGCATATCCA5'

*OTX2*: 5'TCGCTGCAACGATTTCTTCC3' and 5'TCCCTTGGCTGTACCCTGATG3'

*POLK*: 5'ACATTGACTGCAAGTGCTGG3' and 5'CCTTGAGCCCTTCCTTCTGT3'

*RAX3*: 5' TCAGAATGCTCACGACTTTGA3' and 5'GGAAGCAAACCAAGCCTATTTG3'

*SMARCA1*: 5' TCATGCCTCAGTTGTCTTACC3' and 5' AACCTCCATTGTATCGCCC3'

*SIP1*: 5' ATTGTTAGTCGGATGAGCCAG 3' and 5' CAGGCCAGCAAAGCATAAAG 3'

The PCR products were ligated into pGem T plasmid (Promega, Madison, WI) with T4 DNA ligase (Promega)and transformed into Nova Blue™ competent E. coli cells (Novagen, Darmstadt, Germany). To determine the insert's orientation and identity I performed restriction enzyme analysis and obtained sequence data for each clone (Yale DNA Analysis Facility, New Haven, CT).

To produce DNA templates for *in vitro* transcription I linearized the plasmid containing *CHRAC17* sequence with the restriction enzyme SacI for a sense template and SacII for the antisense template. By in vitro transcription of the templates I produced digoxigenin-labeled sense and antisense RNA probes, respectively (Megascript T7™ , Megascript Sp6™, Ambion, Austin, Tx).

For all other genes I linearized the plasmid containing the gene's sequence with the restriction enzyme NcoI or SpeI and by *in vitro* transcription of the templates I produced digoxigenin-labeled sense and antisense RNA probes (Megascript T7™, Megascript Sp6™, Ambion, Austin, Tx).

I generated *Xenopus* embryos as described above. At stage 15 and 28 I collected and fixed whole embryos in MEMFA according to Sive, et al. [4]. I hybridized the whole mount embryos with *in situ* hybridization probes according to Sive, et al., with the following exceptions: I omitted the RNaseA step, I chose the optional overnight monoclonal antibody buffer wash and BM purple color substrate and chose not to clear the embryos with benzylbenzene/benzyl alcohol.

**Microscopy and microphotography**

I observed and photomicrographed sample embryos from each injection using a Leica DFC320 digital camera mounted on a Leica MZFLIII dissecting microscope equipped with an ebq 100 UV light source (Leica Microsystems, Bannockburn, IL). Images were processed using the Leica Applications software installed on a Dell desktop computer running Windows XP. Dr. Jonathan Henry performed sectioning and hematoxylin-eosin staining of embryos unilaterally injected with *CHRAC17* morpholino oligonucleotides (as described in [5]).

**Microinjection of morpholino oligonucleotides:**

I designed morpholino oligonucleotides (MOs) complementary to the 5' untranslated region of *CHD4* or *CHRAC17* messenger RNA flanking the translation start site. I obtained the *CHD4*-specific MO, CHRAC17-specific MO labeled with fluorescein, the inverse sequence MOs, and a standard (negative) control MO that anneals only to a nucleotide sequence found in humans who carry a mutant α-globulin mutation (GeneTools, Philomath, OR). The corresponding sequences are:

*CHD4* MO: GCCGGAGGCCATGCCCAGGAAGGAG

*CHD4* Inverse control MO: GAGGAAGGACCCGTACCGGAGGCCG

*CHRAC1 7*MO: GATCTTCGGGTCTCTCGGCCATTGC (custom oligo with 3' fluorescein *CHRAC17*mo 37-08Dec08A-F)

*CHRAC17* Inverse control MO: 5'CGTTACCGGCTCTCTGGGCTTCTAG

Standard (negative) control MO: 5´CCTCTTACCTCAGTTACAATTTATA3´

I made stock solutions as suggested by Gene Tools by resuspending 300 nmols (2.53 mg) of *CHD4*-specific, *CHRAC17*-specific or Inverse control MOs in 300 µL of water. The resulting concentration is 1 mM (or 8.43 ng/nL). I then diluted the MO stocks in an equal volume of sterile nanopure water, and diluted the resulting solution in an equal volume of water. GeneTools recommends for *Xenopus* oocytes 1-10 nL of a 1mM solution; 10 nL of the dilutions are equivalent to 2.5 nL and 5 nL of a 1 mM solution,

respectively, within the recommended range. To eliminate secondary structure and to remove particulates from the MO solutions I heated them at 65° C for 10 minutes and centrifuged at maximum speed for 10 minutes prior to loading in glass needles. I made glass needles by pulling them to a taper using a Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA).

I obtained and dejellied embryos as described above and transferred them to grooves on an agarose gel flooded with Solution A (below). I injected the immobilized embryos using glass needles calibrated to deliver 5 or 10 nL of MO solutions; needles were mounted on a KITE-L micromanipulator (World Precision Instruments, Sarasota, FL). To deliver MO solution to embryos I applied 30 msec bursts of 8 psi of air pressure to the needles using a MPPI-2 pressure injector (Applied Scientific Instrumentation, Eugene, OR).

After the injections were complete I transferred the embryos to Solution B. At 24 hours post fertilization, I transferred them to Solution C, and changed out Solution C daily thereafter.

At stage 15 and 28 I observed and photographed the gross morphology of the embryos, and collected embryos to fix in MEMFA or extract total RNA and protein from them.

**Whole protein extraction:**

I collected 10-100 embryos and homogenized them by adding homogenization buffer containing protease inhibitors and titurating through a 23g needle twenty times. I centrifuged the homogenates and collected supernatant, twice. I added 10% DTT and incubated at 95 °C for 10 minutes. I stored samples at -20 °C.

I determined concentration of the protein samples by the method of Bradford, using a Beckman Coulter DU530 spectrophotometer. I ran the installed Bradford program and set up a standard curve using a preparation of histone protein. Alternately I measured concentration using the ND1000 Nanodrop spectrophotometer.

**Total RNA extraction:**

I isolated total RNA from embryos injected with *CHRAC17* MO or with standard control MO at various times post fertilization. For each group of injectees I added ten whole embryos to 200 µl Trizol reagent (Invitrogen, Carlsbad, CA). I homogenized them by tituration with 18 to 26 gauge needles, extracted the RNA with chloroform and precipitated the RNA with isopropanol. I dissolved the dried pellets in 100 µl RNase free water.

**RT-qPCR**

To amplify *Otx2* transcripts in whole RNA extracts, I designed primers for RT-PCR as described above in "*in situ* hybridization." The sequences are:

XOTX2FWD: TACAGGCAACATCAGGCTACAGT

XOTX2REV: TTGTCACTGGGTTGGTGCTCATTG

I extracted total RNA as above from embryos at stages 15, 20, 25, 30 and 32. I measured the RNA concentration by ND1000 nanodrop spectrophotometer. I removed genomic DNA from RNA samples using Turbo DNase™ (Applied Biosystems/Ambion, Austin, TX). I performed reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) using the SYBR Green Jumpstart™ Taq Readymix (Sigma-Aldrich, Austin, TX). I programmed a Smart Cycler (Cepheid, Sunnyvale, CA) to anneal at 65 °C and to run 40 PCR cycles.

**Microarray analysis of total RNA:**

I performed whole genome expression analysis on *CHRAC17* MO-injected embryos to identify genes that may be misregulated due to ablation of *CHRAC17* function. I isolated total RNA as above from embryos injected with *CHRAC17* MO or with standard control MO at stage 15 and again at stage 37. I purified the preparations with the RNeasy Mini Kit™ (Qiagen, Hilden, Germany) and resuspended the RNA in RNase free water. I measured the RNA concentration by ND1000 nanodrop spectrophotometer. I adjusted the concentration to 200 ng/μL by adding water.

In partnership with the NIH Microarray Consortium I sent the frozen RNA samples to the Duke University Microarray Facility for hybridization to the Gene Chip *Xenopus laevis* genome 2.0 array (Affymetrix, Santa Clara, CA). The Gene Chip contains

oligonucleotide probes corresponding to 29,900 *Xenopus* mRNAs contained in GenBank databases as of September 2006 and *Xenopus laevis* UniGene build 69 (July 2006). The RNA samples were *in vitro* reverse transcribed to produce cDNA labeled with a fluorophor using the Ambion WT Expression Kit (Affymetrix, Santa Clara, CA). The cDNA was then hybridized to the probes of the Gene Chip; thus, luminosity of a probe was proportional to the expression level of the corresponding gene. After hybridization the luminosity of each probe was quantified using GCOS software (Affymetrix, Santa Clara, CA) to produce probe cell intensity files (.CEL files).

I generated tab delineated probe level summarization (.CHP) files and reports on the quality of the hybridization data from the .CEL files using Gene Console software (Affymetrix, Santa Clara, CA) running the PLIER, RMA and mas5 algorithms. The algorithms make background corrections, normalize the luminosity data and convert the probe level values to probe set expression values. They generate tab delineated files collating the ID of the transcript with the expression value, and in the case of the mas5 algorithm, to a p-value which reflects the probability that the expression value is not the product of artifacts of the microarray method but is a valid representation of the level of expression of the gene in question. I transferred these data to an Excel spreadsheet to calculate the log2 ratios of the luminosities, to represent the expression levels from the CHRAC17 MO-injected embryos divided by those from the standard control MO-injected

embryos. I created a list of all those mRNA species that had been either overexpressed or underexpressed by a factor of two or more for further analysis.

The summarization files are accompanied by summary quality assurance reports reflecting data from control probes on the microarrays and control cDNA's hybridized to them contemporaneously with the experimental hybridizations. These reports indicated that the hybridization of the Gene Chips had produced data of a quality within tolerances.

**Pathway analysis:**

I analyzed the lists of misregulated genes in CHRAC17 morphant embryos at 17 hours post fertilization and 53 hours post fertilization using the Core Analysis of the web-based Ingenuity Pathway Analysis (http://www.ingenuity.com/index.html) site. The analysis uses the Ingenuity® Knowledge Base, annotated by reviewing literature in molecular biology, to identify networks of metabolic or protein/gene interactions in which the list of misregulated genes is overrepresented. It ranks the identified pathways by p-values assigned to it that reflect the likelihood that the overrepresentation is not due to chance. From this it may be inferred that the perturbation of these biological pathways may be responsible for aspects of the CHRAC17 morphant phenotype.

**Western blot:**

I performed Western analysis according to Maniatis [6]. I resolved the whole protein samples extracted from embryos by SDS polyacrylamide gel electrophoresis. I prepared 15% polyacrylamide gels (Amresco, Solon, OH). To 10 µg whole protein samples I added an equal volume of denaturing buffer containing dithiothreitol (Fisher Scientific, Waltham, MA) and heated to 95 °C. I loaded the samples or 10 µg of molecular weight markers to the wells of a 15% polyacrylamide gel and applied voltage to separate the proteins. I blotted the resolved proteins onto a nitrocellulose membrane (Whatman, Dassel, Germany) and stained the blot with Ponceau S to determine the quality of protein and the extent of transfer to the membrane, then rinsed it in RO water and soaked it in Odyssey blocking buffer (Licor, Lincoln, NE).

I probed the membrane with primary antibodies in Odyssey Blocking Buffer with 0.15% Tween 20 at room temperature for two hours or at 4 °C overnight. I washed the blot with large volumes of PBS with 0.1% Tween 20 and incubated it with fluorescent secondary antibodies in Odyssey Blocking Buffer at room temperature for two hours. I visualized the probes by scanning on an Odyssey Infrared Imager (Licor, Lincoln, NE).

The CHRAC17 primary antibody was a generous gift from Dr. Paul Wade. Antibodies to EF1 alpha and nucleolin were obtained from Developmental Studies Hybridoma Bank (University of Iowa, Iowa City, IA).

**Reagents:**

Solution A: 6% Ficoll type 400 in 0.1X MMR, with gentamicin sulfate (20 μg/mL), filter sterilized.

Solution B: 1% Ficoll type 400 in 0.1X MMR, with gentamicin sulfate (20 μg/mL), filter sterilized.

Solution C: 0.1X MMR with gentamicin sulfate (20 μg/mL).

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# CHAPTER 3

## The role of CHRAC17 in *Xenopus* development

### Introduction

The CHRAC chromatin remodeling complex found in eukaryotes from yeast to human catalyzes translation of nucleosomes, promoting regular spacing of nucleosome arrays [1]. It has in common with the ACF remodeling complex the ACF1 protein, and CHRAC can be thought of as an ACF complex with two histone fold proteins added. ACF1 is homologous to the WSTF subunit of the WICH complex, suggesting functional as well as structural relationships between the three complexes.

In *Xenopus* the histone H2B-like subunit of the CHRAC complex is known as CHRAC17, but in other species it is known by various other names. I will describe the CHRAC complexes that have been studied in yeast, *Drosophila, Xenopus,* and mammals (mouse and human), then the non-ISWI containing protein complexes that have been reported to contain CHRAC17.

The structure and functions of CHRAC complexes are highly conserved. The yeast CHRAC homolog, yCHRAC, consists of ISWI (Isw2p), ACF1 (Itc1p) and two histone fold proteins, CHRAC17 (DPB4) and CHRAC15 (DLS1) [2]. The histone fold proteins are homologous to those found in CHRAC complexes of other species, and contain an H2A-like domain and H2B-like domain, respectively; thus they are capable of

heterodimerizing by the histone-like handshake interaction. The CHRAC17 subunit contacts extranucleosomal DNA from 37bp to 53bp away from the nucleosome exit/entry site and remains stationary on the DNA site while the core nucleosome is translocated [3]. Thus it may function to anchor the CHRAC complex to extranucleosomal DNA to facilitate translocation of core nucleosomes. The CHRAC15 subunit was shown to be partially required for normal yCHRAC-dependent chromatin remodeling at a number of gene loci and for yCHRAC-dependent transcriptional repression of some of them [4].

In *Drosophila* the dCHRAC complex contains ISWI, ACF1 and the histone fold proteins CHRAC17 (CHRAC14) and CHRAC15 (CHRAC16). The ACF1 protein structure has been studied extensively in *Drosophila.* It contains a number of conserved domains: from N-terminal to C-terminal, the WAC, DDT, BAZ1, BAZ2, WAKZ domains, two PHD domains, and a bromodomain [5]. Analysis of truncated ACF1 proteins lacking domains illuminates ACF1's roles in remodeling. The N-terminal region including the WAC motif binds DNA [6]; a large domain between ACF1's N-terminal and C-terminal, including the DDT and BAZ motifs, binds ISWI's C-terminal HAND-SANT-SLIDE domain; the PHD domain binds all four histones of nucleosome cores [7]; in general, bromodomains are known to bind acetylated nucleosomes and there's evidence that acetylation of nucleosomes increases remodeling activity of the CHRAC complex[8].

ACF1 embodies the concept that an ATPase subunit in a family of remodeling complexes such as ISWI may perform the same function in all of them, while other subunits modulate that function to impart distinctive activities to each complex. While ISWI alone is capable of ATP-dependent chromatin remodeling, ACF1 greatly enhances ISWI activity. Besides increasing ISWI's remodeling activity, ACF1 changes it qualitatively: *in vitro,* ISWI alone catalyzes the translocation of nucleosomes from the middle of the chromatin substrate toward the ends, while ISWI combined with ACF1 slides nucleosomes in the opposite direction [9].

As in other species the *Drosophila* homologs of the CHRAC17 and CHRAC15 subunits bind each other via their histone fold motifs. X-ray crystallography shows that the heterodimer interacts with ISWI and ACF1's N-terminus, and CHRAC17 coprecipitates with CHRAC activity in a nucleosome sliding assay [10-12]. The surface of the heterodimer that faces the DNA is basic and it binds DNA at low affinity. Neither the N-terminal of CHRAC17 nor that of CHRAC15 is necessary for DNA binding of the heterodimer, but deletion of the C-terminus of CHRAC17 reduces DNA binding with concomitant loss of nucleosome sliding activity while deletion of CHRAC15's C-terminus increases DNA binding. The finding that dynamic, low affinity DNA binding by CHRAC17/15 facilitates chromatin remodeling by CHRAC suggests that the heterodimer may function as a DNA chaperone analogously to the activity of the high mobility group protein, HMGB1, which nonspecifically binds and bends chromosomal DNA.

ACF1 has specific regulatory roles in *Drosophila* development and differentiation. Western analysis and whole-mount immunofluorescence microscopy reveals that it is abundant during the first six hours of development when cell differentiation is not complete [13]. In contrast, 18 hours after egg laying ACF1 is undetectable in differentiated tissues, but remains at high levels in undifferentiated nervous tissue and in primordial germ cells. Unlike ISWI, ACF1 is not found in differentiated neurons.

The loss of ACF1 in embryos diminishes the periodicity of chromatin and reduces the distance between adjacent nucleosomes [14]. Concomitantly, heterochromatic genes and those regulated by the polycomb group proteins are derepressed, while the cell cycle is accelerated through S phase. When ACF1 was ectopically expressed in eye imaginal discs abnormal morphology of photoreceptor cells resulted [13]. The effect seems to be due to the loss of synchronized progression into the S phase of the cell cycle by which cells differentiate and form the regular clusters of photoreceptor cells characteristic of the compound eye. Ablating ACF1 disrupts heterochromatin formation in early embryos and larvae. The implication is that the ACF1-containing complexes are required for appropriate euchromatin and heterochromatin assembly in early development.

ISWI and ACF1 have roles in Wingless signaling. In cultured *Drosophila* cells ISWI and ACF1 knockdown derepressed specific target genes of Wingless signaling [15]. Loss of ISWI function in wing imaginal discs dramatically increased their expression of WG

target genes. Loss of ACF1 function did not derepress the genes, and while ISWI knockdown embryos died by mid pupal stage, ACF1 loss of function didn't affect viability or fertility of affected flies. Interestingly, the combination of ACF1 loss of function and ISWI loss of function produced more severe phenotypes than ISWI knockdown alone, suggesting that ACF1 may have roles in development independent of ISWI. ChIP analysis revealed that the transcriptional activator TCF, which directly binds target promoters in WG signaling, requires ISWI and ACF1 for binding. Conversely, WG signaling reduces the amount of ACF1 bound to target promoters. One interpretation would be that ISWI and ACF1 repress WG targets in the absence of signaling, then switch to a role that facilitates TCF binding in response to WG signaling.

Less is known of the roles of CHRAC17 in *Drosophila* development. It is expressed in early embryogenesis in *Drosophila,* but is abruptly downregulated 6 to 12 hours into development, suggesting a requirement for CHRAC at early stages, possibly in the rapid nuclear divisions of those stages. There is indirect evidence that CHRAC17 may function with ISWI to promote normal eye development in *Drosophila* [11].

The subunits of the xCHRAC complex of *Xenopus* include ISWI, ACF1, p70 and p55 proteins, and CHRAC17. While a second histone fold protein subunit has not been characterized in *Xenopus* CHRAC, its existence is suggested by the presence in other species of a functionally important histone fold heterodimer in the CHRAC complex, and by the fact that in nucleosome sliding assays the CHRAC17/CHRAC15 heterodimer, but

not the monomers, enhance nucleosome sliding activity of the hACF1-ISWI complex[10]. Finally, in human cell lines importin13 mediates the nuclear import of the CHRAC17/CHRAC15 heterodimer, but not of the monomers [16].

HuCHRAC, the human CHRAC complex contains the subunits ISWI (SNF2H), ACF1 and the histone fold proteins CHRAC17 (Pole3) and CHRAC15 (Pole4) [17]. The ACF1 subunit contains the same domain structure as those of other species. Studies in human cells suggest that ACF1 plays a role in DNA replication through heterochromatin [18]. ACF1 and SNF2H colocalize to pericentric heterochromatin at that stage in the cell cycle (late S phase) when heterochromatin is replicated. Loss of ACF1 function impairs replication in late S phase and the impairment appears to result from the very compact nature of heterochromatin in those cells. Targeted mutation of ACF1's BAZ domain demonstrates that ACF1 must bind ISWI to facilitate replication through heterochromatin.

A survey of various human tissues showed that CHRAC17 and CHRAC 15 mRNAs are expressed in all of them, and the ratio of the two mRNAs is constant across the tissues [17]. Studies in mouse fibroblasts further suggest a function of CHRAC in DNA replication, in that expression of *CHRAC17 (Pole3)* was found to be coupled to the cell cycle, peaking in the start of S phase after serum stimulation. Activation of the gene is mediated by the binding of E2F and MYC to a bidirectional promoter that drives CHRAC17 and another gene of unknown function [19].

*CHRAC17 and non-ISWI-containing protein complexes*

CHRAC17 also functions as a subunit of the ATAC (Ada Two A Containing) complex and of DNA polymerase ε. I will first describe the structure and function of ATAC and then address polymerase ε.

ATAC was first described in *Drosophila* [20]. It belongs to a large family of protein complexes that all contain relatives of the HAT (histone acetyltransferase) protein GCN5 (General Control Nonrepressed protein 5). They include the yeast complex Ada (alteration/deficiency in activation, yADA), SAGA (SPT3-TAF-GCN5 Acetylase)-type complexes and ATAC (ADA2A Containing Complex)-type complexes. The complexes are found in all eukaryotes studied and despite a wide assortment of subunit compositions, similarities among them can be discerned.

All GCN5/PCAF-containing complexes contain a HAT subunit that functions to catalyze the acetylation of histones on specific lysine residues. GCN5 is found as a subunit in all species, while in humans there are two paralogous HATs, GCN5 and PCAF, and they are alternative subunits of the ATAC complex. GCN5 co-regulates gene activity by acetylating lysines in the tails of target histones (reviewed in [21]). *In vitro* it acetylates free histone H3 but not assembled nucleosomes. Just as ISWI's activity is modulated by subunits of ISWI complexes, GCN5 in association with subunits of two yeast protein complexes (Ada and SAGA) acetylates a broader spectrum of histone H3 lysine residues than GCN5 alone [22]. As well, the profile of H3 lysine residues

acetylated by the ADA complex differs from that of the SAGA complex. Similarly, the SAGA and ATAC complexes in *Drosophila* regulate different sets of genes [23]. ATAC but not SAGA localize to TPA-induced transcription sites on polytene chromosomes, and in HeLa cells knockdown of ATAC, but not of SAGA, leads to defective TPA-induced gene expression.

ATAC was discovered because an *in silico* search for *Drosophila* homologs to components of the GCN5/PCAF containing complexes revealed that there are two different ADA2 homologs in *Drosophila*, ADA2A and ADA2B [20]. Both can be found in complexes that contain such common subunits as GCN5 and ADA3, but ADA2A is not associated with the same assortment of subunits that ADA2B is known to bind. Instead ADA2A is a component of the ATAC (Ada Two A Containing) complex. The *Ada2a* gene has an interesting regulatory region. Like CHRAC17 in mammals, ADA2A in *Drosophila* is the product of a bicistronic gene; its promoter overlaps that of *Dtl,* which is also a transcription coactivator [24]. *Ada2a* also codes for RPB4 via alternative splicing; RPB4 is a subunit of RNA polymerase II[25]. Thus, three proteins involved in transcription regulation are under the control of a bicistronic promoter. As suggestive as this may be, no functional relationship between the proteins has been established [26]. ADA2 and ADA3 are known to mediate recognition and acetylation by GCN5 of the N-terminal tails of nucleosomal H3 and H4 [27].

All GCN5/PCAF containing complexes except yADA contain homologs of Spt proteins including YEATS2. The YEATS2 subunit is a scaffold subunit that forms a YEATS2-NC2β histone fold module analogous to that of the POLE3-POLE4 heterodimer[28]. All but the yADA complex also contain numerous TATA binding protein (TBP)-associated factors (Tafs).

Orthologs of CHRAC17 and a histone-fold binding partner are found in human ATAC complexes (POLE3 and POLE4) and in *Drosophila* an ortholog to CHRAC14 (CHRAC14), but not a histone-fold binding partner, is known. A study of human ATAC purified by anti-flag YEATS2 immunoprecipitation identified CHRAC17 (POLE3) and CHRAC15 (POLE4) as subunits [28], but this was not confirmed in a study of human ATAC purified by anti-hAda2a immunoprecipitation [23]. Affinity purification of mouse ATAC followed by mass spectroscopy did not identify a CHRAC17 homolog among the subunits [29]. Clearly the adumbration of ATAC's subunit composition is a work in progress. Both CHRAC17 and Nc2β have been reported to form homodimers and CHRAC17 alone is able to efficiently enhance nucleosome-sliding by the ATP-dependent chromatin remodeling complex SWI-SNF [30]. The mechanics of the CHRAC17/POLE4 heterodimer have not been studied in great detail as in the CHRAC17/15 heterodimer of the CHRAC complex; it would be interesting to compare these two closely related yet functionally distinct modules.

While there is not abundant literature characterizing a role for ATAC in development, inferences can be made from what is known of its subunits. For instance, CSRP2BP (SPT3) has been shown in yeast to directly interact with the TBP to regulate its binding to certain promoters [31]. In mammalian cells deficient for CSRP2BP the ATAC complex is destabilized, apoptosis is increased and histone acetylation reduced. CSRP2BP-deficient mouse embryos have delayed development, possibly due to the increased apoptosis they displayed. The YEATS2-NC2β module of ATAC was also shown to bind the TBP and negatively regulate transcription [28]. As well, mass spectrometry and Western analysis of nuclear extracts from cultured human cells determined that ATAC physically interacts with MAP3K7/TAK1, a kinase that is activated by TGFβ and bone morphogenetic protein signaling and that translocates to the nucleus during Wnt-1 signaling. Thus, ATAC may be regulated by extracellular signaling.

The paralogous alternative HAT subunits of ATAC in humans, GCN5 and PCAF, confer distinctive functions upon the complex ([32-34]). GCN5 expression begins by day 8 of gestation while PCAF expression begins on day 12. GCN5 null mutant mice fail to develop mesodermal lineages due to increased apoptosis and die between day 9.5 and 11 of gestation; in contrast, PCAF null mutants have no phenotype. Embryos mutant for both GCN5 and PCAF have a more severe phenotype, suggesting that the paralogs have overlapping roles in development [32].

Several lines of evidence suggest that PCAF has a role in skeletal myogenesis. An *in vitro* transcription system based on an array of core histones and a reporter construct bearing the recognition sequence of the myogenic transcription factor MyoD revealed that PCAF and another acetyltransferase, p300, are required for optimal MyoD-dependent activation of transcription [35]. *In vitro* MyoD and PCAF interact directly and in cultured fibroblasts viral proteins known to disrupt the interactions of MyoD and PCAF repressed muscle differentiation [36]. Exogenous expression of PCAF promotes MyoD-dependent activation of transcription and muscle differentiation, while inactivation of PCAF prevents muscle differentiation. In the interplay between MyoD, PCAF and p300, PCAF acetylates three conserved lysines on MyoD itself, increasing the transcription factor's avidity for its cognate DNA [37]. Mutating the lysines so they cannot be acetylated interferes with MyoD's ability to transactivate myogenic genes and to promote conversion of cultured fibroblasts into muscle cells. In cultured murine myoblasts that lack expression of insulin-like growth factor II and consequently undergo apoptosis in medium lacking growth factors, PCAF can promote survival in the absence of the growth factors [38].

The function of ADA2-like subunits of ATAC and other GCN5-containing complexes have been the focus of many studies. In yeast the ADA2 subunit of the yADA complex enhances GCN5's catalytic activity and the binding of the GCN5/ADA2 complex to the histone substrate [39], a role mediated by the SANT domain of ADA2 [40]. Like

the paralogs GCN5 and PCAF, the two ADA2 paralogs are alternative subunits of the SAGA and ATAC complexes, respectively, and they confer upon their respective complexes distinct functions. In *Drosophila,* mutations in Ada2B, a component of the GCN5-containing complex SAGA, reduce acetylation of histone H3 lysines K5 and K12, but not of histone H4 residues[41]. In contrast, Ada2A mutations don't affect histone H3 acetylation[27], and there is evidence that acetylation of histone H4 is reduced in the mutants, although an earlier study found to the contrary ([41]). Ada2A and Ada2B have different functions in *Drosophila* development [41]; null mutations of either are lethal and impede cell proliferation, but they have differential effects on eye differentiation.

There is evidence that ATAC interacts genetically with ISWI-containing complexes. In *Drosophila* mutant for ISWI binding by Ada2A to polytene chromosomes is reduced, while Ada2B binding is unaffected [42]. Consistent with this, acetylation of H4 lysine K12 is reduced on polytene chromosomes in flies mutant for ISWI or for a subunit of the ISWI-containing chromatin remodeling complex NURF. Under the same conditions acetylation of histone H3 lysines K9 and K14, mediated by SAGA rather than ATAC, is unaffected. Thus, the NURF complex is required for recruitment of ATAC to polytene chromosomes and for the global acetylation of H4. Flies homozygous for mutations in a NURF subunit, Gcn5, or in Ada2a were studied by whole genome expression analysis. It found a significant correlation between downregulated genes between the NURF301 mutants and the *Gcn5* or *Ada2a* mutants.

In contrast to the ATAC complex, DNA polymerase epsilon (Pol ε) has been studied for some time. Among others it has been described in yeast [43, 44], *Drosophila* [45], *Xenopus* [46, 47], and HeLa cells [48]. Pol ε is a holoenzyme of four subunits including the catalytic (cdc20+) subunit, Pole2, CHRAC17 (Pole3) and Pole4. The cdc20+ subunit contains in its N-terminal region the catalytic machinery responsible for its polymerase activity, while the C-terminal contains nonessential elements of unknown function. In yeast, cdc20+ and the second largest subunit, Pole2 (DPB2) are essential, while the CHRAC17 and Pole4 subunits, Dpb4 and Dpb3, are nonessential [44, 49]. In yeast Dpb4 mutants the Polε complex is less stable, suggesting that Dpb4 and Dpb3 function to provide a surface for protein interactions for Polε [50]. The CHRAC17 homologs have been described as subunits of Pol ε in fission yeast [51], *Saccharomyces* [43, 52], *Drosophila* [45], and *Homo* [53].

Pol ε has attracted interest because it appears to combine the function of DNA replication with that of non-replicative functions such as DNA "damage sensing" and repair, maintenance of chromatin structures and regulation of transcription. It is one of three DNA polymerases needed for replication in eukaryotes; the other two are DNA polymerase alpha (Pol α) and DNA polymerase delta (Pol δ). Pol α is known to provide the RNA primer to initiate chromosomal DNA synthesis, but its low processivity and lack of proofreading function (intrinsic 3' exonuclease activity) make it ill suited to the elongation step of DNA replication. Rather, Pol δ takes over replicating the lagging

strand while Pol ε replicates the leading strand (reviewed in [52, 54-56]). Pol δ and Pol ε have proofreading function and high processivity, and Pol ε is further distinguished from the other two by not requiring the proliferating cell nuclear antigen (PCNA) for its high processivity [45]. In *Xenopus* egg extracts it was demonstrated that expression of the four subunits of Pol ε was sufficient for rapid and efficient replication of chromosomal DNA [46, 47].

Numerous studies have established a role for Pol ε in DNA repair. In yeast it is required for activation of S phase checkpoint to prevent mitosis and induction of DNA damage-response genes [57]. The function was localized to the C terminal region which has a structure unique among the DNA polymerases. In nuclear extracts from yeast mutant for Pol ε, base excision repair of DNA was defective, and Pol α and Pol δ modulate the repair activity mediated by Pol ε [58]. Yeast double mutants defective for both Pol δ and Pol ε, but not single mutants, were defective in DNA repair after UV-induced damage [59]. In PCNA-depleted human cell extracts, which are deficient in UV-damage repair, purified Pol ε can efficiently repair UV-damaged DNA [60]. In calf thymus nuclear extract a large protein complex containing Pol ε was shown to catalyze recombinational repair of double strand gaps and deletions in DNA by gene conversion [61].

Evidence that Pol ε has a role in transcriptional silencing comes from studies in yeast [62]. The mating-type gene at locus HMR is silenced because the locus is flanked

by binding sites for the proteins Rap1 and Abf1, and for an origin recognition complex. The proteins affect silencing of the gene by recruiting proteins that form a chromatin structure inaccessible to transcription machinery. Yeast strains were constructed that had point mutations in the locus that ablated the silencing of the gene. But silencing was restored in these strains when Pol ε or PCNA were also mutated. One interpretation is that silencing is interrupted by DNA replication through the silenced chromatin so PCNA and Pol ε may play a role in restoring silencing after the replication fork has passed through the locus.

Finally, there is evidence that Pol ε has a role in sister chromatid cohesion [63]. In yeast mutants with inactive Pol ε there is defective sister chromatid cohesion, and it was shown that Pol ε binds DNA polylmerase sigma (Pol σ), which is involved in sister chromatid cohesion.

In *Xenopus,* three ISWI-containing remodeling complexes have been characterized: ACF , CHRAC and WICH (reviewed in [64]). The modular nature of the complexes suggests that each may have distinct roles in development. The knowledge gleaned from ablation of ISWI function does not distinguish the roles of one ISWI-containing complex from another because ISWI knockdown ablates all of those functions. To help parse out the roles in development of individual ISWI-containing remodeling complexes, I determined to investigate the developmental roles of the CHRAC complex. I hypothesized that the CHRAC complex's role in development would

be distinct from that of the three ISWI-containing complexes combined. To test this, I first studied the expression pattern of CHRAC17 and compared and contrasted it with the pattern of ISWI expression. I then studied the loss of function phenotype of CHRAC17, a protein not found in the ACF or WICH complexes, but present in the CHRAC complex. I knocked down CHRAC17 function in developing embryos using morpholino oligonucleotides and compared and contrasted the loss of function phenotype in the "morphants" with the phenotype resulting from loss of function of ISWI. To study the developmental consequences of CHRAC17 loss of function at the molecular level, I measured the level of expression of putative target genes that might be misregulated in CHRAC17 morphants, using qRT-PCR and whole genome microarray analysis. To identify possible alterations in cellular function in knockdown embryos I performed pathway analysis of the misregulated genes. Finally I visualized the expression patterns of misregulated genes in affected embryos.

I reasoned that if I were to find no differences between ISWI and CHRAC17 in these respects it would suggest that the CHRAC remodeling complex has roles in development that are equivalent or redundant to one or more of the other ISWI-containing complexes found in *Xenopus*. But if I were to find that CHRAC17's expression pattern is different from that of ISWI and that CHRAC17 loss of function leads to only a subset of the developmental defects caused by loss of function of ISWI, the hypothesis that the CHRAC complex's role in development is distinct from that of the three ISWI-

containing complexes combined would be supported. It is likely that the phenotypic differences derive from the fact that both these proteins may be members of other complexes in addition to CHRAC: CHRAC17 is a subunit of the ATAC and DNA polymerase epsilon complexes, while ISWI may also be a subunit of other complexes.

**Results:**

*CHRAC17 is expressed in a distinct spatiotemporal pattern over the course of Xenopus development.*

The expression of a gene in a particular region of a developing embryo suggests that the gene product may be active in a developmental process in that region. I reasoned that where the pattern of expression of *CHRAC17* and that of *ISWI* overlap in whole embryos, the CHRAC remodeling complex will be present and active. Where CHRAC17 is expressed but ISWI is not, it is likely that CHRAC17 is functioning in the ATAC complex and/or Pol ε. Accordingly I visualized *CHRAC17* mRNA expression in *Xenopus* embryos by whole mount *in situ* hybridization. This revealed that *CHRAC17* is expressed as early as stage 15 in *Xenopus* and persists at least as late as stage 43 (Fig. 1). At stage 15 expression is localized to a region parallel to the neural plate, possibly the medial edge of the presomitic mesoderm, and near the presumptive forebrain or eye region (Fig. 2). The posterior-lateral regions of the embryo exhibit diffuse signal; while the signal is faint, it was detected in multiple *in situ* hybridization experiments and likely

represents genuine expression in this region. Sectioning of embryos followed by *in situ* hybridization will be required to confirm the findings on whole embryos.

In stage 28 embryos *CHRAC17* expression is prominent in the brain and facial structures, the region of the olfactory placode, in pharyngeal arches or laterally-situated placodes, in myotomes, and the anterior-ventral region. A band of intense staining along the antior-posterior axis may be notochord; again, *in situ* hybridization of sectioned embryos would be needed to confirm (Fig. 3). An embryo that was fixed at stage 37 and was partially cleared of yolky material after *in situ* hybridization exhibited the fine detail of *CHRAC17* expression in the olfactory, lens, otic, facial epibranchial placodes and possibly in the anterior-ventral lateral line placode, and in narrow stripes running dorso-ventrally in the myotomes (data not shown). A dorsal view of embryos at this stage of development underscores the intense expression levels anterior and lateral to the forebrain and other localized regions of the neural tube (Fig. 4).

This pattern contrasts with that of *ISWI*. *ISWI* is expressed in neural structures but not in myotomes, as *CHRAC17* is, and while *CHRAC17* is prominently expressed in the placodes listed above, there is not a comparable expression in ISWI [65].

At stages 41 and 45 *CHRAC17* expression is more widespread and diffuse (Fig. 1). At these stages it appears that *CHRAC17* is expressed in the optic cup but is no longer expressed in the lens, both of which are completely invaginated at these stages.


**Figure 1** *In situ* hybridization of staged *Xenopus* embryos. Embryos were probed with control probe (left) or *CHRAC17*-specific probes (right). From top to bottom, the embryos were fixed at stages 15, 22, 27, 39, and 43. *CHRAC17* expression begins as early as stage 15 and persists beyond stage 43. By stage 27 expression is most prominent in the head and neural structures and in myotomes. In later stages CHRAC17 expression is very widespread. Abbreviations: ov, optic vesicle, cp, cranial placodes, oc, optic cup.

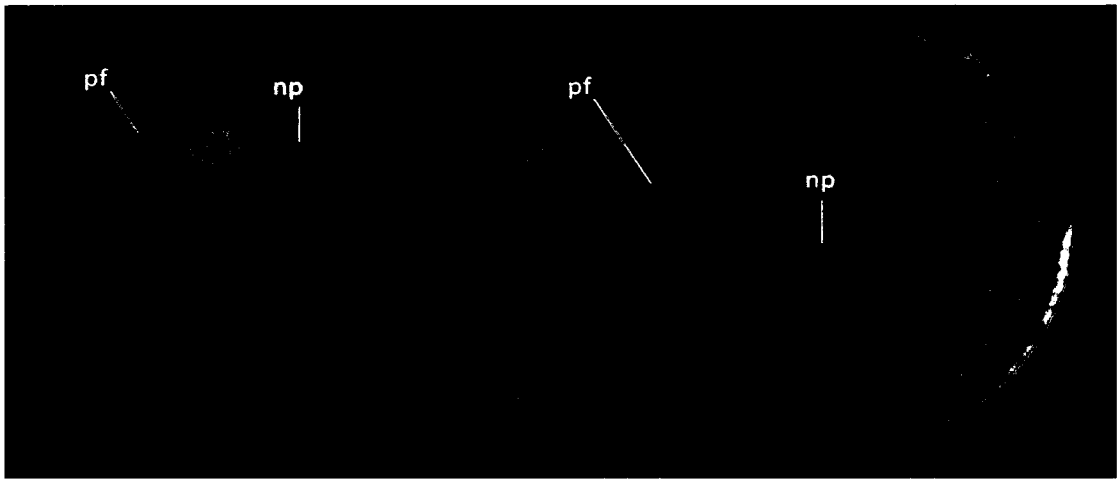


**Figure 2** *In situ* hybridization of stage 15 embryos. Embryos were probed with control probe (left) or *CHRAC17*-specific probe (right). The *CHRAC17* expression is at the margins of the neural folds, the eye primordia, and in the posterior region. Abbreviations: np, neural plate; pf, presumptive forebrain.

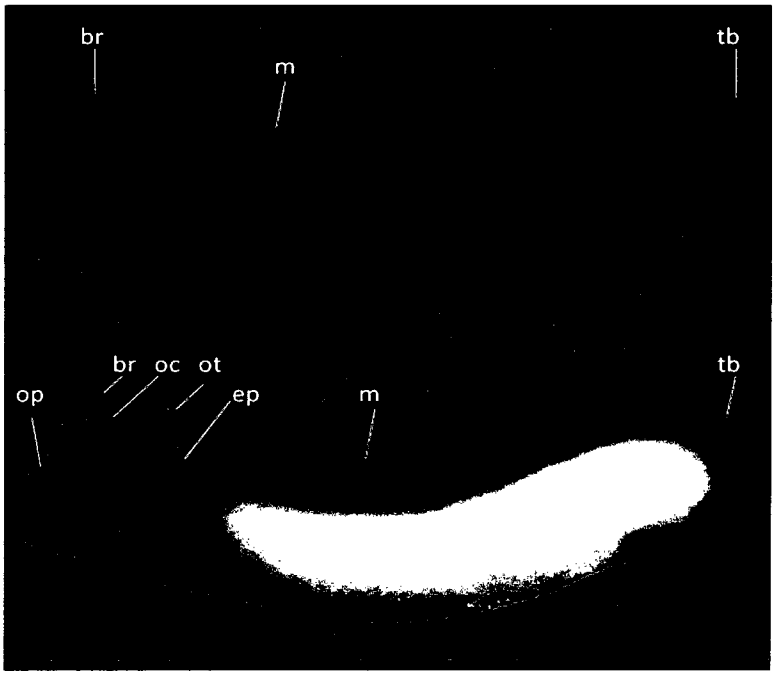


**Figure 3** *In situ* hybridization of stage 28 embryos. Embryos were probed with control (top) or *CHRAC17*-specific probe (bottom). Staining is present in the brain and in olfactory, lens, epibranchial and otic placodes, in myotomes and the anterior-ventral region. Diffuse signal is seen in the tailbud and in the posterior-ventral region. Abbreviations: op, olfactory placode; br, brain; oc, optic cup; ot, otic vesicle, ep, epibranchial placode; m, myotome; tb, taiblud.


**Figure 4** Dorsal view of stage 28 embryos. Embryos were probed with control (top) or CHRAC17-specific probe (bottom). Note the prominent staining anterior and lateral to the brain (br).

*Ablation of CHRAC17 function by morpholino oligonucleotides results in a distinct morphant phenotype.*

In order to determine the developmental roles of CHRAC17 I injected *Xenopus* embryos with *CHRAC17*-specific morpholino oligonucleotides (MOs). I designed the MOs to anneal with the region of *CHRAC17* mRNA that spans the translation start site, thus preventing translation of CHRAC17 protein in injected embryos. As a control for nonspecific MO effects on development, I injected embryos from the same clutch of eggs, fertilized with the same testis, with negative control MOs (see Materials and Methods).

Embryos injected with 84 ng *CHRAC17* MOs had high mortality; the majority were dead by stage 35 (Table 1). All survivors were severely abnormal in morphology. Among embryos injected with 42 ng *CHRAC17* MO there was no observable phenotype

on the gross morphological scale before stage 27. Unlike ISWI knockdowns, >80% of embryos survived beyond gastrula stage on average. At stage 40 a distinct phenotype emerged (Fig. 5). Abnormalities include a reduction of anterior-posterior axis length; to quantify this, the lengths of embryos from their most anterior to their most posterior tips were measured from three experiments (Fig. 8). The average AP length of 106 Inverse control MO injectees was 4.19 mm, while that of 96 *CHRAC17*-specific MO injectees was 3.09 mm, significantly shorter ($p<0.0001$ Student's t-test).

The reduced AP length may be a reflection of the finding above that *CHRAC17* is expressed in the myotomes. As noted above, knockdown of the BPTF subunit of the NURF complex results in axial deformities; the loss of function of the CHRAC complex could analogously lead to abnormal axial development. Knockdown of *CHRAC17* might result in destabilization of the ATAC complex as it destabilizes Pol ε in yeast, leading to developmental defects in myotomes or other mesodermal tissues along the anterior-posterior axis. Knockdown of the ATAC complex subunit Gcn5 in mice leads to increased apoptosis and a failure of mesoderm to develop normally. The alternative subunit, PCAF, has a well defined role in myogenesis. If ATAC function is required for PCAF's myogenic function, development of myotomes may be perturbed in *CHRAC17* knockdown embryos.

Compared to controls, *CHRAC17* morphant embryos show reduced head volumes, particularly in the areas of the forebrain and nasal placode, and possibly including the

ventral eye vesicle. Retinal pigmented epithelium (RPE) was greatly reduced either in density of pigment or size of the RPE, and overall size of the eye vesicle was reduced. Closure of the ventral fissure of the optic vessel is incomplete at this stage, suggesting retarded eye development or coloboma, or a gradient of pigment loss that is greatest anteriorly and ventrally. The edge of the dorsal fin is irregular or serrated compared to controls. Development of the tailbud is delayed or otherwise abnormal in morphants.

Our collaborator Dr. Jonathan Henry (University of Illinois-Urbana) sectioned control and *CHRAC17* morphant embryos collected at stage 41 and stained them with hematoxylin and eosin to study them microscopically. These sections reveal that the eyes develop normally in the control embryos (as expected) but are very poorly developed in the *CHRAC17* morphants (Fig. 6). The eyes of control embryos show normally differentiating retinal layers including a pigmented retinal epithelium lining of the eye, and a layer of differentiating rod and cone cells. The lenses in control embryos also develop normally, with a dense inner sphere of primary fiber cells surrounded by secondary fiber cells, and are fully separated from the overlying ectoderm. In contrast, development of the eyes of *CHRAC17* morphants is very abnormal. The retinas are not normally differentiated and are unlayered. The lenses show no secondary fiber cells and remain attached to the surface ectoderm. In the example shown in Fig. 6, a lens placode remains in the surface ectoderm, or alternatively a small lens body is present that contains some or no internal primary cells.

Histology also reveals abnormal neural tube and notochord development at stage 41 in *CHRAC17* morphants (Fig. 7). The tissues of the neural tube are poorly differentiated and the notochord is defective in shape and in its relative position to the neural tube.

I classified 1300 embryos injected with *CHRAC17*-specific MOs or with control MOs as normal or abnormal using criteria based on the gross morphology of eyes and head and on length of the anterior-posterior axis. I compared the number of normal and abnormal embryos injected with control MO or CHRAC17-specific MO from eight independent experiments (Table 1, Fig. 9). The average percentage of abnormal embryos among control embryos is 16.9% and ranges from 7.8% to 28.6%, and includes an array of nonspecific defects. Among *CHRAC17* morphants the average percentage of abnormal embryos is 70.1% and ranges from 53.8% to 90.0% and these defects are highly uniform and reproducible. Embryos injected with 21 ng of *CHRAC17*-specific MOs immediately after fertilization exhibit a milder phenotype than those injected with 42 ng MOs. This dosage effect is also consistent with the phenotype being caused specifically by the activity of the *CHRAC17* MO.

**Table 1** The frequency of abnormal development in MO-injected embryos

| **Injectate** | **Amount injected** | **Total # of embryos** | **# of abnormal* embryos** | **% of abnormal* embryos** |
|---|---|---|---|---|
| **Uninjected** | | 1111 | 75 | 7% |
| **INV MO** | 42 ng | 851 | 150 | 18% |
| | 84 ng | 100 | 5 | 5% |
| ***CHRAC17* MO** | 21 ng | 119 | 41 | 34% |
| | 42 ng | 856 | 571 | 68% |
| | 84 ng | 349 | 298 | 85% |

*The morphant phenotype includes abnormal eye development combined with abnormal anterior-posterior axis.

After stage 28 normal embryos begin spontaneous movement and later become responsive to tactile stimulation. Interestingly, the *CHRAC17* knockdown embryos remained inert and nonresponsive as late as stage 45. This is not due merely to delayed behavioral development, because days later knockdown embryos are still nonresponsive to tactile simulation. Determination of eye function depends on functional motility in embryos (i.e., testing whether embryos swim from shadows or other obstacles) so I was unable to establish whether vision is normal or abnormal in knockdown embryos. However, given the lack of eye differentiation observed in the morphants (see below), it is clear that these embryos must be entirely blind.

In *Xenopus* each cell of a two-cell stage embryo gives rise to either the right-hand side or the left-hand side of the later embryo. I wanted to determine whether injecting one of the two cells with *CHRAC17*-specific MOs would result in an embryo with normal morphology on the uninjected side and a morphant phenotype on the side that had received the *CHRAC17* MO. This method provides morphological features on the

uninjected side that can be compared to features on the side in which CHRAC17 is ablated. There are some caveats to this technique; for example, midline structures can sometimes still form as a result of signaling from the uninjected side of the embryo; however, the normal side generally serves as a very powerful internal control for the effects of the MOs.

Embryos injected unilaterally with *CHRAC17*-specific MO at the two cell stage are frequently crescent-shaped and the side of the embryo with ablated *CHRAC17* function, identified by fluorescence of the fluorescein tag on the MO, typically form the inner edge of the crescent (Fig. 10). This is consistent with the AP-axis shortening observed in global knockdowns performed at the one-cell stage (Fig. 5).

On the gross morphological level the phenotype of unilaterally injected embryos appears mild compared to that of globally injected embryos, but at the histological scale developmental defects are obvious in these embryos (Fig. 11). Embryos fixed at stage 41 were sectioned and stained as above by Dr. Jonathan Henry. These embryos exhibit developmental abnormalities of the eye, neural tube and notochord on the affected side, but not on the contralateral side (Fig. 12). In some cases there also appears to be abnormal development of mesodermal tissue.

Interestingly, the lack of response to stimulation observed in embryos in which CHRAC17 was bilaterally ablated was not present in unilaterally injected embryos. To the contrary, many of the latter are hyperactive, many displaying what could be

described as convulsive activity. Such embryos convulse upon tactile stimulation and continue the activity long after the usual response would have abated. Seemingly, the activity is initiated spontaneously as well.

In summary, CRAC17 knockdown embryos exhibit a phenotype that overlaps with but is distinct from ISWI knockdown embryos. Complete ISWI knockdown results in death at neurulation, while 80% of CHRAC17 knockdown embryos survive to stage 45. This is consistent with the corresponding expression patterns of these genes as determined by *in situ* hybridization. The extent to which the differences reflect differences between the CHRAC complex's developmental functions and those of other ISWI-containing complexes will require further study.


**Figure 5** The *CHRAC17* morphant phenotype in *Xenopus*. At stage 41, compared to the control MO injectee (top) the *CHRAC17* MO injectee (bottom) demonstrates the characteristic developmental abnormalities in head structures, eyes, proctodeum and anterior-posterior axis.

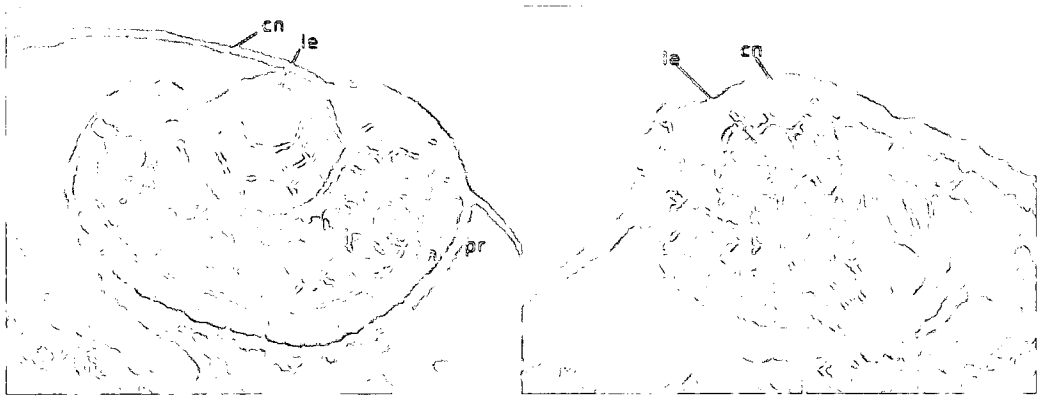


Figure 6 Cross sections of eyes of MO-injected embryos. Stage 41 embryos were injected with negative control MOs (left) or *CHRAC17* specific MOs (right). In the control embryo, the retina contains normal differentiating layers of tissue including the darkly-stained pigmented retinal epithelium encapsulating the eye and, internal to that, the differentiating rod and cone cells. The lens, which is normally separated from the overlying ectoderm at this stage, contains a dense inner sphere of primary fiber cells surrounded by secondary fiber cells. The *CHRAC17* morphant embryo (right) displays very abnormal eye development; the lens has no secondary fiber cells and is attached to the surface ectoderm. The retina lacks the normal layers of differentiating cells. Abbreviations: cn, cornea; gn, ganglion layer; in, inner nuclear layer; ip, inner plexiform layer; ln, lens; on, outer nuclear layer; op, outer plexiform layer; pr, pigmented retinal epithelium.

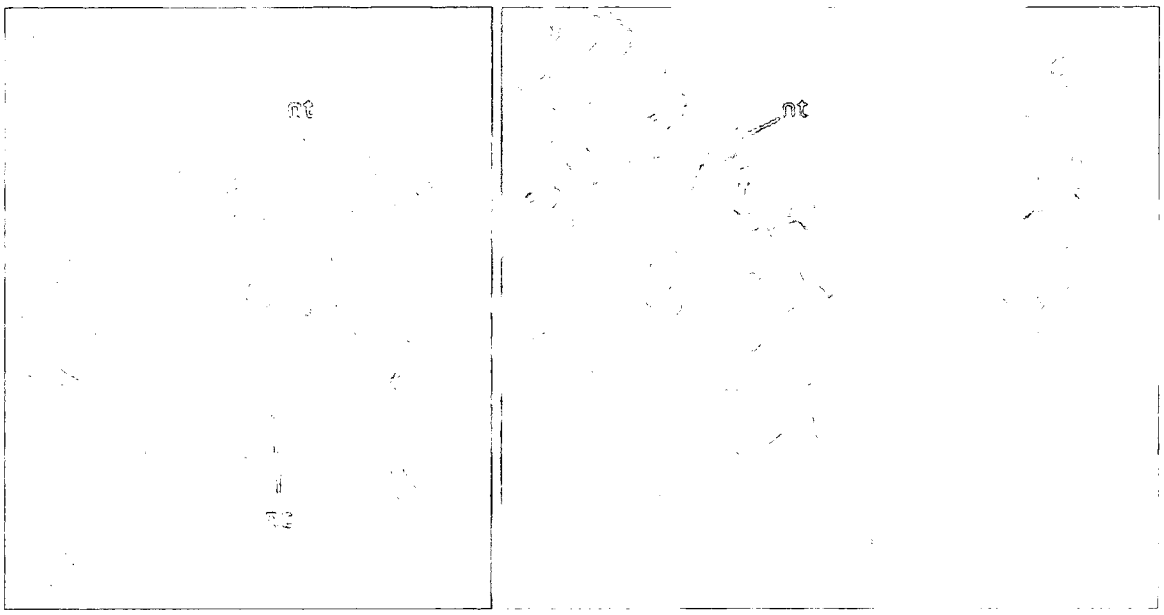


Figure 7 Cross sections of neural tubes of MO-inected embryos. At stage 41 the control embryo (left) exhibits normal development of neural tube (top) and notochord (bottom center). The morphant embryo (right) exhibits defective neural tube (top) and notochord (bottom center). Abbreviations: nt, neural tube, nc, notochord.

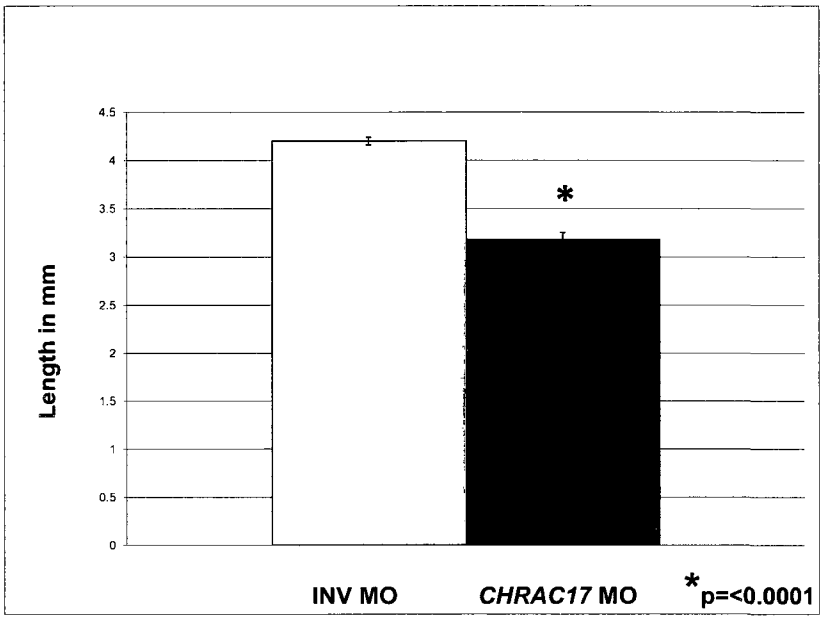


**Figure 8** The average AP-axis length of MO-injected embryos. Morphants are significantly shorter on average than those injected with negative control (INV) MOs.

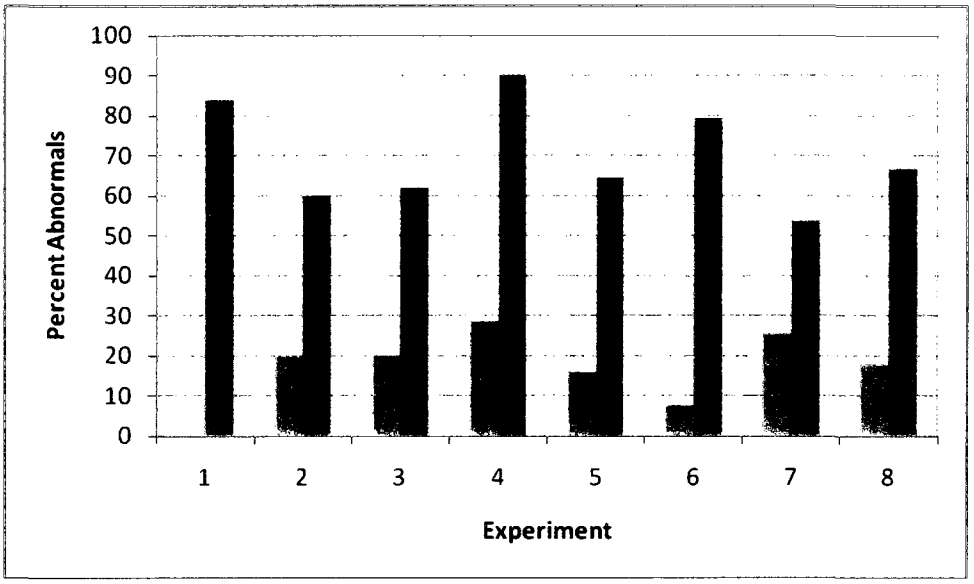


**Figure 9** Numbers of normal vs. abnormal embryos. Stage 37 embryos from eight independent experiments injected with control MO or with *CHRAC17* specific MO were sorted into normal and abnormal groups based on the gross morphology of the eye, face and anterior-posterior axis. For each experiment , the percent of abnormal and normal embryos is shown for the control embryos (blue bars) and the *CHRAC17* morphant embryos (red bars).

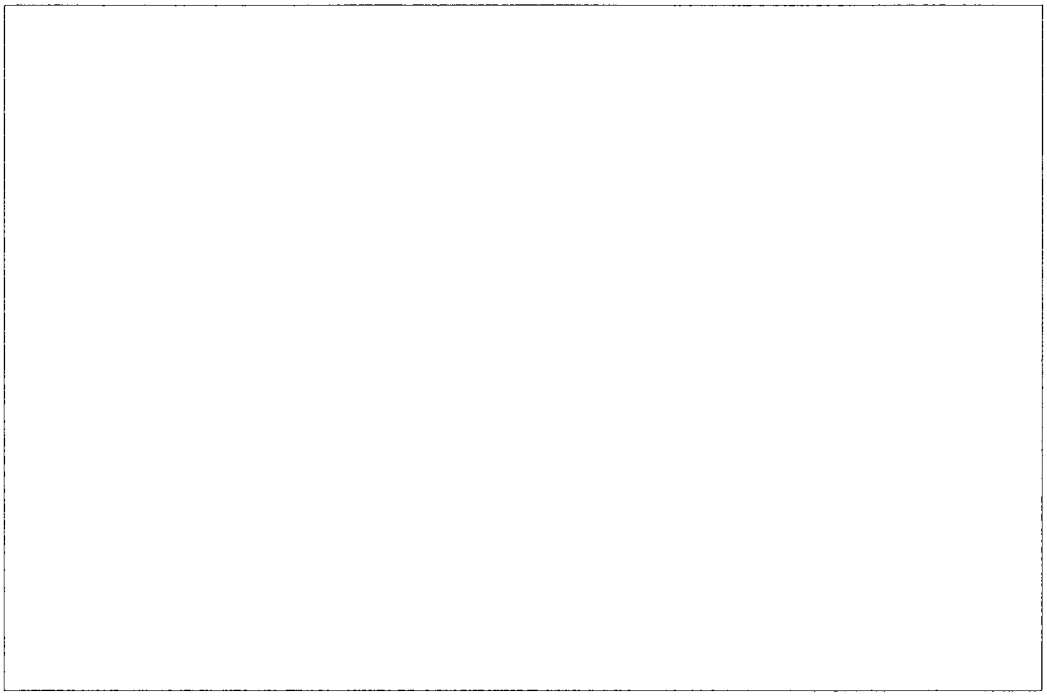

Figure 10 A unilaterally injected *Xenopus* embryo. Fluorescein-labeled *CHRAC17* specific MOs were injected on the left side; this is a dorsal view with head to the left and tail to the right. The typical bowing of these embryos suggests that the affected side is shorter along the anterior-posterior axis, presumably by the same mechanism that results in overall shortened AP axis length in globally injected embryos.

Figure 11 Left and right sides of a unilaterally injected embryo. *CHRAC17* specific MOs were injected on the right side at the two cell stage. At stage 37 the right side exhibits gross morphological abnormalities including absence of eye structures while the left side exhibits normal development.

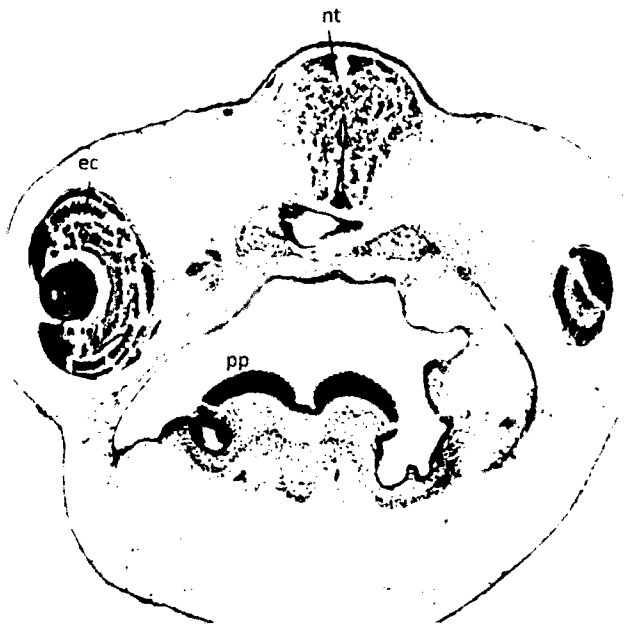


**Figure 12** Histological section of a unilaterally injected embryo. A two-cell stage embryo was injected unilaterally with *CHRAC17* specific MOs. At stage 44 the unaffected side (left) demonstrates normal development while the right side, where CHRAC17 function is ablated, has abnormally developing eye, neural tube, notochord, and pharyngeal structures. Abbreviations: ec, eyecup; nt, neural tube, pp, pharyngeal pouches.

*Loss of CHRAC17 function coincides with depletion of xOTX2 mRNA.*

In order to study the developmental consequences of *CHRAC17* loss of function at the molecular level, I measured the level of expression of putative target genes that might be misregulated in CHRAC17 knockdown embryos. The homeobox gene *xOtx2* is known to be involved in AP-axis formation at gastrulation and later is required for patterning of anterior head structures [66], [67]. I reasoned that a misregulation of *xOtx2* by ablation of CHRAC17 function would be consistent with the pattern of

*CHRAC17* expression in these structures in normal embryos and with the perturbation of those structures in *CHRAC17* morphants.

I injected embryos with negative control MOs or *CHRAC17* specific MOs (42 ng) immediately after fertilization, collected 10 embryos from each group at stages 12, 14, 23, 37 and 45 and extracted total RNA from them. I measured the amount of specific mRNA species in the RNA samples using RT-qPCR (see Materials and Methods).

I found that the level of *xOtx2* mRNA is reduced by 50% in *CHRAC17* morphant embryos at stage 22 and remains depressed in subsequent stages, though expression may begin to recover at later stages (Fig. 13).

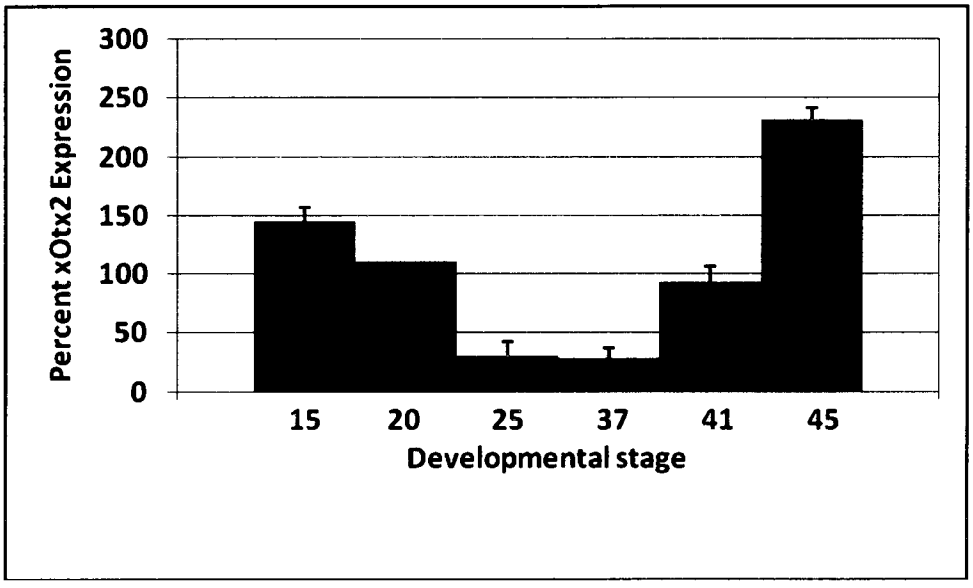


**Figure 13** *xOTX2* expression in staged *CHRAC17* morphant embryos. Total RNA was extracted at the stages shown. Levels of *xOTX2* mRNA were measured by RT-qPCR. The bars represent the level of *xOtx2* mRNA in *CHRAC17* morphants divided by the level of *xOtx2* mRNA measured in the negative control embryos.

*Genes that are misregulated in CHRAC17 morphant embryos are overrepresented in biological pathways involved in development, cell morphology and motility, and hematopoiesis.*

In order to identify other genes regulated directly or indirectly by CHRAC17 I performed whole genome expression analysis of total RNA samples taken from control and *CHRAC17* morphant embryos at stage 15 and stage 37 (see materials and methods). Whole genome expression analysis identified many misregulated genes at stage 15 and stage 37. At stage 15, 414 genes were misregulated with p values <0.001 in *CHRAC17* morphants, while at stage 37, 588 genes showed significant changes in expression levels. Compared to the profiles of misregulated genes in ISWI and WSTF knockdowns, that of *CHRAC17* morphants contain unique genes; the extent to which the profiles may overlap has not been determined.

I focused on several genes found to be misregulated when *CHRAC17* function is ablated , listed on Table 2. Three (*CXCL12, DAB2,* and *MIXL1*) are known to have regulatory functions in development. H1F0 is associated with chromosome condensation and cells entering terminal differentiation.

CXCL12 was first identified as a bone marrow stromal cell-derived factor and pre-B-cell stimulatory factor and named PBSF/SDF-1. It is known as a highly atypical chemokine (reviewed in [68]). While it belongs to a subfamily of proteins characterized by the first two cysteines being separated by one amino acid (CXC), its amino acid

sequence is as similar to the members of another subfamily (CC) as it is to other CXC proteins. The sequence and function are so highly conserved across species it is suspected that almost all of its residues are necessary for biological activity [69]. CXCL12 was thought to be the ligand for a single receptor, CXCR4, unlike the other CC and CXC chemokines but lately a second cognate receptor, CXCR7, has been identified (reviewed in [70]). In *Xenopus* CXCL12 is expressed in developing mid- and hindbrain, otic vesicles, dorsal fin, posterior heart, and later in the proctodeum [69]. It has been shown that the CXCL12/CXCR4 axis is necessary for regulating the massive cell migration that takes place during gastrulation [71]. The paradigm for CXCL12 function is that CXCL12-expressing cells create an extracellular gradient of CXCL12 that attracts CXCR4-expressing cells. CXCR4-expressing cells include adult stem cells that might enter circulation from their niches, and upon encountering the CXCL12 gradient pass through the endothelium to their targets, much as leukocytes are known to do when attracted to sites of inflammation by other chemokines. An intriguing theory is that CXCR4-expressing cardiac neural crest cells might be attracted in this way to the ventriclular septum of the developing heart [69].

The microarray chip data indicated that *CXCL12* was upregulated 6.8-fold at stage 15 and 5.1-fold at stage 37. For a detailed picture of how CHRAC17 ablation affects *CXCL12* expression in developing *Xenopus* I injected one cell of two-cell stage embryos with *CHRAC17*-specific MOs. My collaborator Jasmin Horn (Julius-Maximilians University

Wuerzburg) then performed whole mount *in situ* hybridization with a probe for *CXCL12* mRNA on embryos fixed at stage 15 and stage 37. I found that *CXCL12* mRNA was detectable at stage 37 in the eye, otic vesicle, mid- and hindbrain and dorsal fin, confirming findings of earlier studies [69] (Fig. 16). Consistent with the microarray chip data, the injected (right) side of the embryo has visibly more *CXCL12* expression than the uninjected side.

DAB2 is involved in endocytosis in clathrin-coated structures at the cell surface, where it interacts with the cargo-binding domain of the actin-based molecular motor myosin VI (reviewed in [72, 73]. Myosin VI in turn promotes secretion of vascular endothelial growth factor (VEFG) and the serine protease, prostate-specific antigen [74]. DAB2 is known to mediate TGFβ signaling [75]. In *Xenopus* DAB2 was shown to mediate the induction of VEGF expression by activin-like signaling, and this signaling is essential for the development of intersomitic blood vessels [76].

*Dab2* was upregulated 2.1-fold at stage 37. To visualize how CHRAC17 ablation affects *Dab2* expression in developing *Xenopus* I again unilaterally injected two-cell stage embryos with *CHRAC17*-specific MOs and Jasmin Horn performed whole mount *in situ* hybridization on embryos fixed at stage 15 and stage 37, using a probe specific to *Dab2*. I found that *Dab2* mRNA was detectable at stage 37 in a diffuse pattern consistent with earlier studies [76] (Fig. 17). Consistent with the microarray chip data,

the injected (right) side of the embryo has visibly more *Dab2* expression than the uninjected side.

H1F0 is a linker histone associated with condensed chromatin and terminally-differentiated cells. A global gene expression experiment associated *H1f0* expression with erythroid differentiation [77]. *H1f0* expression was upregulated by a factor of two at stage 15 and by a factor of four at stage 37. *In situ* hybridization detected no difference in its expression level between injected and uninjected sides of stage 15 embryos, possibly an artifact due to overstaining. *In situ* hybridization was not performed on a stage 37 embryo.

MIXL1 is a paired-like homeobox protein and an activin immediate-early response gene. It is expressed in the *Xenopus* embryo's marginal zone and deep vegetal cells [78]. *Mixl1* expression domain largely overlaps that of *brachyury (Xbra)*, at the gastrula stage, but the two genes inhibit the expression of each other so in successive stages their expression patterns become exclusive [79]. This is the result of MIXL1-mediated induction of *goosecoid (gsc)* expression, which in turn directly suppresses expression of *Xbra* [80]. Thus the protein regulates mesoderm development and mediates endoderm differentiation. It has been shown to be required for head formation [79]. *Mixl1* was downregulated by a factor of 1.5 at stage 15. Whole mount *in situ* hybridization of embryos unilaterally injected with *CHRAC17*-specific MOs confirm this, as the uninjected side exhibits higher *Mixl1* expression than the injected side (Fig. 18).

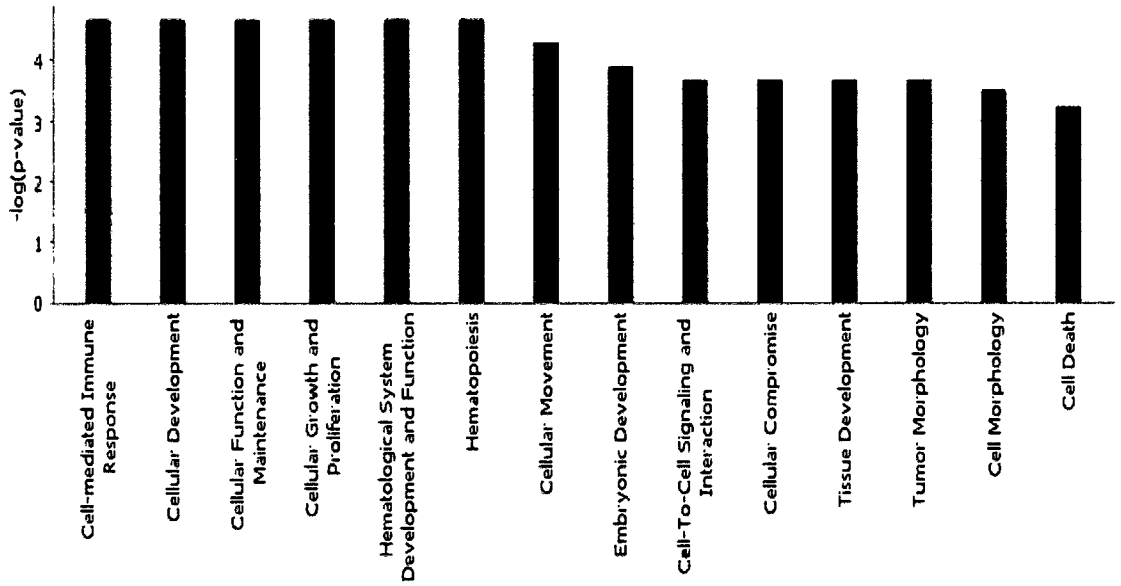


**Figure 14** Biological pathways of misregulated genes in stage 15 morphants. The pathways are in descending order of significance, left to right. Note that many of the pathways are involved in embryonic development, hematopoiesis and in cell motility.

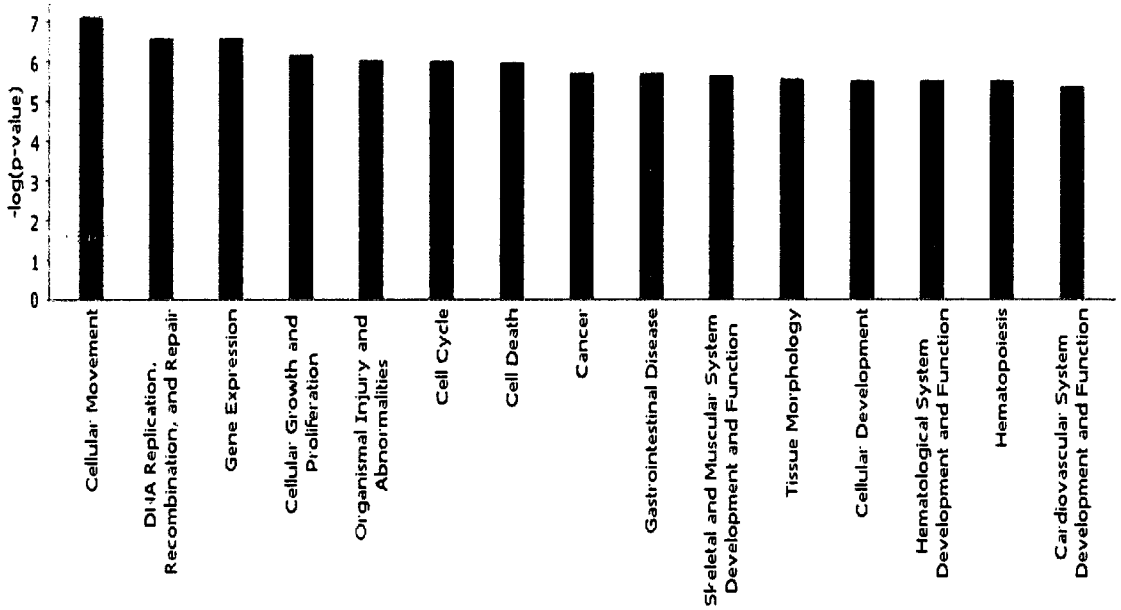


**Figure 15** Biological pathways of misregulated genes in stage 37 morphants. The pathways are in descending order of significance, left to right. Note that again many of the pathways are involved in embryonic development, hematopoiesis, and cell motility.

**Table 2** Selected genes misregulated in *CHRAC17* morphants

| Gene name | Gene function | Degree of misregulation | |
|---|---|---|---|
| | | Stage 15 | Stage 37 |
| *CXCL12* | chemokine, directs motility of cells that express CXCR4 or 7 (endothelial and neuronal cells), regulates hematopoiesis and development of brain, spinal cord and eye | **6.82** | **5.13** |
| *DAB2* | Endocytosis and angiogenesis | | **2.08** |
| *H1F0* | Chromosome condensation at interphase, terminal differentiation | **2.28** | |
| *MIXL1* | Pronephros development, endoderm formation and regulation of mesoderm development | **-2.83** | |

**Figure 16** *CXCL12* expression in a *CHRAC17* morphant. These are the left and right sides of a stage 37-38 embryo injected unilaterally on the right side with *CHRAC17*-specific MOs, then probed with *In situ* hybridization probe specific for *CXCL12* expression. The right side exhibits increased *CXCL12* expression, consistent with the cDNA microarray results.

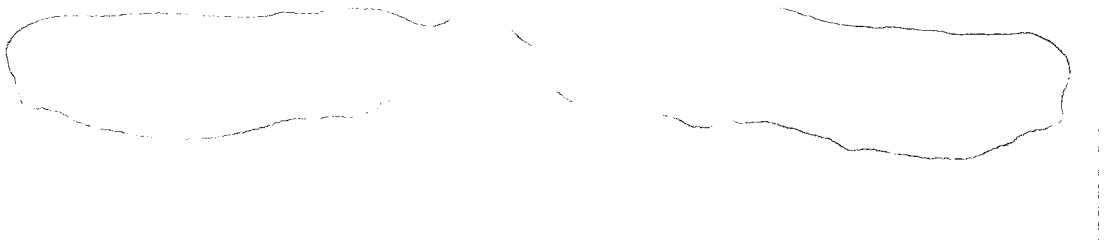

Figure 17 *DAB2* expression in a *CHRAC17* morphant. These are the left and right sides of A stage 37-38 embryo injected unilaterally on the right side with *CHRAC17*-specific MO, then probed with *In situ* hybridization probe specific for *DAB2* expression. The right side has increased *DAB2* expression, consistent with the cDNA microarray results.

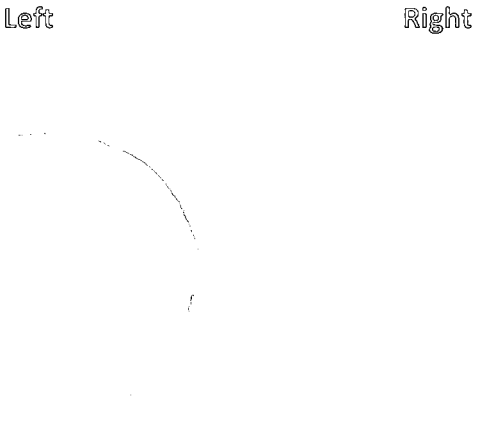


Figure 18 *MIXL1* expression in a *CHRAC17* morphant. These are the left and right sides of a stage 15 embryo injected unilaterally on the right side with *CHRAC17*-specific MOs, then probed with *In situ* hybridization probe specific for *MIXL1* expression. The right side exhibits decreased *MIXL1* expression, consistent with the cDNA microarray results.

**Discussion**

Here I have collected evidence consistent with the hypothesis that CHRAC17, and by inference the CHRAC chromatin remodeling complex and/or other CHRAC17-containing complexes, have critical roles in development, particularly of the nervous system and AP axis. The role of *CHRAC17* overlaps with, but is distinct from, the role of ISWI (representing all ISWI-dependent complexes). The expression pattern of *CHRAC17* mRNA visualized by *in situ* hybridization is distinct from that of ISWI, in that *CHRAC17* is expressed in myotomes and prominently in ectodermal placodes and/or branchial arches, while ISWI expression is primarily confined to neural tissue. ISWI expression has not been detected in the myotomes.

The *CHRAC17* morphant phenotype is dramatically different from that of ISWI. Most ISWI morphants die around the neurulation stage, while 80% of *CHRAC17* morphants survived to at least stage 45. At later stages the length of *CHRAC17* morphants is significantly shorter than that of control embryos, consistent with the finding that *CHRAC17* is expressed in the myotomes. In ISWI morphants that survive (for example due to only partial knockdown of ISWI) no such phenotype is observed.

By stage 37 *CHRAC17* morphants exhibit reduced head volumes relative to controls, particularly in the areas of forebrain and nasal placode, and possibly including the ventral eye vesicle. Retinal pigmented epithelium (RPE) is greatly reduced and the overall size of the eye vesicle is reduced. Closure of the ventral fissure of the optic

vessel is incomplete at this stage. The edge of the dorsal fin is irregular or serrated compared to controls. Development of the tailbud is delayed or otherwise abnormal in knockdowns. This phenotype overlaps with but is distinct from the ISWI phenotype; ISWI knockdown embryos that survive to later stages exhibit specific eye and brain defects that are qualitatively different from the *CHRAC17* morphant phenotypes.

Histology reveals abnormal retina, lens, neural tube and notochord morphologies at stage 41 in *CHRAC17* morphants. Again, this phenotype overlaps with but is distinct from that of ISWI knockdown embryos.

*CHRAC17* morphants are behaviorally inert and nonresponsive as late as stage 45. Late stage ISWI embryos, while totally blind, remain responsive to touch and swim reasonably well.

The morphology of embryos injected unilaterally with *CHRAC17* MOs dramatically exhibit the morphological abnormalities found in *CHRAC17* morphant embryos and suggest that there is little cross-talk between affected and unaffected sides in the development of the impacted structures. Whatever the molecular mechanisms of perturbation of the anterior-posterior axis, neural tube, notochord and eyes in *CHRAC17* morphants, it does not seem to be significantly rescued by the normally developing contralateral side of unilateral injectees. The crescent shape of unilaterally injected embryos is consistent with the reduced anterior-posterior axis length in embryos injected at the one cell stage. On the other hand, the overall phenotype of unilaterally

inected embryos appears milder than that of globally injected embryos; for instance, the behavior phenotype is mitigated and sometimes even reversed in unilateral morphants. This may reflect some cross-talk between the two halves of the embryo.

The abnormalities found in *CHRAC17* morphants are consistent with the expression pattern of *CHRAC17* as revealed by *in situ* hybridization. *CHRAC17* is normally expressed prominently in the most anterior head structures and brain, while in morphants the most anterior head structures are absent or reduced and histologically the neural tube and eyes were found to be defective. The behavioral phenotype found in morphants may be expected to derive from neural tube defects, although more studies would be required to make a cause and affect argument. The reduced anterior-posterior axis length of *CHRAC17* morphants is consistent with the observation that *CHRAC17* is expressed in the myotomes.

RT-qPCR and pathway analysis of the whole genome expression microarray experiment, comparing expression of genes in *CHRAC17* morphants with that of normal controls, reveal a significant overrepresentation of genes involved in embryonic development, hematopoiesis and cell motility, among the misregulated genes in *CHRAC17* morphants.

It will require further studies to establish whether or not some developmental consequences of CHRAC17 knockdown may arise from loss of function of the ATAC complex or of DNA polymerase epsilon, rather than a loss of CHRAC function. There is

no evidence that DNA polymerase epsilon has a role in development, but as cited in the introduction to this chapter, biochemical and genetic studies of the ATAC complex's subunits suggest that it could function in regulatory pathways in development.

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# Chapter 4

## The role of CHD4 in *Xenopus* development

### Introduction

As mentioned in Chapter 1 the CHD4 protein was initially identified as the dermatomysitis-specific autoantigen Mi-2 (antigen recognized by patient Mitchell's autoimmune antibodies 2) [1]. Subsequently, several groups identified a related set of remodeling complexes containing either CHD3 or CHD4 as the ATPase subunit. The genes encoding the proteins may be referred to as *Chd3* and *Chd4*, respectively, and the proteins are also known as Mi-2α and Mi-2β. These complexes include the *Xenopus* Mi-2 complex [2] and the human complexes NuRD/NURD/NRD [3-5].

Since the publication of Chapter 1 much has come to light regarding the developmental roles of the subunits of the NuRD complex. CHD4 regulates the relative amounts of mesoderm and neuroectoderm in developing *Xenopus* [6]. It directly binds the *Sip1* gene which suppresses *Sip1* expression and consequently neural development. Conversely, CHD4 suppression of *Sip1* prevents expression of the *brachyury (Xbra)* gene in the prospective neural plate while still allowing it to be expressed in prospective mesoderm.

The subunits MBD2 and MBD3 were confirmed to be mutually exclusive and were shown to confer different functions on their respective NuRD-like complexes [7].

In cell lines expressing a tagged version of each subunit, the MBD2 complex, but not the MBD3 complex binds the arginine methyltransferase PRMT5. The MBD2 complex also binds import α nuclear transport proteins, suggesting that the MBD2 complex may translocate between the cytoplasm and nucleus. All three MTA proteins (MTA1, MTA2 and MTA3) were found associated with both the MBD2 and MBD3 complexes; there is evidence that the MTA proteins are expressed in a tissue-specific manner, resulting in tissue-specific NuRD complexes so the number of tissue-specific complexes determined by combinations of subunits may be substantial [8], [9]. The same group found numerous post-translational modifications of the NuRD complex, many of them occurring on highly conserved residues, implying that the modifications also may be evolutionarily conserved.

MBD2 but not MBD3 contains a motif that is a known substrate for PRMT5, and *in vitro* the MBD2 complex specifically methylates MBD2 containing the motif, but not MBD2 lacking the motif or MBD3. Chromatin immunoprecipitation in cultured cells revealed that MBD2 and PRMT5 colocalize to two genomic sites, and that PRMT5 methylates its target histone residue H4R3 at these sites.

A critical role for MBD3 in peri-implantation development was revealed by mouse genetics and *ex vivo* studies of MBD3 null inner cell mass cells [10]. MBD3 null embryonic stem cells contain little or no intact NuRD complex. Embryos deficient in MBD3 fail to expand their pluripotent cell population or form a normal epiblast post

implantation, and fail to develop normal extraembryonic ectoderm. Analogously, inner cell masses deficient in MBD3 and cultured in the presence of LIF, which inhibits differentiation and promotes expansion of pluripotent cell populations, fail to expand into a normal cell mass.

A role in gene silencing has been ascribed to the p66 subunit of the NuRD complex, since named Gatad2a. Mice mutant for *Gatad2a* die around the time that the embryo implants and gastrulation begins, consistent with a function in mediating DNA methylation and developmental gene silencing, which begin at the same stage [11]. However, the same study showed that *Gatad2a*-null embryonic stem cells are viable and capable of differentiation in embryoid bodies.

With so much functional diversity resulting from different assortments of the alternative MBD and MTA subunits of the NuRD complex, the question arises as to what functional differences might exist between the CHD3-containing and CHD4-containing complexes. The two complexes may be functionally redundant, or CHD3 and CHD4 may be among those sets of paralogous proteins that are the products of gene duplications and subsequent divergent evolution. To begin to answer these questions I ablated CHD4 function in developing *Xenopus* embryos to observe the consequences of loss of CHD4 function.

**Results**

*Ablation of CHD4 function by Morpholino oligonucleotides results in a distinct morphant phenotype.*

In order to determine the developmental roles of CHD4 I injected *Xenopus* embryos with *CHD4*-specific morpholino oligonucleotides (MOs). I designed the MOs to anneal with the region of *CHD4* mRNA that spans the translation start site, thus preventing translation of CHD4 protein in injected embryos. As a control for nonspecific MO effects on development, I injected embryos from the same clutch of eggs, fertilized with the same testis, with *CHD4* inverse control MOs or with water (see Materials and Methods). A portion of each clutch was left uninjected.

Compared to negative control MO-injected embryos, those injected with *CHD4*-specific MOs exhibited profound developmental abnormalities (Fig. 1). The most anterior and dorsal head structures appeared to be reduced or missing. The eyes were malformed or had retarded development. The embryos exhibited varying degrees of cyclopia. The anterior-posterior axes were severely shortened and malformed.

I counted abnormally developing *Xenopus* in four independent experiments based on morphology of the head, eyes, and anterior-posterior axis(Table 1). Of 152 control embryos 1.3% exhibited abnormalities, while of 353 *CHD4* morphants, 77% were abnormal.

My collaborator Dr. Jonathan Henry of the University of Illinois-Urbana sectioned control and *CHD4* morphant embryos collected at stage 44 and stained them with hematoxylin and eosin to study them microscopically. These sections reveal striking developmental abnormalities in development of neural tube, notochord, eyes, and pharyngeal pouches (Fig. 2). In particular the eyes reflected the varying degrees of cyclopia seen on the gross morphological scale. In the example in Figure 2 the retinas are apparently fused at the midline and the lenses are invaginated far nearer to the midline than in normal controls. While the pigmented retinal epithelium is discernible, other layers of the retina are not present. Overall, the head is much narrower than that of the normal control.

**Table 1** Percentage of abnormal embryos in control and *CHD4* morphants

| Morpholino | Normal | # abnormal | % abnormal |
|---|---|---|---|
| Control | 150 | 2 | 1.3% |
| *CHD4* | 82 | 271 | 76.7% |

Figure 1 The *CHD4* morphant phenotype in *Xenopus*. At stage 41, compared to the control MO-injected embryo (top) the embryo injected with *CHD4*-specfic MO (bottom) demonstrates the characteristic developmental abnormalities following CHD4 loss of function in head, eyes,and anterior-posterior axis.


Figure 2 Cross sections of MO-injected *Xenopus* embryos. Embryos were injected with control or *CHD4*-specific MOs. At stage 41 the embryo lacking CHRAC17 function exhibits abnormally developing eyes, neural tube, notochord, and pharyngeal structures. Abbreviations: ec, eye cup, ln, lens; nc, notochord; nt, neural tube; pp, pharyngeal pouch; pr, pigmented retinal epithelium.

## Discussion

This study provides evidence that CHD4 and by implication the NuRD complex have critical roles in development, particularly in normal development of head structures, eyes, and the anterior-posterior axis. Embryos lacking CHD4 function exhibit dramatic abnormalities including cyclopia and reduced or missing structures in the anterior and dorsal head. The histological findings of the pigmented retinal epithelia of the two developing eyes fused at the midline suggest that part of the perturbation of development must involve failure of midline signals that commonly lead to similar abnormalities. The known involvement of CHD4 with positioning the mesoderm/neuroectoderm boundary [6] may be involved with this developmental abnormality. The findings point to the potential for studies at the molecular level that will be necessary to confirm this.

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