Functional characterisation of Mig30 and IGFBP-rP10 in early *Xenopus laevis* development

Dissertation

Submitted to the

Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Dipl.-Biol. Cornelia Magin

born in Ludwigshafen am Rhein

Dissertation

Submitted to the

Combined Faculties for the Natural Sciences and for Mathematics
of the Ruperto-Carola University of Heidelberg, Germany

for the degree of

Doctor of Natural Sciences

presented by

Dipl.-Biol. Cornelia Magin

born in Ludwigshafen am Rhein

Date of oral examination:……

Functional characterisation of Mig30 and IGFBP-rP10 in early *Xenopus laevis* development

Referees:

Prof. Dr. Herbert Steinbeißer

Prof. Dr. Thomas Holstein

Table of contents

Table of contents 4
1 Summary 7
Zusammenfassung 8
2 Introduction 9
2.1 Short overview of the mammalian IGF system 10
2.1.1 IGFs and their receptors 10
2.1.2 IGFBPs 12
2.1.3 IGFBP-rPs 13
2.2 The IGF system in development and growth 15
2.3 The IGF system in *Xenopus* 17
2.3.1 Mig30 20
2.3.2 IGFBP-rP10 21
2.4 Aim of this work 22
3 Results 23
Mig30 23
3.1 Mig30 gain of function inhibits canonical Wnt signalling 23
3.2 Mig30 gain of function inhibits BMP signalling 26
3.3 Mig30 mutant constructs inhibit Wnt and BMP signalling 27
3.4 Mig30 GOF is not able to induce neural marker expression 29
3.5 Mig30 affects IGFBP5 signalling 30
3.6 Mig30 blocks IGF induced differentiation in RCJ3.1C5.18 cells 34
3.7 Endogenous Mig30 promotes Wnt signalling in an IGF-dependent manner 35
3.8 Mig30 loss of function inhibits BMP-signalling 37
3.9 Loss of Mig30 enhances exogenous IGF signals 38
3.10 Mig30 plays a role in head and neural development 40
3.11 Mig30 plays a role in neural crest development 43
3.12 Mig30 has no effect on FGF signalling 45
3.13 Mig30 does not affect ADMP signalling 46

IGFBP-rP10 48
3.14 Transcriptional regulation of IGFBP-rP10 48
3.15 Characterisation of IGFBP-rP10 gain function 50
3.16 Characterisation of IGFBP-rP10 loss of function 52
3.17 Functional analysis of IGFBP-rP10 55
3.18 IGFBP-rP10 is involved in head development 60
3.19 IGFBP-rP10 is required for early neural development 62
3.20 IGFBP-rP10 has no effect on IGF induced differentiation in RCJ3.1C5.18 cells 65
3.21 IGFBP-rP10 has no effect on ADMP signalling 66
3.22 Expression analysis of Bono1 in early mouse development 68
4 Discussion 72
4.1 Mig30 and IGFBP-rP10 function are connected to the IGF system 72
4.1.1 Gain of Mig30 and IGFBP-rP10 function 72
4.1.2 Loss of Mig30 and IGFBP-rP10 function 76
4.2 Mig30 and IGFBP-rP10 are required for head and neural development 77
4.3 Bono1 is expressed in the decidua 79
5 Materials and Methods 81
5.1 Materials 81
5.1.1 Chemicals 81
5.1.2 Proteins, enzymes, inhibitors, and markers 81
5.1.3 Buffers and Solutions 82
5.1.4 Oligonucleotides 83
5.1.5 Plasmids 84
5.1.6 Antibodies 85
5.1.7 Bacteria and cells 85
5.1.8 Kits 86
5.1.9 Equipment and other materials 86
5.1.10 Computer programs 86
5.2 Molecular Biology 87
5.2.1 Isolation of nucleic acids 87

5.2.2 Restriction of DNA ... 87
5.2.3 cDNA synthesis ... 88
5.2.4 Polymerase chain reaction (PCR) ... 88
5.2.5 *in vitro* transcription of RNA ... 90
5.2.6 Transformation of competent bacteria ... 90
5.2.7 Agarose gel electrophoresis ... 90
5.3 Embryological methods ... 91
5.3.1 *Xenopus laevis* embryo culture and manipulations ... 91
5.3.2 Animal cap assay ... 91
5.3.3 Whole mount *in-situ* hybridisation ... 91
5.3.4 Fixation of mouse embryos for cryosections ... 92
5.3.5 Cryosections ... 92
5.3.6 *In-situ* hybridisation on cryosections ... 92
5.4 Cell culture methods ... 93
5.4.1 Maintaining and differentiation of F9-cells ... 93
5.5 Biochemical and immunological methods ... 93
5.5.1 SDS-PAGE and Western blot ... 93
5.6 Luciferase reporter assay ... 94
6 References ... 95
7 Table of figures ... 107
8 Abbreviations ... 109
Danksagung ... 111

1 Summary

The insulin-like growth factor (IGF) system was intensively studied over the past decades because it has an impact on numerous key aspects of life. In *Xenopus* the IGF pathway is required for head and neural development. Biological actions of IGFs are mediated through their receptors and IGF signals are modulated by insulin-like growth factor binding proteins (IGFBPs) in a complex manner that is poorly understood. Insulin-like growth factor binding protein-related proteins (IGFBP-rPs) are a heterogeneous class of proteins that share a conserved N-terminal insulin-like growth factor binding (IB) domain with conventional IGFBPs. Although it was shown that some IGFBP-rPs can bind IGFs *in vitro*, it is not known if their IGF interaction is physiological relevant.

This work demonstrated for the first time that the *Xenopus* IGFBP-rPs mixer inducible gene30 (Mig30) and insulin-like growth factor binding protein-related protein10 (IGFBP-rP10) are components of the IGF system and that they act as IGF signalling-dependent modulators of the Wnt and BMP signalling pathways. Gain of function experiments in *Xenopus* embryos and rat chondrocytes showed that Mig30 can act as an activator or inhibitor of IGF signalling. For the inhibition of IGF1 the N-terminal IB domain of Mig30 is essential whereas for antagonising IGFBP5 function the C-terminal IgC2 domain is required. Under experimental conditions in which Mig30 and IGFBP-rP10 augment IGF signalling the BMP pathway is inhibited. Only Mig30 however was able to inhibit the Wnt/β-catenin pathway in an IGF-dependent manner.

The endogenous functions of Mig30 and IGFBP-rP10 in *Xenopus* embryos were determined by an antisense morpholino oligonucleotide mediated knockdown strategy. These experiments revealed that both proteins act as inhibitors of IGF signalling. Inhibition of Mig30 and IGFBP-rP10 function promoted Wnt/β-catenin and BMP signalling. Cells in Mig30 and IGFBP-rP10 deficient *Xenopus* embryos failed to differentiate into epidermis and neural tissue and maintained a precursor state which is not yet committed to epidermal or neuronal lineage. The function of Mig30 and IGFBP-rP10 therefore is to regulate in an IGF-dependent manner the spatial and temporal activity of Wnt and BMP signalling. These pathways are essential for the specification of neural plate tissue and neural crest cells as well, as for neuronal differentiation.

Zusammenfassung

Das System der insulinähnlichen Wachstumsfaktoren (IGFs) wurde über die letzten Jahre intensiv erforscht, weil es viele Schlüsselaspekte des Lebens beeinflusst. Die biologischen Funktionen der IGFs werden über ihre Rezeptoren vermittelt und IGF Signale werden von insulinähnlichen Wachstumsfaktorbindeproteinen (IGFBPs) in komplexer Weise moduliert, die noch wenig verstanden ist. Insulinähnliche Wachstumsfaktorbindeprotein-verwandte Proteine (IGFBP-rPs) sind eine heterogene Proteinklasse, die mit den IGFBPs eine konservierte N-terminale Wachstumsfaktor-Bindedomäne (IB) gemeinsam haben. Obwohl es bekannt ist, dass einige IGFBP-rPs IGFs *in vivo* binden können, ist es nicht bekannt ob diese Interaktionen physiologisch relevant sind.

Diese Arbeit demonstriert zum ersten Mal, dass die *Xenopus* IGFBP-rPs, Mig30 und IGFBP-rP10, Komponenten des IGF Systems sind und dass sie als IGF Signal-abhängige Modulatoren der Wnt und BMP Signalwege agieren. Überexpressions Experimente in *Xenopus* Embryonen und Ratten Chondrocyten zeigen, das Mig30 als Aktivator und Inhibitor des IGF Signalweges fungieren kann. Für die Inhibition von IGF1 ist die N-terminale IB Domäne essentiell. Die Funktion von IGFBP5 wird dagegen durch die C-terminale IgC2-Domäne von Mig30 inhibiert. Unter experimentellen Bedingungen in welchen Mig30 und IGFBP-rP10 IGF Signale unterstützen ist der BMP Signalweg blockiert. Der Wnt/ß-catenin Signalweg kann in IGF-abhängiger Weise nur von Mig30 blockiert werden.

Die endogenen Funktionen von Mig30 und IGFBP-rP10 wurden in *Xenopus* Embryonen mittels einer Gen-Knockdown Strategie analysiert. Diese Experimente zeigen, dass beide Proteine als Inhibitoren des IGF Signalweges fungieren. Inhibition von Mig30 und IGFBP-rP10 Funktion begünstigt Wnt/ß-catenin und BMP Signale. Zellen mit reduzierter Mig30 oder IGFBP-rP10 Funktion in *Xenopus* Embryonen können nicht in Epidermis und Neuralgewebe differenzieren und verharren in einem Vorläufer Stadium in dem epidermal und neuronal noch nicht spezifiziert sind. Die Funktion von Mig30 und IGFBP-rP10 besteht darin die räumliche und zeitliche Aktivität von Wnt und BMP Signalen in einer IGF-abhängigen Weise zu kontrollieren. Diese Signalwege sind essentiell für die Spezifikation von Neuralplatte, Neuralleiste und auch für die neuronale Differenzierung.

2 Introduction

The insulin-like growth factor (IGF) system is ubiquitous and plays an important role in normal physiology. Numerous diseases, like cancer, diabetes and malnutrition are linked to abberations in the IGF system. Extensive research over the last decades has increased the knowledge of the diverse biological actions of the insulin-like growth factors (Pollak et al., 2004; Monzavi and Cohen, 2002; Jones and Clemmons, 1995). Biological actions of IGFs are mediated through their receptors and modulated by the members of the insulin-like growth factor binding protein (IGFBP) family (rev in Jones und Clemmons, Firth und Baxter). Insulin-like growth factor binding protein-related proteins (IGFBP-rPs), like IGFBPs contain an insulin-like growth factor binding (IB) domain and can bind IGFs *in vitro* although their physiological relevance in IGF signalling is not known (Kim et al., 1997; Baxter, 1994).

Most work on the IGF system was done in mouse and cell culture models and provided an important insight into the mechanisms of IGF signalling. However the mammalian system is also limited due to functional redundancy (Murphy, 1998). Research on the IGF system in *Xenopus laevis* gave evidence that IGF signalling is required for head and neural induction and plays a role in morphogenetic movements. Mechanistically IGF performs these functions by interaction with Wnt and BMP signalling pathways, which are important in early patterning of the *Xenopus* embryo. An additional advantage of *Xenopus* is that the IGF system is much smaller than the mammalian IGF system, reducing the possibility of redundancy (Pera et al., 2001; Richard-Parpaillon et al., 2002; Pera et al., 2003).

Functional studies of IGFBP-rPs in *Xenopus* showed that IGFBP-rPs also interact with Wnt and BMP signalling (Abreu et al., 2002; Mercurio et al., 2004; Latinkic et al., 2003; Hayata et al., 2002). Recent data indicate IGF-dependent and -independent actions of the IGFBP-rP, mixer inducible gene30 (Mig30; Kuerner, 2008). Like IGFs, Mig30 plays a role in morphogentic movements, neural development and can inhibit Wnt signalling.

This work therefore exploits the advantages of the model system *Xenopus laevis*, to functionally analyse the two IGFBP-rPs Mig30 and IGFBP-rP10. Understanding the signalling mechanism in *Xenopus*, should then help to analyse the biological actions in the more complex mammalian system.

2.1 Short overview of the mammalian IGF system

The mammalian insulin-like growth factor (IGF) system is one of the most studied growth factor and cytokine systems known, because the system is involved in growth, development, cell differentiation, survival and metabolism and found in most, if not all tissues (LeRoith and Yakar, 2007). The system is complex and comprises the two ligands insulin-like growth factor1 (IGF1) and insulin-like growth factor2 (IGF2), three receptors namely IGF1R, IGF2R and InR, six IGFBPs, namely IGFBP1-6 and more than ten IGFBP-rPs. The physiological significance of IGFBP-rPs to the IGF system is controversial. It has been shown that some IGFBP-rPs actually can bind IGFs but for most IGFBP-rPs only IGF-independent actions are described.

2.1.1 IGFs and their receptors

The IGFs, IGF1 and IGF2 are small hormone peptides of approximately 7 kDa, which are closely related to insulin (Claeys et al., 2002). They were first found 1957 in serum, as mediators of growth-stimulating activity of growth hormone (GH; Daughaday et al., 1972). IGFs are widely expressed and found in relatively high doses in circulation, as well as in extravascular fluids. IGFs have characteristics of both circulating hormone and tissue growth factor. Circulating IGFs are mainly produced by the hepatic system (Jones and Clemmons, 1995; Firth and Baxter, 2002). IGFs produced in the periphery only have minor effects on the levels of circulating IGFs, performing their functions by autocrine and/or paracrine mechanisms (Yakar et al., 1999; LeRoith et al., 2001). Regulation of IGFs is complex. Hepatic IGF1 is mainly regulated by growth hormone, insulin and nutritional status. Several factors in addition to growth hormone regulate extrahepatic IGF1 expression. IGF2 is not as tightly regulated by growth hormone, although it is expressed in the liver and extrahepatic tissues. In the regulation of IGF2 expression imprinting plays an important role (LeRoith and Roberts, 2003; Pollak et al., 2004).

IGF signalling is transduced into the cells by binding of IGFs to their specific receptors. In mammals there are three receptors known to interact with IGFs, namely the Insulin-like growth factor 1 receptor (IGF1R), the Insulin receptor (InR) and the Insulin-like growth factor2 receptor (IGF2R).

IGF2R is an approximately 600 kDa, monomeric transmembrane receptor with a large extracellular, but no signalling domain. It specifically binds IGF2 and internalises the growth factor for degradation in the lysosomes, thereby controlling the level of circulating IGF2 (Morgan et al., 1987; Nakae et al., 2001; Ghosh et al., 2003).

IGF1R and InR belong to the family of receptor tyrosine kinases and are recognized as the mediators of IGF signalling. IGF1R and InR are composed of a $\alpha_2\beta_2$ heterotetrameric structure. An αβ-heterodimer is formed by proteolysis of a precursor peptide and subsequent linkage by disulfide bonds. The mature receptor results after linkage of two αβ-heterodimers by secondary disulfide bonds. The α subunits are entirely found extracellular and form the ligand binding domain, while the β-subunits are largely intracellular, containing a transmembrane domain and the tyrosine kinase domains and associated motifs required for signal transduction (Adams et al., 2000; Siddle et al., 2001; Butler et al., 1998).

Ligand binding to the α-subunits of the receptor induces a conformational change that allows ATP binding and leads to autophosphorylation, which is followed by phosphorylation of additional tyrosine residues in the β-subunits, further increasing the kinase activity of the receptor. The phosphorylation of residues in the β-subunits creates high affinity binding sites for signalling/docking proteins. Recruitment of insulin receptor substrates (IRS) and other proteins leads to the activation of the phosphoinositol 3-kinase (PI3K) and/or the mitogen activated protein kinase (MAPK) signalling pathways. The ability of the receptors to recruit and activate a number of different docking proteins is the reason why IGF signalling can regulate so many different processes in diverse tissues. The expression of these docking proteins differs between tissues and throughout development. Additionally these docking proteins are shared, not only by IGF1R and IR receptor but also by other RTKs and members of the cytokine receptor family, adding an additional mode of regulation (Butler et al., 1998; Adams et al., 2000).

IGF1R is ubiquitously expressed, binds IGF1 with high affinity, IGF2 with medium affinity and insulin with low affinity and is therefore the main mediator of IGF signalling (Frasca et al., 1999; Baker et al., 1993; Liu et al., 1993). An alternately spliced form of the IR (IR-A lacking exon 11) can bind IGF2 with high affinity and plays an important role in embryonic development (Frasca et al., 1999;

Pandini et al., 2005). Additionally, hybrid receptors comprised of an insulin αβ hemireceptor and an IGF1 αβ hemireceptor add to the divergent actions of insulin and IGFs (Adams et al., 2000; Siddle et al., 2001)

2.1.2 IGFBPs

In biological fluids IGFs are normally bound to IGFBPs, because free IGFs have a very short half-life of approximately 10 minutes. To date there are six mammalian IGFBPs known, which are important modulators of IGF activity and availability. IGFBPs are small secreted proteins that share a highly conserved structure, with three domains of approximately equal size. The cysteine rich, amino-terminal (N-terminal) domain contains the consensus sequence which is important for IGF binding and is highly conserved. The carboxy-terminal (C-terminal) thyroglobulin type 1(TY) domain is also cysteine rich and takes part in IGF binding. Other functionally important subdomains are located in the C-terminal domain of various IGFBPs. For example an RGD- integrin binding motif, basic motifs with heparin binding activity and a nuclear localization sequence. These motifs are involved in cell and matrix binding, interaction with the serum protein ALS (acid labled sub-unit) and nuclear transport. The central domain is the least conserved domain of IGFBPs, it contains sites for posttranslational modifications and proteolytic cleavage sites and is thought to act as structural hinge between the C- and N-terminal domains (Firth and Baxter, 2002; Hwa et al., 1999).

The most abundant IGFBP in circulation is IGFBP3. IGFBP3 forms a ternary complex with IGFs and the glycoprotein ALS (Acid labile sub-unit) and so transports about 75% of the serum IGFs and prolongs the half-life of IGFs up to 12 hours. About 10% of the serum IGFs are transported in a ternary complex with IGFBP5 (Monzavi and Cohen, 2002; Clemmons, 2007). All six IGFBPs are found in circulation. Free IGFBPs and IGFBPs complexed with IGFs are believed to exit the circulation rapidly, while ternary complexes are thought to be confined to the vascular compartment. So in circulation IGFBPs act as transport proteins and control efflux of IGFs from the vascular space. IGFBPs have comparable affinities for IGFs like the IGF1R, consequently they compete for IGFs with the receptor and thereby inhibit IGF signalling. In some cases IGFBPs can potentiate IGF signalling in a context dependent manner. It is thought that IGFBs increase the local concentration of IGFs in the vicinity of the receptor by association with the cell

surface or extracellular matrix (ECM). The affinities of IGFBPs for IGFs are then lowered by for example proteolytic cleavage or posttranslational modifications. Additionally to their IGF dependent functions IGFBPs exerts functions independently of IGFs and IGF1R (Jones and Clemmons, 1995; Hwa et al., 1999; Firth and Baxter, 2002).

2.1.3 IGFBP-rPs

The mammalian IGFBP-rPs are a group of cysteine-rich proteins, that are primarily characterized by the presence of an N-terminal IB domain. They were grouped together with the six IGFBPs and an IGFBP-superfamily was proposed (Kim et al., 1997; Baxter et al., 1998; Hwa et al., 1999; Rosenfeld et al., 1999). *In vitro* binding studies show that some of these IGFBP-rPs, can indeed bind to IGFs, albeit and unlike IGFBPs, with low affinity (Kim et al., 1997; Oh et al., 1996; Burren et al., 1999). IGFBP-rP1 is the founding member of the IGFBP-rP family, it is also known as Mac25, IGFBP7, TAF (tumour derived adhesion factor), PSF (prostacyclin-stimulating factor) and AGM (angiomodulin; Akaogi et al., 1994; Akaogi et al., 1996; Yamauchi et al., 1994). Like IGFBPs it contains an amino-terminal IB domain, but lacks the C-terminal TY- domain. Its IB domain is followed by a kazal type serine protease inhibitor (KAZAL) domain and at the C-terminus an IgC2-domain is located (Hwa et al., 1999). IGFBP-rP1 was found to be expressed in various tissues and cell types. Depending on the cellular context IGFBP-rP1 can enhance IGF- or insulin-mediated cell growth as well as inhibit insulin signals, because IGFBP-rP1 is able to bind insulin with high and IGFs with low affinity (Akaogi et al., 1996; Oh et al., 1996; Yamanaka et al., 1997). In another cell system, IGFBP-rP1 can selectively reduce differentiation responses to IGFs and insulin without affecting cell proliferation (Haugk et al., 2000). By binding to the heparin sulfate proteoglycan (HSPG) Syndecan-1, IGFBP-rP1 promotes attachment and spreading of rat liver cells (Ahmed et al., 2003; Sato et al., 1999). Additionally IGFBP-rP1 is able to bind to other ECM components, growth factors and to a range of chemokines (Akaogi et al., 1996; Kato et al., 1996; Sato et al., 1999; Usui et al., 2002). The biological role of IGFBP-rP1 remains largely unknown, despite these diverse functional studies.

Two further mammalian proteins share the domain architecture of IGFBP-rP1, IGFBP-rP4 (IGFBP-like) and IGFBP-rP10 (KAZALD1, Bono1). The structural

similarity of these proteins, led to the suggestion of an IGFBP-like subgroup, within the IGFBP-rPs. Mouse studies suggest that IGFBP-rP10 has a function in bone development and bone regeneration (James et al., 2004). IGFBP-rP4 is supposed to be a tumor suppressor (Cai et al., 2005; Smith et al., 2007).

The serine protease HTRA1, like IGFBP-rP1, shows an N-terminal IB and KAZAL domain. It was demonstrated that HTRA1 specifically cleaves IGFBP5 (Hou et al., 2005). Additionally, HTRA1 cleaves the proteoglycans Biglycan, Syndecan-4 and Glypican-4. By cleavage of these proteoglycans, HTRA1 releases cell surface bound fibroblast Growth factors (FGFs) and so stimulates FGF-dependent long-range signalling (Hou et al., 2007).

The members of the CCN family are another group of IGFBP-rPs. The six family members are Cyr61, (Cysteine-rich61, CCN1), CTGF (connective tissue growth factor, CCN2), NOV (nephroblastoma-overexpressed, CCN3), the namesakes of the family and WISP1-3 (Wnt-induced secreted proteins, CCN4-6). With the exception of WISP-2, the CCN family members contain three functional domains, in addition to the IB domain. The IB domain is followed by a von Willebrand factor type C (VWC) domain, a thrombospondin-homology domain and at the C-terminus a cysteine knot (CT) heparin binding domain (Leask and Abraham, 2006; Brigstock, 2003). CCN proteins are matricellular proteins that connect the cell surface and the ECM. Binding to cell surface components, like integrins and proteoglycans, modifies intracellular signalling events. In addition to their adhesive abilities CCN proteins can modulate the activity of a variety of growth factors. So CCN proteins are able to stimulate mitosis, adhesion, apoptosis, ECM production, growth arrest and migration. As a result they play important roles in development, wound healing and disease (Perbal, 2001; Leask and Abraham, 2006).

Another protein that belongs to the IGFBP-rPs, is ESM-1 (endothelial specific molecule-1, IGFBP-rP6). The 20 kDa ESM1 protein requires an attached dermatan sulfate proteoglycan to be functional. ESM1 is involved in the regulation of cell adhesion, in inflammatory disorders and in tumour progression (Lassalle et al., 1996; Sarrazin et al., 2006).

2.2 The IGF system in development and growth

The components of the IGF system are expressed very early in mouse embryogenesis and are already found in blastocysts and early in embryonic as well as in extraembryonic lineages (Morali et al., 2000; Puscheck et al., 1998). IGF2, IGF1R and decidua-derived IGFBP1 are involved in trophoblast invasion during implantation. Placental trophoblast invasiveness is inhibited by decidua-derived IGFBP1. This inhibitory effect is overcome by inhibition of IGFBP1 production by trophoblast-derived IGF2, demonstrating that members of the IGF system play a role very early in development (Firth and Baxter, 2002; Bowman et al.).

Growth, considered as an increase in size, is one important aspect of development. The requirement of the components of the IGF system in embryonic and postnatal growth was first demonstrated by knockout experiments (Baker et al., 1993; Liu et al., 1993). Mice with a deleted IGF1 gene are very small at birth (60% of the size of their wt littermates) and most die after birth. Those that survive show developmental defects in brain, muscle, bone and lung and are infertile (Yakar et al., 1999). The peripubertal growth spurt is lacking in the surviving mice. This shows the role of IGF1 in embryonic and postnatal growth. Knockout of IGF2 also leads to strong growth retardation, but the survivors grow normally after birth indicating that IGF2 has no effect on postnatal growth in mice (Yakar et al., 1999; Monzavi and Cohen, 2002; Maki, 2010). IGF1R is the main mediator of IGF signalling in the embryo, because deletion of the IGF1R gene is lethal, mice die at birth and are 55% smaller than their littermates (Baker et al., 1993; Liu et al., 1993; Maki, 2010). The actions of IGF1 are primarily mediated by the IGF1R, as IGF1R and IGF1 double knock-out mice show the same phenotype as IGF1R deletion alone. Some effects of IGF2 are mediated through IR, as simultaneous deletion of IGF1R and IGF2 genes leads to a more severe phenotype (Yakar et al., 2002). Tissue-specific gene deletion of IGF1 in the liver, resulted in apparently normal mice, with about 80% reduced levels of circulating IGFs (Yakar et al., 1999). Detailed analysis of these mice showed that locally produced IGF1 is the main contributor of somatic growth. (LeRoith and Roberts, 2003; Yakar et al., 2002; Clemmons, 2007).

Analysis of IGF knockout animals showed that besides their effects on embryonic growth, IGFs are involved in developmental bone and brain formation. IGF signalling via the IGF1R is required for normal brain development. IGF1 and IGF1R null mutant mice exhibit brain growth retardation and overexpression of IGF1 in the brain leads to brain overgrowth. Humans with IGF1 deficiencies are microcephalic and mentally retarded. In mice, the IGF1R is ubiquitously expressed in all neural cells, IGF1 is found in the central nervous system and IGF2 is found in the brain and surrounding structures. Signalling via the IGF1R has pleiotropic effects in brain development including proliferation of neural progenitors and pluripotent neural stem cells and survival and differentiation of neurons and oligodendrocytes (D'Ercole, 2008).

Knockout of components of the IGF signalling pathway, strongly affects bone density and bone growth. Bone lengthening is primarily driven by production of hypertrophic chondrocytes, which secrete characteristic matrix, from proliferating chondrocytes. When IGF signalling is blocked, chondrocyte proliferation, maturation and inhibition of apoptosis are inhibited and bone growth is decreased (Canalis, 2009; Wang et al., 2006; Yakar et al., 2002). Bone density is maintained by the matrix secretion of mature osteoblasts. In IGF1R knockout mice the number of osteoblasts is decreased and hence the matrix production required for bone density (Zhang et al., 2002; Zhao et al., 2000).

Surprisingly no major differentiation defects have been detected in mice null for different components of the IGF system. Although it has been shown that IGFs are able to promote differentiation of various cell lines *in vitro*. It was speculated that the mammalian IGF signalling system contains considerable functional redundancy (Murphy, 1998). So null mice for IGFBP3, IGFBP4 or IGFBP5 show only modest growth impairment. Overlapping functions of the proteins are only revealed by triple knockouts (Ning et al., 2006). Analysis of IGFBP2 null mice indicates that the deletion of one IGFBP could cause compensatory changes in others (Wood et al., 2000). This limits the efficiency of loss of function experiments considerably and makes it difficult to assess the physiological role of IGFBPs *in vivo*. If IGFBP-rPs are also affected by redundancy remains to be analysed.

2.3 The IGF system in *Xenopus*

The *Xenopus* IGF system is much smaller compared to the mammalian IGF system. Although there are three IGFs (IGF1, IGF2 and IGF3) found in *Xenopus*, there are only 2 IGFBPs and 6 IGFBP-rPs. (Kuerner and Steinbeisser, 2006). So the possibility of functional redundancy is much smaller in the *Xenopus* system.

All three IGFs and the IGF1-R are expressed during early embryogenesis. Expression is very prominent along the dorsal midline during neurulation and at tailbud stage they are detectable in different anterior head structures (Groigno et al., 1999; Pera et al., 2001; Richard-Parpaillon et al., 2002; Zhu et al., 1998). Overexpression of IGFs gives rise to strongly enlarged cement glands and increasing IGF signals in future neural tissue gives rise to ectopic eye structures, cement glands and expands mid- and hindbrain structures (Richard-Parpaillon et al., 2002; Pera et al., 2001). Injection of IGFs into the ventral mesoderm even causes the formation of ectopic heads including eye structures and cement glands (Pera et al., 2001). Disruption of the IGF pathway (by either IGFR morpholino or a dnIGFR) has the opposite effect and leads to a loss of anterior structures and eyes. This demonstrates that IGF signalling is required for head formation (Pera et al., 2001; Richard-Parpaillon et al., 2002). IGF signalling is also required for neural induction. IGFs induce expression of anterior neural markers in ectodermal explants and embryos, this ability is lost when the pathway is blocked on the level of IGFR (Richard-Parpaillon et al., 2002; Pera et al., 2001).

IGF signalling was found to be sufficient and required for head and neural induction in *Xenopus* (Pera et al., 2001; Richard-Parpaillon et al., 2002). Head development is triggered by inhibition of growth factor signals required for trunk development, namely BMP, Wnt and Nodal (Glinka et al., 1997; Glinka et al., 1998; Piccolo et al., 1999). Inhibition of only one pathway is not sufficient to induce secondary heads, only Cerberus, a triple inhibitor of Wnt, BMP and Nodal signalling, is able to induce secondary head structures (Bouwmeester et al., 1996). A popular mechanism to explain neural induction was the so-called “default model”, which proposes that ectodermal cells acquire neural fate when they don’t receive BMP signals (Harland, 2000; Stern, 2005). More recent intensive research on neural induction showed that Wnt inhibition (Heeg-Truesdell and LaBonne, 2006; Glinka et al., 1997) and functional FGF signalling (Launay et al., 1996; Sasai et al., 1996; Xu et al., 1997) are also required for neural induction. IGF signalling is

now another pathway involved in head and neural induction, and it remains to be elucidated how the integration of the different neural inducing signals may occur (Kuroda et al., 2004).

Two molecular mechanisms were demonstrated that can explain the head and neural inductive activities of IGF signalling. On the one hand IGFs are potent inhibitors of Wnt signalling, acting intracellularly on the level of GSK3 or ß-catenin (Pera et al., 2001; Richard-Parpaillon et al., 2002). How this activity is mediated is not understood, possibly by phosphorylation changes induced on the ß-catenin degradation complex. On the other hand IGFs are inhibitors of BMP signalling. Here again acting intracellularly, on the level of the BMP signal transducer Smad1 (Pera et al., 2003). Normally Smad1 is phosphorylated at its C-Terminus, in a BMP-dependent manner, builds a transcription factor complex with Smad4 that enters the nucleus, and turns on the expression of BMP target genes (Kretzschmar et al., 1997b; von Bubnoff and Cho, 2001). But Smad linker phosphorylation by MAPK or GSK3 blocks nuclear accumulation of Smads and so inhibits BMP signalling (Kretzschmar et al., 1997a; Aubin et al., 2004; Fuentealba et al., 2007; Sapkota et al., 2007). Therefore IGFs block Wnt und BMP signalling not by physical interaction with the paracrine factors but by preventing signals to reach the nucleus.

Additionally the IGF pathway has been recently shown to be involved in convergent extension (CE) movements (Richard-Parpaillon et al., 2002; Carron et al., 2005). CE movements, controlled by the non-canonical Wnt pathway, are essential for trunk and posterior development and for elongation of the anteroposterior axis (Keller et al., 2000; Keller et al., 1985). The head region does not undergo CE movements, there the non-canonical Wnt signalling pathway is repressed by the expression of Otx2 (*Xenopus* orthodenticle homeobox 2). Otx2 is a transcription factor, expressed in anterior head structures, that is required for anterior brain and cement gland formation. Additionally it represses the transcription, of Xbra (*Xenopus* brachyury), a transcription factor required for the expression of the non-canonical Wnt-ligand, Wnt11 and so blocks CE in head regions (Andreazzoli et al., 1997; Pannese et al., 1995; Latinkic et al., 1997; Tada and Smith, 2000). IGF1 blocks CE movements by specific induction of Otx2 expression at early gastrula stage in future anterior head region (Carron et al., 2005).

The only IGFBP known to be expressed early in *Xenopus* is IGFBP5. At four-cell stage transcripts are found at the animal pole, during gastrulation they are detectable on the dorsal side of the embryo and during neurulation it is confined to the floor plate, notochord, and dorsal endoderm. Overexpression of IGFBP5 led to embryos with enlarged head structures and cement glands. This anteriorising effects of IGFBP5 are blocked by inhibition of the IGF1R, indicating that IGFBP5 signalling occurs via the IGF1R (Pera et al., 2001).

IGFBP4 is found at later stages in *Xenopus* embryos in the anterior part of the liver adjacent to the heart and is required for heart development. The mechanism that underlies the function of IGFBP4 in heart development is IGF-independent. IGFBP4 directly interacts with LRP6 (low-density-lipoprotein receptor6) and Frz8 (Frizzled8), thereby inhibiting Wnt signalling (Zhu et al., 2008).

The functions of two CCN family members are described in *Xenopus*. Cyr 61 (CCN1) is involved in gastrulation movements. Some of the effects of Cyr61 on gastrulation movements derive from its ability to support assembly of a fibronectin-rich extracellular matrix and to regulate cell-cell and cell-matrix adhesion. Other effects may be caused by its ability to modulate Wnt signalling. It is suggested that Cyr 61 acts via its IB-domain to elevate low level Wnt signalling, while it inhibits high levels of Wnt signalling via its CT-domain (Latinkic et al., 2003). CTGF is also able to inhibit canonical Wnt signalling. This is probably due to binding to the Wnt co-receptor LRP6, via its CT domain (Mercurio et al., 2004). Additionally it was shown that CTGF binds BMP4 and TGF-ß1 through its VWC domain. Thereby it inhibits BMP4 but activates TGF-ß1 signalling and this function might be required for the deposition of ECM (extracellular matrix) components such as fibronectin and collagen (Abreu et al., 2002).

The serine protease HTRA1 is also found in *Xenopus*, where it stimulates FGF-dependent long-range signalling by cleavage of proteoglycans (Hou et al., 2007). If its ability to specifically cleave IGFBP5 has a physiological relevant function is still unknown (Hou et al., 2005) .

Three additional IGFBP-rPs have been reported in *Xenopus*: Mig30, IGFBP-rP10 and IGFBP-like. All three are structurally related to mammalian IGFBP-rP1 and belong to the IGFBP-like subgroup (Kuerner and Steinbeisser, 2006) .

2.3.1 Mig30

Mig30 belongs to the IGFBP-superfamily and was originally identified in *Xenopus laevis* as target gene of the homeobox gene Mixer, which is required for endoderm development (Hayata et al., 2002). It is a secreted, 36 kDa protein and there is no mammalian homologue. Its protein structure shows a signal peptide followed by the insulin-like growth factor binding (IB) domain, but so far no direct binding of Mig30 to IGFs was shown. The IB domain partially overlaps with a kazal type serine protease inhibitor (KAZAL) domain and at the C-terminus an immunoglobulin C-2 type (IgC2) domain is located (Fig.3). IgC2 type domains are known to be involved in variety of functions, including cell adhesion and recognition .

Expression analysis of Mig30 showed that it is expressed maternally, transcripts decline until zygotic expression starts with midblastula transition. Transcript levels are highest during gastrulation, but are still detectable during neurulation. At early stages Mig30 is found at dorsal animal cells, during gastrulation it is strongly expressed in the anterior mesendoderm and in the prospective prechordal plate, regions known to contribute to forebrain and midline structures at later stages (Smith et al., 1991; Sasai et al., 1994; Smith et al., 1995; Kuroda et al., 2004; Wessely et al., 2001). At neurula stages it is found at the edges of the neural folds and prospective lens and otic placodeal areas. At later stages Mig30 is detectable in the developing eye, otic vesicle, branchial tissues, neural tube, notochord and somites (Hayata et al., 2002; Kuerner and Steinbeisser, 2006).

The biological effects of Mig30 were first investigated by an overexpression approach. Hayata and colleagues showed that overexpressing Mig30 leads to impaired head development and suggested that it plays a role in morphogenetic movements during gastrulation. The involvement of Mig30 in morphogenetic cell behavior was confirmed recently. Additionally, it was shown that gain of Mig30 can stimulate IGF signalling and Mig30, like IGF1 is able to inhibit Wnt intracellularly at the level of ß-catenin. Additionally Mig30 inhibits Chordin and Cerberus activity. Analysis of Mig30 mutant constructs showed discrete activities of the IB and IgC2 domain. Overexpression of Mig30 mutant constructs comprising the signal peptide plus either the N-terminal or the C-terminal domains of Mig30, showed a more severe phenotypes then Mig30 alone. Mig30ΔC which contains the IB and KAZAL

domain led to embryos with enlarged cement glands and ectopic eye structures, reminiscent to activation of IGF pathway phenotypes. For Mig30ΔN the IB and Kazal domain were removed, so the IGc2-domain follows the signal peptide. Microinjection of MigΔN resulted in embryos that show stronger microcephaly than Mig30 injected embryos. Surprisingly both mutants are able to inhibit Wnt signalling and cooperate withIGF signals (Kuerner, 2008).

Knockdown of Mig30 by antisense morpholino oligonucleotides led to a delay in gastrulation and strong defects in anterior neural folding. At later stages blocking Mig30 resulted in microcephaly and shortend trunks and tails, similar to the gain of function phenotype, while cement gland formation is unaffected. Precise spatio-temporal expression is required for normal development, and Mig30 is required for neural plate and neural crest development (Kuerner, 2008).

It remains to be analysed which of these functions are connected to the IGF signalling pathway and which are IGF-independent.

2.3.2 IGFBP-rP10

IGFBP-rP10 is a 44,6 kDa protein, that like Mig30 belongs to the IGFBP-superfamily. IGFBP-rP10 was first identified in mouse and according to its discovery in bone and odentoblasts in teeth, it was originally named Bono1 (bone and odentoblasts1, James et al., 2004). In the mouse it is expressed in most ossification regions of the head including calvarial bones, skull and jaws from day 13 onward. IGFBP-rP10 is also found in osteoblastic cells, where it is known to play a role in proliferation and differentiation (Shibata et al., 2004). The protein structure of IGFBP-rP10 is similar to Mig30 and IGFBP-rP1, it shows a signal peptide followed by an N-terminal IB domain that partially overlaps with a KAZAL (kazal type serine protease inhibitor) domain and an IgC2 (immunoglobulin-like domain of the C2 type) domain at the C-terminus. However the IB domain differs slightly from the conserved core sequence GCGCCXXC of other IGFBPs in that the second glycine is replaced by an aspartate (Kuerner and Steinbeisser, 2006). Since the direct binding of IGFBP-rP10 to IGFs was not tested so far, it remains unclear if this amino acid exchange in the region thought critical for IGF binding has functional consequences.

First studies of IGFBP-rP10 in *Xenopus* show that IGFBP-rP10 is secreted and post-translationally modified by N-glycosylation. By expression analysis it was

found that transcripts are detectable throughout development. At four-cell stage transcripts are found at the animal pole, during gastrulation on the dorsal side of the embryo and later on it is expressed in the notochord, floor plate, somites and fin (Kuerner and Steinbeisser, 2006; de Beer, 2005).

2.4 Aim of this work

The functions of IGFBP-rPs in embryonic development are very poorly understood. Therefore the goal of this thesis work is the functional characterisation of the IGFBP-rPs Mig30 and IGFBP-rP10.

Using the *Xenopus* embryo as a model I will test whether Mig30 and IGFBP-rP10 are components of the IGF pathway and whether they have IGF-independent functions as well. In gain of function and knockdown experiments it will be analysed whether Mig30 and IGFBP-rP10 act as activators or repressors of IGF signalling. In *Xenopus* IGF signalling negatively interferes with the Wnt/β-catenin-and BMP4 signalling pathways which are essential for early embryonic patterning. In case Mig30 and IGFBPrP-10 would modulate the IGF pathway, the embryonic body plan should be perturbed due to interference with the BMP and Wnt pathways.

The comparative analysis of Mig30 and IGFBPrP-10 should reveal redundant and non-redundant function of these proteins.

3 Results

This chapter is divided thematically into two parts. In the first part the results of the analysis concerning Mig30 are described and in the second part the results concerning IGFBP-rP10.

Mig30

Mig30 was first described by Hayata et al. as a target gene of the homeobox gene Mixer. The authors showed that, overexpressing Mig30 leads to impaired head development and that it plays a role in morphogentic movements during gastrulation. Recent studies showed that Mig30 can stimulate IGF signalling and inhibit Wnt signalling. Mig30 also inhibits Chordin and Cerberus activity via unknown mechanism. Additionally it was shown by knockdown studies that Mig30 is necessary for neural plate and neural crest development. Analysis of Mig30 mutant constructs showed that the IB and IgC2 domain have discrete activities. It remains to be demonstrated which of these function are physiological relevant and are connected to the IGF signalling pathway or are IGF-independent (Kuerner, 2008).

3.1 Mig30 gain of function inhibits canonical Wnt signalling

In *Xenopus* the IGF signalling pathway is required for neural and head development. IGF exerts this function by antagonising the Wnt/ß-catenin as well as the BMP signalling pathway. Wnt/ß-catenin signalling is inhibited intracellularly on the level of GSK-3 or ß-catenin. And BMP signalling is antagonised by mediating linker phosphorylation of the signal transducer Smad1 (Pera et al., 2001; Richard-Parpaillon et al., 2002; Pera et al., 2003; Eivers et al., 2004).

Mig30, like IGF signalling affects head and neural development, therefore the effect of Mig30 on the canonical Wnt pathway was tested (Kuerner, 2008). An animal cap (AC) assay was used, because in animal caps no endogenous Wnt is detectable and the responsiveness to Wnt can be analysed. The activation of the Wnt primary response gene Xnr3 was examined in AC explants at early gastrula stage after stimulation with Wnt8 (Brannon et al., 1997; McKendry et al., 1997). Four-cell stage embryos were injected into the animal pole of each blastomere. Injection of 3 pg Wnt8 mRNA induced expression of the Wnt responsive gene

Xnr3, compared to uninjected controls. Co-injection of 500 pg Mig30 mRNA showed a weak effect on the expression of Xnr3. Only high doses of Mig30 mRNA (1 ng) were able to significantly reduce Xnr3 expression (Fig.1A).

To analyse on what level Mig30 inhibits Wnt signalling a luciferase reporter assay was performed. The Topflash reporter construct contains multimeric TCF binding sites, so the transcriptional activity of the ß-catenin/TCF complex can be measured (Korinek et al., 1997). Topflash reporter (20 pg) was animally injected in four-cell stage embryos alone or in combination with different mRNAs. At gastrula stage embryos were collected and luciferase activity was measured. Without ectopic activation Wnt/ß-catenin signal is low in *Xenopus* embryos. Injection of Wnt8 mRNA (10 pg) activates the reporter. This activation is blocked by co-injection of 2 ng Mig30 mRNA (Fig.1B). To exclude extracellular interaction of Mig30 with the Wnt/ß-catenin pathway, signalling was induced by 50 pg ß-catenin mRNA. Co-expression of 3 ng Mig30 efficiently blocks reporter activity (Fig.1C). This confirms previous results, that high doses of Mig30 are able to inhibit canonical Wnt signalling (Kuerner, 2008).

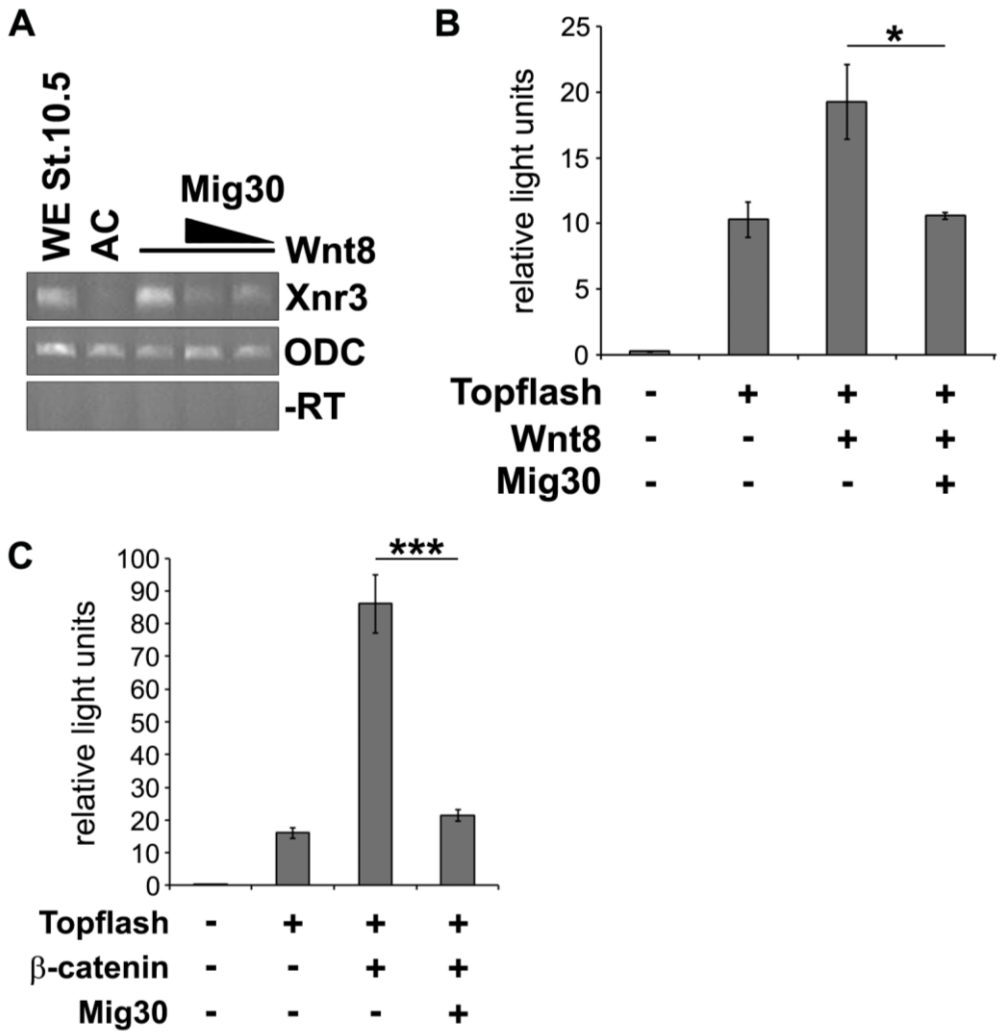


Fig. 1: Mig30 inhibits Wnt signalling
(A) Animal cap assay. 4-cell stage embryos were microinjected as indicated into the animal pole. Injection of 3 pg Wnt8 mRNA alone or in combination with 500 or 1000 pg Mig30 mRNA. Animal caps (AC) were explanted at blastula stage and cultured to early gastrula stage (N.F. stage 10.5). Subsequently, RT-PCR was performed. Expression of the Wnt-responsive gene Xnr3 was analysed. Ornithine decarboxylase (ODC) served as loading control. (WE: sibling whole embryo, -RT: control reaction without reverse transcriptase). (B,C) Luciferase Assay. 4-cell stage embryos were microinjected as indicated into each animal blastomere. N.F. stage 10+ embryos were collected in pools of 4-5 embryos and assayed for luciferase activity in triplicates. (B) Injection with 20 pg Topflash, 10 pg Wnt8 mRNA and 1.5 ng Mig30 mRNA, respectively (C) with 20 pg Topflash, 50 pg β-Catenin mRNA and 3 ng Mig30 mRNA, . Level of significance in F test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.2 Mig30 gain of function inhibits BMP signalling

Inhibition of BMP signalling is a crucial step in head and neural development, in *Xenopus*. This inhibition can be due to active IGF signalling, which mediates linker phosphorylation and subsequent degradation or cytoplasmic retention of the signal transducer Smad1 (Pera et al., 2003; Eivers et al., 2004).

To study if Mig30 can antagonise BMP signals, we used the luciferase reporter construct BRE-luc, which contains multimeric BMP response elements (Hata et al., 2000). Animal injection of the BRE-luc reporter (100 pg) showed no significant promoter activation by endogenous BMPs. The reporter is strongly activated when BMP4 mRNA (100 pg) is co-injected. This BMP4 mediated activation of the reporter is blocked by co-injection of Mig30 mRNA (1 ng). Thus, similar to IGFs, Mig30 can antagonise the BMP signalling pathway.

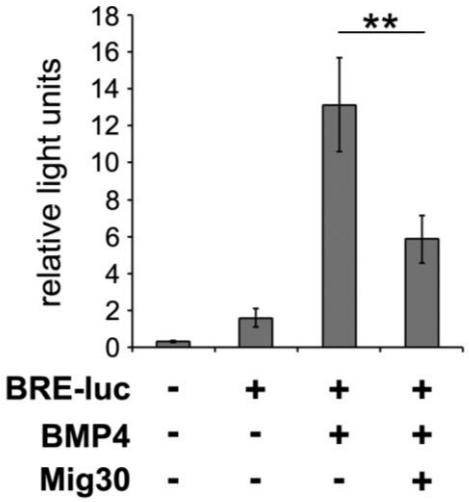


Fig. 2: Mig30 inhibits BMP signalling
Luciferase Assay. 4-cell stage embryos were microinjected as indicated into each animal blastomere. N.F. stage 10+ embryos were collected in pools of 4-5 embryos and assayed for luciferase activity in triplicates. (B) Injection with 100 pg BRE-luc, 100 pg BMP4 mRNA and 1 ng Mig30 mRNA. Level of significance in F test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

3.3 Mig30 mutant constructs inhibit Wnt and BMP signalling

Mig30 mutant constructs were designed to reveal discrete activities of individual domains of Mig30 (Fig.3). Mig30ΔC comprises the signal peptide, the IB and the KAZAL domain, while the C-terminal IgC2 domain was removed. Overexpressing Mig30ΔC leads to embryos with enlarged cement glands and ectopic eye structures, reminiscent to activation of IGF pathway phenotypes (Kuerner, 2008; Pera et al., 2001; Richard-Parpaillon et al., 2002). For Mig30ΔN the IB and KAZAL domain were removed, so the IgC2-domain follows the signal peptide. Microinjection of Mig30ΔN results in embryos that show stronger microcephaly than Mig30 injected embryos (Kuerner, 2008). To determine the effect of the Mig30 mutant constructs on Wnt signalling, again the Topflash reporter construct was used in a luciferase reporter assay. Four-cell stage embryos were injected animally. Injection of 20 pg Topflash alone showed no effect. Co-injection of 10 pg Wnt8 profoundly activated the ß-catenin /TCF promoter. This activation was blocked by co-injection of 1.5 ng Mig30ΔC. Surprisingly injection of Mig30ΔN (1.5 ng) also blocks the activation of the promoter by Wnt8 (Fig.4A). Like for Mig30, extracellular interactions of the mutant constructs with the Wnt/ß-catenin pathway, can be excluded because both, Mig30ΔC and Mig30ΔN also block promoter activity after stimulation with ß-catenin (Fig.4B). This confirms the results recently shown by Kuerner, K.M.

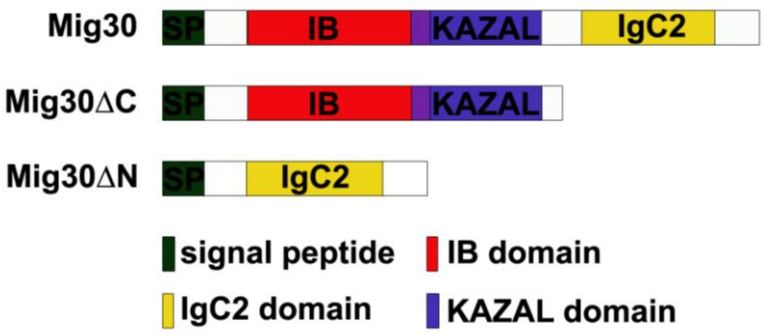


Fig. 3: Schematic diagram of Mig30 and Mig30 mutant constructs
SP: signal peptide (green), IB: Insulin-like growth factor binding domain (red), IgC2: immunoglobulin C-2 type domain (yellow), KAZAL: kazal type serine protease inhibitor domain (blue), overlap of IB and KAZAL (purple; adapted from Kuerner, 2008)

To analyse the effect of the mutant constructs on BMP signalling, the BRE-luc reporter was again utilised. Injection of 100 pg BMP4 together with the reporter (100 pg) lead to a strong activation. However, this activation was very efficiently blocked by the co-injection of 1 ng Mig30ΔC or 1 ng Mig30ΔN (Fig.4C). Thus both, the IB domain and the IgC2 domain seem to be sufficient to inhibit Wnt and BMP signalling.

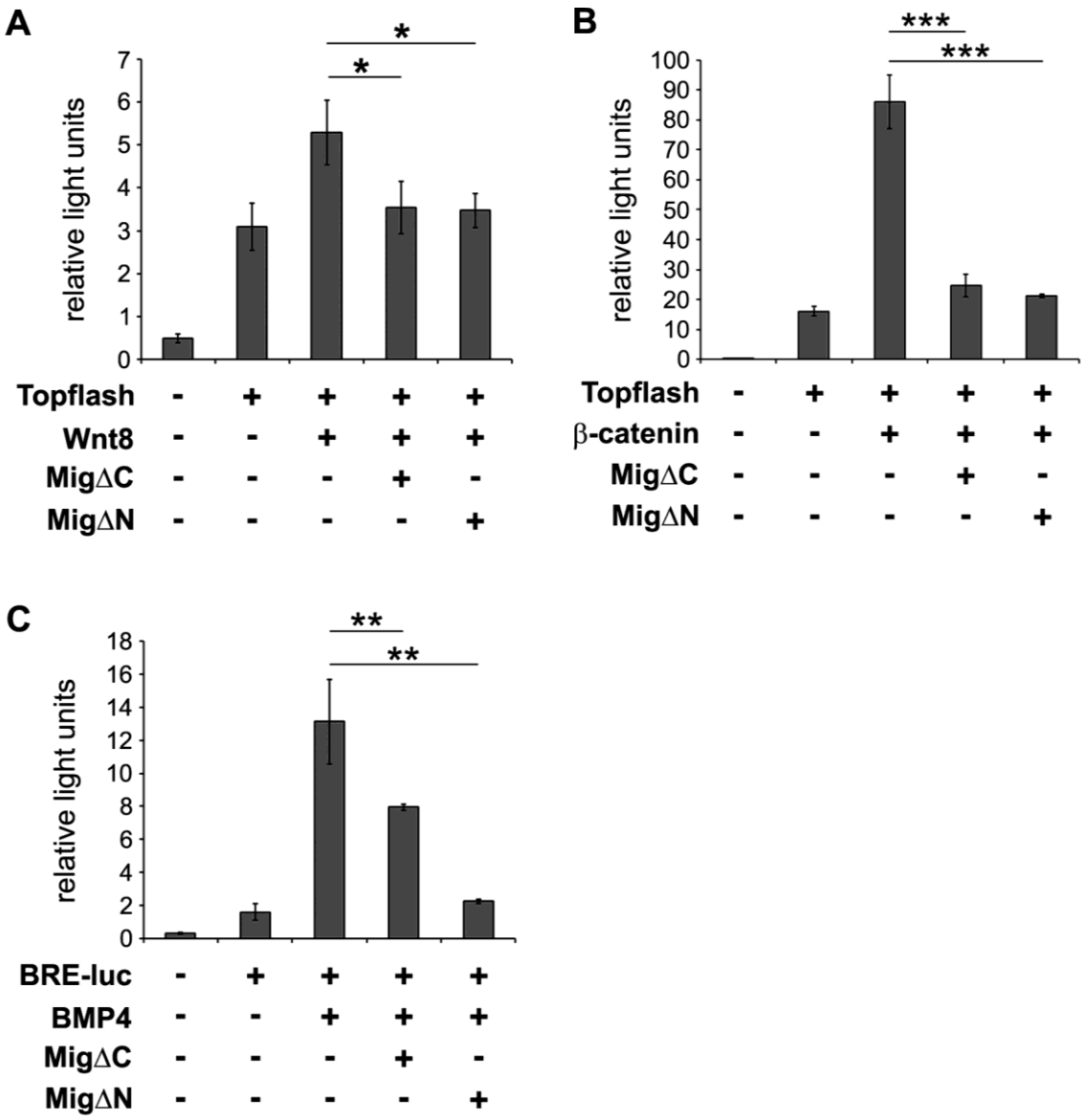


Fig. 4: Mig30 mutant constructs inhibit Wnt and BMP signalling
(A,B) Luciferase Assay. 4-cell stage embryos were microinjected as indicated into each animal blastomere. N.F. stage 10+ embryos were collected in pools of 4-5 embryos and assayed for luciferase activity in triplicates. (A) Injection with 20 pg Topflash, 10 pg Wnt8 mRNA, 1.5 ng Mig30ΔC mRNA and 1.5 ng Mig30ΔN mRNA, (B) with 20 pg Topflash, 50 pg β-Catenin mRNA, 3 ng Mig30ΔC mRNA and 3 ng Mig30ΔN mRNA, respectively. (C) Injection with 100 pg BRE-luc, 100 pg BMP4, 1 ng Mig30ΔC or 1 ng Mig30ΔN, respectively. Level of significance in F test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

3.4 Mig30 GOF is not able to induce neural marker expression

It was shown, that IGF signalling, by blocking Wnt and BMP signalling, is required for head and neural development in *Xenopus*. Gain of IGF signalling induces the expression of neural markers in animal cap explants (Pera et al., 2001; Richard-Parpaillon et al., 2002). If Mig30 promotes IGF signalling as previous data suggests, overexpression of Mig30 should also induce neural marker expression. To examine this, an animal cap assay was performed. Four-cell stage embryos were injected with 1 ng Mig30 mRNA or 1 ng Mig30ΔC mRNA. At blastula stage caps were dissected and grown until control siblings reached neurula stages. Then expression of different marker genes was analysed by RT-PCR (Fig.5). Sox2 marks the neural plate including pre-neural tissue, as well as tissue not yet committed to neural fate (Mizuseki et al., 1998; Wills et al., 2010). Pax6 is marker for developing eyes and neural structures, NCAM is a pan-neural marker, Xag1 is a specific cement gland marker and Bf1 is a marker for the telencephalic primordium, an anterior brain structure (Hirsch and Harris, 1997; Krieg et al., 1989; Sive et al., 1989; Bourguignon et al., 1998). Injection of 1 ng Mig30 had no effect on the neural markers Sox2, Pax6 and NCAM. The brain marker Bf1 was also not affected. The cement gland marker Xag1, which is strongly induced by IGFs is not affected by Mig30 overexpression. This suggests that Mig30 is not able to induce neural marker expression by promoting IGF signalling. Injection of Mig30ΔC (1 ng), like Mig30, is not sufficient to induce the neural markers Sox2, Pax6 and NCAM. Also the expression of the brain marker Bf1 was not induced. However Mig30ΔC is able to induce the expression of the cement gland marker Xag1, according to its overexpression phenotype which shows increased cement glands (Kuerner, 2008).

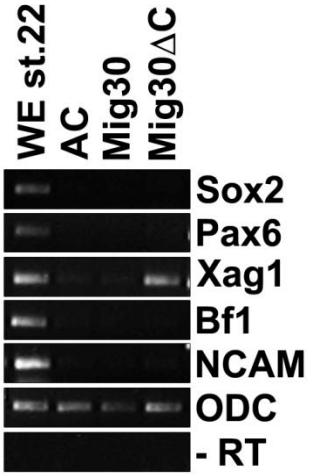


Fig. 5: Overexpression of Mig30 cannot induce neural tissue.
4-cell stage embryos were microinjected into the animal pole of each blastomere with 1 ng Mig30 or MigΔC mRNA. Animal caps were explanted at blastula stage and cultured to N.F. stage 22. Subsequently, RT-PCR was performed. Sox2, a neural plate marker, Pax6 and NCAM, two neural marker, XAg1 a cement gland marker and Bf1 a forebrain marker were analysed. Ornithine decarboxylase (ODC) served as loading control (WE: sibling whole embryo, -RT: control reaction without reverse transcriptase).

3.5 Mig30 affects IGFBP5 signalling

In *Xenopus* there are so far only two IGFBPs identified, and only one of them, namely IGFBP5 is expressed early in development. IGFBP5 is a 38kDa protein that can be cleaved by IGFBP proteases, binds to the ECM and binds IGFs with high affinity (Clemmons, 1997; Parker et al., 1998). In *Xenopus* it is expressed throughout development, at four-cell stage it is found in the animal hemisphere, during gastrulation on the dorsal side and during neurulation it becomes confined to the floor plate, notochord and dorsal endoderm. Gain of IGFBP5 leads to embryos with enlarged head structures and cement glands. IGFBP5 promotes anterior development by stimulating signalling by endogenous IGFs via IGFR (Pera et al., 2001). In other systems IGFBP5 can act either as an IGF antagonist (Stewart and Rotwein, 1996; Kalus et al., 1998) or as an IGF promoting agent (Jones et al., 1993).

To explore the possibility that Mig30 affects IGF signalling by interacting with IGFBP5, the effects of Mig30 on IGFBP5 overexpression were analysed. Four-cell stage embryos were injected animally, at blastula stage animal caps were cut and cultured until N.F. stage 20. Expression of marker genes was analysed by

qRT-PCR. Injection of IGFBP5 (500 pg) lead to an induction of the cement gland marker XAG1 and at this stage to an even stronger induction of Otx2, a forebrain marker, whose expression is required for cement gland formation, this confirms results of Pera et al. (Fig.6, Sive et al., 1989; Blitz and Cho, 1995; Pannese et al., 1995). 500 pg Mig30 had no effect on the expression levels of Xag1 and Otx2, corresponding to the findings that 1 ng Mig30 is not able to induce Xag1 and other neural markers in animal caps (Fig.5). Co-injection of 500 pg Mig30 and IGFBP5 did not affect IGFBP5 induced expression of Xag1, but expression of Otx2 was mildly reduced compared to IGFBP5 alone. However high doses of Mig30 (1 ng) strongly blocked IGFBP5 induced expression of the two markers. Epidermal keratin, a marker for non-neural ectodermal tissue (LaFlamme and Dawid, 1990) was expressed at the same levels during the experiment, indicating that the induction of anterior markers is not due to cell fate changes. Thus high doses of Mig30 are able to block signalling induced by IGFBP5.

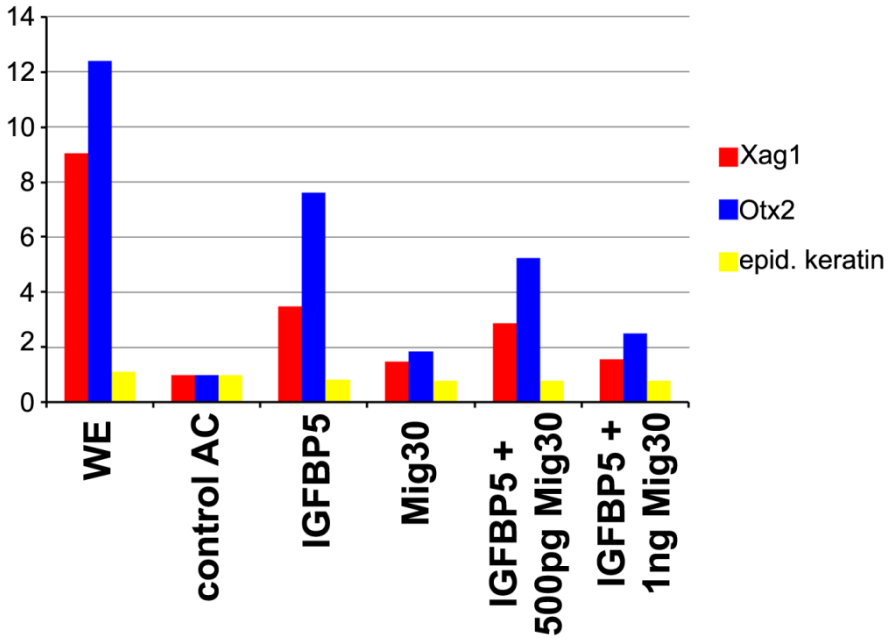


Fig. 6: Mig30 overexpression inhibits IGFBP5
qRT-PCR analysis of animal caps. 4-cell stage embryos were injected animally with 500 pg IGFBP5 mRNA, 500 pg or 1 ng Mig30 mRNA alone or in combination. Animal caps were explanted at blastula stage and cultured to N.F. stage 20. Expression of the cement gland marker Xag1, the anterior neural marker Otx2 and the epidermal marker epidermal keratin were analysed

To elucidate which domain is required for the effect on IGFBP5, the experiment was repeated with Mig30ΔC and Mig30ΔN. The animal caps were allowed to grow longer, so they reached NF stage 24 and so the expression of level Xag1 exceeds the expression level of Otx2. Like gain of IGFBP5, overexpression of Mig30ΔC affects cement gland size. In the animal cap assay injection of 500 pg IGFBP5 strongly induced Xag1 and Otx2 expression (Fig.7A). Low doses of Mig30ΔC (500 pg) had no effect on the expression of Xag1 and Otx2 (Fig.7A), while high doses of Mig30ΔC (1 ng) were able to induce Xag1 expression on its own (Fig.7A and Fig.5). However co-expression of 500 pg Mig30ΔC and IGFBP5 increased the transcript levels of Xag1 and Otx2, indicating a synergy of the two proteins (Fig.7). Unexpectedly, the IGFBP5 induced Xag1 and Otx2 expression was blocked by co-injection of high doses Mig30ΔC (1 ng; Fig.7A). This shows that moderate levels of Mig30ΔC cooperate with IGFBP5, while high doses oppose IGFBP5 signals.

Mig30ΔN lacks the IB domain, and induces a microcephalic phenotype when overexpressed, similiar to phenotypes seen when IGF signalling is inhibited. On its own, moderate doses of Mig30ΔN (500 pg) do not affect the transcript levels of Xag1 and Otx2 in animal caps. But in contrast to Mig30ΔC moderate doses of Mig30ΔN are sufficient to block IGFBP5 induced expression of Xag1 and Otx2 (Fig.7B). This suggests that Mig30 is a biphasic protein, due to the opposing effects of its C- and N-terminal domains.

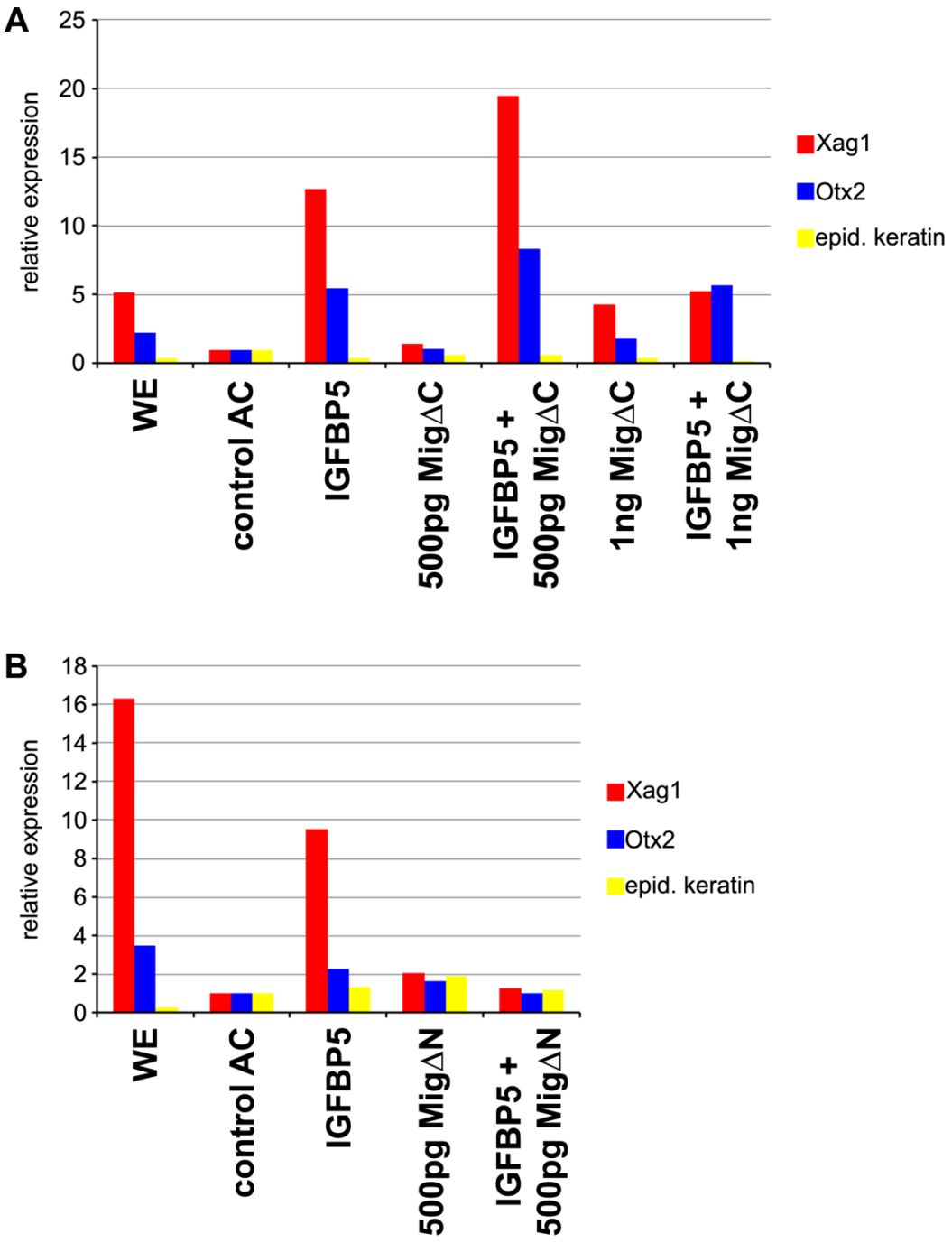


Fig. 7: Mig30 mutant constructs interfere with IGFBP5 signalling
qRT-PCR analysis of animal caps. 4-cell stage embryos were injected animally. Animal caps were explanted at blastula stage and cultured to N.F. stage 24. Expression of the cement gland marker Xag1, the anterior neural marker Otx2 and the epidermal marker Epidermal keratin were analysed (A) Injection of 500 pg IGFBP5 mRNA, 500 pg or 1 ng MigΔC mRNA alone or in combination. (B) Injection of 500 pg IGFBP5 mRNA, 500 pg MigΔN mRNA alone or in combination.

3.6 Mig30 blocks IGF induced differentiation in RCJ3.1C5.18 cells

IGF1 is an important growth factor in growth plate development. It is known from *in vitro* and *in vivo* studies that IGF1 stimulates proliferation and differentiation of growth plate chondrocytes (Wang et al., 1999; Wang et al., 2006; Ohlsson et al., 1994). The mesenchymal RCJ3.1C5.18 (RCJ) cell line, derived from fetal rat calvaria, is widely used as a model for growth plate chondrocyte development (Grigoriadis et al., 1996; McDougall et al., 1996; Lunstrum et al., 1999). RCJ cells spontaneously progress to differentiated growth plate chondrocytes and the differentiation can be enhanced by exogenous IGF1. The action of exogenous IGF1 can be directly studied because RCJ cells do not express IGF1 (Spagnoli et al., 2001).

That the RCJ cells do not express IGF1 on their own, gave us the idea to investigate if Mig30 and the Mig30 mutant constructs directly interfere with IGF1signalling in these cells. Therefore the cells were transiently transfected with pCS2+ Mig30, pCS2+ Mig30ΔC, pCS2+ Mig30ΔN or the empty pCS2+ vector as a control. The cells were cultured in differentiation medium from day 4 onward. At day 7 of culture, cells were serum-starved for 12 h and stimulated with 100 ng/ml IGF1 for another 12 h. The cells were harvested and transfection efficiency controlled. All samples showed that the transfection was successful and the plasmids were transcribed (data not shown). Then the expression of IGFBP5, which was upregulated during chondrocyte differentiation and ALP (alkaline phosphatase), a chondrocyte differentiation marker were analysed by qRT-PCR (Kiepe et al., 2005; Lunstrum et al., 1999; Spagnoli et al., 2001). IGFBP5 and ALP were expressed at low levels in vector transfected cells. The expression increased dramatically when the differentiation process was stimulated by additional treatment with IGF1 (Fig.8). This increase of IGFBP5 and ALP expression after stimulation with IGF1 was strongly reduced when cells were previously transfected with Mig30 (Fig.8). This result clearly shows that Mig30 inhibits IGF1 extracellularly by direct protein-protein interaction. To investigate which domain of Mig30 is required for this interaction the experiment was performed with Mig30ΔC and Mig30ΔN. Mig30ΔC, which contains the N-terminal IB domain showed the same inhibitory effect on IGF1 stimulated IGFBP5 and ALP, as Mig30 (Fig.8).

Mig30ΔN, which comprises the C-terminal IgC2 domain, did not have an effect on the IGF1 stimulated differentiation (Fig.8). This demonstrates that it is the IB domain, of Mig30 that is required for direct interaction with IGF1.

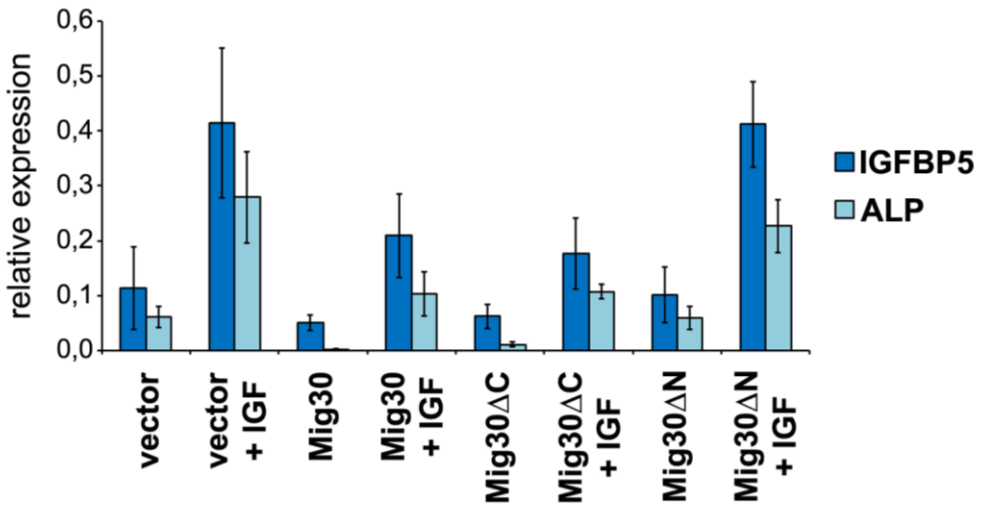


Fig. 8: Mig30 inhibits IGF1 induced differentiation of RCJ3.1C5.18 cells
RCJ cells were transfected as indicated, cultured in differentiation medium from day 4 onward. At day 7 cells were 12 h serum starved and then stimulated with 100 ng/ml IGF1. After 12 h cells were harvest and expression of IGFBP5 and ALP were measured by qRT-PCR.

3.7 Endogenous Mig30 promotes Wnt signalling in an IGF-dependent manner

Gain of function experiments revealed several distinct, partially oppposing activities of Mig30 and the Mig30 mutant constructs. It still remains to be demonstrated which of these functions play a physiological role in the embryo and participate in IGF signalling. To address the endogenous function of Mig30 a knockdown approach with an antisense morpholino oligonucleotide was utilised (Heasman et al., 1994). Mig30 antisense morpholino (Mig30Mo, Mig30 morpholino) is targeted against the transcriptional start codon of Mig30 and efficiently blocks translation of endogenous Mig30. Injection of Mig30 morpholino delays gastrulation, disturbs anterior neural folding and gives rise to microcephalic embryos with reduced eye size (Kuerner, 2008).

Inhibition of the IGF signalling pathway also gives rise to microcephalic embryos with small or no apparent eyes (Pera et al., 2001; Richard-Parpaillon et

al., 2002). Proper head formation in *Xenopus* requires simultaneous inhibition of Wnt and BMP signalling (Glinka et al., 1997), which both can be inhibited by active IGF signalling (Pera et al., 2003). To elucidate the role of endogenous Mig30 in Wnt signalling a Topflash reporter assay was used (Korinek et al., 1997). Dorsal injection of 300 pg Topflash reporter, led to an activation of the promoter by endogenous Wnt signals, which cannot be inhibited by co-injection of 35 ng control morpholino (Fig.9A). However injection of 35 ng Mig30Mo (Fig.9A) strongly inhibited Wnt signalling.

To test if the inhibition of Wnt signalling by Mig30 is dependent on the IGF signalling pathway we exploited the function of dominant negative IGFR (dnIGFR). dnIGFR encodes a secreted form of the xIGF1R devoid of its transmembrane and intracellular tyrosine kinase domain, which efficiently blocks IGF1 and IGF2 signal transduction (Pera et al., 2001). Wnt signalling can be partially rescued by blocking the IGF signalling pathway with dnIGFR (1.5 ng), while blocking the IGF signalling pathway alone has no effect on promoter activation (Fig.9A). This suggests that endogenous Mig30 inhibits IGF signalling and thereby promotes Wnt signalling.

This is supported by the finding that dorsal injection of Mig30Mo (35 ng) inhibits the expression of Myf5 (Fig.9C; Hopwood et al., 1991). Myf5 is a marker for lateral mesoderm, whose transcription depends on zygotic Wnt/ß-catenin signals (Marom et al., 1999; Yang et al., 2002). Hence endogenous Mig30 promotes Wnt signalling, by inhibition of the IGF pathway.

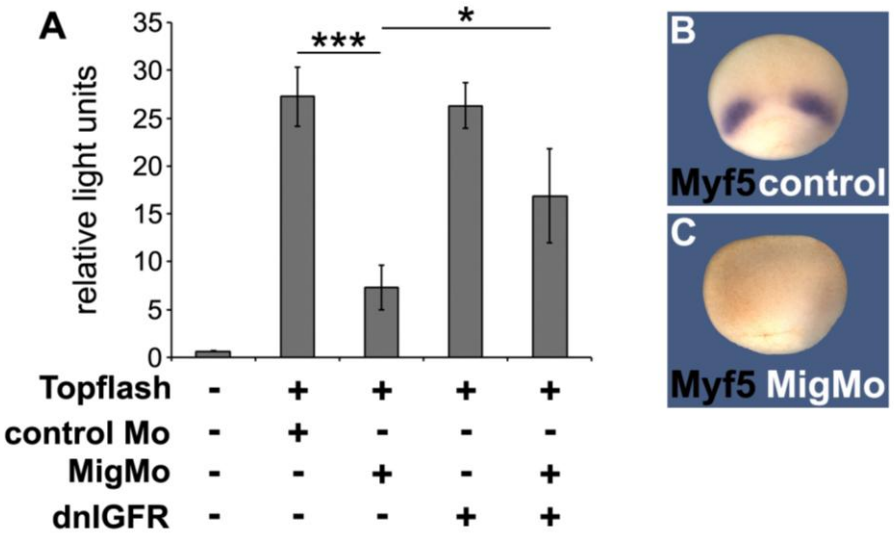


Fig. 9: In Mig30 morphants Wnt signalling is inhibited in an IGF-dependent manner
(A) Luciferase Assay. 4-cell stage embryos were microinjected as indicated into both dorsal blastomeres. N.F. stage 10+ embryos were collected in pools of 4-5 embryos and assayed for luciferase activity in triplicates. Injection of 300 pg Topflash, 35 ng Mig30 morpholino, 35 ng control morpholino and 1.5 ng dnIGFR mRNA, respectively. Level of significance in F test: *, P < 0.05; **, P <0.01; ***, P < 0.001. (B,C) *In-situ* hybridisation for Myf5 at N.F. stage 10.5. (B) uninjected control. (C) after microinjection of 35 ng Mig30 morpholino into both dorsal blastomeres at 4-cell stage

3.8 Mig30 loss of function inhibits BMP-signalling

Next we analysed the effect of endogenous Mig30 on BMP signalling. A luciferase reporter assay was performed with the BRE-luc reporter construct which contains several BMP-response elements (Hata et al., 2000). Embryos were injected at four-cell stage into the two dorsal or ventral blastomeres and assayed at gastrula stage. Ventral injection of 300 pg reporter showed a strong transcriptional activation, by endogenous BMP signals. On the dorsal side of the embryo, BMP signals are inhibited by different BMP inhibitor molecules, like Chordin and Noggin, so the transcriptional activation of the reporter was not as strong as on the ventral side (Sasai et al., 1994; Moon and Christian, 1992). This activation was blocked by co-injection of Mig30 morpholino (35 ng). Additionally the expression of the BMP-responsive gene Vent2 was reduced, when Mig30 morpholino (35 ng) was injected dorsally (Fig.10C; Karaulanov et al., 2004). Thus BMP signalling is blocked in Mig30 morphants, presumably due to an increase in IGF signalling.

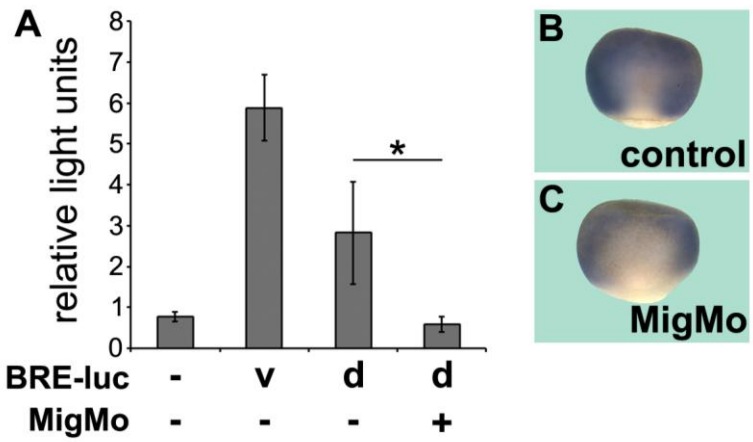


Fig. 10: In Mig30 morphants BMP signalling is inhibited
(A) Luciferase Assay. 4-cell stage embryos were microinjected into both dorsal or ventral blastomeres as indicated. N.F. stage 10+ embryos were collected in pools of 4-5 embryos and assayed for luciferase activity in triplicates. Injection of 300 pg BRE, 35 ng Mig30 morpholino. Level of significance in F test: *, P < 0.05; **, P < 0.01; ***, P < 0.001. (B,C) *In-situ* hybridisation for Vent2 at N.F. stage 10.5. (B) uninjected control. (C) after microinjection of 35 ng Mig30 morpholino into both dorsal blastomeres at 4-cell stage

3.9 Loss of Mig30 enhances exogenous IGF signals

The cement gland, a mucous secreting anterior structure is particularly sensitive to IGF signalling. Hyperactivation of the IGF pathway results in enlargement of the cement gland (Pera et al., 2001; Richard-Parpaillon et al., 2002). Therefore the size of the cement gland can be used as a marker for the level of IGF activity. In case the endogenous Mig30 would act as an inhibitor of the IGF pathway, knockdown with Mig30Mo would sensitise the tissue for ectopic IGF signals. To test this theory low doses of IGF1 mRNA (100 pg) or Mig30Mo (35 ng) or both together were injected into the dorsal blastomeres of a four-cell stage embryos. At tailbud stage (N.F. 25) the size of the cement glands was measured and embryos were grouped according to their cement gland size in small, middle and large (Fig.11). In uninjected control embryos 8% (n=62) showed a large cement gland. When injected with low doses IGF1 only 4% (n=49) showed a large cement gland. Injection of Mig30Mo increased the number of large cement glands to 20% (n=53) but co-injection of IGF1 and Mig30Mo resulted in an increase of large cement glands up to 45% (n=50). This supports the role of endogenous Mig30 as a repressor of IGF signalling.

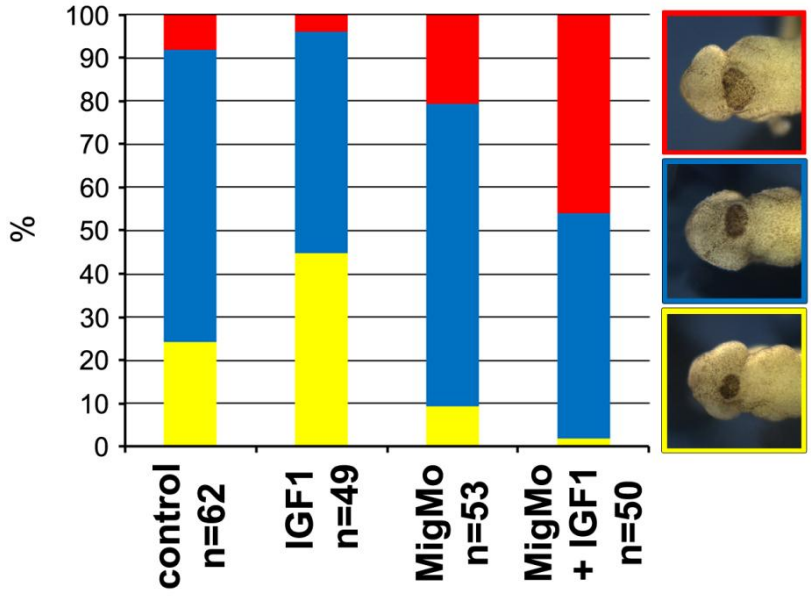


Fig. 11: Loss of Mig30 enhances exogenous IGF signals
Embryos were injected into both dorsal blastomeres with 17.5 ng Mig30Mo, 100 pg IGF1 or in combination. At stage 25 cement gland size was measured. Embryos were grouped according to their cement gland size, small (up to 900 px, shown in yellow), mid-size (901-1400 px, shown in blue) and large (over 1401 px, shown in red).

Cement gland size is also increased when IGFBP5 is overexpressed, because IGFBP5 can stimulate endogenous IGF signalling via IGFR (Pera et al., 2001). To test if Mig30 interferes with IGFBP5 induced signalling, four-cell stage embryos were injected dorsally and allowed to grow until N.F. stage 19, then expression of the anterior marker Otx2, that is required for cement gland induction, was analysed (Blitz and Cho, 1995; Pannese et al., 1995). Dorsal/animal injection of 200 pg IGFBP5 expanded the expression domain of Otx2, confirming previous results (Pera et al., 2001). Co-expression of low doses Mig30 (500 pg) had no effect on IGFBP5 induced Otx2 expression (Fig.12C), which is consistent with the data of the animal cap assay (Fig.7). Surprisingly, in embryos injected with IGFBP5 and Mig30Mo (35 ng) the Otx2 expression domain was further expanded (Fig.12D), than with IGFBP5 alone (Fig.12B), although Mig30Mo alone has no effect on the size of the cement gland. This indicates that IGFBP5 and endogenous Mig30 have opposing effects on IGF signalling.

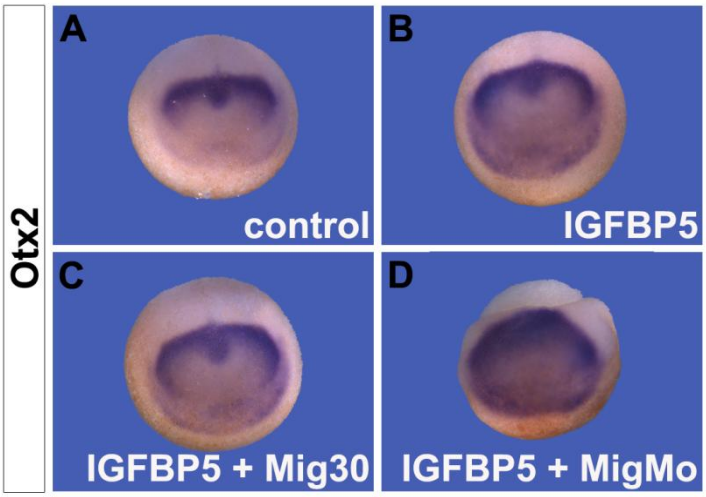


Fig. 12: Loss of Mig30 enhances IGFBP5 signalling
(A-D) *In-situ* hybridisation. 4-cell stage embryos were injected dorsally and at NF stage 19 scored for Otx2 expression. (A) uninjected control (B) 500 pg IGFBP5 (C) 500 pg IGFBP5 plus 500 pg Mig30 and (D) 500 pg IGFBP5 plus 35 ng Mig30 morpholino.

3.10 Mig30 plays a role in head and neural development

The IGF signalling pathway is required for head and neural development, in *Xenopus*. Blocking the IGF signalling pathways reveals embryos with impaired head development (Pera et al., 2001; Richard-Parpaillon et al., 2002). A similar phenotype is caused by the loss of Mig30 function. Both knockdown of the IGFR and Mig30 lead to reduced expression of the neural marker NCAM and Bf1, a marker for telencephalic primordium (Richard-Parpaillon et al., 2002; Kuerner, 2008; Krieg et al., 1989; Bourguignon et al., 1998). Additionally, the expression of the hindbrain marker Krox20 and the eye marker Rx1 is reduced in Mig30 morphants (Kuerner, 2008; Papalopulu et al., 1991; Casarosa et al., 1997). To examine if the loss of head structures in Mig30 morphants is due to inhibition of Wnt signalling, the expression of En2, a marker for the mid-hindbrain boundary, whose expression is dependent on active Wnt signalling was analysed (McMahon et al., 1992; McGrew et al., 1997). Four-cell stage embryos were injected into the right dorsal blastomere with 35 ng Mig30 morpholino, grown until late neurula stage and analysed for En2 expression by *in-situ* hybridisation. In 93% of injected embryos the expression of En2 was strongly reduced (Fig.13B, n=28). This shows that Wnt signalling is inhibited when Mig30 is depleted. Indicating that endogenous

Mig30 inhibits IGF signalling and thereby promotes Wnt signalling. Moreover the results support earlier results that Mig30 plays a role in head development (Kuerner, 2008).

During neural induction, the embryonic neural plate, which gives rise to future neural and non-neural tissue, is specified and set aside from other parts of the ectoderm. It was shown that blocking Wnt signalling is sufficient to induce neural precursors, marked by an expansion of the neural plate (Heeg-Truesdell and LaBonne, 2006). Since Mig30 seems to interfere with Wnt signalling, by inhibition of IGF signalling, the role of endogenous Mig30 on neural plate formation was analysed. Four-cell stage embryos were injected into the right dorsal blastomere with Mig30 morpholino (35 ng), to block translation of endogenous Mig30. The embryos were allowed to grow until neurula stages and examined by *in-situ* hybridisation for expression of neural plate markers. The expression domain of Sox2 a neural plate marker, that marks future neural and non-neural tissue is expanded laterally on the morpholino injected side (73%, n=30; Fig.13D, Mizuseki et al., 1998). Another neural plate marker, Sox3 is also expanded laterally after morpholino injection (77%, n=26; Fig.13F), indicating an inhibition of Wnt signalling (Penzel et al., 1997). The expression domain of Epidermal keratin, a marker for non-neural ectoderm is reduced after morpholino injection (86%, n=29; Fig.13H, LaFlamme and Dawid, 1990; Heeg-Truesdell and LaBonne, 2006). This indicates that neural plate tissue expands on the expense of presumptive non-neural ectoderm.

Since injection of Mig30 morpholino leads to an expansion of the neural plate and hence to an increase of the neural progenitor pool, it was analysed if neural differentiation actually takes place, because neural differentiation, like neural plate induction, requires inhibition of Wnt signalling (Heeg-Truesdell and LaBonne, 2006). N-tubulin a marker for differentiated neural tissue was reduced in 77% of injected embryos, at the site of injection (n=103; Fig.13L, Chitnis et al., 1995). And as previously shown, expression of the pan-neural marker NCAM was also reduced (75%, n=33; Fig.13J, Kuerner, 2008).

This data show that Mig30 plays a role in head and neural development, presumably by inhibiting IGF signalling and thereby promoting Wnt signalling, which is needed to restrict the size of the neural plate.

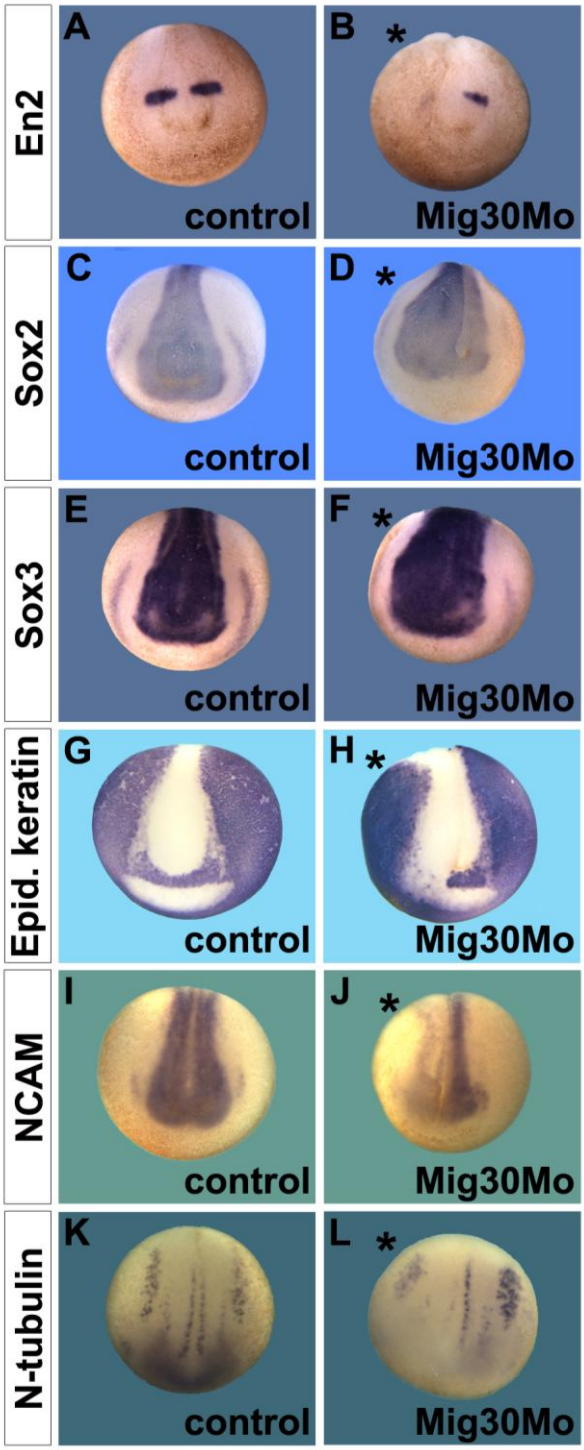


Fig. 13: Knockdown of Mig30 perturbs head and neural development.
In-situ **hybridisation. 4-cell stage embryos were injected into the right dorsal blastomere (marked with *) with 35 ng Mig30 morpholino and analysed for marker expression at N.F. stages 16-18. Control, uninjected sibling embryo. (A,B) En2 (C,D) Sox2 (E,F) Sox3 (G,H) Epidermal keratin (I,J) NCAM and (K,L) N-tubulin.**

3.11 Mig30 plays a role in neural crest development

Neural crest (NC) induction is induced by a combination of secreted signals in which Wnt signalling and the dorsolateral mesoderm of gastrula embryos play a particular role (Wu et al., 2005). For the induction at gastrulation stage Wnt signals are activated and BMP signals are inhibited, while later for maintenance both pathways have to be active (Steventon et al., 2009).

Anterior neural folding is disturbed in Mig30 depleted embryos. The folds mark the edge of the neural plate and contain the pre-migratory neural crest cells (Mayor et al., 1995; Aybar et al., 2003). After injection of 35 ng Mig30 morpholino into the right dorsal blastomere at four-cell stage embryos the transcription of Slug, an early neural crest marker was strongly reduced (78%, n=42; Fig.14B), as shown before (Kuerner, 2008). This supports the findings that Wnt signalling is attenuated in Mig30 morphants, because the induction of neural crest cells requires Wnt signalling (Wu et al., 2005; Steventon et al., 2009). However the transcription of Slug can be rescued by co-expression of ChdSPMig30. ChdSPMig30 is a Mig30 mutant construct, where the signalpeptide of Mig30 was changed against the signalpeptide of Chordin, so Mig30 morpholino cannot bind anymore (Kuerner, 2008). Injection of 75 pg ChdSPMig30 DNA into one dorsal blastomere of four-cell stage embryos had no effect on Slug expression (90%, n=93; Fig.14A), while injection of Mig30 morpholino reduced Slug expression in 45% (n=91; Fig.14A) of the injected embryos. Co-injection of Mig30 morpholino and ChdSPMig30 restored Slug expression nearly back to the level of control cells (Fig.14A). This confirms the specificity of Mig30 morpholino and that endogenous Mig30 is required for neural crest induction.

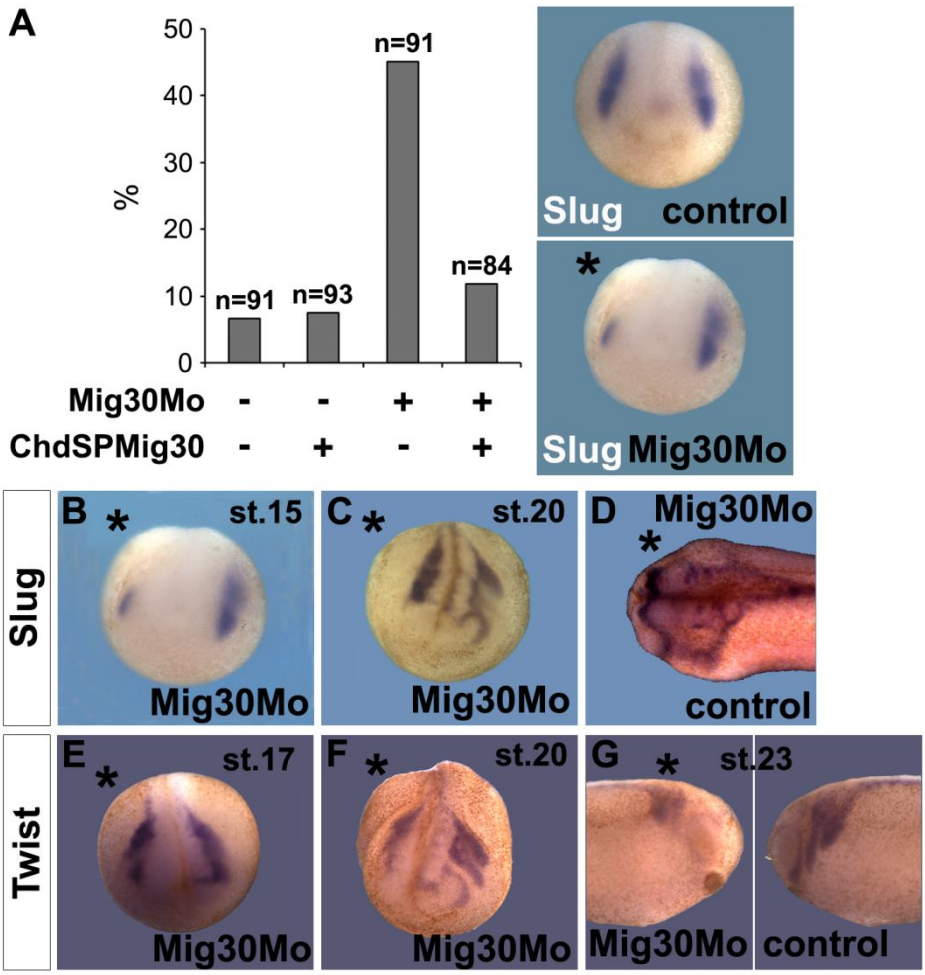


Fig. 14: Mig30 is required for neural crest development.
(A) Mig30Mo rescue experiment. Evaluation of Slug repression after injection of 35 ng Mig30 morpholino , 75 pg ChdSPMig30 DNA or a combination of both. *(B-G) In-situ* hybridisation. 4-cell stage embryos were injected into the right dorsal blastomere (marked with *) with 35 ng of Mig30Mo. Control, uninjected sibling embryo. (B-D) Slug (B) stage 15, anterior view (C) stage 20, anterior view (D) stage 30 dorsal view. (E-G) Twist (E) stage 17, anterior view (F) stage 20, anterior view (G) stage 23, lateral view

Neural crest cells are a highly motile cell population, which emigrate from their place of induction, the neural plate border. Neural crest cells are separated into two distinct populations depending on their position along the anterior-posterior axis. The most anterior ones are called cranial neural crest cells (CNC), which are contributing to facial structures. The CNC cells segregate into the mandibular, hyoid and branchial segments, to migrate (through the pharyngeal pouches) to their final destinations. During their migratory route the neural crest cells become committed to their final cell fate (Kontges and Lumsden, 1996). Dorsal injection of Mig30 morpholino (35 ng) strongly reduces the expression of the neural crest

marker Slug (Fig.14B) at N.F. stage 15. Surprisingly, at the end of neurulation, N.F. stage 20 the expression of Slug reappeared, but the segregation into the three distinct pharyngeal streams was delayed in 62% of the embryos (n=32; Fig.14C). At tailbud stage the expression domain of Slug, at the morpholino injected side did not spread as far dorsolateral as on the uninjected control side (Fig.14D). The same effect was seen with a second neural crest marker, namely Twist (Greaves et al., 1985). The expression of Twist was reduced after injection with 35 ng Mig30 morpholino in N.F stage 17 embryos (Fig.14E). At stage 20 Twist was expressed at the neural plate border but the segregation into the three pharyngeal branches did not take place yet (Fig.14F). At stage 23 the lateral emigration of the neural crest cells just started on the side of morpholino injection while on the control side the neural crest almost reached their final destination (Fig.14G). This indicates that Mig30 plays a role in temporally fine tuning the signals required for proper neural crest induction.

3.12 Mig30 has no effect on FGF signalling

Another pathway known to be involved in neural induction is the FGF signalling pathway (Stern, 2005). Additionally FGF plays an important role in mesoderm formation and is required for expression of the mesodermal marker Xbra (Kimelman and Kirschner, 1987; Amaya et al., 1993). Both, Mig30 and IGF hyperactivity inhibit Xbra expression (Hayata et al., 2002; Kuerner, 2008; Carron et al., 2005). Therefore an animal cap assay was performed to analyse if Mig30 interacts with the FGF signalling pathway. Embryos were injected animally, animal caps were dissected at blastula stage and cultured until control siblings reached N.F. stage 15. Injection of FGF (10 pg) strongly induced XER81 (Munchberg and Steinbeisser, 1999), a marker for active FGF signalling and the mesoderm marker Xbra (Schulte-Merker and Smith, 1995; Isaacs et al., 1994; Smith et al., 1991). Mig30 (1 ng) alone was not able to induce the expression of XER81 and Xbra, indicating that Mig30 cannot induce mesoderm specification on its own. Co-injection of FGF and Mig30 induces expression of XER81 and Xbra, like injection of FGF alone (Fig.15). So Mig30 is not able to inhibit mesoderm formation induced by FGF signalling.

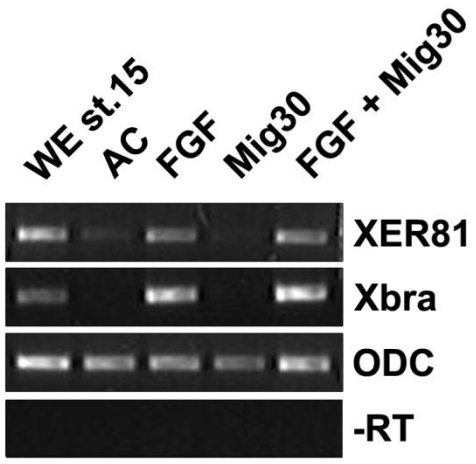
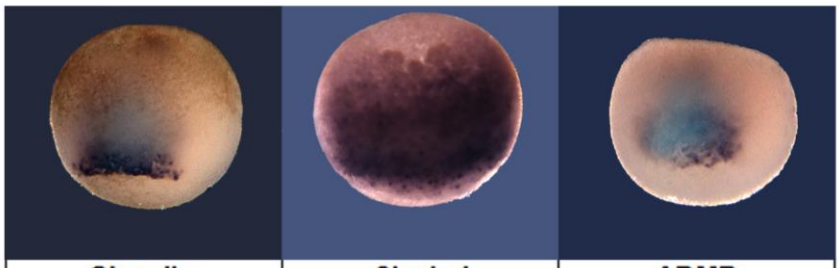


Fig. 15: Overexpression of Mig30 has no effect on FGF signalling
RT-PCR analysis of animal caps. 4-cell stage embryos were injected with 10 pg FGF mRNA, 1 ng Mig30 mRNA or both, at blastula stage animal caps were dissected and grown until control siblings reached N.F. stage 15. Expression of the mesodermal markers XER81 and Xbra was analysed. ODC served as loading control.

3.13 Mig30 does not affect ADMP signalling

At the beginning of gastrulation Mig30 is expressed in the Spemann organiser the source of BMP and Wnt inhibitors. Another protein that is found in the organiser is ADMP (Anti-Dorsalising Morphogenetic Protein) which antagonises dorsal and anterior structures (Moos et al., 1995). To check if Mig30 and ADMP do interact, proteins that are known to be affected by ADMP signalling were analysed by *in-situ* hybridisation after injection with Mig30 mRNA or Mig30 morpholino. Chordin is a BMP inhibitor that is expressed in the Spemann organiser like Mig30, which also blocks ADMP. ADMP blocks Chd and vice versa Chd inhibits ADMP (Fuentealba et al., 2007). In case Mig30 would interfere with ADMP an increase or decrease in Chd expression should be detectable. The experiment showed that Chd expression did not change when Mig30 is overexpressed or knocked down (Fig.16). Also ADMP expression was not altered in a gain or loss of Mig30 function experiment. The third marker analysed was Sizzled. Sizzled is a negative feedback inhibitor of BMP signalling (Yabe et al., 2003; Salic et al., 1997) expressed at the ventral signalling center. Gain of ADMP greatly enhances Sizzled expression, while ADMP knockdown inhibits Sizzled. Inhibition of Mig30 function by injection of 35 ng morpholino into the dorsal or ventral blastomeres of four-cell

stage embryos has no effect on Sizzled expression. When Mig30 is overexpressed Sizzled expression is also not affected. These results show that Mig30 does not interact with ADMP.



		Chordin	Sizzled	ADMP
construct	amount	number of embryos		
control		12	7	11
Mig30 dorsal	1 ng	5	5	5
Mig30 ventral	1 ng	5	6	5
Mig30Mo dorsal	35 ng	6	6	4
Mig30Mo ventral	35 ng	6	6	6

Fig. 16: Mig30 has no effect on ADMP signalling.
(A-C) *In-situ* hybridisation. 4-cell stage embryos were injected as indicated. (A) Chordin, stage 10.5 dorsal view (B) Sizzled, stage 10.5 ventral view and (C) ADMP, stage 10.5, dorsal view.

IGFBP-rP10

IGFBP-rP10 is an IGFBP-rP that belongs to the IGFBP-like subgroup. Protein sequence prediction shows a signal peptide, a N-terminal IB domain that partially overlaps with a KAZAL (kazal type serine protease inhibitor) domain and an IgC2 (immunoglobulin-like domain of the C2 type) domain at the C-terminus.

IGFBP-rP10 was first identified in mouse, where it was detectable from embryonic day 13 onward. It was named Bono1 because it is localised in developing bones and odentoblasts, where its expression correlates with matrix mineralisation (James et al., 2004). Another group identified it in a bone regeneration screen and termed it IGFBP-rP10 (for further use the mouse IGFBP-rP10, is called Bono1). Bono1 transcription is upregulated at the early phase of bone regeneration and induced by BMP2. It is suggested to be involved in the proliferation of osteoblasts (Shibata et al., 2004).

The *Xenopus* homologue of IGFBP-rP10 was identified in a bioinformatic approach (Kuerner and Steinbeisser, 2006). IGFBP-rP10 transcripts are detectable throughout development. At four-cell stage transcripts are found at the animal pole, during gastrulation on the dorsal side of the embryo and later on it is expressed in the notochord, floorplate, somites and fin (Kuerner, 2008).

3.14 Transcriptional regulation of IGFBP-rP10

At the beginning of gastrulation IGFBP-rP10 is expressed on the dorsal side of the embryo, where the Spemann-Mangold organiser is located (Kuerner and Steinbeisser, 2006). The organiser has a global patterning function in development and is able to induce a complete body axis when transplanted (De Robertis, 2006; De Robertis et al., 2000; Niehrs, 2004). Therefore it was analysed if the signals of the organiser or the signals that are required for organiser formation are able to induce IGFBP-rP10 expression. Members of the TGF-ß superfamily play an important role in mesoderm formation. Activin can induce the entire range of mesodermal derivates in animal caps (Green et al., 1990; Green et al., 1992). Transcription of IGFBP-rP10 is induced in animal caps after treatment with Activin (de Beer, 2005). Other important players in mesoderm formation are the Nodal-related proteins, also members of the TGF-ß-superfamily. Injection of 50 pg Xnr1 into the ventral blastomeres of four-cell stage embryos induced ectopic expression IGFBP-rP10 on the ventral site of the embryos (Fig.17B, white arrow).

In animal caps Xnr1 was also able to induce expression of IGFBP-rP10 (Fig.17A). FGF (20 pg), another mesoderm inducing factor, induces the expression of the pan-mesodermal Xbra in animal caps, but has no effect on IGFBP-rP10 (Fig.17A, Isaacs et al., 1994; Isaacs et al., 1992). This demonstrates that IGFBP-rP10 is regulated by members of the TGF-ß family, which are required for mesoderm formation.

IGF activity is present on the dorsal side, like IGFBP-rP10, so it was checked if signalling molecules affected by IGF signalling also have an effect on its transcriptional regulation. Overexpression of IGF1 (200 pg) in animal caps had no effect on IGFBP-rP10 expression (Fig.17A). RT-PCR analysis of animal caps overexpressing Wnt8 (20 pg) showed that Wnt8 induces the expression of Xnr3 but not IGFBP-rP10 (Fig.17A). Overexpression of BMP4 (150 pg) also had no effect on IGFBP-rP10 expression (Fig.17A). This shows that the analysed signalling molecules involved in IGF signalling, namely IGF1, Wnt8 and BMP4 do not have an effect on the transcriptional regulation of IGFBP-rP10.

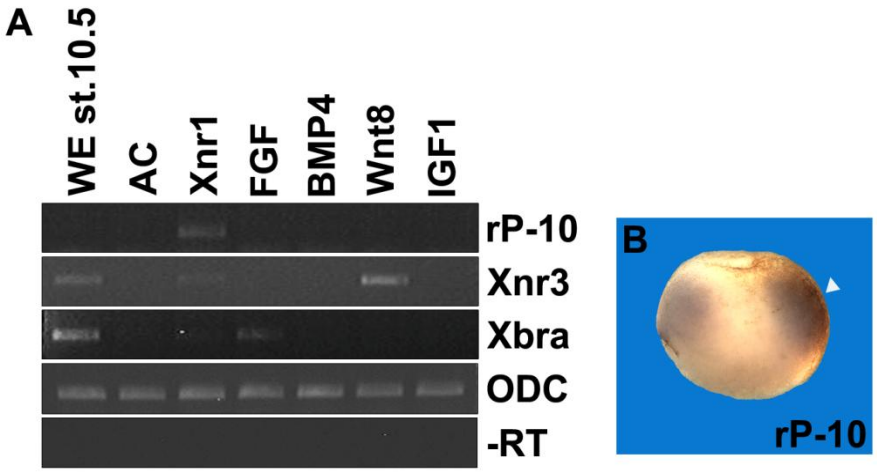


Fig. 17: Transcriptional regulation of IGFBP-rP10
(A) RT-PCR analysis of animal caps. 4-cell stage embryos were injected with 20 pg Xnr1 mRNA, 10 pg FGF mRNA, 150 pg BMP4 mRNA, 20 pg Wnt8 mRNA andor 200 pg IGF1. At blastula stage animal caps were dissected and grown until control siblings reached N.F. stage 10.5. Expression IGFBP-rP10, Xnr3, Xbra were analysed. ODC served as loading control. (B) *In-situ* hybridisation of IGFBP-rP10. White arrow marks ectopic IGFBP-rP10 expression after ventral injection with 50 pg Xnr1.

3.15 Characterisation of IGFBP-rP10 gain of function

To investigate the biological effects of IGFBP-rP10 an overexpression experiment was performed. Four-cell stage embryos were microinjected animally with 4 ng IGFBP-rP10 mRNA and allowed to develop until tailbud stage. In most cases the injected embryos showed no phenotype and looked like the control embryos. Few showed reduced eye or/and head structures (15%, n=41, Fig.18B) reminiscent of gain of BMP4 embryos (Koster et al., 1991). Injection of lower doses IGFBP-rP10 mRNA (2 ng) into animal blastomeres had no visible effect (Fig.18G). Injection of 1 ng IGFBP-rP10 mRNA into one dorsal blastomere at four-cell stage also showed no phenotypic effect (Fig.18I).

Injection into the two ventral blastomeres, restricted IGFBP-rP10 expression to areas where it is not endogenously found. To see if ectopic expression of IGFBP-rP10 is able to induce cement gland structures, like Mig30, an *in-situ* hybridisation was performed with the cement gland marker Xag1 (Sive et al., 1989). Ventral injection of IGFBP-rP10 did not show a phenotypic effect or an effect on cement gland structures and no ectopic cement gland patches were detectable (n=21, Fig.18K).

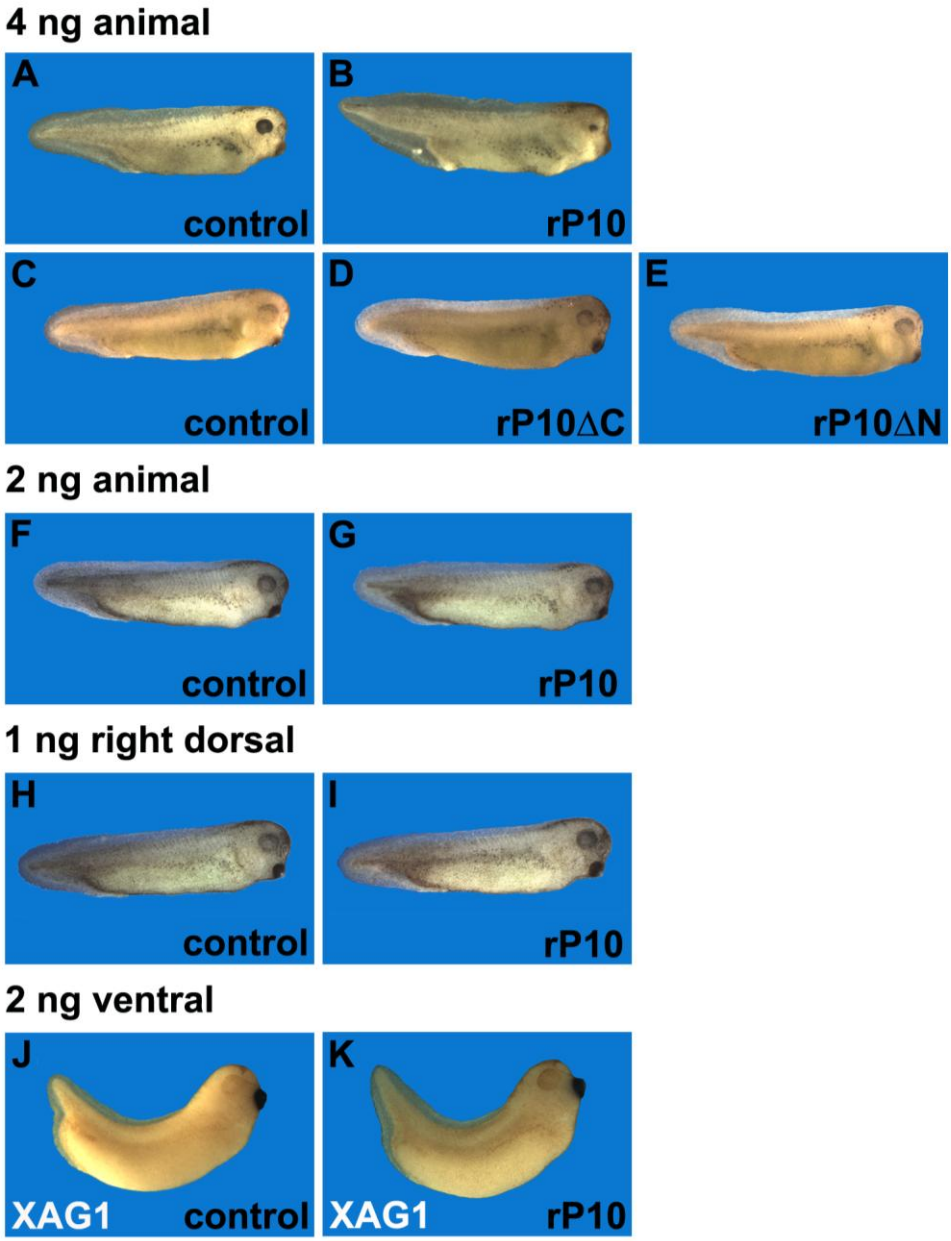


Fig. 18: Phenotypic analysis after IGFBP-rP10 overexpression
(A-I) stage 34-35 embryos, lateral view, control: uninjected control embryos. Animal injection of (B) 4 ng IGFBP-rP10 mRNA (D) 4 ng IGFBP-rP10ΔC mRNA, (E) 4 ng IGFBP-rP10ΔN mRNA and (G) 2 ng IGFBP-rP10 mRNA. (I) Injection of 1 ng IGFBP-rP10 mRNA into the right dorsal blastomere. (J,K) *In-situ* hybridisation. 4-cell stage embryos injected ventrally with 2 ng IGFBP-rP10 were analysed for Xag1 expression. Lateral view.

Since gain of the Mig30 mutant constructs Mig30ΔC and Mig30ΔN showed a more dramatic phenotype than Mig30 alone, the overexpression of the corresponding IGFBP-rP10 mutant constructs was analysed. IGFBP-rP10ΔC (rP10ΔC) comprises the signal peptide, the IB and the Kazal domain, while the C terminal IgC2 domain was removed. IGFBP-rP10ΔN (rP10ΔN) only contains the signal peptide followed by the IgC2 domain (de Beer, 2005). Four-cell stage

embryos were injected animally with 4ng rP10ΔC or rP10ΔN and scored at tailbud stage. Both mutant construct showed no phenotypic effect, the embryos looked like untreated control animals (Fig.:18D,E).

In summary, phenotypic analysis after gain of IGFBP-rP10 showed impaired eye and/or head structures only when high doses of IGFBP-rP10 mRNA were injected.

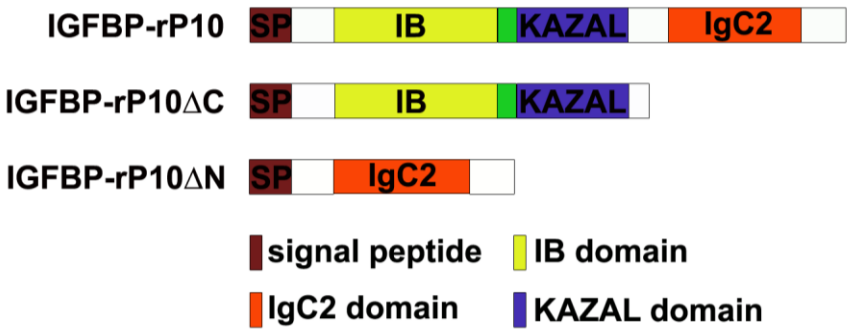


Fig. 19: Schematic diagram of IGFBP-rP10 and IGFBP-rP10 mutant constructs
SP: signal peptide (brown), IB: Insulin-like growth factor binding domain (yellow), IgC2: immunoglobulin C-2 type domain (orange), KAZAL: kazal type serine protease inhibitor domain (blue), overlap of IB and KAZAL (green; adapted from de Beer, 2005)

3.16 Characterisation of IGFBP-rP10 loss of function

To address the endogenous function of IGFBP-rP10 an antisense morpholino oligonucleotide knockdown approach was used. The knockdown of target genes in the tetraploid *Xenopus laevis* by antisense morpholino oligonucleotides is achieved by binding to their target mRNA and specifically blocking its translation. Therefore an antisense morpholino oligonucleotide against the transcriptional start codon of IGFBP-rP10 was designed. To test the specificity of IGFBP-rP10 antisense morpholino (IGFBP-rP10 morpholino, rP10Mo) plasmid DNA of myc-tagged IGFBP-rP10 that contains the specific target sequence was injected alone or in combination with IGFBP-rP10 morpholino. As a control myc-tagged Mig30 plasmid DNA was used. Embryos were collected at gastrula stages and protein amounts were analysed by Western blot using an anti myc-antibody. Injection of 200 pg IGFBP-rP10 DNA strongly induced expression of myc-tagged IGFBP-rP10. This was efficiently blocked by co-injection of 35 ng IGFBP-rP10 morpholino. myc-

tagged Mig30 was also expressed after injection with Mig30 DNA, but this was not blocked by co-injection of IGFBP-rP10 morpholino. This shows that IGFBP-rP10 morpholino efficiently blocks translation of IGFBP-rP10 and is specific for its target.

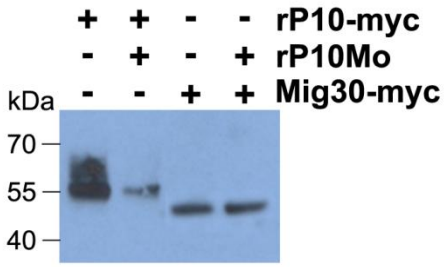


Fig. 20: IGFBP-rP10 specifically blocks IGFBP-rP10 translation
Western Blot. Embryos were injected animally with IGFBP-rP10myc cDNA and Mig30myc cDNA alone or in combination with IGFBP-rP10 morpholino. Protein expression was detected with an anti-myc antibody.

At neurulation stage knockdown of IGFBP-rP10 leads to disturbed anterior neural folding on the side of morpholino injection (35 ng, 85%, n=37; Fig.21B). At tailbud stage (N.F. st. 42) embryos show smaller eyes and reduced head structures after dorsal injection of 35 ng IGFBP-rP10 morpholino into one dorsal blastomere. This phenotype is very similar to the phenotype when IGFBP-rP10 is overexpressed in high doses. Although both loss and gain of IGFBP-rP10 show microcphaly and reduced eye structures, a dorsally curved phenotype was not detected when IGFBP-rP10 was overexpressed.

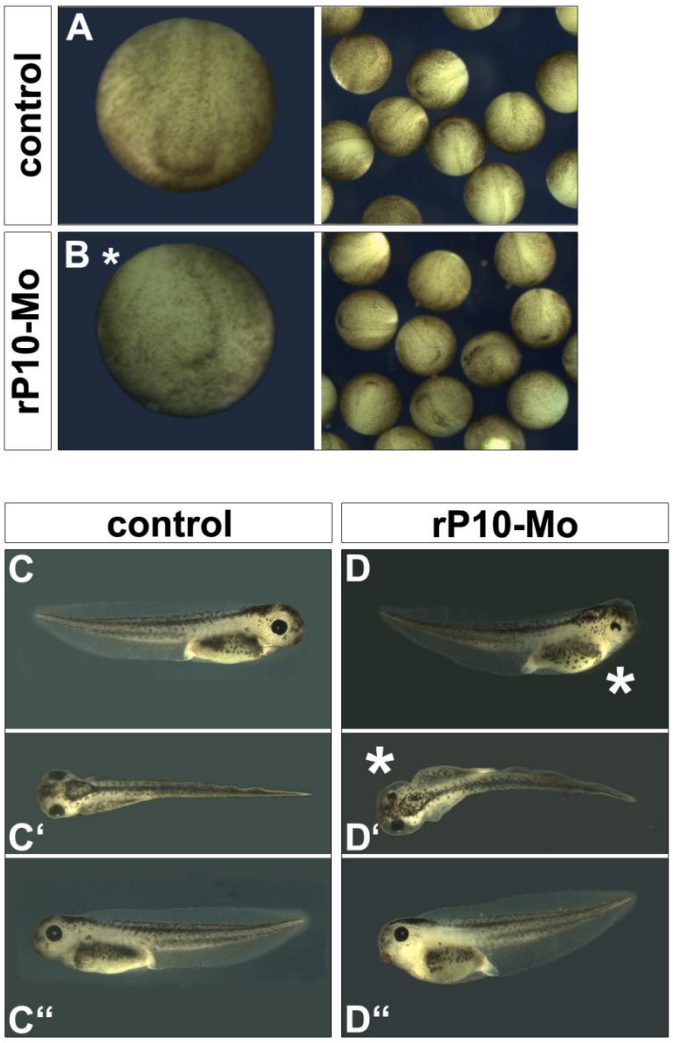


Fig. 21: Knockdown of IGFBP-rP10
4-cell stage embryos were injected into the right dorsal blastomere with 35 ng IGFBP-rP10 morpholino (A,B) Stage 17. Anterior view. (C,D) Stage 40, lateral right. (C', D') Stage40, dorsal view (C'', D'')Stage 40, lateral left uninjected.

To further analyse the phenotypic effects of IGFBP-rP10 knockdown, four-cell stage embryos were in injected dorsally with 35 ng IGFBP-rP10 morpholino into both dorsal blastomeres or only the right blastomere. Injection of IGFBP-rP10 morpholino in both dorsal blastomeres resulted in curved embryos with reduced head and eye structures at tailbud stage (42%, n=43; Fig.:22D). Spina bifida was seen for 12% and about 25% had reduced head and eye structures but were not curved. However the formation of the cement gland was not affected. The effect of the morpholino was strongly reduced when only one dorsal blastomere was injected. Then about 12% showed the strong phenotype (n=40; Fig.22D), 20% still had reduced head and eye structures (Fig.22C), while in about 32% only the eye was affected (Fig.22B). The phenotype of IGFBP-rP10 morphants is very similar to

the phenotype of Mig30 morphants, suggesting that IGFBP-rP10 and Mig30 might be redundant.

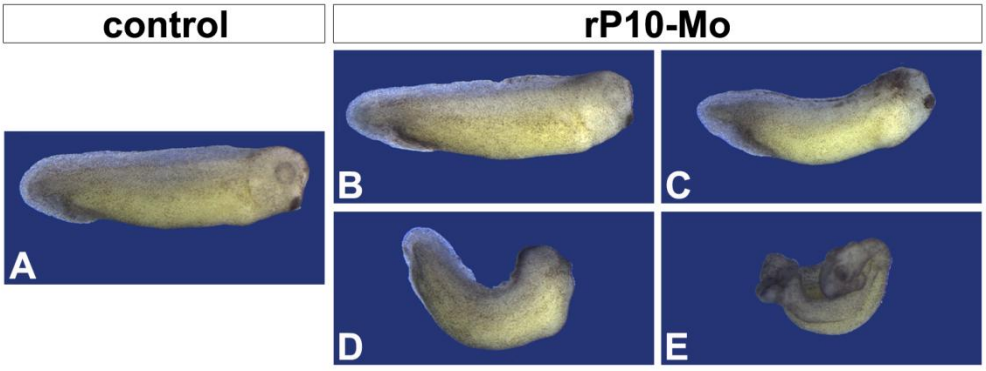


Fig. 22: Knockdown of IGFBP-rP10
(A) Uninjected control (B-E) Embryos were injected with 35 ng IGFBP-rP10 morpholino into one or both dorsal blastomeres and phenotypically scored in different classes (B) reduced eye (C) reduced eye and head structures (D) dorsal curvature, reduced eye and head structure (E) spina bifida. Lateral view.

3.17 Functional analysis of IGFBP-rP10

Since IGFBP-rP10 belongs to the IGFBP-rP superfamily and IGFBP-rP10 morphants phenotypically slightly resemble the loss of IGF signalling the possibility that IGFBP-rP10 interferes with IGF signalling was explored. For an animal cap assay, embryos were injected animally at four-cell stage. Animal caps were dissected at blastula stage and cultured until control siblings reached early gastrula stage. RT-PCR analysis showed that IGFBP-rP10 (800 pg) is able to activate transcription of Otx2, an anterior marker, which is required for cement gland formation and represses convergent extension to the same extend as are low doses of IGF1 (Carron et al., 2005). Co-injection of IGFBP-rP10 and IGF1 did not further increase the level of Otx2 transcription. However IGFBP-rP10 cooperates with IGF1 to reduce the expression of Msx1, a marker whose expression is BMP-dependent. Neither IGFBP-rP10 nor IGF1 have an effect on Msx1 expression on their own (Fig.23). This shows that IGFBP-rP10 can cooperate with IGF1 in the repression of BMP signalling.

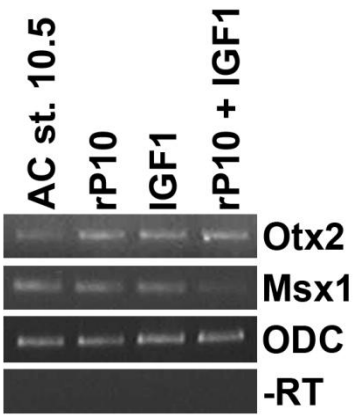


Fig. 23: Overexpression of IGFBP-rP10 induces Otx2 expression
Embryos were injected animally with 200 pg IGF1 mRNA, 800 pg IGFBP-rP10 mRNA or a combination of both. Animal caps were explanted at blastula stage and cultured to N.F. stage 10.5. Subsequently, RT-PCR was performed and expression of the anterior marker Otx2 and the BMP-dependent marker Msx1 were analysed. Ornithine decarboxylase (ODC) served as loading control. -RT, control reaction without reverse transcriptase.

Inhibition of BMP signalling plays an important role in head and neural development (Stern, 2005). IGF signalling contributes to BMP inhibition by linker phosphorylation of the signal transducer Smad1, which then is degraded or retained in the cytoplasm (Pera et al., 2003; Eivers et al., 2004). The IGFBP-rP10 gain of function phenotype resembles the gain of BMP phenotype, therefore the effect of IGFBP-rP10 overexpression on BMP signalling was analysed in a luciferase assay with the BRE-luc luciferase reporter construct, which contains multiple BMP response elements (Hata et al., 2000). Four-cell stage embryos were injected animally, cultured until gastrula stage and then luciferase activity measured. When BRE-luc reporter (100 pg) alone was injected, endogenous signals were not able to activate luciferase activity. Reporter activity was strongly induced when BMP4 was co-expressed (100 pg). This BMP4 induced activation was blocked when IGFBP-rP10 (1 ng) was added. IGFBP-rP10ΔC and IGFBP-rP10ΔN (1 ng each) were also able to block BMP induced reporter activation. This demonstrates that IGFBP-rP10 is able to antagonise BMP signalling.

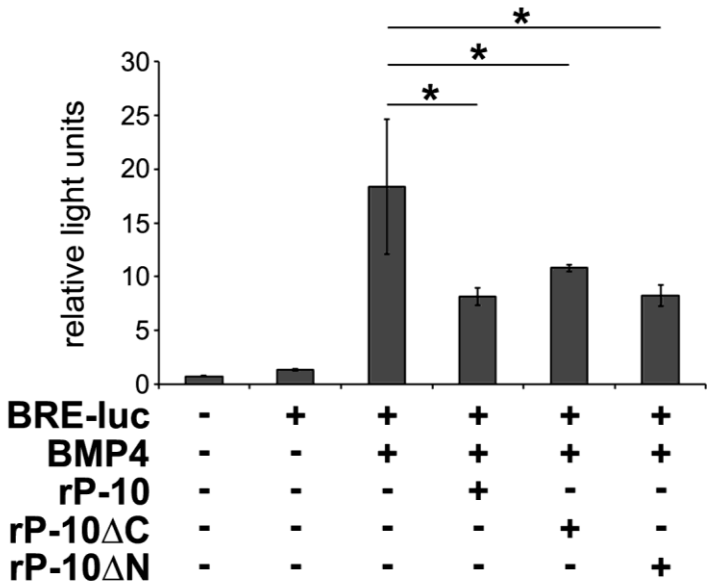


Fig. 24: IGFBP-rP10 overexpresion inhibits BMP signalling
Luciferase Assay. 4-cell stage embryos were microinjected as indicated into each animal blastomere. N.F. stage 10+ embryos were collected in pools of 4-5 embryos and assayed for luciferase activity in triplicates. Injection of 100 pg BRE-luc, 100 pg BMP4 mRNA, 1 IGFBP-rP10, 1 ng IGFBP-rP10ΔC mRNA and 1 ng IGFBP-rP10ΔN mRNA, as indicated. Level of significance in F test: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

In *Xenopus* another important pathway involved in head and neural induction is the canonical Wnt signalling pathway. Activation of the IGF signalling pathway intracellularly antagonises the Wnt/ß-catenin pathway (Pera et al., 2001; Richard-Parpaillon et al., 2002) Gain of Mig30 also inhibits Wnt signalling (Fig.1 and Kuerner, 2008). To check if IGFBP-rP10 also interferes with the Wnt signalling pathway a Topflash reporter construct was used (Korinek et al., 1997). Co-injection of Topflash reporter (20 pg) and Wnt8 (10 pg) lead to a strong transcriptional activation. This activation was blocked by addition of IGF1 (1.5 ng), confirming previous results (Richard-Parpaillon et al., 2002). Injection of IGFBP-rP10 (1.5 ng) had no effect on the Wnt8 induced transcriptional activation. Also IGFBP-rP10ΔC and IGFBP-rP10ΔN do not block reporter activation. To investigate if IGFBP-rP10 is able to interact intracellularly with Wnt signalling the reporter was stimulated with 50 pg ß-catenin. However co-injection of IGFBP-rP10 or the two mutant constructs did not inhibit ß-catenin induced transcriptional activation (Fig.25). This shows that the gain of IGFBP-rP10 does not affect Wnt signalling in this assay.

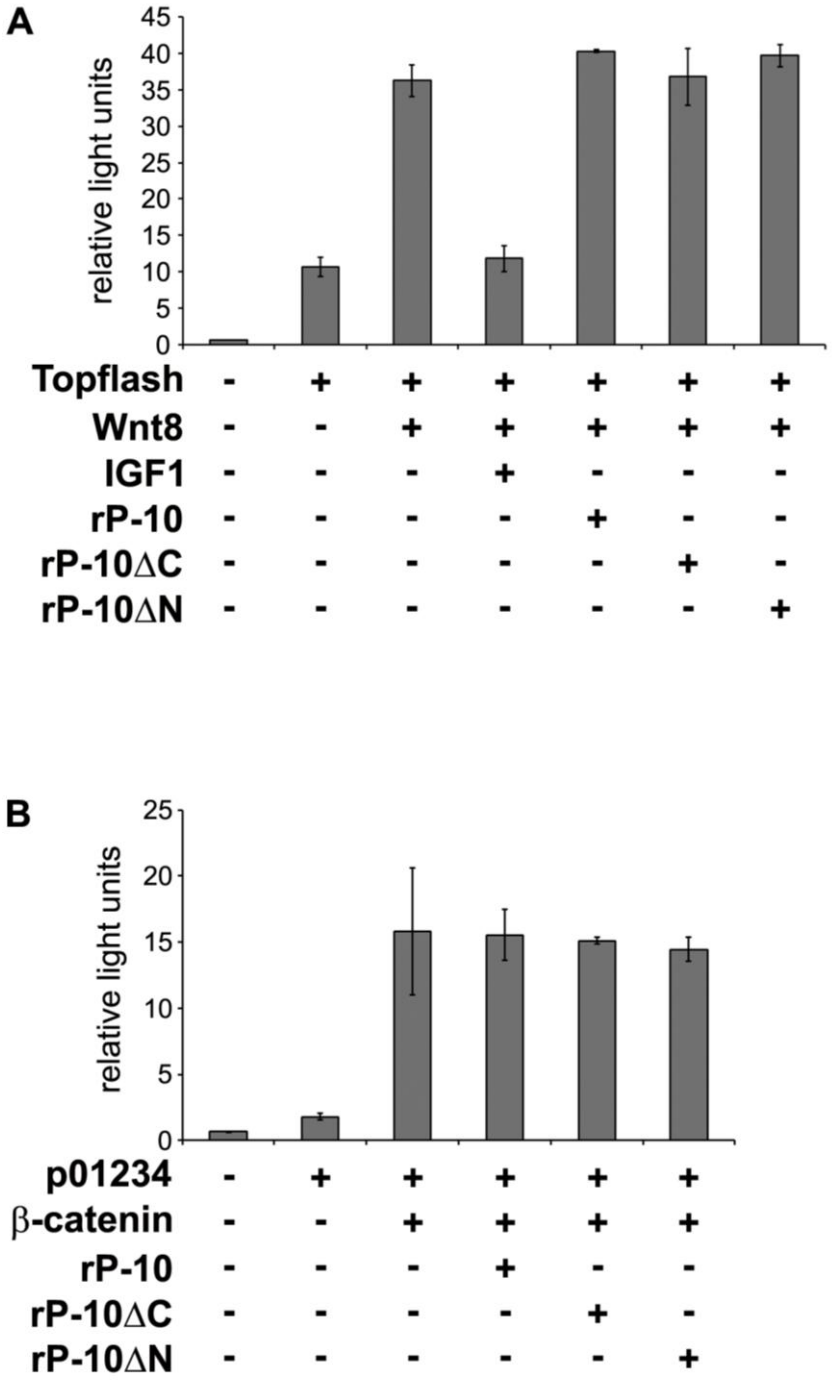


Fig. 25: Gain of IGFBP-rP10 does not inhibit Wnt signalling
(A,B) Luciferase Assay. 4-cell stage embryos were microinjected as indicated into each animal blastomere. N.F. stage 10+ embryos were collected in pools of 4-5 embryos and assayed for luciferase activity in triplicates. (A) Injection with 20 pg Topflash, 10 pg Wnt8 mRNA, 1.5 ng IGF1, 1.5 ng IGFBP-rP10, 1.5 ng IGFBP-rP10ΔC mRNA and 1.5 ng IGFBP-rP10ΔN mRNA (B) with 20 pg Topflash, 50 pg β-Catenin mRNA, 1.5 ng IGFBP-rP10, 1.5 ng IGFBP-rP10ΔC mRNA and 1.5 ng IGFBP-rP10ΔN mRNA.

Next the effect of gene expression after IGFBP-rP10 knockdown in gastrula embryos was analysed by *in-situ* hybridisation. To check if the endogenous IGFBP-rP10 also plays a role in BMP signalling we analysed the expression of

Vent2, which is expressed in cells of the marginal zone and animal cap region, excluding the organiser and whose expression is BMP-dependent (Onichtchouk et al., 1996). Dorsal injection of IGFBP-rP10 morpholino strongly reduced the expression domain of Vent2 (70%, n=27; Fig.26B). This suggests that endogenous IGFBP-rP10 is also able to interfere with BMP-signalling, similar to IGF signalling.

To check if endogenous IGFBP-rP10 also plays a role in Wnt signalling we analysed the expression of Myf5. Myf5 is a marker for lateral mesoderm, whose transcription depends on zygotic Wnt/ß-catenin signals (Marom et al., 1999; Yang et al., 2002). Embryos were injected dorsally with IGFBP-rP10 morpholino (35 ng) and fixed at gastrula stage. Injection of IGFBP-rP10 morpholino completely abolished the expression of Myf5 (90%, n=33; Fig.26D). This suggests that endogenous IGFBP-rP10 promotes Wnt signalling, possibly by interfering with IGF signalling.

The expression of the pan-mesodermal marker Xbra shows a ring-like marginal expression pattern, at N.F stage 10.5. Like Myf5, the expression of Xbra is dependent on functional zygotic Wnt/ß-catenin signalling. Injection of IGFBP-rP10 morpholino (35 ng) into both dorsal blastomeres, inhibits Xbra expression at the side of injection (80%, n=25; Fig.26F). Further supporting the idea that endogenous IGFBP-rP10 promotes Wnt signalling.

Next the expression pattern of Otx2 was analysed. Otx2 is a marker for anterior structures, which inhibits convergent extension movements and is required for cement gland formation (Blitz and Cho, 1995; Pannese et al., 1995). It was shown that at gastrula stages it is specifically upregulated by IGF signalling and overexpressing IGFBP-rP10 also slightly upregulates Otx2 expression in AC (Fig.23, Carron et al., 2005). Knockdown of IGFBP-rP10, by dorsal injection of 35 ng IGFBP-rP10 morpholino did not change the levels of Otx2 transcripts. But the expression domain of Otx2 appeared to be shorter and broader along the prospective A-P axis compared to uninjected control embryos (60%, n=10, Fig.23H).

In summary these knockdown results indicate, that endogenous IGFBP-rP10 plays a role in Wnt and BMP signalling, possibly by interfering with IGF signalling. But it remains to be elucidated by which molecular mechanisms IGFBP-rP10 performs these effects or if it is physiological relevant.

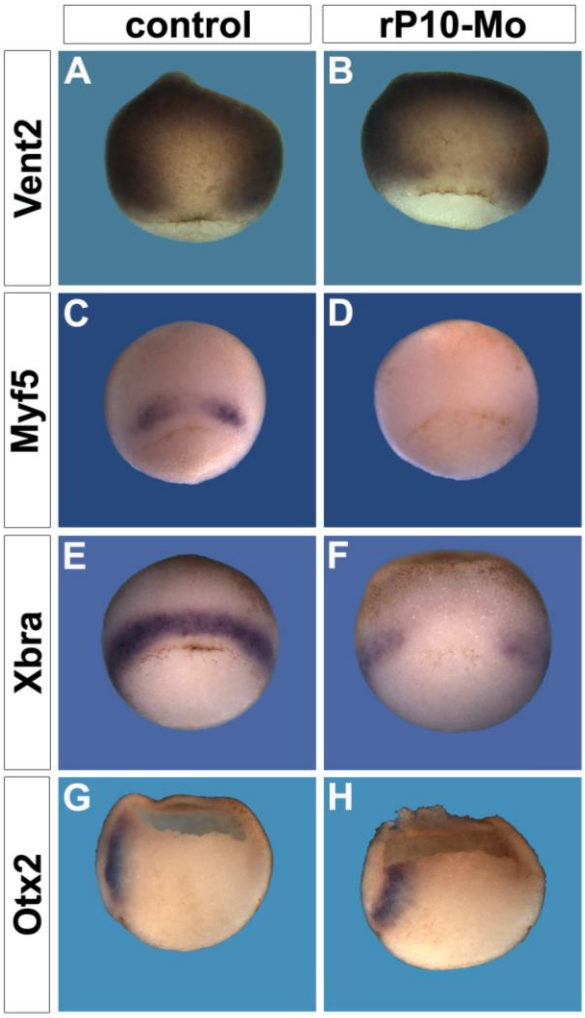


Fig. 26: Knockdown of IGFBP-rP10 reduces transcription of Vent2 and Myf5.
(A-J) *In-situ* hybridisation. 4-cell stage embryos were injected into both blastomeres with 35 ng IGFBP-rP10 morpholino or 1 ng IGFBP-rP10 mRNA, as indicated and analysed for marker expression at N.F. stage 10+. Control, uninjected sibling embryo. (A-C) Otx2 (D-F) Xbra (G,H) Myf5 (G,H) and (I,J) Vent2.

3.18 IGFBP-rP10 is involved in head development

Loss of IGFBP-rP10 leads to phenotypes with impaired head development. Also blocking the IGF pathway or Mig30 function leads to embryos with impaired head structures (Richard-Parpaillon et al., 2002; Pera et al., 2001; Kuerner, 2008), therefore the role of IGFBP-rP10 in head development was analysed. Embryos were injected into the right dorsal blastomere with 35 ng IGFBP-rP10 morpholino, fixed at neurula stages and *in-situ* hybridisation was performed. Injection of IGFBP-rP10 morpholino resulted in strong reduction of the expression of the hindbrain marker Krox20 and the expression of the forebrain marker Bf1 was also reduced (91%, n=12; Fig.27B, Bourguignon et al., 1998; Papalopulu et al., 1991).

In 45% of the injected embryos the size of the eye field was reduced, marked by the expression of the eye field marker Rx1 (n=17; Fig27D, Casarosa et al., 1997). Expression of the Wnt-dependent marker for the mid-hindbrain boundary, En2 was reduced in 88% of injected embryos (n=27; Fig.27F, McGrew et al., 1997; McMahon et al., 1992). This supports previous assumptions that endogenous IGFBP-rP10 interferes with Wnt signalling by inhibition of IGF signalling and shows that IGFBP-rP10 plays a role in brain patterning.

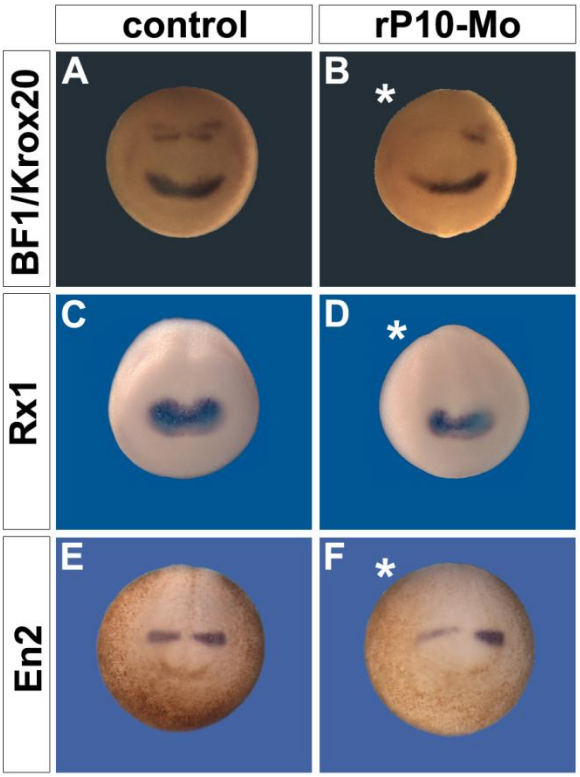


Fig. 27: Knockdown of IGFBP-rP perturbs brain patterning
In-situ **hybridisation. 4-cell stage embryos were injected into the right dorsal blastomere (marked with *) with 35 ng IGFBP-rP10 morpholino and analysed for marker expression at N.F. stages 16-18. Control, uninjected sibling embryo. (A,B) Bf1/Krox20 (C,D) Rx1 and (E,F) En2.**

3.19 IGFBP-rP10 is required for early neural development

Functional IGF signalling and Mig30 functions are not only required for proper development of head structures, but also for neural crest and neural plate induction (Fig.13, Pera et al., 2001; Richard-Parpaillon et al., 2002; Kuerner, 2008). IGFBP-rP10 is expressed on the dorsal side of the embryo during gastrulation, including the regions that later give rise to neural tissue. Additionally anterior neural folding, the place of neural crest induction, is disturbed in IGFBP-rP10 morphants (Fig.21B). Embryos were injected into one dorsal blastomere, with 35 ng IGFBP-rP10 morpholino, grown until neural stages and then analysed for neural crest, neural plate and neural differentiation markers.

Neural crest induction begins at gastrulation stages and requires active Wnt signalling and simultaneous inhibition of BMP signals (Steventon et al., 2009). Injection of IGFBP-rP10 morpholino (35 ng) reduced expression of Slug, an early marker for neural crest fate (75%, n=52; Fig.28, Mayor et al., 1995). To confirm that endogenous IGFBP-rP10 is required for neural crest development, a rescue experiment was performed. Embryos were injected with IGFBP-rP10 morpholino (17.5 ng), which inhibited the expression of Slug in about 50% of the cases (n=16, Fig.28). When 1 ng IGFBP-rP10 mRNA was co-injected, only 25% of the embryos showed reduced Slug expression. The rescue was even more significant when mRNA of the mouse homologue Bono1 (1 ng) was co-injected with the morpholino (12%, n=18; Fig 28). The overexpresion of IGFBP-rP10 or Bono1 had no effect on Slug expression compared to uninjected control embryos. This confirms the specificity of IGFBP-rP10 morpholino and the requirement for IGFBP-rP10 in neural crest development.

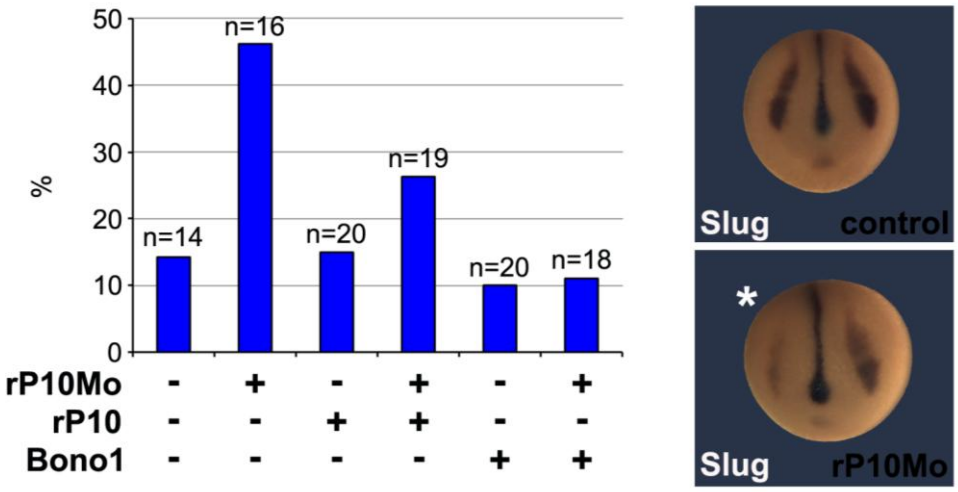


Fig. 28: IGFBP-rP10 is required for neural crest development.
IGFBP-rP10 morpholino rescue experiment. Evaluation of Slug repression after injection of 17.5 ng IGFBP-rP10 morpholino , 1 ng Bono1 mRNA, 1 ng IGFBP-rP10 or a combination of mRNAs and morpholino.

Neural induction is a complex process, which requires the interplay of different signalling pathways. Formation of the neural plate which comprises prospective neural tissue, is an important step in neural induction, which requires simultaneous inhibition of Wnt and BMP signals (Stern, 2005). Injection of IGFBP-rP10 morpholino led to an expansion of the neural plate markers Sox2 (100%, n=18; Fig29B, Mizuseki et al., 1998) and Sox3 (95%, n=20; Fig.29, Penzel et al., 1997). The expression domain of Epidermal keratin (78%, n=19, Fig.29F, LaFlamme and Dawid, 1990), which marks non-neural epidermal cells, was reduced on the morpholino injected side. Thus the knockdown of IGFBP-rP10 expands the neural plate on the expense of of adjacent neural crest and non-neural ectoderm.

To check if the expansion of prospective neural tissue, marked by Sox2 and Sox3 expression, also leads to an increase in neural differentiation, neural differentiation markers were analysed. The expression of the pan-neural marker NCAM was reduced in embryos injected with IGFBP-rP10 morpholino (86%, n=37, Fig.29J, Krieg et al., 1989). N-tubulin, a marker for differentiated neural tissue was also reduced, after IGFBP-rP10 morpholino injection (90%, n=44, Fig.29H, Chitnis et al., 1995). Neural differentiation is a Wnt-dependent process and reduction of neural differentation after loss of IGFBP-rP10 shows that Wnt signalling is inhibited when IGFBP-rP10 is knocked down. Both, neural induction and neural differentiation, require Wnt and BMP signalling tightly controlled for their proper

development. The fact that both are disturbed when endogenous IGFBP-rP10 is inhibited, indicates that IGFBP-rP10 might play a role in Wnt and/or BMP signalling.

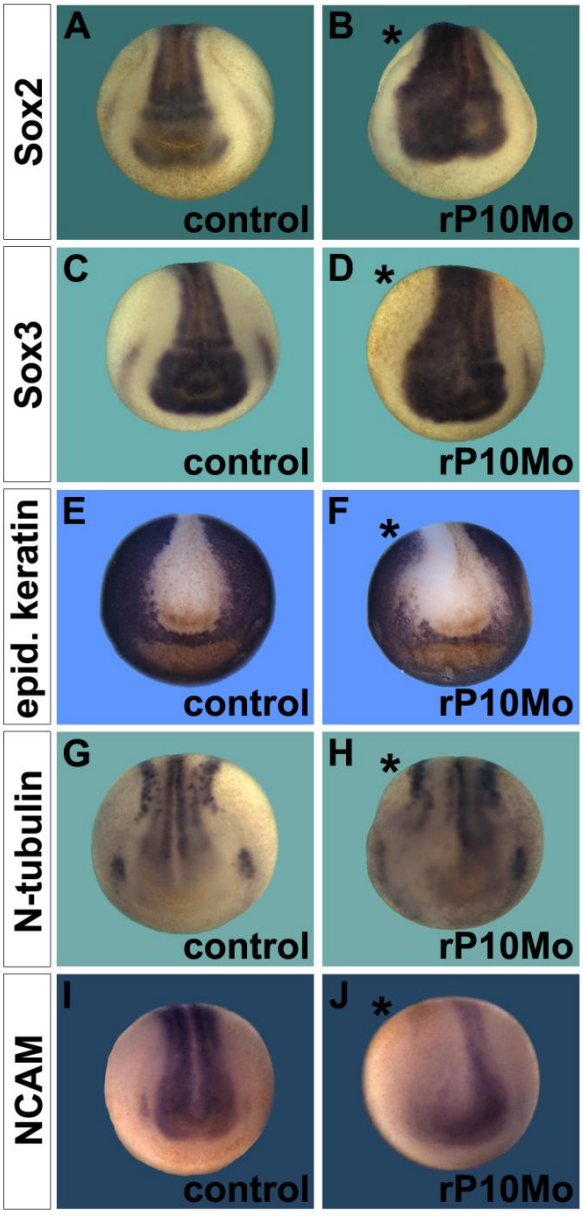


Fig. 29: Loss of IGFBP-rP10 affects neural development.
In-situ **hybridisation. 4-cell stage embryos were injected into the right dorsal blastomere (marked with *) with 35 ng Mig30 morpholino and analysed for marker expression at N.F. stages 16-18. Control, uninjected sibling embryo. (A,B) Sox2 (C,D) Sox3 (E,F) Epidermal keratin (G,H) NCAM and (I,J) N-tubulin.**

3.20 IGFBP-rP10 has no effect on IGF induced differentiation in RCJ3.1C5.18 cells

The IGFBP-rP10 loss of function data indicates that endogenous IGFBP-rP10, like Mig30, inhibits IGF signalling. To analyse if IGFBP-rP10 directly interacts with IGF1, the mesenchymal chondrogenic RCJ3.1C5.18 cells were used (Grigoriadis et al., 1996; McDougall et al., 1996; Lunstrum et al., 1999). The RCJ cell line is a model widely used for growth plate chondrocyte development (McEwen et al., 1999; Weksler et al., 1999; Cohen et al., 2006). RCJ cells spontaneously progress into differentiated growth plate chondrocytes and the differentiation can be enhanced by exogenous IGF1. The action of exogenous IGF1 can be directly studied because RCJ cells do not express IGF1 (Spagnoli et al., 2001).

RCJ cells were transiently transfected with either the empty pCS2+ vector or IGFBP-rP10. Mig30 was used as a positive control. The cells were cultured in differentiation medium from day 4 onward. At day 7 of culture, cells were serum-starved for 12 h and stimulated with 100 ng/ml IGF1 for another 12 h. The cells were harvested and transfection efficiency controlled. All samples showed that the transfection was successful and the plasmids were transcribed (data not shown). Then the expression of IGFBP5, which is upregulated during chondrocyte differentiation and ALP (alkaline phosphatase), a chondrocyte differentiation marker were analysed by qRT-PCR (Lunstrum et al., 1999; Spagnoli et al., 2001; Kiepe et al., 2005). IGFBP5 and ALP are expressed at low levels in cells transfected with empty pCS2+ vector, Mig30 or IGFBP-rP10 (Fig.30). The expression increases dramatically when the differentiation process is stimulated by additional treatment with IGF1 (Fig.30). This increase of IGFBP5 and ALP expression after stimulation with IGF1 is strongly reduced when cells were previously transfected with Mig30 (Fig.30). Transfection with IGFBP-rP10 has no effect on the IGF1 induced expression of IGFBP5 and ALP. This shows that IGFBP-rP10 is not able to interact with IGF1 in this assay. This is possibly due to the amino acid exchange in the IB domain of IGFBP-rP10.

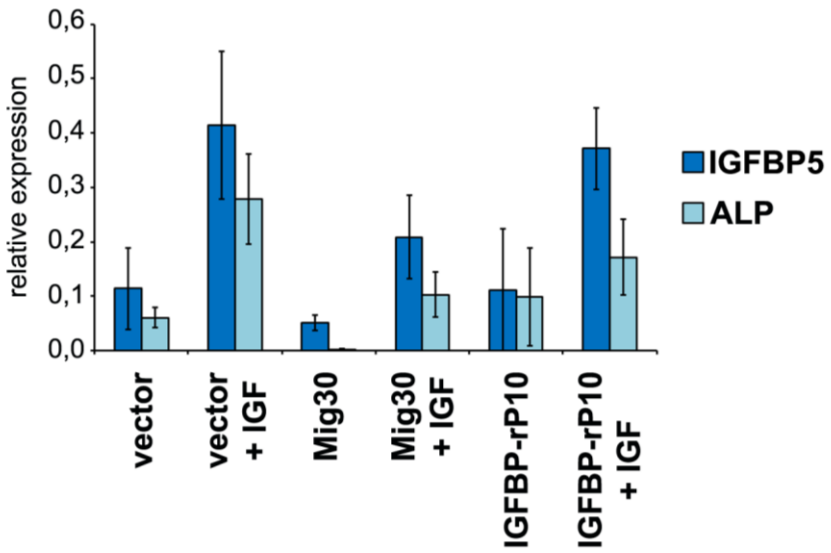
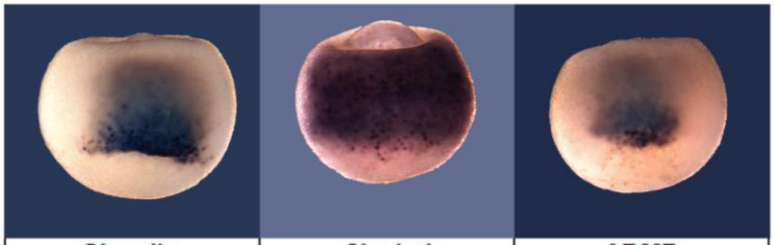


Fig. 30: IGFBP-rP10 has no effect on IGF1 induced differentiation of RCJ3.1C5.18 cells
RCJ cells were transfected as indicated, cultured in differentiation medium from day 4 onward. At day 7 cells were 12 h serum starved and then stimulated with 100 ng/ml IGF1. After 12 h cells were harvested and expression of IGFBP5 and ALP were measured by qRT-PCR.

3.21 IGFBP-rP10 has no effect on ADMP signalling

ADMP is a TGF-ß family member expressed in the Spemann organiser and during neurulation found in neural floor plate and prechordal plate. Overexpression of ADMP leads to embryos with severe anterior and dorsal defects (Moos et al., 1995). Gain of ADMP activates known BMP target genes, like Chordin and loss of ADMP, results in expansion of head structures and a decrease in BMP-dependent markers (Fuentealba et al., 2007). Since gain of function of IGFBP-rP10 also affects BMP signalling and ADMP is also expressed in the Spemann organiser, it was analysed if the two proteins might interact. At gastrula stage Chordin, Sizzled and ADMP expression were analysed by *in-situ* hybridisation after injection with IGFBP-rP10 mRNA or IGFBP-rP10 morpholino. ADMP expression is not altered after gain or loss of IGFBP-rP10 (Fig.30). Chordin expression is blocked by gain of ADMP, but since IGFBP-rP10 has no effect on ADMP, Chd expression was not altered (Fig.30). Sizzled is expressed on the ventral side of the embryo and is a negative feedback inhibitor of BMP signalling. Its expression is not disturbed after overexpression or knockdown of IGFBP-rP10.



		Chordin	Sizzled	ADMP
construct	amount	number of embryos		
uninjected control		13	31	14
rP10Mo dorsal	1 ng	11	25	6
rP10Mo ventral	1 ng	8	20	12
IGFBP-rP10 dorsal	35 ng	8	21	13
IGFBP-rP10 ventral	35 ng	9	21	12

Fig. 31: IGFBP-rP10 has no effect on ADMP signalling.
(A-C) *In-situ* hybridisation. 4-cell stage embryos were injected as indicated. (A) Chordin, stage 10.5 dorsal view (B) Sizzled, stage 10.5 ventral view and (C) ADMP, stage 10.5, dorsal view.

At later stages inhibition of ADMP by antisense morpholino oligonucleotides leads to strongly dorsalised embryos, while overexpressing ADMP has the opposite effect and ventralises embryos (Fuentealba et al., 2007). To check if IGFBP-rP10 might have an effect at later stages the expression of Otx2, Krox20 and Sizzled were analysed at N.F. stages 24-25. Otx2 is a marker for anterior head structures, and is expanded in dorsalised embryos and reduced in ventralised embryos. Krox20 marks two rhombomeres of the hindbrain and their expression is not affected by gain or loss of ADMP. The expression of Sizzled, the negative feedback inhibitor of BMP, signalling is strongly expanded in ventralised embryos and reduced in dorsalised embryos. Overexpression of IGFBP-rP10 (1 ng) by injection into both dorsal or ventral blastomeres had no effect on the expression of the three markers analysed. Also knockdown of IGFBP-rP10 did not affect the expression of Otx2, Krox20 and Sizzled. These results show that IGFBP-rP10 does not interfere with ADMP signalling.

construct	amount	Chd/Szl/Krox number of embryos
uninjected control		28
rP10Mo dorsal	1 ng	16
rP10Mo ventral	1 ng	17
IGFBP-rP10 dorsal	35 ng	16
IGFBP-rP10 ventral	35 ng	15

Fig. 32: IGFBP-rP10 has no effect on ADMP signalling.
***In-situ* hybridisation. 4-cell stage embryos were injected as indicated and analysed for Otx2, Sizzled and Krox20 expression at N.F. stages 24-25, lateral view**

3.22 Expression analysis of Bono1 in early mouse development

Bono1 was first identified in mouse, but not found before embryonic day 13 (James et al., 2004). IGF ligands and receptors on the other hand are present from pre-implantation stages on in embryonic- and extraembryonic cell lineages (Puscheck et al., 1998; Morali et al., 2000). Additionally IGF signalling is involved in the crosstalk between maternal and blastocyst derived tissue during implantation (Paria et al., 2001). In early mouse development at the time of implantation the blastocyst gives rise to three established cell lineages. The trophoectoderm, which forms extraembryonic ectoderm, the epiblast which forms the embryo proper and the primitive endoderm. The epiblast and the primitive endoderm are derived from pluripotent cells of the inner cell mass (ICM) of the blastocyst. The ICM derived primitive endoderm is highly motile, can differentiate into visceral endoderm (VE) or spread over the trophoectoderm and differentiate into parietal endoderm (PE) of the yolk sac. Wnt signalling plays an important role in the proper differentiation of these cell lineages. In pluripotent ICM cells Wnt/ß-catenin signalling is known to be active, while the differentiation to VE cells requires the inhibition of the Wnt/ß-catenin signalling pathway (Ralston and Rossant, 2005; Shibamoto et al., 2004). In *Xenopus* we found that Wnt and BMP signalling are inhibited when IGFBP-rP10 is knocked down. So we analysed if Bono1 is expressed in these extraembryonic endodermal cells. Therefore F9 embryonic carcinoma cells were used, because these cells are an established

model for extra-embryonic endoderm differentiation (Strickland and Mahdavi, 1978; Strickland et al., 1980). F9-cells were treated with retinoic acid or retinoic acid and cAMP to differentiate them into VE- or PE-like cells. After 7 days cells were collected and gene expression analysed by RT-PCR. The marker for endodermal tissue, Sox17 (Sry-related HMG-box transcription factor 17, Kim et al.) is upregulated when the cells were treated with RA or RA/cAMP, which shows that the differentiation in endoderm-like structures was successful (Fig.33). The expression level of Bono1 also increased, especially in PE-like cells treated with RA and cAMP (Fig.33). This indicates that Bono1 might be expressed in extraembryonic endodermal tissue during mouse development.

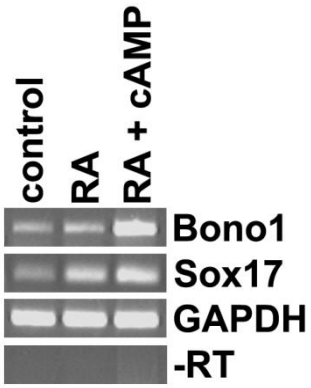


Fig. 33: Bono1 is expressed in differentiated F9-cells
RT-PCR analysis for Bono1 and Sox17 in F9-cells differentiated into visceral and parietal endoderm like cells. GAPDH served as loading control. -RT, control reaction without reverse transcription.

qRT-PCR analysis of 6.5 and 7.5 mouse embryos support the idea that Bono1 might be expressed in extraembryonic endodermal tissues. The expression of Bono1 in 6.5 and 7.5 mouse embryos and/or the surrounding deciduas was analysed. At embryonic day 6.5 Bono1 expression is high in the decidua, but also detectable in embryonic tissue. At embryonic day 7.5 the expression of Bono1 in the decidua is even stronger than at day 6.5, but very weak in embryonic tissues (Fig.34).

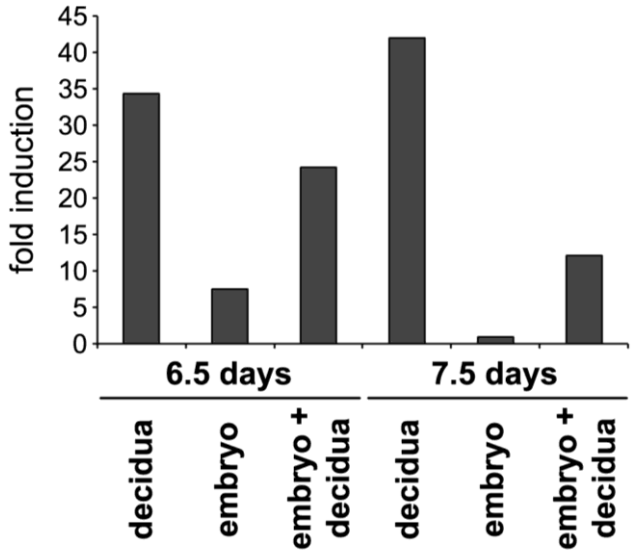


Fig. 34: Bono1 is expressed in the decidua.
qRT-PCR for Bono1 in d 6.5 and d 7.5 mouse embryos and extra-embryonic tissues

To look if Bono1 is really expressed in endodermal tissue in early mouse development, an *in-situ* hybridisation on cryosections was performed. Day 7.5 embryos were dissected including extraembryonic tissue and the surrounding decidua. The maternal tissue was included into the experiment, because a crosstalk between maternal and blastocyt derived signals is required for successful implantation and to prevent abortion. qRT-PCR analysis indicated that Bono1 is present there. The embryos were fixed and sagitally sectioned. Msg1 (melanocyte-specific gene1) specifically marks visceral endoderm (Dunwoodie et al., 1998). No expression was detected in the decidua, placenta or other embryonic structures (Fig.35). Bono1 was not detectable in endodermal tissues or the embryo, but it was found at the border of the decidua. This suggests that Bono1 might play a role in the crosstalk between maternal and embryo derived signals.

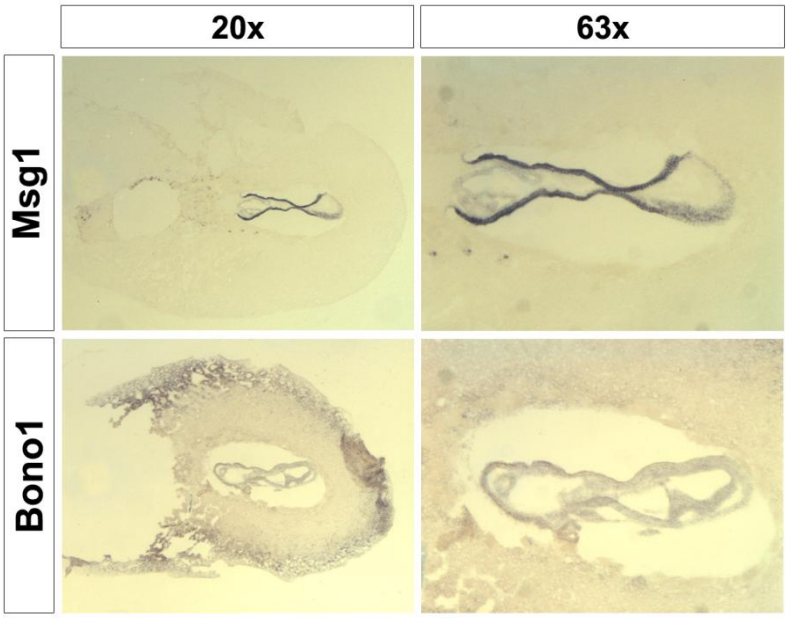


Fig. 35: Bono1 expression in the decidua
In-situ **hybridisation. D 7.5 embryos and the sourrounding deciduas were sagitally sectioned and analysed for Msg1 and Bono1 expression. 20x and 63x magnification.**

4 Discussion

4.1 Mig30 and IGFBP-rP10 function are connected to the IGF system

4.1.1 Gain of Mig30 and IGFBP-rP10 function

Mig30 and IGFBP-rP10 are two structurally related proteins which are classified as IGFBP-rPs. Some IGFBP-rPs are known to be able to bind IGFs *in vitro* (Oh et al., 1996; Kim et al., 1997; Burren et al., 1999). But so far no physiologically relevant role for IGFBP-rPs in IGF signalling has been demonstrated. In the early embryo the expression patterns of both Mig30 and IGFBP-rP10 overlap with the expression pattern of components of the IGF system. This makes them likely candidates for interaction with the IGF system. Additionally previous results in *Xenopus* strongly suggest that Mig30 plays a role in IGF signalling (Kuerner, 2008). Functional characterisation of Mig30 and IGFBP-rP10 should reveal whether Mig30 and IGFBP-rP10 play a role in IGF signalling or whether they have IGF-independent functions. Additionally comparative analysis will show redundant und non-redundant functions of the two proteins.

In the rat chondrogenic cell line RCJ3.1C5.18 ectopic addition of IGF1 induces differentiation (Spagnoli et al., 2001). This effect was used to analyse, whether Mig30 and IGFBP-rP10 are able to inhibit IGF1 function. In cells transfected with Mig30, IGF1 induced differentiation was blocked (Fig.8). This shows that Mig30 inhibits IGF1 function.

Importantly Mig30ΔC, which contains the putative IGF binding (IB) domain also inhibits chondrocyte differentiation. However Mig30ΔN, which lacks the IB domain, had no effect on chondrocyte differentiation (Fig.8). This demonstrates that the IB domain of Mig30 is responsible for the inhibition of IGF activity.

Surprisingly, IGFBP-rP10 had no effect on IGF1 induced chondrocyte differentiation (Fig.30). This difference from Mig30 is possibly due to an amino acid change in the core sequence of the IB domain thought critical for IGF binding (Kuerner and Steinbeisser, 2006).

Alternatively Mig30 and IGFBP-rP10 could modulate IGF activity by interaction with a protein that functions as a regulator of IGF signalling. In *Xenopus* the only protein known to play a role in IGF signalling is IGFBP5. It has been shown that

IGFBP5 potentiates IGF activities and therefore requires a functional IGF1R (Pera et al., 2001). Like the other components of the IGF signalling pathway it is co-expressed with Mig30 and IGFBP-rP10. Indeed, I showed that Mig30 affects IGFBP5 induced signalling. Overexpression of IGFBP5 in animal caps induces the transcription of Otx2 and Xag1 at neurula stages (Blitz and Cho, 1995; Sive et al., 1989; Pannese et al., 1995). This induction is blocked when high doses of Mig30 are co-expressed with IGFBP5 (Fig.6). This shows that Mig30 inhibits IGFBP5 induced signalling.

Overexpression of Mig30ΔC in combination with IGFBP5 shows opposing effects depending on the injected doses of Mig30ΔC. At low doses Mig30ΔC promotes IGFBP5 induced signalling, while at high doses IGFBP5 induced marker expression is repressed. Since Mig30ΔC contains the IB domain, which is required for IGF binding, the result suggests that at low doses Mig30ΔC binds to the free endogenous IGFs thereby prolonging their half-life and so promoting IGF activity. However at high doses of Mig30ΔC all free IGFs are sequestered and signalling cannot take place.

Mig30ΔN, which lacks a functional IB domain, is a strong inhibitor of IGFBP5 induced signalling at low and high doses (Fig.6). Mig30ΔN is comprised of the IgC2 domain, a domain known to be involved in cell-cell adhesion and recognition. IGFBP5 is also known to bind to the cell surface and extracellular matrix components, thereby sequestering IGFs in the vicinity of its receptor and promoting IGF signalling (Jones et al., 1993; Kiepe et al., 2001). When the interaction with the cell matrix is lost, IGFBP5 antagonises IGF signalling (Kalus et al., 1998). This suggests that Mig30ΔN blocks IGFBP5 function by displacing IGFBP5 from the cell matrix and thereby preventing presentation of IGFs to their receptors. Another possibility is that Mig30ΔN binds directly to IGFBP5, thereby preventing binding to IGF and subsequently receptor activation could also be blocked if Mig30ΔN binds to the IGF/IGFBP5 complex.

Opposing effects of different domains within a protein are also shown for the IGFBP-rP Cyr61. Cyr61 acts via its IB domain to elevate low level Wnt signalling, while it inhibits high levels of Wnt signalling via its cysteine-knot domain (Latinkic et al., 2003).

These findings show that the IB and IgC2 domain of Mig30 can have opposing effects on IGFBP5 induced signalling. Furthermore it indicates that the level of Mig30 needs to be precisely controlled for its normal activity.

By affecting two pathways involved in early patterning, namely Wnt and BMP signalling, IGF signalling plays an important role in early *Xenopus* development. IGFs are potent inhibitors of Wnt signalling, acting intracellularly on the level of GSK3 or ß-catenin (Pera et al., 2003; Pera et al., 2001; Richard-Parpaillon et al., 2002). Besides IGFs are inhibitors of BMP signalling. Here again they act intracellularly at the level of the BMP signal transducer Smad1 (Pera et al., 2003).

Mig30 also inhibits Wnt signalling intracellularly at the level of ß-catenin. This was demonstrated by using a reporter assay (Kuerner, 2008). I confirm these results (Fig.1B and Fig.1C) and additionally show that at high doses Mig30 is able to inhibit Wnt8 induced marker expression in animal cap explants (Fig.1A). The intracellular inhibition of the Wnt pathway mediated by Mig30 argues that there is no direct, extracellular interference with Wnt ligands or transmembrane receptors. Such an IGF-independent mode of inhibition was demonstrated for IGFBP4 and CTGF which directly bind to the Fz8 receptor and the co-receptor LRP6 and thereby prevent the binding of Wnt ligands (Zhu et al., 2008; Mercurio et al., 2004). The intracellular action of Mig30 is similar to Wnt inhibition by IGF signals and therefore it is likely that Mig30 inhibits Wnt signalling by promoting IGF signals.

IGFBP-rP10 has no effect on Wnt signalling in the reporter assay, indicating that a functional IB domain and therewith inhibition of IGF function is required for Wnt inhibition (Fig.25). Indeed Mig30ΔC antagonises Wnt8 and ß-catenin induced Wnt signalling in a reporter assay (Fig.4A and Fig.4B). This further supports that the IB domain is required for Wnt inhibition and that Mig30 inhibits Wnt signalling by promoting IGF signals.

Unexpectedly MigΔN inhibits Wnt signalling as efficiently as Mig30 and Mig30ΔC (Fig.4). Here the question arises how can this construct mediate Wnt inhibition and is this inhibition really IGF-dependent? One possible explanation would be that the IgC2 domain also has IGF binding capacities. Such binding capacities of the IgC2 domain are described for a drosophila insulin-related peptide-binding protein, which can bind to insulin-related proteins and human IGFs (Sloth Andersen et al., 2000). Another possible explanation is that Mig30ΔN is

able to interact with an additional cell surface receptor, whose activation channels into the IGF pathway. Several cell surface receptors have been described for IGFBP-rPs but none of these are reported to induce functions specific for IGF signalling (Syndecan1, integrins, HSPGs, LRPs, Notch1, tyrosine kinase receptor TrkA, Ahmed et al., 2003; Leask and Abraham, 2006). It is also possible that adding an excess of our molecule led to an activation of pathways normally not involved in the endogenous signalling events.

Activation of IGF signalling has also been shown to inhibit the BMP pathway (Pera et al., 2003). In animal caps Mig30 is able to inhibit the expression of the BMP-responsive gene Msx1 (Kuerner, 2008). Moreover I show that Mig30 inhibits BMP induced reporter activation (Fig.2). This shows that gain of Mig30 antagonises BMP signalling, further indicating that gain of Mig30 promotes IGF signalling.

Interestingly, overexpression of IGFBP-rP10 also inhibits BMP4 induced signalling in a reporter assay (Fig.24). This argues that the IB domain and therefore inhibition of IGF activity might not be required for BMP inhibition. Unexpectedly Mig30ΔC and IGFBP-rP10ΔC also block BMP signalling. The IB domains of Mig30 and IGFBP-rP10 contain several cysteines. Such cysteine-rich (CR) domains are found in known regulators of BMP signalling, like Chordin, Sizzled and Short gastrulation. (Sasai et al., 1994; Francois and Bier, 1995; Salic et al., 1997). This suggests that the two IGFBP-rPs directly interact with the BMP signalling pathway. This is supported by the finding that Mig30ΔC inhibits Chordin activity (Kuerner, 2008).

However this does not explain how Mig30ΔN and IGFBP-rP10ΔN, which lack the IB domain, inhibit BMP signalling.

In summary the gain of function data show that Mig30 can act as activator or inhibitor of IGF signalling, depending on the system analysed. For the inhibition of IGF1 function in rat chondrocytes the IB domain is essential, whereas antagonising IGFBP5 in *Xenopus* requires the IgC2 domain. In experimental conditions where Mig30 promotes IGF signalling the Wnt and BMP pathways are inhibited. IGFBP-rP10 is not able to block IGF function. In *Xenopus* gain of IGFBP-rP10 inhibits BMP signalling, but has no effect on Wnt signalling.

4.1.2 Loss of Mig30 and IGFBP-rP10 function

To address the endogenous function of Mig30 and IGFBP-rP10 and to avoid unspecific effects possibly induced by protein overexpression, a knockdown approach with antisense morpholino oligonucleotides was utilised.

Knockdown experiments support the finding that Mig30 inhibits IGFBP5 induced signalling. The expression pattern of Otx2 is increased when IGFBP5 is co-expressed with Mig30 antisense morpholino, compared to IGFBP5 overexpression alone (Fig.12).

Knockdown of Mig30 sensitises tissue for ectopic IGF signals. When Mig30 is knocked down in early embryos low doses of IGF1 are sufficient to induce an enlarged cement gland, which is not the case when low doses of IGF1 are expressed on their own (Fig.11). This result supports the finding that Mig30 interferes with IGF1 function. But it indicates that endogenous Mig30 is an inhibitor of IGF signalling, which opposes the gain of function data which suggest that Mig30 promotes IGF signalling.

In Mig30 morphants the transcription of Myf5, which depends on zygotic Wnt/ß-catenin, is blocked, as is the transcription of Vent2, a BMP-responsive gene (Fig.9 and 10, Marom et al., 1999; Hopwood et al., 1991; Karaulanov et al., 2004). This too, indicates that endogenous Mig30 impedes Wnt and BMP signalling rather than antagonise it. Consistent with this, BMP activity is inhibited in Mig30 morphants (Fig.10). Also, Wnt activity is blocked in a reporter assay when Mig30 is knocked down. Importantly, I show that Wnt inhibition in Mig30 morphants is an IGF-dependent process: blocking the IGF signalling pathway by co-expression of a dnIGFR construct could partially rescue Wnt signalling (Fig.10). Taken together these data show that endogenous Mig30 inhibits the IGF pathway and thereby promotes Wnt and BMP signalling.

Endogenous IGFBP-rP10 also seems to be an inhibitor of IGF signalling. In IGFBP-rP10 morphants the expression of the Wnt-dependent marker Myf5 and the BMP-responsive gene Vent2 are strongly reduced (Fig.26). That endogenous IGFBP-rP10 is an inhibitor of IGF signalling is further supported by its role in head and neural development (see chapter 8.2).

In summary, these results show that endogenous Mig30 and IGFBP-rP10 are inhibitors of IGF signalling and thereby promote Wnt and BMP signalling.

4.2 Mig30 and IGFBP-rP10 are required for head and neural development

Anterior development in vertebrates is a complex process, which requires a tightly balanced interplay of different signalling pathways to induce head and neural structures (Niehrs, 2004; Stern, 2005).

The process of head development was considered a default state, inhibiting the signals required for trunk development. The growth factor signals required to be inhibited for proper head formation are Wnt and BMP signals (Glinka et al., 1997; Glinka et al., 1998; Piccolo et al., 1999). Inhibition of only one pathway is not sufficient to induce secondary heads. Only Cerberus, a triple inhibitor of Wnt, BMP and Nodal signalling, and IGFs are able to induce secondary heads (Bouwmeester et al., 1996; Pera et al., 2001). Furthermore it was suggested that the IGF pathway participates in patterning because it regulates the amount of tissue allocated to head and trunk regions (Pera et al., 2001; Richard-Parpaillon et al., 2002).

Knockdown of Mig30 and IGFBP-rP10 led to embryos with reduced head structures, showing that both are required for head development. Analysis of spatially restricted brain markers showed that Mig30 and IGFBP-rP10 also play a role in brain patterning. I could demonstrate that in IGFBP-rP10 morphants the expression of Bf1, a forebrain marker, En2, a marker of the mid/hindbrain boundary and Krox20 a hindbrain marker, are disturbed (Fig.27, McMahon et al., 1992; McGrew et al., 1997; Bourguignon et al., 1998; Papalopulu et al., 1991). The same effect was shown previously in Mig30 morphants for Bf1 and Krox20, and I could show it for En2 (Fig.13, Kuerner, 2008). All three markers are known to require proper Wnt signalling for their expression (Kiecker and Niehrs, 2001). This indicates that by regulation of Wnt signalling, IGFBP-rP10 and Mig30 are involved in brain patterning.

The only BMP expressed in the organiser and known to play a role in patterning is ADMP (Anti Dorsalising Morphogentic Protein, Moos et al., 1995). The function of ADMP is to repress ectopic expression of head organiser genes (Dosch and Niehrs, 2000). Gain and loss of Mig30 and IGFBP-rP10 function do not affect ADMP signalling. (Fig.16, 31 and 32). This indicates that Mig30 and IGFBP-rP10 perform their function in head formation by inhibition of Wnt signalling.

During neural induction distinct regions of the ectoderm become specified to form neural plate and neural crest. The so called “default model” proposes that ectodermal cells acquire neural fate when they do not receive BMP signals. However neural induction is a complex process, which requires the interplay of different signalling pathways. It was shown that Wnt inhibition and functional FGF signalling are also required (Stern, 2005; Xu et al., 1997; Launay et al., 1996; Glinka et al., 1997; Sasai et al., 1996). Another active pathway required and sufficient for neural induction is the IGF signalling pathway. The IGF activities in neural induction and patterning are not easily separated into Wnt- or BMP-dependent defects as the Wnt and the BMP pathway are linked with each other at the level of Smad1 linker phosphorylation (Pera et al., 2003). At present, it is not fully understood how these signals are integrated.

It was shown that blocking Wnt signalling is sufficient to induce neural precursor fate marked by an expansion of the neural plate (Heeg-Truesdell and LaBonne, 2006). In Mig30 and IGFBP-rP10 morphants the expression patterns of Sox2 and Sox3, two markers for not yet committed neural precursors, are expanded (Mizuseki et al., 1998; Penzel et al., 1997). Furthermore, the expression of the epidermis marker Epidermal keratin is reduced (Fig.13 and 29, LaFlamme and Dawid, 1990). Thus knockdown of Mig30 and IGFBP-rP10 expand the neural plate at the expense of non-neural epidermal tissue, which implies that Mig30 and IGFBP-rP10 interfere with Wnt signalling.

Blocking Wnt signalling also inhibits neural differentiation (Heeg-Truesdell and LaBonne, 2006). Consistent with the hypothesis that knockdown of Mig30 and IGFBP-rP10 inhibits Wnt signalling, neural differentiation is blocked when IGFBP-rP10 and Mig30 are knocked down (Fig.13 and 29).

Mig30, unlike IGF signals, is not able to induce neural marker expression in ectodermal explants on its own (Fig.5). Additionally Mig30 does not affect FGF signalling which also plays a role in neural induction and patterning (Launay et al., 1996; Delaune et al., 2005; Sasai et al., 1996; Xu et al., 1997). Mig30 is therefore not able to inhibit XER81 expression induced by FGF signals in ectodermal explants (Fig.15, Munchberg and Steinbeisser, 1999). This finding suggests that Mig30 performs its function in neural induction and patterning by modulating endogenous IGF signals. The influence of IGFBP-rP10 on the FGF pathway has not yet been analysed. However since IGFBP-rP10 and Mig30 functions are

overlapping in neural development it is likely that IGFBP-rP10 also performs its functions by modulating IGF signals.

Wnt signalling is essential at the neural plate border where neural crest precursors are specified (Steventon et al., 2009). This is in line with my observation that in Mig30 morphants the expression of two neural crest markers, namely Slug and Twist, is strongly reduced (Fig.14, Aybar et al., 2003; Greaves et al., 1985). The expression of Slug is also reduced, when IGFBP-rP10 is knocked down. This further shows that Mig30 and IGFBP-rP10 interfere with Wnt signalling.

Surprisingly, at the end of neurulation, expression of Slug and Twist reappeared in Mig30 morphants (Fig.14). A recent study has proposed that neural crest induction is a multi-step process. They showed that the first inductive step requires Wnt activation and BMP inhibition, whereas the maintenance step requires simultaneous activation of both Wnt and BMP signalling, demonstrating a spatial and temporal requirement for neural crest inductive signals (Steventon et al., 2009). The results indicate that Mig30 is involved in this spatial and temporal regulation of neural crest specification.

Based on our knowledge of Mig30 and IGFBP-rP10 function in neural development I propose that during neurulation Mig30 and IGFBP-rP10 time and fine tune Wnt signalling in an IGF-dependent manner. This ensures active Wnt signalling required for neural crest specification and promotes the differentiation of Sox positive neural progenitors.

4.3 Bono1 is expressed in the decidua

The components of the IGF system are expressed very early in mouse embryogenesis and are already found in blastocysts and early in embryonic as well as in extraembryonic lineages (Puscheck et al., 1998; Morali et al., 2000). An important process in early mouse development is the differentiation of the inner cell mass (ICM) in embryonic and extraembryonic cell lineages. The ICM derived extraembryonic primitive endoderm cells are multipotent progenitors that can differentiate into parietal endoderm and visceral endoderm. Precise regulation of Wnt/ß-catenin and TGF-ß pathways is required for these differentiation processes.

In *Xenopus* we found that Wnt and BMP signalling are inhibited when IGFBP-rP10 is knocked down. Therefore I analysed whether Bono1, the mouse homologue of IGFBP-rP10, is expressed early in mouse development and if it is

found in extraembryonic endodermal cells. Bono1 is expressed in F9-cells, after differentiation into VE- or PE-like cells, indicating that Bono1 might be found in endodermal cells. qRT-PCR of d 6.5 and 7.5 mouse embryos and the surrounding decidual tissue suggested that Bono1 might also be found in the decidua. IGF signals are also known to be involved in the crosstalk between maternal and blastocyst derived tissue during implantation. The local response elicited by the implantation of a blastocyst or an IGF1 coated bead into a receptive uterus is expression of BMP2, which induces Bono1 expression in osteoblasts (Paria et al., 2001; Shibata et al., 2004). This could explain the finding that Bono1 is strongly expressed in the decidua and only weakly in endodermal tissues of day 7.5 mouse embryos (Fig.35).

The detection of Bono1 in early mouse embryos, raises the question of whether Bono1 also plays a role in the IGF system during early mouse development.

5 Materials and Methods

5.1 Materials

5.1.1 Chemicals

All chemicals, if not stated otherwise, were obtained from J.T.Baker, Merck, Roth, and Sigma-Aldrich.

Ampicillin	biomol
Aqua-Mount	Lerner
Bactotryptone	BD
Bromphenol Blue	Serva
ethidium bromide	Merck
Freon	Fluka
Gelatine	Sigma
L-cysteine	biomol
LE-Agarose	Biozym
Milk powder	Roth
Mowiol	Calbiochem
Penicillin	PAA Laboratories
Phenol-chloroform-isoamylalcohol	Fluka
RNase free water	Ambion
sheep serum	Sigma
Temed	biomol
tissue-TEK	O.C.T

5.1.2 Proteins, enzymes, inhibitors, and markers

All enzymes were obtained from Fermentas, Roche, and New England Biolabs if not stated otherwise.

10x protease inhibitor complete	Mini Roche
EuroTaq	Biocat
GeneRuler 1kb DNA ladder Plus	Fermentas
Human chorionic gonadotropin	Sigma
PageRuler Prestained protein ladder	Fermentas
Poly-L-Lysine	Sigma
Trypsin	PAA Laboratories
TurboFect	Fermentas
BMpurple AP-Substrate	Roche
Boehringer Blocking Reagent (BBR)	Roche

5.1.3 Buffers and Solutions

20x SSC	3M NaCl, 0.3M Sodium citrate
3x SDS-sample buffer	150 mM Tris-HCl (pH 6,8), 6% SDS, 0.3% Bromophenol Blue, 30% glycerol, 300 mM DTT, 6x loading buffer, 40% glycerol, 0.25% Bromophenol Blue
6x DNA loading buffer	40% glycerol, 0.25% Bromophenol Blue
Blocking solution (Western blot)	5% milk powder in PBS/0.1% Tween-20
DMEM high-glucose medium	PAA Laboratories
Hybridisation buffer (mouse)	50% Formamide, 5x SSC, 0.3 mg/ml Yeast tRNA, 0.1 mg/ml Heparin, 1x Denhardt's solution, 0.1 % Tween, 0.5 mM EDTA
Hybridisation buffer (*Xenopus*, 50 ml)	0.5 g BBR, 25 ml formamide, 12.5 ml 20x SSC, 6 ml H_2O, 5 ml Torula RNA (10 mg/ml), 100 µl Heparin (50 mg/ml), 250 µl 20 % Tween, 500 µl 10 % CHAPS, 500 µl 0.5 M EDTA
LB (2l)	20g bacto tryptone, 10g yeast extract, 20g NaCl
LB-Amp	50µg/ml ampicillin in LB
LB-Amp plate	1.5% agar in LB-Amp
MAB (10x, 200 ml)	23.2 g maleic acid (pH 7.5), 17.4 g NaCl
MBSH buffer	88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Na(NO)_3$, 0.41 mM $CaCl_2$, 10 mM HEPES (pH 7.4), 10 µg/ml streptomycin-sulfate, 10 µg/ml penicillin
MEMFA	0.1M MOPS (pH 7.4), 2 mM EGTA, 1 mM $MgSO_4$, 3.7 % formaldehyde
Mowiol	20 mg Mowiol, 80 ml PBS, 50 ml glycerol
PBS	126 mM NaCl, 2,7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4
PBS for cell culture	PAA Laboratories
pH-9-buffer	100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 0.1 % Tween20
RIPA buffer	0.1 % SDS, 0.5 % Na-deoxycholate, 1% NP-40, 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), proteinase inhibitor
SDS-PAGE running buffer	24.8 mM Tris, 192 mM glycine, 0.1 % SDS
TBE (10x)	890 mM Tris-borate, 0,2 mM EDTA, pH 8,0
Transfer buffer	24.8 mM Tris, 192 mM glycine, 20 % methanol
Tris/NaCl	100 mM Tris-HCl (pH 7.4), 100 mM NaCl
Western blot transfer buffer	24.8 mM Tris, 192 mM glycine, 20% methanol

5.1.4 Oligonucleotides

The following oligonucleotides were ordered from Operon or Sigma-Aldrich.

ODC for	GTCAATGATGGAGTGTATGGATC	RT-PCR
ODC rev	TCCATTCCGCTCTCCTGAGCAC	RT-PCR
Bf1 for	GCGGCAAAGACGGCGGCGACA	RT-PCR
Bf1 rev	CCGGCGCAGTTTGCCGGTGGT	RT-PCR
NCAM for	CCCACAGCCACTACAGCCACCGCA	RT-PCR
NCAM rev	TGCCTTTGGCACCAGGTCCGGC	RT-PCR
Pax6 for	CAG AAC ATC TTT TAC CCA GGA	RT-PCR
Pax6 rev	ACT ACT GCT AAT GGG AAT GTG	RT-PCR
Xag1 for	CTG ACT GTC CGA TCA GAC	RT-PCR
Xag1 rev	GAG TTG CTT CTC TGG CAT	RT-PCR
Otx2 for	GGA TGG ATT TGT TGC ACC AGT C	RT-PCR
Otx2 rev	CAC TCT CCG AGC TCA CTT CTC	RT-PCR
IGFBP-rP10 for	CAC ATG ACG GGC CCT GTG AA	RT-PCR
IGFBP-rP10 rev	CTG CAG CCA GCC AGT CAC TT	RT-PCR
Msx1 for	GTG TGA AGC CGT CCC TGG GC	RT-PCR
Msx1 rev	AGG CGG GTG GGC TCA TCC TT	RT-PCR
Xnr3 (EXON) for	CTA AAG AAC AGT CTC ATC C	RT-PCR
Xnr3 (EXON) rev	GAG CAA ACT CTT AAT GTA GG	RT-PCR
XER81 for	CTC ATGAATCAGAAGAACTCTTCC	RT-PCR
XER81 rev	TGGAATAGCTGTTATCAGAGATGG	RT-PCR
Xbra for	CACAGTTCATAGCAGTGACCG	RT-PCR
Xbra rev	TTCTGTGAGTGTACGGACTGG	RT-PCR
Sox2 for	CGA GTG AAG AGA CCC ATG AAC	RT-PCR
Sox2 rev	TTG CTG ATC TCC GAG TTG TG	RT-PCR
En2 for	TAC GGC CGG AGT TCG GGA GG	RT-PCR
En2 rev	AGA CCC AGG CTG GCC ACA GT	RT-PCR
ODC for	TGC ACA TGT CAA GCC AGT TC	qRT-PCR
ODC rev	GCC CAT CAC ACG TTG GTC	qRT-PCR
Otx2 for	GCA CCA GTC GGT GGG ATA	qRT-PCR
Otx2 rev	GCC CTG GTA AAA GTG GTC CT	qRT-PCR
Xag1 for	CCC CAC TAT ATA TTC TGC CAC TG	qRT-PCR
Xag1 rev	TGT TAT TCT TCA CAT AGG GCA ACA	qRT-PCR
Ep.ker. for	CAT TGG TGC TGG GTC TAA AGA T	qRT-PCR
Ep.ker. rev	TGC AGA GTC ACT GTA GCA TTA TCA	qRT-PCR
Bono1 for	GTC ACA TCC ATA TGA CAC TTG	RT-PCR
Bono1 rev	TGT GAG CAC TGT CAA GCT AG	RT-PCR
Sox17 for	GCC AAA AGA GCT TTT CAG ATA TAA	RT-PCR
Sox17 rev	AGG AAT TAA AGG CAA ATT TTG TG	RT-PCR
GAPDH for	TGC ACC ACC AAC TGC TTA	RT-PCR
GAPDH rev	GGA TGC AGG GAT GAT GTT	RT-PCR
Mig30 for	TGT TTG GCT CTA GGG CTC TG	qRT-PCR

Mig30 rev	CTG GCC TGG CCT ATT GAG T	qRT-PCR
IGFBP-rP10 for	GTG AAT CAG CAC CTC AGA TCC	qRT-PCR
IGFBP-rP10 rev	TCC TTT CCG GTA ATG TTC CA	qRT-PCR
rALP for	AAC AAC CTG ACT GAC CCT TCC C	qRT-PCR
rALP rev	TCA ATC CTG CCT CCT TCC ACT	qRT-PCR
rIGFBP5 for	AGT CGT GTG GCG TCT ACA CTG A	qRT-PCR
rIGFBP5 rev	TTT GCT CGC CGT AGC TCT TTT	qRT-PCR
r18S for	AGT TGG TGG AGC GAT TTG TC	qRT-PCR
r18S rev	GCT GAG CCA GTT CAG TGT AGC	qRT-PCR

5.1.5 Plasmids

Plasmids used in this work

Plasmid	**Source**
pCS2+Mig30	(Kuerner, 2008)
pCS2+Mig30ΔC	(Kuerner, 2008)
pCS2+Mig30ΔN	(Kuerner, 2008)
pCS2+ Mig30-myc	(Kuerner, 2008)
pCS2+IGFBP-rP10	(Kuerner, 2008)
pCS2+ IGFBP-rP10 myc	(Kuerner, 2008)
pCS2+IGFBP-rP10ΔC	(de Beer, 2005)
pCS2+IGFBP-rP10ΔN	(de Beer, 2005)
pCRII Topo Bono1	(James et al., 2004)
pCS2+IGF1	(Pera et al., 2001)
pCS2+IGFBP5	(Pera et al., 2001)
pCS2+dnIGFR	(Pera et al., 2001)
pCS2+BMP4	(Hess et al., 2008)
pCS2+	R. Rupp and D. Turner
pCS2+Xwnt8	(Christian and Moon, 1993)
XBF1	(Bourguignon et al., 1998)
Krox20	(Papalopulu et al., 1991)
pSP64T sense ß-cat	S. Schneider
pBSKS Otx2	(Blitz and Cho, 1995)
TA cloning Xag1	A. Schweickert

pSP72+ Xbra	(Smith et al., 1991)
pSP73 Myf5	R. Rupp
pMX 363 (Slug)	R. Rupp
pBISK II Msg1	(Dunwoodie et al., 1998)
pBSKs Vent2	(Onichtchouk et al., 1996)
pBSK Szl	A. Fainsod
Rx1	(Casarosa et al., 1997)
pSK Xnr1	(Jones et al., 1995)
pSP 64T XeFGF	(Isaacs et al., 1994)
pSP 64T ADMP	(Moos et al., 1995)
pSP35 chordin	(Sasai et al., 1994)
Topflash	(Korinek et al., 1997)
p01234-luc	(Brannon et al., 1997)
pBRE x4-E1b-d-luc	(Hata et al., 2000)
pGL 4.70 (hRluc)	Promega
Sox3	C. Niehrs
p33 En2	C.Niehrs
pNCAM	R. Rupp
Epidermal keratin	N. Papalopulu
N-tubulin	N. Papalopulu
pCS2+ Sox2	(Mizuseki et al., 1998)

5.1.6 Antibodies

α-myc 9E10	mouse	
α-mouse peroxidase	goat	Dianova

5.1.7 Bacteria and cells

E.coli Q 358 XL1, chemocompetent
F9 , mouse embryonal carcinoma (Bernstine et al., 1973)
RCJ3.1C5.18 , fetal rat calvaria (Spagnoli et al., 2001)

5.1.8 Kits

Absolute QPCR SYBR Green Rox Mix	Thermo Scientific
Lumi-lightplus Western Substrate	Roche
mMessage mMachine	Ambion
QIAquick Gel extraction kit	Qiagen
QIAquick PCR purification kit	Qiagen
Super Signal West Femto	Pierce
MasterPure Complete DNA and RNA Purification	EPICENTRE

5.1.9 Equipment and other materials

5415 D tabletop centrifuge	Eppendorf
ABI 7500 Fast Real-Time PCR cycler	Applied Biosystems
CC-12 digital camera	Olympus
Cronex 5 film	Agfa
Cryostat CM 30505	Leica
EasyCast electrophoresis system	Owl scientific
EpiChemie II Darkroom	UVP laboratory product
IM300 Microinjector	Narishige
JC-5 centrifuge	Beckman Coulter
KL 1500 electronic cold light source	Zeiss
Micromanipulator	Micro Instruments
NanoDrop ND-1000 Spectrophotometer	Thermo Scientific
NC2010 Gel cassettes 1.0 mm	Invitrogen
Novex XCell SureLock mini	Invitrogen
Optimax Typ TR x-ray film processor	Protec Medizintechnik
PD-5 Puller for producing microneedles	Narishige
Peltier Thermocycler PTC-200	MJ Research
Pipettes	Gilson
Protran BA 85 membrane	Whatman
Protran Nitrocellulose Transfer Membrane	Whatman
SZX12 stereo microscope	Olympus
Superfrost Plus	Thermo Scientific

5.1.10 Computer programs

Adobe Photoshop CS3	Adobe
Combine ZM	http://www.hadleyweb.pwp.blueyonder.co.uk
ImageJ 1.41n	NIH, USA
EndNote	Thomson Reuters
IBM SPSS Statistics 19	SPSS, Schweiz

5.2 Molecular Biology

5.2.1 Isolation of nucleic acids

5.2.1.1 Isolation of DNA

For isolation of plasmid DNA from bacteria, 2 ml (Miniprep) or 50 ml (Midiprep) of LB-Amp were inoculated with a single colony and cultered overnight, at 37°C shaking. If not mentioned otherwise, the nucleic acids were isolated with the appropriate kits (see 5.1.8 Kits) according to the manufacturers' instructions.

5.2.1.2 Isolation of total RNA

For isolation of total RNA from embryos, animal caps and cells, the tissues were collected and all additional buffers where removed before freezing at -80°C. Then total RNA was isolated using the MasterPure Complete DNA and RNA Purification from EPICENTRE. Including 1 h DNAse-digestion removing DNA-contamination.

5.2.1.3 Phenol-Chloroform purification

Phenol-chloroform extraction was used to separate nucleic acids from proteins and lipids. The aqueous solution was mixed with 1 volume phenol-chloroform-isoamylalcohol und centrifuged for 2 min at 13.200 rpm. The upper phase was transferred to a new tube, mixed with 1 volume chloroform, and centrifuged again. The upper phase was transferred to a new tube and nucleic acid was precipitated with ethanol or isopropanol.

5.2.1.4 Precipitation of nucleic acids

Alcohol precipitation was used to purify and/or concentrate RNA or DNA from aqueous solution. The nucleic acid solution was mixed with 1/10 volume 3M Sodium Acetate (pH 5.2), 2.5 volume ethanol (99%) or 1 volume isopropanol were added, and the mixture was incubated several hours at -20°C.

5.2.2 Restriction of DNA

Plasmids were digested using FastDigest restriction enzymes (Fermentas). The enzymes were chosen according to the further use of the linearised plasmids. The following reaction was set up:

10	µg	DNA
10	µl	FastDigest restriction buffer
5	µl	FastDigest restriction enzyme
x	µl	H_2O
100	µl	

Reactions were incubated for 10 to 30 minutes at 37°C and subsequently stopped by heat inactivation. DNA was precipitated with ethanol and restriction was analysed by agarose gel electrophoresis.

5.2.3 cDNA synthesis

Total RNA was reverse transcribed with RevertAIDH-Minus M-MuLV reverse transcriptase (Fermentas) using random hexamer primer (Fermentas). 1 µg RNA was mixed with 0.2 µg of random hexamer primer and incubated at 70°C for 10 min. 4 µl of 5x reaction buffer, 2 µl 10 mM dNTP mix and 0.5 µl RiboLock RNAse inhibitor were added, the reaction filled to 19 µl total volume by nuclease free water and incubated for 5 min at 25°C. After that 1 µl reverse transcriptase was added and the reaction was incubated at 42°C for 2 h. The reverse transcription was stopped by incubation at 70°C for 15 min. For further use the reaction was filled to a total volume of 50 µl with nuclease free water and stored at -20°C.

5.2.4 Polymerase chain reaction (PCR)

5.2.4.1 RT-PCR

To detect the expression of a target gene in different setups reverse transcription (RT)-PCR was used. Total RNA was isolated, using the EPICENTRE, RNA isolation Kit (see 5.2.1.2), and reverse transcribed (see 5.2.3).

RT-PCR was performed in a 10 µl reaction:

1	µl	cDNA
0.2	µl	EuroTaq
1	µl	10x reaction buffer
0.4	µl	$MgCl_2$
1	µl	2mM dNTPs
2	µl	Primermix (10µM each)
4.4	µl	H_2O

For amplification of the template cDNA the following PCR program was used:

cycles	time	temperature	
1	1 min	95 °C	
25-30	30 sec	95°C	dependent on primers
	45 sec	57-65°C	
	30 sec	72°C	
1	2 min	72°C	
1	∞	4°C	

After amplification the reactions were mixed with loading buffer and analysed by agarose gel electrophoresis.

5.2.4.2 qRT-PCR

To quantify gene expression quantitative Realtime-RT-PCR was performed on an ABI 7500 Fast Realtime PCR cycler. The qPCR reaction was setup in 10 µl.

4 µl	Primer Mix (1 µM each)
1 µl	cDNA
5 µl	Absolute QPCR SYBR Green Rox Mix
10 µl	

For amplifaction the following program was used

cycles	time	temperature
1	20 min	25 °C
1	1 min	95 °C
40	15 sec	95 °C
	1 min	63 °C
1	10 sec	25 °C

dissociation stage

5.2.5 *in vitro* transcription of RNA

5.2.5.1 Cap-mRNA

Cap-mRNA was synthesised with the mMassage mMachine kit according to the manufacturer's instructions. A 10 µl reaction was set up using 0.5 µg linearised plasmid and 1 µl enzyme mix. The Cap-mRNA was purified with phenol-chloroform extraction followed by isopropanol precipitation.

5.2.5.2 Digoxigenin (DIG)-RNA

DIG-RNA was synthesised with the DIG RNA Labeling Kit according to the manufacturer's instructions. A 20 µl reaction was set up using 1 µg linearised plasmid, 2 µl DIG labeling mix and 1 µl appropriate enzyme. The DIG-RNA was ethanol precipitated, resuspended and stored in 2x SSC/50% formamide.

5.2.6 Transformation of competent bacteria

50 µl competent bacteria were transformed with 100-200 µg purified plasmid DNA in 100 µl final volume. Chemocompetent cells were heatshocked for 45 sec at 42°C and subsequently placed on ice for 5 min. 900 ml LB medium were added and bacteria were cultured for 1 h shaking at 37°C. 100-200 µl of transformed bacteria were plated on LB-amp agar plates and cultured overnight at 37°C.

5.2.7 Agarose gel electrophoresis

1% to 2% agarose gels containing 5 µg/ml were prepared. DNA or RNA samples were mixed with 6x DNA loading buffer and loaded onto the gel. DNA or RNA fragments were then separated electrophoretically in TBE buffer at 100 V. To visualise nucleic acids, the gel was illuminated by UV light and imaged on a gel documentation system.

5.3 Embryological methods

5.3.1 *Xenopus laevis* embryo culture and manipulations

Xenopus females were injected with 500 IU human chorionic gonadotropin (Sigma). Approx. 15 h later eggs were collected and *in vitro* fertilised. Embryos were dejellied in 2% cysteine solution and microinjected in 1x MBSH solution. Embryos were staged according to (Nieuwkoop and Faber, 1967). The dorsal blastomeres of four-cell stage embryos were identified according to (Klein, 1987).

5.3.2 Animal cap assay

Four-cell stage embryos were injected animally. At st. 9 the vitelline membrane was removed and the animal cap excised with forceps. Animal caps were cultured for further use in 0.5x MBSH until control siblings reached the desired stage.

5.3.3 Whole mount *in-situ* hybridisation

To detect the spatial expression pattern of endogenous mRNA whole mount *in-situ* hybridisation was performed. Therefore embryos were fixed in MEMFA for several hours at RT and then stored in methanol. For rehydration embryos were sequentially washed 10 min in 75%, 50% and 25% MeOH in PBS. Short washing in PBS-T, was followed by a 20 min proteinase K digestion (10 µg/ml in PBS-T). After 2x 5 min washing in PBS-T the embryos were refixed in 4% paraformaldehyde in PBS. Paraformaldehyde was removed by 4x 5 min washing in PBS-T. Next embryos were equilibrated in hybridisation buffer and then prehybridised for 2-3 h at 65°C. DIG-RNA was added and allowed to hybridise o/n at 65°C.

On the second day embryos were washed with 50% formamide/5x SSC/0.1% CHAPS and then with 25% formamide/3.5x SSC/0.1% CHAPS for 5 min at RT. Two times washing for 5 min RT in 2x SSC/0.1% CHAPS., were followed by two times washing 25 min at 37°C. Next a serial washing in 0.2x SSC/0.1% CHAPS took place (5 min RT, 2x 30 min 60°C, 2x 5 min RT, 2x 10 min RT). The last washing steps were 4x 10 min in MABT before 1 h incubation in MABT containing 2% BBR. After 1 h incubation in MABT containing 2% BBR and 20% heat inactivated sheep serum, the buffer was replaced by fresh buffer additionally containing anti-DIG Fab_2 fragments (Fermentas 1:10 000). Embryos were

incubated o/n at 4°C after which excess and unspecific bound antibody was removed by 6x washing in MABT for 1 h.

For staining, embryos were equilibrated in pH-9-buffer for 15 min, after which the reaction was started by incubation in the dark in BM-purple (Roche)/pH-9-buffer. The reaction was stopped by washing in PBS. The embryos were refixed in 3.7% formaldehyde in PBS. When required pigments where removed by bleaching in 50% formamide/0.5x SSC/1% H_2O_2.Fixation of mouse embryos for cryosections

5.3.4 Fixation of mouse embryos for cryosections

Pregnant mice seven days after plug-check, were sectioned and all embryos (d 7.5) including extraembryonic tissue and decidua were collected. The embryos were rinsed in PBS and then placed in formaldehyde for fixation at 4°C o/n. After second rinse in PBS, embryos were placed in 20% sucrose solution until they sink to the bottom of the tube. The embryos were then embedded in mounting media (1:1 tissue-TEK (O.C.T) and Aqua-Mount (Lerner)) in the required position for sectioning. The mounting media was hardened fast by placing it in an ethanol/dry ice mixture. The sample was then placed for 1 h in the cryostat.

5.3.5 Cryosections

20 µM sections were cut at -20°C, collected on Superfrost Plus precoated glass slides and dried o/n. Dried cryosections were stored at -20°C.

5.3.6 *In-situ* hybridisation on cryosections

All vessels and slides were treated against RNA-contamination. All buffers and solutions used were prepared with DEPC water. Sections were taken from storage at -20°C and warmed to RT, and dried at 50°C for 15 minutes. After fixation for 20 minutes in 4% paraformaldehyde in PBS, the slides were washed 2x 5 min in PBS. Sections were penetrated by 5 µg/mL Proteinase K (Roche) in PBS for 8.5 min at RT. Following 1x 5 min wash with PBS, the sections were postfixed for 15 min in 4% paraformaldehyde in PBS. Paraformaldehyde was removed by 2-3x dipping the slides into DEPC treated water. To acetylate and neutralise positive charged proteins, slides were incubated with 0.25% acetic anhydride for 10 min under constant stirring. Following a 5 min wash in PBS, slides were prehybridised in hybridisation buffer for 1-4 h at 65°C in a humid chamber. DIG-labelled *in-situ*

probes were mixed with fresh hybridisation buffer and slides were incubated at 65°C o/n in a humid chamber. The second day 2x washing in 2x SSC at 60-65°C for 15 min, was followed by 2x washing at RT. Next RNAse treatment for 30 min, with 200 µl RNAse A (10 mg/ml) and 2 µl RNAse T1 (100 U/ml) in 200 µl 2x SSC at RT. To wash away unhybridised RNA, 2x 5 min 2x SSC, were followed by 2x 30 min washing in 0.2x SSC at 65°C and 2x 5 min 0.2x SSC at RT. To block unspecific antibody binding slides were incubated 1 h in PBT/10% sheep serum after 2x 30 min equilibration in PBT. Anti-DIG-AP-Fab was diluted in PBT/10% sheep serum 1:2000, and binding took place at 4°C o/n. On the third day 2x 30 min washing in PBT, was followed by equilibration in pH-9-buffer.The detection was performed in the dark with BM-purple substrate. When staining was detected the reaction was stopped by adding PBS. Slides were dried and mounted using Mowiol.

5.4 Cell culture methods

5.4.1 Maintaining and differentiation of F9-cells

F9-cell were maintained on gelatin-coated cell culture plates in high glucose DMEM supplemented with 10% fetal calf serum and 100 µg/ml penicillin and streptomycin at 37°C in a humified 5 % CO_2 incubator. At 80-90% confluency cells were subcultured. Cells were cultured for 7 days to differentiate into visceral endoderm (VE)-like by addition of 1 µM retinoic acid (RA) and into parietal endoderm (PE) by addition of 1 µM RA plus 100 µM cAMP (cyclic adenosine monophosphate)

5.5 Biochemical and immunological methods

5.5.1 SDS-PAGE and Western blot

Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using Novex Xcell SureLock mini chamber. A 12% separating gel topped with 6% stacking gel was prepared by polymerisation as described (Laemmli, 1970). Stained protein molecular weight standards and proteins were then separated at constant voltage of 160 V in SDS-PAGE running buffer.

After SDS-PAGE proteins were transferred to a nitrocellulose membrane by wet transfer at constant amperage (400 mA, 80 min) in Western blot transfer buffer. Transfer was checked by PoinceauS staining and the membrane was blocked in 5% milkpowder in PBST. Incubation of first antibody was o/n at 4°C. After 6 washing steps in PBST at RT, the membrane was incubated for 1 h at RT in the secondary antibody. After removing unspecific and excess antibody, by washing 6 x in PBST the specific signal was visualised by chemoluminiscence using SuperSignal West Femto Maximum Substrate (Thermo Scientific). The luminescence was registered on X-Ray film, which was developed using a X-Ray film processor (Protec Medizintechnik).

5.6 Luciferase reporter assay

Embryos were microinjected with either luciferase reporter TOPflash (Korinek et al., 1997) or pBREx4-E1b-dLuc (Hata et al., 2000) and synthetic mRNAs. Early gastrula embryos were collected in pools of 4-5 and assayed for luciferase activity in triplicates. Light units were normalized by co-injection of 2 pg Renilla luciferase plasmid (Promega). Error bars show standard deviation from mean of three independent samples. Significant differences were analysed by one-way ANOVA and post hoc Scheffé's ***F*** test. One representative result from reproducible and independent experiments is presented.

6 References

Abreu, J.G., Ketpura, N.I., Reversade, B., and De Robertis, E.M. (2002) Connective-tissue growth factor (CTGF) modulates cell signalling by BMP and TGF-beta. Nat Cell Biol 4:599-604.

Adams, T.E., Epa, V.C., Garrett, T.P., and Ward, C.W. (2000) Structure and function of the type 1 insulin-like growth factor receptor. Cell Mol Life Sci 57:1050-1093.

Ahmed, S., Yamamoto, K., Sato, Y., Ogawa, T., Herrmann, A., Higashi, S., and Miyazaki, K. (2003) Proteolytic processing of IGFBP-related protein-1 (TAF/angiomodulin/mac25) modulates its biological activity. Biochem Biophys Res Commun 310:612-618.

Akaogi, K., Okabe, Y., Funahashi, K., Yoshitake, Y., Nishikawa, K., Yasumitsu, H., Umeda, M., and Miyazaki, K. (1994) Cell adhesion activity of a 30-kDa major secreted protein from human bladder carcinoma cells. Biochem Biophys Res Commun 198:1046-1053.

Akaogi, K., Okabe, Y., Sato, J., Nagashima, Y., Yasumitsu, H., Sugahara, K., and Miyazaki, K. (1996) Specific accumulation of tumor-derived adhesion factor in tumor blood vessels and in capillary tube-like structures of cultured vascular endothelial cells. Proc Natl Acad Sci U S A 93:8384-8389.

Amaya, E., Stein, P.A., Musci, T.J., and Kirschner, M.W. (1993) FGF signalling in the early specification of mesoderm in Xenopus. Development 118:477-487.

Andreazzoli, M., Pannese, M., and Boncinelli, E. (1997) Activating and repressing signals in head development: the role of Xotx1 and Xotx2. Development 124:1733-1743.

Aubin, J., Davy, A., and Soriano, P. (2004) In vivo convergence of BMP and MAPK signaling pathways: impact of differential Smad1 phosphorylation on development and homeostasis. Genes Dev 18:1482-1494.

Aybar, M.J., Nieto, M.A., and Mayor, R. (2003) Snail precedes slug in the genetic cascade required for the specification and migration of the Xenopus neural crest. Development 130:483-494.

Baker, J., Liu, J.P., Robertson, E.J., and Efstratiadis, A. (1993) Role of insulin-like growth factors in embryonic and postnatal growth. Cell 75:73-82.

Baxter, R.C. (1994) Insulin-like growth factor binding proteins in the human circulation: a review. Horm Res 42:140-144.

Baxter, R.C., Binoux, M., Clemmons, D.R., Conover, C., Drop, S.L., Holly, J.M., Mohan, S., Oh, Y., and Rosenfeld, R.G. (1998) Recommendations for nomenclature of the insulin-like growth factor binding protein (IGFBP) superfamily. Growth Horm IGF Res 8:273-274.

Bernstine, E.G., Hooper, M.L., Grandchamp, S., and Ephrussi, B. (1973) Alkaline Phosphatase Activity in Mouse Teratoma. Proc Natl Acad Sci U S A 70:3899-3903.

Blitz, I.L., and Cho, K.W. (1995) Anterior neurectoderm is progressively induced during gastrulation: the role of the Xenopus homeobox gene orthodenticle. Development 121:993-1004.

Bourguignon, C., Li, J., and Papalopulu, N. (1998) XBF-1, a winged helix transcription factor with dual activity, has a role in positioning neurogenesis in Xenopus competent ectoderm. Development 125:4889-4900.

Bouwmeester, T., Kim, S., Sasai, Y., Lu, B., and De Robertis, E.M. (1996) Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of Spemann's organizer. Nature 382:595-601.

Bowman, C.J., Streck, R.D., and Chapin, R.E. Maternal-placental insulin-like growth factor (IGF) signaling and its importance to normal embryo-fetal development. Birth Defects Res B Dev Reprod Toxicol 89:339-349.

Brannon, M., Gomperts, M., Sumoy, L., Moon, R.T., and Kimelman, D. (1997) A beta-catenin/XTcf-3 complex binds to the siamois promoter to regulate dorsal axis specification in Xenopus. Genes Dev 11:2359-2370.

Brigstock, D.R. (2003) The CCN family: a new stimulus package. J Endocrinol 178:169-175.

Burren, C.P., Wilson, E.M., Hwa, V., Oh, Y., and Rosenfeld, R.G. (1999) Binding properties and distribution of insulin-like growth factor binding protein-related protein 3 (IGFBP-rP3/NovH), an additional member of the IGFBP Superfamily. J Clin Endocrinol Metab 84:1096-1103.

Butler, A.A., Yakar, S., Gewolb, I.H., Karas, M., Okubo, Y., and LeRoith, D. (1998) Insulin-like growth factor-I receptor signal transduction: at the interface between physiology and cell biology. Comp Biochem Physiol B Biochem Mol Biol 121:19-26.

Cai, Z., Chen, H.T., Boyle, B., Rupp, F., Funk, W.D., and Dedera, D.A. (2005) Identification of a novel insulin-like growth factor binding protein gene homologue with tumor suppressor like properties. Biochem Biophys Res Commun 331:261-266.

Canalis, E. (2009) Growth factor control of bone mass. J Cell Biochem 108:769-777.

Carron, C., Bourdelas, A., Li, H.Y., Boucaut, J.C., and Shi, D.L. (2005) Antagonistic interaction between IGF and Wnt/JNK signaling in convergent extension in Xenopus embryo. Mech Dev 122:1234-1247.

Casarosa, S., Andreazzoli, M., Simeone, A., and Barsacchi, G. (1997) Xrx1, a novel Xenopus homeobox gene expressed during eye and pineal gland development. Mech Dev 61:187-198.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D., and Kintner, C. (1995) Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. Nature 375:761-766.

Christian, J.L., and Moon, R.T. (1993) Interactions between Xwnt-8 and Spemann organizer signaling pathways generate dorsoventral pattern in the embryonic mesoderm of Xenopus. Genes Dev 7:13-28.

Claeys, I., Simonet, G., Poels, J., Van Loy, T., Vercammen, L., De Loof, A., and Vanden Broeck, J. (2002) Insulin-related peptides and their conserved signal transduction pathway. Peptides 23:807-816.

Clemmons, D.R. (1997) Insulin-like growth factor binding proteins and their role in controlling IGF actions. Cytokine Growth Factor Rev 8:45-62.

Clemmons, D.R. (2007) Modifying IGF1 activity: an approach to treat endocrine disorders, atherosclerosis and cancer. Nat Rev Drug Discov 6:821-833.

Cohen, M., Kam, Z., Addadi, L., and Geiger, B. (2006) Dynamic study of the transition from hyaluronan- to integrin-mediated adhesion in chondrocytes. EMBO J 25:302-311.

D'Ercole, J.A.a.Y., Ping (2008) Expanding the Mind: Insulin-Like Growth Factor I and Brain Development. Endocrinology 149:5958-5962.

Daughaday, W.H., Hall, K., Raben, M.S., Salmon, W.D., Jr., van den Brande, J.L., and van Wyk, J.J. (1972) Somatomedin: proposed designation for sulphation factor. Nature 235:107.

de Beer, D. (2005) Funktionelle Charakterisierung von IGFBP-rP10 in Xenopus laevis Embryonen. Fakultät für Biowissenschaften. Ruprecht Karls Universität, Heidelberg.

De Robertis, E.M. (2006) Spemann's organizer and self-regulation in amphibian embryos. Nat Rev Mol Cell Biol 7:296-302.

De Robertis, E.M., Larrain, J., Oelgeschlager, M., and Wessely, O. (2000) The establishment of Spemann's organizer and patterning of the vertebrate embryo. Nat Rev Genet 1:171-181.

Delaune, E., Lemaire, P., and Kodjabachian, L. (2005) Neural induction in Xenopus requires early FGF signalling in addition to BMP inhibition. Development 132:299-310.

Dosch, R., and Niehrs, C. (2000) Requirement for anti-dorsalizing morphogenetic protein in organizer patterning. Mech Dev 90:195-203.

Dunwoodie, S.L., Rodriguez, T.A., and Beddington, R.S. (1998) Msg1 and Mrg1, founding members of a gene family, show distinct patterns of gene expression during mouse embryogenesis. Mech Dev 72:27-40.

Eivers, E., McCarthy, K., Glynn, C., Nolan, C.M., and Byrnes, L. (2004) Insulin-like growth factor (IGF) signalling is required for early dorso-anterior development of the zebrafish embryo. Int J Dev Biol 48:1131-1140.

Firth, S.M., and Baxter, R.C. (2002) Cellular actions of the insulin-like growth factor binding proteins. Endocr Rev 23:824-854.

Francois, V., and Bier, E. (1995) Xenopus chordin and Drosophila short gastrulation genes encode homologous proteins functioning in dorsal-ventral axis formation. Cell 80:19-20.

Frasca, F., Pandini, G., Scalia, P., Sciacca, L., Mineo, R., Costantino, A., Goldfine, I.D., Belfiore, A., and Vigneri, R. (1999) Insulin receptor isoform A, a newly recognized, high-affinity insulin-like growth factor II receptor in fetal and cancer cells. Mol Cell Biol 19:3278-3288.

Fuentealba, L.C., Eivers, E., Ikeda, A., Hurtado, C., Kuroda, H., Pera, E.M., and De Robertis, E.M. (2007) Integrating patterning signals: Wnt/GSK3 regulates the duration of the BMP/Smad1 signal. Cell 131:980-993.

Ghosh, P., Dahms, N.M., and Kornfeld, S. (2003) Mannose 6-phosphate receptors: new twists in the tale. Nat Rev Mol Cell Biol 4:202-212.

Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., and Niehrs, C. (1998) Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature 391:357-362.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C., and Niehrs, C. (1997) Head induction by simultaneous repression of Bmp and Wnt signalling in Xenopus. Nature 389:517-519.

Greaves, D.R., Patient, R.K., and Lilley, D.M. (1985) Facile cruciform formation by an (A-T)34 sequence from a Xenopus globin gene. J Mol Biol 185:461-478.

Green, J.B., Howes, G., Symes, K., Cooke, J., and Smith, J.C. (1990) The biological effects of XTC-MIF: quantitative comparison with Xenopus bFGF. Development 108:173-183.

Green, J.B., New, H.V., and Smith, J.C. (1992) Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. Cell 71:731-739.

Grigoriadis, A.E., Heersche, J.N., and Aubin, J.E. (1996) Analysis of chondroprogenitor frequency and cartilage differentiation in a novel family of clonal chondrogenic rat cell lines. Differentiation 60:299-307.

Groigno, L., Richard-Parpaillon, L., and Boujard, D. (1999) Expression pattern of insulin receptor mRNA during Xenopus laevis embryogenesis. Mech Dev 86:151-154.

Harland, R. (2000) Neural induction. Curr Opin Genet Dev 10:357-362.

Hata, A., Seoane, J., Lagna, G., Montalvo, E., Hemmati-Brivanlou, A., and Massague, J. (2000) OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. Cell 100:229-240.

Haugk, K.L., Wilson, H.M., Swisshelm, K., and Quinn, L.S. (2000) Insulin-like growth factor (IGF)-binding protein-related protein-1: an autocrine/paracrine factor that inhibits skeletal myoblast differentiation but permits proliferation in response to IGF. Endocrinology 141:100-110.

Hayata, T., Tanegashima, K., Takahashi, S., Sogame, A., and Asashima, M. (2002) Overexpression of the secreted factor Mig30 expressed in the Spemann organizer impairs morphogenetic movements during Xenopus gastrulation. Mech Dev 112:37-51.

Heasman, J., Crawford, A., Goldstone, K., Garner-Hamrick, P., Gumbiner, B., McCrea, P., Kintner, C., Noro, C.Y., and Wylie, C. (1994) Overexpression of cadherins and underexpression of beta-catenin inhibit dorsal mesoderm induction in early Xenopus embryos. Cell 79:791-803.

Heeg-Truesdell, E., and LaBonne, C. (2006) Neural induction in Xenopus requires inhibition of Wnt-beta-catenin signaling. Dev Biol 298:71-86.

Hess, K., Steinbeisser, H., Kurth, T., and Epperlein, H.H. (2008) Bone morphogenetic protein-4 and Noggin signaling regulates pigment cell distribution in the axolotl trunk. Differentiation 76:206-218.

Hirsch, N., and Harris, W.A. (1997) Xenopus Pax-6 and retinal development. J Neurobiol 32:45-61.

Hopwood, N.D., Pluck, A., and Gurdon, J.B. (1991) Xenopus Myf-5 marks early muscle cells and can activate muscle genes ectopically in early embryos. Development 111:551-560.

Hou, J., Clemmons, D.R., and Smeekens, S. (2005) Expression and characterization of a serine protease that preferentially cleaves insulin-like growth factor binding protein-5. J Cell Biochem 94:470-484.

Hou, S., Maccarana, M., Min, T.H., Strate, I., and Pera, E.M. (2007) The secreted serine protease xHtrA1 stimulates long-range FGF signaling in the early Xenopus embryo. Dev Cell 13:226-241.

Hwa, V., Oh, Y., and Rosenfeld, R.G. (1999) Insulin-like growth factor binding proteins: a proposed superfamily. Acta Paediatr Suppl 88:37-45.

Isaacs, H.V., Pownall, M.E., and Slack, J.M. (1994) eFGF regulates Xbra expression during Xenopus gastrulation. EMBO J 13:4469-4481.

Isaacs, H.V., Tannahill, D., and Slack, J.M. (1992) Expression of a novel FGF in the Xenopus embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. Development 114:711-720.

James, M.J., Jarvinen, E., and Thesleff, I. (2004) Bono1: a gene associated with regions of deposition of bone and dentine. Gene Expr Patterns 4:595-599.

Jones, C.M., Kuehn, M.R., Hogan, B.L., Smith, J.C., and Wright, C.V. (1995) Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. Development 121:3651-3662.

Jones, J.I., and Clemmons, D.R. (1995) Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev 16:3-34.

Jones, J.I., Gockerman, A., Busby, W.H., Jr., Camacho-Hubner, C., and Clemmons, D.R. (1993) Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J Cell Biol 121:679-687.

Kalus, W., Zweckstetter, M., Renner, C., Sanchez, Y., Georgescu, J., Grol, M., Demuth, D., Schumacher, R., Dony, C., Lang, K., and Holak, T.A. (1998) Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. EMBO J 17:6558-6572.

Karaulanov, E., Knochel, W., and Niehrs, C. (2004) Transcriptional regulation of BMP4 synexpression in transgenic Xenopus. EMBO J 23:844-856.

Kato, M.V., Sato, H., Tsukada, T., Ikawa, Y., Aizawa, S., and Nagayoshi, M. (1996) A follistatin-like gene, mac25, may act as a growth suppressor of osteosarcoma cells. Oncogene 12:1361-1364.

Keller, R., Davidson, L., Edlund, A., Elul, T., Ezin, M., Shook, D., and Skoglund, P. (2000) Mechanisms of convergence and extension by cell intercalation. Philos Trans R Soc Lond B Biol Sci 355:897-922.

Keller, R.E., Danilchik, M., Gimlich, R., and Shih, J. (1985) The function and mechanism of convergent extension during gastrulation of Xenopus laevis. J Embryol Exp Morphol 89 Suppl:185-209.

Kiecker, C., and Niehrs, C. (2001) A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. Development 128:4189-4201.

Kiepe, D., Andress, D.L., Mohan, S., Standker, L., Ulinski, T., Himmele, R., Mehls, O., and Tonshoff, B. (2001) Intact IGF-binding protein-4 and -5 and their respective fragments isolated from chronic renal failure serum differentially modulate IGF-I actions in cultured growth plate chondrocytes. J Am Soc Nephrol 12:2400-2410.

Kiepe, D., Ciarmatori, S., Hoeflich, A., Wolf, E., and Tonshoff, B. (2005) Insulin-like growth factor (IGF)-I stimulates cell proliferation and induces IGF binding protein (IGFBP)-3 and IGFBP-5 gene expression in cultured growth plate chondrocytes via distinct signaling pathways. Endocrinology 146:3096-3104.

Kim, H.S., Nagalla, S.R., Oh, Y., Wilson, E., Roberts, C.T., Jr., and Rosenfeld, R.G. (1997) Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): characterization of connective tissue growth factor as a member of the IGFBP superfamily. Proc Natl Acad Sci U S A 94:12981-12986.

Kim, P.T., Hoffman, B.G., Plesner, A., Helgason, C.D., Verchere, C.B., Chung, S.W., Warnock, G.L., Mui, A.L., and Ong, C.J. Differentiation of mouse embryonic stem cells into endoderm without embryoid body formation. PLoS One 5:e14146.

Kimelman, D., and Kirschner, M. (1987) Synergistic induction of mesoderm by FGF and TGF-beta and the identification of an mRNA coding for FGF in the early Xenopus embryo. Cell 51:869-877.

Klein, S.L. (1987) The first cleavage furrow demarcates the dorsal-ventral axis in Xenopus embryos. Dev Biol 120:299-304.

Kontges, G., and Lumsden, A. (1996) Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development 122:3229-3242.

Korinek, V., Barker, N., Morin, P.J., van Wichen, D., de Weger, R., Kinzler, K.W., Vogelstein, B., and Clevers, H. (1997) Constitutive transcriptional activation by a beta-catenin-Tcf complex in APC-/- colon carcinoma. Science 275:1784-1787.

Koster, M., Plessow, S., Clement, J.H., Lorenz, A., Tiedemann, H., and Knochel, W. (1991) Bone morphogenetic protein 4 (BMP-4), a member of the TGF-beta family, in early embryos of Xenopus laevis: analysis of mesoderm inducing activity. Mech Dev 33:191-199.

Kretzschmar, M., Doody, J., and Massague, J. (1997a) Opposing BMP and EGF signalling pathways converge on the TGF-beta family mediator Smad1. Nature 389:618-622.

Kretzschmar, M., Liu, F., Hata, A., Doody, J., and Massague, J. (1997b) The TGF-beta family mediator Smad1 is phosphorylated directly and activated functionally by the BMP receptor kinase. Genes Dev 11:984-995.

Krieg, P.A., Sakaguchi, D.S., and Kintner, C.R. (1989) Primary structure and developmental expression of a large cytoplasmic domain form of Xenopus laevis neural cell adhesion molecule (NCAM). Nucleic Acids Res 17:10321-10335.

Kuerner, K.-M. (2008) Functional analysis of the insulin-like growth factor binding protein-related protein Mig30 in Xenopus laevis. Fakultät für Biologie. Eberhard Karls Universität Tübingen, Tübingen.

Kuerner, K.M., and Steinbeisser, H. (2006) Expression analysis of IGFBP-rP10, IGFBP-like and Mig30 in early Xenopus development. Dev Dyn 235:2861-2867.

Kuroda, H., Wessely, O., and De Robertis, E.M. (2004) Neural induction in Xenopus: requirement for ectodermal and endomesodermal signals via Chordin, Noggin, beta-Catenin, and Cerberus. PLoS Biol 2:E92.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

LaFlamme, S.E., and Dawid, I.B. (1990) Differential keratin gene expression during the differentiation of the cement gland of Xenopus laevis. Dev Biol 137:414-418.

Lassalle, P., Molet, S., Janin, A., Heyden, J.V., Tavernier, J., Fiers, W., Devos, R., and Tonnel, A.B. (1996) ESM-1 is a novel human endothelial cell-specific molecule expressed in lung and regulated by cytokines. J Biol Chem 271:20458-20464.

Latinkic, B.V., Mercurio, S., Bennett, B., Hirst, E.M., Xu, Q., Lau, L.F., Mohun, T.J., and Smith, J.C. (2003) Xenopus Cyr61 regulates gastrulation movements and modulates Wnt signalling. Development 130:2429-2441.

Latinkic, B.V., Umbhauer, M., Neal, K.A., Lerchner, W., Smith, J.C., and Cunliffe, V. (1997) The Xenopus Brachyury promoter is activated by FGF and low concentrations of activin and suppressed by high concentrations of activin and by paired-type homeodomain proteins. Genes Dev 11:3265-3276.

Launay, C., Fromentoux, V., Shi, D.L., and Boucaut, J.C. (1996) A truncated FGF receptor blocks neural induction by endogenous Xenopus inducers. Development 122:869-880.

Leask, A., and Abraham, D.J. (2006) All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci 119:4803-4810.

LeRoith, D., Bondy, C., Yakar, S., and Liu, J.L. (2001) The somatomedin hypothesis:2001. Endocr Rev 22:53-74.

LeRoith, D., and Roberts, C.T., Jr. (2003) The insulin-like growth factor system and cancer. Cancer Lett 195:127-137.

LeRoith, D., and Yakar, S. (2007) Mechanisms of disease: metabolic effects of growth hormone and insulin-like growth factor 1. Nat Clin Pract Endocrinol Metab 3:302-310.

Liu, J.P., Baker, J., Perkins, A.S., Robertson, E.J., and Efstratiadis, A. (1993) Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). Cell 75:59-72.

Lunstrum, G.P., Keene, D.R., Weksler, N.B., Cho, Y.J., Cornwall, M., and Horton, W.A. (1999) Chondrocyte differentiation in a rat mesenchymal cell line. J Histochem Cytochem 47:1-6.

Maki, R.G. (2010) Small is beautiful: insulin-like growth factors and their role in growth, development, and cancer. J Clin Oncol 28:4985-4995.

Marom, K., Fainsod, A., and Steinbeisser, H. (1999) Patterning of the mesoderm involves several threshold responses to BMP-4 and Xwnt-8. Mech Dev 87:33-44.

Mayor, R., Morgan, R., and Sargent, M.G. (1995) Induction of the prospective neural crest of Xenopus. Development 121:767-777.

McDougall, S., Fu, Y.H., Lowe, G.N., Williams, A., Polendo, R., Benya, P.D., Iida-Klein, A., Fang, M.A., and Hahn, T.J. (1996) Surface adhesion-mediated regulation of chondrocyte-specific gene expression in the nontransformed RCJ 3.1C5.18 rat chondrocyte cell line. J Bone Miner Res 11:1130-1138.

McEwen, D.G., Green, R.P., Naski, M.C., Towler, D.A., and Ornitz, D.M. (1999) Fibroblast growth factor receptor 3 gene transcription is suppressed by cyclic adenosine 3'.5'-monophosphate. Identification of a chondrocytic regulatory element. J Biol Chem 274:30934-30942.

McGrew, L.L., Hoppler, S., and Moon, R.T. (1997) Wnt and FGF pathways cooperatively pattern anteroposterior neural ectoderm in Xenopus. Mech Dev 69:105-114.

McKendry, R., Hsu, S.C., Harland, R.M., and Grosschedl, R. (1997) LEF-1/TCF proteins mediate wnt-inducible transcription from the Xenopus nodal-related 3 promoter. Dev Biol 192:420-431.

McMahon, A.P., Joyner, A.L., Bradley, A., and McMahon, J.A. (1992) The midbrain-hindbrain phenotype of Wnt-1-/Wnt-1- mice results from stepwise deletion of engrailed-expressing cells by 9.5 days postcoitum. Cell 69:581-595.

Mercurio, S., Latinkic, B., Itasaki, N., Krumlauf, R., and Smith, J.C. (2004) Connective-tissue growth factor modulates WNT signalling and interacts with the WNT receptor complex. Development 131:2137-2147.

Mizuseki, K., Kishi, M., Shiota, K., Nakanishi, S., and Sasai, Y. (1998) SoxD: an essential mediator of induction of anterior neural tissues in Xenopus embryos. Neuron 21:77-85.

Monzavi, R., and Cohen, P. (2002) IGFs and IGFBPs: role in health and disease. Best Pract Res Clin Endocrinol Metab 16:433-447.

Moon, R.T., and Christian, J.L. (1992) Competence modifiers synergize with growth factors during mesoderm induction and patterning in Xenopus. Cell 71:709-712.

Moos, M., Jr., Wang, S., and Krinks, M. (1995) Anti-dorsalizing morphogenetic protein is a novel TGF-beta homolog expressed in the Spemann organizer. Development 121:4293-4301.

Morali, O.G., Jouneau, A., McLaughlin, K.J., Thiery, J.P., and Larue, L. (2000) IGF-II promotes mesoderm formation. Dev Biol 227:133-145.

Morgan, D.O., Edman, J.C., Standring, D.N., Fried, V.A., Smith, M.C., Roth, R.A., and Rutter, W.J. (1987) Insulin-like growth factor II receptor as a multifunctional binding protein. Nature 329:301-307.

Munchberg, S.R., and Steinbeisser, H. (1999) The Xenopus Ets transcription factor XER81 is a target of the FGF signaling pathway. Mech Dev 80:53-65.

Murphy, L.J. (1998) Insulin-like growth factor-binding proteins: functional diversity or redundancy? J Mol Endocrinol 21:97-107.

Nakae, J., Kido, Y., and Accili, D. (2001) Distinct and overlapping functions of insulin and IGF-I receptors. Endocr Rev 22:818-835.

Niehrs, C. (2004) Regionally specific induction by the Spemann-Mangold organizer. Nat Rev Genet 5:425-434.

Nieuwkoop, P.D., and Faber, J. (1967) Normal table of Xenopus laevis (Daudin). North-Holland Publishing Co, Amsterdam.

Ning, Y., Schuller, A.G., Bradshaw, S., Rotwein, P., Ludwig, T., Frystyk, J., and Pintar, J.E. (2006) Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. Mol Endocrinol 20:2173-2186.

Oh, Y., Nagalla, S.R., Yamanaka, Y., Kim, H.S., Wilson, E., and Rosenfeld, R.G. (1996) Synthesis and characterization of insulin-like growth factor-binding protein (IGFBP)-7. Recombinant human mac25 protein specifically binds IGF-I and -II. J Biol Chem 271:30322-30325.

Ohlsson, C., Isaksson, O., and Lindahl, A. (1994) Clonal analysis of rat tibia growth plate chondrocytes in suspension culture--differential effects of growth hormone and insulin-like growth factor I. Growth Regul 4:1-7.

Onichtchouk, D., Gawantka, V., Dosch, R., Delius, H., Hirschfeld, K., Blumenstock, C., and Niehrs, C. (1996) The Xvent-2 homeobox gene is part of the BMP-4 signalling pathway controlling [correction of controling] dorsoventral patterning of Xenopus mesoderm. Development 122:3045-3053.

Pandini, G., Mineo, R., Frasca, F., Roberts, C.T., Jr., Marcelli, M., Vigneri, R., and Belfiore, A. (2005) Androgens up-regulate the insulin-like growth factor-I receptor in prostate cancer cells. Cancer Res 65:1849-1857.

Pannese, M., Polo, C., Andreazzoli, M., Vignali, R., Kablar, B., Barsacchi, G., and Boncinelli, E. (1995) The Xenopus homologue of Otx2 is a maternal homeobox gene that demarcates and specifies anterior body regions. Development 121:707-720.

Papalopulu, N., Clarke, J.D., Bradley, L., Wilkinson, D., Krumlauf, R., and Holder, N. (1991) Retinoic acid causes abnormal development and segmental patterning of the anterior hindbrain in Xenopus embryos. Development 113:1145-1158.

Paria, B.C., Ma, W., Tan, J., Raja, S., Das, S.K., Dey, S.K., and Hogan, B.L. (2001) Cellular and molecular responses of the uterus to embryo implantation can be elicited by locally applied growth factors. Proc Natl Acad Sci U S A 98:1047-1052.

Parker, A., Rees, C., Clarke, J., Busby, W.H., Jr., and Clemmons, D.R. (1998) Binding of insulin-like growth factor (IGF)-binding protein-5 to smooth-

muscle cell extracellular matrix is a major determinant of the cellular response to IGF-I. Mol Biol Cell 9:2383-2392.

Penzel, R., Oschwald, R., Chen, Y., Tacke, L., and Grunz, H. (1997) Characterization and early embryonic expression of a neural specific transcription factor xSOX3 in Xenopus laevis. Int J Dev Biol 41:667-677.

Pera, E.M., Ikeda, A., Eivers, E., and De Robertis, E.M. (2003) Integration of IGF, FGF, and anti-BMP signals via Smad1 phosphorylation in neural induction. Genes Dev 17:3023-3028.

Pera, E.M., Wessely, O., Li, S.Y., and De Robertis, E.M. (2001) Neural and head induction by insulin-like growth factor signals. Dev Cell 1:655-665.

Perbal, B. (2001) NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol 54:57-79.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T., and De Robertis, E.M. (1999) The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. Nature 397:707-710.

Pollak, M.N., Schernhammer, E.S., and Hankinson, S.E. (2004) Insulin-like growth factors and neoplasia. Nat Rev Cancer 4:505-518.

Puscheck, E.E., Pergament, E., Patel, Y., Dreschler, J., and Rappolee, D.A. (1998) Insulin receptor substrate-1 is expressed at high levels in all cells of the peri-implantation mouse embryo. Mol Reprod Dev 49:386-393.

Ralston, A., and Rossant, J. (2005) Genetic regulation of stem cell origins in the mouse embryo. Clin Genet 68:106-112.

Richard-Parpaillon, L., Heligon, C., Chesnel, F., Boujard, D., and Philpott, A. (2002) The IGF pathway regulates head formation by inhibiting Wnt signaling in Xenopus. Dev Biol 244:407-417.

Rosenfeld, R.G., Hwa, V., Wilson, L., Lopez-Bermejo, A., Buckway, C., Burren, C., Choi, W.K., Devi, G., Ingermann, A., Graham, D., Minniti, G., Spagnoli, A., and Oh, Y. (1999) The insulin-like growth factor binding protein superfamily: new perspectives. Pediatrics 104:1018-1021.

Salic, A.N., Kroll, K.L., Evans, L.M., and Kirschner, M.W. (1997) Sizzled: a secreted Xwnt8 antagonist expressed in the ventral marginal zone of Xenopus embryos. Development 124:4739-4748.

Sapkota, G., Alarcon, C., Spagnoli, F.M., Brivanlou, A.H., and Massague, J. (2007) Balancing BMP signaling through integrated inputs into the Smad1 linker. Mol Cell 25:441-454.

Sarrazin, S., Adam, E., Lyon, M., Depontieu, F., Motte, V., Landolfi, C., Lortat-Jacob, H., Bechard, D., Lassalle, P., and Delehedde, M. (2006) Endocan or endothelial cell specific molecule-1 (ESM-1): a potential novel endothelial cell marker and a new target for cancer therapy. Biochim Biophys Acta 1765:25-37.

Sasai, Y., Lu, B., Piccolo, S., and De Robertis, E.M. (1996) Endoderm induction by the organizer-secreted factors chordin and noggin in Xenopus animal caps. EMBO J 15:4547-4555.

Sasai, Y., Lu, B., Steinbeisser, H., Geissert, D., Gont, L.K., and De Robertis, E.M. (1994) Xenopus chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. Cell 79:779-790.

Sato, J., Hasegawa, S., Akaogi, K., Yasumitsu, H., Yamada, S., Sugahara, K., and Miyazaki, K. (1999) Identification of cell-binding site of angiomodulin (AGM/TAF/Mac25) that interacts with heparan sulfates on cell surface. J Cell Biochem 75:187-195.

Schulte-Merker, S., and Smith, J.C. (1995) Mesoderm formation in response to Brachyury requires FGF signalling. Curr Biol 5:62-67.

Shibamoto, S., Winer, J., Williams, M., and Polakis, P. (2004) A blockade in Wnt signaling is activated following the differentiation of F9 teratocarcinoma cells. Exp Cell Res 292:11-20.

Shibata, Y., Tsukazaki, T., Hirata, K., Xin, C., and Yamaguchi, A. (2004) Role of a new member of IGFBP superfamily, IGFBP-rP10, in proliferation and differentiation of osteoblastic cells. Biochem Biophys Res Commun 325:1194-1200.

Siddle, K., Urso, B., Niesler, C.A., Cope, D.L., Molina, L., Surinya, K.H., and Soos, M.A. (2001) Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors. Biochem Soc Trans 29:513-525.

Sive, H.L., Hattori, K., and Weintraub, H. (1989) Progressive determination during formation of the anteroposterior axis in Xenopus laevis. Cell 58:171-180.

Sloth Andersen, A., Hertz Hansen, P., Schaffer, L., and Kristensen, C. (2000) A new secreted insect protein belonging to the immunoglobulin superfamily binds insulin and related peptides and inhibits their activities. J Biol Chem 275:16948-16953.

Smith, J.C., Price, B.M., Green, J.B., Weigel, D., and Herrmann, B.G. (1991) Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. Cell 67:79-87.

Smith, P., Nicholson, L.J., Syed, N., Payne, A., Hiller, L., Garrone, O., Occelli, M., Gasco, M., and Crook, T. (2007) Epigenetic inactivation implies independent functions for insulin-like growth factor binding protein (IGFBP)-related protein 1 and the related IGFBPL1 in inhibiting breast cancer phenotypes. Clin Cancer Res 13:4061-4068.

Smith, W.C., McKendry, R., Ribisi, S., Jr., and Harland, R.M. (1995) A nodal-related gene defines a physical and functional domain within the Spemann organizer. Cell 82:37-46.

Spagnoli, A., Hwa, V., Horton, W.A., Lunstrum, G.P., Roberts, C.T., Jr., Chiarelli, F., Torello, M., and Rosenfeld, R.G. (2001) Antiproliferative effects of insulin-like growth factor-binding protein-3 in mesenchymal chondrogenic cell line RCJ3.1C5.18. relationship to differentiation stage. J Biol Chem 276:5533-5540.

Stern, C.D. (2005) Neural induction: old problem, new findings, yet more questions. Development 132:2007-2021.

Steventon, B., Araya, C., Linker, C., Kuriyama, S., and Mayor, R. (2009) Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction. Development 136:771-779.

Stewart, C.E., and Rotwein, P. (1996) Growth, differentiation, and survival: multiple physiological functions for insulin-like growth factors. Physiol Rev 76:1005-1026.

Strickland, S., and Mahdavi, V. (1978) The induction of differentiation in teratocarcinoma stem cells by retinoic acid. Cell 15:393-403.

Strickland, S., Smith, K.K., and Marotti, K.R. (1980) Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. Cell 21:347-355.

Tada, M., and Smith, J.C. (2000) Xwnt11 is a target of Xenopus Brachyury: regulation of gastrulation movements via Dishevelled, but not through the canonical Wnt pathway. Development 127:2227-2238.

Usui, T., Murai, T., Tanaka, T., Yamaguchi, K., Nagakubo, D., Lee, C.M., Kiyomi, M., Tamura, S., Matsuzawa, Y., and Miyasaka, M. (2002) Characterization of mac25/angiomodulin expression by high endothelial venule cells in lymphoid tissues and its identification as an inducible marker for activated endothelial cells. Int Immunol 14:1273-1282.

von Bubnoff, A., and Cho, K.W. (2001) Intracellular BMP signaling regulation in vertebrates: pathway or network? Dev Biol 239:1-14.

Wang, J., Zhou, J., and Bondy, C.A. (1999) Igf1 promotes longitudinal bone growth by insulin-like actions augmenting chondrocyte hypertrophy. FASEB J 13:1985-1990.

Wang, Y., Nishida, S., Sakata, T., Elalieh, H.Z., Chang, W., Halloran, B.P., Doty, S.B., and Bikle, D.D. (2006) Insulin-like growth factor-I is essential for embryonic bone development. Endocrinology 147:4753-4761.

Weksler, N.B., Lunstrum, G.P., Reid, E.S., and Horton, W.A. (1999) Differential effects of fibroblast growth factor (FGF) 9 and FGF2 on proliferation, differentiation and terminal differentiation of chondrocytic cells in vitro. Biochem J 342 Pt 3:677-682.

Wessely, O., Agius, E., Oelgeschlager, M., Pera, E.M., and De Robertis, E.M. (2001) Neural induction in the absence of mesoderm: beta-catenin-dependent expression of secreted BMP antagonists at the blastula stage in Xenopus. Dev Biol 234:161-173.

Wills, A.E., Choi, V.M., Bennett, M.J., Khokha, M.K., and Harland, R.M. (2010) BMP antagonists and FGF signaling contribute to different domains of the neural plate in Xenopus. Dev Biol 337:335-350.

Wood, T.L., Rogler, L.E., Czick, M.E., Schuller, A.G., and Pintar, J.E. (2000) Selective alterations in organ sizes in mice with a targeted disruption of the insulin-like growth factor binding protein-2 gene. Mol Endocrinol 14:1472-1482.

Wu, J., Yang, J., and Klein, P.S. (2005) Neural crest induction by the canonical Wnt pathway can be dissociated from anterior-posterior neural patterning in Xenopus. Dev Biol 279:220-232.

Xu, R.H., Kim, J., Taira, M., Sredni, D., and Kung, H. (1997) Studies on the role of fibroblast growth factor signaling in neurogenesis using conjugated/aged animal caps and dorsal ectoderm-grafted embryos. J Neurosci 17:6892-6898.

Yabe, T., Shimizu, T., Muraoka, O., Bae, Y.K., Hirata, T., Nojima, H., Kawakami, A., Hirano, T., and Hibi, M. (2003) Ogon/Secreted Frizzled functions as a negative feedback regulator of Bmp signaling. Development 130:2705-2716.

Yakar, S., Liu, J.L., Stannard, B., Butler, A., Accili, D., Sauer, B., and LeRoith, D. (1999) Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci U S A 96:7324-7329.

Yakar, S., Rosen, C.J., Beamer, W.G., Ackert-Bicknell, C.L., Wu, Y., Liu, J.L., Ooi, G.T., Setser, J., Frystyk, J., Boisclair, Y.R., and LeRoith, D. (2002) Circulating levels of IGF-1 directly regulate bone growth and density. J Clin Invest 110:771-781.

Yamanaka, Y., Wilson, E.M., Rosenfeld, R.G., and Oh, Y. (1997) Inhibition of insulin receptor activation by insulin-like growth factor binding proteins. J Biol Chem 272:30729-30734.

Yamauchi, T., Umeda, F., Masakado, M., Isaji, M., Mizushima, S., and Nawata, H. (1994) Purification and molecular cloning of prostacyclin-stimulating factor from serum-free conditioned medium of human diploid fibroblast cells. Biochem J 303 (Pt 2):591-598.

Yang, J., Mei, W., Otto, A., Xiao, L., Tao, Q., Geng, X., Rupp, R.A., and Ding, X. (2002) Repression through a distal TCF-3 binding site restricts Xenopus myf-5 expression in gastrula mesoderm. Mech Dev 115:79-89.

Zhang, M., Xuan, S., Bouxsein, M.L., von Stechow, D., Akeno, N., Faugere, M.C., Malluche, H., Zhao, G., Rosen, C.J., Efstratiadis, A., and Clemens, T.L. (2002) Osteoblast-specific knockout of the insulin-like growth factor (IGF) receptor gene reveals an essential role of IGF signaling in bone matrix mineralization. J Biol Chem 277:44005-44012.

Zhao, G., Monier-Faugere, M.C., Langub, M.C., Geng, Z., Nakayama, T., Pike, J.W., Chernausek, S.D., Rosen, C.J., Donahue, L.R., Malluche, H.H., Fagin, J.A., and Clemens, T.L. (2000) Targeted overexpression of insulin-like growth factor I to osteoblasts of transgenic mice: increased trabecular bone volume without increased osteoblast proliferation. Endocrinology 141:2674-2682.

Zhu, L., Ohan, N., Agazie, Y., Cummings, C., Farah, S., and Liu, X.J. (1998) Molecular cloning and characterization of Xenopus insulin-like growth factor-1 receptor: its role in mediating insulin-induced Xenopus oocyte maturation and expression during embryogenesis. Endocrinology 139:949-954.

Zhu, W., Shiojima, I., Ito, Y., Li, Z., Ikeda, H., Yoshida, M., Naito, A.T., Nishi, J., Ueno, H., Umezawa, A., Minamino, T., Nagai, T., Kikuchi, A., Asashima, M., and Komuro, I. (2008) IGFBP-4 is an inhibitor of canonical Wnt signalling required for cardiogenesis. Nature 454:345-349.

7 Table of figures

Fig. 1: Mig30 inhibits Wnt signalling 25
Fig. 2: Mig30 inhibits BMP signalling 26
Fig. 3: Schematic diagram of Mig30 and Mig30 mutant constructs 27
Fig. 4: Mig30 mutant constructs inhibit Wnt and BMP signalling 28
Fig. 5: Overexpression of Mig30 cannot induce neural tissue. 30
Fig. 6: Mig30 overexpression inhibits IGFBP5 31
Fig. 7: Mig30 mutant constructs interfere with IGFBP5 signalling 33
Fig. 8: Mig30 inhibits IGF1 induced differentiation of RCJ3.1C5.18 cells 35
Fig. 9: In Mig30 morphants Wnt signalling is inhibited in an IGF-dependent manner 37
Fig. 10: In Mig30 morphants BMP signalling is inhibited 38
Fig. 11: Loss of Mig30 enhances exogenous IGF signals 39
Fig. 12: Loss of Mig30 enhances IGFBP5 signalling 40
Fig. 13: Knockdown of Mig30 perturbs head and neural development. 42
Fig. 14: Mig30 is required for neural crest development. 44
Fig. 15: Overexpression of Mig30 has no effect on FGF signalling 46
Fig. 16: Mig30 has no effect on ADMP signalling. 47
Fig. 17: Transcriptional regulation of IGFBP-rP10 49
Fig. 18: Phenotypic analysis after IGFBP-rP10 overexpression 51
Fig. 19: Schematic diagram of IGFBP-rP10 and IGFBP-rP10 mutant constructs. 52
Fig. 20: IGFBP-rP10 specifically blocks IGFBP-rP10 translation 53
Fig. 21: Knockdown of IGFBP-rP10 54
Fig. 22: Knockdown of IGFBP-rP10 55
Fig. 23: Overexpression of IGFBP-rP10 induces Otx2 expression 56
Fig. 24: IGFBP-rP10 overexpresion inhibits BMP signalling 57
Fig. 25: Gain of IGFBP-rP10 does not inhibit Wnt signalling 58
Fig. 26: Knockdown of IGFBP-rP10 reduces transcription of Vent2 and Myf5. 60
Fig. 27: Knockdown of IGFBP-rP perturbs brain patterning 61
Fig. 28: IGFBP-rP10 is required for neural crest development. 63
Fig. 29: Loss of IGFBP-rP10 affects neural development. 64
Fig. 30: IGFBP-rP10 has no effect on IGF1 induced differentiation of RCJ3.1C5.18 cells 66
Fig. 31: IGFBP-rP10 has no effect on ADMP signalling. 67

Fig. 32: IGFBP-rP10 has no effect on ADMP signalling. 68
Fig. 33: Bono1 is expressed in differentiated F9-cells 69
Fig. 34: Bono1 is expressed in the decidua. 70
Fig. 35: Bono1 expression in the decidua 71

8 Abbreviations

(v/v)	volume volume ratio
(w/v)	weight volume ratio
°C	degree Celsius
µg	microgram
µl	microlitre
AC	animal cap
Amp	ampicillin
A-P	anteroposterior
C-	carboxy
cAMP	cyclic adenosine monophosphat
cDNA	copy DNA
CE	convergent extension
CG	cement gland
CNC	cranial neural crest
CNS	central nervous system
DIG	Digoxygenin
DNA	deoxyribonucleic acid
dNTP	2'-desoxy-nucleoside-5'-triphosphate
ECM	extracellular matrix
EDTA	ethylen-diamin-tetraacetate
H2O	water
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-ethansulfonic acid
ICM	inner cell mass
ISH	whole mount *in-situ* hybridisation
kb	kilo basepairs
kDa	kilo Dalton
l	litre
LB	Luria Bertani Medium
M	Molar
MBS	Modified Barth's Solution
MEM	Modified Eagle´s Medium
min	minute
ml	millilitre

mM	millimolar
Mo	antisense morpholino oligonucleotide
mRNA	messanger RNA
MW	molecular weight
N-	amino
N	number
N.F.	Nieuwkoop and Faber
NC	neural crest
ng	nanogram
nl	nanolitre
o/n	overnight
ODC	ornithin decarboxylase
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain ceaction
pg	Picogram
qRT-PCR	quantitative reverse transcription-PCR
RA	retinoic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcription PCR
SDS	sodium-dodecyl-sulfate
SP	signal peptide
SSC	standard saline citrate
st.	Stage
TBE	Tris-boracic acid-EDTA
TEMED	N,N,N’,N’-Tetramethylethylendiamin
U	units
V	Volt
we	whole embryo
wt	wildtype

Danksagung

An dieser Stelle möchte ich allen danken, die durch ihre Unterstützung zum Gelingen dieser Arbeit beigetragen haben.

Mein besonderer Dank gilt Prof. Dr. Herbert Steinbeißer und der ganzen Arbeitsgruppe für die gute Zusammenarbeit und angenehme Arbeitsatmosphäre. Dank euch hatte ich die letzten Jahre immer Spaß an meiner Arbeit.

Frau Dr. Daniela Kiepe und Fr. Ulrike Hügel möchte ich herzlich danken für die Betreuung der Chondrocyten Experimente.

Prof. Dr. M. Treier und seiner Arbeitsgruppe gilt mein Dank für die Mausembryonen und die technische Hilfe im Umgang mit dem Cryostat.

Anne Glaser, Sandra Puskaric, Dr. Claudia Durand, Dr. Eva Decker danke ich für die praktische Hilfe bei der Maus *in-situ* Hybridisierung und dem Luciferase Assay.

Und am Ende möchte noch allen danken die mit konstruktiven Diskussionen und Anregungen, vor allem technischer Art, beim Schreiben und Formatieren dieser Arbeit zur Seite standen.

Danke!