The role of *Xenopus* BRG1, a conserved subunit of SWI/SNF class of remodeling complexes, during early frog development.

Vorgelegt von

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1 SUMMARY

BRG1 is a conserved subunit of the SWI/SNF family of ATP dependent chromatin remodeling complexes. These complexes play an important role in the transcription of various genes by making promoters accessible to the transcription machinery. Mutations in BRG1 have been connected to various cancers. In addition, a BRG1 knock-out in mice is lethal at the periimplantation stage, while BRG1 heterozygote mice are predisposed to exencephaly and tumors of epithelial origin, showing the importance of BRG1 in normal development and disease.

In this study, I used *Xenopus laevis* to study the role of BRG1 because this system allows manipulation of endogenous protein levels by the use of antisense oligonucleotide mediated knock-down as well as interference analysis at early stages of development by overexpression of wild type and dominant negative protein variants. Since BRG1 is conserved among all vertebrates, I initially studied the role of BRG1 in *Xenopus* development by overexpression of wild type and dominant negative human BRG1. Overexpression of dominant negative human BRG1 gave a ventralized phenotype suggesting a role of BRG1 in dorsal-ventral patterning. The specificity of phenotypes was confirmed by using wild type human BRG1. On the other hand, overexpression of wild type and dominant negative variants of human BRM showed no developmental phenotypes.

Prompted by these results, a frog *brg1* cDNA was cloned by searching the *Xenopus laevis* EST database, using human BRG1 as a query. In addition, monoclonal antibodies specific to xBRG1 were raised and characterized. The expression pattern of Xbrg1 was found to be ubiquitous until gastrula stage and is tissue specific from neurula stage onwards. A *Xenopus* homologue of INI1, a subunit of SWI/SNF chromatin-remodeling complex, was cloned using database search. The expression pattern of Xini1 was found to be similar to Xbrg1.
Using site directed mutagenesis, a dominant negative construct of xBRG1 was made by mutating the conserved lysine into arginine (K770R). Loss and gain of function studies showed that BRG1 is involved in AP axis formation during *Xenopus* development. The gain of function studies were done by overexpressing wild type and dominant negative xBRG1, while loss of function studies were done using highly specific antisense morpholino oligos. Specificity of morpholino treatment was further proven by the rescue of ventralized phenotypes of morphant embryos by overexpression of human BRG1. It was found that BRG1 knock-down affects several tissues as assessed by *in-situ* hybridization using tissue specific markers.

To determine the molecular explanation for these pleiotropic effects, several genes involved in early patterning of *Xenopus* embryo during organizer formation were analyzed. The analysis was done using whole mount *in-situ* hybridization, revealing the spatial gene expression pattern. This analysis revealed that BRG1 mostly affects WNT signaling dependent genes required for dorsal mesoderm formation while leaving pan-mesodermal genes unaffected. Furthermore the genetic interaction of BRG1 with the WNT pathway was confirmed by epistasis experiments showing that overexpression of β-CATENIN can rescue the xBrg1 antisense morpholino oligos dependent ventralized phenotypes as well as formation of secondary axis by overexpression of β-CATENIN could be prevented by BRG1 knock-down.

Since the whole embryo represents a complex situation whereby many signaling pathways interact with each other and influence the outcome, the animal cap system was used to analyze the effect of BRG1 on various signaling pathways by analyzing corresponding direct target genes. Animal cap assays showed that the effect of BRG1 is signal specific. Moreover, among the affected signaling pathways, BRG1 knock-down affected only specific genes. These results showed that the BRG1 effect is gene and signal specific.
The importance of WNT signaling has also been shown in cancer as well as in haematopoietic and embryonic stem cell self renewal. Given the importance of the WNT signaling, the role of BRG1 on the WNT signaling pathway was further investigated. Treatment of animal cap cells with various doses of Wnt8 mRNA showed the differential requirement of the WNT signal for maximal stimulation of direct target genes. The direct target genes of the WNT pathway showed various degrees of reduction in their maximal stimulation upon BRG1 protein knock-down. The requirement of BRG1 for proper stimulation of the WNT target genes was further confirmed by overexpression of xBRG1 under sub-optimal conditions of WNT stimulation.

A major conclusion from these experiments is that BRG1 protein defines signaling thresholds for WNT-mediated activation of target genes. This implies that chromatin remodeling complexes are part of the machinery, which translates inductive signals into spatial gene expression domains.
2 INTRODUCTION

An animal starts its life as a single cell, i.e. a fertilized egg. Repeated division of this cell gives rise to a multicellular organism in a complex but precise manner. The development of a multicellular organism involves complex series of genetic, cellular and physiological events. These events occur in a correct order in specific cells, and at the appropriate times to orchestrate the proper development of an organism. Selective gene expression controls the four essential processes to develop an embryo; a) cell proliferation, producing many cells from one, b) cell differentiation, creating cells with different characteristics at different positions, c) cell interaction, coordinating the behavior of one cell with that of its neighbors, and d) cell movement, rearranging the cell to form structured tissues and organs toward establishing the body plan.

Transient and stable gene expression patterns are governed by various external stimuli, causing epigenetic changes in the genome via direct or indirect mechanisms. The external stimuli are relayed to the genome of the cells via various signaling pathways. The major signaling pathways, which control embryonic patterning and cell specification, are conserved throughout evolution. Thus, the study of these pathways using one model system would allow making predictions in other systems.

2.1 Advantage of Xenopus as a model system

Over several years, the African clawed frog, *Xenopus laevis* has been one of the most successful vertebrate model systems to study the various signaling pathways involved in the patterning and cell specification that give rise to a normal embryo.

The advantages of the *Xenopus* model system have been well appreciated over time. It has been used extensively to study events in early embryogenesis. The embryonic development of *Xenopus* is rapid. From fertilization to feeding stage tadpole, it takes only about 48 hours. The eggs of
*Xenopus* are about 1.5 mm in diameter and so are easy to handle. Moreover, a single female lays up to 1500 eggs per day, thus providing sufficient material for study. Other than these features, eggs can be laid at the desire by hormone induction. The eggs are easy to culture in semi-sterile conditions and do not require external nutrients aside from the yolk present in the embryo.

In *Xenopus*, fate maps were created, showing the statistical contribution of each blastomeres of the 32-cell stage embryo, which can be used to trace the origin of the cells in various tissues (Dale and Slack, 1987).

Maternal mRNAs deposited in the egg control the early developmental events of all embryos, which include the patterning of the basic body plan, the determination of cell fate, and the early patterning of the major organs and body musculature. These maternal mRNAs are the source of early patterning events occurring prior to the start of transcription. These events can be studied easily in *Xenopus* embryos because of the long duration taken to complete these events and in addition, the embryos are easily accessible due to external development. These features allow to study and learn about the cellular and molecular mechanisms of early patterning including the role of specific extracellular growth factors, cell surface receptors and intracellular signaling pathway components. Many of the factors originally identified in *Xenopus* were later shown to be involved in other critical biological processes and oncogenesis. Other than these features, *Xenopus* has given an excellent contribution to our understanding of cell biological and biochemical processes, including chromosome replication, chromatin, cytoskeleton, and nuclear assembly and cell cycle progression.

Another experimentally important aspect of the *Xenopus* model system is the availability of the animal cap assay system. The animal cap is derived from the roof of the blastocoel, which is made up of prospective ectodermal cells. The animal cap system allows the study of various signaling pathways in an isolated, but still endogenous tissue. Animal caps have been used mostly for induction assays. Animal cap cells consist of multipotential embryonic stem cells, which
can be differentiated into various organs upon differential treatments (Ariizumi and Asashima, 2001; Fukui et al., 2003). This property of animal cap cells provides an ideal system to study the complex mechanisms involved in organ differentiation outside the embryo.

2.2 Early development of *Xenopus*

2.2.1 Fertilization and cleavage

The mature *Xenopus* egg has a dark, pigmented animal region and a pale, yolk-rich vegetal region. The egg is enclosed in a protective vitelline membrane, which is embedded in a gelatinous coat. The unfertilized egg of *Xenopus* is radially symmetrical about the animal-vegetal axis and this symmetry is broken with the fertilization. At fertilization, one sperm enters the egg in the animal region. The egg and sperm nuclei fuse to form the diploid zygote nucleus. The vitelline membrane lifts off the egg surface and in about 15 minutes the egg has rotated within it under the influence of gravity so that the heavier yolky, vegetal region is now downward. Within 90 minutes of fertilization, changes in the egg become distinguishable opposite to the site of sperm entry. The plasma membrane and the cortex-, a gel-like layer of actin filaments and associated material about 5 μm thick beneath the membrane, rotate about 30° relative to the rest of the cytoplasm, which remains stationary. This cortical rotation is towards the site of sperm entry, the opposite vegetal cortex move towards the animal pole (Gerhart et al., 1989).

The first cleavage occurs along the animal-vegetal axis in 90 minutes of fertilization, and divides the embryo into equal left and right halves. The second cleavage is also along the animal vegetal axis but at right angle to the first and divides the embryo in the 4-cells at which stage dorsal and ventral sides could be easily distinguished by the pigmentation and the size of the blastomeres. The third cleavage is equatorial, at right angle to the first two, and divides the embryo into four animal cells and four vegetal cells. After about 12 cell divisions a
spherical mass of cells with a fluid filled cavity, known as the blastocoel, is formed, although it can first be seen at the 4-8-cell stage of the embryo. This spherical mass of the cells is called as blastula (Gerhart and Keller, 1986; Keller, 1991).

**2.2.2 Gastrulation**

Gastrulation in *Xenopus* is initiated on the future dorsal side of the embryo, just below the equator. Gastrulation converts the blastula into a three-layered structure with clearly recognizable anterio-posterior and dorso-ventral axis and bilateral symmetry. At the blastula stage, the mesodermal and endodermal germ layers are located in the equatorial and vegetal region and are essentially on the outside of the embryo, while the ectoderm is confined to the animal region. Gastrulation involves extensive cell movements and rearrangement of the tissues of the blastula.

Gastrulation is initiated by the invagination on the surface of the blastula on the dorsal side, forming a small slit like infolding-the blastopore. The dorsal blastopore lip is initially composed of cells called the bottle cells (Jonas and Spannhof, 1971; Keller, 1981; Spannhof and Dittrich, 1967). The layer of the future endoderm and mesoderm in the marginal zone involutes through the dorsal lip of the blastopore and converge and extend along the anterio-posterior axis beneath the ectoderm while the ectoderm spreads downward to cover the whole embryo by a process known as epiboly (Keller and Danilchik, 1988). The number of deep cell layers increases during gastrulation through three rounds of successive cell divisions in the animal hemisphere as well as numerous deep layer cells integrates into one layer. The most superficial layer expands by cell division and flattening. The result of these expansions is the epiboly of the superficial and deep cells of the animal cap and the non-involuting marginal zone over the surface of the embryo (Keller, 1980; Keller et al., 1985). The layer of dorsal endoderm is closely applied to the mesoderm; the space between it and the yolky vegetal cells is known as the archenteron and is the precursor of the
gut cavity. The inward movement of the endoderm and mesoderm eventually spreads to form a complete circle around the blastopore. By the end of gastrulation, the blastopore has closed. The dorsal mesoderm lies beneath the dorsal ectoderm, and the lateral mesoderm begins to spread in a ventral direction on either side. The inner surface of the archenteron becomes completely covered by a layer of endoderm, forming the gut. At the same time, the ectoderm spread to cover the whole embryo. During gastrulation, dorsal mesoderm develops into two main structures, the notochord and the somites. The notochord is a stiff, rod-like structure that forms along the dorsal midline and eventually becomes incorporated into the vertebrae. The somites form by segmentation of the paraxial mesoderm lying immediately at either side of the notochord. Somites are formed in pairs, and segmentation proceeds in an antero-posterior direction.

Figure 2.1 Schematic representation of early development of *Xenopus laevis* (adapted from De Robertis et al., 2000).
2.2.3 Neurulation and organogenesis

Gastrulation is succeeded by neurulation, the formation of the neural tube, and the early embryonic precursor of the central nervous system. While notochord and somites are developing, the neural plate ectoderm above them begins to develop into the neural tube. At this stage, the embryo is called a Neurula. The early signs of neural development are the formation of the neural folds, which form on the edges of the neural plate. These rise up fold towards the midline and fuse together to form the neural tube, which sinks beneath the epidermis (Keller et al., 1992a; Keller et al., 1992b; Keller, 1980; Keller et al., 1985). The anterior neural tube gives rise to the brain; further back, the neural tube overlying the notochord will develop into the spinal cord. The main structures that can be recognized at this stage are the neural tube, the notochord, the somites, the lateral plate mesoderm, and the endoderm lining the gut. The unsegmented lateral plate mesoderm, lying lateral and ventral to the somites, gives rise to tissues of the heart and kidney, as well as to the gonads and gut muscle, while the most ventral mesoderm gives rise to the blood islands. The endoderm lining the gut will bud off organs such as the liver and lungs. At the tail bud stage, the brain is already divided up into a number of regions while the eye and ear have begun to develop at the anterior end. There are three branchial arches, of which the anterior most will form the lower jaw. More posteriorly, the somites and notochord are well developed. The post-anal tail of the tadpole is formed last. It develops from the tail bud, which, at the dorsal lip of the blastopore, gives rise to the continuation of notochord, somites and neural tube. Nieuwkoop and Faber have divided the early development of *Xenopus* into various stages (Nieuwkoop and Faber, 1967).

After organogenesis is completed, the mature tadpole hatches out of its jelly covering and begins to swim and feed. Later, the tadpole larva undergoes metamorphosis to give rise to the adult frog.
2.3 Role of signaling events in establishment of early pattern formation

2.3.1 Organizer formation

At the gastrula stage, the dorsal side of the embryo can be recognized by the presence of the dorsal blastopore lip. The importance of this dorsal lip was shown in a transplantation experiment carried out by Spemann and Mangold in 1924, using salamander gastrulae. They isolated dorsal lips from the embryos and transplanted it in the host gastrula stage embryo on the ventral side opposite to dorsal lip of the host embryo. This transplantation of dorsal lip resulted in an embryo with a complete secondary axis (Spemann, H., and Mangold. 1924). These experiments were also later repeated in *Xenopus* and were found to have the same effect. These experiments revealed the importance of dorsal lip cells in axis formation. In another complementary experiment, embryos were dissected into dorsal and vegetal halves at gastrula stage. The dorsal half of the gastrula gave rise to all dorsal structures while the ventral half of the gastrula embryo remained undifferentiated tissue showing that the differentiation of the embryo is determined by the dorsal side of the gastrula embryo (De Robertis et al., 2000).

The group of Nieuwkoop in 1969 carried out another important set of experiments. These experiments utilized the property of *Xenopus* embryo explants to be cultured in isolation in normal buffered saline. They showed that animal caps develop into epidermis, while vegetal explants neither develop into recognizable tissues nor develop posterior endodermal character (Nieuwkoop, 1963). When animal caps were grafted onto vegetal explants, mesoderm and pharyngeal endoderm developed. Using pigmentation and $^3$H-thymidine labeled cells as markers, it was concluded that mesoderm and head endoderm develop exclusively from the animal cap tissue and were therefore induced by vegetal cells. Explants of dorsal vegetal cells induce dorsal mesoderm, giving rise to notochord and muscles, as well as head endoderm. Explants from the ventral vegetal cells induce ventral mesoderm and gives rise to blood and mesenchyme.
These experiments gave rise to the three signal model (Heasman, 1997) for mesoderm patterning, consisting of an early pair of signals differing qualitatively between dorsal and ventral vegetal blastomeres, that acted in the blastula stage to divide the early marginal zone of mesoderm into two distinct territories: the dorsal and ventral mesoderm. The third signal, a dorsalizing inductive signal from the dorsal mesoderm, would then impose dorsal (paraxial) and intermediate fates on neighboring ventral mesoderm in the gastrula stage (Heasman, 1997). In subsequent experiments it was shown that vegetal cells start inducing signals as soon as 16-32 cell stage, arguing for maternal proteins for meso-endoderm induction since zygotic transcription starts only after mid blastula transition. Later it was shown that induction of both dorsal and ventral mesoderm is induced by gradients of several nodal related signals released by endoderm at the blastula stage and thus modifying the three-signal model into a two-signal model (Agius et al., 2000). These experiments also showed a gradient of Xnrs from the dorsal to the ventral side of the embryo.

*Xenopus* nodal related genes (*xnrs*) are potent mesoderm inducers (Jones et al., 1995) and the events leading to the generation of the gradient of Xnrs can be traced back to the fertilization stage. Fertilization of an egg with sperm starts a rotation in the cortex of the egg with respect to the yolky cytoplasm leading to the asymmetry in the egg. This event of cortical rotation brings about stabilization of \(\beta\)-CATENIN on the dorsal side of the embryo (Rowning et al., 1997). Increased stabilization of \(\beta\)-CATENIN leads to activation of the canonical WNT signaling pathway on the dorsal side of the embryo (Schneider et al., 1996). When the embryos were UV irradiated, dorsal structures of the embryo were abolished giving rise to a ventralized embryo. UV treatment of the embryo causes disruption of microtubules and thus prevents the accumulation of \(\beta\)-CATENIN, which mediates WNT signaling on the dorsal side of the embryo (Moon and Kimelman, 1998).

Vg1, a TGF-\(\beta\) factor was the first known asymmetrically localized RNA in the egg. Vg1 is localized to the vegetal pole of the embryo (Melton, 1987; Weaks
and Melton, 1987). VG1 precursor protein is abundant in vegetal cells, but the processed mature form has not been readily detected and no activity has been demonstrated for the putative VG1 mature protein. By using an engineered VG1 fusion (Bvg1) that promotes formation of mature VG1 protein in vivo, it was shown that VG1 could be involved in mesoderm induction (Dale et al., 1993; Thomsen and Melton, 1993). VegT, a T-box transcription factor is localized to the vegetal pole of the *Xenopus* oocytes (Zhang and King, 1996). VG1 and VegT are both potent inducers of endoderm. It was shown that depletion of maternal VegT leads to the absence of endoderm (Cui et al., 1996; Joseph and Melton, 1998; Zhang et al., 1998). Besides Vg1, endoderm expresses Xnrs. It was found that in wild type embryos, microinjection of VegT and Vg1 induces only low levels of *xnrs* transcription. However, when β-CATENIN is also provided, a high level of Xnr expression is achieved (Agius et al., 2000). It has been shown that the expression of Xnrs in the endoderm occurs in a gradient, having higher expressions in dorsal endoderm and lower levels of expression in ventral endoderm. This gradient is probably established due to higher levels of Wnt signaling on the dorsal side of the embryo. The dorsal endoderm thus establishes a signaling center called “Nieuwkoop center” (De Robertis et al., 2000). In the dorsal-animal cap marginal region, the β-CATENIN signal induces the expression of Chordin and Noggin. Chordin and Noggin are BMP antagonists. This region of embryo has been named as the “BCNE center” for Blastula Chordin and Noggin Expression center (Kuroda et al., 2004). The Nieuwkoop center and BCNE center show only a limited overlap. Both blastula centers are formed simultaneously, as soon as zygotic transcription starts and require the beta-catenin signal on the dorsal side of the embryo, but the Nieuwkoop center also requires Vg1 and VegT mRNAs localized in the vegetal pole of the fertilized egg.

A high level of Xnr expression in the Nieuwkoop center induces the formation of dorsal mesoderm and low level of Xnrs induces the formation of ventral mesoderm at the gastrula stage of the embryo (Agius et al., 2000). The
dorsal mesoderm is known as “Organizer” (De Robertis et al., 2000). BMP-2, a maternal component, is expressed in the ventral mesoderm of the embryo and has been shown to induce ventral mesoderm in animal cap explants. It has also been shown that dorsal injections of BMP-2 could ventralize the embryo (Clement et al., 1995; Nishimatsu and Thomsen, 1998; Plessow et al., 1991), showing it to be a potential inducer of ventral mesoderm.

![Figure 2.2 Signaling events during the formation of Organizer (adapted from the De Robertis et al., 2004).](image)

Nieuwkoop center cells form anterior endoderm at gastrula and BCNE center cells give rise to prospective brain and floor plate, as well as the notochord region of the Spemann organizer at gastrula. Both signaling centers are required for brain formation as Nieuwkoop center cells involute to come into intimate contact with the future brain to provide a “double assurance” mechanism for brain formation (De Robertis and Kuroda, 2004).

The organizer acts as an inhibitory center for early maternal signals. Molecules secreted by the organizer can be divided based on their inhibitory properties. Among the molecules secreted by the organizer, *frzb-1* (Leyns et al., 1997; Wang et al., 1997), *dickkopf-1* (Glinka et al., 1998) and *crescent* (Pera and De Robertis, 2000) act as zygotic WNT signaling inhibitors. *cerberus* (Piccolo et al., 1999) acts to inhibit WNT, NODAL as well as BMP signaling, while *chordin* (Sasai et al., 1994) and *noggin* (Smith and Harland, 1992) that are first
expressed in the BCNE center and later in the organizer, inhibit BMP signaling. *follistatin* also inhibits BMP signaling (Fainsod et al., 1997), while *lefty/antivin* are antagonists to the TGF-β/NODAL receptor (Cheng et al., 2000; Meno et al., 1999; Meno et al., 1996; Thisse and Thisse, 1999). The inhibition of BMP signaling by *chordin* and *noggin* sets up a gradient of BMP signaling in mesodermal tissue that is required for the differentiation of head and tail structures. Similar to WNT signaling inhibition, inhibition of NODAL signaling by organizer-secreted molecules is also required for head formation. Thus, the organizer plays a central role in the patterning of the embryo.

On one hand maternal WNT signaling is required for early patterning as described above, zygotic WNT signaling is involved in ventral mesoderm formation in combination with BMP signaling (Hoppler and Moon, 1998).

### 2.3.2 Morphogens and signaling thresholds

The dynamic gradients of NODAL and WNT/β-CATENIN activity revealed detailed steps in early patterning. The idea of morphogen gradients and thresholds has long been an important one in developmental biology (Green, 2002). Morphogens are defined as substances, whose nonuniform distribution in a field of cells differentially determines the fate and phenotype of those cells. A graded morphogen provides polarity and a scalar value that can be interpreted by cells according to threshold values to provide a coordinate system. The morphogen gradients for Activin (Green and Smith, 1990) and FGF (Green et al., 1992; Slack, 1987; Slack et al., 1987) have been investigated in the animal cap assay system. To produce distinct cell types, morphogen interpretation relies on the sharpening of threshold responses to cellular stimuli. This suggests some kind of cooperation leading to a steep sigmoid response in the interpreting cell (Lewis et al., 1977; Slack, 1993). In *Xenopus*, the detailed analysis of *xbra* regulation has shown that the initial dose window of *xbra* induction by increasing the dose of ACTIVIN is bounded by an initially relatively “fuzzy” dose threshold, but it progressively sharpened with time over a few hours (Green et al., 1994;
Gurdon et al., 1999). One of the mechanisms of sharpening of a threshold is cell contact dependent and consists of a positive feed back loop as manifested by “community effect” (Gurdon et al., 1993; Standley et al., 2001). The other mechanism involves inhibition as in the case of \textit{xbra} by Goosecoid, which is induced at higher concentrations of ACTIVIN (Artinger et al., 1997). However other than these mechanisms, chromatin environment may also play an important role in setting up fine activation thresholds as pointed out by some \textit{in-vitro} studies (Laybourn and Kadonaga, 1992).

2.4. Evidence for regulation of embryonic patterning in \textit{Xenopus} by chromatin environment

The animal cap cells are able to form the mesodermal tissue from the morula through to the blastula stage (Woodland and Jones, 1987). At early gastrula, animal cap cells lose the ability to form mesodermal tissue (Green et al., 1990). The mechanism of mesodermal competence has been studied in detail using ACTIVIN to induce the mesoderm in the animal cap cells. It has been shown that loss of mesodermal competence is programmed cell autonomously and occurs even in the single cell (Grainger and Gurdon, 1989). One of the factors required for loss of mesodermal competence of animal cells was shown to be somatic linker histones (Steinbach et al., 1997). In this report it was shown that accumulation of somatic linker histone H1, which is required for forming higher order chromatin structures and acts as a general repressor of transcription (Paranjape et al., 1994), causes the loss of mesoderm forming capacity of animal cells. In other words, the repressive chromatin environment blocks the conversion of ectodermal cells into mesodermal cells. In a recently published report, the authors showed that BRG1 overexpression could induce \textit{xbra} induction even in the gastrula stage animal caps after bFGF induction (Hansis et al., 2004). It has been also shown that treatment of embryos with TSA, an inhibitor of HDAC, before gastrulation, results in the loss of muscles in the embryos (Rupp et al., 2002; Steinbach et al., 2000). These experiments showed
the role of chromatin environment in the correct expression of various genes required for normal development of the embryo. Below is a brief overview of chromatin structure and chromatin remodeling mechanisms required for regulation of gene transcription.

2.5 Chromatin structure and chromatin remodeling complexes

Based on his light microscopic observation of nuclear material the German anatomist Walter Flemming in 1882 (Fleming et al., 1882) first established the term chromatin, which is derived from the Greek word “Khorma” which means color. The details of chromatin were studied using biochemical and electron microscopic techniques. When chromatin structures were digested with micrococal nuclease that breaks down unprotected DNA, about 200 bp DNA fragments were obtained (Clark and Felsenfeld, 1971). When interphase nuclei are broken open very gently and their contents were examined under the electron microscope, most of the chromatin is in the form of a fiber with a diameter of about 30 nm. When this chromatin is subjected to treatments that cause it to unfold partially, it can be seen under the electron microscope as a beaded structure termed “beads on a string” (Olins and Olins, 1974). In this, the string is DNA, and each bead is a nucleosome core particle. The term “nucleosome” was given by the group of Chambon (Oudet et al., 1975), while a proposal was made about 31 years ago by Roger Kornberg that the structure of chromatin is based on a repeating unit of eight histone molecules and about 200 bp, laid the basis for subsequent chromatin research (Kornberg, 1974).

2.5.1 Chromatin structure

The nucleosome represents the basic building block of chromatin. A nucleosome is composed of DNA and histone proteins. The histone proteins are present as core histones and as linker histones. In each nucleosome, 147 base pairs of DNA are wrapped around a core of histone proteins.
Albert Kossel first isolated histones, the basic proteins from nuclei and termed them “histon” in 1884. Histones represent the major class of DNA binding proteins. As a universal component of the chromosomes, histones were thought to be roughly equal to that of DNA; In fact, they were long viewed as the genetic material itself. Upon extraction in acid, which prevents proteolysis, five types of histones, designated as H1, H2A, H2B, H3 and H4 were found (Phillips and Johns, 1965). Among them H2A, H2B, H3 and H4 form the core of the nucleosome and H1 serves as the linker histone.

Figure 2.3 Diagrammatic representation of DNA Packaging into chromatin (adapted from Hansen, 2002).

The histone core consists of a \((\text{H3})_2(\text{H4})_2\) tetramer flanked by two H2A-H2B dimer thus forming an octamer. Each core histone contains two functional domains: a signature “histone fold” motif sufficient for both histone-histone interaction as well as DNA-histone interaction within the nucleosome and second an amino terminal and carboxy terminal “tail domain” containing sites for post-translational modifications (Arents et al., 1991). Linker histone H1 is unrelated in sequence to core histones. H1 consists of a globular domain flanked by amino
and carboxyl terminal tail domains while globular domain interacts with the nucleosome, the H1 tail domains are believed to be required for chromatin folding (Parseghian and Hamkalo, 2001; Ramakrishnan, 1997).

The low-resolution (7-Å) structure of the nucleosome was determined in 1984, and it revealed that the histone octamer forms a helical ramp around which 1.7 turns of a left handed DNA super helix is wrapped. The high-resolution structure at 2.8 Å was resolved in the year 1997 (Luger et al., 1997). It shows that where the DNA enters and leaves the nucleosome, it is bound by N-terminal extensions of the H3 histone fold. Each of the heterodimers binds about 30 bp of DNA. The DNA is contacted at 10 bp intervals as the minor groove faces protein. The average number of base pairs per helical turn of DNA—the helical periodicity of DNA, was found to be 10.2 bp.

A value of 10.2 bp allows the minor and major grooves from neighboring turns of the DNA super helix to line up, forming channels through which the histone tail can pass. This also leaves the major grooves accessible, enabling them to

Figure 2.4 High-resolution Nucleosome structure at 2.8 Å (adapted from Luger et al., 1997).
participate in cellular processes, acting, for example, as DNA integration-hotspots. In conjunction with the linker histone, H1, which is present in stoichiometric amounts with histone octamer, nucleosomal arrays fold into higher-order structures.

2.5.2 Chromatin remodeling

This packaging of the DNA provides on one hand a means to prevent the DNA from becoming an unmanageable tangle; it also serves as a means to regulate various processes like DNA replication, repair, recombination and transcription. The regulation of these processes are mainly governed either by covalent modifications of histone tails or by energy dependent nucleosomal structural alterations that may change the nucleosome position with respect to the DNA sequence, or may displace histone subunits. Regulation of nucleosomal structure requires enzymatic proteins.

There are two classes of nucleosome remodeling enzymes, also referred to as chromatin remodeling enzymes. The first class of enzyme is responsible for covalent modification of histone tails such as histone acetyltransferase and deacetylas. It was proposed that distinct histone modifications on one or more tails act sequentially or in combination to form a ‘histone code’ that is read by other proteins to bring about distinct downstream events (Jenuwein and Allis, 2001; Strahl and Allis, 2000). The second class of enzymes utilize the energy of ATP hydrolysis to alter or disrupt the nucleosomal structure by affecting DNA-histone interactions (Becker and Horz, 2002; Kadam and Emerson, 2002; Katsani et al., 2003; Narlikar et al., 2002; Tsukiyama, 2002).

2.5.2.1 Histone modifications

The known post-synthetic modifications of histones that cause a change in the state of chromatin are phosphorylation, methylation, ubiquitination, ADP-ribosylation along with acetylation and deacetylation.
2.5.2.1.1 Acetylation

The discovery of HAT provided a link between histone acetylation and gene activation (Brownell and Allis, 1995; Brownell and Allis, 1996; Brownell et al., 1996). Based on their protein sequence and functional conservation, HATs can be grouped into three main families: The GNATs, the MYST and the hormone receptor co activator p160 (SRC) family. (Gregory et al., 2001; Vaquero et al., 2003). Other HATs such as CBP/p300, TAFII250, TAFIIC and NUT1 do not belong to any family. Even though most of them have been shown to be involved in transcriptional activation, SAS (some thing about silencing) has been implicated in transcriptional repression (Reifsnyder et al., 1996) and has been proposed to be in the MYST family of HATs.

The effect of acetylation is partly explained by a decrease in the positive charge of the histone while other hypothesis propose that the acetylated lysine’s are recognized by bromodomain containing proteins, which then affect local chromatin structure. Many biological processes such as chromatin assembly, DNA repair and apoptosis, dosage compensation or cell cycle progression are affected by histone acetylation (Carrozza et al., 2003; Hassan et al., 2001; Kristeleit et al., 2004; Neely and Workman, 2002).

2.5.2.1.2 Deacetylation

The connection between acetylation and transcription is further shown by the fact that deacetylation can cause repression. The isolation of a human histone deacetylase, HDAC1, which was homologous to RPD3 (Furukawa et al., 1996) demonstrated the connection of deacetylation and repression. The HDACs have been divided into three groups. Class I enzymes includes HDACs 1-3 and 8 whereas class II includes HDAC 4-7, 9 and 10. The class III enzymes are related to the silencing regulator SIR2 (Imai et al., 2000).

All of the known deacetylases occur in multi-protein complexes with important functions. The complexes are able to deacetylate histones in
nucleosomes, whereas the isolated deacetylase subunits cannot deacetylate histones. Other members of the deacetylase complex include chromodomain proteins, retinoblastoma protein-associated proteins, and SIN3 (Wang et al., 2004a).

2.5.2.1.3 Methylation

Histone methylation was first described by Murray in 1964 (Murray, 1964). Arginine residues can be mono- or dimethylated by PRMTs and lysine residues can be mono- di- or tri-methylated by SET-domain containing histone methyltransferases (HMTs). While tri-methylation of histone is associated with silencing of gene transcription, di-methylation of histone has been shown to be associated with transcriptionally active gene transcription. The group of Thomas Jenuwein discovered SUV39H1, a homologue of *Drosophila* Su (var) 3-9 (Aagaard et al., 2000; Zhang and Reinberg, 2001), which supported a direct connection between heterochromatin formation, gene silencing and specific histone lysine methylation. The histone methylation is generally related to gene silencing with some exceptions (Lachner and Jenuwein, 2002; Lachner et al., 2003).

2.5.2.1.4 Phosphorylation

This modification occurs on serine or threonine residues. Labile forms of phosphorylation involving P-N linkage of lysines or arginine have also been described (Smith et al., 1978). Histone phosphorylation has also been observed on metaphase chromosomes during condensation. (Green, 2001; Mahadevan et al., 2004; Nowak and Corces, 2004).

2.5.2.1.5 Ubiquitination

This modification of protein is primarily a signal required for protein turnover and has also been involved in various physiological processes such as spermiogenesis, DNA repair, and transcription. Ubiquitinated H2A and H2B were
preferentially found in transcriptionally active chromatin, supporting a positive role of this modification in gene expression (Zhang, Y., 2003).

2.5.2.1.6 **ADP-ribosylation and other modification**

Nishizuka and colleagues first described Poly-ADP ribosylation (Nishizuka et al., 1968). It can be catalyzed on arginine or glutamine residues by the poly (ADP-ribose) polymerase. All four core histones and linker histone H1 can be used as a substrate for this reaction (Rouleau et al., 2004). Other than this, biotinylation (Camporeale et al., 2004) as well as SUMOlation (Shiio and Eisenman, 2003) of histones has also been reported.

![Figure 2.5 The known histone post translational modifications (adapted from Khorasanizadeh, 2004).](image)

**2.5.2.2 ATP dependent chromatin remodeling**

The complexes involved in energy dependent chromatin remodeling are multi-protein complexes, containing 2-12 subunits (Becker and Horz, 2002). Each complex has a catalytic subunit, carrying the ATPase activity. These ATPases are highly conserved throughout evolution. In addition to the ATPase motif, proteins in the SNF2 family also contain sequence motifs similar to those found.
in DNA and RNA helicase protein families. Proteins with these helicase motifs have been divided into multiple superfamilies based upon amino acid sequences found within the motifs. By this method, the SNF2 family has been assigned to the helicase superfamily 2, which also includes the ERCC3, RAD3, PRIA, ELF4A, and PRP16 protein families (Eisen et al., 1995).

Our lab has established a family of SNF2-like nuclear ATPases by browsing the annotated human genome database. Furthermore, the homologues of these ATPases have also been established in *Xenopus* showing that vertebrates share a common family of ATPase chromatin remodeling complexes (Linder et al., 2004). The enzymes in the SNF2 family can be subdivided into several subfamilies according to the sequence motifs outside of their ATPase domain.

![Sequence similarity tree of the human SNF2-domain containing proteins](image)

Figure 2.6 Sequence similarity tree of the human SNF2-domain containing proteins (adapted from Linder et al., 2004).

Based on this analysis, seven subfamilies have been assigned, out of which nucleosome-remodeling activity has been shown only for SWI2/SNF2-related enzymes (Sudarsanam and Winston, 2000), ISWI type enzymes (Langst and Becker, 2004), and for CHD family members (Brehm et al., 2000). In addition the recently identified INO80 also shows nucleosome remodeling activity (Shen et al., 2000). Other members are known to be involved in DNA repair, recombination, as well as in transcription (Becker and Horz, 2002).
2.5.2.2.1 ISWI, a SANT-like domain-containing member of the SNF2 family

The ATPase ISWI was first discovered in *Drosophila melanogaster* because of the similarity of its ATPase domain to that of BRM (Elfring et al., 1994). NURF (Gdula et al., 1998; Martinez-Balbas et al., 1998; Xiao et al., 2001), CHRAC (Varga-Weisz et al., 1997) and ACF (Ito et al., 1999) are various chromatin-remodeling complexes, which contain the ISWI ATPase. Later ISWI containing complexes were identified in human (Barak et al., 2003; Poot et al., 2000), mouse (Lazzaro and Picketts, 2001) and *Xenopus* (Guschin et al., 2000). Two SANT-like domains in the C-terminus of the protein distinguish them from the other members of SNF2 family (Aasland et al., 1996). Homozygous null mutation of ISWI is lethal in *Drosophila* (Deuring et al., 2000). The homozygous deletion of SNF2H, a murine homologue of ISWI, is lethal, but mice with heterozygous deletion of SNF2H were normal (Stopka and Skoultchi, 2003).

2.5.2.2.2 The CHD class of remodelers are characterized by the presence of a chromodomain

Among this class of remodelers, CHD3 (MI-2α) and CHD4 (MI-2β) are mostly studied members of this family. The members of this class contain two PHD fingers in addition to the characteristic chromodomain. MI-2 was identified as a dermatomyositis-specific autoantigen. It has been shown to reside in the NURD complex (for nucleosome remodeling and deacetylation)(Knoepfler and Eisenman, 1999).

Like ISWI, the MI-2 ATPase is an active enzyme for nucleosome remodeling, able to disrupt histone-DNA interactions and to induce nucleosome sliding on DNA fragments (Brehm et al., 2000). Interestingly, MI-2β has also been shown to combine deacetylation and ATP dependent remodeling (Wade et al., 1999).
2.5.2.2.3 The SWI/SNF complexes

2.5.2.2.3.1 SWI/SNF complexes

The yeast SWI/SNF complex was the first chromatin-remodeling complex to be described (Stern et al., 1984). The genes encoding its various subunits were originally identified in two independent screens for mutants affecting either mating type switching or growth on sucrose (Sudarsanam and Winston, 2000; Workman and Kingston, 1998) and hence were named “switching defective” and “Sucrose non-fermenting”. The biochemical evidence for a direct connection between chromatin and SWI/SNF function was provided by the findings that the SWI/SNF complex could alter nucleosome structure in an ATP-dependent manner (Vignali et al., 2000; Workman and Kingston, 1998).

A closely related yeast chromatin-remodeling complex is called RSC for “Remodel the Structure of Chromatin” (Cairns et al., 1994; Cairns et al., 1999). This complex contains about 15 subunits, sharing two identical and at least four homologous subunits with the ySWI/SNF complex (Cairns et al., 1998; Wang, 2003). STH1 is the paralogue of the SWI2/SNF2 ATPase subunit in the RSC complex. Furthermore, RSC8, RSC6 and SFH1 in RSC correspond to SWI3, SWP73 and SNF5 in ySWI/SNF, respectively. RSC complexes are about 10 times more abundant than SWI/SNF complexes.

Despite these structural similarities, there are several important functional differences between ySWI/SNF and RSC. In contrast to ySWI/SNF, RSC functions are required for yeast viability. A Genome wide gene expression analysis revealed that ySWI/SNF and RSC regulate different, largely non-overlapping sets of target genes. RSC complexes have also been shown to be involved in sister chromatid cohesion and chromosome segregation, which indicates the broader role of these complexes in chromatin dynamics (Baetz et al., 2004; Hsu et al., 2003; Huang et al., 2004; Wong et al., 2002).
Homologous ATPase complexes have also been isolated in *Drosophila* and mammals. Human cells contain two distinct SWI2/SNF2 like ATPase subunits, named hBRM and BRG1, which are equally similar to yeast SWI2/SNF2 and STH1. In contrast, *Drosophila* contains only a single protein corresponding to yeast SWI2/SNF2 or STH1, called BRM (Papoulas et al., 1998; Tamkun et al., 1992). In higher eukaryotes, the remodeling complexes of SWI/SNF class are referred to as BAP (BRM associated proteins) in *Drosophila* and hSWI/SNF-BAF (BRG1/hBRM- Associated factors) in mammals, whereas the RSC orthologue are referred to as PBAP or hSWI/SNF-PBAF in *Drosophila* or mammals, respectively. BRM was originally discovered as a suppressor of polycomb and therefore was classified as a trithorax-group protein. Two BRM associated proteins, the common subunit Moira (MOR) and the BAP selective subunit OSA, are also encoded by trxG genes (Brizuela and Kennison, 1997; Collins et al., 1999; Collins and Treisman, 2000; Crosby et al., 1999; Kennison and Tamkun, 1988; Vazquez et al., 1999).

Most of the *Drosophila* and mammalian subunits are equally similar to their counterparts in ySWI/SNF and RSC. The two exceptions are OSA/BAF250 and Polybromo/BAF180, which are the signature subunits of the ySWI/SNF or RSC type subfamilies respectively. OSA/BAF250 is related to the ySWI/SNF subunit Swi1, whereas there is no homologue in RSC (Collins et al., 1999; Collins and Treisman, 2000; Dallas et al., 2000). Conversely, Polybromo/BAF180 is structurally related to the RSC1, RSC2 and RSC4 proteins, but lacks a counterpart in ySWI/SNF. In addition, BAF can contain either BRG1 or BRM as the core motor subunit, whereas PBAF contains only BRG1 (Mohrmann et al., 2004; Xue et al., 2000). In mammals there appear to be additional tissue specific subunits of SWI/SNF remodelers. A number of studies have reported additional sub-complexes in which the SWI/SNF type remodelers are associated with other factors such as BRCA1 (Bochar et al., 2000) or components of the histone deacetylating SIN3 complex (Sif et al., 2001). It has been shown that BAF53b is
### Table 1 (Roberts and Orkin, 2004; Wang, 2003)

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Drosophila</th>
<th>Human</th>
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<tbody>
<tr>
<td><strong>SWI/SNF</strong></td>
<td>BAP</td>
<td>PBAF</td>
</tr>
<tr>
<td><strong>SWI2/SNF2</strong></td>
<td>STH1</td>
<td>BRM</td>
</tr>
<tr>
<td><strong>SNF5</strong></td>
<td>SFH1</td>
<td>SNR1</td>
</tr>
<tr>
<td><strong>SWI3</strong></td>
<td>RSC8</td>
<td>Moira</td>
</tr>
<tr>
<td><strong>SWI1</strong></td>
<td>OSA</td>
<td>BAF250</td>
</tr>
<tr>
<td><strong>ARP7</strong></td>
<td>RSC6</td>
<td>BAP60</td>
</tr>
<tr>
<td><strong>SWP73</strong></td>
<td>RSC3</td>
<td>BAP111</td>
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<tr>
<td><strong>RSC9</strong></td>
<td>ENL</td>
<td>ENL</td>
</tr>
<tr>
<td><strong>RSC5,7,10,13-15</strong></td>
<td>TFG3</td>
<td>RSC9</td>
</tr>
<tr>
<td><strong>SNF11</strong></td>
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part of a brain specific complex (Olave et al., 2002a), while BAF60c could be a part of heart specific complex since it shows a heart specific expression pattern in early embryonic stage; later it is also expressed in somites (Lickert et al., 2004). Various SWI/SNF complexes have been summarized in table 1.

2.5.2.2.3.2 Interaction motifs in the SWI/SNF class of remodelers

The distinguishing feature of the SWI/SNF class of chromatin remodelers is the presence of a bromodomain in the ATPase subunit, which is absent in ISWI, CHD/MI-2 and INO80 type remodelers (Eberharter and Becker, 2004).

Bromodomains are 90 amino acid long modules. Bromodomains recognize acetylated lysines in histone tails (Dhalluin et al., 1999; Hassan et al., 2002; Hudson et al., 2000; Jacobson et al., 2000; Marmorstein and Berger, 2001; Owen et al., 2000; Zeng and Zhou, 2002). PBAP and PBAF contain another bromodomain protein called polybromo or BAF180. Polybromo contains six bromodomains, which are required for targeting of remodelers to hyperacetylated yeast chromatin domains (Mohrmann et al., 2004), however, in Drosophila it was found that the deletion of the bromodomain of BRM neither affects BRM function nor chromatin binding (Elfring et al., 1998).

SWI/SNF remodeling complexes harbor a number of distinct DNA binding domains. Polybromo and BAP111 each contain a high mobility group (HMG) domain. HMG domains are highly conserved protein folds, which contact the DNA in the minor groove and induce a sharp bend (Thomas and Travers, 2001). BAP111 and its human orthologue BAF57 are common subunits of SWI/SNF

![Diagram of BAF180 and Brg1 proteins](image-url)
remodelers in higher eukaryotes, but are absent in yeast. Studies on *Drosophila* established that BAP111 is required for normal BRM complex function in vivo (Papoulas et al., 2001).

In addition to its highly conserved HMG domain, Polybromo harbors two, less well conserved, C2H2-type zinc fingers. The putative double C2H2 zinc finger motif is present in *Drosophila* and *C. elegans* polybromo, but absent in chicken and human. The C-terminus of BAP170 also contains a highly conserved, double zinc finger motif comprising a canonical C2H2 zinc finger, and a second one in which the spacing between the two cysteine residue is somewhat larger. The strict conservation of the zinc finger motifs in BAP170 suggests that they might be functionally important (Mohrmann et al., 2004).

BAP 170 contains a second DNA binding motif in its N-terminus, an AT rich interaction domain (ARID). ARID domain proteins are also present in yeast Swi1, *Drosophila* OSA and mammalian BAF250, which define the ySWI/SNF, BAP and BAF subclass, respectively (Collins et al., 1999). ARID domains are sometimes referred to as BRIGHT domains and have also been implicated in sequence-specific as well as sequence-independent DNA binding (Gregory et al., 1996; Herrscher et al., 1995; Wilsker et al., 2002). The ARID harbors a helix-turn-helix region and, as reflected by its name, preferentially binds AT-rich sequences. ARID domain in OSA binds DNA without sequence specificity while dead ringer is an example of a sequence-specific DNA binding ARID containing protein. The ARID of BAF 250 has been implicated in transcriptional co-activation of hormone receptors (Inoue et al., 2002), suggesting that it acquires specificity through interactions with other cofactors.

Actin is known for many cellular functions in the cytoplasm of eukaryotic cells, including processes like muscle contraction, cell motility, or cytokinesis. Actin is highly expressed and is in fact the most abundant protein in many cell types. The purification of actin by biochemical means led to the striking finding that it was a tightly bound subunit of SWI/SNF family of remodeling complexes
(Olave et al., 2002b; Papoulas et al., 1998; Zhao et al., 1998), which was for a long time considered as a contaminant. Actin Related Proteins (ARPS), consist of a large and diverse group of proteins that share between 10% to 80% sequence similarities with actin (Schafer and Schroer, 1999). The actin fold is conserved in the Arps, but there is much less conservation in flanking regions (Robinson et al., 2001). The presence of stoichiometric amounts of actin and Arps in diverse chromatin remodeling complexes has been firmly established (Olave et al., 2002b). Yeast SWI/SNF and RSC contain ARP7 and ARP9, whereas *Drosophila* BAP and PBAP and human BAF and PBAF contain one ARP, BAP55 and BAF53 respectively and actin (Cairns et al., 1998; Peterson et al., 1998; Wang et al., 1996a; Wang et al., 1996b). It has been proposed that Actin and ARPs can modulate binding of the remodeling complex to chromatin or to the nuclear matrix, stimulate the DNA-dependent ATPase activity, promote complex assembly and stability, histone binding, or remodeling and translocation (Boyer and Peterson, 2000; Rando et al., 2002; Shen et al., 2003; Szerlong et al., 2003).

### 2.5.2.2.3.3 Differential targeting of SWI/SNF remodelers

Results in *Drosophila* suggest that BRM is involved in transcription of most genes (Armstrong et al., 2002), however, one way in which variation in subunit composition can direct functional differentiation is through unique gene targeting. Immunolocalization on larval salivary gland polytene chromosomes revealed that OSA and Polybromo, the defining subunits of BAP and PBAP, each display distinct, albeit overlapping genome-wide distributions (Mohrmann et al., 2004). Genome wide expression studies in yeast revealed that ySWI/SNF and RSC each regulate different sets of target genes (Angus-Hill et al., 2001; Holstege et al., 1998; Sudarsanam et al., 2000).

### 2.5.2.2.3.4 Nucleosomal remodeling by SWI/SNF complexes

Nucleosomal remodeling by SWI/SNF complexes is either by displacement of histone octamers, sliding of histone octamers or by stable
alteration of nucleosomes as shown in figure 2.7. (Boeger et al., 2004; Bruno et al., 2003; Fan et al., 2003; Haushalter and Kadonaga, 2003; Langst and Becker, 2004). It has also been shown that BRG1 opens centrally located sites more then an order of magnitude better then SNF2H, an ISWI homologue. This capability of BRG1 is caused by its ability to create DNA loops on the surface of a nucleosome, even when that nucleosome is constrained by adjacent nucleosomes (Fan et al., 2003). It has been also shown that SWI/SNF-complexes move nucleosomes to the DNA ends in the absence of histone H1. In the presence of histone H1, SWI/SNF slides octamer to more central positions and does not promote nucleosome movements off the ends of the DNA (Ramachandran et al., 2003). These observations shed light on the roles of H1 and SWI/SNF in modulating chromatin structure.

Figure 2.8 Nucleosomal remodeling by SWI/SNF complexes (modified after Vignali et. al., 2000).
2.5.2.3.5 Function of RSC class of remodelers

RSC complexes have been shown to be involved in sister chromatid cohesion and segregation (Baetz et al., 2004; Hsu et al., 2003; Huang et al., 2004; Wong et al., 2002). The accurate transmission of the genome during cell division requires chromatin restructuring. Various studies revealed that several RSC subunits, namely STH1, SFH1, RSC3 and RSC9 are essential for cell cycle progression through G2 and mitosis (Angus-Hill et al., 2001; Damelin et al., 2002). Current evidence suggests that cell cycle failure in RSC mutant is not due to defective transcription of cell cycle control genes (Ng et al., 2002) rather it appears that the spindle checkpoints might play a critical role in the G2/M arrest caused by RSC mutants (Tsuchiya et al., 1992). An independent genetic screen for haploinsufficient modifiers of chromosome segregation fidelity further supported this notion (Baetz et al., 2004). Thus, RSC mediates chromatin restructuring, independent of transcription regulation and appears to be directly required for chromosome segregation.

Although the molecular mechanisms remain unclear, recent studies suggest that RSC is required for the loading of cohesin on chromosome arms, and for kinetochore function (Baetz et al., 2004; Hsu et al., 2003; Huang et al., 2004). The cohesin complex is the molecular machine responsible for the controlled pairing of sister chromatids prior to their segregation (Nasmyth et al., 2000; Yokomori, 2003). PBAF complexes have been shown to be localized at the kinetochore (Xue et al., 2000).

2.5.2.3.6 Function of mammalian SWI/SNF complexes

Biochemical studies revealed important functional differences between BRM and BRG1. BRG1 binds to Zn-finger proteins through a unique N-terminal domain, which is not present in BRM. Conversely, BRM interacts with two ankyrin repeat proteins that are critical components of notch signal transduction (Kadam and Emerson, 2003). Thus, BRG1 and BRM complexes may direct distinct cellular processes through recruitment to specific promoters mediated by
protein-protein interaction that is unique to each ATPase. Moreover, PBAF, but not BAF, is necessary for ligand-dependent transactivation by several nuclear hormone receptors, such as VDR and PPAR (Lemon et al., 2001). However, other researchers reported that the BAF complex, but not PBAF complex, is required for glucocorticoid receptor dependent transcription (Trotter and Archer, 2004).

Studies on mammalian SWI/SNF remodelers also uncovered functional differences. Gene inactivation experiments in mice revealed that the in-vivo importance of the very similar mammalian paralogs, BRM and BRG1, is quite different. BRM knock-out mice are viable and display only a subtly altered control of cell proliferation (Reyes et al., 1998), while in contrast, BRG1 knock-out mice die already during early embryogenesis and animals with mono-allelic BRG1 expression are predisposed to tumors, suggesting a role in neoplasias (Bultman et al., 2000). These differences might be the results of distinct timing of expression of BRM and BRG1 (LeGouy et al., 1998) or perhaps BRG1 may compensate for loss within the BAF complex, but conversely, BRM cannot replace BRG1 in the PBAF complex. Such a scenario would implicate that inactivation of BRM will only lead to a partial loss of BAF function, whereas loss of BRG1 will completely abrogate PBAF. Alternatively, the difference between inactivation of either BRM or BRG1 might reflect the functional difference between BAF and PBAF.

2.5.2.2.3.7 SWI/SNF complexes in disease

A very important development has been the realization that inactivation of SWI/SNF complexes plays a critical causal role in the development of human cancers. Mice with mono-allelic BRG1 expression are also predisposed to tumors, albeit at a low incidence (Bultman et al., 2000). Tumors are of epithelial origin, localize in subcutaneous tissues and display glandular structures. Loss of BRG1 or mutations in BRG1 have been identified in human tumor cell lines and in some primary tumors (Wong et al., 2000). In non-small cell lung cancers, the
loss of BRG1 expression correlates with a poorer prognosis (Reisman et al., 2003). BRG1 has been implicated further in tumorogenesis by its association with proteins with an established role in lung cancers, including pRb, BRCA1, MLL (Klochendler-Yeivin et al., 2002; Roberts and Orkin, 2004). BRG1 is not only an anti-proliferation factor but some cells also require its function for cell growth or differentiation. Thus, the BRG1 containing SWI/SNF complexes act in a cell type specific manner. Whereas, some cells cannot survive without its activity, in others it activates the senescence program.

The human snf5 gene, a universal component of SWI/SNF complexes, was deleted or mutated in atypical teratoid and malignant rhabdoid tumors (ATRTs and MRTs), very aggressive cancers of early childhood. ATRTs and MRTs typically occur in the kidneys and the brain. hSNF5/INI1 mutations were also found associated with chronic myeloid leukemia, chronoid plexus carcinoma, medulloblastoma and central primitive neuroectodermal tumors (Biegel et al., 2002; Versteege et al., 2002; Versteege et al., 1998). In addition to somatic mutations, germ line mutations have been reported, which predispose carriers to various cancers, including MRTs. Typically, the wild type allele is either lost or mutated in the resulting tumor, consistent with a typical tumor suppressor phenotype. Indeed, gene inactivation studies in mice revealed that heterozygous mice survive but are prone to soft tissue tumors, resembling MRTs (Roberts et al., 2000; Roberts et al., 2002). These results pointed out the role of SNF5 in tumor inhibition. Re-expression of hSNF5 in MRT cells causes an accumulation of cells in G0/G1, cellular senescence and apoptosis through direct transcriptional activation of the tumor suppressor p16\textsuperscript{INK4a} (Ae et al., 2002; Baetz et al., 2004; Versteege et al., 2002; Zhang et al., 2002). It was also shown that hSNF5 is critical for the recruitment of BRG1 to the p16\textsuperscript{INK4a} promoter and transcriptional activation (Oruetxebarria et al., 2004). Authors also showed that the induction of cellular senescence by hSNF5 was strictly dependent upon the p16\textsuperscript{INK4a}pRb tumor suppressor pathway by using siRNA knock-down experiments.
To circumvent the periimplantation lethality seen in case of INI1 knock-out mice (Guidi et al., 2001), a reversibly inactivating conditional allele was used in mice to study hSNF5 function (Roberts et al., 2000). This experiment demonstrated that most normal, nonmalignant cells require SNF5 for their survival. They also showed that loss of SNF5 function resulted in a highly penetrant and extremely short latency development of lymphomas and rhabdoid tumors. These results indicate that oncogenic transformation due to a loss of SNF5 functions might be limited to certain cell types or to cells that contain additional mutations.

2.6 Objectives of this work

BRG1 is essential in normal development of mice as shown by homozygous deletion of \textit{brg1}. BRG1 has also been found to be associated with various human cancers. Furthermore, BRG1 heterozygous mice display tumors of epithelial origin. These observations indicate important roles of BRG1 in normal development and disease.

The periimplantation lethality of mice by homozygous deletion of \textit{brg1} precludes the investigation of the role of BRG1 in early development of mice. Thus, the function of BRG1 will be studied during early development of \textit{Xenopus}.

Furthermore the role of BRG1 will be addressed to ascertain its role in global versus selective transcriptional regulation. The role of BRG1 will also be addressed in embryonic induction and patterning.
3 MATERIALS AND METHODS

3.1 Reagents
Fine chemicals: Fluka, Merck, Sigma, USB.

Bio-chemicals
Agar (Difco); Agarose (Gibco/BRL); Ampicillin, Streptomycin, Bacto trypton, Yeast extract (Difco); Chicken serum, lamb serum (Gibco/BRL); Human choriogonadotrophin (Sigma); Levamisol (Vector Laboratories).

Enzymes and proteins
Alkaline phosphatase (Roche), BSA fraction V, Chymostatin, Leupeptin, Pepstatin (Sigma); DNase1 (Stratagene); Klenow enzyme (Roche); MMTV reverse transcriptase (Gibco/BRL); Restriction endonuclease with 10x restriction buffer system (New England Bio Labs, Roche, Fermentas); RNaseA (Sigma); RNAsin (Promega); T3, T7 and SP6 RNA polymerase with 5x incubation buffer (Promega); Taq DNA polymerase with 10x PCR buffer (Perkin Elmer); Pfu polymerase with 10x PCR buffer (Stratagene); Proteinase K ( Sigma); RNase free DNase I (Promega); Pre-standard protein molecular weight standard low and high range (Gibco/BRL).

Immunocchemical
Sheep anti-mouse IgG coupled with alkaline peroxidase (1:5000, Roche); Sheep anti-Digoxygenin Fab fragment coupled with alkaline phosphatase (Roche); BM Purple solution (Roche).

3.2 Devices
Branso Digital Sonifier; 250-D
FRENCH Pressure Cell Press
Gel filtration columns QuickSpin G-50 (Roche).
Glass injection needles: Glass 1BBL W/FIL 1.0 mm (World Precision Instrument). Injector Plt-100 (Digitimer Ltd.).
Incubator: Driblock DB1 and DB20 (Teche).
Micro needle Puller P-87 (Sutter Instrument).
Micromanipulator: Mm-33 (Science Products).
Microscopes: Stereomicroscopes Stemi SV6 and Stemi SV11 (Zeiss).
Microsurgery: Gastromaster (Xenotek Engineering).
Nylon membrane: Hybond TM N (Amersham).
PVDF membrane: Millipore
Software: Adobe Photoshop 6.0; Illustrator 9.0 (Adobe); McVector 6.0 (Oxford Molecular Group); Microsoft Office 98 (Microsoft).
Spectrophotometer: GeneQuant II (Pharmacia Biotech).
Thermocycler: Primus 96 plus (MWG).
Centrifuges: Eppendorf centrifuge 5417C (Eppendorf); centrifuge 2.0 RS (Haereus); Sorvall RC-5B (Dupont).

3.3 Nucleic acids

3.3.1 Size standard
DNA standard: Gene Ruler™ 1kb DNA ladder (Fermentas). The DNA ladder yields the following 14 discrete fragments (in base pairs): 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250.
DNA standard: Gene Ruler™ 100bp DNA ladder plus (Fermentas). The DNA ladder yields the following 14 discrete fragments (in base pairs): 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100.

3.3.2 Oligonucleotides
All oligonucleotides were synthesized from MWG Biotech or by Biomer.

Random Hexamer
RR13: 5’- NNNNNC -3’ (N =G, A, T or C)

Primers used in RT-PCR analyses
**Xenopus nodal related 3:** (Armstrong et al., 1998)
Forward: 5'- TGAATCCACTTGTGCAGTTCC -3'  
Reverse: 5'- GACAGTCTGTGTTACATGTCC -3'

**Xenopus siamois:** (Armstrong et al., 1998)  
Forward: 5' - CCATGATATTTCATCCAACTGTGG -3'  
Reverse: 5'- GTTCTGTTTCTAGATCTGGTAC -3'

**Xenopus brachyury:** (Sun et al., 1999)  
Forward: 5' - TTCTGAAGGTGAGCATGTCG -3'  
Reverse: 5'- GTTTGACTTTTGCTAAAAGAGACAGG -3'

**Xenopus fd-1'**: (Friedle et al., 1998)  
Forward: 5'- AGGAGATGAAACTGGAGGGAGCTTAA -3'  
Reverse: 5'- GCCAAGGTAGCCATCATTAGAGAGAC -3'

**Xenopus goosecoid:** (Prothmann et al., 2000)  
Forward: 5'- ACAACTGGAAGCACTGGA -3'  
Reverse: 5'- TCTTATTCCAGAGGAACC -3'

**Xenopus nodal related 6:** (Yang et al., 2002)  
Forward: 5'- TCCAGTATGATCCATCTGTGC -3'  
Reverse: 5'- TTCTCGTTTCTTGTGCCTT -3'

**Xenopus myf-5:** (Shi et al., 2002)  
Forward: 5'- CTATTCCAGAATGGAGATGGT -3'  
Reverse: 5'- GTCTTGGAGACTCTCAATA -3'

**Xenopus myodb:** (Steinbach et al., 1998)  
Forward: 5'- TGACAGCCCAAATGACTCGAGACTT -3'  
Reverse: 5'- AGCAACCAGCGAGGCAGGCTGAA -3'

**Xenopus histone h4:** (Niehrs et al., 1994)  
Forward: 5'- CGGGATAACATTCCAGGGTATC -3'  
Reverse: 5'- ATCCATGGCGTAACTGTCTTCCT -3'
3.3.3 Plasmids

3.3.3.1 Plasmids for \textit{in-vitro} transcription

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Linearized by</th>
<th>Polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCS2+ wt \textit{hbrg1}</td>
<td>Nar1</td>
<td>Sp6</td>
</tr>
<tr>
<td>pCS2+ dn \textit{hbrg1}</td>
<td>Nar1</td>
<td>Sp6</td>
</tr>
<tr>
<td>pCS2+ wt \textit{hbrm}</td>
<td>Asp718I</td>
<td>Sp6</td>
</tr>
<tr>
<td>pCS2+ dn \textit{hbrm}</td>
<td>Asp718I</td>
<td>Sp6</td>
</tr>
<tr>
<td>pCS2+ wt \textit{xiswi}</td>
<td>Not1</td>
<td>Sp6</td>
</tr>
<tr>
<td>pCS2+ dn \textit{xiswi}</td>
<td>Not1</td>
<td>Sp6</td>
</tr>
<tr>
<td>pCS2+ wt \textit{xbrg1}</td>
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<td>Sp6</td>
</tr>
<tr>
<td>pCS2+ wt \textit{xbrg1}</td>
<td>Not1</td>
<td>Sp6</td>
</tr>
</tbody>
</table>

3.3.3.2 Plasmids for dig-labeled RNA \textit{in-situ} hybridization probes

<table>
<thead>
<tr>
<th>RNA antisense Probe</th>
<th>Plasmids linearized by</th>
<th>Polymerase</th>
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<tbody>
<tr>
<td>Xnr3</td>
<td>EcoR1</td>
<td>T7</td>
</tr>
<tr>
<td>Chordin</td>
<td>EcoR1</td>
<td>T7</td>
</tr>
<tr>
<td>Goosecoid</td>
<td>EcoR1</td>
<td>T7</td>
</tr>
<tr>
<td>Xbra</td>
<td>EcoR1</td>
<td>T7</td>
</tr>
<tr>
<td>Xvent2</td>
<td>Rupp lab</td>
<td></td>
</tr>
<tr>
<td>Xpo</td>
<td>HindIII</td>
<td>T7</td>
</tr>
<tr>
<td>XmyoDb (pRR3)</td>
<td>HindIII</td>
<td>T7</td>
</tr>
<tr>
<td>Xmyf-5 (pRR65)</td>
<td>Rupp lab</td>
<td></td>
</tr>
<tr>
<td>N-\text{\textbeta}-tubulin</td>
<td>BamHI</td>
<td>T3</td>
</tr>
<tr>
<td>Nkx2.5</td>
<td>HindIII</td>
<td>T7</td>
</tr>
<tr>
<td>Pax8</td>
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<td>Troponin</td>
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<td>Mlc35</td>
<td>BamHI</td>
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<tr>
<td>HoxB9</td>
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3 Materials and methods

<table>
<thead>
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<td>Shh</td>
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<td>T3</td>
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<td>Xbrg1</td>
<td>Spe1</td>
<td>T7</td>
</tr>
<tr>
<td>Xini1</td>
<td>EcoR1</td>
<td>T3</td>
</tr>
</tbody>
</table>

3.4 Bacterial manipulation
Preparation of competent cells and transformation was performed according to standard methods (Sambrook et al., 1989).

3.5 Embryological Methods

3.5.1 Solutions

Cystein: 2% L-Cystein in 0.1 x MBS or 0.1 x MMR (pH 7.8 at 23°C, adjusted with 5 M NaOH).
Human Chorionicgonadotropin (HCG): 1000 I.U/ml HCG in ddH2O.
MEMFA: 0.1 M MOPS, 2mM EGTA, 1 mM MgSO4, 3.7% formaldehyde (pH 7.4 at 23°C).
1 x Modified Barth’s Saline (MBS): 5 mM HEPES, 88 mM of NaCl, 1 mM of KCl, 0.7 mM CaCl2, 1 mM MgSO4, 2.5 mM NaHCO3 (pH 7.6 at 23°C). Add the CaCl2 before use.
0.1 x MBS/Gentamycin: 0.1 x MBS +10 μg/ml Gentamycin in cell culture water.
0.5 x MBS/BSA: 0.5 x MBS, 1 mg/ml BSA, 10 μg/ml Gentamycin in cell culture water.
0.5 x MBS/CS: 0.5 x MBS with 20% chicken serum and stored at -20°C.
1 x Marc’s Modified Ringer’s Solution (MMR): 0.1 M NaCl, 2 mM of KCl, 1 mM MgCl2, 2 mM CaCl2, 5 mM HEPES (pH 7.5 at 23°C)
0.1 x MMR: 0.1 x MMR +10 μg/ml Gentamycin.
0.1 x MMR/Ficoll: 0.1 x MMR, 6%(w/v) Ficoll, 10 μg/ml Gentamycin.
0.4 x MMR/Ficoll: 0.4 x MMR, 6%(w/v) Ficoll, 10 μg/ml Gentamycin.
3.5.2 Experimental animals

Adult wild type (Nasco) *Xenopus laevis* frogs were used. The frogs were kept in water at 16-19°C and at a population density of 5 L water per frog. Frogs were fed three times weakly.

3.5.3 Superovulation of the female *Xenopus laevis*

*Xenopus laevis* females were stimulated for laying eggs by injecting 500 units of human chorionic gonadotropin into the dorsal lymph sac and incubated at 15-23°C overnight. Frogs layed eggs about 12 hours after injection.

3.5.4 Preparation of testis

The male frog was anaesthetized by 0.1 g 3-Aminobenzoesaueure-ethylester per 100 ml ddH₂O for 20 min, cooled down in ice and killed by decapitation. The two testes were taken from the abdominal cavity by opening and pulling out the yellow fat body, to which they are connected by connective tissue. The testis were either used immediately or stored in MBS/CS plus antibiotics for maximum of 7 days.

3.5.5 *In-vitro* fertilization of eggs and culture of the embryos

Freshly laid eggs were *in-vitro* fertilized either by piercing testis with a forceps or by mincing a portion of testis to release sperms. Afterwards they were cultured in 0.1 x MBS or 0.1 x MMR at 15-23°C in 110 mm petriplates.

3.5.6 Jelly coat removal

In order to prepare embryos for injection with mRNA (A) or morpholino oligos, after approx.1 hpf, the jelly coat was removed in a 2% cystein solution for 5 min under agitation. Embryos were then washed three times with 0.1 x MBS and were cultured in 0.1 x MBS or 0.1 x MMR at 15-23°C.
### 3.5.7 Injection of embryos

The glass injection needles were pulled with Microneedle Puller (Sutter Instrument, model P-87). They were filled with 1-2 $\mu$l nucleotide acid containing solution just before the injection. The needles were placed into the holder of the injection equipment (Medical System, model Pi-100). The injection volume was adjusted by manual breaking of the needle tip and choice of the injection pressure and/or the injection duration. Totally, 2.5-10 nanoliter solution was injected per embryo. Embryos were usually injected at 2-4-cell stage. After injection, embryos were incubated in 0.1 x MMR at 15-23°C until the desired developmental stages. The medium was changed every day to increase survival.

### 3.5.8 Preparation of explants

For the preparation of explants, injected embryos were transferred in 3 cm cell culture dishes covered with 1% agarose. The culture medium for explants was 0.5 x MMR. The animal caps were removed with Gastromaster (Xenotek Engineering) after seven hrs post fertilization. The caps were transferred individually into a 96-well plate covered with 50 $\mu$l 1% agarose and filled with 0.5 x MMR.

### 3.6 Histological methods

#### 3.6.1 Solution

**AP buffer**: 100 mM Tris HCl (pH 9.5), 50 mM MgCl$_2$, 100 mM NaCl, 0.1% Tween 20, 5mM Levamisol.

**AP staining solution**: 4.5 $\mu$l NBT, 3.5 $\mu$l BCIP in 1 ml AP buffer or BM purple from Roche.

**MEMFA**: 0.1 M MOPS, 2 mM EGTA, 1 mM MgSO$_4$, 3.7% formaldehyde (pH 7.4 at 23°C).

**PBS**: 137 mM of NaCl, 2.7 mM of KCl, 8 mM Na$_2$HPO$_4$, 1.7 mM KH$_2$PO$_4$ (pH 7.2 at 23°C).

**PBT**: PBS plus 2 mg/ml BSA plus 0.1%(v/v) triton-X-100.
3.6.2 Fixation of embryos

Embryos were fixed in MEMFA for 2 h under rotation on a vertical wheel. The embryos were washed thrice with PBS. The vitelline membrane was manually removed with a pair of sharp forceps from the embryos used for immunohistochemistry. These embryos were finally washed once with methanol and then stored in methanol at -20°C until use. Embryos for in-situ hybridization were incubated for several hours in 100% ethanol. Rinsed once more with 100% ethanol and then stored in 100% ethanol at -20°C until use.

3.6.3 Immunocytochemistry

1. Fix embryos in MEMFA for two hours at room temperature with rotation. Rinse in PBS, remove vitelline membrane manually, and store the embryos in 100% methanol.
2. Rehydrate by 75%, 50%, 25% methanol in PBS with each wash of 5 minutes.
3. Incubate in PBT for 15 min.
4. Block protein-binding sites by incubating embryos in PBT plus 10% heat inactivated goat serum at room temperature for one hour.
5. Remove blocking solution and add primary antibody; incubate overnight at 4°C.
6. Wash vials by filling them for at least 5 times with each wash for one hour by PBT.
7. Blocked as above (step 4) for one hour.
8. Add secondary antibody coupled with AP in blocking solution and incubate at 4°C overnight.
9. Washing was done with at least five changes, 4 ml of PBT per wash for one hour each.
10. Wash twice in AP buffer for 5-20 minutes.
11. Staining was performed in 1 ml staining solution in dark.
12. After staining, embryos were washed in PBS and fixed overnight. The embryos were stored in PBS at 4°C.
3.7 Protein Methods

3.7.1 SDS-PAGE and Western blotting

SDS-PAGE (SDS-polyacrylamide gel electrophoresis) and Western blotting were carried out according to standard protocols (Sambrook et al., 1989). The signals were detected by ECL kit (Amersham) as per manufacturer’s instruction. In short, 7.5% gels were used to resolve the proteins with a constant voltage of 120V and 400mA for one and a half hour. The protein was transferred on to the PVDF membrane by the wet transfer system from BioRad for 90 minutes at 120V and at 4°C. The membrane was soaked into 5% milk in TBST to block nonspecific binding sites. The membrane was washed once with TBST for 15 minutes and twice in TBST for 5 minutes each. The membrane was incubated with primary antibody against xBRG1 antibody XB3F1 at a dilution of 1:20 for overnight at 4°C. The membrane was washed as described above and then incubated with anti-Rat IgG and IgM Peroxidase conjugated antibody at a dilution of 1:5000 for one hour at room temperature. The membrane was washed once in TBST for 15 minutes and then for four times in TBST each for 5 minutes. The signal was detected by ECL solution. To detect β-CATENIN signal, membrane was stripped in stripping buffer for 30 minutes at 60°C, blocked and proceeded as described above with antibody p14L (Schneider et al., 1993), specific for X β-CATENIN.

3.7.2 Immunoprecipitation

Xenopus embryos extracts was prepared in RIPA lysis buffer containing protease inhibitors by incubating them for 1 hour on ice. Protein G beads were blocked in equal volume of 20% BSA for two hours at 4°C. Blocked beads were incubated with anti-BRG1 antibody XB3F1 rat monoclonal antibody supernatant for another two hrs at 4°C. Antibody coated beads were used for Immunoprecipitation of endogenous or in-vitro translated xBRG1 by incubating them with whole embryo lysate or in-vitro translated protein for overnight at 4°C.
Next day beads were washed three times with 150 mM RIPA lysis buffer containing protease inhibitors and two times with 500 mM RIPA lysis buffer. The immunoprecipitated proteins were detected by western blotting using the antibody as indicated in figures.

Immunoprecipitation of the protein after formaldehyde cross-linking was done as described above with some modifications. The beads coating was done with 20% BSA along with 2.5 μg/μl sonicated herring sperm DNA. After immunoprecipitation, beads were given two additional washings with LiCl solution. For crosslinking, 100 embryos were incubated in 1 ml of 1% formaldehyde under various conditions as shown in figures. The cross-linked embryos were washed twice with ice cold PBS. Four milliliters of RIPA lysis buffer with protease inhibitors was added to the embryos and embryos were sheared with 200 μl pipette tips. These sheared embryos were applied to French press at 1100 psi twice.

**Composition of RIPA buffer**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NP40</td>
<td>10 ml</td>
</tr>
<tr>
<td>5% Sodium deoxycholate</td>
<td>10 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>1 ml</td>
</tr>
<tr>
<td>5M NaCl</td>
<td>3 ml</td>
</tr>
<tr>
<td>0.5M EDTA</td>
<td>1 ml</td>
</tr>
<tr>
<td>1M Tris pH8.0</td>
<td>5 ml</td>
</tr>
<tr>
<td>Double distilled H₂O</td>
<td>70 ml</td>
</tr>
</tbody>
</table>
### 3.8 Molecular biological methods

#### 3.8.1 Isolation of nucleic acids

##### 3.8.1.1 Mini-preparation with Qiagen kit

Plasmid DNA mini-preparations were carried out using Qiagen mini-preparation kit’s standard protocol.

##### 3.8.1.2 Isolation of RNA

The embryos or explants were collected at the desired developmental stage in 1.5 ml eppendorf tubes. Pipette off the buffer as much as possible and add Trizol (GibcoBRL), 60 μl per explant and 200 μl per embryo, vortex for 5-10 min at room temperature. If not used immediately, trizol samples were stored at 70°C. Two micro liter of chloroform was added per 10 μl of trizol amount and then shaken vigorously for 15 seconds and incubated for 3 minutes. The samples were then centrifuged for 20 minutes at 11900 RCF at 4°C. The upper phase was collected and incubated with 0.5 volume of Isopropanol at room temperature for 10 minutes and then centrifuged at 4°C for 10 minutes at 11900 RCF. The pellet was washed with 70% ethanol. The ethanol was removed and dried. The RNA was dissolved in RNase free water in a volume of 2 μl per explant or 25 μl per embryo.
3.8.2 Analysis and manipulation of nucleic acids

3.8.2.1 Gel electrophoresis of nucleic acids

DNA or *in-vitro* synthesized RNA was isolated in horizontal agarose gel. Depending upon fragment size, 1% or 1.7% agarose gel was used. After electrophoresis, the gels were photographed. 100bp or 1kb DNA ladder was used as size standard.

3.8.2.2 Isolation of DNA fragments from agarose gel

In order to isolate DNA fragments after electrophoresis from agarose gel, the appropriate band was cut out under long-wave UV light. The DNA was extracted from the gel with Qiagen gel-extraction kit.

3.8.2.3 Cloning methods

The cloning of DNA has been performed according to standard methods (Sambrook et al., 1989).

3.8.3 Polymerase chain reaction (PCR)

3.8.3.1 PCR amplification of *xbrg1* c-DNA fragment for cloning

The reaction was accomplished in a total volume of 50 μl. The reaction mixture contained 1 μl of the cDNA template, 25 pmol each primer, 0.5 mm dNTPs, 1 U advantage taq polymerase and 1x buffer.

Following Primers were used:

**Primers for xbrg1**

**Forward Primer**

5’ TGTCGGTGATAGTTGGTGGAAAAC 3’

**Reverse Primer**

5’ AGGGGGTAAAGGAATGTGATG 3’

Since normal PCR gave a smear in case of xbrg1, Touch down PCR was carried out to obtain full-length c-DNA for xbrg1 as following:
3 Materials and methods

Step 1.  95°C  1 Min.
Step 2.  95°C  15 Seconds
         60°C  30 Seconds
         68°C  6 Min
for 5 cycles
Step 3.  95°C  15 Seconds
         60°C  30 Seconds (temp decrease @ 0.5°C per cycle)
         68°C  6 Min
for 20 cycles
Step 4.  95°C  15 Seconds
         50°C  30 Seconds
         68°C  6 Min
for 10 cycles
Step 5.  72°C  10 Min

The PCR obtained a band of about 5Kb. The band was excised from the
gel and cloned into PCRII TOPO vector. The clones were checked by EcoR1
restriction digestion. The ORF only of \textit{xbrg1} was further sub cloned into pCS2+
vector with a flag tag at the C-terminus. The primers used were having EcoRV
and Xho1 restriction sites, which were used to clone \textit{xbrg1} into Stu1 and Xho1
restriction sites of pCS2+ vector. The primers were following:
Forward primer:
\begin{verbatim}
5' GAGATATCCACGATGTCCACCCCAGACCCT 3'
\end{verbatim}
Reverse Primer:
\begin{verbatim}
5' CCGCTCGAGTTATTTATCGTCATCGTCTTTGTAGTCGTCTTTCTTACCTTCC
ACTTCC 3'
\end{verbatim}
The pCS2+ vector containing full-length \textit{xbrg1} was sequenced by MWG.

3.8.3.2 RT-PCR

In RT-PCR assay, RNA was initially reverse-transcribed to yield cDNA.
The cDNA samples were normalized by PCR amplification of H4 (histone4) in the
linear range. The desired target cDNA species were amplified using specific
3 Materials and methods

primers. PCR was carried out in the exponential phase of amplification and PCR samples were loaded side by side in the agarose gel to compare their intensity.

3.8.3.3 Northern blotting

Northern blotting was carried out using standard protocol (Sambrook et al, 1989). For the detection of Xbrg1 transcripts, Northern hybridization was done using a RNA probe made from N-terminal sequence of xbrg1. The probe was about 2 kb long. The blots were exposed for three days.

3.8.4 In-vitro transcription

3.8.4.1 In-vitro reverse transcription

A cDNA pool was generated from total cellular RNA by using random oligonucleotides and reverse transcriptase (RTase) (Steinbach and Rupp, 1999). The reaction was accomplished in a total volume of 20 µl. The reaction contains 2µl of total RNA, 4µl RT buffer, 1µl RTase, 2µl dNTPs, 1µl RNAsin and 100 pmol random hexamer primers as well as 200 U reverse transcriptase. The reaction was as following: total RNA was mixed with random hexamers and RNase free water and incubated for 5 minutes at 70°C. The reaction was cooled down in ice and rest of the components were mixed. The samples were incubated at 25°C for 10 min and then transferred to 42°C for 60 minutes. The samples were incubated at 70°C for 10 min to inactivate reverse transcriptase. The sample was cooled down to 4°C. The synthesized cDNA were stored at -20°C.

3.8.4.2 In-vitro transcription for microinjection

Capped mRNA used for microinjection was in-vitro transcribed with RNA polymerase. The reaction was setup in a total volume of 50 µl containing 2.5 µg linearized plasmid DNA, 1 x transcription buffer, 0.5 mM dNTPs, 2.5 mM RNA cap structure analogue, 10mM DTT, 20U RNAsin and 40U Sp6 or 60U T3 or T7 RNA Polymerase and incubated for at least 2.5 hours at 37°C except xbrg1.
which were incubated at 20°C. The template was digested with 10 U of RNase
free DNase I by incubating the samples for 30 min at 37°C. The volume was filled
up with RNase free water to 90 μl, extracted and centrifuged with one volume of
PCI (13500 x g, eppendorf centrifuge 5415C, 14000 rpm, 23°C, 5 min). About 80
μl of the supernatant was applied on the Quick Spin column and eluted by
centrifugation (1100 x g, 23°C, 4 min). It was again centrifuged (13500 x g, 23°C,
5 min), and supernatant was transferred into a new 1.5 ml reaction tube. The
RNA was precipitated with 0.1 volume of 3 M sodium acetate and 2.5 volume of
ice-cold 100% ethanol for 30 min at -20°C. After centrifugation (13500 x g, 4°C,
20 min), the pellet was washed with 0.5 ml of 70% ethanol, air-dried and
dissolved in 10 μl RNase free water. The concentration was determined by
GeneQuant II (Pharmacia Biotech).

3.8.4.3 In-vitro transcription of dig labeled RNA probes

Plasmid was linearized and used to generate antisense RNA by using
RNA polymerases. The reactions were set up as following: in a total volume of 20
μl; 1 μg linearized plasmid DNA, 1 x transcription buffer, 0.1 mM Dig NTPs, 20 U
RNAsin and 20 U SP6 or T3 or T7 RNA Polymerase, incubated at 37°C for 2 h.
To obtain the antisense probe for xbrg1, -36 to+ 618 fragment of xbrg1 was
cloned into the pCRII-TOPO (Invitrogen Life Technologies) vector. It was
linearized with Spe1 and transcribed using T7 promoter to obtain antisense
probe.

PCR primers for the xbrg1 (-36 to +618 fragment)

Forward Primer
5’ TGTCGGTGATAGTTGGTGGAAAAC 3’
Reverse Primer
5’ TGTTTCTGGTTGAAGGAGTAGGG 3’
3.8.5 Site-directed mutagenesis

The dominant negative BRG1 was produced by doing a point mutation at position 770 and dominant negative ISWI was obtained by doing a point mutation at position 204, converting lysine into arginine.

**Mutagenesis Primers for xBRG1 (K\(^{770}\) - R)**

Forward Primer
5’ GAGATGGGCCTGGGAGGACTATTCAGACCATT 3’
Reverse Primer
5’ AATGGTCTGAATAGTCTTCCAGGCCCATCTC 3’

**Mutagenesis Primers for xISWI (K\(^{204}\) - R)**

Forward Primer
5’ GAAATGGGTCTAGGAGGACTTTGCAGACCATC 3’
Reverse Primer
5’ GATGGTCTGCAAAGTCCTTCATCCATCTTC 3’

For BRG1, mutagenesis was done on EST 234591 cDNA clone, which spans the location of aa 770. Successful mutation was confirmed by sequencing. This mutated fragment was replaced with the wild type fragment in the wild type xbrg1 clone into the XmaC1 sites and orientation was checked by restriction enzyme analysis and by sequencing.

Site-directed mutagenesis was done with the Quick Change site-directed mutagenesis kit (Stratagene, Cat.200518) according to the instruction manual. In brief, the Quick Change site-directed mutagenesis method was performed using Pfu DNA polymerase and a temperature cycler. The basic procedure utilizes a super coiled double-stranded DNA (dsDNA) template (50 ng each). The oligonucleotide primers, each complementary to opposite strands of the vector, are extended during temperature cycling by Pfu DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling (96°C 1 min, 55°C, 1 min, 72°C 2 min/kb, 20
cycles), the product was treated with DpnI. The DpnI endonuclease (target sequence: 5'-G_mATC-3') is specific for methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for mutation-containing synthesized DNA. DNA isolated from almost all E.coli strains is dam methylated and therefore susceptible to DpnI digestion. The nicked vector DNA containing the desired mutations was then transformed into XL1-Blue super competent cells. After the transformation, the competent cells repair the nicks in the mutated plasmid. The small amount of starting DNA template required to perform this method, the high fidelity of the Pfu turbo DNA polymerase, and the low number of thermal cycles all contribute to the high mutation efficiency and decreased potential for generating random mutations during the reaction. The mutated plasmids were confirmed by sequencing.

3.8.6. Designing and synthesis of antisense morpholino oligonucleotides

Following Morpholino antisense oligo was designed against xBrg1
5' CCATTGGAGGGTCTGGGGTGGACAT 3'

The antisense morpholino oligo were designed against Xbrg1 on the basis of following guidelines:
1) An optimal target sequence chosen was 25-base pair long to obtain maximum efficiency.
2) were within in the region from the 5´cap through the first 25 bases of coding sequence.
3) had approximately 50% GC content.
4) had little or no self-complementarity.
5) having less then seven total guanine and less then three contiguous guanines in a 25- mer oligo.

Based on above criteria following oligo was synthesized from Gene-Tool, LLC.

Following is the sequence of standard control morpholino oligos supplied by company:
5' CCTCTTACCTCAGTTACAATTTATA 3'
3.8.7. Expression and Purification of GST-xBRG1 fusion protein for the generation of monoclonal antibody

For the generation of monoclonal antibodies against xBRG1 a fragment covering aa 202 to aa 282 was sub-cloned into pGEX4T3 vector to generate a GST fusion protein. The primers used were following:

**Primers for GST- xBRG1 fusion protein construction in PGEX: aa202-282**
Forward Primers:
5' CGGGATCCCCCATGCCTGGGATGCAACAA 3'
Reverse Primers:
5' GGAATTCGGTCAAGTGGCAGGAGCAGCCATT 3'

The \textit{gst-xbrg1} construct was transformed in to BL21 E.Coli host strain. Five clones were tested for optimization of the fusion protein production. The clones were grown until the cell density reached at O.D. 0.7. The cells were induced by 1mM IPTG for 2hrs. This initial screening yielded two clones, giving good yield of fusion protein as determined by SDS page analysis. These two clones were further tested. The clone yielding better amount of fusion protein was chosen for large-scale production of fusion protein.

This clone was grown in 2.5 liter of culture volume until the cell density of equivalent to O.D; 0.7 was reached. The bacterial cells were induced as described. After induction, bacterial pellet was collected by centrifugation at 6000 x g for 10 minutes in aliquots of 500 ml culture volume. The pellet was frozen at -20°C. Afterwards, pellet from 1 liter culture was resuspended in ice cold PBS. This 50ml culture pellet was divided into two parts and then each aliquot was sonicated at 60% amplitude on Branso Digital Sonifier 250-D of 1' cycle for 3' on ice to keep sample cool. Sonicated samples were then incubated with 20% Triton-X at final concentration of 1% for 30' at 4°C. The samples were centrifuged at 12000 x g for 15' and supernatant was collected. The supernatant was incubated with 50% glutathione sepharose 4b beads for overnight at 4°C. On
next day, the slurry was centrifuged at 500 x g for 5’. The Glutathione sepharose beads were washed three times with ice cold PBS. The slurry was incubated with 1mL of 8 M urea buffer for two hrs at 20°C. The beads were removed by centrifugation of the samples at 2000 x g for 10’. The protein amount was measured in supernatant by Bradford method as well as by running BSA standards on SDS-PAGE. The protein was about 8 mg/mL by comparing with BSA standards on gel as well as by Bradford method.

The GST-xBRG1 fusion protein was used to immunize rats for the production of xBRG1 specific antibodies, which was carried out in collaboration with Prof. Elisabeth Kremmer.

3.8.8 RNA *in-situ* hybridization

The RNA *in-situ* hybridization was performed as described previously (Harland, 1991) with slight modifications. The method used is described below:

**Day 1:**
1. The fixed embryos were rehydrated by serial changes of 75%, 50% and 25% ethanol series.
2. Washed 3 times in PBSw.
3. Treated with 10 μg/ml Proteinase K in PBSw (1 ml per vial) for 30 min at room temperature.
4. Rinsed with PBSw.
5. Washed 2 times in PBSw, 5 min each
6. Fixed with 4% PFA in PBSw (0.5ml per vial). Stand for 5 min and then rock for 15 min.
7. Short rinsed with PBSw.
8. Washed 5 times in PBSw.
9. Incubation in 50% PBSw / 50% hybridization solution, followed by 100% hybridization solution for about 3 min each step without agitation at RT.
10. Added 0.5 ml of fresh hybridization solution.
11. Embryos were incubated at 65°C for 1 hour to inactivate endogenous phosphatases.
12. Embryos were pre-hybridized at 60°C for 2-6 hours.
13. Heated 30 ng of probe to 95°C for 2-5 min. Add to 100 μl of pre-warmed hybridization solution.
14. Add the probe to the embryos in pre-hybridization solution.
15. Incubate at 60°C overnight (hybridization).

**Day 2:**
16. Pre-warmed 2 x SSC / 0.1%CHAPS to 37°C, and 0.2 x SSC / 0.1% CHAPS to 60°C.
17. Short sequential washes of embryos at 60°C with:
   a) Hybridization solution
   b) 50% hybridization solution / 50% 2 x SSC / 0.1% CHAPS
   c) 2 x SSC / 0.1% CHAPS
18. Washed 2 times in two x SSC / 0.1%CHAPS for 30 min at 37°C.
19. Short rinse with 0.2 x SSC / 0.1% CHAPS
20. Washed 2 times in 0.2 x SSC / 0.1% CHAPS for 30 min at 60°C.
21. Rinsed in 50% TBS / 50% 0.2 x SSC / 0.1% CHAPS.
22. Washed once in TBS.
23. Rinsed in TBS / 0.1% Triton-X (TBSX).
24. Incubated in antibody buffer (0.5 ml per vial) for 2 hours at 4°C.
25. In parallel, pre-absorb AP-conjugated anti-DIG antibodies (1/5000 diluted) against *Xenopus* proteins present in antibody solution.
26. Added 0.5 ml of pre absorbed antibody solution to embryos (i.e. final dilution of antibodies was 10^{-4}).
27. Incubate overnight at 4°C.

**Day 3:**
28. Rinsed with TBSX
29. Washed 6 times in TBSX for 1 hour each.
30. Rinsed with AP-buffer.
31. Equilibrated in AP-buffer for 15 minutes.
32. Replaced AP-buffer with 0.5 ml BM-Purple staining solution.
33. Incubate overnight at room temperature in dark with slight rocking (color reaction).

**Day 4:**
34. Stop reaction by washing twice in PBS for 10 min at room temperature with rocking.
35. Refix embryos in MEMFA for 2 hours, short rinse with H₂O or PBS, store in H₂O or PBS at 4°C.

**Solutions:**
Antibody-buffer: 80% (v/v) TBSX, 15% (v/v) heat-inactivated lamb serum, 5% (v/v) *Xenopus* egg extract.
DEPC-H₂O: dd H₂O with 0.1% (v/v) Diethylpyrocarbonate (DEPC) agitated at room temperature and incubated overnight and then autoclaved twice.
10 mM DIG NTP mixture: 10 mM CTP, GTP, ATP, 6.5 mM UTP and 3.5 mM Dig-11-UTP.
Hybridizing solution: 5 x SSC, 50% (v/v) formamide, 1% (w/v) Boehringer block, 0.1 % (w/v) Torula RNA, 0.01% Heparin, 0.1% Tween-20, 0.1 %CHAPS, 5 mM EDTA.
Lamb Serum: Heat-inactivated lamb serum (30 min with 56°C), stored at -20°C.
MEMFA: 0.1 M MOPS, 2 mM EGTA,1 mM MgSO₄, 3.7% formaldehyde (pH 7.4 at 23°C).
PBS: 137 mM of NaCl, 2.7 mM of KCl, 8 mM Na₂HPO₄, 1.7 mM KH₂PO₄ (pH 7.2 at 23°C).
PBSw: 1 x PBS, 0.1 % Tween-20 (pH 7.5 at 23°C).
PCI: 50% (v/v) phenol, 48% (v/v) chloroform, 2% (v/v) isoamyl alcohol.
PCR buffer (Taq): 10 mM of trichloroethylene HCl, 50 mM of KCl, 15 mM MgCl₂ (pH 8.4 at 23°C).
3 Materials and methods

PCR buffer (Pfu): 10 mM of trichloroethylene HCl, 10 mM of KCl, 2 mM MgCl₂, 10 mM (NH₄)₂SO₄, 0.1% (v/v) triton X-100, 100 μg/ml nuclease free BSA in DEPC H₂O (pH 8.4 at 23°C).

5 x RNA polymerase transcriptions buffer: 200 mM of trichloroethylene HCl, 30 mM MgCl₂, 10 mM of spermidin, 50 mM of NaCl (pH 7.5 at 23°C).

20 x SSC: 3 M NaCl, 0.3 M sodium citrate (pH 7.0 at 23°C).

TBS: 50 mM of trichloroethylene HCl, 150 mM of NaCl (pH 7.5 at 23°C).

TBSX: 1 x TBS, 0.1% triton-X (pH 7.5 at 23°C).

TE: 1 mM EDTA, 10 mM of Tris HCl (pH 8.0 at 23°C).

TBE: 100 mM of trichloroethylene HCl, 83 mM borate, 0.1 mM EDTA (pH 8.6 at 23°C).

Transcription buffer: 20 mM of Tris HCl, 10 mM of Spermidin, 3 mM MgCl₂, 50 mM of NaCl (pH 7.5 at 23°C) in DEPC H₂O.

AP-buffer: 100 mM Tris HCl, 50 mM MgCl₂, 100 mM NaCl.

**Xenopus egg extract for in-situ hybridization**

De-jelly unfertilized eggs with 2% Cystein, wash 3 times, add 1 volume of PBS then lysed by 10 strokes of a dounce homogenizations, and centrifuged (7500 x g, Sorvall RC-5b, rotors SS-34, 4°C, 10 min). The supernatant was transferred into a fresh centrifuge tube and centrifuged twice under the same conditions. The supernatant was aliquoted and stored at -20°C.
4 Results

4.1 Dominant negative human BRG1 causes head and eye defects in *Xenopus* embryos

In order to study the role of BRG1 during normal development of the *Xenopus* embryo, I used wild type and dominant negative versions of human BRG1. The dominant negative version of human BRG1 was constructed by introducing a point mutation, which converts a conserved lysine at position 783 into arginine (Khavari et al., 1993). The wild type and dominant negative versions of human *brg1* (*hbrg1*) c-DNA (de la Serna et al., 2000) were cloned into the pCS2+ vector (Rupp et al., 1994).

![Schematic representation of experimental procedure](image)

Figure 4.1. A schematic representation of experimental procedure carried out for microinjection in *Xenopus laevis* embryos. The embryos were fertilized and microinjected at 2-4-cell stage. The dorsal and ventral side of the embryo can be distinguished by pigmentation as shown in animal view of 4-cell stage embryo. The embryos were cultured for another two days until the embryos reached NF St. 35. The embryos were fixed in MEMFA and phenotypes were scored.

These clones were used to synthesize mRNA by *in-vitro* transcription using the Promega *in-vitro* transcription system. The synthesized RNA was injected into
either dorsal or ventral marginal of *Xenopus* embryo at the four-cell stage. The injections were targeted to the equatorial zone at the boundary of pigmented animal half and un-pigmented vegetal half as represented in figure 4.1. The injected embryos were cultured for 50 hrs post-fertilization at 23°C until the embryos reached NF stage 35 (Nieuwkoop and Faber, 1967).

Interestingly overexpression of dominant negative hBRG1 (dn hBRG1) into dorsal marginal zone produced embryos with a head and eye defect as well as a shorter anterio-posterior axis. As shown in figure 4.2, overexpression of dn hBRG1 gave a spread of phenotypes. The eye was always reduced, even in the least affected embryo, while more strongly affected embryos had severely reduced head as well as reduced anterio-posterior axes. Some embryos also displayed reduced pigmentation. Other than these defects, affected embryos were non-motile in response to mechanical as well as chemical stimuli. Finally, no heart beating was observed. The defects produced by overexpression of dn hBRG1 were dose dependent; about 63% embryos were affected with 250 pg of dominant negative Hbrg1 mRNA while about 81% embryos were affected by 500 pg of dn Hbrg1 injections (Table 4.1.). The overexpression of wt hBRG1 and GFP alone did not produce any abnormalities. The embryos injected with wild type and dominant negative variants of Hbrg1 mRNA into the ventral marginal zone of four-cell stage embryos, also did not produce any phenotype and were normal when compared to Gfp alone-injected embryos or with un-manipulated embryos. These results showed that the overexpression of dn hBRG1 interferes with the dorsal program of the embryos while the ventral program remains unaffected.

BRM is a closely related ATPase to BRG1. In order to test if overexpression of human BRM also interferes with normal development of *Xenopus* embryos, the wild type human *brm* and dominant negative human *brm* (de la Serna et al., 2000) were cloned into pCS2+ vector and used to *in-vitro* synthesize mRNA.
4 Results

Figure 4.2 Overexpression of dn hBRG1 on the dorsal side of the embryo produces embryo with head and eye defect. Severely affected embryos (A) show head and eye defects as well as shortened AP axes and reduced pigmentation while mildly affected embryos (C) shows only eye defect. In some cases, head and eye are defected but pigmentation remains normal (B). On the other hand, expression of wt hBRG1 does not produce phenotypes (D). All the injections were done with Gfp as a lineage tracer. The expression of GFP has been shown in adjoining figures. The expression of GFP shows that the expression of wt or dn hBRG1 does not affect cell viability.

The *in-vitro* transcribed mRNA was again injected into the dorsal and ventral marginal zones of *Xenopus* embryo at four-cell stage at 500 pg/embryo. The phenotypes were compared with the embryos overexpressing GFP alone and with un-manipulated embryos. The injections of dominant negative (n=18) and wild type (n=20) variants of human BRM displayed no abnormalities.
These results demonstrate that while BRG1 is required for anterior-posterior axis formation, BRM is either not involved or dispensable for AP axis formation.

Table 4.1 Overexpression of human BRG1 in dorsal marginal zone affects anterior-posterior axes formation

<table>
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<th>wt Hbrg1 (pg/emb)</th>
<th>Gfp (pg/emb)</th>
<th>Total Number</th>
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<th>Head and Eye defect (%)</th>
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4.2 Phenotypes produced by dn human BRG1 are specific

The gain of functions phenotypes produced by the mis-expression of dn hBRG1 was checked for specificity. To do so, wt Hbrg1 was co-injected along with dn Hbrg1. The dominant negative Hbrg1 at a dose of 250 pg of mRNA per embryo was injected either alone or in combination with wild type human brg1 in an increasing ratio. While the embryos injected with dominant negative Hbrg1 alone showed the head defects as described above, co injection with wild type human brg1 showed recovery of eyes and a normal anterior-posterior axis in the best cases while in other cases they showed partial recovery of eyes. The percentage of rescued embryos increased with increasing amount of Hbrg1 (Table 4.1). These results demonstrate that the phenotypes produced by dominant negative human BRG1 are due to the specific interference of BRG1 function in the normal embryo.
4.3 Cloning of Xenopus brg1

The results obtained with human BRG1 prompted us to search for a functional Xenopus homologue of BRG1 in order to further study the role of BRG1 in Xenopus development in detail. Since BRG1 is an evolutionarily conserved molecule from yeast to vertebrates, the Xenopus EST database was searched using hBRG1 as a query.

Figure 4.3 Schematic representation of EST found by searching Xenopus EST database using hBRG1 as a query (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/x_tropicalis). AW766934 spans the N-terminus and BG234591 spans the C-terminus of the xBRG1 protein while a third EST shows a significant overlap with AW766934 and BG234591. Forward and reverse primers were designed using EST sequence to obtain the full-length xBRG1 protein.

The searches obtained three overlapping ESTs, namely AW766934 covering aa 1-566, BG234591 covering aa 603-1600 and the third one BQ728178. BQ728178 spans both the ESTs having 100% identity to AW766934 in the region common to both the ESTs and about 95% identical with BG234591. Conceptual translation indicated that the three ESTs together covered the entire open reading frame of xbrg1 (Figure 4.3).

The nucleotide sequence of EST clones corresponding to the N-terminus and the C-terminus portion of the protein was used to design forward and reverse primers for the cloning of xbrg1. The embryos at NF st.20 were used to obtain total cellular RNA. The cDNA was synthesized using oligo dT primers to obtain a full-length cDNA pool.
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Figure 4.4 Full-length sequence of the xbrg1 open reading frame. The 4803 nucleotides of the xbrg1 c-DNA are shown. The amino acid sequence is shown in the single letter amino acid code. Letters shown in Green represent SNF2 domain, which spans amino acid residues 742 to 1,037 while letters shown in blue represent bromodomain, which spans from amino acid residues 1,414 to 1,504. In the ATPase domain, lysine (K) shown in black at position 770 was mutated to arginine (R) to obtain the dominant negative xBRG1.
Figure 4.5 (A) Clustalw alignment of Xenopus BRG1 amino acid sequence with human (AAB40977), chicken (CAA62831), Zebrafish (AAP22968) BRG1. The alignment shows the significant homology with the BRG1 protein of other vertebrates and shows that the conservation of the amino acid sequence is just not in the ATPase domain but is through out the entire length of the protein.

Touch down PCR was carried out to obtain the predicted full-length gene of 5kb length as described in the materials and method section of this report. The sequencing revealed an open reading frame of 4803 bp for xbrg1 (Fig4.4.).
The nucleotide sequence was translated into amino acid sequence using Mac vector 6.5.3 software. The open reading frame translated into a 1600 aa long protein. The amino acid sequence was used to search the pfam database to determine functional domains in the protein.

The pfam analysis showed a SNF2 domain extending from aa 742 to aa 1037 and a bromodomain, the characteristic domain of SWI/SNF family members, between aa 1414 to aa 1504 as shown in figure 4.4.

The xBRG1 protein sequence was aligned with the full-length sequence of human BRG1 (AAB40977) (Khavari et al., 1993), Chick BRG1 (CAA62831) (Goodwin, 1997), and Zebrafish BRG1 (AAP22968) (Gregg et al., 2003), which revealed high sequence identity (Figure 4.5A). The identity was about 87% with human BRG1, 88% with Chick BRG1 and 85% with Zebrafish BRG1 (Fig 4.6 H). Notably the identity between the ATPase domain of Xenopus BRG1 and Human BRG1 was about 97% while the identity between Xenopus and human BRG1 bromodomain was about 96%. The absence of the poly-glutamine stretch, present in hBRM in the N-terminal region of the protein, as well as a C-terminal end sequence (EED in BRG1 while DDE in BRM protein) showed that the c-DNA obtained corresponds to BRG1 (figure 4.5B).

![Figure 4.5 (B) Clustlaw alignment of C-terminal of BRG1 and BRM proteins of Xenopus and Human. Notably, C-terminus end in BRG1 is EED while in BRM, it is DDE.](image-url)
4.4 Xbrg1 is maternally expressed and has a tissue-specific expression pattern

The Northern blot showed that Xbrg1 is expressed as a single transcript of about 8 kb and is present both before and after the start of zygotic transcription (figure 4.6.1).

Figure 4.6 Normal expression pattern of Xbrg1 at 4-cell, a time before zygotic transcription begins, shows that Xbrg1 transcript are provided maternally (A), while at gastrula stage Xbrg1 is expressed ubiquitously (B). At Neurula, it starts expressing specifically in prospective neural tissues and shows strong expression in eye anlage (C). At st.28 Xbrg1 transcript are present in neural tube, eye, otic vesicles, pronephros, branchial arches (D) and at st.35 expression become more concentrated to the head region with expression in retina (E), at st45 its expression is detected only in the heart (F). Head section of a st. 35 embryo passing through the eye shows expression of Xbrg1 in retina(r) and neural tube (nt) but not in iris (i). (G) Degree of amino acid identity between BRG1 homolog of human (AAB40977), chicken (CAA62831), Zebrafish (AAP22968) and Xenopus (H). Northern blot analysis of Xbrg1 shows that there is a single transcript of approximately 8kb in size (I).

In order to determine the spatial pattern of Xbrg1 expression in embryonic tissues, RNA in-situ hybridization was performed. NF st. 4, NF st. 11, NF st. 14,
NF st. 28, NF st. 35 and NF st. 45 of embryonic development were probed with a Xbrg1 specific antisense probe. The -36 to +618 fragment was cloned into the pCR II-TOPO (Invitrogen Life Technologies) vector to obtain the antisense RNA probe as described in materials and methods section of this report. A sense strand probe was used as a negative control. Specific in-situ staining was observed with the antisense Xbrg1 probe from 2-4 cell stages onwards. At 2-4 cell stage, only maternal transcript are present since in Xenopus embryos the zygotic transcription starts at the mid blastula transition (MBT) (Newport and Kirschner, 1982). Up to the gastrula stage, Xbrg1 is expressed ubiquitously in the embryo. From neurula stage of embryos, Xbrg1 has a restricted expression pattern. As shown in figure 4.6, the expression of Xbrg1 was detected in the prospective eye field and prospective neural tube showing a region of high-level expression of Xbrg1 at Neurula stage. NF st. 28 embryos show that Xbrg1 is expressed strongly in eye, brain tissue and branchial arches, while it has a weaker staining in pronephros and in neural tube. Interestingly, its expression could not be detected in somites, which express Xbrm (Linder et al., 2004).

By NF stage 35, expression could be seen in retina, but not in the lens tissue, as also seen in embryonic cross-sectioning of head of NF stage 35 embryos passing through the eyes. The expression of Xbrg1 shows an increase in pronephros and in brain tissue while reduces in neural tube as compared to NF st. 28 embryos. The expression was also present in the tail region. At NF stage 45 expression of Xbrg1 has disappeared in head, eye and neural tissue, and was maintained exclusively in heart.

Thus, Xbrg1 is a maternally expressed gene, and is present ubiquitously at gastrula stage and later shows a tissue specific expression pointing towards a role of xBRG1 in tissue specific transcription.
4.5 Generation and characterization of monoclonal antibodies for xBRG1

In order to carry out future experiments related to chromatin Immunoprecipitation, Co-IP and western blotting, monoclonal antibodies specific to BRG1 were raised in collaboration with Prof. Elizabeth Kremmer (GSF).

As described in the material and methods section, a specific fragment of xbrg1 was cloned into GST vector and the fusion protein obtained was used to immunize rats. Several clones were obtained namely XB3F1, XB4D8, XB5B9, XB6A1, XB6D8, and XB7G9. These clones were tested first on in-vitro translated xBRG1 protein. All antibodies detected xBRG1 specific band on western blot.
Further, these supernatants were tested on whole embryo lysate obtained from stage 11 embryos.

The western analysis showed that detection of xBRG1 in supernatant by XB3F1 was more specific than other supernatants. XB3F1 was able to detect endogenous xBRG1 without any nonspecific band except a very low molecular weight band, which was present in all supernatants. On the other hand, supernatants XB6D and XB7G6 detected xBRG1 along with several non-specific bands (figure 4.7.). The XB3F1 clone generated supernatant was used in future studies based on these results. The supernatant from the stabilized XB3F1 clone showed only the specific xBRG1 band of an expected size of about 200 kDa.

Since these antibodies will also be used for chromatin immunoprecipitation and Co-IP, BRG1 antibody clone XB3F1 was tested for its efficiency to immunoprecipitate endogenous BRG1. Immunoprecipitation efficiency of antibody clone XB3F1 and XB6D8 was tested first on \textit{in-vitro} translated xBRG1 protein.

As shown in figure 4.8, both XB3F1 and XB6D8 supernatants were able to immunoprecipitate xBRG1. XB3F1 shows specific immunoprecipitation compared to Immunoprecipitation using XB6D8. Further, XB3F1 was tested whether it can carry out Immunoprecipitation after cross-linking of DNA and protein by formaldehyde treatment used for chromatin Immunoprecipitation. DNA and protein were cross-linked by treating the embryos with formaldehyde in three conditions varying in temperature and duration. These conditions were following: a) 5’ at RT b) 15’ at RT c) 10’ at 37°C. The Immunoprecipitation was carried out as described in materials and methods. As shown in figure 4.8, XB3F1 was able to immunoprecipitate xBRG1 in all cross-linking conditions with approximately 10% efficiency.
4.6 Optimization of \textit{in-vitro} transcription for \textit{xbrg1}

In order to obtain the \textit{in-vitro} transcribed mRNA of \textit{xbrg1}, \textit{xbrg1} cDNA was cloned in pCS2+ vector. The pCS2+\textit{xbrg1} plasmid construct was linearized by Not1 restriction digestion.
In-vitro transcription was carried out using SP6 polymerase at 37°C. Interestingly, when the transcripts were size fractionated on formaldehyde gels, the transcript was about 2 kb shorter than the expected band of 5 kb. In order to obtain the full length RNA, two approaches were tried. First, it was checked whether another promoter like T3 or T7 gives the full-length transcript. For this, \textit{xbrg1} in pCR II-TOPO (Invitrogen Life Technologies) vector was used to transcribe RNA. pCR II-TOPO vector has T7 promoter upstream of \textit{xbrg1}. In-vitro transcription was done using standard protocol at 37°C, which obtained full-length transcript of about 5 kb in size. Although the use of T7 promoter obtained

![Figure 4.9 Conditions were optimized for in-vitro transcription of \textit{xbrg1}. The in-vitro transcription of pCS2+ wt and \textit{dn xbrg1} using Sp6 polymerase gives smaller transcripts than the expected transcript of 5 kb in length at 37°C(A). The in-vitro transcription of pCR II-TOPO \textit{xbrg1} was carried out using T7 polymerase (B), which shows full-length transcript. Further, in-vitro transcription of pCS2+ wt \textit{xbrg1} was carried out using sp6 polymerase at different temperatures (C). In-vitro transcription at 20°C shows optimal transcription.](image_url)
full-length mRNA but SP6 promoter used in pCS2+ vector, has been shown to have higher transcriptional efficiency as well as transcript obtained from SP6 promoter has highest translational efficiency. Thus, another approach was used to obtain the full-length transcript. The in-vitro transcription was carried out at various temperatures that is at 10°C, 20°C, and at 30°C. As shown in figure 4.11, at 10°C the transcript was of full-length but the amount was extremely low, while at 30°C, the amount was sufficient but transcript were spreaded on the formaldehyde gel indicating several short transcripts while at 20°C the transcript amount was sufficient as well as transcript was also full length (figure 4.9.). Thus, in future experiments, in-vitro transcripts for xbrg1 were generated at 20°C using SP6 RNA polymerase.

4.7 Xenopus BRG1 is required for anterior-posterior axis formation

To carry out in depth analysis of developmental functions of xBRG1, a dominant negative construct similar to dn hBRG1 was made. To obtain dominant negative version of xBRG1, a lysine at the conserved ATP binding site (GKT), at position 770 was point mutated to arginine (K770-R) as has been described for hBRG1(Khavari et al., 1993) by a single point mutation using site directed mutagenesis.

The in-vitro synthesized, wild type Xbrg1 and dominant negative Xbrg1 mRNAs were injected at a dose of 500 pg/embryo in to dorsal marginal zone at four-cell stage along with Gfp as lineage tracer. The control embryos were injected with in-vitro synthesized Gfp mRNA. The phenotypes were assessed at NF stage 35. As shown in figure 4.10, dominant negative Xenopus BRG1 produced 61% embryos with head defects as seen in case of dominant negative hBRG1 overexpression. Overexpression of wild type hBRG1 did not produce phenotypes but on contrary, overexpression of wild type xBRG1 resulted in 53% embryos with reduced head structures (Table 4.2). Superficially, the head defects shown by wild type and dominant negative xBRG1 looked similar, but on closer inspection it was found that in case of wild type xBRG1 the cement gland was
preferentially reduced, while eyes remained unaffected. In contrast, over-
expression of dominant negative *Xenopus* BRG1 affected primarily eye
formation, while cement glands differentiated normally.

**4.8 Ventral overexpression of wild type xBRG1 produces partial
secondary axis**

Wild type and dominant negative xBRG1 was overexpressed on ventral
side of the embryos by injecting 500pg mRNA along with Gfp as a lineage tracer.
As shown in figure 4.10, overexpression of dominant negative xBRG1 did not
produce any abnormalities, consistent with dominant negative hBRG1
overexpression on ventral side of the embryo. In contrast, overexpression of wild
type *Xenopus* BRG1 on the ventral side produced partial secondary axis at a
frequency of about 65% (Table 4.2.). The overexpression of GFP did not produce
any defect.

The results obtained from overexpression of xBRG1 on the dorsal side of
the embryo revealed a possible role of BRG1 in anterior-posterior patterning of
*Xenopus* embryo, which are principally consistent with the results obtained by
hBRG1 overexpression.

Phenotypes produced by dorsal or ventral overexpression of wild type and
dominant negative xBRG1 could not be produced by overexpression of wild type
and dominant negative overexpression of xISWI, another SNF2 domain
containing protein, either on the dorsal side or on the ventral side of the *Xenopus*
embryos. For these experiments, wild type and dominant negative Xiswi *in-vitro*
synthesized mRNA were injected into the dorsal marginal zone or into the ventral
marginal zone.

Dominant negative xISWI variant was obtained by a single point mutation
into ATPase domain. The point mutation converted the conserved lysine residue
at position 204 into arginine. This mutation renders its ATPase domain inactive,
as reported previously for *Drosophila* ISWI (Deuring et al., 2000). The mRNA at a
dose of 500 pg/embryo was injected along with GFP at 100ng/embryo as a lineage tracer at four-cell stage. The phenotypes were scored at NF st.35. The embryos injected with wild type (n=24) and dominant negative (n=20) version were normal and displayed no abnormalities.

Figure 4.10 Overexpression of wt xBRG1 (B) and dn xBRG1 (C) on dorsal side of the *Xenopus* embryo shows defect in head structure. The overexpression wt xBRG1 shows the defects in cement gland development while dn xBRG1 shows defect in eye development as shown in close up in left corners of figures. Overexpression of wt xBRG1 on ventral side produces a secondary axis (E), dn xBRG1 failed to do so (E). While embryos overexpressing GFP on Dorsal (A) and ventral (D) side were normal. Corresponding legend shows GFP expression, used for lineage tracing. Nucleotide binding motif comparison of RAD3 helicase with hBRM, hBRG1 and xBRG1. The conserved lysine in ATP binding motif is shown in green. This conserved lysine at position 770 in xBRG1 was converted to arginine to produce dominant negative xBRG1 (G).
4.9 Reduction of endogenous xBRG1 causes severe head and axial defect

Gain of function phenotypes produced by overexpressing wild type and dominant negative versions of xBRG1 illustrated a role played by BRG1 in anterior-posterior axis formation. In order to further confirm the role of xBRG1 in anterio-posterior axis formation, loss of function studies were performed.

*Xenopus* BRG1 protein translation was inhibited using morpholino antisense oligo. *Xenopus* is an allotetraploid vertebrate and thus has two non-allelic functional copies of each gene. The EST database search gave an EST
namely BG554361, which includes the N-terminus sequence of the second non-allelic copy of *xbrg1*. A morpholino antisense oligo was designed, which targets both copies of the gene and thus blocking translation from the transcript of both copies. To assess the translational blocking efficiency of xBrg1 antisense morpholino oligos, embryos were injected at two-cell stage into both blastomeres of the embryos at a dose of 20 ng/embryo and at 80 ng/embryo of xBrg1 specific morpholino oligos. The control morpholino oligos were injected at 80 ng/embryo. Embryos were collected at gastrula stage and lysed in RIPA buffer. For western blot analysis, lysate equivalent to one embryo per lane were loaded onto the gel. The blots were probed with xBRG1 specific XB3F1 antibody. Western analysis showed a significant reduction in the xBRG1 endogenous protein levels by both doses of xBrg1 antisense morpholino oligo. The blots were stripped and reprobed with *Xenopus* β-CATENIN specific antibody P14L (Schneider et al., 1993) as a loading control (Figure 4.11.).

For phenotypic analysis, xBrg1 antisense morpholino oligos were injected at various doses into dorsal marginal zone at 4-cell stage. The phenotypes were scored at NF stage 35. The phenotypes obtained were dose dependent and showed loss of eyes at lower doses while loss of eyes and reduced anterior-posterior axes at higher doses of morpholino oligos (Figure 4.11.). Ten nanograms of morpholino oligo injections affected 85% embryos while 20 ng of morpholino injection produced 89% affected embryos (Table 4.3.). At higher doses, the antisense morpholino oligos caused gastrulation defects. On the other hand, embryos injected with control morpholino did not show any phenotypes even at the dose of 40 ng/embryo. These embryos were also injected with GFP as a lineage tracer. The presence of GFP also shows that the injected cells were alive. This result point out a role of xBRG1 in dorso-anterior patterning. On the other hand, when antisense morpholino were injected into ventral marginal zone of the embryo, embryos showed lesion on ventral side of the embryo at different stages of embryo, depending on the amount of antisense morpholino oligos.
Figure 4.11 xBr1 MO were designed to target the +1 to +25 of xbrg1 sequence (A). Translational inhibition of endogenous brg1 by xBr1 MO antisense oligos (B). The blots were probed with XB 3F1, xBRG1 specific antibody. xCATENIN was used as a loading control. The xBr1 MO were injected at 2-cell stage to check the efficacy of MO to block the endogenous BRG1 translation. The embryos were lysed at NFst.11. The xBr1 MO produces phenotypes in a dose dependent manner. xBr1 MO were injected at 2.5 pg/emb (C), 5 pg/emb (D), and at 10 pg/emb (E). While control morpholino were injected at 40 ng/emb (F).

In contrast, injections of xBr1 antisense morpholino oligos at two-cell stage were lethal to the embryo after gastrulation and showed lysed embryos.
4.10 Phenotypes produced by xBrg1 antisense morpholino oligonucleotides are rescuable

In order to check whether the phenotypes produced by Brg1 morpholino are specific, phenotypes produced by xBrg1 morpholino oligos were rescued by overexpression of wild type hBRG1. \( hbrg1 \) contains four mismatches in the target region of morpholino antisense oligo and, thus, cannot be inhibited by the xBrg1 antisense morpholino. Wt Hbrg1 mRNA was co-injected with xBrg1 antisense morpholino oligos into the dorsal marginal zone at 4-cell stage.

As shown in figure 4.12, the embryos injected with 10 ng/embryo xBrg1 antisense morpholino into dorsal marginal zone displayed embryos having loss of eyes, reduced head and severely reduced anterior-posterior axes, but when xBrg1 morpholino oligos were co-injected with 500 pg mRNA of Hbrg1, embryos displayed a range of phenotypes.
Table 4.3
Phenotypes generated by BRG1 depletion can be rescued by hBRG1 and xβ-CATENIN

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<td>-</td>
<td>22(91)</td>
<td>2</td>
</tr>
<tr>
<td>Brg1MO 10 ng/emb+ Wt Xiswi 500 pg/emb/DMZ</td>
<td>38</td>
<td>-</td>
<td>17(44)</td>
<td>21(with head and eye defect)</td>
</tr>
<tr>
<td>Brg1MO 10 ng/emb+ β-catenin 250pg/emb/DMZ</td>
<td>21</td>
<td>12</td>
<td>8(38)</td>
<td>1</td>
</tr>
<tr>
<td>Wt Hbrg1 500pg/emb/DMZ</td>
<td>15</td>
<td>11</td>
<td>2(13)</td>
<td>2</td>
</tr>
<tr>
<td>β-catenin 250pg/emb/DMZ</td>
<td>32</td>
<td>26</td>
<td>3(9)</td>
<td>3</td>
</tr>
<tr>
<td>Control Mo 40 ng/emb /DMZ</td>
<td>17</td>
<td>17</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dn Hbrg1 500 pg/emb + β-catenin 250 pg/emb/DMZ</td>
<td>22</td>
<td>15</td>
<td>7(46)</td>
<td>-</td>
</tr>
<tr>
<td>Dn Hbrg1 500pg/emb/DMZ</td>
<td>22</td>
<td>7</td>
<td>15(68)</td>
<td>-</td>
</tr>
<tr>
<td>Uninjected</td>
<td>24</td>
<td>24</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

The co-injection of Hbrg1 mRNA resulted in only 15% affected embryos compared to 85% affected embryos obtained with Brg1 morpholino alone (Table 4.3.). All of these embryos were showing at least partial recovery of eye differentiation while some embryos showed complete recovery of eyes and
anterior-posterior axes. Interestingly, xISWI and hBRM overexpression failed to rescue the phenotypes generated by Brg1 morpholino oligos.

These results showed that the phenotypes produced by the xBrg1 morpholino oligos were specifically produced by interference with xBRG1 protein function.

4.11 BRG1 knock-down affects expression of various differentiation markers

The BRG1 knock-down experiments displayed severely reduced head structures and anterior-posterior axis formation. To find out the effect of BRG1 knock-down on organogenesis, various tissues were analyzed by in-situ hybridization. *Xenopus* embryos were injected at 4-cell stage into dorsal marginal zone with 5 ng per embryo of xBrg1 antisense morpholino oligonucleotides. The embryos were fixed at stage 35.

Embryos were probed with neural specific β-tubulin in-situ probe (Oschwald et al., 1991). In-situ analysis showed that N-β tubulin stains neural tissue in head region as well as in neural tube. The expression levels were high in head region and lower in neural tube. BRG1 knocked-down embryos displayed strongly reduced N-β tubulin staining in head region, while staining was absent in neural tube region. These results showed that BRG1 knock-down reduced the amount of neural tissue formation (figure 4.13.).

Next I examined the expression of Nkx2.5, a tinman homologue and a known regulator of heart differentiation and morphogenesis (Tonissen et al., 1994). As shown in figure 4.13, the expression was reduced in xBrg1 MO injected embryos, while the expression of Nkx2.5 remained unaltered in control
Figure 4.13 The effect of xBRG1 knock-down on various tissues was tested by in-situ hybridization. Brg1 morphant embryos shows down regulation of neural specific marker N-β-tubulin, heart specific marker Nkx2.5, Msr, a marker for blood vessels, and Shh, which is expresses in floor plate of notochord and Nasal pits while the expression of α-globin, a marker for ventral blood islands shows significant expansion. The expression of Mlc35 is also reduced by xBRG1 knock-down, showing defective muscles in morphant embryos.
morpholino injected embryos. These results showed that BRG1 is involved in heart formation.

To get further insight into vascular system, I analyzed Brg1 morphant embryos for the expression of the mesenchyme-associated serpentine receptor Msr, whose transcripts are expressed in procardiac tube and forming blood vessels, localized in the inner endothelial layer (Devic et al., 1996). As shown in figure 4.13, it was found that BRG1 knock-down has affected blood vessel formation in morphant embryos while it remains normal in control morpholino injected embryos.

Alpha globin is expressed in ventral blood islands (Banville and Williams, 1985; Tracey et al., 1998). As shown in figure 4.13, it was found that the expression of Alpha globin is increased in Brg1 morphant embryos compared to control embryos, which could be due to ventralization of the embryo or due to unequal distribution of blood-island due to reduced blood vessel formation.

Furthermore, I checked the expression levels of sonic hedgehog (Shh), which is expressed in the floor plate of notochord and in the nasal pits (Ekker et al., 1995). BRG1 knock-down into Xenopus embryos shows down regulation of Shh staining, showing defective notochord formation (figure 4.13.).

It has been reported that BRG1 and BRM are involved in muscle differentiation, a study carried out using fibroblast cell lines (de la Serna et al., 2001). To confirm the role of BRG1 in muscle differentiation, in-vivo muscle differentiation was observed in Brg1 morphant embryos. As a muscle marker, Mlc35 was used, which stains differentiated muscles in the embryos at late stage. The in-situ pattern shows that Mlc 35 staining is reduced (figure 4.13.). In addition, chevron pattern of the somatic muscle is perturbed in BRG1 knock-down embryos. It confirms that the BRG1 is involved in muscle differentiation.

These results show that BRG1 knock-down in Xenopus embryos causes late defects in multiple tissues. The effects could be due to the direct effect on organogenesis or due to defect in early patterning of embryo. To understand the
cause of these phenotypes, the genes involved in early patterning of the embryos were investigated.

**4.12 BRG1 knock-down causes down regulation of genes required for early patterning of the dorsal mesoderm**

To understand the role of xBRG1 in early patterning, the embryos were injected with 20 ng of antisense morpholino oligos at 2-cell stage. The target genes were checked by *in-situ* hybridization at relevant embryonic stages.

*Xenopus* nodal related 3 (*xnr3*) is a direct target gene of WNT/β-CATENIN pathway (McKendry et al., 1997). The expression levels of Xnr3 were checked in Brg1 morphant embryos. Xnr3 is expressed maximally at NF st.9 and thus control morpholino as well as Brg1 morpholino injected embryos were fixed at this stage. As shown in fig 4.14, the expression level of Xnr3 in Brg1 morphant embryos is significantly reduced while the size of its expression domain remains same compared to control morpholino injected embryos.

Chordin, a BMP antagonist, is expressed transiently in the BCNE center (*Blastula Chordin Noggin Expression center*) and later in the organizer. The expression of Chordin in the BCNE center is under the β-CATENIN mediated WNT signal (Kuroda et al., 2004). To test the expression of Chordin in BCNE center, embryos were fixed at blastula stage. As shown in figure 4.14, the expression of Chordin was notably reduced by knocking-down BRG1 in the embryos compared to control embryos.

This result not only gives a second read out for Wnt target genes as well as explains neural defects seen in whole embryos since the BCNE center later gives rise to prospective brain and floor plate. Chordin is expressed at the dorsal blastopore lip at the gastrula stage. As shown in legend of figure 4.14, the expression of Chordin was reduced in the ectodermal cells while remained unaffected in the lip. Next, the expression of Goosecoid, a homeobox gene,
Figure 4.14 Whole mount in-situ hybridization of control and Brg1 morphant embryos. Control morpholino (40 ng/emb) or Brg1 morpholino (20 ng/emb) were injected at 2-cell stage in both the blastomeres. Embryos shown for Xnr3 and chordin were collected at stage 9, while for Goosecoid were collected at stage 10 and embryos shown for Chordin (in legend with st. 9 Chordin), Xvent2, Xbra, Myf-5, Myod and Xpo were collected at late gastrula stage. Note that upon BRG1 knock-down, staining for Xnr3, Goosecoid, and Chordin is reduced while staining for Xvent-2 and Xbra remains unchanged but staining for Xpo was increased and extends in organizer. Myf-5 and Myod, the genes responsible for myogenesis are also down regulated in Brg1 morphant embryos.

which is expressed in the organizer and demarcates the organizer domain (Cho et al., 1991), was examined. The embryos were fixed at NF st10. After BRG1 knock-down, Goosecoid expression domain was reduced in size but interestingly the expression level in the remaining domain was not altered (figure 4.14.). These results show the reduced territory of organizer. The goosecoid promoter is
known to have two binding sites, one each for WNT signal and NODAL signal. Siamois/Twin binding on the promoter relays WNT signal. Therefore, it was reasoned that since WNT signal is downregulated as shown by the downregulation of Xnr3 and Chordin, I see a reduction in expression domain of Goosecoid but probably NODAL signaling remains unaffected and thus maintains the intensity of expression.

Xbra, a *Xenopus* homologue of the T-box gene brachyury, is expressed in the pan-mesoderm (Smith et al., 1991). To find out the effect of BRG1 knock-down on the mesoderm formation, the expression of Xbra was checked at NF st.11. As shown in figure 4.13, the expression level of Xbra remains unchanged in the Brg1 morphant embryos compared to control embryos showing that the mesoderm formation remains unaffected by BRG1 knock-down.

Xvent-2, a homeobox gene, has been shown to be required for ventralization of mesoderm (Onichtchouk et al., 1996). Xvent-2 is a direct target of BMP signaling (Schuler-Metz et al., 2000). To find out the effect of BRG1 knock-down on ventral mesoderm formation, the expression of Xvent-2 was checked. The expression levels of Xvent-2 remain comparable to control embryos in Brg1 morphant embryos (figure 4.14). These results show that BRG1 knock-down does not affect BMP signaling and thus ventral mesoderm formation remains unaffected.

*Xenopus*-posterior (Xpo), encodes protein with a ‘CCHC’ finger domain, (Sato and Sargent, 1991) and has been proposed to be involved in anterior-posterior axis organization. Xpo is one of several ventralizing genes, which can be induced by xCAD-2 (Pillemer et al., 1998). As shown in figure 4.14, the expression of Xpo is limited to the posterior ectoderm and mesoderm in the control embryos but is expanded in Brg1 morphant embryos in the organizer territory.

These results shows that knock-down of xBRG1 selectively affect Wnt target genes and shows defective organizer formation during gastrulation of
Xenopus embryo. BRG1 knock-down affects BCNE center formation as shown by the downregulation of Chordin, pointing towards the defective neurogenesis. These results although showed that BRG1 is required during early patterning of the embryo possibly by the modulation of Wnt target genes expression, but does not exclude the possibility of late function of BRG1 in organogenesis.

4.13 BRG1 knock-down affects the expression levels of Myod and Myf-5

As shown in figure 4.13, BRG1 knock-down affects Mlc35 expression, and thus is involved in frog muscle differentiation. In order to find out the mechanism of the loss of muscle differentiation, the expression level of Myod and Myf-5 were checked. MyoD and MYF-5 are basic helix loop helix transcription factors and are responsible to initiate the muscle development (reviewed in Rupp et al., 2002). The embryos were injected with 20 ng of Brg1 morpholino at two-cells stage into both blastomeres. The expression level was assessed at NF st. 11.

BRG1 knock-down from the embryos by antisense morpholino oligos clearly caused a reduction of Myf-5 and Myod expression. The expression level of Myod was reduced in the ventral mesoderm, while the expression of Myf-5 was undetectable (figure 4.14.). These results show that BRG1 acts genetically upstream of MyoD and Myf-5.

4.14 Functional interdependence of xBRG1 and β-CATENIN

Epistasis experiments were performed in order to further confirm that BRG1 acts in Wnt pathway.

Xenopus β-CATENIN was used to rescue the phenotypes produced by BRG1 knock-down. The mRNA encoding Xβ-catenin was either co-injected with Brg1 morpholino or alone into the dorsal marginal zone at 4-cell stage. Similarly, Brg1 morpholino oligos alone were also injected in the same way. The injections
were done with Gfp as a lineage tracer. The phenotypes were scored at the tadpole stage.

Injection of Brg1 morpholino alone shows AP axis defect as described before. Embryos co-injected with β-catenin mRNA and Brg1 morpholino oligos were significantly rescued, characterized by the presence of eyes, almost normal heads and restored length of anterior-posterior axes (figure 4.15.).

![Figure 4.15 BRG1 and β-CATENIN are in the same signaling pathway. The phenotypes produced by xBrg1 MO injection (A) could be rescued by overexpression of x β-CATENIN (B) while overexpression of xβ-CATENIN caused no abnormalities (C). All the embryos were co-injected with Gfp mRNA as a lineage tracer and the images are shown in the corresponding legends.](image)

The rescue shows only 38% percentage affected embryos compared to 85% affected embryos with Brg1 morpholino alone (Table 4.3.). The embryos injected with β-catenin mRNA alone did not show any significant phenotypes. β-CATENIN overexpression also rescued the phenotypes produced by dn hBRG1, bringing down the percentage of strongly affected embryos from 68% to 46%
The results show that phenotypes induced by functional inhibition of BRG1 can be rescued by overexpression of β-CATENIN.

It has been shown that overexpression of β-CATENIN on ventral side produces a complete secondary axis (Funayama et al., 1995) as also shown in figure 4.16. Overexpression of β-CATENIN on ventral side of the embryo gives rise to secondary organizer by forming dorsal mesoderm at the expense of ventral mesoderm. As shown in figure 4.16, when β-CATENIN was overexpressed in Brg1 morphant embryos, it failed to produce secondary axes in the embryos, while β-CATENIN alone induced a complete secondary axes in about 90% embryos. In the same manner, co-injection of dn Hbrg1 with β-catenin also blocked secondary axis formation as only 36% of embryos showed secondary axes (Table 4.4.).
These results show that both BRG1 and β-CATENIN are in the same pathway and β-CATENIN genetically interacts with BRG1.

Table 4.4
Depletion of BRG1 prevents secondary axes formation by β-CATENIN

<table>
<thead>
<tr>
<th></th>
<th>Total Number</th>
<th>Normal</th>
<th>Head And Eye defect</th>
<th>Secondary Axes (%)</th>
<th>Gastrulation Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brg1Mo 10ng/emb/vmz</td>
<td>14</td>
<td>12</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>β-catenin 250pg/emb/vmz</td>
<td>42</td>
<td>1</td>
<td>-</td>
<td>38(90)</td>
<td>3</td>
</tr>
<tr>
<td>Brg1Mo 10ng/emb/vmz+β-Cat. 250pg/emb/vmz</td>
<td>20</td>
<td>18</td>
<td>-</td>
<td>1(5)</td>
<td>1</td>
</tr>
<tr>
<td>dn Hbrg1 500pg/emb/vmz +β-cat. 250pg/emb/vmz</td>
<td>19</td>
<td>7</td>
<td>-</td>
<td>7(36)</td>
<td>5</td>
</tr>
<tr>
<td>Uninjected</td>
<td>71</td>
<td>71</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

4.15 Gene - and signal specific role of xBRG1

The previous results reported here indicated that BRG1 is involved in primary patterning of the Xenopus embryos by primarily regulating Wnt signaling dependent activation of organizer-specific genes. During embryogenesis, several signaling pathways are involved and influence the gene expression of the various downstream target genes.

These signaling pathways are interconnected with each other in a complex network, which makes it realistically impossible to do the in-vivo study of certain protein on various individual pathways. To overcome this problem, animal
Figure 4.17 Schematic representation of animal cap assay. The morpholino were injected at 2-cell stage into both blastomeres. The inducer RNA was injected into two blastomeres at 8-cell stage into the animal pole. The embryos were cultured until 7hpf and the animal caps were excised from blastula stage embryo. The animal caps were further cultured for four hours and then lysed to extract total RNA.
cap in-vitro induction assay was used. As stated before, animal caps are ectodermal cells and their use reduces the complexity of the problem by allowing to test the effect of individual signals on the expression of direct target genes.

The signaling pathways in the animal cap cells are induced by overexpression of specific ligand for the specific pathway. Embryos were injected with 20 ng of Brg1 morpholino at two-cell stage into both blastomeres. At the 8-cell stage, uninjected as well as Brg1 morpholino (B-MO) injected embryos were injected with inducer mRNA into animal pole. The caps were excised at seven hpf and further cultured for 4 hours, before they were lysed to extract total RNA, which was used to assess the induction of representative direct target genes of different signaling pathways.

The direct target genes were selected based on previously reported cycloheximide insensitive genes or the presence of response element in the promoter. Cycloheximide treatment of the cells blocks protein synthesis and thus blocking the effect of intermediate proteins in the signaling pathway on the outcome of induction of the gene.

To directly check the role of BRG1 on WNT signaling, the animal caps were induced with 50 pg of Wnt8 mRNA. Wnt8 activates WNT signaling pathway via interaction with its receptor, which in turns blocks the phosphorylation of β-CATENIN and thus preventing its degradation. This stabilized β-CATENIN is transported to the nucleus where it releases the Tcf3 mediated repression and thus activating Wnt target genes. In addition to xnr3 (McKendry et al., 1997), siamois (Brannon et al., 1997; Brannon and Kimelman, 1996; Lemaire et al., 1995) is also among other known WNT target genes. The promoter of Siamois has been shown to have a Lef/Tcf3 binding sites

The expression of Siamois was checked by semi-quantitative RT-PCR in the un-injected and morpholino treated animal caps along with untreated and morpholino treated caps after induction by Wnt8 mRNA. As shown in figure 4.18,
Siamois, is not expressed in the uninduced cap, but can be strongly induced by Wnt8 mRNA. B-Mo injection alone did not induce expression of Siamois, but reduced the induction of *siamois* about two-three fold.

Figure 4.18 Role of BRG1 on various signaling pathways was assessed using animal cap assay. The induction of direct target genes for WNT, eFGF and ACTIVIN signaling pathway was assessed in the presence and absence of Brg1 MO. The genes tested were Siamois for WNT pathway, Xbra and MyoD for eFGF and Xfd-1′ and Goosecoid for ACTIVIN signaling pathway. BRG1 shows a gene and signal specific effect.

XFD-1′ is a fork head/HNF-3 related transcription factor. Promoter studies have revealed the presence of an Activin response element (ARE), which is both necessary and sufficient for transcriptional activation of a reporter gene in animal cap explants incubated with ACTIVIN A. (Kaufmann et al., 1996). The expression of Xfd-1′ in animal caps could be detected after the injection of five pg of Activin
mRNA. When BRG1 depleted animal caps were induced by Activin mRNA injection, the transcription of *xfd-1'* remained unaffected (figure 4.18).

The promoter of *goosecoid* has been shown to have two signaling inputs one from the WNT signaling through Siamois/Twin and the other from the NODAL signaling. (Germain et al., 2000; Laurent et al., 1997). In order to test the effect of BRG1 knock-down on *goosecoid* transcription under induction by nodal signal only, animal caps were induced by 5 pg of Activin mRNA. As shown in figure 4.18, Activin could induce *goosecoid* as reported previously (Green and Smith, 1990). In contrast to the effect of BRG1 knock-down seen in whole embryos, the induction of *goosecoid* remained unaffected in the absence of BRG1 pointing out that BRG1 is not required for ACTIVIN signaling.

To find out the role of BRG1 in FGF/MAPK signaling, caps were induced by injecting 10 pg of eFGF mRNA at 8-cell stage into animal pole in uninjected and Brg1 morpholino injected embryos. The expression of Xbra was assessed, which has been shown to be a direct target of eFGF (Smith et al., 1991). As shown in figure 4.18, eFGF induces *xbra* in the animal caps but remains unaffected in the absence of BRG1. Similarly, eFGF has also been shown to induce *myod* transcription in the presence of cycloheximide (Fisher et al., 2002), as I have also shown in figure 4.18, *myod* could be induced by eFGF. Unlike Xbra, Myod transcripts were undetectable in the absence of BRG1. These results pointed out that BRG1 is required for the transcription of *myod*.

The *in-vitro* induction experiments confirmed that BRG1 is predominantly involved in WNT signaling pathway. As seen, BRG1 knock-down did not affect activin target genes while selective affects eFGF target genes, showing that the BRG1 affect is gene and signal specific.

### 4.16 Modulation of WNT-dependent gene-activation by xBRG1

Xnr3 and Chordin expression was strongly affected in the embryos upon BRG1 knock-down (figure 4.14), while Siamois expression was mildly down-
regulated in animal cap assay (figure 4.18). Furthermore, \( \beta \)-CATENIN rescue experiments showed that the increased WNT response could compensate for reduced BRG1 activity (figure 4.15). Thus, it is possible that effect on *siamois* down regulation was masked by over stimulation of WNT pathway. This was investigated by testing the BRG1 knock-down effect on the transcriptional readout of weaker WNT signal.

![Figure 4.19. BRG1 sets up a signaling threshold for the optimal gene expression. The expression of Xnr3, Siamois, and Xnr6 was tested for the normal gene expression using two doses of Wnt8 in the presence and absence of Brg1 MO. The requirement of BRG1 for the normal gene expression was further confirmed by co-injection of Xbrg1 mRNA along with sub-optimal dose of Wnt8.](image)

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The animal caps were induced either by 50 pg of Wnt8 mRNA or by 20 pg of Wnt8 mRNA providing stronger and weaker WNT responses. The induction of xnr3, siamois and xnr6 genes was tested. xnr5 and xnr6 have been shown to be direct WNT target genes (Yang et al., 2002).

As shown in figure 4.19, both 50 pg and 20 pg of Wnt8 mRNA injection could induce xnr3 and siamois while xnr6 could only be induced with 50pg of wnt8 mRNA demonstrating that different WNT targets require different amount of inducer in order to become induced. When the expression of these genes were tested in the absence of BRG1, it was found that BRG1 knock-down causes down regulation of Siamois at 20 pg of Wnt8 induction which could be partially rescued by induction of caps with 50 pg of Wnt8 mRNA.

On the other hand, BRG1 knock-down completely inhibited the expression of Xnr6 even when the caps were induced by 50 pg of Wnt8 mRNA. On the contrary, BRG1 knock-down was unable to affect the expression of Xnr3, even when the caps were induced by 20 pg of Wnt8.

These observations were further confirmed by overexpression of xBRG1 in animal caps. The caps were induced by 2.5 pg Wnt8 mRNA, a sub-optimal dose for the full activation of WNT target genes. As shown in figure 4.19, 2.5 pg of Wnt8 mRNA partially induced siamois and xnr3 expression in the animal caps. When 2.5 pg of Wnt8 mRNA was co-injected with 500 pg of Xbrg1 mRNA, Siamois expression was slightly increased, while Xnr3 expression was strongly amplified. Notably, Xbrg1 mRNA alone was not able to induce either of the genes in animal caps (figure 4.19.). These results show that BRG1 sensitize the cells for WNT signaling by reducing the activation threshold. This means that certain WNT target genes can be activated at lower Wnt doses then others under reduced BRG1 protein levels.
4.17 Cloning of *Xenopus ini1*

INI1 is a component of the BRG1/BRM complexes. Homozygous deletion of *ini1* in mouse is lethal and mice die in periimplantation stage (Guidi et al., 2001), like mice deficient of BRG1. However, it has been shown that some of the target genes of BRG1 remain unaffected in the absence of INI1 (Doan et al., 2004).
Figure 4.20. Full length sequence of xini1 open reading frame. 1137 bp long open reading frame has no recognized domains. The corresponding amino acid sequence has been shown in single letter code in bold.
Thus, in order to further study the role of INI1, I cloned *Xenopus ini1* by using human INI1 as a query to search the *Xenopus laevis* EST database. The database search gave a highly homologous EST namely, CA973918, which was fully sequenced. *Xenopus ini1* ORF is of 1037 bp. xINI1 does not contain any functionally characterized domain, similar to other vertebrate INI1. The full-length sequence of xini1 is shown in figure 4.20.

Figure 4.21 Clustlaw alignment of *Xenopus* INI1 with mouse and human INI1. Alignment shows high conservation spreaded throughout the protein sequence.

*Xenopus* INI1 amino acid sequence was aligned with mouse (Q9Z0H3) (Bruder et al., 1999) and human (AAA81905) (Kalpana et al., 1994) INI1 using Clustlaw software. The alignment shows high homology between the *Xenopus*,
mouse and human INI1. The conservation of the protein is throughout the entire length of protein, as shown in figure 4.21.

4.18 Expression pattern of Xini1

The expression pattern of *Xenopus* ini1 was checked using a dig-labeled antisense probe. *In-situ* hybridization was carried out as described in the materials and methods section of this report. It was found that the expression of *Xenopus* ini1 could be detected at 2-cell and 4-cell stage, which represents maternal transcript of the gene.

At neurula stage embryo, Xini1 is expressed in the prospective neural tube and in the eye anlage. At NF stage 28, Xini1 is expressed in the head region specifically in the eye, pronephros, and branchial arches and in the neural tube. By NF stage 40, the expression of Xini1 remains limited to eye, pronephros and branchial arches and disappears from the neural tube (figure 4.22).

![Image of expression pattern](image)

Figure 4.22 Endogenous expression pattern of Xini1. Xini1 is expressed at two (A) and 4 (B) cell stage, a stage when zygotic transcription does not start showing that ini1 is maternally provided like Xbrg1. At Neurula, it starts expressing specifically in prospective neural tissues and in eye anlage (C). At st.35 Xini1 transcript are present in neural tube, eye, otic vesicles, pronephros and branchial arches (D) and at st.40 expression become more concentrated to the head region with expression in retina (E).

As shown here, the expression pattern of Xini1 was found to be similar to Xbrg1.
5 DISCUSSION

ATP dependent BRG1 chromatin remodeling complexes are large complexes of about 2 MDa in size. These complexes play a role in opening up local chromatin structures by either looping or by disrupting the nucleosomes around the promoters of various genes, making these promoters accessible for the transcriptional machinery. It has been shown in many previous reports that BRG1 containing chromatin remodeling complexes play a role in both repression and activation of genes. Most of the studies came from either Yeast or Drosophila (reviewed by Becker and Horz, 2002). The role of BRG1 has not been studied in vertebrates since knock-out studies in mouse could not reveal the role of these complexes in normal development (Bultman et al., 2000). In this study, I have shown by gain and loss of function experiments that BRG1 is required for the anterio-posterior body axis formation by specifically modulating the cellular Wnt signaling response during early patterning of the *Xenopus* embryo. In addition, I have shown that BRG1 modulated transcription is gene and signal specific and sets up a signaling threshold for normal Wnt-dependent gene induction.

5.1 Cloning and expression pattern

*Xenopus brg1* and *Xenopus ini1* were cloned by using the human homologues as a query to search the database. The members of ATPase family show high degree of sequence conservation among the vertebrates as also seen in case of *Xenopus* homologues. In case of *Xenopus* BRG1, the conservation of this protein is not just in the ATPase domain; rather it is extended throughout the protein as also shown by *Xenopus* INI1, which is also conserved throughout the whole sequence. This high conservation of sequence identity represents an opportunity to use the existing database and provides a method for *in-silico* cloning for other members of the ATPase complexes.
Xenopus BRG1 shows about 85% identity with its human counterpart. There were several missing regions in either of the sequences, which could be due to different splice variants of protein. The presence of a bromodomain, which represents a signature domain of SWI/SNF family members established this clone as a member of SWI/SNF family member (Horn and Peterson, 2001). The absence of a polyglutamine stretch, which is present in the BRM coding region, was one of the distinguishing features of it. Moreover, the sequence motif EED at the C-terminus of BRG1 is conserved in all the vertebrate homologues cloned until date, while BRM has sequence motif DDE at the C-terminus. Whether these differences are of functional importance is not known. These sequence features identify this cDNA as the Xenopus homologue of BRG1. Cloning of INI1 was undertaken to further understand the function of various subunits of BRG1 complex using database searches and was found to have significant homology with other vertebrate counterparts.

Xbrg1 transcript are present in the maternal mRNA pool of the embryo, which is consistent with the reported maternal expression of BRG1 protein checked by immunobloting of protein extract from various stages of oocyte (Gelius et al., 1999). In the same way, the Zebrafish brg1 has also been reported to be expressed maternally (Gregg et al., 2003). The expression of Xbrg1 at gastrula stage was ubiquitous, which becomes locally restricted from neurula stages onward, which is also one of the conserved features of the Brg1 expression in Zebrafish. Similarly, mouse Brg1 has also been shown to have widespread expression, which later becomes restricted to a number of tissues. In mice, highest Brg1 expression is found in the spinal cord, the brain, parts of the peripheral nervous system and in retina (Randazzo et al., 1994). This pattern is comparable to Zebrafish and Xenopus (Gregg et al., 2003). The Zebrafish retina shows expression in differentiating neuroepithelium and at later stage restricted to ganglion cells and inner nuclear layers (Gregg et al., 2003). The similar expression is also shown by Xbrg1 as seen in cross-sections of st. 35 embryos. Interestingly, at stage 45, the expression of Xbrg1 was found only in the heart. In a recent report, the expression of Smarcd3, a mouse homologue of BAF60c, a
subunit of BRG1/BRM complex, was also found to be restricted to the heart region until embryonic stage E9.25 (Lickert et al., 2004). Thus, the expression pattern of Xbrg1 is very similar to the previously reported Brg1 expression patterns in mice and Zebrafish, which concludes that expression of Brg1 is conserved among vertebrates.

The expression pattern of Xini1 was also found to be similar to that of Xbrg1. The expression pattern of Xbaf57 (Domingos et al., 2002) has also been reported to be similar to that of Brg1 and Ini1. Thus, xBRG1, xINI1 and xBAF57, which are conserved in core SWI/SNF complexes form an synexpression group (Niehrs and Pollet, 1999) while specific subunits like BAF60c, which are part of tissue specific SWI/SNF remodeling complexes, show a locally restricted expression.

5.2 Role of BRG1 in Dorso-ventral patterning

5.2.1 Methodological consideration

In order to study the role of chromatin remodeling molecules in the early development of *Xenopus laevis*, I used both loss and gain of function methods to find out the role of BRG1 in normal development. The gain of function studies were carried out by microinjection of mRNA at 4-cell stage for either wild type or dominant negative versions of BRG1. Microinjection of mRNA at 4-cell stage leads to overexpression of the protein in the embryo already prior to the onset of zygotic transcription. Overexpression of a core subunit of a protein complex might have several consequences. The exogenous protein may compete with its endogenous counterpart for incorporation into the complex during assembly. Alternatively, if this protein is normally limiting, more complexes may be assembled. Increased number of dominant negative BRG1 complexes will be competing with the endogenous protein complexes and thus will be inhibiting the endogenous function of BRG1. On the other hand increased number of wild type BRG1 containing complexes will co-operate with the endogenous function of BRG1 and thus potentiate their function. Proteins involved in complexes with
BRG1 to obtain full functional capability, are also involved in making complex with BRM. Thus during the gain of function studies, it is possible that overexpressed protein sequesters a subunit of the complex and thus makes it unavailable for other complexes which can lead to non-specific effects. This possibility of non-specific consequences was ruled out by rescue experiments, in which the phenotype of dominant negative BRG1 protein was reverted by overexpression of wild type BRG1 protein but not by overexpression of BRM protein.

Gain of function studies provide useful information about the potential function of a protein, however only loss of function studies can reveal essential functions of a protein. The methods generally used for loss of function studies in *Xenopus* are maternal knockouts or modified antisense oligonucleotides. siRNA procedure has also been used in *Xenopus* embryos for loss of function studies (Anantharam et al., 2003; Nakano et al., 2000; Zhou et al., 2002) but our attempts to use siRNA mediated knock-down failed, similar to some other *Xenopus* labs. Maternal knock-out studies (Heasman et al., 1991) are technically difficult with limited success and do not distinguishes between zygotic or maternal functions of the protein. The most commonly used method of knocking-down proteins is by microinjection of modified oligonucleotides in the desired region of embryo. Among the modified oligonucleotides, most common ones are phosphoorthothioate antisense oligonucleotides and morpholino antisense oligonucleotides. Phosphoorthothioate antisense oligonucleotides are comparatively cheap, but they are known to produce non-specific effects. Moreover, it is often also difficult to choose the correct target site for these oligonucleotides. In recent times, morpholino antisense oligonucleotides have been used (Heasman et al., 2000), which offer the advantage of high specificity, easier selection of target sites, and efficient knock-down of the desired protein. Based on these mentioned advantages and disadvantages of various methods available, I chose to carry out loss of function studies using morpholino antisense oligonucleotides.
In this study, I reported that the full-length transcript of Xbrg1 could only be obtained by \textit{in-vitro} transcription at lower temperature, than the usual 37°C. It has been reported previously that the \textit{in-vitro} transcription using T3, T7 or SP6 at 37°C may result in truncated transcripts and that this problem could be overcome by lowering the temperature (Krieg, 1990). Here, the full-length transcript of Xbrg1 could be obtained by T7 promoter, but not by SP6 polymerase. Thus, for the transcription of \textit{xbrg1}, the use of SP6 RNA polymerase is problematic. Since the pCS2+ vector, which is used to overexpress proteins in \textit{Xenopus}, contains an SP6 promoter upstream of translation start site, it was essential to use SP6 polymerase. In order to obtain full-length transcripts using SP6 promoter, lower temperatures were used for transcription reaction. As per some available reports (Ambion inc), some of the bacteriophage derived RNA polymerase show the tendency of falling off and terminating RNA synthesis, leading to prematurely terminated transcripts. The underlying reason for this premature termination has not been resolved until date.

5.2.2 Specific role of BRG1 in anterio-posterior axis formation

I have shown in the report that BRG1 participates in the formation of AP axis. These results are shown first, by overexpression of human BRG1, which could be rescued and thus representing the specific phenotypes. Later these results were also confirmed by overexpression of dominant negative \textit{Xenopus} BRG1. These results show that human BRG1 and \textit{Xenopus} BRG1 act in a similar manner, suggesting functional conservation. On the other hand, wild type human BRG1 did show no phenotype on dorsal as well as on ventral overexpression, while \textit{Xenopus} BRG1 produced head defects upon dorsal overexpression and induced an incomplete secondary axis upon ventral overexpression. These results show that \textit{Xenopus} BRG1 is more potent than human BRG1. This might be due to either higher stability of \textit{Xenopus} BRG1 or due to some sequence divergence, xBRG1 may be capable to form more stable complexes due to its compatibility with other endogenous components of BRG1.
complex. Overall, these results using human and *Xenopus* BRG1 variants confirmed the role of BRG1 in AP axis formation.

Apart from these gain of function studies, loss of function studies provided further evidence of the importance of BRG1 in head formation and dorsal ventral patterning. Notably, the xBrg1 MO induced phenotypes could not be rescued by hBRM and xISWI, demonstrating that the function of BRG1 is not redundant and could not be taken over by other members of the SNF2 family. Thus, the results obtained with gain and loss of function studies unambiguously established the role of BRG1 in AP axis formation.

Overexpression of wild type and dominant negative human BRM, as well as variants of xISWI did not cause any developmental phenotypes. These results confirmed that the observed phenotypes with BRG1 are not due to injection artifacts or due to toxic effects of RNA. These results obtained from overexpression of dominant negative and wild type human BRM are in consent with the recent reports showing that BRM knock-out mice do not show any phenotypes, probably due to compensation by BRG1 (Reyes et al., 1998). Moreover, it is now known that BRG1 is found in BAF and PBAF complexes while BRM is found in BAF complexes only. Thus, it is possible that loss of BRM, which will affect BAF complexes only, is compensated by BRG1, while the loss of BRG1, which affect both BAF and PBAF complexes, can not be fully compensated by BRM. Moreover other studies have also pointed out that BRG1 and BRM have transcriptional specificity (Kadam and Emerson, 2003). Furthermore, in a recent report it was shown that the transcription of sox2, a gene expressed in early neural cells, is dependent on BRM and not on BRG1 (Kondo and Raff, 2004) showing the differential requirement of BRM and BRG1.

The unsuccessful attempts to achieve developmental phenotypes by ISWI overexpression may be explained differently. In particular, it is known that xISWI is a highly abundant molecule in *Xenopus* oocytes (Guschin et al., 2000) and, thus, I might not be able significantly inhibit its function by overexpression of dominant negative protein. These results were also in consent with a recent
report showing that mice heterozygous for ISWI were normal, but homozygous null mutant mice dies in early embryonic stages (Stopka and Skoultchi, 2003).

5.3 BRG1 is required for normal expression of WNT target genes

When the phenotypes obtained from dorsal overexpression of wild type and dominant negative xBRG1 were observed closely, the overexpression of dominant negative BRG1 was found to cause preferentially loss of eyes, while wild type xBRG1, preferentially caused loss of cement glands. In a recent report, it was shown that the local mis-expression of Wnt8 in the head territory under control of the cerberus promoter causes preferentially loss of cement glands (Silva et al., 2003). A detailed model for head formation has been discussed elsewhere (Niehrs, 1999; Niehrs, 2004). Moreover, overexpression of xBRG1 on the ventral side produces a partial secondary axis, which indicates the formation of weak organizer on the ventral side. The role of WNT signaling has been convincingly shown in organizer formation as the overexpression of $\beta$-CATENIN on the ventral side causes secondary axis (Funayama et al., 1995) formation as well as inhibition of WNT signaling on dorsal side by UV treatment causes ventralization of the embryo (Kao and Elinson, 1988). The role of WNT pathway in dorso-ventral patterning has been confirmed by interfering with $\beta$-CATENIN by maternal knock-downs as well as by morpholino antisense mediated knock-down (Heasman et al., 2000; Wylie et al., 1996). The phenotypes observed in these cases were similar to BRG1 knock-down. Thus, our results strongly support dose-sensitive interactions between BRG1-containing remodeling complexes and the canonical WNT signaling pathway.

In-situ hybridization analysis of genes involved in dorsal mesoderm formation showed that indeed BRG1 affects WNT target gene expression, as evidenced by the downregulation of Xnr3 and Chordin. Reduced expression of Goosecoid also supports this conclusion. The goosecoid promoter is known to have two signal inputs i.e. WNT signal and NODAL signal. WNT signaling is relayed to the gsc promoter by the distinct WNT-targets siamois/twin, while
NODAL signaling is mediated by SMAD2/SMAD4 heterodimers (Germain et al., 2000). Reduced Siamois expression as checked in animal cap assay shows the reduced WNT signal input for activation of \textit{goosecoid}. Importantly, when \textit{goosecoid} was induced by ACTIVIN signaling alone in animal caps, its induction was unaffected by BRG1 knock-down, indicating that reduced expression of Goosecoid is due to reduced WNT signaling input. Interestingly, a nodal related gene \textit{xnr6}, which has been shown to be transcribed in pre MBT stages under the control of WNT signaling pathway (Yang et al., 2002) was found to be downregulated in animal cap assay under the reduced protein levels of BRG1.

These results suggest that while NODAL signaling remains unaffected, the reduced expression of Xnr6 reduces the nodal input on the \textit{goosecoid} promoter, resulting in reduced expression of Goosecoid. Taken together, four distinct WNT target genes were tested and all of them were misregulated by BRG1 over-/underexpression. I can conclude, therefore that the BRG1-CRC is essential for proper transcriptional regulation of WNT /\textit{\beta}-CATENIN target genes.
5.4 BRG1 containing complexes have gene and signal specific functions

It has been claimed previously that BRG1 acts as a global transcriptional activator (Armstrong et al., 2002). The loss of function studies presented here point to rather selective functions of BRG1. Reduced levels of BRG1 protein results in lower expression of Xnr3, Chordin, Myod and Myf-5, while expression of Xbra and Xvent-2 remains unchanged and Xpo expression is increased. Although it has been shown that WNT signaling affects Xbra expression (Schohl and Fagotto, 2003), I did not find any change in the expression of Xbra. This might be explained by a compensation of loss of WNT signaling input by other signaling events like FGF signaling, required for Xbra expression (Smith et al., 1991). Consistent with this assumption, when animal caps were induced by eFGF for the induction of Xbra, BRG1 knock-down does not affects the transcriptional levels of Xbra. The effect of BRG1 knock-down is not only gene specific but also signal specific as seen in animal cap assay. On one hand, BRG1 knock-down affects WNT signaling, shown by reduced expression of Siamois and Xnr6, ACTIVIN/NODAL related signaling remains unaffected, exhibited by Goosecoid and Xfd-1´.

The effect on FGF/MAPK signal appears also to be highly gene specific, given that the Xbra mRNA levels remain normal while expression of Myod was strongly reduced. The explanation for reduced transcription of myod came from a recent report showing that BRG1 and MyoD interact with each other in the presence of activated MAPK (Simone et al., 2004). It has been also shown that MyoD promotes its own transcription by autocatalysis (Steinbach et al., 1998; Thayer et al., 1989). Thus, it might be possible that BRG1 interacts with MyoD to promote the transcription of myod and thus when BRG1 is knocked out, the autocatalysis of myod will be blocked, leading to reduced transcription as seen in animal cap induction experiments.
BRG1 knock-down does not affect BMP signaling as checked by expression of Xvent-2, a gene under direct control of BMP signaling. These results were unexpected since the weakening of organizer would mean that weak organizer would no longer be able to inhibit invasive BMP signaling mediated ventral mesoderm formation but it is possible that the remaining activity of organizer is still sufficient to prevent the expression of Xvent-2 in the organizer territory. Although the expression of Xpo in organizer territory confirms that the organizer has been weakened, its increased expression could also be due to the repressive function of BRG1 as has been reported previously (Martens and Winston, 2003). Taken together, these results pointed out that BRG1 acts in a gene and signal specific manner.

5.5 Effects of BRG1 knock-down on organogenesis

The pleiotropic effects of BRG1 knock-down was shown in this report (figure 4.13) by reduced expression of several differentiation markers including neural n-β-tubulin, heart specific Nkx2.5, muscle specific Mlc35 and blood vessel specific Msr. These effects could be explained partly by perturbation of the primary embryonic patterning, but it does not exclude a requirement of BRG1 for tissue specific transcription factors. The effect on neural tissue development observed by neural specific beta-tubulin may be explained partly by the reduced expression of Chordin in blastula stage. *chordin* is a gene that is expressed in the BCNE center. The BCNE center gives rise to future neural tissue (De Robertis and Kuroda, 2004). A reduction of Chordin expression will impair the BCNE center, which will ultimately affect neural tissue development. A recent report has also demonstrated a role of *Xenopus* BRG1 in neurogenesis (Seo et al., 2005) although it claims that BRG1 knock-down does not affect neurogenesis during early patterning based on the fact that Sox2 expression was unaffected by the BRG1 ablation. SOX2 is a member of HMG box transcription factor family, involved in early stages of neurogenesis (Bylund et al., 2003). However, a recent report has shown that Sox2 expression is controlled by BRM and not by BRG1 (Kondo and Raff, 2004) in a study carried out in Rats.
As I have shown that BRG1 knock-down affects heart formation, a recent report supported my observation and provided a mechanism. It was shown that BAF60c, which is a component of BRG1 complex is expressed specifically in the heart and when knocked-down using siRNA, heart formation is severely affected. Furthermore, it was shown that BAF60c is required for transcription of Nkx2.5, which is dependent on BRG1. Furthermore, it has been reported that knock-out of BAF180, a signature subunit of PBAF complexes, in mice also causes heart defects (Wang et al., 2004b). The blood vessel formation is severely inhibited in BRG1 MO treated embryos. The process of angiogenesis requires among other factors, MMP-2. It has been shown that the transcription of \textit{mmp-2} is dependent on BRG1 (Ma et al., 2004). This observation points out a possibility that when BRG1 is knocked-down, the transcription of \textit{mmp-2} might get inhibited and this may affect angiogenesis. These observations point out a role for BRG1 containing chromatin-remodeling complex in tissue specific transcription. In later stages of development, the expression of Mlc35 was also reduced, confirming the reported role of BRG1 in myogenesis in which authors reported that dominant negative BRG1 interferes with the MyoD mediated muscle program shown by the down regulation of Myogenin and Mrf-4 (de la Serna et al., 2001). Here in this study, I have shown that both Myod and Myf-5 are down regulated upon BRG1 knock-down and thus showing another level at which myogenesis is affected by BRG1. A TCF binding site has been found in the \textit{myf-5} promoter, as well as it has been shown that \textit{myf-5} can be activated immediately after activation of animal caps by Wnt stimulation in the presence of cycloheximide (Shi et al., 2002), suggesting it to be a direct target of Wnt signaling pathway. As shown, upon BRG1 knock-down, Myf-5 expression is completely inhibited showing that BRG1 is required to activate the transcription of \textit{myf-5}, possibly by interfering with Wnt signaling. Thus, it can be concluded that BRG1 is involved in several differentiation programs at key regulatory levels.
5.6 Signaling thresholds at the nucleosomal level

I have shown that xnr6 could be induced by 50 pg of Wnt8 mRNA injection while siamois and xnr3 become induced already by 20 pg of Wnt8 mRNA. These results imply that different target genes require different doses of Wnt signal to obtain their normal level of transcription. The transcription of xnr6 was completely inhibited, while siamois transcription was reduced by BRG1 knock-down after the induction of Brg1 morphant caps with 50 pg of Wnt8 mRNA. These results show that xnr6 requires the highest amount of BRG1 for activation, while siamois could be induced in the reduced levels of BRG1. Notably, its Wnt dependent activation was not completely rescued even at the highest dose of Wnt8 used in this study. On the other hand, the transcription of siamois could be completely inhibited at reduced Wnt8 mediated induction.

Figure 5.2 BRG1 acts to set up signaling threshold for normal gene expression.

These results implied that BRG1 helps to translate signal quantity into promoter activity. Unexpectedly, it was found that Xnr3 transcription could not be affected by BRG1 knock-down in animal caps unlike in the embryo. Context dependency could be one explanation, since animal cap cells are prospective ectoderm, while in the embryo Xnr3 is expressed in dorsal mesodermal cells.
Another possibility is that the amount of Wnt8 used to induce the *xnr3* transcription is still high enough to counteract the reduced amount of BRG1 in the animal cap assay. Thus, *xnr3* requires the least amount of BRG1 for remodeling of its promoter, and may be fully activated already at very low levels of WNT signal.

This possibility was strengthened by the observation that xBRG1 could hyperstimulate the expression of Xnr3 in sub-optimal WNT induction conditions much stronger than Siamois induction. Thus, it can be concluded that BRG1 protein levels set up a threshold for the activation of WNT target genes at a given signal strength as presented in the model (figure 5.2).

### 5.7 Specific recruitment of BRG1 to target genes

From my results, it has become clear that BRG1 modulates the transcriptional read out of the canonical Wnt-signaling pathway required for early patterning of the *Xenopus* embryo. In order to do this, BRG1 is expected to bind to WNT-target gene promoters. A report showing interaction of BRG1 and β-CATENIN, provides a mechanism how BRG1 may become recruited to these promoters (Barker et al., 2001). However, this result was disputed since interaction of BRG1 and β-CATENIN could not be reproduced by other labs, which tested the interaction of *in-vitro* translated BRG1 and β-CATENIN (Kadam and Emerson, 2003). However, the epistasis experiments carried out during this project showed that β-CATENIN could rescue the Brg1 morpholino mediated phenotypes. Additionally, I could also show that BRG1 is required for the induction of secondary axis formation by β-CATENIN. These results in combination confirmed that BRG1 and β-CATENIN interact genetically. Recently, a report could show convincingly that BAF60c enhances the interaction of BRG1 and β-CATENIN (Lickert et al., 2004). This observation suggested that BRG1 is specifically recruited to the WNT target genes promoter by the interaction of BAF60c and β-CATENIN. In another case, the recruitment of BRG1 on the *myod* promoter could be explained by the interaction of BRG1 and MyoD in the
presence of activated MAPK (Simone et al., 2004). From these observations, it was clear that various subunits of BRG1 complex are required for recruitment of this complex to a variety of promoters other than having their role in obtaining the maximum activity of nucleosomal remodeling by BRG1 complex.

5.8 Conclusions and outlook

In this study, the role of BRG1 was studied in early development of *Xenopus*. It was shown that BRG1 is required for AP axis formation by affecting the early patterning of the embryo. It was also shown that BRG1 affects early patterning of *Xenopus* embryo by modulating WNT signal target genes. Furthermore, it was shown that BRG1 affects myogenesis. In this study, I found that BRG1 affects specific signaling pathways. BRG1 affected the WNT and eFGF-signaling pathway, while the ACTIVIN signaling pathway remained unaffected. I could also show that BRG1 sets up a signaling threshold for the stimulation of WNT target genes.

These observations provide an entry point for the analysis of BRG1 containing remodeling complexes *in vivo*. It will be interesting to show the recruitment of BRG1 on its target promoters using chromatin immunoprecipitation (ChIP). Moreover, it will be interesting to investigate, when BRG1 is required – i.e. before the assembly of pre initiation complex (PIC) as has been shown for some genes (Soutoglou and Talianidis, 2002), or after the assembly of PIC. These experiments can further be extended to find out, whether BRG1 stays bound to promoter, or leaves it after initiating the transcription.

Furthermore, the functions of various other subunits of BRG1 complex including INI1 can now be explored using the same strategy, used in this study. INI1 is a common component of BRG1 and BRM complex. Therefore, it will be interesting to find out if INI1 knock-down affects all BRG1 target genes or other genes as well. Furthermore, it can also be tested if some of the BRG1 target genes remain unaffected by INI1 knock-down as has been reported. These
studies could explore the role of other subunits of BRG1 complex in early development. Given the importance of INI1 in rhabdoid tumor development, it may also provide further mechanistic explanation of the role of INI1 in this disease. Other than INI1, other interesting subunits to study will be BAF180 and BAF250 due to their specific presence in PBAF and BAF complexes respectively.

As it has been shown that BRG1 knock-down affects several tissues, the role of BRG1 in organogenesis can also be studied. It has been shown that BAF60c and BAF180 are required for heart formation. In *Xenopus*, animal cap system can provide an easy method to study the organogenesis outside the organism. Animal caps contain multipotent embryonic stem cells. The animal cap tissue can be differentiated into various organs and cell types by specific induction protocols, using variable Activin and Retinoic acid concentrations (Ariizumi and Asashima, 2001). By this strategy, it is now possible to study the specific role of chromatin remodeling complexes in organ development.
6 LITERATURE


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Flemming W.,1882 Zell substanz,Kern und Zelltheilung.Leipzig:Vogel


6 Literature


## ABBREVIATIONS

<table>
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<tr>
<th>Abbreviation</th>
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<td>A</td>
<td>Adenine</td>
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<td>Ab</td>
<td>Antibody</td>
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<td>APS</td>
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<td>BCIP</td>
<td>5-Bromo-4-chloro-3-indolyl-phosphate</td>
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<td>BCNE</td>
<td>Blastula Chordin Noggin Expression center</td>
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<td>BMP</td>
<td>Bone morphogenic protein</td>
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<td>bp</td>
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<td>HCG</td>
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<td>HEPES</td>
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