Xmc, a novel *Xenopus laevis* organiser gene regulating gastrulation movements

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Abstract

The *Xenopus* (or Spemann-Mangold) organiser is a powerful signalling centre responsible for establishing the main body plan. It instructs indifferent, competent tissue to follow certain paths of development: it neuralises ectoderm and dorsalisises mesoderm and endoderm. In addition to its inductive capabilities, the organiser governs the morphogenetic movements occurring during gastrulation. Gastrulation in vertebrates is a highly dynamic process, driven mainly by *convergent extension* of internal mesodermal cells, by which all three germ layers are patterned within an embryo with recognizable antero-posterior and dorso-ventral polarity.

In search for genes expressed in the organiser, an *in situ* hybridisation screen was carried out on a cDNA subtracted library enriched for organiser genes. Such library was constructed by Suppressive Subtractive Hybridisation, subtracting a Dorsal Marginal Zone cDNA population with an egg cDNA population. 6% of the genes isolated in the screen exhibited differential expression in the organiser.

One of them was termed *Xmc*, an acronym for *Xenopus marginal coil*. *Xmc* encodes a protein containing two widely spaced evolutionarily non-conserved coiled coils. Immuno-localisation studies reveal *Xmc* protein is found in vesicular aggregates in the cytoplasm and associated with the inner plasma membrane. *Xmc* is expressed in a dynamic fashion around the blastoporal circumference, in mesodermal cells undergoing morphogenetic movements, in a pattern similar to *bona fide* FGF target genes. Likewise, *Xmc* expression can be induced by ectopic XeFGF signalling and the early mesodermal expression is dependent on FGF receptor-mediated signaling. Morpholino-mediated translational “knock-down” of *Xmc* results in embryos that display a reduced elongation of the antero-posterior axis and in a pronounced inhibition of convergent extension movements in embryos and dorsal marginal zone explants.
The execution of convergent extension movements normally relies to accurate formation and differentiation of mesodermal tissue. Xmc loss-of-function does not interfere with mesoderm induction or maintenance per se.

These results suggest that Xmc is a novel FGF target gene that is specifically required for morphogenetic movements during gastrulation in Xenopus, but is dispensable for the concomitant patterning of mesoderm.
Alla mia famiglia
...for I myself am not aware of having any wisdom, neither great, nor little

...he thinks he knows something not knowing,

while I, as one who does not know, do not think I do.

(Plato, Apology, fr.21b-d)

Per Aspera Ad Astra
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<tr>
<td>BSA</td>
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<td>DAPI</td>
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<td>fluorescin isothiocyanate</td>
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<td>OD</td>
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<td>PEG</td>
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<td><em>pfu</em> pol.</td>
<td><em>Pyrococcus furiosus</em> DNA polymerase</td>
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<td>rpm</td>
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<td>SDS</td>
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<td>Taq pol.</td>
<td><em>Thermus aquaticus</em> DNA polymerase</td>
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<td>TBS</td>
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Chapter One
Introduction
Echoing heredity of Spemann-Mangold's experiment.

"What has been achieved is but the first step; we still stand in the presence of riddles, but not without hope of solving them. And riddles with the hope of solution-what more can a man of science desire?"

(Hans Spemann, Croonian Lecture, 1927)

In 1924 Hilde Mangold, in Hans Spemann's laboratory, conducted an experiment which was to mark a decisive turn in classical embryology and to raise the issues of cell fate determination and informative induction (Spemann and Mangold, 1924).

The experiment consisted in grafting the dorsal upper lip of the gastrula blastopore, die obere Urmundlippe, to the ventral margin of other gastrula embryos (Fig.1.1). This transplantation would give rise to the formation of a secondary body axis at the site of the graft containing a notochord and a neural plate in normal alignment and proportions. Such types of transplantations had been previously performed, also by Spemann himself (Lewis, 1907, Spemann 1918), but since the donor and the host embryos came from the same newt species, it was not possible to distinguish which elements of the secondary axis originated from the donor or from the host. The innovative addition of Hilde Mangold's experiment to get around this limitation was 'heteroplastic transplantation', the use of donor and host embryos from species of different pigmentation. This allowed the observation that the majority of structures of the supernumerary axis derived from the host. The donor graft always differentiated as notochord, as mesenchyme, sometimes as a minor part of the floor plate of the neural tube and as minor parts of somites and gut roof. But the host gave origin to most of the neural tube tissue, the kidneys, most of the somites and the gut.
Fig. 1.1 Dorsal lip transplantation, seminal experiment of Hilde Mangold.
The dorsal blastopore lip of a light pigmented embryo (oval in red) is excised and grafted onto the ventral side of a host darkly pigmented embryo. The graft originated in the host embryo a supernumerary axis. The supernumerary axis is composed of donor and host cells as assessable from the mosaic pigmentation of the induced structures. Host contributes mostly neural tube, somites, pronephros and gut to the secondary axis, whereas the graft contributes mostly notochord and floor plate. Tissues of the secondary axis are marked g, h or g+h to indicate graft or host origin. Original section adapted from Spemann and Mangold, 1924
These observations let the authors suggest that the grafted lip not only had the ability to differentiate into axial mesodermal derivatives, but could also induce naïve neighbouring tissues of the host to form a variety of tissues that they otherwise would not. It became clear that cells near the resident lip of every embryo achieved their differentiation in the same way, according to their distance from the resident lip. The lip was then christened “organizer”, as the part of the embryo “which is able to set up an organisation field of a certain orientation and extent when introduced in the midst of indifferent tissue” (from a translation by V. Hamburger). By describing the organizer, a source of inductive signals was found, which transfers information to indifferent, but competent cells of the three prospective germ layers, to direct subsequent paths of development. Since then the Holy Grail of modern embryology has been the identification at the molecular level of such signals. Generations of scholars have devoted their careers to the molecular characterization of the organizer and significant progress has been made.

The first molecule to light up the organizer in an in situ hybridization was goosecoid (Cho et al., 1991). Gsc was a great organizer marker, with organizer activity, capable of reproducing embryonic twinning when overexpressed in Xenopus embryos. Following the discovery of gsc, many other organiser genes have been isolated, mainly transcription factors and secreted proteins (reviewed in Harland and Gerhart 1997). Nowadays, genes specifically expressed in the organizer can be counted by the dozen.

In this limited review, I would like to describe the wealth of properties of this potent signalling centre. After having described how it originates in the gastrula embryo, a definition of its trunk and head instructing regions will be provided. This will be followed by an account of the corresponding molecular networks responsible for establishing the dorso-ventral patterning and the distinction between trunk and head formation.
In Chapter Four, the characterisation of a gene likely to have a role in convergent extension movements is presented. In light of this, particular emphasis will be devoted in this general introduction to the description of morphogenetic activities of the organiser and to the organiser molecules responsible for them.

I will mainly concentrate here on the organiser in *Xenopus*, providing where appropriate, some evolutionary parallels with other vertebrate and invertebrate species.

**Evolution of Spemann-Mangold Organizer**

The discovery of the *Xenopus* Organizer and of the mechanism of cell-cell interaction and 'induction' inaugurated a search for similar organising centres and similar mechanisms in other animals. On the basis of their ability to form a secondary axis when heterotopically grafted in young gastrulae, and on the basis of stainings with gsc orthologues, structures functionally equivalent to the amphibian organiser have been identified in other vertebrates (Fig.1.2). In mouse, this structure is the 'node', positioned at the anterior tip of the primitive streak (Beddington, 1994). In the teleost zebrafish, the organiser corresponds to the 'shield', a thickening at the dorsal margin that forms at the onset of gastrulation and that is very much resembling the dorsal lip of *Xenopus* (Oppenheimer, 1936, Shih and Fraser, 1996). In chick, two structures have axis inducing ability: 'Koller's sickle' (Izpisua-Belmonte et al., 1993), located at the posterior marginal zone of the early gastrula and 'Hensen's node', located at the anterior tip of the primitive streak (Waddington, 1933) and induced from the former. Despite the considerable anatomical differences, these vertebrate structures clearly identify elements conserved during evolution.
Fig. 1.2. Equivalent organiser structures in four different vertebrate classes
This picture illustrates the position of the organiser (red oval) in four vertebrate models; in the frog (lateral view, dorsal to the left), the organiser extends from the blastopore lip; in fish (here viewed from the dorsal side of the equator) it is centred on the embryonic shield; in chicken (view from epiblast side, inner circle, area pellucida, outer circle, area opaca, posterior to the bottom) Koller's sickle is located at the posterior margin and will induce Hensen's node at later stages; in mouse it is situated at the anterior tip of the primitive strak (the bottom of the cylinder).
Organiser function then seems to be a distinguishing characteristic of the chordate phylum. In fact, it may be shared by evolutionary older phyla, like the Cnidaria, as unveiled by studies in Hydra. This latter was used as a system for organiser transplantation studies performed as early as 1909 by Ethel Browne. This latter transplanted a small graft of hypostome (mouth tissue) to the flank of Hydra, giving rise to a complete ectopic head, including tentacles, induced in the host. More recent molecular analysis in this diploblastic organism further shows that the Hydra organiser has significant conservation to the signalling pathways of the gastrula organiser (Hobmayer et al., 2000).

These analogies certainly pose interesting evolutionary questions and it is anticipated that more comparative studies with diverse model organisms will help unravel the evolution of developmental control mechanisms.

Formation of the organizer - the animal vegetal axis.

The organizer constitutes about 10% of the cells of a gastrula embryo. Its formation at the equatorial mesodermal region between the animal and vegetal poles is controlled by the combinatorial action of two maternal components: a ‘vegetal’ mesendoderm inducer, the TGF-β and FGF signalling pathways and a ‘dorsal modifier’, the Wnt/β-catenin signalling pathway (reviewed in Nieto 1999, Bouwmeester 2001 and Yasuo & Lemaire 2001). The mesendoderm inducer, secreted by all vegetal cells, sets up the germ layer-specific competence domains (ectoderm, mesoderm and endoderm), while the dorsal modifier, secreted by the Nieuwkoop centre, sets up the dorsoventral axis of the embryo. I will describe in this chapter both of these actions starting with the one of the dorsal modifier.

Essential for the release of the dorsalising signal is the formation of the Nieuwkoop center (dorsal endoderm), which constitutes tiers C and D on the
dorsal side of the 32 cell stage embryo. It is generated during cortical rotation, by the dorsal accumulation of the Wnt signal transducer β-catenin. The enrichment of β-catenin is enhanced by the concomitant displacement along microtubules of Dishevelled (Dsh), which is another upstream component of the Wnt signaling pathway (Miller et al., 1999). The presence of Dsh reinforces the accumulation of β-catenin because it inhibits the constitutive activity of a serine/threonine kinase, glycogen synthase kinase-3β (GSK-3β). In complex with axin and the tumour suppressor Adenomatous Polyposis Coli (APC), GSK-3β normally phosphorylates β-catenin targetting it to proteolytic degradation by the proteasome (Moon and Kimelman 1998). Stabilized cytosolic β-catenin translocates to the nucleus, where it forms a complex with the HMG-box proteins Tcf-3/Lef-1, to transactivate the expression of early zygotic target genes, such as siamois, twin and Xnr-3. In turn, these latter trigger transcription of early Spemann-Mangold organizer genes in the dorsal marginal zone (Harland and Gerhart, 1997; Moon and Kimelman, 1998).

As introduced above, formation of the Spemen-Mangold organizer is also dependent on a mesendoderm inducer setting up the germ layer-specific competence domains. Important experiments by Nieuwkoop (Nieuwkoop, 1969a and b) demonstrated that a signal emitted by the most vegetal cells, the future endoderm, induces the overlying prospective ectoderm to form mesoderm at the equatorial ring of the embryo (reviewed in Nieuwkoop 1977). Later experiments have showed that this signal was present in the cell as early as the 32-cell stage, clearly before the onset of transcription. Such signal was mimicked, at least in vitro, by members of the TGF-β and FGF families of secreted factors (Woodland and Jones, 1987).

Therefore, it is widely accepted that the endogenous mesoderm-inducing signal must be provided as a maternal transcript or a maternally deposited protein encoding a secreted factor. Endoderm development is also dependent on TGF-β
signalling, suggesting that a common pathway may induce mesoderm and endoderm (Henry et al., 1996).

A good candidate for this factor is Vg1, a member of the TGF-β family isolated by Weeks and Melton (1987). Vg1 maternal transcripts are localized to the vegetal cytoplasm during oogenesis. Artificially processed Vg1 has mesoderm and endoderm inducing activity. However, the active form of Vg1 has never been detected in vivo and ectopically expressed wild-type Vg1 does not induce mesoderm or endoderm (Tannahill and Melton, 1989). This might argue that the processing of Vg1 must be tightly regulated, and that the mature form of Vg1 is present in vivo at undetectable levels. An extension of the role of Vg1 has been provided by studies using dominant negative forms of the protein. Such studies imply a role for Vg1 in the development of dorsal mesoderm and endoderm (Joseph and Melton, 1998).

An interesting alternative and a serious challenge to the general accepted view of mesoderm induction by a maternal vegetal secreted factor has been VegT. This is a member of the family of T box transcription factors, isolated from many different labs and proven to have a role in the establishment of the mesoderm and endoderm germ layers (Lustig et al., 1996; Stennard et al., 1996; Zhang and King, 1996; Horb and Thomsen, 1997). VegT is stored maternally, as a transcript localized to the vegetal hemisphere of eggs and embryos. Just before gastrulation, zygotic VegT transcripts are found throughout the mesoderm. Work by Zhang et al. (1998) shows that VegT depleted oocytes do not form any type of mesoderm or endoderm. Furthermore vegetal explants derived from VegT depleted embryos have no mesoderm inducing activity on responsive ectodermal cells (Zhang et al., 1998). As a transcription factor VegT does not act until the start of zygotic transcription at mid-blastula (4000 cells). Therefore zygotic inducing factors, downstream of VegT, not maternal signalling factors, would initiate the endogenous signal for mesoderm formation.
The zygotic action of VegT in mesoderm determination is regulated via TGF-β factors. Several groups have shown that VegT directly activates members of the Nodal subclass of TGF-βs: Xnr-1, Xnr-2 and -4 and Derrière (Kofron et al., 1999; Agius et al., 2000). The transcription of these factors does not occur in VegT depleted embryos (Kofron et al., 1999) and they are able to rescue the phenotype of VegT depleted embryos. While Xnrs can efficiently rescue a complete embryonic axis, derrière only rescues trunk/tail structures, in agreement with its loss-of-function phenotype which only disrupts posterior structures (Sun et al., 1999a). This indicates that this latter may not be overlapping with the Xnrs and that it has a divergent signalling pathway, activating axial and tail genes but repressing or not regulating head genes.

Xnr-1, -2 and -4 have also been implicated in dorsal signaling since they are expressed later in the Spemann organizer and rescue the dorsal axis of irradiated embryos to various extent (Jones et al., 1995; Joseph and Melton, 1997). It is known that β-catenin is also required for the induction of zygotic mesoderm-inducing signals in Nieuwkoop conjugates (Wylie et al., 1996; Zhang et al., 1998). An up-dated scenario of this induction would see both VegT and β-catenin acting together to set up a dorsal to ventral gradient of Nodals in the endoderm. On the ventral side, VegT (and Vg1) would lead to the production of lower nodal-related signals sufficient for the formation of ventral mesoderm and expression of genes such as Xwnt-8 and BMP-4; on the dorsal side VegT and the contribution of a functional β-catenin pathway would establish a high nodal-concentration for the induction of a bona fide Spemann-organizer (expressing genes like chordin, noggin and frzb-1). This would imply that β-catenin signaling is present not only in the most dorsal cortex, but it is extended more internally in the embryo. In fact, genes like cerberus and Xhex, which are expressed at the dorso-anterior endoderm also require an input of β-catenin pathway (Jones et al., 1999; Zorn et al., 1999)
The concerted action of Xnrs and β-catenin results in the alignment of dorsoanterior endoderm, the prechordal plate, the notochord and presumptive floorplate territories, which are the progeny of Spemann's organizer.

How is endoderm specified and how diverging is its specification from the one of mesoderm?

The vegetal endoderm has a deceptively homogenous appearance. As has been outlined above, endoderm has been implicated in the induction of mesoderm (Nieuwkoop, 1969a and b), but it is also involved in axis formation (Vincent et al., 1986) and in germ-cell lineage establishment (Holwill et al., 1987). The recent identification of zygotic endoderm genes has helped define a molecular pathway leading to endoderm formation. Such genes include Sox17α and β, encoding HMG proteins (Hudson et al., 1997), the paired-like homeobox genes Mix.1, Mixer, Milk (also named Bix2), Bix1/3/4 (Rosa, 1989; Lemaire et al., 1998; Henry and Melton, 1998; Ecochard et al., 1998), the divergent homeobox gene HNF1β, and GATA4/5/6, encoding proteins with zinc finger motifs (Vignali et al., 2000; Jiang and Evans, 1996). Many of them have been implicated in the endoderm differentiation by overexpression and dominant negative studies. Recent work by Janet Heasman and collaborators has established a gene hierarchy for endoderm formation, which is initiated by VegT (Xanthos et al. 2001). They confirm that VegT is required for the expression of zygotic endoderm genes, but they also prove that TGF-β signaling (namely Xnrs) are required for the expression of these genes, downstream of VegT. Furthermore, co-culture experiments between normal and VegT depleted endodermal masses show that the latter can receive a signal from the untreated vegetal poles restoring expression of endodermal genes (although this signal is not as robust as for mesodermal induction). This would imply that in addition to being a cell-autonomous event, endoderm formation may rely on cell-cell interaction (Xanthos et al., 2001). Endoderm formation would start by simple and direct initiation of VegT and would become more
tightly regulated by a heterogenous network of zygotic transcription factors and signaling molecules, downstream of VegT and by inductive interactions between neighbouring cells.

The organizer has a structure of its own

The activity of the organizer is most potent during gastrulation. Spemann was able to characterize a structural and temporal order in the organizer. Only grafts from early gastrula stage organizer result in the induction in gastrula host embryos of complete neural axis, including head and most anterior structures. As the age of the graft increased, the capacity of inducing secondary axis with heads was gradually lost and the organizer was only able to duplicate trunk/tail structures (Fig. 1.3; Spemann, 1931). Therefore it was possible to distinguish a head and a trunk organizer differing in their inductive abilities, their morphogenesis and their self-differentiation. These differences arise from the change in time of the cellular composition of the lip, due to the involution and ingression of mesodermal and endodermal cells of the marginal zone inside the lip. The early lip, head organizer, contains cells derived from the vegetal part (ventral band) of the marginal zone, whereas the late lip contains the animal edge (dorsal band) of the marginal zone, which constitutes the limit of ingression.

The head organizer differentiates into head tissues, which are located more ventrally, such as the prechordal plate mesoderm and the pharyngeal endoderm. The mesodermal subregion of the head organizer induces head parts such as anterior neural plate (forebrain, midbrain), and gill slits.

The trunk-tail organizer originates in the dorsal band of the marginal zone and its developmental fate is essentially dorsal mesoderm, mostly the notochord.
Fig. 1.3 Structural and temporal order in the organiser
Spemann was able to identify a structural and temporal order in the organiser (1931). The capability of an organiser graft to induce a complete secondary axis is gradually lost as the age of the graft increases. Grafts from early gastrula can induce a complete neural axis, including head and most anterior structures. Transplantation of late gastrula lips only gives rise to trunk and tail structures. Therefore, a 'head' and a 'trunk' organiser can be distinguished. Picture borrowed from 'Developmental Biology', S.Gilbert, Third Edition.
Its endodermal fraction differentiates into the gut roof. The trunk organizer displays a remarkable morphogenesis. Its population of cells engages in convergent extension (a more accurate description of these movements is given in a subsequent section). The trunk organizer induces several kinds of development in the ectodermal, mesodermal and endodermal competence groups.

In the ectoderm, it induces the middle and posterior part of the neural plate, the parts differentiating into hindbrain, spinal cord and trunk neural crest derivatives. Formation of somites is induced in the mesoderm. Whereas in the endoderm the trunk-tail organizer induces the hypocord from the gut roof (Cleaver et al., 2000).

But there is a third region in the embryo, which has been attributed organiser function. This is the deep yolky endoderm, most recently found, least characterized and suggested to be equivalent to the mouse anterior visceral endoderm (AVE), implicated in anterior patterning of mouse neuroectoderm before gastrulation (Bouwmeester et al., 1996; Thomas and Beddington, 1996). It is the site of expression of \textit{cerberus} and \textit{dickkopf}, two head inducers and it has been therefore defined to have a role in head induction. It is eventually fated to become liver and anterior gut. It anteriorises endoderm and it probably induces heart from the ventral band of the marginal zone mesoderm (Schneider and Mercola, 1999). This region also displays some morphogenetic role. Its cells must engage in changes of affinities for the formation of Brachet's cleft, between the marginal zone and the deep cells at the start of gastrulation (Winklbauer and Schurfeld, 1999).

In summary, the organizer can be distinguished in three qualitatively different parts. These differ for the genes they express, their self-differentiation, for the inductions they promote and for their morphogenesis, but only their concerted action ensures the development of a complete body axis.
Although the head and trunk-tail organizer share some expressed genes and secreted inducers, the trunk organizer has some properties of its own. The deep yolky endoderm serves as an important enhancer to the head organizer, but it also specifies other structures, such as heart and liver.

I will describe the molecular pathways that are responsible for head and trunk induction in the following sections.

**Anti-BMPs as trunk inducers; dorso-ventral patterning**

Within the 75 years of study of the organizer, several secreted proteins have been isolated mediating organizer’s inductive signals: initially Noggin (Smith and Harland, 1992), Follistatin (Hemmati-Brivanlou et al., 1994) and Chordin (Sasai et al., 1994). These molecules antagonize signalling by bone morphogenetic proteins (BMP) and do so by sequestering them into inactive complexes thereby preventing them from binding to their receptors (Fig. 1.4; reviewed in Harland and Gerhart, 1997).

When expressed at the ventral side of *Xenopus* noggin and chordin encoding mRNAs induce secondary embryonic axes of trunk character. These supernumerary axes contain a spinal cord and somites but rarely a notochord, suggesting that for the establishment of a complete secondary axis, other factors are required (these factors are Wnt inhibitors, as discussed below). Head structures in these axes, including optic vesicles, are typically absent.

The activity of chordin and noggin induces neural tissue in ectodermal explants and dorsal mesoderm in ventral mesoderm explants (Lamb et al., 1993; Sasai et al., 1995; Piccolo et al., 1996). Dorsalisation or neural induction is also obtained when these tissues are treated with Follistatin (Hemmati-Brivanlou et al., 1994). The neuralising activity of noggin and chordin can be counteracted by BMP-4.
Fig. 1. Spemann-Mangold organiser's antagonism versus BMP ventral signals. Ventralising BMP signals are secreted form the ventral side of the embryo and are antagonised by organiser secreted factors such as Chordin, Noggin and Follistatin. The antagonism is direct with these latter secreted factors binding BMPs in the extracellular space of ectoderm, mesoderm and endoderm thereby contributing to the pattern of the three germ layers.
(Sasai et al., 1995). In a converse experiment, blocking BMP signalling with a dominant-negative BMP receptor (Graff et al., 1994) or with antisense BMP-4 RNA (Steinbeisser et al., 1995) results in induction of anterior neural tissue in animal cap ectodermal explants (Sasai et al., 1995). In addition to their neural inducing activity, chordin and noggin also induce endoderm in animal explants (Sasai et al. 1996). In agreement with this, the supernumerary axis induced by overexpression of chordin mRNA contains a secondary gut, pointing to BMP inhibitors as patterning all three germ layers.

Another molecule with trunk organizer function is ADMP, anti-dorsalising morphogenetic protein (Dosch and Niehrs, 2000). ADMP is a member of the BMPs and is closely related to BMP3. It is expressed in the chordamesoderm and its overexpression elicits ventralisation in *Xenopus* (Moos et al., 1995). Unlike other BMPs, however, ADMP is not inhibited by a dominant negative BMP type I receptor, Noggin or Chordin, but by Follistatin, indicating that it utilises a distinct TGF-β receptor pathway. A dominant negative form of ADMP, CmADMP leads to dorsoanteriorised embryos, with enlarged heads and shortened trunks and co-injection with BMP inhibitors induces ectopic heads on the ventral side. This suggests that ADMP is required to antagonise head formation; the failure of anti-BMPs (trunk inducers) to induce complete secondary axes could be explained by the concomitant induction in the chordamesoderm of ADMP which, in turn, represses head organizer genes.

The molecular mechanism by which some of these trunk inducers work was resolved with the purification of Chordin (Piccolo et al., 1996) and Noggin (Zimmerman et al., 1996) proteins and the demonstration that they directly bind BMPs.

Chordin binds to BMP4 with an equilibrium dissociation constant, \( K_d \) of 300pM, which is about the same as the affinity of BMP for its cognate receptors on cell membranes (Piccolo et al., 1996). Noggin complexes with BMP4 (\( K_d = 20pM \)) and
BMP2, and less strongly with BMP7. Follistatin has an affinity for BMP4 (Fainsod et al., 1997), but is also a potent inhibitor of activin (Hemmati-Brivanlou and Melton, 1994, Sasai et al., 1995).

More light has been subsequently shed on the mechanism of action of chordin, in analogy with mechanisms utilised by homologous molecules in *Drosophila melanogaster* for the establishment of dorso-ventral patterning. Vertebrates and invertebrates share a common dorsal-ventral patterning system. Chordin and BMP are homologues of the Drosophila Short-gastrulation (Sog) and Decapentaplegic (Dpp), respectively, the activity of this latter being repressed by Sog. Nevertheless, the positioning of these molecules along the dorso-ventral axis of the respective organisms is inversed. As early as 1822, Geoffrey Saint-Hilaire originally proposed that the dorsal-ventral domain is inversed between vertebrates and invertebrates. In chordates, the *sog/chd* domain is dorsal, while in arthropods it is ventral (DeRobertis and Sasai 1996; Arendt and Nuebler-Jung, 1994). Similarly, the *dpp/bmp* domain is ventral in vertebrates and dorsal in invertebrates. These proteins are interchangeably active in either phylum, but their functions are reversed, with the *Xenopus* dorsalising factor *chd* promoting ventralization of cell fates in *Drosophila*, and its invertebrate ventraliser homologue, sog, causing dorsal development in *Xenopus*. The same applies for the orthologue couple *bmp* and *dpp* (Holley et al., 1995).

In *Drosophila*, genetic analysis suggests that the gene *tolloid*, encoding a member of the astacin family of metalloproteases increases *dpp* signalling (Ferguson and Anderson 1992a; Ferguson and Anderson 1992b); it does so because its product cleaves Short-gastrulation, relieving the inhibitory effect on Dpp (Marques et al., 1997). In *Xenopus*, a related gene was isolated and called *xolloid* (Goodman et al., 1998; Piccolo et al., 1997). Micro-injection of its mRNA causes a ventralisation of the *Xenopus* embryos, in a phenotype similar to the one resulting from injection of intermediate doses of BMPs or from inhibition of Spemann-Mangold...
organiser. A biochemical approach has shown that the Xolloid zinc metalloprotease cleaves and inactivates Chordin, thereby releasing reactivated BMPs (Piccolo et al., 1997). Xolloid cleaves Chordin at two of its four cysteine-rich domains, CR1 and CR3, which are also the sites at which BMPs bind Chordin with highest affinity. (Larrain et al., 2000). As shown by cross-linking studies (Piccolo et al., 1996), a possible scenario for this mechanism sees a Chordin monomer bind a BMP dimer via CR1 and CR3 domains, allowing the diffusion of BMPs to distant sites, without being sequestered by BMP receptors. Once the complex meets the metalloproteases, active BMPs would be released (Fig. 1.5).

An additional, intriguing novelty in dorso-ventral regulation has been introduced by studies on twisted gastrulation (tsg), confirming once again the astonishing functional equivalence of molecules among vertebrates and invertebrates.

In Drosophila embryos, tsg is involved in specifying the dorsal-most cell fate (Mason et al., 1994). It is now demonstrated that Tsg acts as an enhancing co-factor in Chordin/Sog antagonism of BMP/Dpp signalling (Chang et al., 2001; Ross et al., 2001; Scott et al., 2001). In Drosophila, Tsg loss-of-function results in defects in the amnioserosa (a most dorsal structure), the formation of which requires high levels of Dpp. So, it was originally thought that Tsg would potentiate Dpp activity. It is now demonstrated that Tsg acts as an enhancing co-factor in Chordin/Sog antagonism of BMP/Dpp signalling in Drosophila, frog and zebrafish (Scott et al., 2001, Ross et al., 2001 and Chang et al., 2001). It appears that synergy between Tsg and Sog makes a more effective inhibitor of BMP signalling than either of them alone.
Fig. 1.5 Possible model for silencing BMP signalling through Chordin. Chordin blocks BMP signalling efficiently by binding a BMP dimer because of its CR1 and CR3 domains ($K_D=3 \times 10^{-10}\text{ M}$). Xolloid digestion (represented by the black arrows) releases the CR1 fragment, which binds BMP with a 10-fold lower affinity and is then less efficient in blocking BMP signalling.
Co-immunoprecipitation studies provide a molecular explanation for these observations. Tsg forms a ternary complex with Sog and Dpp, allowing diffusion of Dpp through the embryo and preventing its association with cell-surface receptors along the way. The degradation activity by the protease tolloid on Sog is facilitated by the presence of Tsg in the complex (Scott et al., 2001). This would result in a quick degradation of Sog and release of Dpp throughout the embryo. But near the source of Sog synthesis, Sog molecules are in high abundance and ready to recapture any released Dpp molecule. Moving away from the signal source, Sog degradation restricts the inhibitory complex, allowing Dpp to hook on its receptors. This mechanism would not only form a smooth gradient of Dpp activity, but also helps reaching a peak of Dpp activity away from Sog source (Fig. 1.6).

The description of these mechanisms pinpoints the role of extra-cellular antagonism in embryonic patterning. The trunk organizer activity resides in the inhibition of BMP signalling, which is by itself an antagonist of trunk formation. A similar mechanism, but with different additional molecules is also observed in the definition of anterior regions of the embryo.

**More inhibition to form a head**

A landmark in understanding the molecular nature of the head organizer was the isolation of *cerberus*, a multifunctional secreted protein, which induces ectopic heads (Bouwmeester et al., 1996). Its characterisation provided evidence that specification of rostral identity in the vertebrate embryo is dictated by signals originating in the dorso-anterior (primitive) endoderm (reviewed in Bouwmeester and Leyns 1997), in addition to signals from the prechordal plate mesoderm previously characterised as instrumental in establishing anteroposterior neural patterning (reviewed in Bally-Cuif and Boncinelli 1997).
Fig. 1.6 Tsg involved in Dpp antagonism

Tsg, Sog (Chd) and Dpp (BMP) form a tripartite complex, in which Tsg and Sog inhibit Dpp. The protein-degrading enzyme Tolloid breaks down Sog (when it is found in the complex), thus releasing Dpp in its active form. This process is accelerated by Tsg. However, in regions where Sog concentration is high, the degradation of Sog will have a minimum effect, because enough available Sog is around to form new complexes with Tsg and Dpp. Degradation of the tripartite complex in regions where Sog is scarce effectively releases Dpp.
Cerberus is in fact expressed in the anterior endoderm of the Xenopus embryo. In mouse, transcripts of its homologue reside in the anterior visceral endoderm (AVE), the topological equivalent of the frog anterior yolky endoderm (Thomas and Beddington 1996).

Expression of cerberus at ventral vegetal blastomeres induces small ectopic heads with no trunk-axial structures and a cyclopic eye. In addition, the injected embryos display a duplicated heart and liver. Data from radial injections of cerberus indicate that it suppresses trunk mesoderm, promotes cyclopia, presumably by inhibiting formation of the prechordal plate and induces anterior neuroectodermal structures such as brain, olfactory placodes and cement gland (Bouwmeester et al., 1996).

But which additional properties confer cerberus head inducing activity as opposed to other organiser secreted factors? Epistatic analyses initially performed by Niehrs and his collaborators demonstrate that cerberus is a potent inhibitor of Wnt signalling as it can abolish secondary axis formation by Xwnt8 (Glinka et al., 1997). In general, they show for the first time that the functional division between trunk- and head-organizer activity can be attributed to inhibition of BMP signalling for trunk formation only and dual repression of BMP as well as of Wnt signalling for trunk and head formation (Glinka et al., 1997; Fig. 1.7).

Subsequent biochemical and epistatic studies from Eddy De Robertis and collaborators have given evidence that cerberus is a multivalent antagonist not only of BMP and Wnt signals, but also of Nodal signalling (Piccolo et al., 1999). Their accurate biochemical analysis defines the independent regions in Cerberus structure responsible for binding to each of the ligands. In mouse, Robertson and colleagues demonstrated that nodal signalling is required in the AVE for head formation (Varlet et al., 1997), which would be in disagreement with nodal antagonism by cerberus. Paradoxically also in Xenopus, cerberus expression is
Fig. 1.7 Outline of secreted molecules implicated in extracellular antagonism for the establishment of trunk and head regions.
The functional division in the organiser between trunk and head formation resides in inhibition of BMP signalling for trunk formation and repression of BMP as well as of Wnt and Nodal signalling for trunk and head formation.
dependent on an early nodal signalling in the endoderm mimicked by injection of Xnr-1 mRNA and not DNA (expressed only after MBT) correlating with Xnr-1 expression in endoderm at late blastula (Jones et al., 1995). Later on development, though, cerberus would feedback negatively on Nodal signalling in order to initiate head fate and inhibit trunk fate.

In addition to cerberus, several other molecules are known to inhibit Wnt glycoproteins. These include the secreted Frizzled Related Proteins, sFRPs (Frzb1, Sizzled, Crescent) and the structurally unrelated WIFs molecules (Fig. 1.8). Frzb-1 is a member of the sFRP gene family encoding secreted forms of the ligand binding CRD domain of the frizzled WNT receptors (Leyns et al., 1997; Wang et al., 1997).

It complexes with Xwnt-1 and -8 (but not Xwnt3A, -5A, or -11), blocking their receptor binding. At early gastrula, frzb-1 is expressed in the dorsal marginal zone whereas Xwnt-8 is expressed in the ventrolateral marginal zone. When overexpressed alone, frzb-1 essentially phenocopies the dominant negative Xwnt-8 mis-expression phenotype; embryos are anteriorised and contain enlarged notochords (and concomitantly reduced somitic tissue and XmyoD expression).

In contrast to other organiser secreted factors inhibiting the ventralising activity of BMPs, such as chordin and noggin, frzb-1 does not act as a neural inducer of ectoderm or a meso-endoderm inducer of animal cap cells.

The dorsal mesodermal marker chordin is expanded at late gastrula in frzb-1 injected embryos, indicating that one of Xwnt-8 functions is to prevent expansion of organiser tissue into the lateral marginal zone. This way frzb-1 would counteract the limits set by Wnts on the effects of other signals from the organiser. Most importantly, frzb-1, if co-injected together with BMP inhibitors, induces secondary heads connected to a short trunk (Piccolo et al., 1999).
Fig. 1.8 Dual BMP and Wnt antagonism
Diagram representing the inhibition of BMP and Wnt by secreted factors in the Xenopus gastrula. In addition to Follistatin, Chordin and Noggin inhibiting BMP secreted in the ventral side, the organiser is a source of other secreted factors (Frzb-1, Dickkopf, Cerberus), which inhibit the activity of Xwnt-8. The combination of this inhibition allows head induction. Sizzled is the only Wnt inhibitor expressed on the ventral side; together with the other Wnt inhibitors, it fine-tunes and restricts Wnt activity to the lateral regions. Cerberus is also a Nodal inhibitor (not shown). The Wnt inhibitory action of WIF at the presomitic mesoderm is also not shown. The representation of the expression of BMP and Xwnt-8 in concentric circles is arbitrary and for clarity; this is a vegetal view of a gastrula embryo; in reality BMP4 overlaps with Xwnt-8, and so does Frzb with Chd. (Diagram adapted from Salic et al., 1997, see main text for details).
Sizzled (Salic et al., 1997), although clearly related to Frzb by sequence and function, is only 18% identical and has a completely different expression pattern. Sizzled occupies the ventral marginal zone of gastrulating embryos, but can dorsalise embryos after MBT and antagonise the ventralising action of Xwnt-8. Similarly to Frzb-1, sizzled overexpression perturbs expression of XmyoD, which is normally dependent on Xwnt-8.

WIF-1 (Wnt inhibitory factor 1, Hsieh et al., 1999), another molecule binding to Wnts and inhibiting their activities, is a secreted protein first isolated in humans and present in Xenopus, mouse and zebrafish. It contains a WIF specific domain, five EGF repeats and a hydrophilic carboxy-terminal region. Predominantly expressed during somitogenesis in the unsegmented paraxial presomitic mesoderm, if over-expressed, it phenocopies the anteriorisation and dorsalisation of other Wnt inhibitors. This suggests that WIF may regulate Wnt activity later temporally.

One conclusion can be drawn from these data, that these secreted proteins contribute to a regionalisation of the marginal zone. They fine tune spatially and temporally the activity level of the Wnt pathway, so that Wnts would be fully active laterally and their activity inhibited dorsally and ventrally.

Another crucial gene in head formation is dickkopf. It was isolated in a gain-of-function screen as a clone that co-injected with the dominant negative BMP receptor (tBR) was able to induce complete secondary axes (Glinka et al., 1998). Unlike frzb-1 and cerberus, dickkopf-1 induces heads with two eyes, pointing to a different mechanism of action of the protein. dickkopf encodes a secreted protein with cysteine rich knots belonging to a new family of at least three members, dkk-1, -2 and -3, present in different species. Like cerberus, its expression resides in the anterior endoderm and prechordal plate. Dickkopf not only induces a complete secondary axis with a head if co-injected with tBR. It is also clearly required for head formation as injection of antibodies specific for Dickkopf
results in severe microcephaly. This strong head formation coordinator is a potent antagonist of Wnt signalling as it inhibits the ability of mRNA Xwnt-8 to induce secondary axes as well as the ability of Xwnt-8 plasmid DNA to posteriorise the embryonic axis of *Xenopus*. Thus far a biochemical mechanism of head induction had been elucidated only for *cerberus*, found to directly bind BMPs, Nodals and Wnts. Very recently a mechanism of action has also been elucidated for *dickkopf*. At the time of its isolation, *dickkopf* was found to act upstream of dishevelled, the intracellular component of Wnt signalling, suggesting the it interacted somehow directly with the Wnt ligands or their seven-transmembrane *frizzled* receptors (Glinka et al., 1998). Niehrs and collaborators (Mao et al., 2001) have now demonstrated that it binds to LDL-receptor-related protein 6 (LRP6), a co-receptor for the canonical frizzled receptor (Pinson et al., 2000; Tamai et al., 2000; Wehrli et al., 2000). Thus, in contrast to the previously characterised Wnt inhibitors of the sFRP, Cerberus and WIF classes, all functioning by directly binding to and sequestering Wnt ligands, Dkks employ a novel, indirect method of action by binding to the putative Wnt co-receptor LRP6.

In summary, trunk formation requires active WNT signalling and inhibition of BMP signals. Head formation requires antagonistic action against growth factors of both the TGF-β and WNT superfamilies. In this scenario, Nodals initially induce and later inhibit the head organizer. BMPs (a) ventralise dorsal mesoderm and (b) antagonize neuralisation of ectoderm. WNTs (a) ventralise dorsal mesoderm, (b) posteriorise neuroectoderm and (c) antagonise neuralisation of ectoderm. So, in trying to explain head and neural induction, it is possible to describe a two-inhibitor model (Fig. 1.9; Niehrs, 1999), which is a molecular interpretation of Nieuwkoop’s two-signal concept (Nieuwkoop et al., 1952). In this model anterior neuralisation of ectoderm is induced by an ‘activator’ released from prechordal plate mesoderm.
Fig. 1.9 Two inhibitor model-molecular interpretation of Nieuwkoop's two signal model. Neuralisation is prevented in the ectoderm by the Wnts and BMPs. Their neutralisation, by Wnt and BMP inhibitors secreted from the anterior endoderm and prechordal plate mesoderm (head organiser activity) allows anterior neural induction (Nieuwkoop's activator). More posteriorly, progressive decrease in concentration of Wnt inhibitors in the chordamesoderm results in posteriorisation of neural fates by Wnts, FGFs and probably retinoic acid. Diagram modified from Niehrs, 1999.
Later in gastrulation, a ‘transformer’ released by the chordamesoderm further specifies the activated neuroectoderm in an anterior-posterior gradient, conferring increasing posterior neural character (hindbrain, spinal cord).

The two-inhibitor model offers the activator and transformer a molecular identity. BMP and Wnt signals from ectoderm and ventral mesoderm prevent neuralisation by default. Their neutralisation by BMP inhibitors (from posterior chordamesoderm) and concomitantly by Wnt inhibitors (released from anterior endoderm and prechordal plate, i.e. the head organiser) leads to anterior neural induction. This constitutes Nieuwkoop’s activator. More posteriorly, in the chordamesoderm, Wnt inhibitors are progressively less active. This allows posterior signals to posteriorise neural and ventralise mesodermal fates. These signals include Wnt signals, but as traditionally known, also FGFs and probably retinoic acid (for review see Niehrs, 1999 and Doniach, 1995). Together these factors constitute Nieuwkoop’s transformer activity. The role of Nodal inhibitors (cerberus) in the anterior endoderm is that of further preventing it from forming trunk mesoderm.

It is remarkable that of four distinct families of Wnt inhibitors, none of them have orthologues in the fly or worm genome (and this is also true for noggin and Xnr-3, for instance). This suggests that the extra-cellular modulation of growth factor activity (with the exception of the Dpp/BMP and Sog/Chd pairs in dorso-ventral patterning) underwent an expansion in the vertebrate lineage.

Furthermore, there is no apparent evolutionary conservation between all of the secreted antagonists described so far. A possible explanation for this could be the difficulty in the evolution of entire signalling pathways from extracellular ligand to transcription factor. The generation of a new antagonist and adaptation of its regulatory action to modulate existing signalling pathways is probably less demanding.
A machine driving movements

"it is not birth, marriage or death
but gastrulation that is truly
the most important time in your life"
(Lewis Wolpert)

In addition to its inductive capabilities, the organizer controls the complex cellular movements and rearrangements occurring during gastrulation. This latter is the most crucial step in the establishment of the vertebrate body plan. It is a dynamic process, which through the coordinated morphological movements of the three germ layers shapes an unpatterned, spherical early zygote into an elongate, bilaterally symmetrical embryo with recognizable antero-posterior and dorso-ventral polarity. Although germ layers and cell fates are already specified at the late blastula stage, cellular rearrangements at the onset of gastrulation implement the regionalisation of the Spemann organizer into head and trunk organizer compartments (Fetka et al., 2000). Gastrulation starts at 10 hr of development, when the embryo contains ~20000 cells, with the appearance of the black blastoporal line, visible at ~50° of latitude from the vegetal pole. In analyzing the organisation of a gastrula embryo, it is possible to distinguish five different regions, cylindrically symmetric about the animal-vegetal axis and each characterized by a particular type of motile activity of its constituent cells (Fig. 1.10).

One region is the animal cap. Its cells derive from the pigmented animal cap of the egg and engage in epiboly, an isotropic expansion in the direction of the vegetal pole, which covers ~40% of the late gastrulae surface.

A second group of cells playing a role are the bottle cells. These are an anulus of epithelial cells, which extensively contract their medium facing apices, while elongating basally, thus acquiring a bottle shape. As a consequence of their apical
Fig. 1.10 Tissues involved in *Xenopus* gastrulation.
This diagram illustrates the regions involved in morphogenetic movements during early gastrula (top panel) and late gastrula (bottom panel). Movements are shown from a surface viewpoint (left column) and from a midsagittal viewpoint (right column).
Abbreviations: A, animal pole; AC, animal cap; AR, archenteron roof; BC, bottle cells; BLC, blastocoel; BPL, blastopore pigment line; DZ, deep zone cells; IMZ, involutin marginal zone; IMZ-D, IMZ deep layer; IMZ-S, IMZ superficial layer; LI, limit of involution; NIMZ, non-involutin marginal zone; PBC, prospective bottle cells; RBC, respread bottle cells; VB, vegetal base; YP, yolk plug.
contraction scattered pigment granules concentrate along a line, resulting in the appearance of the black blastoporal line.

The marginal zone provides a pivotal contribution. In light of the movements of gastrulation, the marginal zone can be divided into two subzones, the involuting and non-involuting marginal zones (IMZ and NIMZ, respectively). The former is the more vegetal half of the marginal zone and is the one that will turn inside the embryo. The latter corresponds to the more equatorial subregion of the marginal zone. It does not involute internally, but spreads over the surface vacated by the IMZ. The cell population of both the IMZ and NIMZ undertake convergent extension, converging towards the midline and extending along the animal-vegetal axis.

Internal to the IMZ lies a further group of cells, which are apposed to the vegetal core. These constitute the deep zone. Such cells engage in spreading migration along the blastocoel wall and roof.

Finally, the large yolky cells of the vegetal hemisphere. They are also internalised, extending conically from the sub-blastoporal vegetal epithelial surface to the blastocoel (BLC) floor. Their surface area is gradually decreased, covered by the blastopore lip. The epithelial surface of the vegetal cells eventually forms the floor of the archenteron (AF).

**Epiboly of the animal cap**

Isotropic expansion of animal cap cells starts two hours before the appearance of the blastoporal line and proceeds all the way through gastrulation. Deep cells of the cap radially intercalate to form fewer layers of greater area, whereas cells of the superficial epithelium divide and flatten (Keller, 1978; Keller, 1980). Little is known about the autonomy of the epiboly movements. Isotropic expansion has long been considered as an autonomous activity of the animal cap, as explanted caps corrugate and form folds on their own (Spemann, 1938; Gerhart and Keller,
1986), but no-one has documented spreading of cells in these circumstances by appropriate time-lapse recordings.

Convergent extension of the marginal zone

Without exaggeration, it is the major force driving gastrulation in the whole embryo. The terms ‘convergence’ and ‘extension’ describe the narrowing and lengthening of the dorsal tissues of the embryo. It involves involution of the marginal zone, closure of the blastopore, elongation along the anteroposterior axis and accumulation of cells in the dorsal midline (Keller et al., 1985). Such movements represent the type of ‘mass movement’ (Keller et al., 1992) driven by convergent extension dynamics in which local cell motility is mechanically integrated to produce forces that modify the shape of cell populations. In a gradient of developmental timing, dorsal mesodermal cells along the anteroposterior axis go through a series of cell behaviours: radial intercalation, cell elongation and mediolateral intercalation (Fig. 1.11). Cells at the anterior end of the axis experience these first and then movements are extended more caudally as development proceeds (Wilson and Keller, 1991). Although these movements have been described and analyzed in whole embryos, their mechanics and the cellular behaviours underlying them can be best understood in cultured ‘open-faced’ explants, which expose cells to direct observation.

Convergent extension is achieved through cell intercalations in two directions. First, deep cells of the marginal zone intercalate perpendicular to the surface of the embryo, so that several layers of deep cells form fewer layers of greater area (radial intercalation). Following radial intercalation, cells intercalate along the mediolateral axis to align in a longer, narrower array (mediolateral intercalation). This produces convergence and extension. Mediolateral intercalation involves a restriction of cell protrusive activity: from random and multi-polar to bipolar, towards the medial and lateral ends of the cells (Lane and Keller 1997).
Early

Mid

Late Gastrula

**Radial intercalation**

**Mediolateral intercalation**

Fig. 1.11 Convergence and extension occur by radial and medio-lateral intercalation. This diagram shows cell intercalations underlying convergence and extension movements of the IMZ in open-faced explants. Changes in the shape of open-faced explants are shown in the centre column; cell behaviours producing these movements are shown on the sides. Radial intercalation by IMZ starts first at early gastrula and it is largely completed by midgastrula. Cells intercalate perpendicular to the surface of the embryo such that several layers of deep cells form fewer layers of greater area.

With some overlap with radial intercalation, medio-lateral intercalation starts at midgastrula in the IMZ and continues until the end of neurulation. Cells intercalate along the mediolateral axis to form a longer, narrower array. Note cell protrusions.

The inner, deepest surface is shown uppermost and the epithelial layer is at the bottom. Open arrows in the centre column illustrate tissue deformation. Small arrows on the cells in right and left columns indicate the direction of cells movements.

Diagram borrowed from Keller et al., 1992.
During this period epithelial cells, too, spread, divide and intercalate mediolaterally to form a narrower and longer array (Wilson and Keller, 1991). Radial intercalation starts at early gastrula (st. 10) and is largely complete by midgastrula (st. 10.5). Mediolateral intercalation begins with some overlap to radial intercalation and continues until near the end of neurulation (st. 18).

**Dynamics of the bottle cells**

The change in shape from columnar to bottle-like of these cells through apical connection seem to have an important role in the initial involution of the epithelial sheet at the blastoporal groove and the initial small involution of the IMZ. They seem to play a role in orienting the direction of involution, but not to be essential for gastrulation to continue, because removal of bottle cells, after their formation does not interfere with the involution of the marginal zone and closure of the blastopore. (Keller, 1981).

**Deep zone cells**

During their spreading migration, they lead the IMZ along the walls and roof of the blastocoel. They are important for this initial involution, without which convergent extension would occur on the surface of the gastrula, to produce an exogastrula.

**The vegetal base**

This region has always been thought to undergo the least deformation during gastrulation. Recent evidence from Winklbauer reviews the general assumption that the force driving mesoderm and endoderm in the interior of the embryo is exclusively provided by the mesoderm and that vegetal cells are internalised passively. They show that the vegetal mass is morphogenetically active and dominates the initial phase of mesendoderm internalisation (Winklbauer and Schierfeld, 1999). As a sort of pregastrula movement, an active distortion of the vegetal mass, ‘vegetal rotation’, causes a dramatic expansion of the blastocoel roof and a concomitant turning of definitive mesendoderm towards the marginal
zone (Fig. 1.12). These vegetal cellular translocations have been suggested to constitute a pre-requisite for the initial partitioning of head and trunk organisers (Fetka et al., 2000). At late blastula stage, organiser progeny (dorsoanterior endoderm, prechordal plate, notochord and presumptive floorplate) is initially aligned in a diagonal stripe and is induced by the vegetal VegT/Xnr pathway in synergy with the dorsalising β-catenin pathway. As a result of vegetal rotation, an anterior posterior pattern is established according to which the dorsoanterior endoderm (expressing the trunk antagonist cerberus) occupies an anterior position and is separated from definitive mesoderm (prechordal plate and notochord).

In summary morphogenetic movements involved at gastrulation are formation of bottle cells, epiboly of the animal cap, migration of the deep mesoderm along the blastocoel roof, convergent extension of the non-involuting and involuting marginal cells and vegetal rotation of the vegetal mass. The correct timing, placement and intensity of these five region specific cellular activities are the conditions for a successful gastrulation and also for organiser partitioning.

*Links to the cytoskeleton*

Certainly, the execution of the above described movements must involve a strict regulation of cell-adhesion and cell polarity, in an intensive interaction with the cytoskeletal architecture.

The modulation of cell-adhesion during cellular movements is extremely important: cells need to maintain or lose their adhesiveness in order to remain as a compact population of cells or freely move with respect to one another. Migrating cells can adhere to each other or to the extracellular matrix. In the latter case, contacts between cells and the extracellular matrix are mediated by the transmembrane receptors-integrins (Hynes, 1992).
Fig. 1.12 Vegetal rotation and organiser regionalisation.
Organiser progeny (dorsoanterior endoderm, prechordal plate, notochord and floor plate) is induced by the concerted action of the vegetal VegT/Xnr pathway and the dorsalising β-catenin pathway. Cerberus acts as a local feedback antagonist of Xnrs in the vegetal endoderm. Vegetal rotation movements (black arrows) in the vegetal endoderm constrict the vegetal outer surface and expand the blastocoel roof. This results in a passive displacement of the organiser progeny to the marginal zone (indicated by blue arrow). Abbreviations: pp, prechordal plate; n, notochord; f, floorplate; DAE, dorsoanterior endoderm.
These contacts become important at later stages of gastrulation when mesodermal cells migrate on a network of extracellular matrix fibrils synthesised by the cells of the blastocoel roof.

Cell-cell adhesion is mediated by cadherins, Ca\(^{2+}\) dependent transmembrane adhesion proteins (Huber et al., 1996). These cadherins are involved in signalling pathways, along which they interact via catenins with actin filaments to modulate cell rearrangements inside the cell mass. PAPC, paraxial protocadherin, has been shown to regulate morphogenetic movements (Kim et al., 1998). It is a divergent Ca\(^{2+}\) dependent cadherin, which engages in homotypic cell-adhesion in contrast to homophilic interactions between classical cadherins and does not interact with the cytoskeletal actin network via α- and β-catenins. Its dominant negative form specifically blocks paraxial morphogenesis \textit{in vivo} and in activin-treated animal-caps, suggesting a role for PAPC in the regulation of convergent extension of somitic precursors.

In addition to cadherins, integrins and extracellular matrix proteins, several other genes encoding a variety of proteins have been demonstrated to regulate cell movements by affecting cell-adhesion.

One is \textit{Xrnd1}, a \textit{Xenopus} homologue of human Rho-related protein Rnd1 (Wunnenberg-Stapleton et al., 1999). Its transcripts reside in tissues undergoing morphogenetic movements (particularly involuting marginal zone, somitic mesoderm and neural crest) and, if overexpressed in blastomeres, they cause a disruption in cell-adhesion, with injected cells falling into the blastocoel. This effect is reversed if \textit{Xrnd1} is co-injected with \textit{XRhoA}, indicating that these proteins have antagonistic functions in regulating cell-adhesion. This gave a clue to whether Rhos are mediators of tissue morphogenesis in \textit{Xenopus}. Rho GTPases are intracellular switches that integrate diverse signalling events with changes in the actin cytoskeleton and had been already implicated in a variety of morphogenetic processes during \textit{Drosophila} development, such as gastrulation.
and dorsal closure (Settleman, 2000). It could be hypothesized then, that Rho together with other small GTPases of the Rho family, like Rac and Cdc42 are actors in the morphogenesis scenario.

Recently, a new protein with a hydrophobic central domain and a N-acetyltransferase motif called Camello has been demonstrated to inhibit gastrulation in *Xenopus* by decreasing cell-adhesion as demonstrated by microscopic analysis and blastomere aggregation assays (Popsueva et al., 2001). Cell fractionation and confocal microscopy data suggest that camello is localized in the secretory pathway (Posueva et al., 2001).

Microtubule rearrangements are required for convergent extension of dorsal and axial structures, but are dispensable for initial bottle cell formation and alignment of pre-involuting mesoderm. An intact microtubular system is required for the transition from multi-polar to bipolar cell morphology in mesodermal cells undertaking mediolateral intercalation as shown in embryos treated with nocodazole (Lane and Keller, 1997).

In the case of vegetal mass rotation, vegetal cells form lamellipodia and filopodia extensions, suggesting that the movements could arise from changes in the actin cytoskeletal framework.

Convergent extension movements must be spatially confined in the embryo. They need to extensively occur at the posterior side of the embryo, but they are excluded at its most anterior regions. Otx2 is a transcription factor expressed in *Xenopus* and mouse embryos in cells that are destined to become the most anterior structures. It not only promotes the induction of anterior structures such as cement gland, but it also has a second function: it prevents cells that express it from undergoing convergent extension movements (Pannese et al., 1995).

XclpH3, a direct transcriptional target of Otx2, which is homologous to the human Calponin, accomplishes this second function. The phenotype resulting from the overexpression of this molecule phenocopies the gastrulation
phenotype of ectopic Otx2 expression. How XclpH3 exerts its activity is not completely clear. It is not excluded that its inhibitory effect could result from a direct link to the actin cytoskeletal architecture, binding to both actin and myosin preventing generation of contractile force by actin filaments.

Which are the molecules and the signalling pathways instructing convergent extension movements?

Until very recently, much of the attention in analysing gastrulation had been devoted to convergent extension movements. Despite a wealth of descriptive analyses concerning the cellular behaviour underlying such movements, relatively little is known about the molecular mechanisms that govern the patterning, timing and execution of these important cellular rearrangements. A crucial problem in unravelling the molecular basis of morphogenetic movements has been the difficulty to dissect the execution of these movements from the cell fate specification events occurring at the same time during gastrulation.

Only recently signaling cascades have been identified, that regulate aspects of these complex cellular rearrangements. 'Non-canonical' Wnt/Frizzled signaling pathways, appear to be implicated as essential regulators of convergent extension behaviour during gastrulation.

Wnt ligands can be classified into two classes: the transforming, axis inducing, dorsalising class (some of which are Xwnt-8, Xwnt-8b, Xwnt3a) and the non-transforming class (for example Xwnt-5a, Xwnt-4, Xwnt-11). The dorsalising Wnts signal through the canonical or dorsalising pathway. This involves phosphorylation of Dishevelled (Dsh) and stabilisation of cytoplasmic β-catenin, with subsequent translocation in the nucleus of this latter in complex with the TCF/LEF family of transcription factors for the transcriptional activation of the target genes siamois and Xnr-3 (Carnac et al., 1996; McKendry et al., 1997).
Signalling of the members of the non-transforming class is much less well understood, but they play a role in cell polarity in *Drosophila melanogaster* and affect morphogenetic movements in vertebrates (Moon et al., 1993a; Moon et al., 1993b; Du et al., 1995; Ungar et al. 1995). They trigger a calcium release (Slusarski et al., 1997a; Slusarski et al., 1997b) and activate protein kinase C (PKC) and Ca\(^{2+}\)/calmodulin-dependent kinase (CamKII). Further downstream components of this pathway are not known (Fig. 1.13.)

Xwnt11 has been implicated in regulating convergent extension movements through activating Jun N-terminal kinase (JNK) in *Xenopus* and in zebrafish (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000). This Wnt/JNK pathway is thought to resemble the 'planar polarity' pathway in *Drosophila melanogaster* (Boutros and Mlodzik, 1999) and shares Dishevelled with the canonical pathway. A dominant negative form of Xwnt11, dwnnt11, specifically downregulates a hyperphosphorylated form of Dishevelled (Dsh) and interferes with convergence extension movements of axial and paraxial cells without affecting mesodermal differentiation. Rescue of convergent extension is possible by overexpression of dwnnt11 and mutant forms of Dsh that can effectively signal down the planar polarity cascade, but which cannot relay the canonical Wnt/β-catenin signalling (Tada and Smith, 2000). Furthermore, cells lacking Dsh function fail to undergo convergent extension and this failure derives from defects in cell polarity. Cells at the DMZ overexpressing a mutant form of Dsh perturbing the planar polarity cascade are not able to stabilize their random protrusions along the mediolateral axis and therefore fail to intercalate, to converge and to extend (Wallingford et al., 2000).

Overexpression of Xwnt5a and of a dominant negative form of Frizzled-8 (Nxfz-8) inhibits elongation of Keller explants (Wallingford et al., 2001). The effect of Nxfz-8 on convergent extension is rescued by co-expression of Xdsh, which is shared by both the canonical and non-canonical Wnt signalling pathways.
Fig. 1.13 Bifurcation of Wnt pathway

The canonical Wnt pathway (left) is triggered by dorsalizing, transforming Wnt ligands, prototyped by Xwnt-8; it promotes cell fate specification signalling through β-catenin. Dishevelled represents a crossroad at which a second, non-canonical pathway is followed, which via the activity of RhoA and Jun-N-terminal kinase, regulates cell polarity. Yet another Wnt pathway has been identified in vertebrates governing morphogenesis. It does not signal via the canonical β-catenin pathway and it is not equivalent to the planar polarity pathway. It elicits calcium release and involves activation of G-trimeric proteins and PKC and CamKII. Red arrows and inhibitory lines describe the additional proposal by Kuehl et al. (2001) that the Wnt-beta catenin pathway is also required for convergent extension (in addition to fate specification). In this function it would be inhibited by the Wnt-calcium pathway at multiple levels. Note the promiscuity of signalling components in the different pathways. See main text for details.
Dominant negative (DN)-GSK3, a potent activator of the canonical pathway is not able to restore convergent extension movements in explants injected either with Xwnt5 or Nxfz-8 (Wallingford et al., 2001). This suggests that these molecules also regulate morphogenesis, through the non-canonical Wnt pathway.

In Drosophila, overexpression of either wild-type Dsh or wild-type Frizzled inhibits the non-canonical Wnt signalling pathway, without perturbing the canonical Wingless transduction. Similar effects are observed with overexpression of Xdsh in dorsal mesoderm (Wallingford et al., 2000). Consistent with a role for Xfz-8 in the non-canonical cascade, overexpression of wild-type Xfz-8 in the dorsal mesoderm also strongly inhibits convergent extension movements, in a similar fashion as its dominant negative form (Wallingford et al., 2001). Similar results are obtained over-expressing wild-type Xfz-7 (Djiane et al., 2000; Medina et al., 2000; Medina and Steinbeisser, 2000).

Interestingly, XwntSa can trigger an increase of intracellular Ca\textsuperscript{2+} concentration by stimulating the phosphatidyl-inositol signalling pathway via heterotrimeric G-protein subunits (Slusarski et al., 1997a and b). This would raise the intriguing possibility that calcium signalling may play a inhibitory role in coordinating convergent extension, perhaps by modulating another non-canonical Wnt signalling cascade.

A very recent report from Kuehl et al. confirms this hypothesis, but arguing that a Wnt/Ca\textsuperscript{2+} pathway would block convergent extension by negatively regulating endogenous Wnt/\beta-catenin pathway (Kuehl et al., 2001). Surprisingly they attribute an essential role in the execution of convergent extension to the Wnt/\beta-catenin/Lef-1 pathway and its downstream target, Xnr-3. This latter being down regulated by Xwnt5a and able to specifically restore convergent extension in ventralised embryos, without restoring dorsal markers. The antagonistic regulation of convergent extension by the Wnt/Ca\textsuperscript{2+} pathway would result from
potential negative regulation of the β-catenin pathway at two levels. PKC would block the β-catenin pathway upstream of β-catenin and would phosphorylate Dsh, CamKII would inhibit Lef-1 downstream of β-catenin in the same pathway (red arrows in Fig. 1.13).

The FGF signalling pathway has also been shown to regulate gastrulation movements. Overexpression of a dominant negative FGF receptor in Xenopus embryos, results in gross defects in gastrulation movements (Amaya et al. 1991; Isaacs et al., 1994). Genetic evidence from mice confers Brachyury also a role in morphogenetic movements during gastrulation (Wilson et al., 1995). Overexpression in Xenopus of a dominant-negative form of Xbra, a known downstream target of FGF, inhibits elongation of activin-treated animal caps and causes loss of cell adhesion in ectodermal cells (Conlon and Smith, 1999). Xbra is also required for transcription of Xwnt-11. However, the concomitant absence of mesoderm in these phenotypes has made it difficult to assess whether FGFs have a direct role in the control of morphogenesis, in addition to their essential role in mesoderm induction and maintenance. Some genetic evidence in mice corroborated a distinct role for the FGF pathway in the control of morphogenetic movements (Deng et al., 1994; Yamaguchi et al., 1994; Ciruna et al., 1997; Sun et al., 1999b).

Amaya and co-workers have recently provided the first molecular dissection of distinct FGF signaling cascades involved in either mesoderm induction/maintenance or convergence and extension movements, by their studies on Xsprouty2, a gene strongly expressed at the dorsal marginal zone (Nutt et al., 2001). They show that overexpression of the FGF-dependent Xsprouty2, severely inhibits establishment of the embryonic antero-posterior axis through convergent extension in whole embryos. DMZ explants from embryos injected with Xsprouty2 fail to elongate and so do animal caps expressing Xsprouty2 in
response to activin. These inhibitory effects exerted on convergent extension occur leaving mesodermal induction and patterning intact (Nutt et al., 2001). It is further demonstrated that Xsprouty2 functions intracellularly in the FGF signalling pathway to block calcium mobility, but not MAPK phosphorylation. So, there might be at least two distinct FGF signalling pathways: a Sprouty-insensitive Ras/MAPK pathway required for the transcription of most mesodermal genes and a Sprouty-sensitive pathway for the coordination of cellular morphogenesis (Fig.1.14).

The inhibitory effect of Xsprouty2 on calcium efflux (Nutt et al., 2001) is against the promotion of the same process by the Wnt pathway mentioned above in the case of Xwnt5a, which also inhibits elongation of Keller explants if overexpressed (Slusarski et al., 1997b). This means that Ca\(^{2+}\) signalling, controlled by both FGF signalling (Mohammadi et al., 1992; Peters et al., 1992) and the non-canonical Wnt signalling may modulate the execution of convergent extension movements, but that its function must be tightly regulated. Finally, the question remains of why an inhibitor of gastrulation, such as Xsprouty2, is expressed in the cells that normally undergo the gastrulation movements. The answer to this question might lie in the tight regulation required for the coordination of such movements. Convergent extension is initiated laterally and then continues medially and along the antero-posterior polarity (Wilson and Keller, 1991; Shih and Keller 1992). This progression must be achieved by molecules ensuring a correct timing. Sprouty might be one such molecule, that, where overexpressed, is responsible for the disruption of convergent extension.

Two main signalling pathways have so far been identified as implicated in gastrulation movements: the Wnt and the FGF pathway. It is not excluded that the realisation of gastrulation movements relies on a tight interplay between the two pathways. It would be of ultimate interest to unravel such correlations.
Sprouty independent (mesodermal specification)

Fig. 14 The role of the FGF pathway in the regulation of morphogenetic movements

Sprouty2 is an FGF target and an inhibitor of convergent extension, but it does not affect mesodermal differentiation. It blocks calcium mobility, but it does not perturb MAPK phosphorylation. It therefore dissects the FGF pathway into two branches: a Sprouty insensitive Ras/MAPK pathway required for activation of mesodermal genes and a Sprouty sensitive pathway for the coordination of cellular morphogenesis.
Aims of this work

This doctoral thesis had as principal aim the isolation of new genes expressed in the Spemann-Mangold organiser of *Xenopus laevis*.

Among the various possibilities for the identification of new genes, *in situ* hybridisation screen was chosen to isolate genes exclusively on the basis of their pattern of expression, with no bias as to their function. In order to increase the percentage of genes in the organiser, the screen would be conducted on a subtracted library, enriched in genes of this tissue by Suppressive Subtractive Hybridisation.

The initial part of the project would then give the possibility to establish expertise in how to approach a large-scale screen and in the preparation of the starting material for such screen.

The project would then develop in the characterisation of mainly one of the genes isolated. The type of screen conducted gives no direct information about the activity of the isolated clones. Therefore, from the analysis of the pattern of expression, a function for a new candidate would be hypothesised and available gain and loss-of-function approaches in *Xenopus laevis* would be undertaken to investigate it.

*Xmc*, one gene isolated was strongly expressed in mesodermal tissues (mainly of somitic fate) undergoing morphogenetic movements. This was suggestive of a possible role in the co-ordination of gastrulation.

By morpholino mediated translational ‘knock-down’, the requirement of *Xmc* in the execution of gastrulation movements would be investigated.

Successful gastrulation relies on the accurate execution of cellular movements, but also on precise specification of mesodermal fate. The ultimate goal of this work was then to dissect whether *Xmc* loss-of-function caused a direct impairment of cellular rearrangements or indirectly affected morphogenetic movements due to the inhibition of mesoderm formation.
Chapter Two

Materials and Methods
Embryo manipulations

In vitro fertilisation

Egg laying in frog female was induced by injection of 500-600 units of human chorionic gonadotropin (Sigma). Eggs were collected in Petri dishes by gently squeezing the lower side of the animals' back. Testes for fertilization were prepared by surgical removal and stored for up to four days at 4° C. Testes were homogenized using a forceps in 1x MMR (100mM NaCl, 2mM KCl, 2.4 mM CaCl₂, 1mM MgCl₂, 5mM Hepes, pH 7.4) After addition of the testes, eggs were stored for 5 minutes at RT and subsequently immersed in 0.1 X Barth for 15 minutes (1x Barth: 88mM NaCl, 1mM KCl, 0.4 mM CaCl₂, 0.3mM Ca(NO₃)₂, 0.8 mM MgSO₄, 2.4 mM NaHCO₃, 10mM Hepes, pH 7.4). The solution was then substituted with a 2% cysteine solution in 0.1 X Barth (pH 7.9-8.0) for separation of the eggs (dejellying procedure).
Successful fertilization was monitored by the correct orientation of the eggs, rotation of the vegetal cortex.

Animal cap and dorsal marginal zone explants

Animal caps and dorsal marginal zones were excised at st. 8 and st. 10.5-11, respectively from dechorionated embryos in 1x Barth using a hair mounted Pasteur pipette. After excision they were cultured in 0.3 x Barth, occasionally in the presence of streptomycin and penicillin.

Micro-injections

Embryos were injected at the two or four cell stage in 1x Barth on agar coated plates and subsequently cultured in 0.1x Barth. The injector used was a Narishige model IM 300.
To prepare mRNA, all cDNAs in pCS2+ (or pCS2'-HA) constructs were linearized with NotI (except for XeFGF and XFD, linearised with BamHI and EcoRI, respectively and human GFP, linearised with Kpnl) and transcribed with SP6 RNA polymerase. Capped mRNA for microinjections was synthesized using the Ambion Message Machine Kit, according to the manufacturer’s instructions. Briefly, ~3μg linearised DNA (in a total volume of 6μl water) were incubated for two hours at 37° C with 1μl reaction buffer, 10μl ribonucleotide mix (dNTPs/CAP mix) and 2μl enzyme mix. After RNA synthesis, the plasmid DNA was degraded by digestion with 2μl RNAase free DNAase for 15 minutes at 37° C. The reaction was then diluted with 200μl water and stopped by adding 18μl ammonium acetate stop solution. The products were phenol/chloroform extracted and ethanol precipitated. The RNA was stored and diluted in nuclease free water.

Construction and screening of the subtracted library

The subtracted cDNA library, enriched for zygotically-activated organizer genes was generated following reagents and instructions of the PCR-Select cDNA Subtraction kit (Clontech). A detailed description of the principle of Suppressive Subtractive Hybridisation and a scheme of the procedure is given in Chapter Two.

In brief, ~2μg of poly (A)+ mRNA were isolated from ~300 stage 10.5-11 Dorsal Marginal Zone (DMZ) explants (tester population, see Figs. 4.2 for the size of the DMZ explant) and ~10μg poly (A)+ mRNA from fertilized eggs (driver population). cDNA was synthesized from both populations, digested with RsaI (a four base-cutter enzyme) and common cDNAs were subtracted in two consecutive rounds of hybridisation, resulting after selective PCR amplification in a ~20-fold enrichment of differentially expressed genes.
The subtracted cDNA library was cloned (by T/A cloning) into Topo PCR II (according to the manufacturer’s instructions, Invitrogen), a dual promoter (SP6 and T7) vector and subsequently plated. Individual colonies were picked and inserts were amplified by PCR using SP6 and T7 primers. The PCR amplicons served as templates to synthesize both SP6 and T7 DIG RNA probes (Roche DIG RNA Labeling Kit). Probes were applied on whole and cut embryos (st. 10.5 and 13) for in situ hybridization (cut embryos were additionally used to allow a better penetration of the RNA). Clones that rendered interesting patterns of expression were sequenced (Fig. 3.2). The in situ hybridisation protocol followed for the screen was exactly the same as the one normally utilised for manual in situ hybridisation (described below), but it was programmed and performed on an automated machine (In situ Pro available from Abimed), which allows processing of 96 probes per run.

Histological techniques

In situ hybridisation

Whole mount in situ hybridisation embryos was performed as previously described (Bouwmeester et al., 1996). The digoxigenin (DIG) labelled probes were prepared in the following way. Xmc and Xcad1/2 cDNAs (in pBluescript) were linearised with EcoRI and NotI, respectively. After phenol extraction and ethanol precipitation, antisense RNA was synthesised from the templates with T7 pol. Xbra cDNA (in pSP64T) was linearised with Sall and the antisense transcribed with SP6 pol.

The labelling in vitro transcription reaction was performed with the DIG RNA labeling kit (SP6) from Boehringer in a 20μl reaction starting with ~3μg of linearised DNA:
Template DNA 3μl (~3μg)
Water 2μl
10x transcr. buffer (0.4mM Tris, pH 8) 2μl
DIG RNA labelling mixture 10μl
RNAguard (RNAase inhibitors) 1μl
SP6 polymerase 2μl

The reaction was incubated for 2 hours at 37° C.

The prepared embryos, stored in methanol, were rehydrated through sequential 75, 50, 25% methanol series in PBSw (PBS with 0.1% Tween-20; 1xPBS: 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.75mM KH₂PO₄, pH 7.4). After further washes in PBSw, embryos were treated with proteinase K (10μg/ml) in PBSw for 12 minutes. The reaction was stopped by washing with the same buffer. Embryos were then refixed in 4% paraformaldehyde in PBSw for a total of 20 minutes. The samples were washed in PBSw, equilibrated to the hybridization solution (50% formamide, 5X SSC, 10mg/ml torula RNA, 50mg/ml heparin, 20% Tween-20, 10% Chaps, 0.5M EDTA; 20X SSC: 3M NaCl, 0.3M sodium citrate, pH 7.0) and preincubated for 1hr at 65° C to kill endogenous phosphatases. ~200ng of RNA probe were applied to the embryos for an incubation at 55 ° C overnight. Later embryos first underwent two half an hour washes at 37° C (2X SSC, 0.1% Chaps) and then at increased temperature and decreased concentration of salt (60°, 0.2X SSC, 0.1 % Chaps). Embryos were subsequently equilibrated to the antibody solution by washes in TBS-X (TBS with 0.1 % Triton X-100; TBS: 0.1 mM Tris-base, 1.5mM NaCl). Embryos were blocked in the antibody solution (TBS-X, 15% goat serum, 5% egg extract) at 4° C for two hours, rocking. The pre-absorbed antibodies (alkaline phosphatase-coupled antibodies against DIG) were then added for an overnight incubation at 4° C. After extensive washes in TBS-X, the hybridized DIG probes were visualized by colour reaction with BM purple (Boehringer). For long term storage, stained embryos were kept in methanol.
**Histological sections**

For histological sections, embryos stained through in situ hybridisation and dehydrated in methanol were carefully placed in plastic moulds containing fresh paraffin wax at 60° C. Embryos were then allowed to cool to RT before removing and mounting on plastic microtome platforms for sectioning. 6 to 8μm thick longitudinal sections were cut, placed on coated glass slides and placed on 42° C heat platform to allow "stretching-out" of the embryo sections. After repetitive alternated washes of five minutes in xylene and ethanol, sections were mounted on coverslips using the mounting Permount solution (Fischer). Pictures on sections were taken using a Zeiss dissection microscope fitted with camera.

**Immunostainings**

Immunofluorescence was done essentially as described in Klingbeil et al., 2001b. In brief, two-cell embryos were injected animaly with ~800 pg mRNA coding for C-terminally HA-tagged Xmc or the HA-tagged Xmc deletion constructs. Animal caps were explanted at stage 9, fixed in 4% PFA/PBSw for 45 minutes and incubated overnight at 4° C with anti-HA mouse monoclonal antibody (BabCO, 1:1000). After several washes in PBSw, caps were incubated for 1 hr at RT with FITC-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc, 1:250) Cortical actin was visualized with rhodamin phalloidin (Molecular Probes). Caps were mounted upside down on glass slides with mowiol. Image analysis was performed using a LEICA TCS NT laser-scanning confocal microscope.
Morpholinos

Morpholino oligos have their riboside moieties substituted with nitrogen morpholino moieties and are phosphorodiamidate linked. Anti-Xmc morpholino oligonucleotides were purchased from Gene-Tools, LLC and solubilized in water at a concentration of ~15 pM. The morpholino comprised 25 nucleotides with the following sequence: 5'-TGCAGCCATTATGTATATGAAAAA-3' (as designed by the manufacturer). For microinjection the oligo was diluted in sterile nuclease-free water and injected at a concentration between 4 and 10ng per blastomere.

Molecular biology techniques

Total and poly A' RNA isolation

Total RNA from whole embryos, or embryonic explants was isolated using the following procedure. The biological material was homogenized in Trizol reagent (Gibco, 1ml for ~20-30 explants) and incubated at room temperature for 5 minutes. RNA was extracted by adding half volume of chloroform and subsequently precipitated with an equal volume of isopropanol. The precipitated RNA pellet was washed with 75% ethanol and redissolved in nuclease free water. Purification of poly A' RNA from total RNA was achieved using the Qiagen Oligotex kit, which makes use of an affinity resin to isolate polyadenylic acid sequences.

cDNA synthesis

~1μg RNA from total RNA extractions were usually utilized as template for 1st strand cDNA synthesis in a final volume of 6μl water. Reverse transcription was
carried out using random hexamers and MMLV-reverse transcriptase from Gibco and the supplied additional reagents in the following reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X RT buffer</td>
<td>2μl</td>
</tr>
<tr>
<td>d[N₆] (random hexamers, 100μM)</td>
<td>1μl</td>
</tr>
<tr>
<td>dNTP (5mM)</td>
<td>1μl</td>
</tr>
<tr>
<td>Water</td>
<td>2μl</td>
</tr>
<tr>
<td>DTT (0.1M)</td>
<td>1μl</td>
</tr>
<tr>
<td>RNAguard (RNAase inhibitors)</td>
<td>0.3μl</td>
</tr>
<tr>
<td>BSA (1mg/ml)</td>
<td>1μl</td>
</tr>
<tr>
<td>MMLV-RT</td>
<td>1μl</td>
</tr>
</tbody>
</table>

The reaction was incubated at 55° C for 30 minutes.

**RT-PCR**

Semi-quantitative RT-PCR was conducted essentially as described in Bouwmeester et al., 1996. cDNAs were synthesised as above. The following protocol was adopted for the PCR reaction:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>100ng cDNA</td>
<td></td>
</tr>
<tr>
<td>10X PCR buffer (Boehringer, with Mg)</td>
<td>2.5μl</td>
</tr>
<tr>
<td>dNTP (2mM)</td>
<td>2.5μl</td>
</tr>
<tr>
<td>³²P-α-dCTP</td>
<td>0.1μl</td>
</tr>
<tr>
<td>Forward primer (20μM)</td>
<td>1μl</td>
</tr>
<tr>
<td>Reverse primer (20μM)</td>
<td>1μl</td>
</tr>
<tr>
<td>Water</td>
<td>15.65μl</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>0.25μl</td>
</tr>
</tbody>
</table>

Cycling conditions: step 1: 94° C for 1 minute; step 2: 55° for 1 minute; step 3: 72° C for 1 minute. Where not otherwise stated RT-PCR products were generally obtained through 28 cycles. The amplicons were run on 5%-6% polyacrylamide gels, dried and exposed to Kodak X-omat film in an X-ray cassette at -80° C. Amplicons resulting from non-radioactive PCR were loaded on 1% EtBr agarose gels.
Primers used for amplification:

**ODC:**
F-5'-CCCATCTCTTACTTGTCCAT-3'  
R-5'-TCAGCAACATAAAGGCAATCT-3'  

**VegT:**
F-5'-TTGGTTCAGCTCAAGGCTCA-3'  
R-5'-GTCCTGAGGTTTTGCCTCAAGA-3'  

**Cerberus:**
F-5'-GCTGAACTATTTGATTCGAGGA-3'  
R-5'-ATGGGTTGTATTCTGTGGGGC-3'  

**Pintallavis:**
F-5'-CCACTTGCTATGATGACTGTTCT-3'  
R-5'-TGGATGTCTCCAGAAAGGATC-3'  

**Xmc:**
F-5'-CTGCTGTTACAGACCAAGG-3'  
R-5'-ACCTGTCTTTGCCCACTC-3'  

**Xbra:**
F-5'-GGATCGTTATCACCTTCG-3'  
R-5'-GTGTAGTCTGTAGCTGCA-3'  

**a-actin:**
F-5'-TCCCTGACGGTCTGCTGGTA-3'  
R-5'-TCTCAAGTGCCAAAGCCACATA-3'  

**XmyoD:**
F-5'-AGCTCCAAGTCTCCAGGGCATGAA-3'  
R-5'-AGGAGAGAATCCAGGTGGAAACA-3'  

**Cloning**

Growth and handling of *E.coli* cells and preparation of competent *E.coli* cells were performed as described in Sambrook et al., 1989. Restriction digestions were carried out standardly in 50μl total volume reaction, with the appropriate buffer (according to the instructions of the restriction enzyme manufacturer's, New England Biolabs) and with 1-2 units of enzyme. The vector digest was supplemented with 1 unit calf intestinal alkaline phosphatase (Boehringer) to inhibit religation.
After agarose gel extraction (Qiagen Quiaquick kit), vector and insert, generally in a 1:1 and 1:2 ratio were set up in a ligation reaction overnight at RT (ligation buffer and T4 DNA ligase from Gibco). 2μl of the ligation reaction were then used for transformation of DH5α cells by electroporation.

PCR is essentially carried out as described for semi-quantitative RT-PCR. For amplification of fragments used for functional studies, pfu pol. was used as polymerase.

Mini-preps and maxi-preps were performed with the reagents and according to the protocols provided by Qiagen.

Sequencing was done by the EMBL sequencing facility.

**Plasmids constructs**

A full-length Xmc cDNA in pBS(K) was isolated from a *Xenopus* tailbud cDNA library by standard filter hybridization. For microinjection and for immunolocalization studies the Xmc ORF was sub-cloned in frame into the EcoRI and *XhoI* sites of pCS2⁺ and pCS2⁺-HA, respectively. The latter construct is a modified version of pCS2⁺, which contains a HA-tag inserted between the *XhoI* and *XbaI* sites. The C-terminal deletion of Xmc (*Xmc.NH2*, amino acids 1-210) as well as the N-terminal deletion of Xmc (*Xmc.COOH*, amino acids 301-515) were generated by PCR using pfu as polymerising enzyme and the following primer pairs, respectively: 5’-GCTCGTGAAATTCATGCTGGCTGATTTGATAAAAAGC-3’, 5’-GCTCGGCTCGAGTCCACCTGAATTGTGCTATTAAATACAGT-3’ for Xmc.NH2 and 5’GCTCGTGAAATTCATGCTGGCATTACTTTAAATATGCTG-3’ and T7 for Xmc.COOH. The primers contain restriction sites for EcoRI and *XhoI* (underlined), respectively to facilitate directional cloning into the complementary sites of pCS2⁺ and pCS2⁺-HA.
Plaque lifting and screening

Standard techniques were used to identify a desired recombinant phage clone from a tailbud library (in Lambda ZAP II vector, Stratagene). The plates containing phage plaques were chilled at 4° C for two hours. A nylon membrane was laid on top of the agarose for 3 minutes. After the lift, the membrane was submerged in each of the following buffers for 30 seconds and then air dried: denaturation buffer (1.5M NaCl, 0.5 M NaOH), neutralisation buffer (1.5 M NaCl, 0.5 M Tris.HCl, pH 7.5) and 2x SSC (1.5M NaCl, 0.1M sodium citrate).

After plaque lifting, re-hydrated filters (in 2x SSC), were pre-hybridised before addition of radioactive probes in pre-hybridisation solution for 1 hour at 50° C (5x SSPE, 5x Denhardt's, 0.5% SDS, 10% dextran sulphate).

Double stranded DNA used for probing phage libraries was radioactively labelled with ^32P-α-dCTP using the Prime-It II labeling kit (Stratagene), according to manufacturer's instructions.

25ng of DNA probe were mixed with 10µl random hexamers and heat denatured at 100° C for 5 minutes. The tube was allowed to cool down to RT and 10µl dCTP buffer (buffered aqueous solution containing 0.1mM of each dATP, dGTP, dTTP), 5µl ^32P-α-dCTP and 1µl of exo(-) Klenow were added. The labelling was carried out for 30 minutes at 37° C. Following the incubation period, the reaction is stopped by adding 2µl of stop mix (0.5 M EDTA, pH 8.0). Probes were precipitated by adding 20µl 7.5M NH₄Ac and 150 µl 100% ethanol at -80° C for 15 minutes. After spinning the DNA down by 15 minutes centrifugation, the pellet was dissolved in 200µl water. The solution was then boiled for 5 minutes at 100° C and applied to pre-hybridised filters (see above). Positive signals on the X-ray film were used to locate the corresponding plaques on the plate. An agar plug corresponding to each of the positive plaques was removed using a Pasteur pipette and transferred to an eppendorf containing 1ml of standard SM buffer.
(50mM Tris-HCl, 100mM NaCl, 10mM MgSO₄, 0.01% gelatine) and 20μl chloroform. Elution of the phages from the agarose plug was allowed to occur at room temperature for two hours. Length of the phagemid insert was checked by PCR (M13 forward and reverse primers). The phage solution was then either plated out for a re-screen, or used to inoculate a culture for in vivo excision of the full-length pBluescript SK(-) phagemid from the ZAP library, using helper f1 phage, according to the manufacturer's instructions (Stratagene).

**Yeast two-hybrid screen**

**Yeast strain and media**

The yeast strain utilised was PJ69-4α (James et al., 1996). This strain contains three different inducible markers, each driven by promoters recognising the Gal4 binding domain. A GAL1-HIS3 and a GAL2-ADE reporter for the selection of protorophic transformants in adenine (Ade) and histidine (His) selective media. The third inducible marker is GAL7-lacZ for the identification of positive clones by colour reaction with X-Gal.

Standard YPD medium (20g peptone, 10g yeast extract, 20 glucose, pH 6.5 for 1 liter) was used for non selective yeast-growth. For selective growth, DOB medium (1.7g yeast nitrogen base, 20g dextrose, 5g ammonium sulphate) was supplemented with a dropout supplement mixture lacking the appropriate nutrients (purchased by BIO 101).

**Yeast constructs**

A Xenopus oocyte library (a gift of Hank Farr at Washington University and constructed as in Hollenberg et al., 1995.) was cloned in a vector (f1VP16) containing the the Leu2 (leucine) marker gene, downstream of the VP16 activation domain (VP16AD).
Full-length Xmc was cloned as a bait downstream of the Gal4 binding domain (GAL4BD) in pGBDU, a vector containing the Ura3 (uracil) marker gene (James et al., 1996). Xmc was amplified using the following primers: F-5’-GCTCGTGAAATTCATGGCTGCAGTTGATGAAAGC-3’ and R-5’-CTCGTGATCTCGAGTTATAGCTCTGTGTGAATACC-3’ and cloned in the EcoRI and BamHI sites of pGBDU.

Yeast transformation

Yeast transformations were performed following the Clontech Matchmaker-Two Hybrid System® manual. Cells were grown overnight at 30° C to a final OD₆₀₀ =0.5±0.1 and centrifuged at room temperature for five minutes at 2500 rpm. Cells were resuspended in 1.5 ml 1x TE/LiAc (1xTE pH7.4: 10mM Tris-HCl pH 7.4, 1mM EDTA, pH8.0). 0.1µg DNA were used for 0.1 ml competent cells with 0.1 mg carrier salmon DNA (Sigma) for single plasmid transformation. For library transformation, bait containing yeast was grown overnight and ~20µg library DNA (~1x 10⁷ cfu) were used. 600µl PEG/LiAc were added to the DNA/cells mixture and vigorously vortexed for 1 minute. After 30 minutes incubation at 30° C, 70µl DMSO was added and the solution mixed thoroughly. Cells were then heat-shocked at 42° C for 15 minutes and subsequently chilled on ice for 3 minutes and centrifuged for 5 seconds at 14K rpm. Pellets were resuspended in 0.5ml TE and a volume of 100µl was used for plating.

Yeast mating

Colonies of one mating type were dissolved in YPD and the solution was distributed in microtitre plates. Individual colonies of the opposing mating type to be tested were added to each well; the well stirred mixture was incubated overnight at room temperature and then streaked on selective plates.
**Screening methodology**

The cDNA library was screened for proteins which interact with Xmc full-length. To ascertain integrity of expression in yeast, the Gal4 Xmc fusion protein was expressed in the yeast strain and detected by Gal4 antibody.

Yeast co-transformed with the bait and the library in PJ69-4a was first plated on Ura', Leu' and Ade' plates to select for adenine prototrophy. Prototrophic transformants were then re-streaked on Ade and His selective plate, to also check their ability to grow in the absence of His. Finally, the positive clones were assayed for the presence of β-galactosidase activity on Ade and His selective plates, enriched with X-gal (40mg/ml) The library plasmids were subsequently isolated by repetitive plating of the clones on minimal Leu selective media enriched with 5-fluoroorotic acid (5-FOA), a drug which helps select out Ura3 marker constructs. After loss of the Ura3 plasmid, the positive colonies were further checked for self-activation on Leu', Ade' and His' plates and also individually mated with their opposite mating type PJ69-4a, already containing the Xmc-Ura3 plasmid for a further check for Ade and His prototrophy and β-galactosidase activity.

The ultimate positive clones were PCR amplified (F-5'- TCTGGATATGGCCGACTTCGAGTTT-3' and R-5'-TCGGCCATCTCCACACCAGTTATTTT-3') and the amplicons subjected to restriction analysis with the rare cutter *HaeIII*, to group clones with identical restriction pattern. Eventually clones underwent sequence analysis (Sequence primer: F-5'- TCTGGATATGGCCGACTTCGAGTTT-3').
Chapter Three

A screen for genes of the organiser
In situ hybridisation screen and suppressive subtractive hybridisation

Many organiser genes might have not yet been identified. It is a very exciting goal to isolate and characterise new ones.

In the past, two main strategies have been developed to identify genes in Xenopus, which are a powerful alternative to large-scale mutagenesis screens undertaken in the more genetically accessible mouse or fish system or in invertebrates such as Drosophila and Caenorhabditis elegans. One of them is expression cloning which has proven very successful in screening for activities. It is based on assaying pools of mRNAs for a desired activity and splitting a positive pool into smaller components to isolate the ultimate responsible molecule, a procedure called sib selection. By this method cDNAs involved in dorso-ventral patterning were isolated, such as Xwnt-8 (Smith and Harland 1991), Noggin (Smith and Harland, 1992), Xnr-3 (Smith et al., 1995) and Gremlin (Hsu et al., 1998) or the homeobox genes Siamois (Lemaire et al., 1995) and Twin (Laurent et al., 1997). Similarly, expression cloning has been utilised for the identification of genes involved in other aspects of embryonic patterning (for example Mixer, Henry and Melton, 1998) or morphology (Xombi, Lustig et al., 1996). Additionally this method can be employed to isolate genes able to modify other activities, like in the case of dickkopf-1, which co-operated with tBR to induce a complete secondary axis (Glinka et al., 1998) and Xmd1, a Rho-like GTPase which suppressed tBR-induced secondary axis and is involved in morphogenesis (Wuennenberg-Stapleton et al., 1999). Recently a large-scale expression cloning screening has been carried out in search of group of genes implicated in vertebrate organogenesis (Grammer et al., 2000).

An alternative strategy to expression cloning is the isolation of genes on the basis of their spatial expression, with no initial bias as to their activity. It is possible to extensively screen a collection of cDNAs derived from the tissue where genes are expressed. This was the case of the first molecule isolated in the organiser,
Goosecoid (Cho et al., 1991), but also of Cerberus, PAPC, Sox-2, frizzled, Fkh-like (Bouwmeester et al., 1996).

Large-scale in situ hybridisation screenings have been carried out using randomly picked clones from whole embryo cDNA libraries as starting material (Gawantka et al., 1998).

However, isolation of genes implicated in processes such as patterning and organogenesis, which are mediated by programs of differential gene expression, can be facilitated by the utilisation of cDNA libraries enriched for differentially expressed genes. The general method for identifying genes specifically present in one of two cDNA populations is subtractive hybridisation. A typical subtraction experiment involves mixing the cDNA from one population (tester, which is the target one) to excess of cDNA from another population (driver), denaturation by heat or alkali to form separate strands and reformation of hybrid double helices by reannealing the complementary strands. Driver cDNA is provided in excess, so that sequences in the tester can form hybrids with the common sequences in the driver, leaving the tester enriched in its unhybridised differentially expressed genes. Normally the separation of unhybridised sequences (target) from common hybridised sequences is accomplished by affinity chromatography (Lisistyn, 1995).

A substantial advance from this general subtractive method has been the development of suppressive subtractive hybridisation (SSH, Diatchenko et al. 1996). SSH is a PCR-based subtraction method, which ensures a dramatic selective enrichment for one of two populations being compared, and offers significant advantages over conventional subtraction procedures and over other PCR-based subtraction methods like representational difference analysis (RDA). It overcomes the requirement for high quantities of mRNA and for physical separation of single stranded (ss) from double stranded (ds) cDNAs. The previously established RDA technique is also a procedure of subtraction.
combined with amplification by PCR and it was originally developed as a tool to isolate the differences between genomic DNA populations (Lisitsyn and Wigler 1993; Lisitsyn, 1995). Genomic fragments display a high complexity (i.e. high total length of unique sequences). Therefore, the concentration of each particular fragment in the tester-driver subtraction mixture is low and the formation of hybrid DNA fragments does not reach completion. RDA adopts ‘representation’ to overcome the limitation of the complexity. Representation refers to the sampling of the DNA by digestion with a restriction endonuclease, which, therefore, reduces the complexity of the DNA samples. The differences in restriction sites between tester and driver are converted to differences in DNA sequence content between them. This increases the efficiency of subsequent subtractive hybridisations. RDA has also been advantageously applied to cDNA populations, which, representing only 1-2% of the total genome, contain sufficiently few sequences for RDA to be used without having to extensively reduce the complexity (Hubank and Schatz 1994).

The enrichment of the target population by RDA is achieved by subtractive hybridisation of tester with excess driver and by preferential PCR amplification of self-hybridised tester sequences via distinct primers. However, RDA does not solve the problem of the wide differences in abundance of individual mRNA species present in a given cell.

An exclusive advantage of SSH, in addition to representation and the suppression PCR effect that selectively suppresses amplification of undesired sequences, is the solution of the problem of differences in mRNA abundance and the easy isolation of low-abundance transcripts. This is achieved because of a hybridisation step that normalises (equalises) sequence abundance during the course of subtraction by standard hybridisation kinetics. I will describe more in depth here below the principle and the procedure of SSH.
**Principle and procedure of SSH**

An outline of the procedure is illustrated in Fig. 3.1.

First, poly A+ RNA is isolated from the tissues being compared. The ‘tester’ contains the differentially expressed sequences, while the ‘driver’ is the reference RNA, by which the subtraction is driven. Double stranded cDNAs are synthesized independently from the tester and driver mRNAs and are digested with a four base-cutting restriction enzyme that yields blunt ends. Second, the tester cDNA fragments are divided into two samples (1 and 2) and ligated to two different adapters, resulting in two populations of tester. The ends of the adapters are designed without phosphate groups, so that only the longer strand of each adapter can be covalently attached to the 5’ ends of the cDNA. The SSH technique uses two hybridisations. First an excess of driver is added to each sample of the tester and they undergo a first round of hybridisation: they are mixed, heat denatured and allowed to reanneal. During this first hybridization the single stranded cDNA tester fraction (a) is normalised, which means that concentrations of high- and low-abundance cDNAs become roughly equal. Normalisation occurs because the annealing process generating homohybrid (b) and heterohybrid (c) cDNAs is faster for more abundant molecules, owing to the second order of hybridisation kinetics. Furthermore, the single stranded tester cDNAs in the tester fraction (a) are significantly enriched in cDNAs for differentially expressed genes, as ‘common’ nontarget cDNAs form heterohybrids (c) with the driver. Then follows a second round of hybridisation. The two samples from the first hybridisation are mixed together. Only the remaining normalised and subtracted single stranded tester cDNAs are able to reassociate and form (b), (c) and new (e) hybrids. At this point, addition of a second portion of denatured driver further enriches fraction (e) for differentially expressed genes. The entire population of molecules is then subjected to PCR to amplify the desired differentially expressed sequences.
Fig. 3.1 Outline of SSH procedure. Diagram adapted from Diatchenko et al., 1996.
First the adapter ends are filled in, creating the complementary primer-binding sites needed for amplification. This results in several types of hybrids containing different combinations of adapter sequences at their ends. The newly formed (e) hybrids are different from hybrids (b) and (c) formed during first and second hybridizations, because they have different adapter sequences at their 5' ends. One is from sample 1 and the other is from sample 2. The difference between the sequences allows selective PCR amplification of this population, which is the subtracted and normalized fraction, made up of differentially expressed cDNAs (e). Two PCR amplifications are performed. The first PCR is performed using a primer complementary to the outer part of adapter 1 and 2 (black box in Fig. 3.1). In all PCR cycles, exponential amplification can occur only with type (e) molecules. Type (b) molecules contain long inverted repeats on the ends and form stable 'panhandle-like' structures after each denaturing-annealing PCR step. The 'panhandle-like' structure cannot serve as template for exponential amplification, because intramolecular annealing of longer adapter sequences is both highly favoured and more stable than intermolecular annealing of the much shorter PCR primers. This is the suppression PCR effect. Type (a) and (d) molecules do not contain primer binding sites, and type (c) molecules can be amplified only at a linear rate. Although there is a primer binding sequence on both ends of type (e) molecules, the overall low homology at their two ends (arising from the different internal sequences of the adapter, white and blue boxes) negates the suppression PCR effect. Type (e) molecules are, therefore, exponentially amplified.

A second PCR round, using 'nested' primers complementary to the internal regions of the adapter (white and blue boxes only present together in type (e) molecules) further enriches for differentially expressed sequences. Thus, due to normalisation and the suppression PCR effect, the subtracted population is left enriched in target differentially expressed genes.
Construction of an organiser specific library and screening methodology

Employing suppressive subtractive hybridisation I have constructed a cDNA library enriched for genes differentially expressed in the organiser. A DMZ cDNA population was compared to a newly fertilised egg cDNA population, in order to subtract from the former all the genes that it shared with the latter and retain mainly DMZ differentially expressed zygotic genes. Approximately 2µg of DMZ poly A+ RNA (300 DMZ explants) and 10µg poly A+ RNA of fertilised egg were utilised to synthesise their respective cDNA populations and these latter underwent then the subtraction procedure.

In search of genes with expression in the organiser, the library was screened by in situ hybridisation.

Since the library had no directionality, for in situ hybridisation purposes, it was cloned into a vector (TOPO PCR II, Invitrogen) with double promoter, SP6 and T7. Plating the library resulted in ~683 individual clones. All clones were amplified by PCR and the amplicons served as templates for the synthesis of labelled transcripts for use as probes for in situ hybridisation using both polymerases. The transcripts were subsequently applied on albino embryos. For a compromise between large-scale screening and depth of analysis, the embryonic stages monitored were limited to gastrula (st. 10.5) and to early neurula (st. 13), since genes expressed during gastrulation can commonly still be expressed at neurula stages. The in situ hybridisation analysis was automated by the use of a robot, available from ABIMED. The robot allowed the application of the standard manual in situ hybridisation protocol and the screening of 96 probes per run in total. The antisense probes giving patterns of expression in the organiser would be selected and their cDNA sequences compared to EST databases (Fig.3.2).
Fig. 3.2 Screen methodology
Analysis of subtraction efficiency

Before embarking the large-scale screening we wanted to estimate the efficiency of the subtraction performed. This is possible by comparing the abundance of known genes before and after the subtraction and was done by PCR analysis of the expression of the genes in the two fractions. Differences in intensities of PCR fragments, after the same number of PCR cycles, indicate relative reduction or enrichment of genes. Under the conditions used (Diatchenko et al., 1996), the number of PCR fragments increased ~20-fold during five cycles of amplification.

After a successful subtraction, one would expect a reduction in the library in the abundance of genes common to the two populations compared (e.g. a housekeeping gene) and of genes primarily expressed in the driver population. I then compared between the unsubtracted and subtracted fraction the abundance of ornithine decarboxylase (ODC) and, since subtraction was driven with maternal material, of a primarily maternal gene, VegT. As shown in Fig 3.3, transcripts of these genes are significantly reduced or eliminated in the subtracted fraction, reflecting an approximate 100-fold reduction. The abundance of organiser genes, instead, is high in both fractions. In particular, a roughly equal amplification of the organiser gene cerberus in the subtracted fraction requires five fewer cycles with respect to the unsubtracted fraction (18 vs. 23), indicating an approximate 20-fold enrichment.

These observations indicate that the subtraction had worked efficiently, enriching the library for organiser differentially expressed genes.
Fig. 3.3 Reduction of housekeeping and maternal genes abundance after subtraction
PCR was performed on subtracted and unsubtracted library fractions. Even at the highest number of PCR cycles the housekeeping gene *ode* and the maternal gene *vegT* are absent in the subtracted fraction, indicating a significant enrichment in this latter for differentially expressed genes. As expected the abundance of organiser specific genes *pintallavis* and *cerberus* remains high after the subtraction procedure. Equal amplification of *cerberus* in the subtracted fraction requires five fewer cycles than in the subtracted fraction (18 vs. 23).
**Screen outcome**

The *in situ hybridisation* outcome of individual clones was carefully analysed and the clones systematically grouped according to their predominant pattern of expression. Out of 683 clones screened, 6% \((n=41)\) exhibited differential staining in the organiser or its derivatives. 13% exhibited undetectable staining, 7% displayed ubiquitous expression and 74% showed other differential expression patterns. Half of these displayed prominent neural staining (Table 3.1).

The repeated occurrence during the screening of various previously isolated organiser genes \((n=10, \text{ see Table 3.2.})\) provided us with an additional confirmation of the good quality of the library.

In addition to known organiser genes, 5 new genes displayed differential expression in the organiser. These were isolated and cloned (Table 3.3, Klingbeil et al., 2001a,b).

Isolated three times, one clone, so far called 153, has no significant homology to anything known in the database. The protein it encodes has a nuclear localisation (preliminary data from another lab member). A second gene, singly isolated is likely to be the true homologue of human *sall1*, a gene mutated in Townes-Brocke syndrome (Kohlhase et al., 1998).

*Xwig1*, (*Xenopus wound-induced gene 1*, characterised by another member of the lab, Klingbeil et al., 2001) is another gene isolated for its expression in the organiser. Protein localisation studies show *Xwig1* is anchored in cytoplasmic structures, putatively the endoplasmic reticulum. During embryonic development, *Xwig1* is mainly expressed in epithelial cells that form constrictions during morphogenetic processes, such as the blastopore closure and optic vesicle invagination. Remarkably, transcription of *Xwig1* is activated in response to epidermal wounding.
<table>
<thead>
<tr>
<th>Staining Type</th>
<th>Occurrence</th>
</tr>
</thead>
<tbody>
<tr>
<td>No staining</td>
<td>15%</td>
</tr>
<tr>
<td>Ubiquitous pattern</td>
<td>10%</td>
</tr>
<tr>
<td>Drosal</td>
<td>5%</td>
</tr>
<tr>
<td>Neural</td>
<td>30%</td>
</tr>
<tr>
<td>Other specific stainings</td>
<td>35%</td>
</tr>
</tbody>
</table>

Table 3.1 Screen outcome
<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>No. of isolates</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Endodermin</td>
<td>α2-macroglobin</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Xlim-1</td>
<td>Tx factor</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Pintallavis</td>
<td>Tx factor</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>Cerberus</td>
<td>Secreted factor</td>
<td>9</td>
</tr>
<tr>
<td>5</td>
<td>Chordin</td>
<td>Secreted factor</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>Xfd-12'</td>
<td>Tx factor</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>Xsox-2</td>
<td>Tx factor</td>
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<tr>
<td>10</td>
<td>Xotx-5</td>
<td>Tx factor</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 3.2 List of known organiser genes encountered during the screening

Such transcriptional response is downstream of the transient phosphorylation of extracellular signal-regulated protein kinases (ERKs) and is partly mediated by Elk-1, but independent of FGF signalling.

Xgravin-like (Xgl, Klingbeil et al., 2001), is a putative a-kinase anchoring protein, homologous to human Gravin and SSeCKS, which together constitute a family of scaffolding proteins binding PKA and protein kinase C (Nauert et al., 1996).

Xgravin-like has a very dynamic expression during embryonic development. At gastrulation, Xgl is expressed in posterior mesoderm and the blastopore lip. At neurulation, Xgl transcripts are in the forebrain, at two neuroectodermal stripes and in the notochord. At tailbud stages, expression resides in the mandibular neural crest and the roof of the spinal cord from where neural crest cells migrate into the intersomitic region. Expression is also detected in the heart.

A final gene isolated is Xmc (Xenopus marginal coil). This is the gene I selected for my doctoral work and its analysis constitutes the subject of the remainder of this doctoral thesis. Characterisation of Xmc, dealt with in depth in Chapter Four, revealed that it is implicated in the regulation of gastrulation movements.
<table>
<thead>
<tr>
<th>Clone #</th>
<th># of isolates</th>
<th>Gene product</th>
<th>Protein localization</th>
<th>Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>XSALL1</td>
<td>tx factor (Spalt)</td>
<td>nuclear</td>
<td>hSALL1, Townes Brocks syndrome</td>
</tr>
<tr>
<td>2</td>
<td>#153</td>
<td>novel</td>
<td>nuclear</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Xwig1</td>
<td>ER protein</td>
<td>ER</td>
<td>zebrafish and fly EST</td>
</tr>
<tr>
<td>4</td>
<td>Xmc</td>
<td>coiled coil protein</td>
<td>cytoplasmic</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Xgravin-like</td>
<td>PKA and PKC anchoring protein</td>
<td>cytoplasmic</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.3 List of new genes isolated by subtraction screening and their expression in the organiser.
Chapter Four

Xmc, a gene governing gastrulation movements
Introduction

In the last section of the general introduction, much attention was dedicated to the description of the morphogenetic properties of the organiser. The main cellular movements contributing to the correct positioning of the gastrula germ layers were listed and explained. These movements are epiboly of the animal cap, dynamics of the bottle cells, traction of deep zone cells along the blastocoel, convergent extension along the mediolateral axis and rotation of the vegetal mass.

An account of the initial, but very interesting findings about the molecular deciphering of the most important gastrulation movement, convergent extension, was given. These findings all indicate that the execution of gastrulation movements involves the utilisation of alternative branches of existing signalling pathways, namely so far the non-canonical Wnt-pathway and a putatively non-canonical FGF pathway. These alternative pathways signal via distinct components and do not interfere with mesodermal fate specification.

This chapter covers the characterisation of a novel gene identified in the screen, which turned out to play a role in the execution of gastrulation movements and most probably of convergent extension. This gene is largely expressed in the marginal zone, strongly on the dorsal side. The predicted protein contains two coiled-coil domains at the N-terminus and C-terminus, respectively. It was thus called *Xenopus marginal coil (Xmc)*.

*Xmc* expression in the marginal zone mesoderm is regulated by FGF signalling. Loss-of-function studies by morpholino mediated translational knock-down provided a clue to the function of *Xmc*, revealing that in *Xmc*-depleted embryos gastrulation movements are impaired. Analysis of *Xmc* loss-of-function in DMZ explants reveal a direct inhibition of convregent extension movements without interference with mesodermal specification.
Xmc, could thus be a downstream FGF target having a distinct role in the regulation of gastrulation movements in *Xenopus*, independent of mesoderm induction/maintenance.
Identification and structure of Xmc

Starting from a ~500 bp partial clone isolated from the subtracted library a full-length Xmc cDNA, comprising ~2.0 kb was isolated from a tailbud cDNA library by standard filter hybridisation procedures.

Xmc encodes a predicted protein of 515 amino acids, with a molecular weight of ~56 kDa (Fig. 4.1). Blast searches showed that Xmc has no homology to any other vertebrate protein in the nr or EST database. Prosite and SMART motif predictions revealed the presence of two putative coiled coil domains, one at the amino-terminus (amino acids 79-166) and the other at the carboxy-terminus (amino acids 454-492; Schultz et al., 2000). A short amino acid stretch preceding the carboxy-terminal coiled-coil domain was found, by Prosite predictions only to have limited homology to the phosphatidyl-inositol phospholipase C (PIPLC) X domain (amino acids 338-389). This motif is important for the catalytic activity of eukaryotic PIPLCs.

Spatial and temporal expression of Xmc

The spatio-temporal expression of Xmc was analyzed by RT-PCR and in situ hybridization.

Xmc transcripts are first detected at stage 9, after MBT, as illustrated in Fig.4.2A. At stage 10.5 Xmc is expressed throughout the marginal zone, with strong expression on the dorsal side of the embryo (Fig.4.2B). Sections on embryos stained at this stage show that Xmc is in the mesodermal layer (Fig.4.2C). Expression peaks during mid-gastrulation (st.11-12), when most morphogenetic movements are taking place. Xmc transcripts at this stage are abundant around the blastopore and more concentrated at two patches flanking the future notochord (Fig.4.2D-E).
Fig. 4.1 Xmc is a protein with novel domain composition. Schematic representation of Xmc and two deletion constructs, Xmc.NH2 and Xmc.COOH. Indicated are the two coiled coil domains (blue) and the phosphatidylinositol phospholipase C (PIPLC) X domain (red).
At the end of gastrulation, expression is maintained around the closing blastopore and gets confined to the posterior side of the embryo (Fig. 4.2F). There, it is maintained throughout neurulation. At early neurula stage, when the neural plate starts being defined (Fig. 4.2G and H, dorsal and lateral view, respectively), Xmc transcripts are strongly detectable in the posterior half of the embryo and more weakly in two stripes presumably marking the neural/non-neural boundary. Sections at this stage reveal staining in mesenchymal tissue and in the lateral plate mesoderm (Fig. 4.2I-J). Until st. 25, expression is confined to the posterior side and also clearly resides in the somites, as confirmed by histological sections at this stage (Fig. 4.2K-M). Staining is also slightly detectable in the branchial arches (Fig. 4.2L). At stage 36, expression is maintained in the somites and is also present at the chordoneural hinge (Fig. 4.2N).

The early expression of Xmc and its prominently posterior localization are reminiscent of the expression of XeFGF (Isaacs et al., 1995), and of previously characterised posterior determinants, Xcad1, Xcad2 (Pillemer et al., 1998) and Xbra (Smith et al., 1991) which are known FGF target genes. To confirm this similarity, the spatial expression of Xmc, Xcad2 and Xbra were compared at st. 10.5 by whole mount in situ hybridization and histological sections (Fig. 4.3). In general the expression of all three genes is throughout the marginal zone; while Xbra is more uniformly distributed around the blastoporal circumference, Xcad2 expression is more confined to the ventral-lateral side of the marginal zone. Xmc expression, on the contrary, is stronger on the dorsal side. Sagittal sections along the dorso-ventral axis reveal a comparable distribution of the staining along the embryonic layers, showing a predominance in mesoderm. The red arrows in Fig. 4.3 mark the upper limit of expression at the equatorial region and indicate the most internal limit of mesodermal staining.
Fig. 4.2 Dynamic spatio-temporal expression of Xmc during embryonic development.
(A) RT-PCR analysis of Xmc expression during embryonic development (stages according to Nieuwkoop and Faber, 1967). Histone H4 serves as internal loading control. (B-N) Whole-mount in situ hybridization analysis of Xmc expression. Spatial expression during early (B,C), mid (D,E) and late (F) gastrulation (vegetal views), neurulation (G-J, anterior to the left, G, dorsal and H, lateral view, respectively), and tailbud and tadpole stage (K-N). Asterisks indicate the elevated bilateral dorsal expression and stippled lines indicate the margin of organizer explants used for the subtraction screen. Abbreviations: me, mesenchyme; lp, lateral plate and so, somite.
Fig. 4.3 Comparative expression of Xmc, Xcad2 and Xbra.
Spatial expression of Xmc (A), Xcad2 (B) and Xbra (C) in whole mount-stained mid-gastrula embryos (vegetal view) and transverse sections thereof. White lines indicate the plane of sectioning. The red arrows mark the animal limit of marginal zone expression and black arrowheads indicate the blastopore (dorsal is to the right).
In general, *Xmc* presents a predominant expression at the marginal zone mesodermal cells of the early embryo, which undergo morphogenetic movements and is maintained at the posterior half of the embryo and in somitic mesoderm during later stages of development.

**FGF signalling is sufficient and required for the induction of Xmc**

The similarity and overlap of the expression profiles of *Xmc, Xcad2* and *Xbra* prompted me to investigate whether FGF signalling could also govern the expression of *Xmc*. To test the sufficiency of FGF to up-regulate expression of *Xmc* in naïve ectoderm, *XeFGF* was overexpressed at the animal pole of two-cell stage embryos, caps were explanted at stage 8 and cultured until sibling embryos reached ~st. 11. *Xmc* expression was then monitored by RT-PCR and *in situ* hybridization. *Xmc* was indeed up-regulated by *XeFGF* and so was *Xbra*, a known FGF target (Isaacs et al., 1994; Fig. 4.4A and B), used as positive control. To investigate whether endogenous FGF signalling is required for *Xmc* expression, the FGF signal transduction pathway was inactivated by injecting the dominant-negative FGF receptor (*XFD*; Amaya et al., 1991). Abolishment of FGF signalling results in a marked reduction of *Xmc* expression at early gastrula stage. At neurula stage *Xmc* transcripts are reduced, but still detectable at ventro-lateral territories. This maintenance of Xmc staining at later stages could suggest that alternative factors intervene in the late regulation of Xmc; one possibility are BMPs (Fig. 4.4C).

Together these results provide evidence that Xmc is a target of the FGF pathway in Xenopus marginal zone mesoderm.
Fig. 4.4 FGF signaling is necessary and sufficient to activate Xmc expression. (A) RT-PCR analysis for Xmc and Xbra expression in control caps and XeFGF-injected animal caps. EF-1α serves as loading control. (B) Whole mount in situ hybridization for Xmc and Xbra expression on control and XeFGF-injected albino cap explants. Embryos were injected with ~160 pg of XeFGF mRNA in one animal blastomere at the two-cell stage. (C) Expression of Xmc in control and dnFGFR-injected mid gastrula (stage 11-12) and early neurula embryos (stage 14). Embryos were injected with 400 pg of dnFGFR mRNA in four blastomeres at the four-cell stage.
Subcellular distribution of Xmc

The molecular nature of Xmc gave no indication as to its biological function. To determine the subcellular localization of Xmc, a carboxy-terminally-tagged Xmc-HA fusion protein was generated and ectopically expressed in animal caps. The Xmc-HA chimera is distributed in the cytoplasm in vesicles that are often enriched in the proximity of the cell membrane (Fig. 4.5A-C).

The subcellular distribution of two deletion constructs of Xmc (Fig.4.1) was also analysed. A deletion construct comprising only of the N-terminal coiled coil, Xmc-NH2, if overexpressed in animal caps, is recruited to the membrane without vesicular aggregates (Fig.4.5D-F). Remarkably, cells expressing this chimeric construct tended to round up and subsequently dissociated, as illustrated in Fig.4.5F (inset). The construct containing the carboxy-terminal coiled-coil domain and the preceding PIPLC-X domain, Xmc-COOH, is mislocalized throughout the cytoplasm, revealing in some cases a somewhat perinuclear staining (Fig. 4.5 G-I).

Interaction partners for Xmc

In parallel to the analyses carried out, a two-hybrid screen was conducted to look for interaction partners for Xmc. Xmc fused to the binding domain of the yeast Gal4 transcription factor (Gal4BD), was used to screen a Xenopus oocyte cDNA library cloned downstream of the VP16 transcriptional activation domain (VP16AD). An interaction between a library clone and Xmc would bring the Gal4BD and VP16AD together to drive transcription. The yeast strain used, PJ69-4α (James et al., 1996), had three reporter genes, under the control of inducible promoter elements recognising the Gal4BD. Two of the reporter genes were responsible for the synthesis of adenine
Fig. 4.5. Subcellular localization of Xmc and deletion constructs in animal cap explants.
Confocal images showing Xmc-HA, Xmc.NH2-HA and Xmc.COOH-HA distribution (green channel), cortical actin visualized by rhodamin phalloidin (red channel) and the overlay. Embryos were injected with 800 pg of mRNA in two animal blastomeres at the two-cell stage. (A-C) Full-length Xmc is localized in a vesicular pattern in the cytoplasm (red arrowheads) and associated with the inner cell membrane (white arrows). (D-F) Xmc.NH2 is quantitatively recruited to the plasma membrane. At high doses, cells tend to round up and dissociate (see inset). (G-I) Xmc.COOH is localized in a diffuse perinuclear manner.
(Ade) and histidine (His) for isolation of positive transformants for their prototrophy in Ade and His selective media; the third is a lacZ reporter gene which allows easy isolation of positive clones by colour reaction with X-gal. Of about $1 \times 10^7$ transformants, 458 were able to grow in the absence of Ade. 278 were prototrophic in both Ade and His selective media. About one fourth of these ($n=77$) were β-galactosidase positive.

After bait plasmid loss, all the positive clones were tested for self-activation and were individually mated with opposite mating type PJ69-4a (which was transformed with the bait), to confirm the interaction. This diminished the number of positive clones to 49. Library plasmid DNA was extracted from the yeast and sequenced.

Sequence analysis of the DNA revealed that 45% ($n=22$) of the positive clones were homologous to an as yet unknown human cDNA clone. About 16% ($n=8$) were identical to Eg5, a kinesin related motor protein (Houliston et al., 1994). The rest of the clones presented very insignificant homology to other scattered unknown human cDNAs and were not considered relevant. Kinesins are motor proteins capable of utilising chemical energy from ATP hydrolysis to generate mechanical force (Manning and Snyder, 2000). Eg5 is a kinesin related protein, localised in microtubules and particularly enriched in spindle poles which is required for spindle formation in Xenopus egg extracts (Houliston et al. 1994), (Sawin et al. 1992). It is also associated with microtubules on Xenopus egg cortices during cortical rotation (Marrari et al., 2000).

Of the Eg5 clones interacting with Xmc ($n=8$), all were overlapping with the stalk region of Eg5 (Fig. 4.6). This region is the long α-helical stalk domain between the motor domain of the kinesin and its carboxy-terminal tail.

It can be deduced that the α-helical region of the kinesin might putatively engage in dimerisation with one or both of the coiled coil domains of Xmc.
Fig. 4.6 Eg5 is a putative interactor for Xmc.
Alignment of Eg5 with open reading frames of the clones individually found to interact with Xmc during the two hybrid screening. The clones span the α helical stalk domain of the kinesin related protein Eg5.
Interfering with \textit{Xmc} function causes gastrulation defects and affects posterior structures

In order to gain more insight into the biological function of \textit{Xmc}, gain-of-function and loss-of-function studies were performed. Overexpression of \textit{Xmc} RNA even at high doses (up to 1-2ng) resulted in no prominent abnormalities in whole embryos, although \textit{Xmc} mRNA could be translated in a protein of correct size in a rabbit reticulocyte lysate assay (data not shown). Loss-of-function experiments were then conducted employing morpholino antisense oligos. Morpholino oligos operate by silencing the translation of the target mRNA. As opposed to previously used antisense oligos, which rely on the degradation of the target mRNA, morpholinos are not toxic to cells and they are strongly nuclease resistant. They are designed to bind to the 5' UTR region of a transcript and to block initiation of translation. They have been shown to be effective in inhibiting the translation of targeted mRNAs in \textit{Xenopus} and in zebrafish (Heasman et al., 2000; Nasevicius and Ekker, 2000).

In a first test to prove that the designed morpholino oligo (from Gene Tools, LLC) was able to suppress translation of \textit{Xmc} mRNA, I checked whether the antisense oligo was able to suppress translation of an exogenously provided \textit{Xmc-GFP} chimera, which is easily scorable in whole embryos under UV-light (Fig. 4.7A-C). Embryos were injected with 800 pg \textit{Xmc.GFP} mRNA with or without 8 ng morpholino in two blastomeres at the two-cell stage. Co-injection was indeed potent in abolishing \textit{Xmc.GFP} translation (Fig. 4.7A-C, compare B and C).

This suggests that the morpholino would also abolish translation of an endogenous \textit{Xmc} transcript.

The effects of knocking-down the function of \textit{Xmc} were then studied in whole embryos by injecting the morpholinos at the DMZ, where \textit{Xmc} is predominantly expressed. Morpholino oligos (8-10ng) were injected equatorially at the dorsal
Fig. 4.7 Morpholino oligos suppress translation of Xmc-RNA
Fluorescent images of control (A), Xmc.GFP-injected (B) and Xmc.GFP+ Xmc-morpholino-injected (C) neurula embryos. Embryos were injected with 800 pg Xmc.GFP mRNA with or without 8 ng morpholino in two animal blastomeres at the two-cell stage. Co-injection efficiently abolishes Xmc.GFP translation (compare B and C).
side of the embryo (two times at the 4 cell stage, at the crescent-shaped region of pale pigmentation bisected by the first cleavage furrow). Following injection, three main categories of phenotype were scored at tailbud stages; the most frequent one, 60% of injected embryos (n=164), consisted of a marked kink of the axis (see Fig.4.8B-B'). 13% showed a shorter trunk and a somewhat smaller head (Fig.4.8C-C'). 21% of morpholino-injected embryos did not gastrulate properly; the body shape was lost and the blastopore did not close (Fig.4.8D-D'). On average (6 individual experiments), only 6% of injected embryos of a given batch appeared normal (Fig.4.8A-A'). Injection of two non-related morpholino oligos at similar concentrations did not result in any phenotypic alteration. The abnormalities observed are suggestive of defects in gastrulation movements.

A critical requirement when using morpholino oligos to target novel, uncharacterized RNAs is to verify the specificity. To test the specificity of the Xmc-morpholino oligos, an Xmc rescue construct was co-injected, which lacks the 5’ UTR and which can no longer be targeted by the morpholino. Co-injection of Xmc-RNA, lacking the 5’ UTR (1200pg), together with the morpholino resulted in a considerable reduction of the frequencies of the phenotypes described above (Fig. 4.9). The frequency of the “kinked” phenotype was reduced from 60% to 25% (n= 175) The embryos showing an open blastopore only constituted 8% and embryos with shortened trunk were reduced to 10%. This result indicates that the rescue construct is able to reverse the effects exerted by the morpholino and implies that the morpholino is acting specifically on Xmc transcripts.

It is interesting to note that at early gastrula stages (10-10.5), dorsal lip formation was delayed (~1-2 hrs) in embryos injected with the morpholino with respect to control uninjected embryos. In addition, the formed lip had an irregular shape and later the size of the blastopore was enlarged (data not shown). This, together with the phenotypes described above, further suggests that Xmc might be eliciting its role in the regulation of gastrulation movements.
Fig. 4.8 Morphological defects in whole embryos injected with Xmc-morpholino

Lateral (A-D) and dorsal (A’-D’) views of stage 25 control embryos (A-A’) or embryos injected with ~10ng of Xmc morpholino in two marginal blastomeres at the four-cell stage (B-B’-D-D’). Injection of Xmc morpholinos results in three classes of phenotypic alterations. Class I embryos (B-B’) display a marked kink in the primary axis, class II embryos (C-C’) have a shortened axis and class III embryos (D-D’) display severe gastrulation defects.
Fig. 4.9 Quantitation of the phenotypic alterations. Histogram showing the frequencies of the phenotypes and the reduction after co-injection of Xmc morpholino with the Xmc.rescue construct.
**Xmc loss-of-function inhibits convergent extension, but does not inhibit mesoderm formation**

For a more careful analysis of the gastrulation defects, lineage tracing experiments were conducted. Xmc morpholino oligos were co-injected together with a GFP tracer in one dorsal blastomere of four-cell stage embryos. In 89% of the embryos displaying a kinked axis (n=128), the kink was directed towards the injected site as revealed by GFP staining (Fig. 4.10A and B), suggesting that Xmc loss-of-function might specifically affect the elongation of the medio-lateral axis and the execution of convergent extension movements (morpholino-injected cells are not lost due to cytotoxicity).

To further investigate this possibility, the behaviour of DMZ tissue was examined by the use of Keller explants (Keller et al., 1992). This is a generally employed method for the analysis of convergent extension. Control explanted DMZs undergo convergent extension movements, the tissue elongates transforming the explant into a head and tail region (32/32, Fig.4.11A). Elongation was inhibited by the morpholino; the DMZ explant did not change shape and remained as a compact ball (27/30, Fig. 4.11B). Co-injection of Xmc-morpholino and of Xmc-rescue RNA restored convergent extension, resulting in explants with a mesodermal tail and elongation of some degree (24/26, Fig. 4.11C). These observations would indicate that Xmc might regulate gastrulation movements by controlling convergent extension movements.

To investigate whether Xmc loss-of-function caused a direct impairment of cellular rearrangements or indirectly affected morphogenetic movements due to the inhibition of mesoderm formation, the expression of several mesodermal marker genes was analysed.
Fig. 4.10 Xmc-morpholino specifically affects medio-lateral elongation at the site of its injection.

(A-B) Lineage tracing experiments showing that when co-injecting Xmc morpholino and GFP in one dorsal blastomere, the direction of the kink along the anteroposterior axis follows the site injected with the morpholino, as indicated by GFP. Embryos injected with GFP alone, displayed no phenotype (A). Blue line marks the shape of the embryo.
Fig. 4.11 Xmc-morpholino affects convergent extension movements without interfering with mesodermal patterning.

Morphological analysis of control (A), Xmc morpholino-injected (B) and Xmc morpholino + Xmc.rescue mRNA-injected (C) DMZ explants. (D) RT-PCR analysis for Xbra and XmyoD expression in DMZ explants cultivated till stage 11 and α-actin expression in DMZ explants cultured till stage 25. (E) In situ hybridization for Xbra at st. 10.5-11 in whole embryos injected with Xmc morpholino. Red arrows indicate the blastopore. In all cases, embryos were injected with ~8 ng Xmc morpholino with or without ~1 ng Xmc.rescue mRNA.
The inhibition of DMZ elongation occurred without affecting the expression of the panmesodermal marker Xbra and of the dorsal-lateral mesodermal marker XmyoD (Fig. 4.11D and E). To test whether Xmc loss of function would interfere with the maintenance of mesodermal cell fate, the expression of α-actin was monitored, as a characteristic marker of differentiated muscle, a somitic mesodermal derivative. α-actin expression was also not altered in morpholino-injected DMZs (Fig. 4.11D).

Taken together these results suggest that Xmc has a direct role in the regulation of gastrulation movements, independent of mesodermal differentiation. This distinction is important as normally the regulation of morphogenetic movements and the one of cell fate specification have been difficult to discern at the molecular level.
Chapter Five
Discussion and
Conclusions
Strengths and limits of *in situ* screens

The principal aim of the project reported here was the preferential isolation of genes expressed in the organiser. In order to accomplish this objective, *in situ* screening was chosen amongst the available methods. This type of screening allows isolation of new genes based on their expression pattern, independently of their activity or function.

The screen was performed using a specific library, enriched for organiser genes by suppressive subtractive hybridisation (SSH, Diatchenko et al., 1996). 6% of the genes examined were differentially expressed in the organiser (Table 3.1). This number included a number of genes already known (Table 3.2) and some that had not as yet been identified (Table 3.3).

Utilisation of SSH has certainly increased the percentage of differentially expressed genes isolated. This procedure allowed the significant reduction of undesired genes by ~100-fold and the enrichment of target organiser genes by ~20-fold (Fig. 3.3). The relatively satisfactory number of organiser specific genes amongst the total number of genes screened would have been diminished if a primary, unsubtracted library had been used.

The list of genes isolated in the screen includes a good variety of possible functions. This means that the library constructed could represent a source of non-redundant molecules. But above all it means that in general the organiser is a *reservoir* of a large variety of molecules each in their own way contributing to the exertion and maintenance of the organiser properties. Nevertheless, the screen appears far from saturation. The number of organiser genes that remain to be isolated must still be very high, although it is hard to estimate. Dorsal lip/organiser libraries are constantly being constructed and it would not be surprising to find that many genes isolated for their expression in this region may have no homologies to anything known. This would open up the possibility
that many different molecules participating in various processes and signalling pathways are still to be characterised and that the coverage of the understanding of the organiser function and complexity is far from totality.

The description of gene expression has become an important component of the characterisation of novel genes. By educated guessing, it is often possible, based on the regions of expression or on the similarity of a pattern to those of known genes to grasp the function of an unknown clone. One of the most interesting aspects of in situ screens is that, grouping genes according to their shared, complex expression pattern is often paralleled by a remarkable functional correlation. Of course, such correlation requires confirmation of physiological relevance through further gain- and loss-of-function experiments.

Furthermore, many genes with important regulatory functions, but displaying no particular patterned expression may be easily missed in an in situ hybridisation screen.

The screen was carried out on a library of one specific tissue, with a relatively satisfactory outcome in relation to its scale. Of course this approach was far from global dimensions and was not so large compared to other previously conducted screens (Gawantka et al., 1998). In situ screens have the potential for a global analysis of embryonic gene expression throughout different tissues, providing a chance for a comprehensive, comparative interpretation of tissue/functional relatedness based on shared differential gene expression.

Very recently there has been an impetus for the establishment of genetic methods also in frogs with the advent of *X.tropicalis* as a new vertebrate genetic model system (Amaya et al., 1998).

The diploidy of its genome and its shorter generation time (4-6 months versus 1-2 years in *X.laevis*) make transgenesis techniques originally developed for *X. laevis*
not only conceivable, but practical in *X.tropicalis*, giving the possibility to embark on large genetic screens.

The combination of the existing methods with the power of genetic manipulations would create a vast range of approaches, which could ensure a saturating analysis, and thorough understanding of vertebrate embryonic development and its complexity.

Gastrulation in vertebrates is a dynamic process, which in addition to an accurate programme of cell fate specification involves the co-ordination of cell-adhesivity, cell polarity and cell movement.

Although the results described argue for a requirement of *Xmc* in the regulation of gastrulation, it is not clear in which of these aspects of gastrulation it is particularly involved.

Here I will try to recapitulate the results and discuss them in view of the possible mechanistic action of *Xmc*.

**Xmc codes for a novel cytosolic coiled coil protein**

One very interesting observation during the study of Xmc was the subcellular localisation of Xmc. Tagged versions of Xmc, overexpressed in animal caps localise in large vesicles in the cytoplasm or smaller ones associated with the plasma membrane (Fig.4.5). Such intracellular distribution was observed for GFP and HA-tagged chimeras, and was irrespective of whether the protein was amino- or carboxy-terminally tagged.

Xmc encodes a protein with a novel composition of domains. It contains two coiled coil domains and a putative phosphatidyl-inositol phospholipase C (PIPLC) X domain, preceding the carboxy-terminal coiled coil. α-helical coiled
coils comprise one of the most abundant oligomerisation motifs in proteins. Despite their relatively simple architecture, they constitute a versatile protein-folding motif that displays a variety of different, specialised functions (reviewed in Burkhard et al., 2001). Many nuclear proteins involved in transcriptional regulation contain coiled coil dimerisation domains, like Fos and Jun. Coiled coil domains are also found in a large variety of cytoplasmic proteins, such as cytoskeletal proteins, like α-keratin and motor proteins that interact with the cytoskeleton, such as myosin, kinesin and dynein. In the majority of cases coiled coils constitute the principal oligomerisation motifs and additional domains determine biological function. It is likely that the coiled coil domains of Xmc act as oligomerisation motifs. Assuming that the intracellular distribution of chimeric Xmc reflects the endogenous localisation it is possible to speculate that Xmc might be involved in some kind of intracellular trafficking.

The two-hybrid analysis carried out, aiming at finding interaction partners for Xmc, revealed the kinesin-related motor protein Eg5 (Houliston et al., 1994) as one most prominent interactor. Xmc interacts with the α-helical coiled coil region of this protein.

Eg5 is plus-end-directed microtubule motor associated with microtubules and required for spindle formation (Sawin et al., 1992). It has also been observed associated with microtubules on Xenopus egg cortices during cortical rotation (Marrari et al., 2000). In light of the role of Xmc in the execution of gastrulation movements, it is easy to speculate the possibility that the interaction of Xmc with Eg5 might bring it in association with microtubules to promote cellular movements. However, this is pure speculation; a confirmation of any physiological relevance of the interaction found through the two-hybrid assay awaits further analyses, as coiled coil domains are known to engage in spurious dimerisations in yeast.
First of all, in addition to immunoprecipitation studies between the two molecules, *in vivo* analyses should be carried out. It would be interesting to check the spatial co-expression of Eg5 in the regions of the embryo, where Xmc is normally expressed and to check intracellular co-localisation of Xmc and Eg5 in Xenopus culture cells or in fixed DMZ explants and their co-localisation with microtubular structures.

Which of the coiled coils of Xmc engages in the interaction with the α-helical coiled coil region of Eg5, or whether both of them are required for the interaction is worth determining using the deletion mutants constructed (Fig. 4.1).

Eventually, whether Eg5 plays a role for microtubular movements during gastrulation in the DMZ should be investigated.

**A role for Xmc in convergent extension movements**

Morpholino-mediated ‘knock-down’ experiments revealed that Xmc is essential for gastrulation movements (Fig. 4.8 and 4.9).

The lineage tracing experiments (Fig. 4.10) and the analysis of elongation of DMZ explants (Fig. 4.11 A-C) have been informative as to the possible effect of Xmc-loss-of-function on convergent extension movements.

Morpholino co-injection in whole embryos with GFP as a lineage tracer indicates a requirement of Xmc in convergent extension, because it shows how Xmc loss-of-function on one side of the embryo specifically inhibits elongation of the mediolateral axis only on that side.

The DMZ elongation assay proves that the actual cells normally undergoing convergence and extension are unable to do so in the absence of Xmc.

*Xmc* is expressed around the blastoporal circumference with elevated levels in two domains flanking the presumptive notochord (Fig. 4.2) and is not included in the presumptive notochord region. The cells of *Xmc* expression are fated to give
rise to somitic/paraxial mesoderm and not to axial structures. The above experiments and the pattern described would then suggest a participation of Xmc in convergent extension movements of trunk somitic cells towards the midline, rather than of axial/notochordal cells.

Whether Xmc loss-of-function could also inhibit other gastrulation dynamics, like the migration of deep zone cells on the blastocoel or formation of bottle cells needs further investigation. These failures could be observed by scanning electron microscopy of morpholino-injected embryos. Microscopic studies could also be useful to test whether Xmc-morpholino-injected cells lose the ability to form protrusions like lamellipodia or filopodia.

**Xmc regulating cell-adhesion?**

It is possible that Xmc regulates the adhesiveness of mesodermal cells. The modulation of cell-cell adhesion is of paramount importance during cellular rearrangements. Cells continuously have to adjust their adhesive strength in order to be able to move with respect to their neighbours. An emblematic example of the importance of the modulation of cell-adhesion is the sorting of notochord and somitic cells during morphogenesis in *Xenopus* and zebrafish, accomplished by the co-ordinated function of *spadetail* (*spt*), *floating head* (*flh*), *axial* and *paraxial protocadherin* (*axpc* and *papc*; (Yamamoto et al., 1998; Kodjabachian et al., 1999; Kim et al., 1998). PAPC and Spt are expressed in somitic cells, but excluded from notochordal cells with PAPC controlling adhesion of somitic cells under positive regulation of Spt. Through the negative action of Flh, Spt and therefore PAPC are absent in notochordal cells, where adhesivity is differentially controlled by AXPC. Furthermore, in aggregation and disaggregation studies in *Xenopus*, AXPC and PAPC segregate from each other.
Xmc does not encode a cadherin molecule and its wild-type form protein is not uniformly spread throughout the membrane. However, the membrane recruitment of the N-terminal coiled coil domain of Xmc (Xmc.NH2) and the observed loss of cell adhesion in ectodermal cells in response to ectopic expression of this deletion construct (Fig. 4.5D-F) is compatible with a role in modulating cell-adhesion. In addition, its expression in somitic mesoderm cells and exclusion from notochordal/axial cells would support the hypothesis that Xmc is involved in the regulation of differential adhesive properties between these types of cells, similarly to spt, flh and papc. How molecularly (whether linked to microtubules as speculated above) Xmc might exert this role remains elusive.

Aggregation and disaggregation experiments done on animal caps or DMZs injected with Xmc-morpholino could confirm the requirement of Xmc in disruption of cell-adhesion. Changes in abundance of cadherins at the transcriptional level after Xmc loss-of-function could also be checked on this regard.

**In which molecular pathway is Xmc acting?**

A problem in the molecular understanding of morphogenetic movements has always been the inability to distinguish the execution of these movements from the concomitant mesodermal specification. A crucial point in the study of Xmc was the discovery that its inhibitory effects on convergent extension do not perturb the induction and maintenance of mesodermal fate (Fig.4.11 D and E). Amaya and co-workers have recently presented compelling evidence that a non-canonical Sprouty-sensitive FGF signaling pathway controls aspects of convergent extension behaviour (Nutt et al., 2001). Overexpression of Xsprouty2 results in embryos with gastrulation defects, but with normal mesoderm
specification and patterning, very reminiscent of the Xmc loss-of-function phenotype (Fig. 4.11 D and E and Fig. 4 and 5 in Nutt et al., 2001). Both genes require a functional FGF receptor mediated signalling for their expression during gastrulation (Fig. 4.4). Surprisingly, Xspry2 expression cannot be efficiently activated by an ectopic FGF source, in contrast to Xmc expression, which can be directly activated by XeFGF in animal caps. This suggests that additional factors or competence factors are required for efficient expression of Xspry2. Xmc expression is not co-localised with FGFs during later stages of embryonic development, indicating that Xmc may only be an FGF target gene during gastrulation.

Xsproutyl acts as an intracellular antagonist of FGF-dependent Ca^{2+} signalling. On the contrary, our loss-of-function studies suggest that Xmc has a more positive, agonist role in the regulation of gastrulation movements. However, Xmc alone is not sufficient to promote gastrulation movements in animal cap or ventral marginal zone explants (data not shown).

Further analysis is required to dissect the biochemical pathway followed by Xmc, whether the induction of this latter downstream of the FGF receptor is via the Ras/MAPK pathway or an alternative pathway. A Ras/MAPK dependence could be tested by over-expressing a dominant negative form of Ras protein and observing the effects on Xmc induction. Ras/MAPK signals are essential for the establishment of mesodermal tissue. Xmc interferes with gastrulation movements without affecting mesodermal patterning (Fig. 4.11). As a consequence, Xmc loss-of-function should leave activation of MAPK unaffected. This would be readily testable by monitoring activation of MAPK in animal caps treated with a source of FGF and Xmc-morpholino by immunoblotting with antibody recognising the double-phosphorylated form of MAPK (dp-erk).

It is not excluded that Xmc might be involved in a calcium sensitive pathway. This could be tested through studies on calcium efflux, monitoring the effects of
Xmc over-expression or loss-of-function on calcium store mobilisation after addition of an FGF source (in a similar way to the studies performed for the analysis of Xspry2, Nutt et al., 2001). The possibility of Xmc being involved in calcium signalling is also not to rule out in view of its small PIPLC X domain at its carboxy-terminal region. Such domain is normally involved in the hydrolysis of phosphatidyl-inositol, resulting in the activation of PKC and subsequently in mobilisation of intracellular calcium stores as for the adaptor molecule PLC-γ (Ryan and Gillespie, 1994).

The non-canonical Wnt/planar polarity pathway has an instructive role in the regulation of convergent extension behaviour in *Xenopus* and zebrafish (Tada and Smith, 2000, Wallingford et al., 2000, Heisenberg et al., 2000). Wnt signalling can trigger a transient increase of intracellular Ca\(^{2+}\) by stimulating the phosphatidylinositol-signalling pathway presumably mediated by heterotrimeric G-proteins (Slusarski et al., 1997a and b). These observations suggest that intracellular Ca\(^{2+}\) levels, modulated by both FGF signalling and non-canonical Wnt signalling are important for the regulation of convergent extension movements.

Of great interest to unveil the co-ordination of convergent extension during gastrulation in *Xenopus*, it will be to understand a possible interplay between components of the FGF and Wnt signal transduction pathways. Epistatic experiments can be envisaged to understand the agonistic or antagonistic relationships between Xmc, Xspry2 and Xwnt11, for example examining whether Xmc can rescue the inhibitory effect of Xspry2, dnwnt11, and of dominant negative forms of dishevelled. Whether Xmc and Xspry2 are physically interacting within the FGF signalling pathway could also be investigated.

A prevailing theme from the molecular studies of convergent extension is that there is a redundancy and a degree of promiscuity in the usage of signalling molecules for the accomplishment of the different developmental functions.
Different signals involved in certain aspects of cell fate specification and patterning, such as ventro-lateral specification (Wnts) or anterior-posterior patterning (FGFs), show an incredible versatility in co-ordinating also cell biological aspects of morphogenetic movements, such as cell-polarity and cell-adhesion, down alternative branches of their pathway. It would not be very surprising to discover a synergism of molecules usage between the different pathways, to ensure the accomplishment of several functions during gastrulation.

**Final remarks- Organiser, gastrulation and organicism**

'\textit{The first principle required for the notion of an object conceived as a natural purpose is that the parts, with respect to both form and being are only possible through their relationship to the whole....Secondly, it is required that the parts bind themselves mutually into the unity of a whole in such a way that they are mutually cause and effect of one another}\\
\textit{I.Kant, 'Critique of Judgement}"

The relatively simple transplantation experiment performed 75 years ago by Hilde Mangold was a culminating point in classical embryology and paved the way for the discovery of the enormous importance of the organiser, its extraordinary properties and its complexity. At that time, organicism, explicitly recognised not only by Spemann, but also by figures such as Harrison and Waddington, was the absolute philosophical basis of embryology. Organicism is an organismic, holistic view of embryos and their development. According to this view, properties of the whole and its complexity cannot be simply ascribed directly to their constituent parts, but they emerge because of the interactions among the parts. The whole is greater than the sum of its parts. Such properties that are not those of any part, but that arise through the interactions of parts are called emergent properties. The radical shift of emphasis to the cellular and subcellular levels and, from the 1950s on, to the molecular level, shook the foundations of organicism and its axiomatic beliefs. The introduction of more
reductionist trends in biology (i.e. the essential notion of gene and more radically the advent of molecular biology) resulted in a transition from mere descriptive embryology to developmental mechanics. The wholeness originates from regulation of a dynamic, changing assemblage of interacting parts and there are specific laws for each level of organisation of an embryo.

The biology of the organiser and gastrulation stands out as an emblematic example for the case of organicism (and emergent properties) and its partial redefinition and evolution in light of molecular biology.

The organiser presents itself as a multivalent and rich 'context', which can exert its functions based on the properties and dynamics of its tissues, their specification and their inductive properties, which in turn rely on the extraordinary quantity and variety of molecules in tight co-ordination with one another.

Gastrulation is based on mass movements of entire tissues, which integrate individual cell motility and shape. Now we are in the position to try and dissect the genes and molecules underlying the cellular behaviours.

Tissues, cells, cell components, molecules and genes within specific frames of time and space are wholes on their own and are components of larger wholes. Each of these levels is grounded on its regularities, principles and laws, but laws of one level are deterministically integrated with or dependent on laws of lower and higher levels.

Developmental biology is all of this and only a polyhedral (holistic and reductionist) approach can grant access to the understanding of its large and fascinating complexity.
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