Regulation of Integrin Function by XGIPC

By

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I hereby declare that I am the soul author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Integrins are a large family of transmembrane cell adhesion receptors that are found on the surface of eukaryotic cells. Integrins act predominantly as cell surface receptors for extracellular matrix (ECM) proteins, but also have bidirectional signaling properties that allow them to play fundamental roles in development and cancer metastasis. It has become clear that during gastrulation – a period during which cells participate in morphogenetic movements that lead to the generation of a tripoblastic embryo – the integrin repertoire of each cell is in constant flux. This change in cell surface receptors is mediated through intracellular pathways, which in turn, are regulated by associations with cytoplasmic proteins. One such molecule, GIPC (GAIP-interacting protein, C-terminus), is thought to have a role in regulating α5β1 integrin surface expression, as well as integrin-mediated inside-out and outside-in signaling pathways by mediating the integrin's ability to interact with the ECM protein, fibronectin (FN).

I use *Xenopus laevis* as my experimental model system to study GIPC-regulated integrin function. Xenopus provides a useful model system for regulation of integrin function as α5β1-FN interactions are spatially and temporally regulated. Additionally *Xenopus* embryos are amenable to molecular manipulations *in vivo,* and the tissue can be excised from embryos and cultured *in vitro*. I have investigated the function of GIPC using site-directed mutagenesis to alter the PDZ domain site in *Xenopus* GIPC (XGIPC). Expression of dominant negative XGIPC results in the interruption of gastrulation movements in the early embryo. Yeast two-hybrid and co-immunoprecipitation assays demonstrate that XGIPC physically interacts with the cytoplasmic domain of the α5 and α6 integrin subunit. Furthermore, I have determined that the interaction of XGIPC with α5β1 is required for assembly of a FN matrix. Cell migration and convergent

extension assays demonstrate that XGIPC likely plays other undefined roles in modulating α5β1 function. XGIPC was found to be required for efficient trafficking of α5β1, as determined by α5β1 internalization assays in A6 cells. Together, my data indicate a critical role for XGIPC in modulating α5β1 integrin function during early embryonic morphogenesis.

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Abbreviations

BCR	Blastocoel Roof
BSA	Bovine Serum Albumin
CAMs	Cell Adhesion Molecules
CCBD	Central Cell Binding Domain
CHO	Chinese Hamster Ovary
DFA	Danilchik's for Amy
DTT	Dithiothreitol
ECM	Extracellular matrix
ELB	Embryo Lysis Buffer
F-actin	Filamentous actin
FBS	Fetal Bovine Serum
FN	Fibronectin
FZD	Frizzled receptor
G-actin	Globular actin
GAIP	G Alpha Interacting Protein
GFP	Green Fluorescent Protein
GIPC	GAIP Interacting Protein, C terminus
HA	Hemagglutinin
HCG	Human Chorionic Gonadotropin
HRP	Horse Radish Peroxidase
IGF	Insulin-like Growth Factor
IGF-1R	Insulin-like Growth Factor-1 Receptor
MBS	Modified Barth's Saline
MIB	Mediolateral Cell Intercalation Behaviour
MSS^{-}	Modified Stearn`s Solution

MSS^{+}	Modified Stearn`s Solution supplemented with $MgCl_2$ and $CaCl_2$
NI	Non-injected
PBS	Phosphate Buffer Saline
PDZ	PSD-95 (post-synaptic density protein), Dlg (*Drosophila* disc-large protein), ZO-1
PMSF	Phenylmethylsulfonyl Fluoride
RGD	Arginine-Glycine-Aspartic Acid
RGS	Regulators of G protein Signaling
ROI	Region Of Interest
SC	Synthetic Complete
TBS	Tris Buffer Saline
TCA	Trichloroacetic Acid
TGF-β	Transforming Growth Factor
TrkA	Tyrosine Kinase Receptor A
WI	Water-injected
XGIPC	*Xenopus* GAIP-inteacting Protein, C-terminus
$XGIPC_{mut}$	*Xenopus* GAIP Interacting Protein, C terminus, mutated form

Chapter 1 Introduction

1.1 *Xenopus laevis* embryogenesis

Morphogenesis, from the Greek terms *morphe* (shape) and *genesis* (creation), refers to the processes by which groups of cells undergo coordinated movements, giving rise to a defined form or structure during development. During early amphibian embryogenesis, morphogenesis transforms the hollow, spherical blastula into a tripoblastic embryo; this period of tissue rearrangement is referred to as gastrulation (Figure 1.1). At the onset of gastrulation, *Xenopus* embryos house a large cavity called the blastocoel, which is formed by a thin blastocoel roof (BCR) and a vast blastocoel floor (Gilbert, 2006). The BCR is fated to become ectoderm, the blastocoel floor to become endoderm, and the transition zone between these two regions, the marginal zone, is mainly comprised of presumptive mesoderm (Figure 1.1A) (reviewed by Keller and Gerhart, 1986). Gastrulation commences with the invagination of presumptive mesoderm over the blastopore lip (Figure 1.1B). Presumptive head mesoderm is the first to involute, followed by future axial and paraxial mesoderm until all mesoderm has become internalized (Figure 1.1C) (reviewed by Gerhart and Keller, 1986). Once internalized, mesoderm adheres to the BCR and translocates across the apical surface to the animal pole of the embryo (Figure 1.1D).

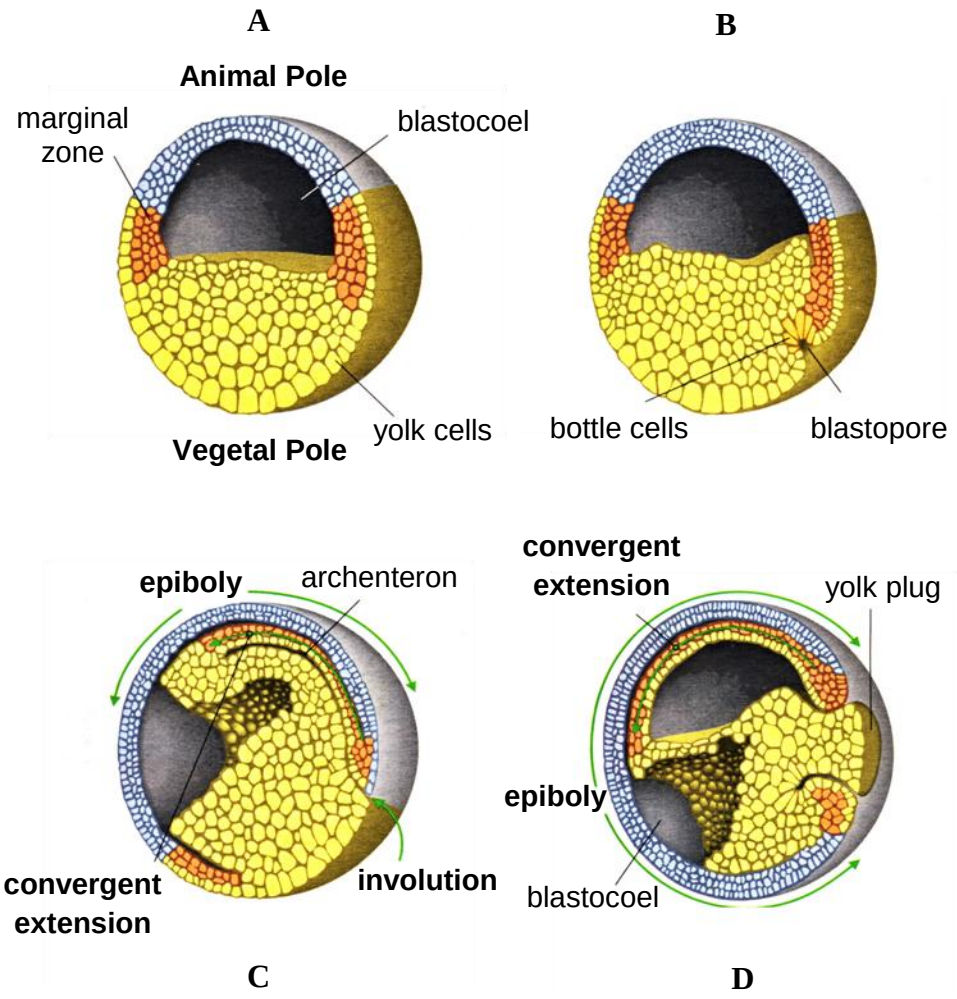


Figure 1.1 Tissue rearrangements during *Xenopus laevis* gastrulation. In the late blastula, presumptive mesoderm (orange) lies between the presumptive ectoderm (blue) of the BCR and the presumptive endoderm (yellow) of the blastocoel floor (A). Gastrulation commences with the involution of mesoderm over the blastopore lip, indicated by the formation of bottle-shaped cells (B). Involution of mesoderm forms a layer underlying the ectodermal layer, which converges and extends along the anteroposterior axis. Concurrently, presumptive endoderm involutes through the blastopore to line the future gut of the embryo (called the archenteron). Ectoderm spreads by a process called epiboly (C). Cell migration and convergent extension of mesodermal cells over the apical surface of the BCR displaces the blastocoel until it is obliterated at the end of gastrulation (D). (Figure adapted from Wolpert et al., 2007)

Mesodermal cell attachment and subsequent translocation on the BCR requires the interaction with the extracellular matrix (ECM) protein, fibronectin (FN). FN is both spatially and temporally regulated, as it is secreted by all cells of the embryo, but only forms a fibril network on the BCR at the onset of gastrulation (Lee et al, 1984; Johnson et al., 1992). FN

assembly and subsequent FN-dependent cell behaviours during *Xenopus* gastrulation are mediated through a single surface receptor called the α5β1 integrin (Benjamin Hoffstrom, PhD thesis, University of Virginia, 2002). Comprehensive studies of FN in *Xenopus* have defined a simple *in vivo* model for assaying integrin function during gastrulation.

The cell movements driving the morphological changes during gastrulation are also spatially and temporally regulated. Involuted mesoderm cells that contact the FN matrix are capable of spreading and migrating, while other gastrula cells are not (Ramos et al., 1996). Two known FN-dependant processes are coupled with mesoderm translocation. First, presumptive head mesodermal cells display directed migratory behaviour (Winklbauer and Nagel, 1991). Second, through their interactions with FN, axial and paraxial mesodermal cells are induced to undergo convergent extension. Convergent extension is the lengthening and narrowing of the tissue in the anteroposterior axis. This extension results from the acquisition of FN-induced polarized protrusions, which allow mesodermal cells to intercalate, mediolaterally wedging between neighbouring cells (Figure 1.2) (reviewed by Keller, 2002). These two FN-dependent morphogenetic processes establish the basic body plan of the embryo.

1.2 FN-induced mesodermal behaviour requires integrin α5β1

The changes in cell behaviours that drive tissue rearrangements during gastrulation have an absolute requirement for FN matrix and for the FN cell surface receptor, integrin α5β1. The beginning of gastrulation corresponds with the assembly of a FN matrix in response to FN-α5β1 interactions. Once the matrix has been assembled, mesodermal cells receive signals from the blastopore lip area that are required for presumptive head mesodermal cells to gain the ability to

spread and “crawl” across the BCR with the aid of actin-rich cell protrusions called lamellipodia (Winklbauer and Keller, 1996). FN contributes to this process by providing guidance cues to direct mesodermal cells towards the animal pole as demonstrated in isolated mesodermal explants from *Xenopus* (Winklbauer and Nagel, 1991), and isolated mesodermal cells from *Pleurodeles* (Shi et al., 1989) and *Ambystoma* (Nakatsuji and Johnson, 1983). When BCRs are cultured to allow deposition of FN matrix onto a supportive surface, head mesoderm cells migrate in a directionally biased manner, thus demonstrating the existence of a substrate-dependent mechanism regulating cell behaviour. Furthermore, FN matrix assembly can be inhibited, without interrupting FN secretion, using GRGDSP (the canonical integrin binding sequence) peptides. This causes mesodermal cell migration to become randomized, suggesting that intact FN fibrils are necessary for mesoderm guidance (Winklbauer and Nagel, 1991).

In contrast to cell migration, convergent extension is a mechanism by which cells rearrange via intercalation, extending the overall shape of an embryo along its anteroposterior axis (Figure 1.2) (reviewed by Keller et al., 1992). Unlike mesoderm migration, which is reliant upon cell-substrate adhesion involving FN, convergent extension is dependent upon cell-cell adhesion (Zhong et al, 1999). Typically, FN fibrils are localized to all tissue boundaries of the early embryo (Davidson et al., 2004). The expression of cell polarity genes mediates the assembly of FN fibrils along these boundaries (Goto et al. 2005). The polarized deposition of fibrils is required for the subsequent induction of mediolateral cell intercalation behaviour (MIB) that result in convergent extension (Marsden and DeSimone, 2003). MIB is the activity of bipolar filo-lamelliform protrusions directed along the mediolateral axis (Shih and Keller, 1992). These protrusions allow for another family of cell surface receptors, the cadherins, to mediate adhesions that allow cells to tractor across each other, a process that drives intercalation and,

therefore, convergent extension. Despite the fact that MIB requires cadherin adhesion, it has an absolute requirement for signals stemming from α5β1 integrin ligation at tissue boundaries (Marsden and DeSimone, 2001).

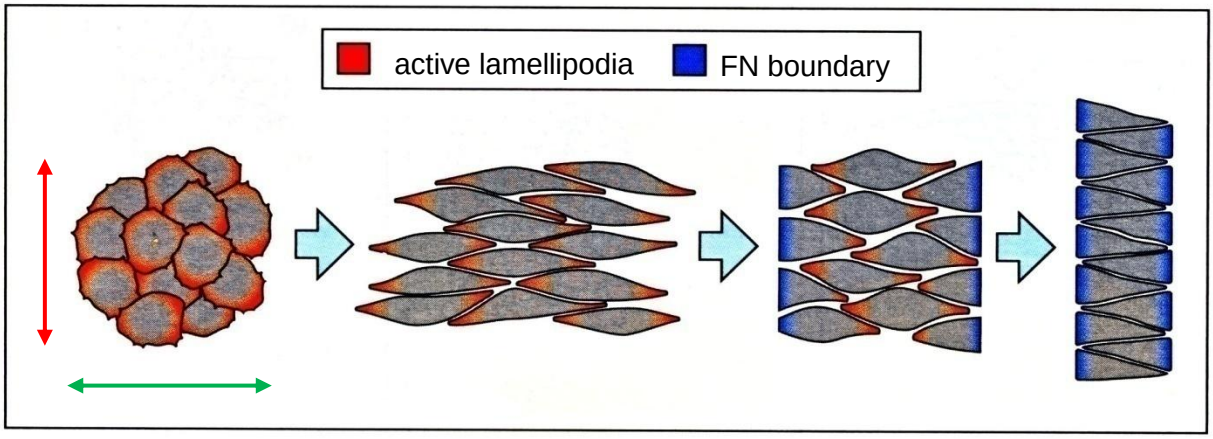


Figure 1.2 Convergent extension. FN is required for acquisition of cell polarity in the mediolateral direction (green arrow). Lamellipodia at the ends of cells actively exert traction on adjacent cells, thereby wedging between neighbouring cells, and elongating the tissue in the anteroposterior axis (red arrow). (Figure adapted from Wolpert et al., 2007)

Given the relatively simple interactions between a single integrin receptor (α5β1) and single ECM molecule (FN), the *Xenopus laevis* gastrulae provide a highly characterized experimental model to study the regulation of integrin α5β1.

1.3 Integrins

It has been clearly demonstrated that FN is a key player in the morphogenetic processes driving *Xenopus* gastrulation and that it does so through its interactions with the integrin receptor, α5β1 (reviewed by Wu, 1997). The α5β1 integrin is one member of a superfamily of transmembrane receptors (reviewed by Dzamba et al., 2002). Integrin-mediated adhesion to the

ECM plays a role in cell morphology and other biological functions including cell migration, proliferation, and gene expression (Cox and Huttenlocher, 1998; Schwartz and Assoian, 2001; reviewed by Giancotti and Ruoslahti, 1999). Additionally, integrins can mediate direct cell-cell interactions (Hynes, 1987).

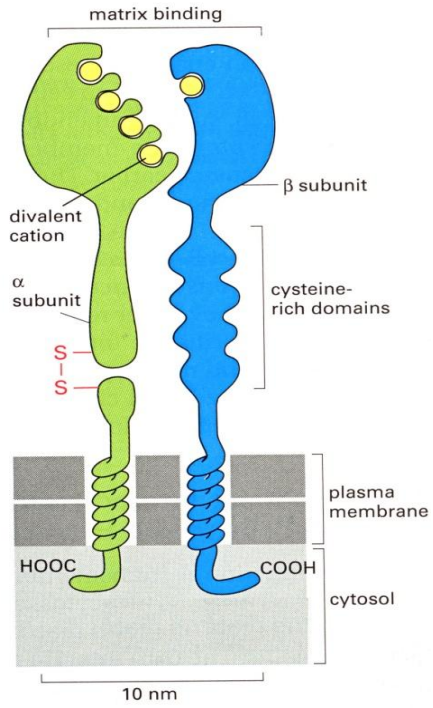


Figure 1.3 Integrin structure. Integrins are heterodimeric cell surface receptors composed of noncovalently-linked α and β subunits. Both subunits have large extracellular domains, which bind extracellular ligands, such as FN; small transmembrane domains; and relatively short cytoplasmic domains, which bind intracellular anchor proteins. The α subunit is characterized by a large extracellular domain containing four divalent-cation binding sites. In some integrins, this domain is connected to the transmembrane domain via a disulfide bond. The extracellular domain of the β subunit contains a single divalent-cation-binding site and a cysteine-rich region. (Figure from Alberts et al., 2002)

Integrins are heterodimeric receptors that are composed of non-covalently associated α and β subunits. The combination of an α and a β subunit defines an individual receptor. Based on their subunit composition, integrins can be loosely grouped into sub-families of defined function.

For instance β1 integrins typically mediate interactions with ECM proteins, while β2 integrins typically associate with other cell surface proteins (reviewed by Coppolino and Dedhar, 2000). Integrins are found in all metazoans; the number of α- and β-subunits encoded in the genome typically increases with organism complexity (reviewed by Calderwood, 2004). For example, mammals contain at least 18 α subunits and 8 β subunits, which can combine to form at least 24 known integrins (Figure 1.4) (reviewed by Bokel and Brown, 2002). In contrast, the *Xenopus* genome contains a select group of integrins, including α2 and α3 (Meng et al., 1997), α4 (Whittaker and DeSimone, 1998), α5 (Joos et al., 1995), α6 (Lallier et al., 1996), αV (Joos et al., 1998), and β1, β2, β3, β6 (Ransom et al., 1993) only (Figure 1.4, asterisks). A single integrin has even been found to be conserved in species with very simple tissue organization, such as coral and sponges (Brower et al., 1997; Pancer et al., 1997).

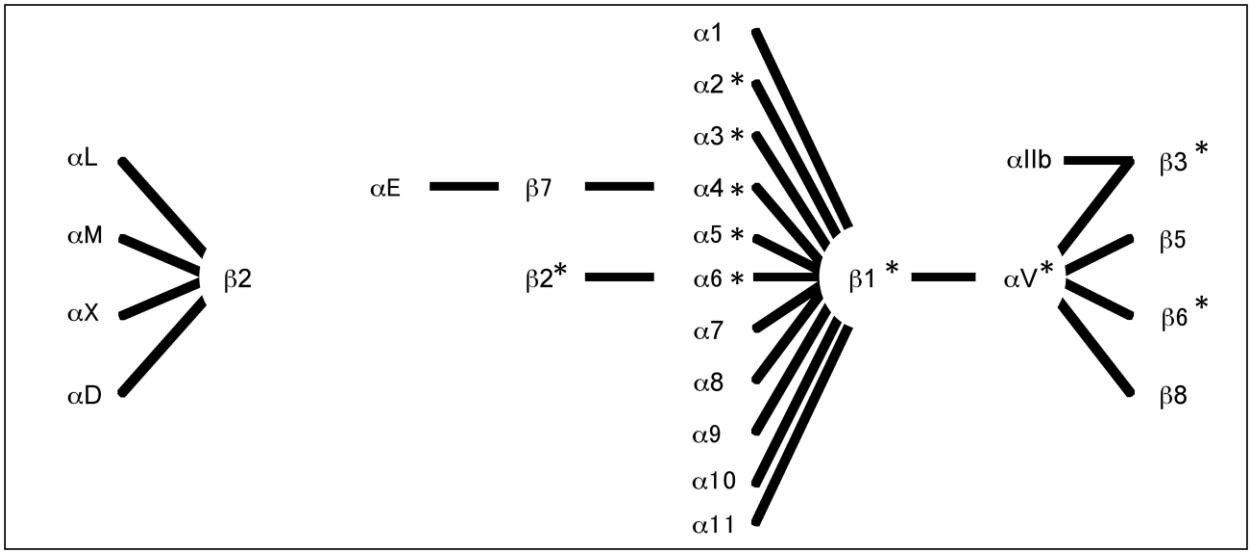


Figure 1.4 Integrin α and β associations of known vertebrate integrins. The spider diagram displays the known possible vertebrate αβ subunit combinations (adapted from Hemler et al., 1992). Asterisks indicate integrin subunits known to be present in *Xenopus laevis*.

While function-based sub-families have been defined, integrin-ligand interactions are unpredictably complex. Firstly, an individual integrin may recognize several extracellular ligands, whereas another integrin may recognize only a single ligand. For example, the αVβ3 integrin has been reported to bind at least seven different ECM ligands, whereas the α5β1 integrin binds exclusively to the ECM protein FN (reviewed by Dzamba et al, 2002). Conversely, individual ligands, including the matrix proteins FN, laminins, collagens, and vitronectin, are capable of binding to multiple integrin receptors (reviewed by Giancotti and Ruoslahti, 1999). For instance, FN is capable of binding both α5- and αV-containing integrins through unique sequences within its central cell-binding domain (CCBD) (reviewed by Dzamba et al, 2002). Integrin-ligand interactions are further complicated as many integrin subunits have alternate splice forms, which are typically cell type-specific (Hynes, 1992). For example, the variation in C-terminal amino acids of the α6A (SDA) and α6B (SYS) subunits in mice is sufficient to alter the intracellular binding specificity, allowing each splice variant to interact with unique intracellular binding partners (El Mourabit et al., 2002).

1.4 Integrin activation

While cell-cell and cell-ECM adhesions are mediated by ligand interactions with the extracellular domain of integrins, the cellular responses, such as cell migration, require the integrins' intracellular domains. Integrins' short cytoplasmic tails are able to bind to cytosolic proteins, thereby providing a link between the extracellular environment and the cytoskeleton (reviewed by Critchley, 2000; Liu et al., 2000). As such, integrins supply a transmembrane

connection for the bidirectional transmission of mechanical forces and biochemical signals (reviewed by Calderwood, 2004).

Modulation of both force and biochemical signals across the plasma membrane is achieved by tightly regulating spatial and temporal control of integrin affinity for ECM ligands. Changes to integrin affinity can occur without changes in integrin gene expression (reviewed by Dzamba et al., 2002). An increase in integrin affinity for a ligand, referred to as integrin activation[1], is possible due to rapid reversible conformational changes in the integrin's extracellular domain. In addition to affinity modulation, a number of affinity-independent mechanisms contribute to the regulation of integrin-mediated adhesion; these include integrin clustering, lateral diffusion of integrins, interactions with and reorganization of the cytoskeleton, and changes in receptor expression patterns (reviewed by Calderwood 2004). Affinity-dependent and –independent mechanisms of mediating integrin-mediation adhesion can act collaboratively; for example, integrin activation stimulates receptor clustering, which further enhances integrins' adhesion to extracellular ligands (Li et al., 2003).

1.5 Integrin-mediated outside-in signaling

The binding of external ligands to integrins can transmit signals into cells - a process referred to as "outside-in" signaling (reviewed by Schwartz et al., 1995). Outside-in signaling can result in the reorganization of the cytoskeleton, gene expression, and cell differentiation (reviewed by Liu et al., 2000). As the cytoplasmic tails of integrins are incapable of enzymatic

[1] Refers to the changes required to enhance the integrin's ligand-binding activity (terminology is therefore based on the primary function of adhesion receptors). Integrins also have important roles as signaling receptor (see sections 1.5 and 1.6); "activation of signaling receptors" refers to changes induced by ligand binding that enhance signal transduction (reviewed by Calderwood, 2004).

activity, outside-in signal transduction is made possible by the association of integrins with adaptor proteins, which mechanically link integrins with the cytoskeleton, kinases, and/or transmembrane growth factor receptor mediated signaling pathways (reviewed by Giancotti and Ruoslahti, 1999). Additionally, outside-in signaling, and the subsequent association with cytoskeletal components, creates a positive feedback loop: integrins binding the ECM become clustered in the plane of the membrane. Integrins then recruit both cytoskeletal and signaling molecules to form a complex that promotes the assembly of actin filaments. These actin filaments then reorganize into larger stress fibres that enhance integrin clustering, which in turn increases FN matrix binding, thus completing the loop (reviewed by Giancotti and Ruoslahti, 1999). As a result of this feedback system, ECM proteins, integrins, and cytoskeletal proteins aggregate on both the extracellular and intracellular sides of the membrane. In cell culture, these aggregates can be seen by immunofluorescence microscopy and are referred to as ECM complexes and focal adhesions, respectively (Fernandez-Valle et al., 1998). While ECM complexes and focal adhesions are an artefact of tissue culture and do not exist *in vivo*, many of the described molecular interactions that occur in focal adhesions appear to exist in tissues.

There is evidence to suggest that integrin-binding of extracellular ligands provides the initial cues for the establishment of cell polarity in *Xenopus*, likely due to outside-in signaling (Davidson et al., 2006). An outside-in signal through the integrin receptor provides the link between cell adhesion and cell polarity pathways (see section 1.3), which together regulate cell rearrangements during gastrulation. In support of this, Marsden and DeSimone (2001) found that integrin-FN interactions are required for the establishment of cell polarity, which is necessary for cell intercalation. Furthermore, mesodermal tissues lacking FN do not demonstrate the bipolar, elongated mediolateral alignment typical of cells undergoing MIB (Marsden and DeSimone,

2003). As previously discussed, cells unable to undergo MIB cannot converge and extend, thus inhibiting gastrulation (Goto et al., 2005).

1.6 Inside-out signaling in *Xenopus*

Early *Xenopus* embryos ubiquitously express the α5β1 integrin on the surface of cells (Joos et al., 1995). All cells of the late blastula are able to attach to FN through α5β1 recognition of the Arg-Gly-Asp (RGD) sequence found in the central cell binding domain of FN (Ramos & DeSimone, 1996; Pierschbacher & Ruoslahti, 1984). At the onset of gastrulation, inside-out signaling induces the α5β1 integrin to recognize the synergy site – a site adjacent to the RGD sequence that works cooperatively to support cell adhesion (Ramos et al., 1996). The involuted presumptive mesodermal cells then rapidly switch from a state of static attachment to one of spreading and migrating across the BCR (Ramos et al., 1996). Although the mechanism regulating the change in α5β1-FN binding remains elusive, it has been shown *in vitro* that following exposure to Activin-A, a member of the TGF-β family of growth factors, mesodermal cells rapidly switch from non-motile attachment to motile spreading and migrating (Smith et al., 1990; Smith and Howard, 1992). The molecular mechanism by which Activin-A induces α5β1 to rapidly switch from binding only the RGD site to binding the RGD/synergy sites is not known. It has been suggested that exposure to Activin-A causes α5β1 already present on the surface of cells to become activated; this activation allows for the recognition of the synergy site (Ramos and DeSimone, 1996). Other model systems, such as human T cells, exhibit similar inside-out signaling mechanisms whereby activation of the integrin receptor leads to increased adhesion of cells to FN without changing surface level expression (Chan et al., 1991).

1.7 Integrin trafficking

An alternate possibility exists that, instead of surface expressed α5β1 becoming activated, integrin activation requires receptor recycling. It is well established that adhesion receptors undergo endocytic-exocytic transport, or "recycling" (Caswell and Norman, 2006). A basic mechanism for cell migration has been proposed where integrins are internalized via endocytosis at the retracting edge of the cell, thereby facilitating detachment. The purpose of this endocytosis is to recycle rather than degrade the receptor (Bretscher, 1992; Sczekan and Juliano, 1990). Internalized integrins are thought to be transported in vesicles to the leading edge of the migrating cell, where they are then exocytosed back onto the cell surface. However, evidence for this model of receptor recycling is limited. Recent experimentation has unveiled an alternative mechanism by which integrins are both endocytosed and recycled within the leading-edge-region of the cell. This allows for ECM receptors to remain spatially restricted, resulting in a polarized distribution of integrins within the leading edge of the cell (Caswell, 2007).

Studies using Chinese hamster ovary (CHO) cells have demonstrated that α5β1 integrins are constantly internalized (Bretscher, 1989). This internalization of α5β1 can occur through clathrin-coated pits or by non-clathrin dependent endocytosis (reviewed by Caswell and Norman, 2008). As mentioned, matrix-bound integrins are associated with actin filaments at focal adhesions; internalized integrins have been shown to be actively transported along these filaments. For instance, several integrin β-subunits have been shown to be transported along actin filaments to the tips of the filopodia where the receptors aid in stabilizing the protrusions. Furthermore, this transport of β1 containing integrins has been found to be important for initial cell spreading and adhesion (reviewed by Bretscher, 1996). A number of mechanisms have been

suggested as regulators of integrin trafficking, including various kinases and GTPase family members (reviewed by Pellinen and Ivaska, 2006). For example, cancer-profiling studies have implicated a number of integrin-associated proteins thought to be involved in receptor trafficking and in the mis-regulation of cell adhesion during tumour metastasis.

1.8 GIPC

One cytoplasmic protein known to directly interact with α-subunit cytoplasmic domains and influence integrin behaviour is GIPC (GAIP interacting protein, C-terminus) (El Mourabit et al., 2002; Tani and Mercurio, 2001). GIPC was originally identified by its interaction with the C-terminus of G alpha interacting protein (GAIP), a regulator of G protein signaling (RGS) protein (De Vries et al., 1998b). GIPC is highly conserved across diverse species and in mammals is represented by three family members GIPC 1, 2, and 3 (reviewed by Katoh, 2002). GIPC is a 36 kD protein containing a central PDZ domain, which is thought to be involved in protein-protein interactions (De Vries et al, 1998b). GIPC's ability to interact with GAIP is due to the PDZ-binding motif found in the C-terminus of GAIP. The binding specificity of the PDZ-domain can be illustrated by deleting or mutating the C-terminal amino acid of GAIP, which causes its interaction with GIPC to be abolished (De Vries et al., 1998b). More recently, GIPC has been found to interact with a number of other proteins through its PDZ-domain.

The list of identified PDZ-binding motif-containing partners for GIPC consists of numerous transmembrane proteins, including the growth factor-type receptors: insulin-like growth factor 1 (IGF-1) receptor (Booth et al., 2002; Ligensa et al., 2001; Wu et al., 2006), Frizzled-3 (FZD3) Wnt receptor (Tan et al., 2001), TGF-β Type III receptor (Blobe et al., 2001),

the tyrosine kinase receptor TrkA (Lou et al., 2001), Syndecan 4 receptor (Gao et al., 2000), neuropilin 1 (Cai and Reed, 1999), and β1-adrenergic receptor (Hu et al, 2003). In addition to the growth factor-type receptor interactions listed above, GIPC also binds to cell adhesion molecules (CAMs), including semaphorin M-SemF (Wang et al., 1999) and integrin α subunits (El Mourabit et al., 2002; Tani and Mercurio, 2001). These proteins bind GIPC via one of two C-terminal consensus sequences, which are referred to as Class 1 and Class 2 PDZ-binding motifs (Ligensa et al., 2001). In Class I PDZ-binding motifs, represented by (S/T/Y, x, V/A), the -2 position is occupied by a hydroxyl-group containing amino acid (S/T/Y), the -1 position is unspecific, and the 0 position is occupied by either valine or alanine (V/A) (Ligensa et al., 2001). All known GIPC interactions utilize Class I PDZ interactions with the exception of Syndecan-4, which contains a Class II PDZ-binding motif (F/Y, *x*¸ A/F/V), and TrkA, which interacts with GIPC through its juxtamembrane region (Lou et al., 2001).

In addition to interacting with the partners listed above, GIPC is able to dimerize, thereby acting as a scaffolding protein to generate protein complexes (Gao et al., 2000). By acting as a scaffolding protein, GIPC likely serves as a connection between distinct signaling pathways. This is supported by evidence that GIPC ties TrkA and FZD3 to heterotrimeric G protein signaling (Lou et al., 2001; Tan et al., 2001).

1.9 A possible role for GIPC in receptor trafficking

A role for GIPC in endocytic trafficking has been suggested based on its localization to endocytic vesicles, including clathrin-rich invaginations and endocytic compartments (Dance et al., 2004; De Vries et al., 1998a; Lou et al., 2002). For instance, cell culture studies have shown

that GIPC aids in maintaining a relatively constant level of receptors at the cell surface during ligand-induced internalization, indicating that GIPC is required for recycling endocytosed choriogonadotropin receptors back to the cell surface (Hirakawa et al., 2003). Furthermore, GIPC has been found to bind myosin VI, a motor protein that associates with clathrin-coated pits and/or vesicles and regulates clathrin-mediated endocytosis (Buss et al., 2002). GIPC-myosin IV complexes associate with the actin cytoskeleton to facilitate the translocation of endocytic vesicles and their contents. GIPC-mediated endocytosis and myosin VI recycling of cell surface receptors has been suggested to regulate receptor-initiated signaling pathways (Hasson, 2003).

1.10 GIPC-integrin interactions

Human GIPC1 has been found to interact with the human integrin subunits α5 (El Mourabit et al., 2002), α6A and mouse α6B (Tani and Mercurio, 2001) through Class 1 PDZ-binding motifs at the C-terminal of the α-subunit cytoplasmic domains. The exact role of GIPC-integrin associations remains unclear, but it has been suggested that in order to stabilize integrin-mediated multi-protein complexes, such as focal adhesions, GIPC is recruited to the C-terminus of the integrin, and in turn, recruits other signaling and/or scaffolding molecules (El Mourabit et al., 2002). Alternatively, as GIPC is hypothesized to be involved in receptor trafficking, and since integrins are known to be internalized in an endocytic/exocytic recycling manner (section 1.7), an interesting possibility exists that GIPC may be involved in regulating integrin trafficking.

1.11 GIPC in *Xenopus* (XGIPC)

In *Xenopus*, two GIPC family members with sequences highly similar to those of the mammalian GIPC gene family have been identified: Kermit 1 and Kermit 2. Kermit 1 was initially identified during a yeast two-hybrid screen of the *Xenopus* oocyte cDNA library for molecules that directly interacted with the C-terminus of *Xenopus* frizzled (XFZD) proteins (Tan et al., 2001). While Kermit 1 is not homologous to any known genes within the GenBank database, it shares a high degree of similarity with mammalian GIPC1 (De Vries et al., 1998b). At the amino acid level, Kermit 1 is found to be 74% identical to human GIPC1, 48% identical to *Drosophila* Kermit-like gene, and 35% identical to *C. elegans C35D10.2* (Tan et al., 2001). Given the diversity of these organisms, it is likely that Kermit 1 is highly conserved across species and represents a GIPC family member.

In *Xenopus*, *in situ* hybridization assays first detect Kermit 1 expression at gastrulation, at which point expression is high in the dorsal marginal zone (the region of cellular involution). Kermit 1 continues to be expressed during late gastrulation, neurulation, and tadpole stages. Knockdown of Kermit 1 using antisense morpholino oligonucleotides has been shown to block neural crest induction in ectodermal explants, but does not interrupt neural crest formation in whole embryos (Tan et al., 2001). Wu et al. (2006) proposed that this may be due to the presence of a redundant protein in embryos. This group went on to identify Kermit 2, a protein previously identified as XGIPC during a yeast two-hybrid screen for insulin-like growth factor-1 (IGF-1) receptor binding proteins in *Xenopus* oocytes (Booth et al., 2002). Kermit 2 was also isolated in the DeSimone laboratory (University of Virginia) in 2002 during a search for PDZ domain

binding proteins and was referred to at that time as XGIPC (unpublished data). I will refer to this molecule as XGIPC for the remainder of this thesis.

At the amino acid level, XGIPC is 64% identical to Kermit 1. Despite this similarity, XGIPC does not interact with XFZD3, nor does it have a redundant role in IGF signaling overlapping that of Kermit 1 (Wu et al., 2006). Wu et al. (2006) demonstrated that XGIPC is ubiquitously expressed throughout early embryonic stages, including gastrulation. During neurulation, XGIPC becomes localized to the anterior region of the embryo, including the cement gland, neural plate border, and the presumptive eye region, where it associates with IGF receptors (Wu et al., 2006). IGFs are neural inducers that work synergistically with bone morphogenetic protein (BMP) antagonists to induce neurulation (Pera et al., 2003). Knockdown of XGIPC using antisense morpholino oligonucleotides results in the disruption of anterior development; in particular, the expression of eye-specific markers is strongly reduced.

The over-expression of dominant negative IGF-1R inhibits anterior neural patterning (Pera et al., 2001) without inhibiting gastrulation. Additionally, translational knockdown of XGIPC using morpholinos was shown to cause gastrulating embryos to develop truncated anteroposterior axes, which is typical of FN-null embryos (Wu et al., 2006), suggesting that XGIPC has a greater role during embryogenesis than mediating IGF-1R.. Given that the FN-receptor, integrin α5β1, has been shown to interact with GIPC in other systems and is the sole active integrin during gastrulation, it is an interesting possibility that XGIPC may be involved in regulating α5β1 activity.

1.12 Experimental objectives of this study

This study aims to determine whether XGIPC mediates the α5β1 integrin interaction with FN during gastrulation and whether it does so by modulating inside-out signaling, outside-in signaling, or integrin trafficking. The *Xenopus* gastrula provides a useful model to investigate a role for XGIPC in regulating α5β1 integrin function. First, the spatially and temporally restricted activities of α5β1 are well characterized. Secondly, *Xenopus* embryos are large and robust, making them amenable to molecular manipulation *in vivo*. Additionally, tissue can be excised from *Xenopus* embryos and cultured *ex vivo*, where they continue to undergo "normal" cell movements, allowing for the anaylsis of *in vivo* processes in a controlled environment.

To study the endogenous function of XGIPC in *Xenopus* embryos, I have utilized a dominant negative XGIPC, which contains a mutation in the PDZ-domain. This PDZ-domain mutation is anticipated to disrupt interactions with the α5β1 integrin. The first objective of this study was to determine if XGIPC directly interacts with the α5β1 integrin through the α-subunit cytoplasmic tail. To accomplish this *in vitro* and *in vivo*, I have used yeast two-hybrid and co-immunoprecipitation assays. My next aim was to determine if XGIPC, through its interaction with the α5-subunit, affects the FN-dependent cell behaviours that drive gastrulation. This has been done by monitoring the progression of gastrulation, as measured by blastopore closure. To determine a role for XGIPC in mediating the changes in cell behaviour necessary for morphogenetic rearrangements, embryos were microinjected with dominant negative XGIPC and used for cell and tissue explant assays designed to monitor the integrin's ability to undergo inside-out and outside-in signaling. As GIPC is thought to have a role in vesicular trafficking, my next goal was to learn if XGIPC has a role in the internalization of α5β1 integrins to

endocytic vesicles. *Xenopus* kidney epithelial (A6) cell cultures expressing wild-type and dominant negative XGIPC were used to monitor the requirement for XGIPC in the turnover of α5β1 receptors at the cell surface.

Chapter 2 Methods and Materials

2.1 Plasmid constructs and generation of *in vitro* transcripts

A full-length cDNA representing *Xenopus* GIPC (XGIPC) tagged with a hemagglutinin (HA) epitope in the expression vector PCS2 was obtained as a gift from Ronald Booth (Ottawa Health Research Institute; Accession AAL58320). Site-directed mutagenesis was used to mutate the amino acid sequence ALGL, within the PDZ domain of XGIPC, to AAEL, thereby generating the $XGIPC_{mut}$ construct (Hyder Al-Attar, personal communication). For use in yeast 2-hybrid assays, XGIPC and $XGIPC_{mut}$ were fused to an activation domain from bacterial sequence B42 in the prey pJG4-6 plasmid (Gyuris et al., 1993) using *Eco*R1 and *Xho*1 restriction sites (Hyder Al-Attar, personal communication). Inserts were confirmed by sequencing (Appendix A.2).

XGIPC- and $XGIPC_{mut}$-green fluorescent protein (GFP) fusion constructs were generated. The DNA sequences encoding XGIPC and $XGIPC_{mut}$ were digested using *Eco*RI and *Bam*HI restriction sites, and ligated into the *Eco*RI and *Bam*HI sites of pEGFP-N1, effectively fusing GFP to the N-terminus of the XGIPC and $XGIPC_{mut}$ constructs (vector was a gift from J. Miller; University of Minnesota).

Clones representing the *Xenopus* integrin subunits α5, α6, and αV were a gift from Douglas DeSimone (University of Virginia). Cytoplasmic domains of the α5, α6 and αV subunits were isolated by PCR using standard techniques; the α5, α6, and αV subunit tail sequences and the associated primers (Na et al., 2003) are shown in Table 2.1. For use in yeast two-hybrid

assays, the cytoplasmic domains of the α5, α6, and αV subunits were subcloned as fusion constructs with the DNA binding domain of LexA in the bait plasmid pEG202, as previously described (Duncker et al., 2002) using *Eco*R1 and *Xho*1 restrictions sites (the pEG202-α5 construct was prepared by Hyder Al-Attar, personal communication). Inserts were confirmed by sequencing (Appendix A.2).

Table 2.1 Subunit tail amino acid sequences and primer DNA sequences.

	Subunit cytoplasmic tail amino acid sequence	Primer DNA sequence
α5	KVGFFKRSYQYGTAMEKAELKPQAASEA	For 5' GGCTTCTTTAAACGCTCTTACC3' Rev5' TTAAGCCTCTGARGCAGCCTG3'
α6	KVGFFRRDKKDQFDATYHKAEIHAQPSDKERLTSDA	For 5'CGGCTTCTTCAGGAGAGATAAG3' Rev 5' TTATGCATCAGAAGTTAGCC3'
αV	KVGFFKRFRPPQEETEREQLQPQENGEGITFT	For 5' GGAATTCAAACGTGTTCGACCCCCACAG3' Rev 5' GGGCTCGAGATTATGTGTCCGTAATTC3'

Underlined amino acids correspond to conserved PDZ-binding motifs previously shown to interact with GIPC in other model systems (De Vries et al., 1998b; Tani and Mercurio, 2001). The αV cytoplasmic tail does not contain a conserved PDZ-binding motif.

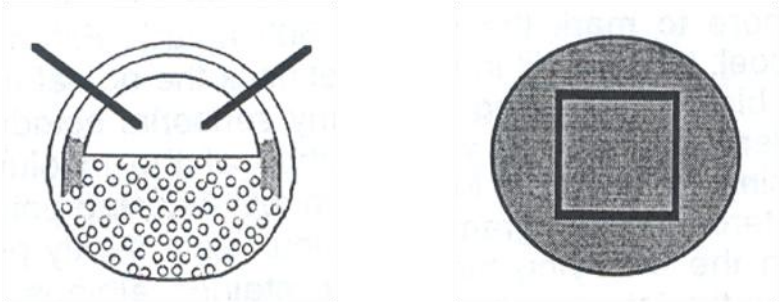
2.2 Xenopus embryos, microinjections and microsurgery

Sexually mature wild-type and albino *Xenopus laevis* adults were purchased from Nasco (Fort Atkinson, Wisconsin). Animals were housed in the Department of Biology Aquatic Facility at the University of Waterloo. Individual female frogs were injected with 800 units of human chorionic gonadotropin (HCG) (Sigma, Oakville, Ontario) to induce spawning. Eggs were obtained manually from *Xenopus* females and fertilized *in v*itro by standard methods (Sive et al.,

1996). Embryos were staged according to Nieuwkoop and Faber (1967). Fertilized embryos were dejellied in 2% cysteine in water (EMD, Mississauga, Ontario).

Microinjection needles were pulled using a Narishige PC-10 puller (East Meadow, NY). Microinjections were performed using a Narishige IM300 pressure injector (East Meadow, NY). Embryos were microinjected with 1 ng/nl of mRNA in 0.5 X Modified Barth's Saline (1X MBS; 88mM sodium chloride (NaCl), 1mM potassium chloride (KCl), 0.7mM magnesium sulphate ($MgSO_4$), 1mM HEPES, 5mM sodium bicarbonate ($NaHCO_3$), 0.1mM calcium chloride ($CaCl_2$), pH 7.6) with 4% Ficoll 400 (Sive et al., 1996). Following microinjection, embryos were cultured in 0.1 X MBS.

Embryos undergoing microsurgery were transferred to a plasticine-coated Petri dish containing 1X MBS. Vitelline membranes were removed manually with forceps. Embryo explants consisting of a square of tissue centred on the animal pole and extending 45^o to the equator were cut using forceps (Figure 2.1) (Sive, 1996). The excised explants, called animal caps, were used in a number of assays (see sections 2.4, 2.5, 2.6).



(A) (B)

Figure 2.1 Animal cap excision. (A) Depiction of a blastula (stage 8) in cross-section. Bolded black lines depict cut made through the animal hemisphere to isolate animal caps. (B) Depiction of blastula from animal view. Black square outlines area to excised (adapted from Sive, 1996)

2.3 Embryo and cell imaging

Embryo and explant images were taken using the Zeiss Lumar.V12 microscope (Zeiss, Burnaby, BC), a Canon PowerShot A620 digital camera, and Zeiss Axiovision 4 software. Embryonic and A6 cells were visualized using a Zeiss Axiovert 200 inverted microscope (Zeiss, Burnaby, BC) equipped with a Ludl motorized stage and Qimaging retiga 1494 digital camera. Images were recorded using OpenLab software (Improvision; Waltham, MA).

2.4 FN staining of animal caps

Assembly of fibrillar FN matrix was monitored using immunocytochemistry. Briefly, XGIPC and $XGIPC_{mut}$ over-expressing embryos, alongside non-injected and water-injected control embryos, were cultured as previously described (section 2.2) until stage 12 and then fixed in 2% trichloroacetic acid (TCA). Fixed embryos were washed in Tris Buffer Saline (TBS; 50mM Tris-HCl pH 7.5, 160mM NaCl) and 0.1% Tween20 (TBST; Fisher Scientific, Ottawa, ON) and animal caps were excised as previously described. Animal caps were stained with a monoclonal antibody directed against FN (4B12; Ramos et al., 1996) in TBST containing 1 ug/ml of Bovine Serum Albumin (BSA). Primary antibodies were detected using Alexa Fluor 488 Conjugated Goat Anti-mouse secondary antibody (Invitrogen, Burlington, ON). Stained animal caps were mounted on glass slides and imaged using a Zeiss Axiovert 200 microscope as described above.

2.5 Cell migration assays

Changes in integrin α5β1 behaviour that result in the switch from cell adhesive to migrating states can be monitored using cell migration assays. Embryos were microinjected with XGIPC and $GIPC_{mut}$ mRNA and cultured until stage 8 as described previously. Animal caps were then isolated and dissociated into individual cells in calcium and magnesium free Danilchik's for Amy (DFA^-; 50mM NaCl, 100mM D-gluconic acid, 5mM Na_2CO_3, 5mM KCl, 6mM HEPES) solution. Individual cells were cultured in the presence or absence of 50 pM Activin-A (R&D Systems, Burlington) until sibling embryos reached stage 10. FN substrates were prepared on a Petri dish by diluting human plasma FN (Calbiochem, Mississauga, ON) to 50 ug/ml in phosphate buffer saline (PBS; 130mM NaCl, 3mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4), supplemented with 1mM $CaCl_2$ and 1mM $MgCl_2$ (PBS^+). Individual, induced cells were plated on FN substrates in Modified Stearn's Solution (MSS^+) (3.75mM NaCl, 0.01mM Na_2SO_4, 0.25mM HEPES, 0.12mM KCl, 30mM Na_2HPO_4, 0.07mM KH_2PO_4, 1mM $CaCl_2$, 1 mM $MgCl_2$, with 0.5 mg/ml BSA, pH 8.3). Cells expressing microinjected constructs were identified using GFP-expression. The cell migrations were monitored using a Zeiss Axiovert 200 microscope as described above.

2.6 Animal cap extension assays

Animal caps of embryos microinjected with XGIPC and $XGIPC_{mut}$ mRNA were excised from stage 8 embryos as previously described. Animal caps from experimental and control embryos were cultured in 0.5X MBS, 0.1% BSA, in the presence or absence of 50 pM Activin-

A. As a control for normal development, sibling embryos were cultured in 0.1X MBS solution. Overnight explant extension was recorded and imaged as described in section 2.3.

2.7 Actin staining of animal cap cells

To determine if XGIPC has a role in mediating F-actin polymerization, embryos were microinjected as described previously and cultured until stage 8. Animal caps were then excised and dissociated as described in section 2.5. Dissociated cells were cultured until sibling embryos reached stage 10 and then plated on 50 ug/ml FN for 2 hours to allow for adhesion to the substrate. Attached cells were fixed using 3.7% formaldehyde (Fisher Scientific, Ottawa, ON) in MSS^+ for 30 minutes. Fixed cells were rinsed with TBS^+ before being permeablized with TBST. Intracellular actin was detected using 10 µg/ml rhodamine-phalloidin (Sigma, Oakville, ON) in TBS. Excess rhodamin-phalloidin was rinsed from cells using TBS. Stained cells were imaged as described above.

2.8 Yeast two-hybrid analysis

Fusion constructs (described in section 2.1) were used to co-transform DY1 *Saccharomyces cerevisiae* cells, which contains the reporter plasmid, pSH18-34 (strain: Duncker Yeast 1; a gift from Dr. Bernard Duncker) (Semple et al., 2006). A positive control of known prey-bait interaction DY-1(pSH18-34)(pJG4-6-Rad53)(pEG202-Dbf4(FL)) was a gift from Dr. Bernard Duncker (University of Waterloo).

Table 2.2 Yeast two-hybrid prey-bait combinations

Prey fusion construct (pJG4-6 vector)	Bait fusion construct (pEG202 vector)
XGIPC	α5
$XGIPC_{mut}$	α5
XGIPC	α6
$XGIPC_{mut}$	α6
XGIPC	αV
$XGIPC_{mut}$	αV
Rad53	Dbf4(FL)

Transformants were grown in synthetic complete (SC) media plates lacking uracil, tryptophan, and histidine at 30^{o}C to a concentration of 5 X 10^6 cells/ml. Cells were washed and then resuspended in 2% galactose-1% raffinose and lacking uracil, tryptophan, and histidine for 6 hours to induce prey expression (Semple et al., 2006). Following induction, 5 X 10^6 cells were harvested and the interactions between fusion proteins were quantified using β-galactosidase assays using the substrate *o*-nitrophenyl-β-D-galactopyranosidase (ONPG) (Burke et al, 2000). β-Galactosidase activity was calculated using the formula: $1000 \times A_{420nm} \div (t \times v \times A_{600nm})$, where t represents time in minutes, v represents volume in millilitres, and A represents absorbance (Semple et al., 2006). Two colonies of each transformant were assessed during independent yeast two-hybrid assays; assays were preformed in triplicate. Yeast protein extraction was carried out as described previously (Varrin et al., 2005) and the expression of bait and prey fusion proteins was confirmed by immunoblotting (refer to section 2.9.1).

2.9 Western blotting

2.9.1 Confirmation of fusion protein expression during yeast two-hybrid assays

Fusion protein expression in yeast two-hybrid assays was confirmed by western blotting using standard protocols (Sambrook, 2001). Briefly, yeast protein extracts were prepared as previously described (Varrin et al., 2005), quantified by Bradford Assay, separated using a SDS-PAGE gel (Sambrook, 2001) and electrophoresed onto nitrocellulose. HA-tagged prey fusion proteins were detected using mouse monoclonal anti-HA (12CA5, Roche, Mississauga, ON) primary antibody and Horse radish peroxidise (HRP) conjugated anti-mouse secondary antibody (Jackson Labs; West Grove, PA). LexA-tagged bait fusion proteins were detected using rabbit polyclonal anti-LexA (Sigma, Oakville, ON) primary antibody and HRP-conjugated anti-rabbit secondary antibody (Jackson Labs; West Grove, PA). Bands were visualized using the ECL system (GE Healthcare; Mississauga, ON) and exposure to RXB x-ray film (Labscientific; Livingston, NJ).

2.9.2 Confirmation of protein expression in *Xenopus* embryos

Prior to conducting experimental assays, embryos were microinjected with serial dilutions of mRNA and Western blot analysis was conducted to ensure equal protein expression (Appendix D). Protein extracts were prepared from embryos homogenized in embryo lysis buffer (ELB) (20mM Tris (pH 7.5), 140mM NaCl, 10mM glycerol, 1mM DTT, 2mM sodium-orthovanadate, 25mM NaF, 1% Nonidet P-40, and 1X Complete Protease Inhibitor (Roche,

Mississauga, ON)). Equivalent amounts of embryo lysate were separated using a SDS-PAGE gel (Sambrook, 2001) and electrophoresed onto nitrocellulose. HA-tagged XGIPC expression was detected using anti-HA (12CA5; Roche, Mississauga, ON) primary antibody and visualized as described above (section 2.9.1).

2.10 Co-immunoprecipitation assays

For immunoprecipitates, anti-β1 antibody (8C8) (a gift from Peter Hausen, Max-Plank Institute, Tubingen) was conjugated to Protein G PLUS/Protein A-Agar Suspension beads (Protein G/A beads; Calbiochem, Mississauga, ON) by incubating at 4^{o}C for 3 hours. Embryos were microinjected with HA-tagged XGIPC and XGIPC$_{mut}$ mRNA and cultured to stage 11. Stage 11 embryos were homogenized in PBS^{+}–Lysis Buffer (PBS^{+}, 1% Triton X-100, 12.5µl/ml phenylmethylsulfonyl fluoride (PMSF), 1X Complete Protease Inhibitor Cocktail (Roche, Mississauga, ON), 0.097mM sodium-orthovanadate). Homogenized embryos were incubated on ice for ten minutes and then centrifuged at 4^{o}C for ten minutes. Cleared lysate was diluted 1:3 in PBS-Lysis Buffer and incubated with 10µl Protein G PLUS/Protein A-Agar Suspension beads at 4^{o}C for 30 minutes to clear non-specific binding proteins. Lysate-Protein G/A bead mixture was centrifuged for 5 minutes at 4^{o}C. The supernatant was then removed and incubated with 8C8 previously bound to Protein G/A beads for 3 hours at 4^{o}C. Lysate-antibody-Protein G/A bead solution was centrifuged at 4^{o}C and 8C8-bound protein complexes were washed four times in cold PBS-Lysis Buffer. IPs were subjected to Western blotting as described in section 2.9; HA-tagged XGIPC was detected using anti-HA (12CA5; Roche, Mississauga, ON) primary antibody.

2.11 Transfection of XGIPC-GFP into *Xenopus* A6 cells

Xenopus A6 cells (ATCC# CCL-102; cells were a gift from Dr. John Heikkila, University of Waterloo) were maintained in 66% L-15 media (Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (FBS) (Wisent, St. Bruno, QC), 1% L-glutamine (Wisent, St. Bruno, QC), 1% Pennicillin/Streptomycin (Wisent, St. Bruno, QC), 1% sodium pyruvate (Wisent, St. Bruno, QC) and maintained at room temperature. Cells were plated and allowed to grow to 60-80% confluence. 1.0µg of purified plasmid was transfected into cells using 20 µl LipoFectamine (Invitrogen, Burlington, ON) for 6 hours, according to standard protocols. Transfection media was then removed and cells were cultured in fresh 66% L-15 media.

2.12 Localization of XGIPC in A6 cells

To determine where XGIPC localizes within the cell, A6 cells were transfected with XGIPC-GFP and $XGIPC_{mut}$-GFP DNA as described above. Forty-eight hours following transfection the 66% L-15 media was removed and cells were rinsed with FBS-free 66% L-15 media. Cells were detached with Trypsin/EDTA (Wisent; 0.05% Trypsin, 0.53mM EDTA; Wisent, St. Bruno, QC) and neutralized using 66% L-15 media with FBS and replated on 60mm glass bottom dishes. Neutralized cells were fixed using 10% formaldehyde (Fisher Scientific, Ottawa, ON) in FBS-free 66% L-15 for 30 minutes. Fixed cells were rinsed three times using PBS^{+} and imaged as described above.

2.13 Internalization assay

Integrin internalization was monitored in A6 cells that were transfected with XGIPC and $XGIPC_{mut}$ encoding DNA as described above. Transfected cells were incubated for 1 hour at 4^oC to slow cell membrane dynamics, including integrin turnover. Cells at 4^oC were incubated with anti-α5β1 antibody (P8D4) at 4^oC for 1 hour. Cells were washed three times with serum-free 66% L-15 medium to remove unbound antibodies. Cells were then incubated at room temperature to allow for cell membrane dynamics. Cells were fixed and rinsed as described previously and blocked in staining solution (TBS, 0.1% Triton X-100 (Fisher Scientific, Burlington, ON) and 1% Lamb Serum (Invitrogen, Burlington, ON)) for 1 hour. P8D4 was detected using a goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Burlington, ON) in staining solution for 1 hour. Following incubation with the secondary antibody, A6 cells were washed three times with staining solution. Transfected cells were identified by GFP-expression and internalized integrins were imaged as described above.

Chapter 3 Results

3.1 Development of a dominant negative XGIPC

We have generated a mutation in the PDZ-domain of XGIPC by mutating the lysine (L; TTA) and glycine (G; GGA) residues of the consensus sequence ALGL to alanine (A; GCA) and glutamic acid (E; GAA), respectively (a gift from Hyder Al-Attar). As the mutant AAEL-containing construct has been demonstrated to abolish interactions between GIPC and human α-subunits, we anticipated it to act as a dominant negative ($XGIPC_{mut}$) when over-expressed in *Xenopus* cells (Tani and Mercurio, 2001). The mutation in $XGIPC_{mut}$ was confirmed by sequencing the Open Reading Frame (ORF) from wild-type XGIPC and $XGIPC_{mut}$ cDNA (Appendix A.1).

3.2 XGIPC interacts with α5 *in vitro.*

To determine if XGIPC is capable of interacting with the C-terminus of the *Xenopus* α5 subunit I conducted yeast two-hybrid assays. *Xenopus* α5, α6, and αV cytoplasmic tail coding sequences were cloned into the yeast two-hybrid bait vector, pEG202. The α6 subunit contains a conserved PDZ-binding motif and was used as a positive control (Table 2.1). The αV subunit does not contain a known PDZ-binding motif and was used a negative control (Table 2.1). The bait constructs were separately transformed with prey plasmid, expressing either XGIPC or $XGIPC_{mut}$, into DY-1, which had been previously transformed with the lacZ reporter-plasmid

pSH18-34 (Semple et al., 2006). Prey and bait fusion construct sequences were confirmed by sequence analysis (Appendix A.2). An established positively interacting prey:bait combination (DY-1(pSH18-34)(pJG4-6-Rad53)(pEG202-Dbf4(FL)), referred to here as the non-relevant control, was included as a positive control for β-galactosidase activity. The expression of prey and bait proteins were monitored by Western blot analysis (Figure 3.1; Figure 3.2) confirming strong expression in yeast transformants. The β-galactosidase activity of the prey:bait transformants listed in Table 2.2 were used as an indication of the strength of interaction between prey and bait.

The strongest β-galactosidase signal was obtained when XGIPC was tested as prey with the α6 subunit as bait (Figure 3.3A), indicating that XGIPC is most likely to interact with the α6 subunit. As XGIPC-α6 β-galactosidase activity was the highest value obtained (5086.7 as calculated using the formula described in section 2.8), it was standardized to a value of 1.0, and the β-galactosidase activity of the other bait and prey pairs are reported as a ratio of this standardized value. When XGIPC was co-expressed with the α5 subunit, a strong β-galactosidase signal was observed (Figure 3.3B), although it was 40% weaker than that of the XGIPC:α6 combination ($P<0.08$, as determined by Student T-Test) (Figure 3.4). Co-expression of XGIPC with the αV subunit resulted in weak β-galactosidase activity that was statistically different ($P<0.001$) from that of the XGIPC:α6 and XGIPC:α5 combinations, indicating that XGIPC does not interact with the αV subunit (Figure 3.3C).

When an XGIPC containing an LG to AE mutation in the PDZ domain ($XGIPC_{mut}$) was tested as prey, regardless of the bait construct with which it had been co-expressed, the β-galactosidase signal was weak. The β-galactosidase activity obtained from the $XGIPC_{mut}$:α5 combination was significantly different from that of the XGIPC:α5 combination, indicating that

$XGIPC_{mut}$ does not interact with the α5 subunit ($P<0.0001$) (Figure 3.3A). Similarly, the β-Galactosidase signal obtained from the $XGIPC_{mut}$:α6 combination was significantly different from that of the XGIPC:α5 combination ($P<0.002$) (Figure 3.3B). The weak β-Galactosidase activity obtained from the $XGIPC_{mut}$:αV combination was similar to that of the XGIPC:αV combination ($P<0.64$), confirming that $XGIPC_{mut}$ is also unable to interact with the αV subunit (Figure 3.3C). These results indicate that XGIPC is capable of interacting with the α5 and α6 subunits through its PDZ domain.

Figure 3.1 Co-expression of XGIPC with α5, α6, and αV subunits in yeast transformants. Whole cell extracts were analysed on Western blots to confirm prey and bait expression. HA-tagged XGIPC was expressed in all transformants (upper panel). The α5, α6, and αV subunits expressed as bait were expressed in each transformant (lower panel). Two individual transformants for each prey:bait combination are shown.

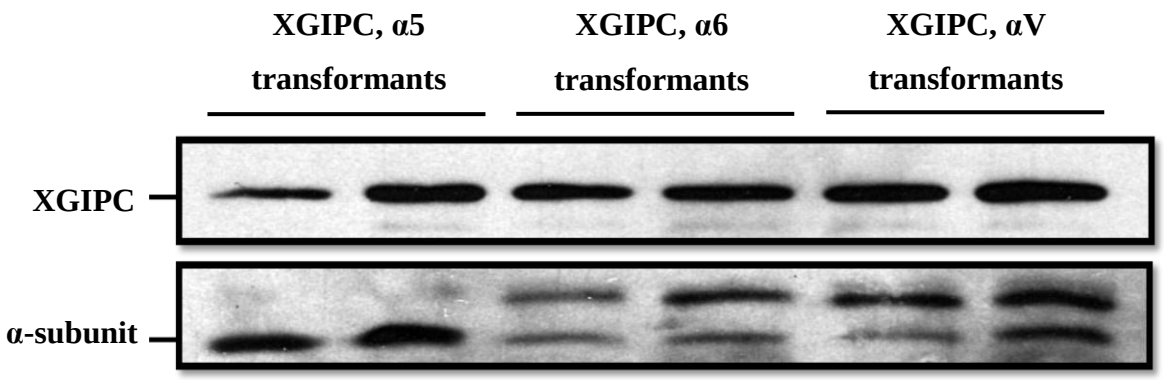

XGIPC, α5 transformants
XGIPC, α6 transformants
XGIPC, αV transformants
XGIPC
α-subunit

Figure 3.2 Co-expression of XGIPC$_{mut}$ with α5, α6, and αV subunits in yeast transformants. Whole cell extracts were analysed on Western blots to verify prey and bait expression. HA-tagged XGIPC$_{mut}$ was expressed in all transformants (upper panel). The α5, α6, and αV subunits expressed as bait were expressed in each transformant (lower panel). Two individual transformants for each prey:bait combination are represented.

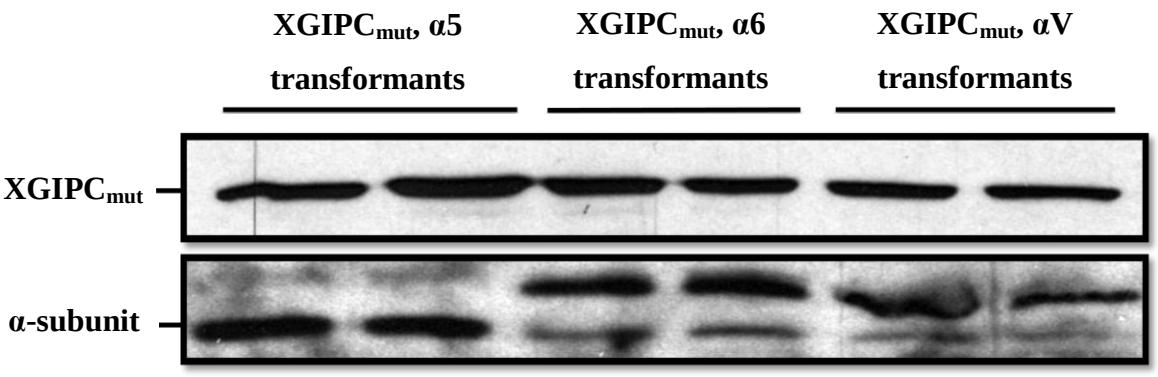
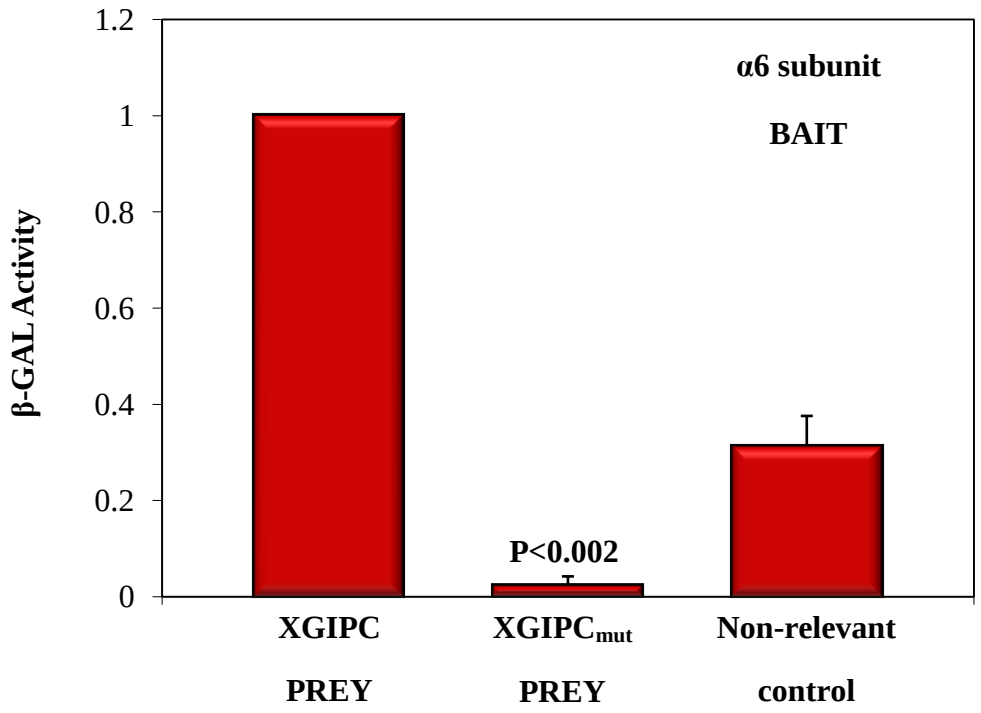
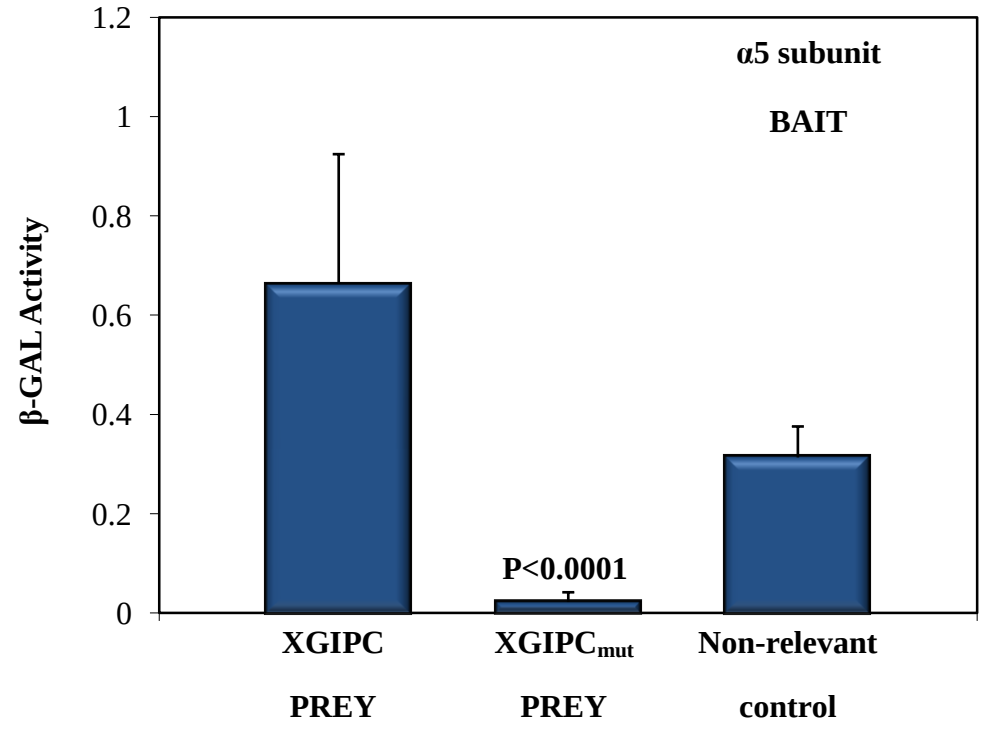

XGIPC$_{mut}$, α5 transformants
XGIPC$_{mut}$, α6 transformants
XGIPC$_{mut}$, αV transformants
XGIPC$_{mut}$
α-subunit

Figure 3.3 XGIPC interacts with integrin α5 and α6 subunits *in vitro*. Yeast two-hybrid assays were conducted using α5, α6 and αV as baits in combination with XGIPC and $XGIPC_{mut}$ as prey. Signal intensities were normalized to the α6:XGIPC β-galactosidase signal (A). Mutation in the XGIPC PDZ-domain abolished interaction with the a6 bait construct ($P<0.002$, as determined by Student T-test). Similarly, α5 subunits interacted with XGIPC, but did not interact with $XGIPC_{mut}$ ($P<0.0001$) (B). As XGIPC does not interact with αV subunit, the $XGIPC_{mut}$ construct has no significant effect to the observed β-galactosidase activity ($P<0.64$) (C). The non-relevant control is included as a positive control for β-galactosidase activity. Error bars represent standard deviations.

(A)


1.2
1
0.8
0.6
0.4
0.2
0
β-GAL Activity
α6 subunit
BAIT
P<0.002
XGIPC
PREY
XGIPCmut
PREY
Non-relevant
control

(B)


1.2
1
0.8
0.6
0.4
0.2
0
β-GAL Activity
α5 subunit
BAIT
P<0.0001
XGIPC
PREY
XGIPCmut
PREY
Non-relevant
control

(C)

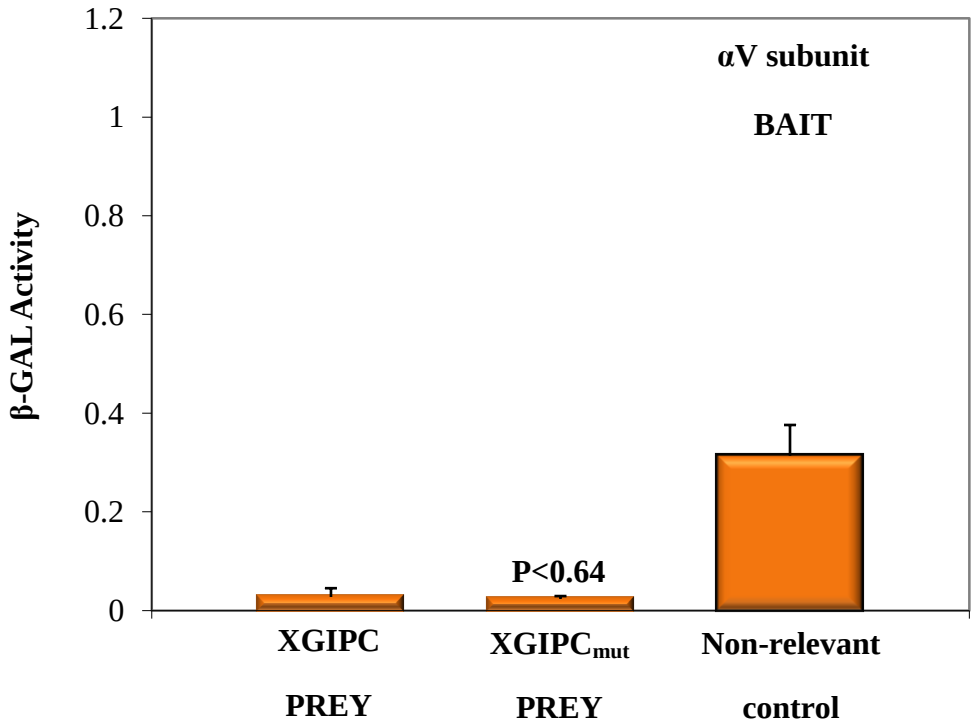
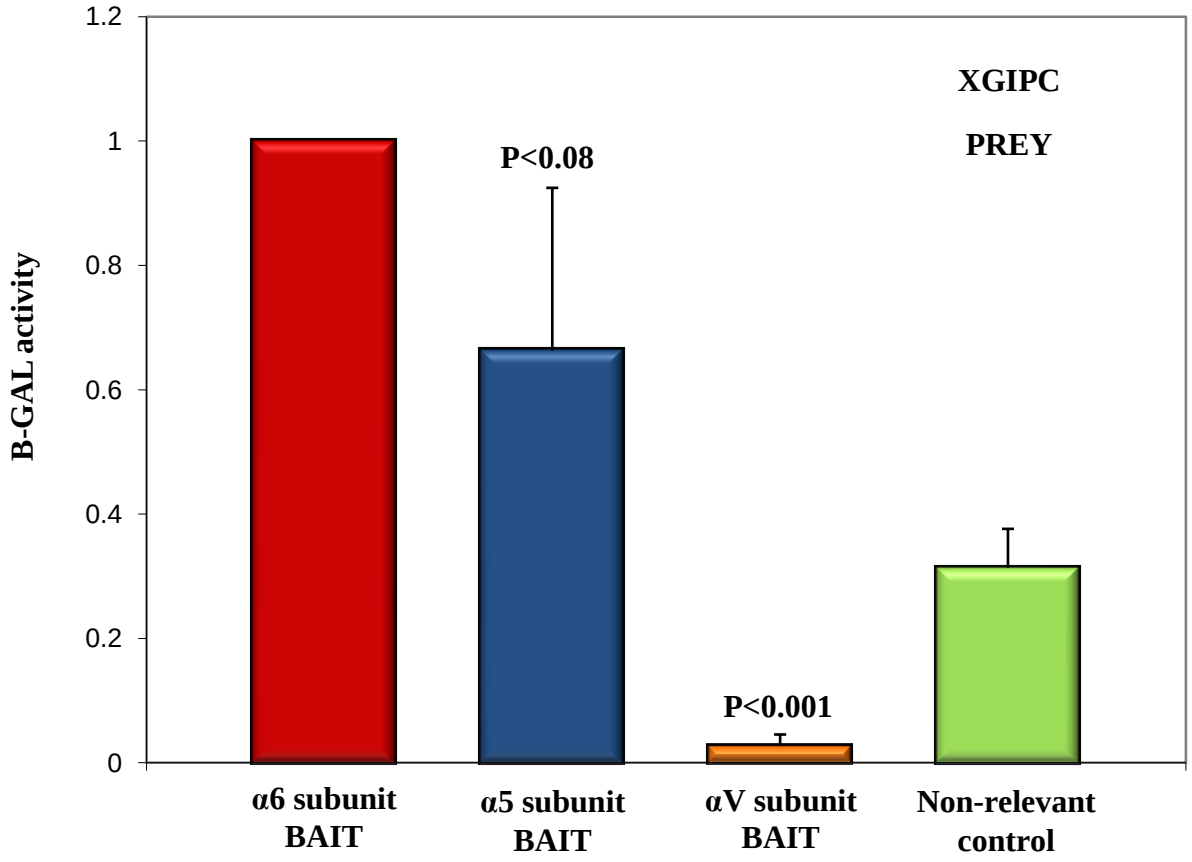

1.2
1
0.8
0.6
0.4
0.2
0
β-GAL Activity
αV subunit
BAIT
P<0.64
XGIPC
PREY
XGIPCmut
PREY
Non-relevant
control

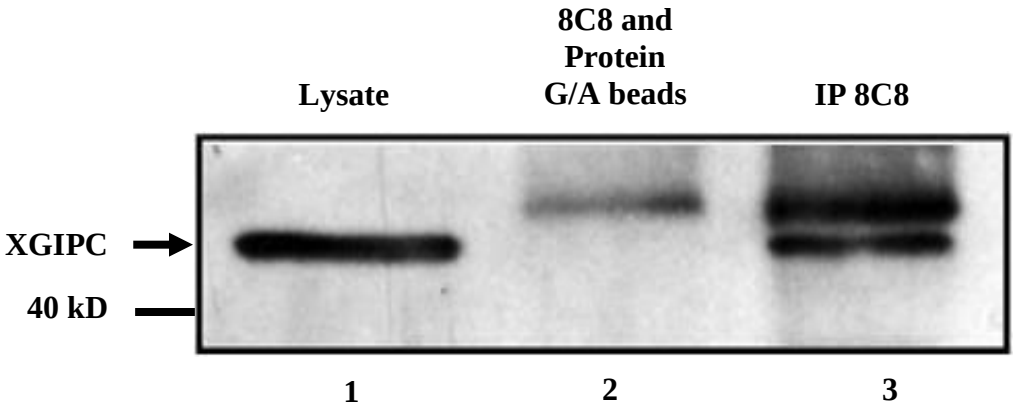
Figure 3.4 XGIPC interacts more strongly with the α6 subunit than with the α5 subunit. Co-expression of XGIPC prey with α6 bait resulted in the strongest β-galactosidase signal (α6 subunit, red bar). The α5 subunit (α5 subunit, blue) showed a weaker interaction with XGIPC ($P<0.08$) than the α6:XGIPC combination. Co-expression of the XGIPC-prey construct with the αV-bait construct resulted in weak β-galactosidase activity relative to the α6:XGIPC combination ($P<0.001$) (αV subunit, orange). The non-relevant control reveals that the interactions between XGIPC and the α5 and α6 subunits is comparatively strong (Non-relevant control, green).


B-GAL activity
1.2
1
0.8
0.6
0.4
0.2
0
XGIPC
PREY
P<0.08
P<0.001
α6 subunit BAIT
α5 subunit BAIT
αV subunit BAIT
Non-relevant control

3.3 Interaction of XGIPC with α5β1 *in vivo*.

To further investigate XGIPC's interaction with the α5β1 integrin, I used co-immunoprecipitation assays to determine if XGIPC interacts with the α5β1 integrin *in vivo*. Attempts to co-immunoprecipitate α5β1 with over-expressed HA-tagged XGIPC failed. To overcome this problem, both the α5 subunit and XGIPC were over-expressed in embryonic cells. Over-expression of the α5 subunit has been shown to be sufficient to drive an increase in surface expression of mature α5β1 integrins (Na et al, 2003). Integrins containing the β1 subunit were immunoprecipitated from embryo lysate using an anti-β1 antibody (8C8) (Gawantka et al., 1992) and HA-tagged XGIPC was detected on western blots using an anti-HA primary antibody (12CA5, Roche). XGIPC was detected in embryonic lysate at approximately 40 kD (Figure 3.5, lane 1, arrow). A protein band, of higher molecular weight than XGIPC, was detected in a control lane of 8C8 antibody and Protein G/A without lysate (Figure 3.5, lane 2) and likely represents Protein G/A as it binds the secondary antibody. HA-tagged XGIPC was detected in 8C8 immunoprecipitates (Figure 3.5, lane 3), indicating that a physical interaction between XGIPC and α5β1 exists *in vivo*. These results indicate that XGIPC interacts with α5β1 integrin in *Xenopus* embryos.

Figure 3.5 XGIPC interacts with α5β1 integrin *in vivo*. Integrins were immunoprecipitated from embryo lysates with an antibody directed against the β1 subunit (8C8) and detected on western blots with anti-HA primary antibody (12CA5, Roche). HA-tagged XGIPC (approximately 40 kD) is detected in lysate (lane 1, arrow). Lane 1 represents 3% of IP input. A protein band, likely Protein G/A, is detected in the control lane (8C8 antibody and Protein G/A) (lane 2). XGIPC co-precipitates with α5β1 (lane 3, lower molecular weight band).


8C8 and
Protein
G/A beads
Lysate
IP 8C8
XGIPC
40 kD
1
2
3

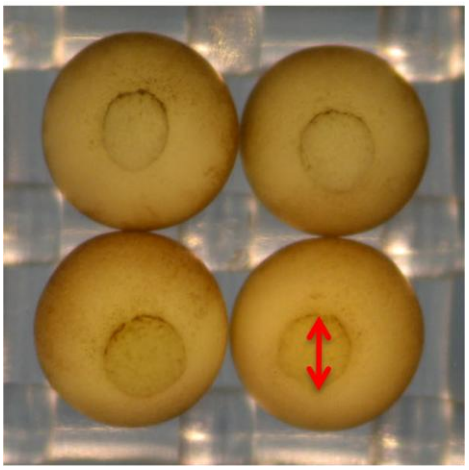
3.4 XGIPC is required for gastrulation.

Active α5β1 integrin is required for the morphogenetic processes that drive gastrulation; disruption of these tissue rearrangements results in delays in blastopore closure (Boucaut et al., 1984; Marsden and DeSimone, 2001). As XGIPC has been found to interact with the α5 subunit, I wished to determine if XGIPC has a role in gastrulation. To do this, gastrulation was monitored by the progression of blastopore closure in non-injected control embryos, and compared to embryos over-expressing XGIPC and $XGIPC_{mut}$ transcripts.

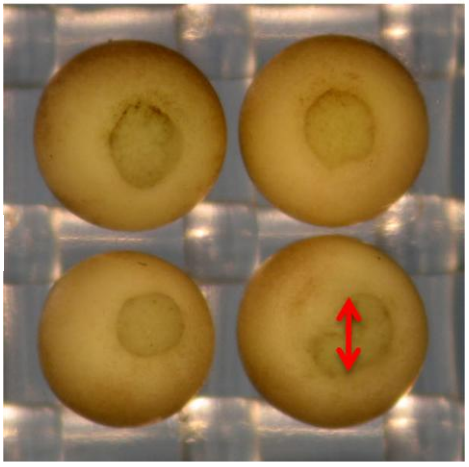
At the onset of gastrulation, the blastopore lip is observed on the dorsal surface of all embryos, indicating tissue rearrangements initiated normally (data not shown). When control embryos reached mid-gastrulation they exhibited normal blastopores, indicating a proper progression of gastrulation (Figure 3.6, *NI*, double-arrow). Embryos microinjected with XGIPC mRNA displayed blastopores indistinguishable from those of control embryos (Figure 3.6, *XGIPC*, double-arrow). In contrast, embryos microinjected with $XGIPC_{mut}$ mRNA exhibited a pronounced delay in blastopore closure, signifying an inhibition of gastrulation (Figure 3.6, $XGIPC_{mut}$, double-arrow). Taken together, these results suggest that the PDZ domain of XGIPC is essential for proper gastrulation.

Figure 3.6 XGIPC is required for gastrulation. Non-injected embryos (NI) exhibited normal blastopore closure (double-arrow). Likewise, embryos microinjected with XGIPC mRNA (XGIPC) showed normal progression of blastopore closure (double-arrow). Embryos microinjected with $XGIPC_{mut}$ mRNA ($XGIPC_{mut}$) failed to close their blastopore (double-arrow).

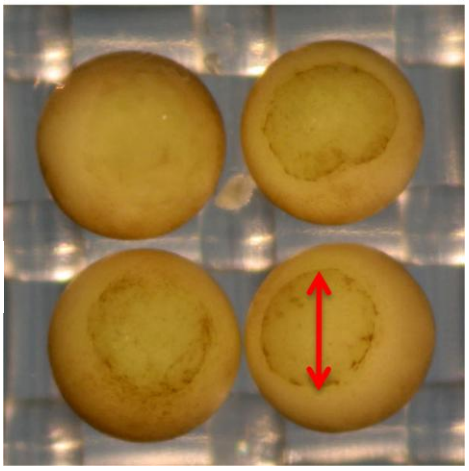
NI



XGIPC



XGIPC$_{mut}$

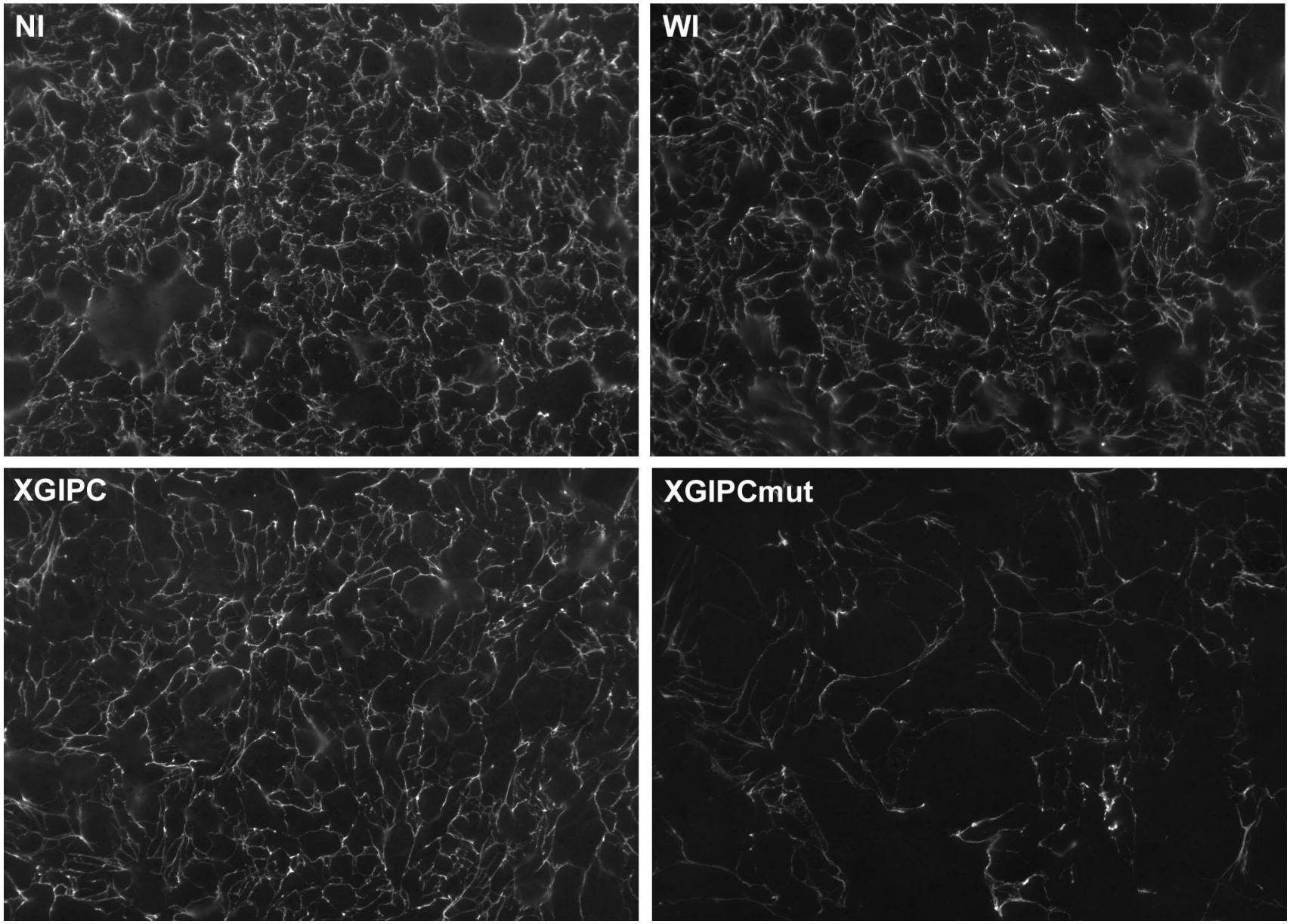


3.5 XGIPC regulates FN matrix assembly.

The delays in blastopore closure exhibited by embryos expressing the dominant negative $XGIPC_{mut}$ are reminiscent of embryos that lack FN matrix due to inhibited α5β1 integrin function (Marsden and DeSimone, 2001; Davidson et al., 2002). To determine if XGIPC mediates FN matrix assembly through its interaction with the α5β1 integrin, the BCRs of gastrula-stage embryos expressing either XGIPC or $XGIPC_{mut}$ mRNA were examined for FN matrix assembly (Figure 3.7).

The apical surfaces of BCRs excised from non-injected control embryos exhibited a typical, mature fibrillar FN matrix (Figure 3.7, *NI*) (Lee et al., 1984). Embryos microinjected with water as a control for injection artefacts displayed a similar FN matrix (Figure 3.7, *WI*). Explants microinjected with XGIPC mRNA also exhibited a well-developed network of fibrils across the apical surface of the BCR, although fibrils were slightly less dense than control embryos (Figure 3.7, *XGIPC*). The FN fibrils on $XGIPC_{mut}$-expressing BCRs appeared as long, sparse tendrils (Figure 3.7, *XGIPCmut*). From these data it can be concluded that the expression of XGIPC containing a mutation in the PDZ domain results in an inhibition of FN matrix assembly across the apical surface of the BCR.

Figure 3.7 XGIPC is required for FN matrix assembly. FN matrix assembly on the BCRs of stage 12 embryos was detected using immunofluorescence with the MAb 4B12. Staining of control non-injected (NI) and water-injected (WI) BCRs showed elaborate FN fibril formation. Embryos microinjected with XGIPC mRNA exhibited a mature FN matrix (XGIPC). Microinjection of $XGIPC_{mut}$ mRNA severely inhibited FN fibril formation ($XGIPC_{mut}$).

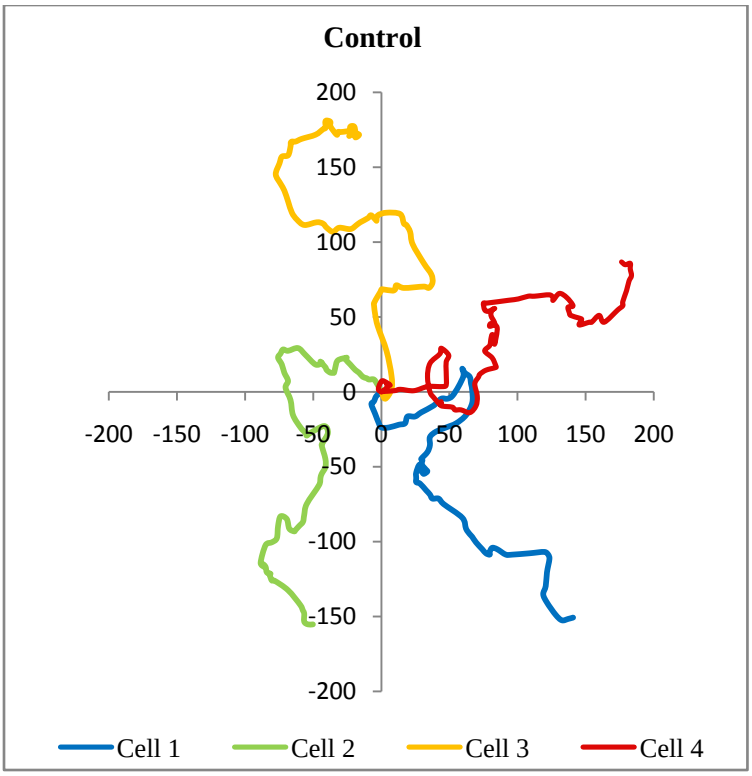

NI
WI
XGIPC
XGIPCmut

3.6 XGIPC expression is required for inside-out signaling.

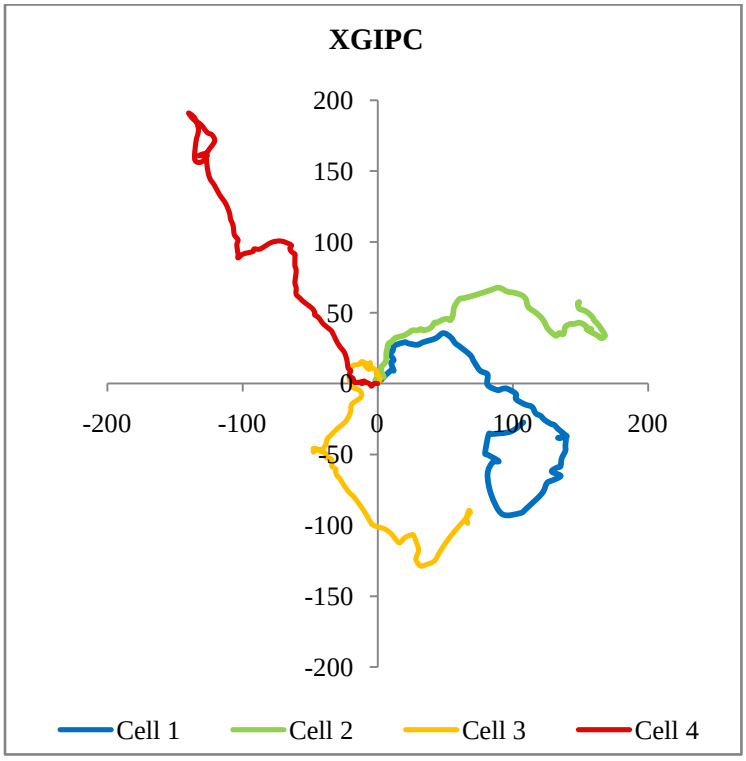
Prior to gastrulation, dissociated embryonic cells remain in a state of static adhesion characterized by α5β1 being ligated to the RGD-binding sequence of FN (Ramos et al., 1996). At the onset of gastrulation, inside-out signaling causes a change in α5β1 binding to include the synergy site adjacent to the RGD sequence. When bound to both the RGD and synergy sites, presumptive mesodermal cells acquire the ability to spread and migrate (Ramos and DeSimone, 1996). This change in behaviour can be mimicked through the treatment of BCR cells with Activin-A. To investigate whether XGIPC has a role in mediating this inside-out signaling, the change in integrin behaviour was analyzed using cell migration assays.

Activin-A induced control cells obtained from non-injected embryos actively migrated away from their point of origin in a directionally-biased manner, referred to as persistent migration (Figure 3.8A) (Winklbauer et al., 1996). Control cells had an average displacement (linear distance travelled from point of origin) of 157µm ±18 µm. Cells expressing microinjected XGIPC mRNA also migrated persistently away from their point of origin resulting in an average displacement of 134µm ±13µm (Figure 3.8B). Cells expressing microinjected $XGIPC_{mut}$ mRNA display a tightly coiled migration pathway that deviate an average of 25µm ±7µm from the point of origin (Figure 3.8C). The displacement of cells expressing dominant negative XGIPC is statistically different from those of control and XGIPC-expressing cells ($P<0.001$, Student T-test). Taken together, this data suggests that XGIPC plays essential roles in mediating changes in integrin function that result from activin induction.

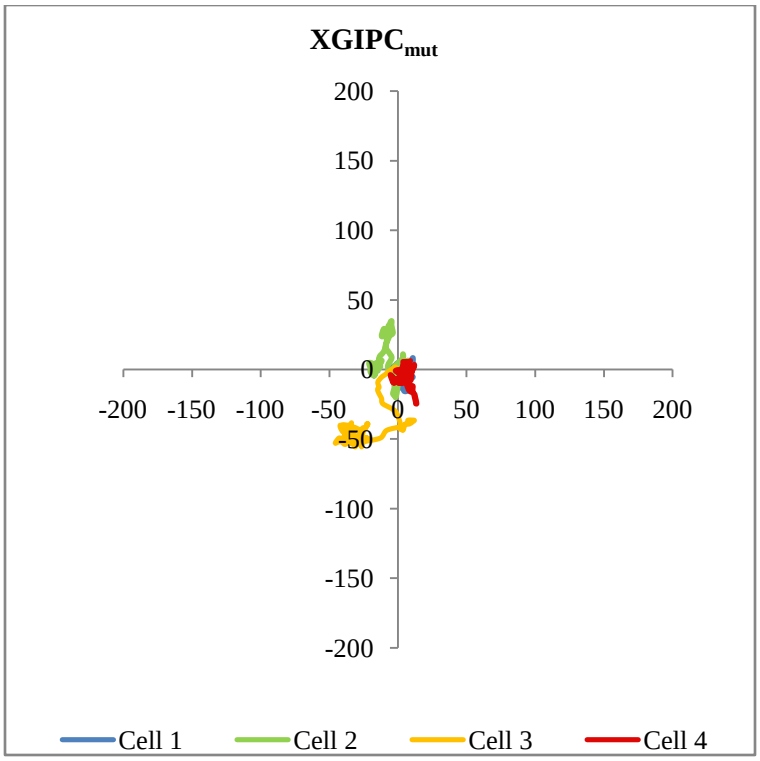
Figure 3.8 'Spider' graphs representing individual cell migration pathways. Graph axes represent micrometres (µm). Migration pathways of control cells indicate persistent migration away from the cells' point of origin. Control cells had an average displacement of 157µm ±18µm (A). Microinjected XGIPC-expressing cells also demonstrate persistent cell migration and have an average displacement 134µm ±13µm (B). $XGIPC_{mut}$-expressing microinjected cells indicate a non-persistent pathway resulting in an average displacement of 25µm ±7µm (C). Standard error determined using N=15 (3 replicates; 5 cells per replicate). Displayed cell migration pathways are representative.


Control
200
150
100
50
0
-50
-100
-150
-200
-200 -150 -100 -50 0 50 100 150 200
Cell 1
Cell 2
Cell 3
Cell 4

(A)


XGIPC
200
150
100
50
0
-50
-100
-150
-200
-200 -100 0 100 200
Cell 1
Cell 2
Cell 3
Cell 4

(B)


XGIPC$_{mut}$
200
150
100
50
0
-50
-100
-150
-200
-200
-150
-100
-50
0
50
100
150
200
Cell 1
Cell 2
Cell 3
Cell 4

(C)

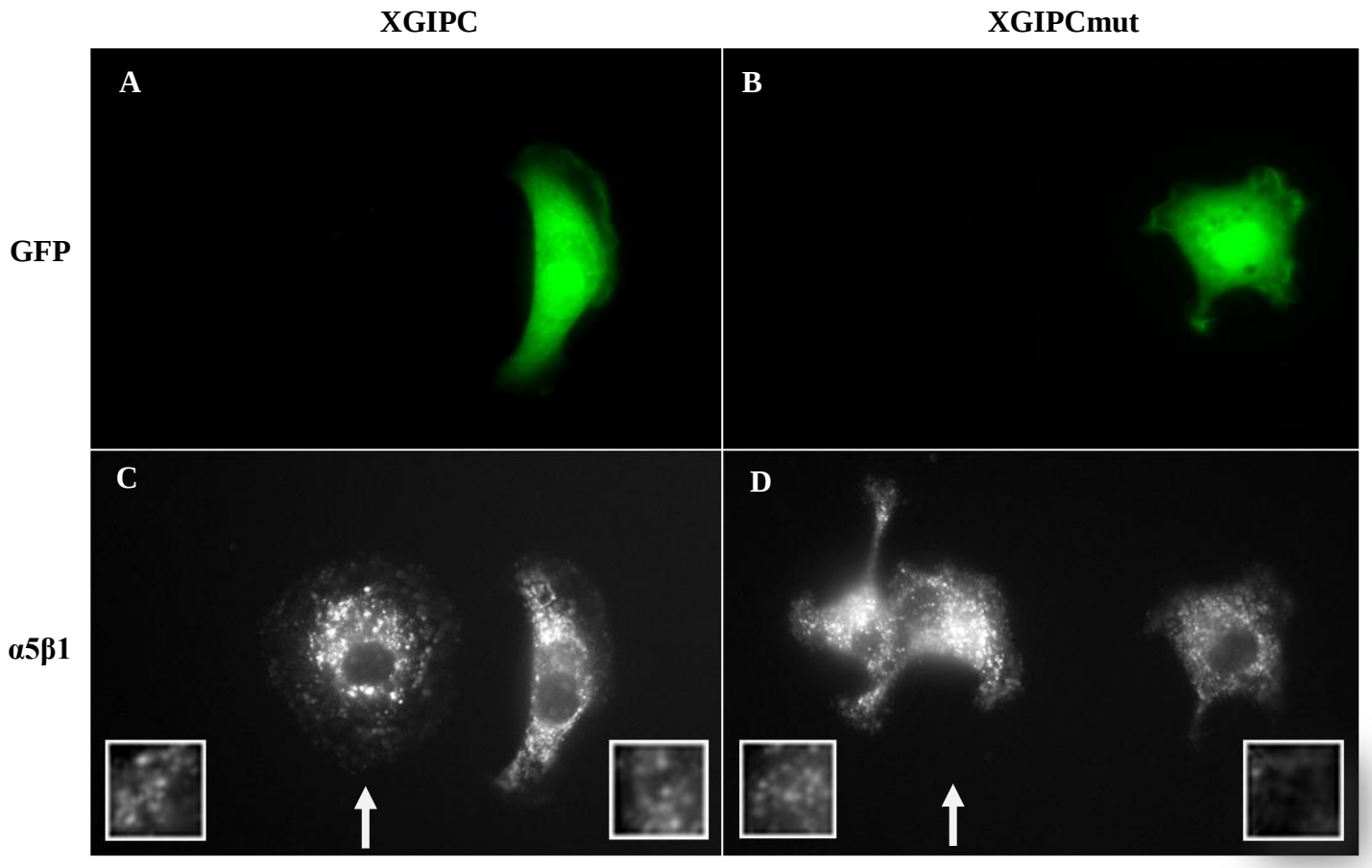
3.7 XGIPC facilitates α5β1 endocytosis.

Integrin endocytic recycling is becoming increasingly recognized as a means of regulating inside-out signaling-dependent processes, including cell adhesion, spreading, and motility (reviewed by Pellinen and Ivaska, 2006). To examine whether XGIPC has a role in mediating the internalization of integrin α5β1, the endocytosis of α5β1 was monitored in A6 cells transfected either XGIPC or $XGIPC_{mut}$ DNA.

The turnover of integrins was monitored by assaying the internalization of a surface bound antibody directed against the α5β1 receptor. Cells were transfected with GFP-tagged XGIPC constructs. In A6 cell cultures, the rate of successful transfection was approximately 30%; non-transfected cells within the same dish serve as controls for GFP-tagged XGIPC construct-expressing cells. Following transfection cell cultures were exposed to the MAb P8D4 directed against the *Xenopus* α5β1 receptor (Davidson et al., 2002). Internalization of α5β1 was detected using a fluorescently tagged secondary antibody (see section 2.13).

An estimate of integrin-containing endocytic vesicles was measured by calculating pixel densities from defined cytoplasmic regions of interest (ROIs). The average pixel density per ROI in control cells was 39.8 pixels per $25\mu m^2$ (±11.5 pixels). Cells expressing XGIPC mRNA exhibited similar pixel densities to that of control cells (40.4 pixels per $25\mu m^2$, ±28.7 pixels). Cells expressing $XGIPC_{mut}$ displayed reduced endocytic activity as measured by ROI pixel densities (11.5 pixels per $25\mu m^2$, ±9.4 pixels). This suggests that XGIPC regulates the endocytosis of integrin α5β1 in A6 cells and that this internalization is retarded by a mutation in the PDZ domain of GIPC.

Figure 3.9 XGIPC facilitates α5β1 endocytosis. *Xenopus* A6 cells were transfected with GFP-tagged XGIPC (A) and $XGIPC_{mut}$ (B). Recycling of α5β1 integrin was detected as described in section 2.13. Non-transfected cells in the same dish served as controls (C and D, white arrows). Pixel densities were determined from randomly selected 25μm^2 ROIs (representative insets in C and D). In XGIPC transfected cells the average ROI pixel densities were 40.4 pixels per 25μm^2, ±28.7 pixels (C, right inset), as compared to control ROI pixel densities 39.8 pixels per 25μm^2 ±11.5 pixels (C, left inset). In $XGIPC_{mut}$ transfected cells, the average ROI pixel densities were 11.5 pixels per 25μm^2, ±9.4 pixels (D, right inset). A ROI from the control cell for $XGIPC_{mut}$ transfected cells also displayed (D, left inset). Standard error using N=15 (3 replicates, 5 cells per replicate). Displayed cells are representative.


XGIPC
XGIPCmut
A
B
GFP
C
D
α5β1

3.8 XGIPC is required for actin polymerization.

To determine if XGIPC affects the polymerization of the actin filaments required to localize α5β1 to their leading edge, gastrula-stage BCR cells expressing GFP-tagged XGIPC constructs were plated onto a FN matrix, induced with Activin-A, and stained with rhodamine-phalloidin (Figure 3.10).

Embryos were microinjected with GAP43-GFP as a control for RNA expression. Cells expressing GAP43-GFP adhered and spread on FN, resulting in a polygonal cell morphology, typical of motile cells (Figure 3.10*A*, *B*) (Cousin et al., 2008). Control cells displayed directionally-biased lamelliform protrusions (Figure 3.10*B*, red arrows), that are rich in filamentous actin as detected with Rodamine-Phalloidin staining (Figure 3.10*C*, red arrows). Likewise, cells derived from XGIPC microinjected embryos (Figure 3.10*D*) show similar shapes (Figure 3.10*E*). F-actin staining of these cells illustrates large, actin-rich lamelliform protrusions (Figure 3.10*F*, red arrows). In both control and XGIPC-expressing cells, actin-sparse retracting protrusions were observed opposite to the actin-rich lamellipodia (Figure 3.10*C*, *F*, yellow arrows). Cells expressing $XGIPC_{mut}$ (Figure 3.10G) displayed a rounded morphology (Figure 3.10*H*). F-actin staining revealed fine filopodial protrusions encompassing the cell in a non-directionally biased manner, characteristic of immotile cells (Figure 3.10*I*, blue arrows) (Cousin et al., 2008). Interestingly, I also observed ameboid-like lobopodia in cells derived from $XGIPC_{mut}$-microinjected embryos (Figure 3.11A, *B*, orange arrows). Unlike the lamellipodia seen in control and XGIPC-expressing cells, lobopodia do not adhere to the FN matrix (data not shown) and are devoid of F-actin filaments (Figure 3.11*C*). This data clearly indicates that XGIPC is required for normal cytoskeletal dynamics in embryonic cells migrating on FN.

Figure 3.10 XGIPC facilitates cell spreading and actin polymerization in lamellipodia. Activin-A **i**nduced BCR cells plated on FN were stained with rhodamine-phallodin to detect F-actin. Microinjected cells were identified by GFP-expression (A,D,G). Control cells expressing GAP43 exhibited a triangular morphology (A,B) and actin-rich lamellipodia (red arrows) (B,C). Cells expressing XGIPC also had a triangular morphology (D,E) and actin-rich lamellipodia (red arrows) (E,F). Yellow arrowheads indicate retracting tails of control and XGIPC-expressing cells. $XGIPC_{mut}$-expressing cells displayed an oval morphology (G,H) and showed inhibition of directionally-biased F-actin polymerization (blue arrows) (H,I).

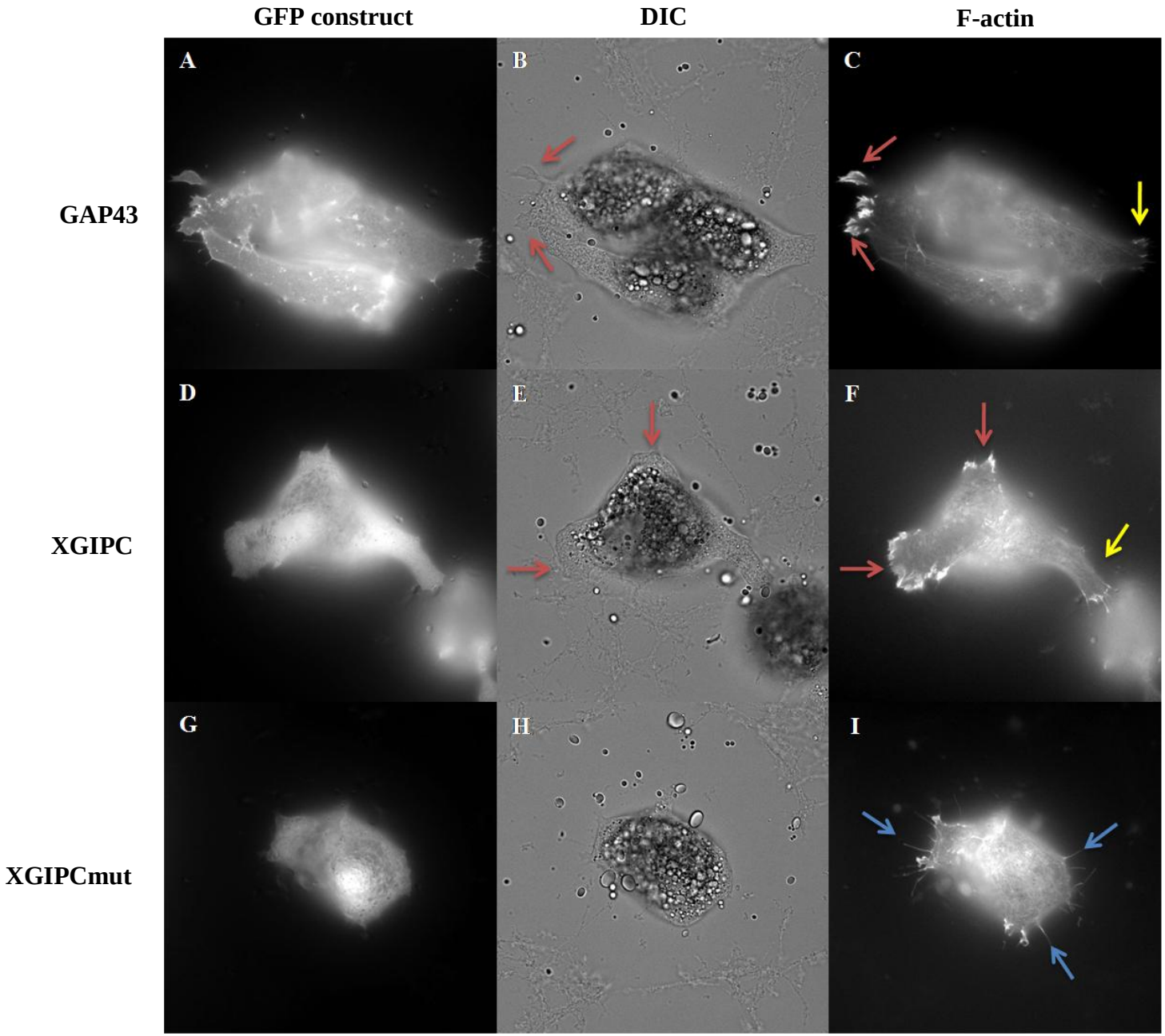
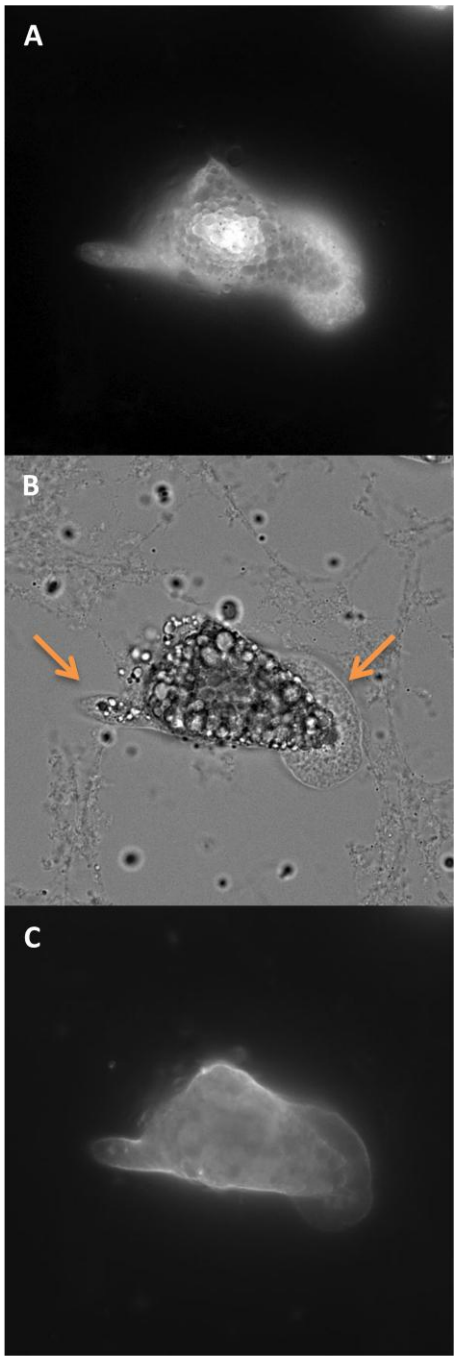

GFP construct
DIC
F-actin
GAP43
XGIPC
XGIPCmut
A
B
C
D
E
F
G
H
I

Figure 3.11 $XGIPC_{mut}$-expression leads to weak cell adhesion to FN. Activin-A treated gastrula-stage cells expressing $XGIPC_{mut}$ (A) developed abnormal, bulging protrusions (B, orange arrows) that do not contain polymerized actin (C).



XGIPC$_{mut}$

DIC

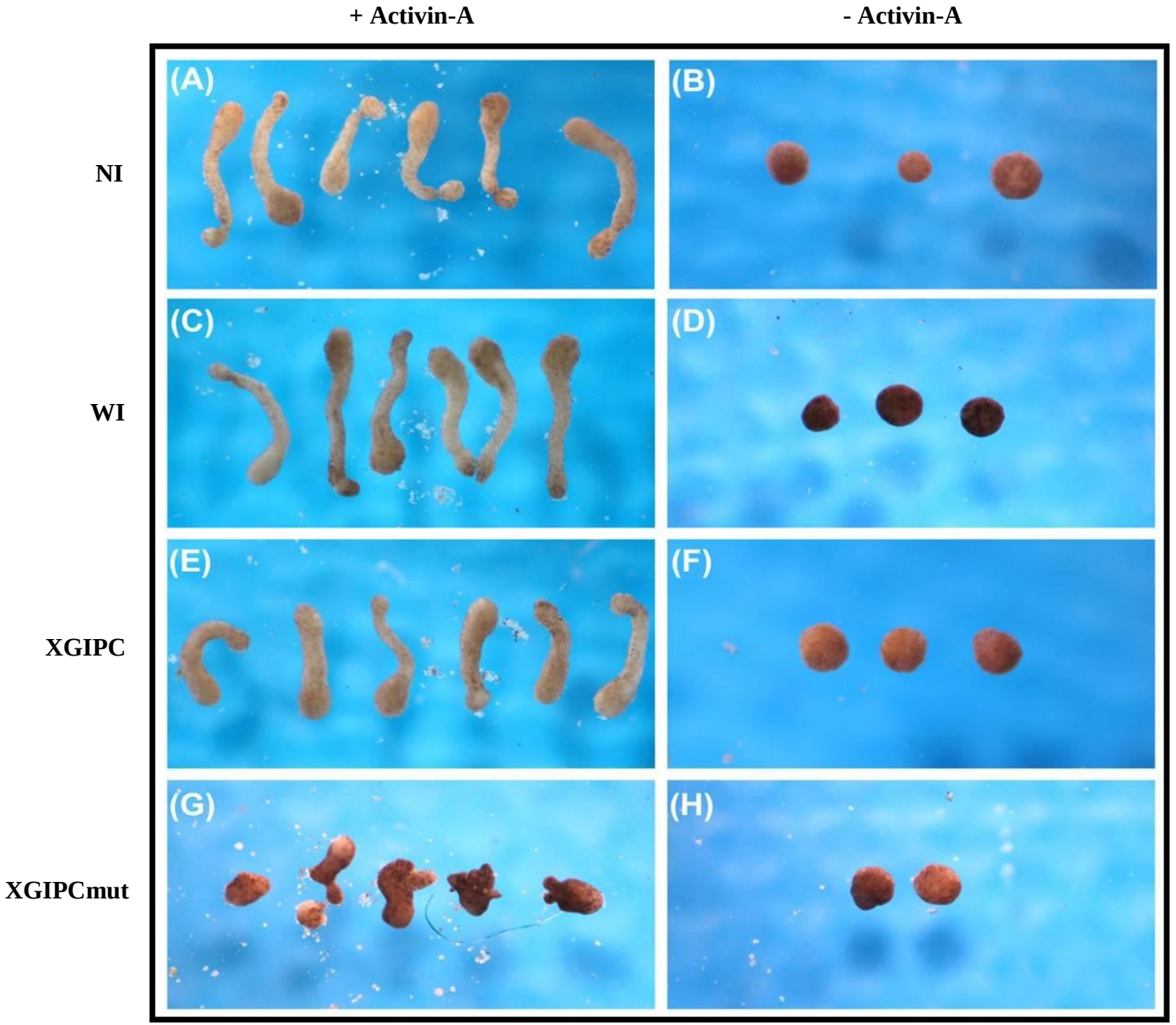
F-actin

3.9 XGIPC expression is not permissive for convergent extension.

As XGIPC was found to have a role in mediating actin polymerization in FN-plated embryonic cells, it raised the question of whether XGIPC has a general role in interpreting outside-in signals stemming from integrin-FN ligations. Animal cap explants treated with Activin-A recapitulate the normal convergent extension movements observed in the embryo and can be used as a simple assay for outside-in integrin signaling. In animal cap explants treated with Activin-A, a signal is propagated from the FN matrix that leads to the polarization and intercalation of cells necessary for convergent extension (Cousin et al., 2008).

Animal caps excised from blastulae were induced with Activin-A and cultured until sibling embryos reached tailbud stage. Animal caps of non-injected and water-injected embryos elongated in the presence of activin (Figure 3.12A, C). Animal caps not exposed to Activin-A did not elongate (Figure 3.12B, D). Explants expressing XGIPC mRNA also elongate in response to mesodermal induction, although not to the extent of control animal caps (Figure 3.12E). Animal caps expressing microinjected XGIPC that were not treated with Activin-A failed to extend indicating that XGIPC alone is not permissive for convergent extension movements (Figure 3.12F). Explants expressing $XGIPC_{mut}$ mRNA do not extend in the presence of Activin-A (Figure 3.12G), indicating that $XGIPC_{mut}$ inhibits extension, and that depletion of functional XGIPC cannot rescue the defects in convergent extension seen in panel E. Additionally, explants from $XGIPC_{mut}$-expressing embryos exhibit defects in healing (Figure 3.12G). Animal caps expressing $GIPC_{mut}$ that were cultured without Activin-A fail to extend (Figure 3.12H). From these experiments it can be concluded that XGIPC is required for extension of Activin-A induced animal caps.

Figure 3.12 XGIPC is not permissive for convergent extension. Stage 8 animal caps were cultured in the presence or absence of Activin-A, until sibling embryos reached stage 22. Induced explants from non-injected embryos (A), water-injected embryos (C), and XGIPC-injected (E) embryos elongated. Sibling explants did not extend in the absence of Activin-A induction (B,D,F). Expression of $XGIPC_{mut}$ inhibits animal cap explant elongation, indicating a failure in convergent extension (G). Uninduced $XGIPC_{mut}$ expressing explants did not elongate (H).


+ Activin-A
- Activin-A
NI
WI
XGIPC
XGIPCmut
(A)
(B)
(C)
(D)
(E)
(F)
(G)
(H)

Chapter 4 Discussion

The primary objective of this study was to establish and characterize a role for XGIPC in the regulation of α5β1 integrin function in *Xenopus*. Using *in vivo* and *in vitro* assays, I have established that XGIPC interacts with the α5β1 integrin, and through this interaction is able to moderate the cell behaviour changes required for *Xenopus* gastrulation. I propose that XGIPC regulates integrin-mediated inside-out signaling by recycling active α5β1 to the cell surface where it is able to initiate cell migration. Additionally, I have shown XGIPC to interact with the cytoplasmic tail of the α6 integrin subunit *in vitro*.

4.1 XGIPC interacts with the α5 cytoplasmic subunit

I have shown that XGIPC physically interacts with the *Xenopus* α5 subunit, both *in vitro* and *in vivo*. Yeast two-hybrid assays demonstrate that this interaction is specific to the central PDZ domain of XGIPC as binding was abolished when the canonical binding site was mutated from ALGL to AAEL. The *Xenopus* α6 subunit, which contains the C-terminus amino acid sequence SDA, was utilized as a positive control, as previous yeast two-hybrid assays have shown GIPC to bind to the SDA consensus sequence of the human α6A subunit splice variant (Tani and Mercurio, 2001). Like the *Xenopus* α6 subunit, the *Xenopus* α5 subunit contains a conserved Type 1 PDZ-binding motif, SEA. Therefore, the interaction between XGIPC and the α5 subunit likely occurs via the conserved C-terminus PDZ-binding sequence, SEA. Quantification of yeast-two hybrid results indicated that the interaction between the α6 subunit

and XGIPC was stronger than that between the α5 subunit and XGIPC (Figure 3.4). In *Xenopus*, this variance might be accounted for by the difference between the α5 subunit's SEA C-terminal sequence and the α6 subunit's SDA C-terminal sequence. Alternatively, the difference in GIPC-α5 and GIPC-α6 binding efficiency may be related to sequences upstream of the PDZ- binding motif (Tani and Mercurio, 2001). It is interesting that simultaneously expressed GIPC ligands are able to bind with varying affinities to GIPC. This may reflect a generalized stochastic mechanism by which interactions are regulated.

The αV subunit, which does not contain a conserved PDZ-binding consensus motif, served as a negative control. Accordingly, the co-expression of XGIPC and $XGIPC_{mut}$ with the αV subunit in yeast two hybrid assays did not result in significant β-galactosidase activity, confirming a lack of interaction with XGIPC.

To further investigate the interaction between XGIPC and the α5 subunit, I confirmed that this binding occurs *in vivo* using co-immunoprecipitation assays. HA-tagged XGIPC was detected in α5β1 protein complexes, indicating that XGIPC physically interacts with the α5-subunit. Earlier attempts to immunoprecipitate native XGIPC were not successful. Therefore to immunoprecipitate the XGIPC-α5 subunit complex, both XGIPC and the α5 subunit were over-expressed in *Xenopus* embryos. Previous studies investigating GIPC interactions with cell surface receptors using co-immunoprecipitation assays have also had to over-express the proteins of interest to confirm interaction (Ligensa et al., 2001; Tan et al., 2001; Wang et al., 1999; Wu et al., 2006). This may be due to GIPC's association with endocytic vesicles (De Vries et al., 1998b). However, *in vivo* the majority of GIPC-binding receptors are found at the cell membrane and not in the cytoplasm; therefore, the amount of endogenous receptor-bound GIPC in the cytoplasm may be below detectable levels. Additionally, the α5β1 integrin is known to be

internalized in both clathrin-dependent and clathrin-independent mechanisms (Norman and Caswell, 2008). As GIPC is only known to associate with clathrin-coated vesicles during receptor turn-over, it is possible that at endogenous levels XGIPC-α5 subunit interactions are not abundant enough to be detected using co-immunoprecipitation assays.

4.2 XGIPC is required for gastrulation and FN matrix assembly.

By monitoring blastopore closure as a measure of gastrulation, I have demonstrated that XGIPC is required for the normal progression of development (Figure 3.6B). Mutation of XGIPC's PDZ-domain results in a severe inhibition of blastopore closure (Figure 3.6C), indicating that this domain is essential for the normal progression of gastrulation. Marsden and DeSimone (2001) have shown that delays in blastopore closure, as well as the development of embryos with truncated anteroposterior axes, are the results of inhibiting FN matrix assembly. Similar to the findings of Marsden and DeSimone (2001), the embryos expressing dominant negative $XGIPC_{mut}$ display delays in blastopore closure and are also found to exhibit a disruption of FN matrix assembly (Figure 3.7D). In contrast to the long, dense, interwoven FN fibrils seen on the BCRs of control and XGIPC-expressing embryos, embryos expressing $XGIPC_{mut}$ exhibit thin, spindly fibrils. The observation that there is still some FN matrix assembly may reflect the inability of the dominant negative $XGIPC_{mut}$ to completely block FN matrix assembly, thereby allowing minimal amounts of fibrillogenesis to occur. However, the resulting fibrils are too sparse to support a normal role for FN. My results demonstrate that a mutation in the PDZ-domain of XGIPC blocks FN matrix assembly, although XGIPC's exact role in this process is unclear.

At the onset of gastrulation, the α5β1 integrin becomes activated by an unknown inducer allowing α5β1 to bind to the RGD site of soluble FN dimers initiating matrix assembly. As this study has shown XGIPC to be required for FN matrix assembly, as well as demonstrated that XGIPC directly interacts with the α5β1 integrin, it is likely that XGIPC regulates FN matrix assembly through its interactions with the α5β1 integrin. In support of this hypothesis, as FN has no known binding site for XGIPC it is unlikely that FN secretion is directly affected by the dominant negative construct. Additionally, as both α5β1 and FN are required for normal tissue rearrangements during gastrulation (Davidson et al, 2002) it is likely that XGIPC's role in gastrulation is through its interaction with the α5 subunit.

While the mechanisms that regulate the initial activation of α5β1 integrin that allows it to bind FN remain unclear in Xenopus, De Vries et al. (1998) found that two pools of GIPC exist: a cytosolic pool and a membrane-bound pool. It is now clear that the membrane-bound pool is directly linked to endocytosis, however, the role of the cytosolic pool remains unclear (Lou et al., 2001). It is possible that this cytosolic pool of GIPC has a role in changes in integrin binding state.

4.3 XGIPC is required for inside-out signaling.

There are two temporal phases during gastrulation that require inside-out signaling: first, the assembly of the FN matrix (discussed above), and second, the induction of cell migration. The second phase of integrin activation has been well characterized. Following the attachment of involuted mesodermal cells, α5β1 integrins are induced to bind both the RGD and synergy sites, thereby acting as permissive signals for cells to spread and migrate on the FN matrix (Ramos and

DeSimone, 1996; Ramos et al., 1996). By assaying the ability of XGIPC- and $XGIPC_{mut}$-expressing cells to persistently migrate on FN, I have established that XGIPC expression is required for the propagation of inside-out signals (Figure 3.8). Activin-induced cells from embryos microinjected with XGIPC migrated away from their point of origin in a largely unidirectional manner, indicating that the α5β1 integrin had undergone activation. In contrast, cells derived from embryos microinjected with $XGIPC_{mut}$ were unable to migrate in a persistent manner and instead hovered around their point of origin. The inability of $XGIPC_{mut}$-expressing cells to transition from a state of static adhesion to one of active migration indicates that the XGIPC interaction with the α5 subunit is required for the presence of activated α5β1 on the cell surface.

It has been previously suggested that *in vitro*, Activin-A induces α5β1 already on the cell surface to undergo a conformational change that would allow the integrin to accommodate the synergy site, thereby initiating cell migration (Ramos and DeSimone, 1996). However, it is also possible that the change in α5β1 binding specificity is regulated through integrin trafficking. By measuring the internalization of α5β1 integrins in *Xenopus* A6 cells, I have shown XGIPC to be required for efficient α5β1 endocytosis.

Cold A6 cells have impaired cell membrane dynamics preventing endocytosis of the α5β1 integrin. Therefore integrins decorated with antibodies remain at the cell surface. Warming these labelled cells releases cold-restricted endocytosis and α5β1 is internalized in endocytic vesicles (Appendix B). By imaging immunofluorescent α5β1 integrins, I can directly relate pixel values within a cytoplasmic region of interest (ROI) to the number of internalized integrins within a given area. It is apparent that the internalization of α5β1 in XGIPC-expressing cells is similar to that in control cells, whereas cells expressing $XGIPC_{mut}$ demonstrate a reduction in

α5β1 internalization (Figure 3.9). The internalization assay needs optimization as the present image resolution limits my ability to accurately estimate pixel densities. Future studies will address this limitation by using confocal or de-convolution microscopy to generate images with greater Z axis resolution.

As XGIPC is required for α5β1 endocytosis, as well as being required for cells to acquire migratory behaviours (Figure 3.8), I propose that upon induction, α5β1, facilitated by XGIPC, is endocytosed. The internalized receptor may be modified to an active form, and recycled back to the cell membrane. Alternatively, the endocytosed receptors may be replaced by an active form of newly exported integrin. In either case, an active form of α5β1 is expressed on the cell surface and able to bind FN's RGD and synergy sites, leading to mesodermal cell migration.

This proposed mechanism is consistent with several lines of evidence. First, GIPC was initially identified by a yeast two-hybrid screen for GAIP-interacting protein (De Vries et al., 1998b). GAIP is a membrane-anchored protein found on clathrin-coated vesicles, proposed to have a role in vesicular trafficking (De Vries et al., 1996; De Vries et al., 1998a). GIPC has also been found to be associated with small vesicles normally located near the cell membrane, suggesting a role for GIPC in regulating vesicular trafficking (De Vries et al., 1998b). Later work from the same group demonstrated that in human cell lines GIPC is recruited to endocytic vesicles at the cell periphery and is associated with TrkA, a neuron growth factor receptor. Upon association with GIPC, the TrkA receptor is internalized through clathrin-coated pits and becomes localized to early endosomes. siRNA knockdown experiments further demonstrate GIPC is required for efficient TrkA trafficking to early endosomes (Varsano et al., 2006). Similarly, α5β1 is known to be internalized through clathrin-coated pits and transported in clathrin-coated vesicles (reviewed by Caswell and Norman, 2008). I have demonstrated that

XGIPC is required for efficient α5β1 endocytosis and that by disrupting α5-binding to XGIPC`s PDZ-domain, integrin internalization is reduced (Figure 3.7). Therefore, it is likely that the mechanism of α5β1 integrin turnover is similar to that of the TrkA receptor. Furthermore, I have obtained preliminary data that suggests that when XGIPC is expressed at low levels it is localized to endocytic vesicles in *Xenopus* A6 cells (Appendix C). Co-localization studies are required to ascertain that XGIPC is directly associated with integrin-containing clathrin-coated vesicles in *Xenopus* cells (see section 4.8).

A second line of evidence supporting this proposed mechanism comes out of molecular dynamic simulations that have demonstrated that post-translational modification of integrins is necessary for changes in binding affinity. Liu et al. (2008) have shown that the region of β_1 integrin subunits important for ligand binding, the I-like domain, likely undergoes glycosylation. Glycosylation would alter β_1 folding, which is sufficient to alter the binding affinity of the β_1-containing heterodimer (Liu et al., 2008). Internalized receptors are recycled through the early endosome where they intermix with newly assembled integrins originating from the trans-golgi network. As such, recycling the α5β1 integrin may explain the change in cell behaviours that follow inside-out signaling in *Xenopus* cells. In this scenario, either new or modified α5β1 integrins that are exported back to the cell surface may be permissive for binding to the RGD and synergy sites of FN. This change in integrin cell surface expression may be sufficient to transit cells out of a static adhered state and into a migratory one. Since the alteration of glycosylation state is the only known structural change apart from alternative splicing that can modify integrin behaviour, an analysis of the glycosylation state of surface-expressed α5β1 integrins in cells expressing XGIPC and dominant negative $XGIPC_{mut}$ would begin to address this question.

4.4 XGIPC-mediated outside-in signaling is required for actin polymerization.

Changes in integrin adhesion leading to cell motility occur predominantly in the polarized cell protrusions on the leading edge of migrating cells. This concentration of active integrins is the result of spatially regulated α5β1 recycling (Caswell et al., 2007). Integrin recycling to polarized protrusions is accomplished by transporting integrin-bound endocytic vesicles along actin filaments to the tips of filopodia where they aid in the stabilization of the cytoplasmic protrusions. In this way, actin functions as a "track" for recycling internalized integrins. Subsequent release of cell adhesion at the trailing edge of the cell results in the cell being pulled forward.

I have shown that the expression of the dominant negative $XGIPC_{mut}$ results in one of two cell phenotypes: One, cytoplasmic protrusions fail to attach to the underlying matrix and instead exhibit actin-devoid, lobopods (Figure 3.11). Alternatively, actin fails to polymerize in lamellipodia and cells instead develop unpolarized filopodial protrusions, which allow cells to adhere to the underlying FN matrix, but renders them immotile (Figure 3.10; Figure 3.8). Both of the observed dominant negative XGIPC phenotypes could arise from a reduction in cell matrix adhesion as a result of a failure in $XGIPC_{mut}$ to recycle activated α5β1 integrins. Active α5β1 is required for actin polymerization, initiating a feedback loop in which further vesicle-bound activated α5β1 is targeted along actin fibres to locomotory protrusions. My results can be explained by a model in which XGIPC acts as a molecular chaperone for α5β1-endocytosis, leading to targeted cell surface expression of activated α5β1. As activated α5β1 is required for actin polymerization, XGIPC indirectly participates in the organization of the actin cytoskeleton. An alternative possibility exists that XGIPC acts to directly activate the integrin. However, at

this time, we do not have the ability to distinguish between recruitment of active receptors and changes in receptor activation. As such, my data reaffirm the requirement for wild-type XGIPC to either activate α5β1 directly, or to target activated α5β1 integrins to polarized protrusions, to aid in cell migration.

4.5 XGIPC is required for convergent extension

As actin polymerization was observed to be inhibited when $XGIPC_{mut}$ was expressed, it is possible that XGIPC acts as a scaffolding protein facilitating the assembly of integrin associated complexes responsible for actin polymerization. Therefore, it was of interest to determine if XGIPC has a general role in outside-in signaling. To address this question, I have assayed the convergent extension of explants expressing XGIPC and $XGIPC_{mut}$ mRNA. *In vitro*, Activin-A induces explants to assemble a FN matrix, which in turn is required for the initiation of cell polarization, MIB, and convergent extension (Marsden & DeSimone, 2001). This outside-in signaling pathway results in the elongation of the explanted tissue from a spherical mass to a narrow array. Explants expressing XGIPC were able to converge and extend in the presence of Activin-A. However, XGIPC alone cannot induce the elongation of tissue in the absence of activin indicating that it has no direct permissive role. Explants over-expressing XGIPC displayed a slight inhibition of extension relative to the control explants, likely due to protein over-expression. This is supported by previous studies showing that the over-expression of XGIPC can have inhibitory effects on normal cell behaviours (Awan et al., 2002; Gao et al., 2000). This result brings up the possibility that XGIPC is a negative regulator of convergent extension. However, this possibility was dismissed as explants microinjected with dominant

negative XGIPC also fail to extend in the presence of Activin-A. This likely reflects an inability of these explants to assemble a FN matrix in the presence of the dominant negative construct. Combined, these results indicate that XGIPC acts upstream of integrin mediated outside-in signaling. Therefore, I conclude that the failure of dominant negative $XGIPC_{mut}$-expressing explants to converge and extend is a side-effect of an inhibition of inside-out signaling. A disruption of inside-out signaling inhibits FN matrix formation (Figure 3.7), which in turn, is required for the acquisition of cell polarity, cell intercalation, and the ability to converge and extend.

4.6 Why not IGF-signaling?

A caveat to this work is that the only study to investigate an *in vivo* role for XGIPC found this molecule to be involved in mediating IGF-1 receptor (IGF-1R) function (Wu et al., 2006). The IGF family of molecules includes two secreted proteins, IGF-1 and IGF-2, both of which bind to the same IGF-1R receptor. Binding to IGF-1R leads to the activation of two prominent intracellular pathways: the Ras-Raf-MAPK pathway, and the PI3 kinase pathway.

Wu et al. (2006) demonstrated that XGIPC is specifically required for IGF-induced anterior neural patterning, in particular eye development. Wu et al. (2006) established that the inhibition of anterior neural patterning that results from deletion of XGIPC can be partially rescued by PI3 kinase, indicating that XGIPC has a role downstream of IGF-1R, but upstream of PI3 kinase activation. This suggests that XGIPC regulates IGF signaling by stabilizing surface expression of IGF-1R. However, these authors admit that such a role for XGIPC is unlikely as depletion of XGIPC did not significantly reduce the level of IGF-1R under the same conditions

that reduced PI3 kinase activation. Likewise, XGIPC depletion did not affect IGF-induced Ras-Raf-MAPK activation. Interestingly, in addition to eye defects, translational knock-down of XGIPC using morpholinos was shown to disrupt gastrulation, resulting in truncated embryos closely resembling embryos with defects in FN matrix assembly. Therefore, XGIPC must play a greater role during embryogenesis than simply mediating IGF signaling.

Further arguments against a role for XGIPC-mediated IGF signaling during gastrulation come out of the demonstration that dominant negative expression of IGF-1R does not affect gastrulation, but does inhibit neural differentiation (Pera et al., 2001). In particular, IGF-signaling plays a crucial role in head formation and eye patterning as demonstrated by over-expression of IGF-1, which leads to the formation of ectopic heads and eyes (Pera et al., 2001; Richard-Parpaillion et al, 2002). Somewhat surprisingly, Richard-Parpaillion et al., (2002) demonstrated that the over-expression of IGF-1 causes an inhibition of convergent extension in Activin-A treated animal caps. This was unexpected because IGF-1R transcript levels are at low levels until after neurulation commences (Richard-Parpaillion et al., 2002). The likely explanation for this phenomenon is that the over-expression of IGF-1 causes an inhibition of Wnt-signaling. The inhibition of Wnt-signaling alters cell fate, and mesoderm, which normally undergoes convergent extension, becomes non-extending anterior neural tissue (Pera et al., 2001). Despite these arguments, it would be prudent to explore a potential role IGF may play in FN assembly as in other systems IGF is known to regulate integrin function (Kabir-Salmani, 2004). This is easily accomplished by over-expressing the dominant negative IGF-1R receptor and assaying for FN assembly.

4.7 Conclusions

Through its' interaction with the α5 integrin subunit, XGIPC is able to spatially and temporally regulate the function of α5β1 during *Xenopus* gastrulation. The regulation of integrin function by XGIPC can be divided into two phases. First, at the onset of gastrulation, XGIPC is required for α5β1-mediated FN matrix assembly, and although its role in this mechanism is unclear, it is possible that XGIPC mediates α5β1's ability to bind soluble FN by modulating integrin trafficking. Second, XGIPC is necessary for α5β1-mediated cell migration and development of actin-rich lamellipodia. XGIPC has been found to facilitate efficient endocytosis of the α5β1 integrins to endocytic vesicles. Mutations in the PDZ domain of XGIPC not only inhibit migratory behaviours but also endocytosis of α5β1. Therefore XGIPC recycling of the α5β1 integrin is central to cell migration. Additionally, I have concluded that XGIPC has no direct role in outside-in signaling; instead XGIPC indirectly affects convergent extension by inhibiting the inside-out signaling that leads to FN matrix assembly. Based on these observations, I conclude that XGIPC, through its direct interaction with the α5 subunit, is able to regulate α5β1-mediated inside-out signaling function during *Xenopus* gastrulation.

4.8 Future directions

This study has clearly demonstrated that XGIPC has a role in regulating integrin-mediated inside-out signaling during gastrulation. To ensure that defects in FN matrix assembly and cell migration resulting from the expression of dominant negative XGIPC are specific, a XGIPC translational knock-down using morpholinos is required. By eliminating endogenous

XGIPC expression and reconstituting the embryo with either wild-type or dominant negative XGIPC I will be able to ensure the specificity of the above described XGIPC interactions. Wu et al., (2001) have previously demonstrated that reconstitution with wild-type XGIPC following morpholino knockdown can rescue embryonic development; therefore, I also anticipate the rescue of FN matrix assembly following knock-down of XGIPC translation.

This study has demonstrated that the expression of functional XGIPC is required for the assembly of FN on the apical surface of the BCR, but we have not yet investigated the effect of XGIPC expression on FN secretion *in vivo*. Although the possibility that dominant negative XGIPC inhibits FN secretion is remote, there is some evidence that inhibition of integrin cell surface expression disrupts FN secretion (Bischof et al., 1995). Future experimentation is needed to confirm that XGIPC mediates FN assembly, not secretion. A measure of FN secretion can be conducted by immunoblotting the blastocoel contents of control and experimental gastrulae.

While we have evidence for XGIPC-mediated integrin trafficking, we need to address if XGIPC influences the surface expression of α5β1 *in vivo*. To investigate this, integrin receptors will be surface labelled with biotin, immunoprecipitated with antibodies directed against α5β1, and surface receptors will be detected by Western blotting with HRP streptavidin.

To confirm that the inhibition of FN matrix assembly displayed when expressing dominant negative $XGIPC_{mut}$ is a result of disrupting α5β1-mediated signaling and not a consequence of inhibiting IGF signaling, IGF-1R over-expression will be used to demonstrate that it does not affect FN matrix assembly.

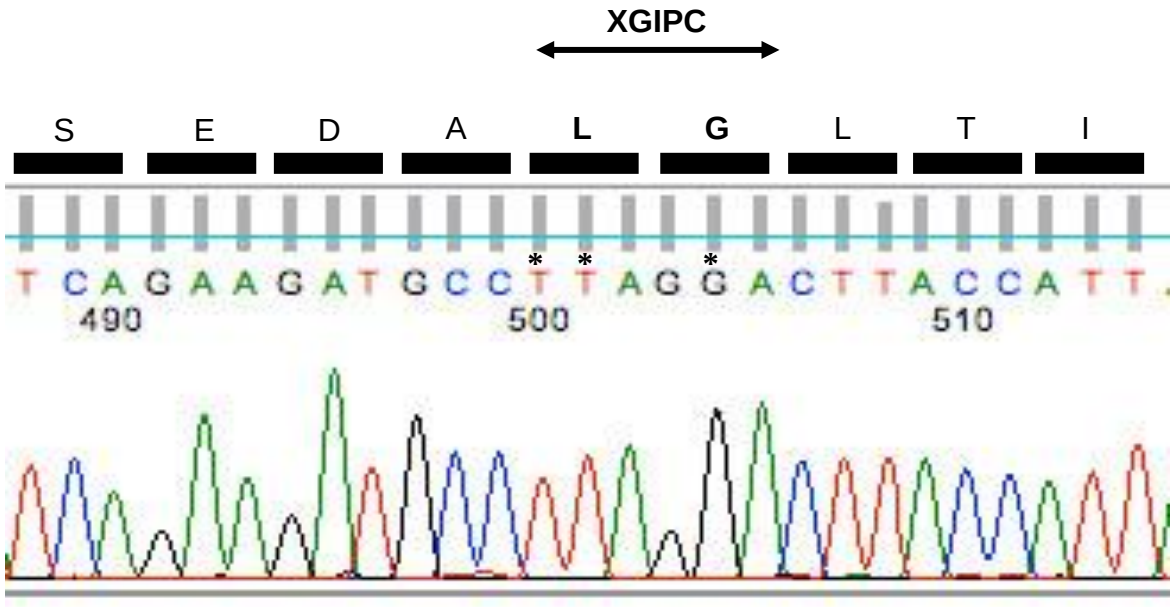
This thesis has presented evidence supporting a role for XGIPC in the endocytosis and recycling of α5β1. Further work is required to ascertain if XGIPC is associated with clathrin-

coated vesicles in *Xenopus* cells. A polyclonal rabbit antibody will be available in the coming months (Santa Cruz Biotech, Santa Cruz, CA) that may be utilized to confirm the co-localization of XGIPC with clathrin-coated integrin-containing endocytic vesicles. Additionally, this antibody can be used in morpholino experiments to confirm knock-down expression of XGIPC.

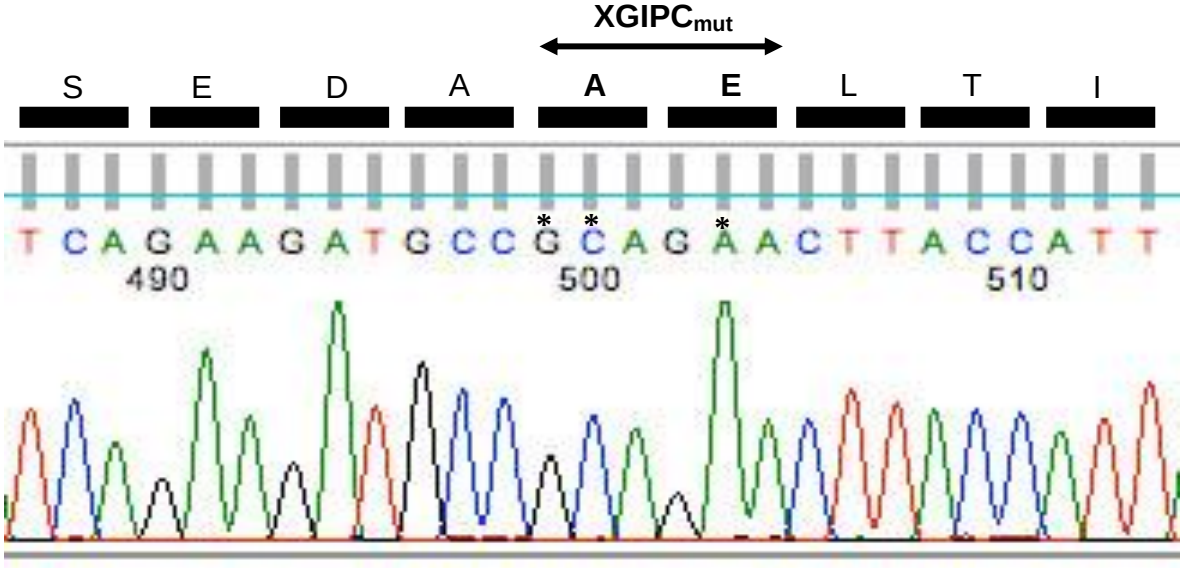
Continued work on the role of XGIPC in the regulation of integrin signaling is of interest as human GIPC1 and GIPC2 are up-regulated in several types of gastric cancer. While several proteins have been identified as GIPC1- and GIPC2-interacting proteins in gastric cancer cell lines, the role of GIPCs in gastric cancer, particularly in diffuse-type gastric cancer, remains elusive. Further work will elucidate a specific role for XGIPC in regulating integrin adhesion in *Xenopus* embryos and lead to a better understanding of the mis-regulation of cell adhesion in metastatic cancer.

Appendix A

Figure A.1 Confirmation of XGIPC and XGIPC$_{mut}$ PDZ-domain coding sequences. To generate the dominant negative XGIPC$_{mut}$ coding sequence (B), the coding sequence of XGIPC's PDZ-domain (A) was mutated at positions 500 (T→G), 501 (T→C), and 505 (G→A). Nucleotides substitutions are indicated with astericks. These mutations translate into an amino acid exchange from leucine (L) to alanine (A) (TTA→GCA) and from glycine (G) to glutamic acid (E) (GGA→GAA).


XGIPC
S E D A L G L T I
T C A G A A G A T G C C T T A G G A C T T A C C A T T
490
500
510

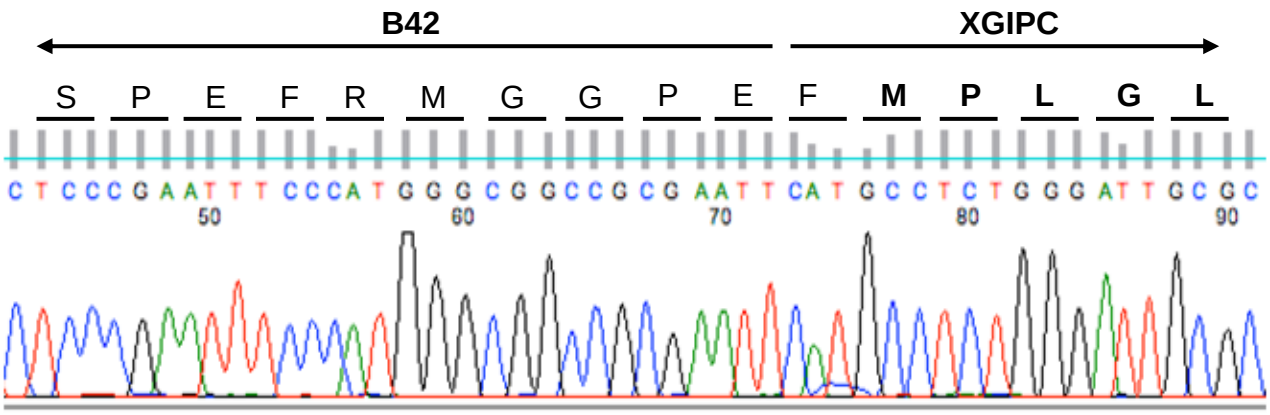
(A)


XGIPCmut
S E D A A E L T I
T C A G A A G A T G C C G C A G A A C T T A C C A T T
490
500
510

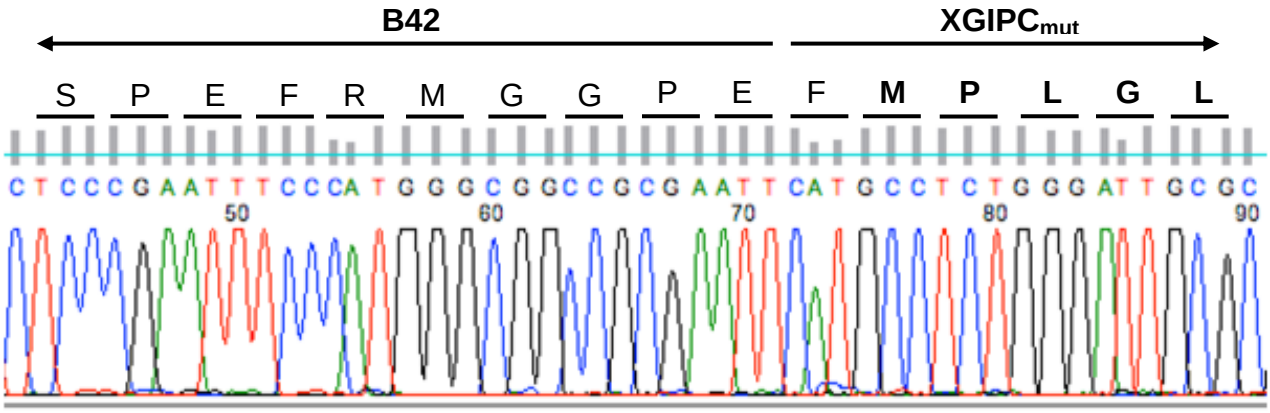
(B)

Figure A.2 Confirmation of the open reading frame in yeast two-hybrid prey and bait fusion constructs. The coding sequences for XGIPC and $XGIPC_{mut}$ were cloned into the prey plasmid pJG4-, which contains the activation domain from a bacterial sequence B42 (U89961). Sequencing has shown that XGIPC (A) and $XGIPC_{mut}$ (B) coding sequences to have been properly cloned into the open reading frame. The coding sequences for the cytoplasmic domains of the α5, α6, and αV subunits were cloned into the bait plasmid pEG202, which contains the DNA-binding domain of LexA (U89960). Sequencing has confirmed that α5 (C), α6 (D), and αV (E) coding sequences were accurately inserted into the open reading frame.

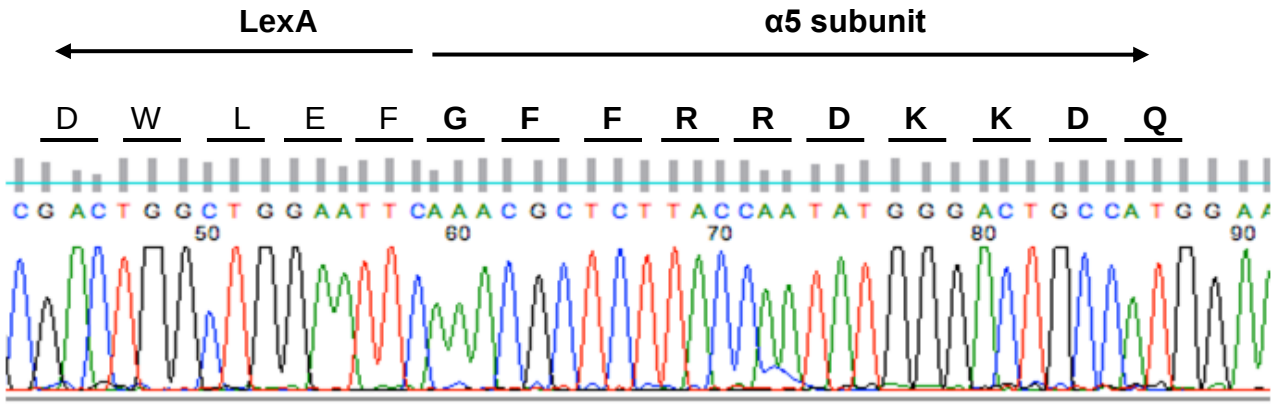
(A)


B42
XGIPC
S P E F R M G G P E F M P L G L
C T C C C G A A T T T C C C A T G G G C G G C C G C G A A T T C A T G C C T C T G G G A T T G C G C
50
60
70
80
90

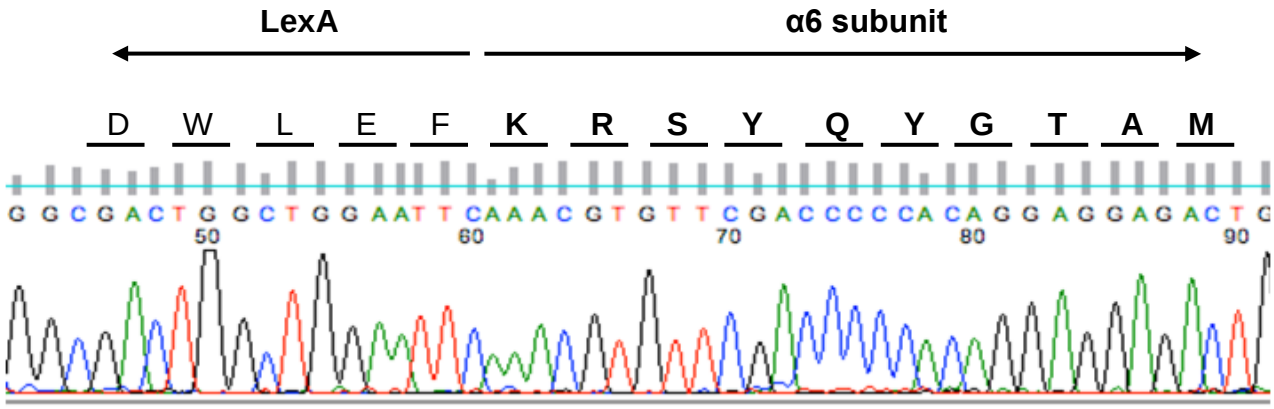
(B)


B42
XGIPCmut
S P E F R M G G P E F M P L G L
C T C C C G A A T T T C C C A T G G G C G G C C G C G A A T T C A T G C C T C T G G G A T T G C G C
50
60
70
80
90

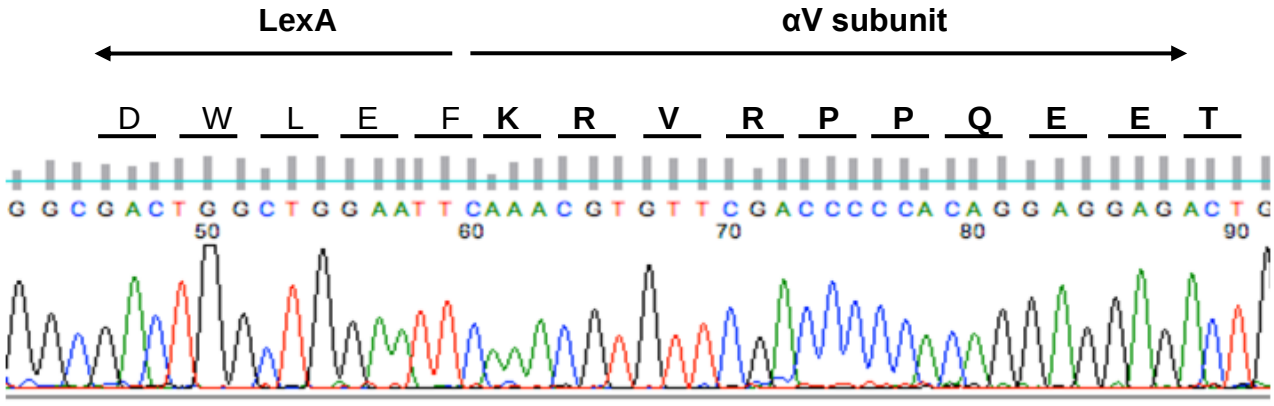
(C)


LexA
α5 subunit
D W L E F G F F R R D K K D Q

(D)


LexA
α6 subunit
D W L E F K R S Y Q Y G T A M

(E)

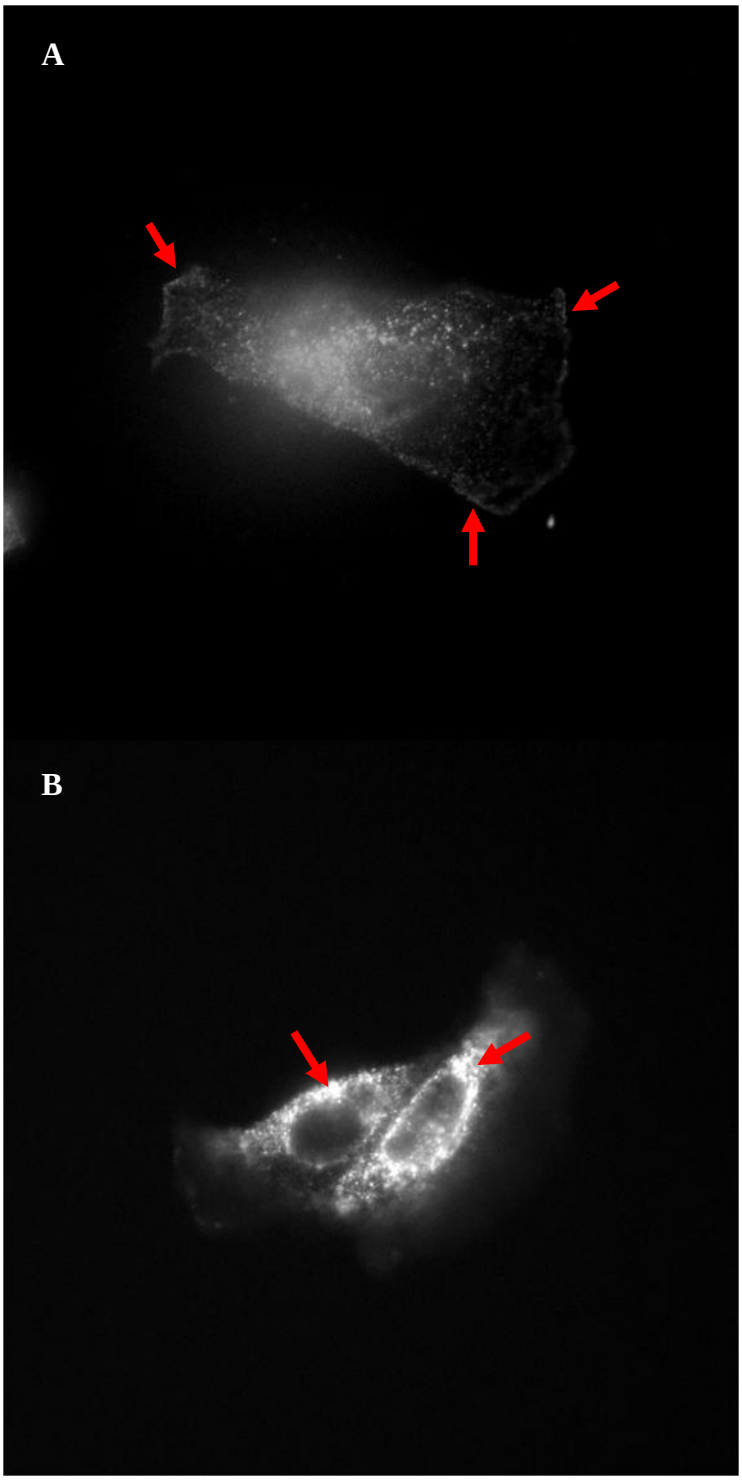

LexA
αV subunit
D W L E F K R V R P P Q E E T

Appendix B

Figure B.1 Release of cold-restriction allows α5β1 integrin internalization. Cold *Xenopus* A6 cells were surface-labelled with anti-α5β1 antibody (P8D4). Cold cells display impaired cell membrane dynamics and labelled α5β1 integrins are largely localized to the cell surface (A, red arrows). Warming cells to room temperature for 1 hour allows for α5β1 internalization to the peri-nuclear region of the cell (B, white arrows).

0 minutes

60 minutes

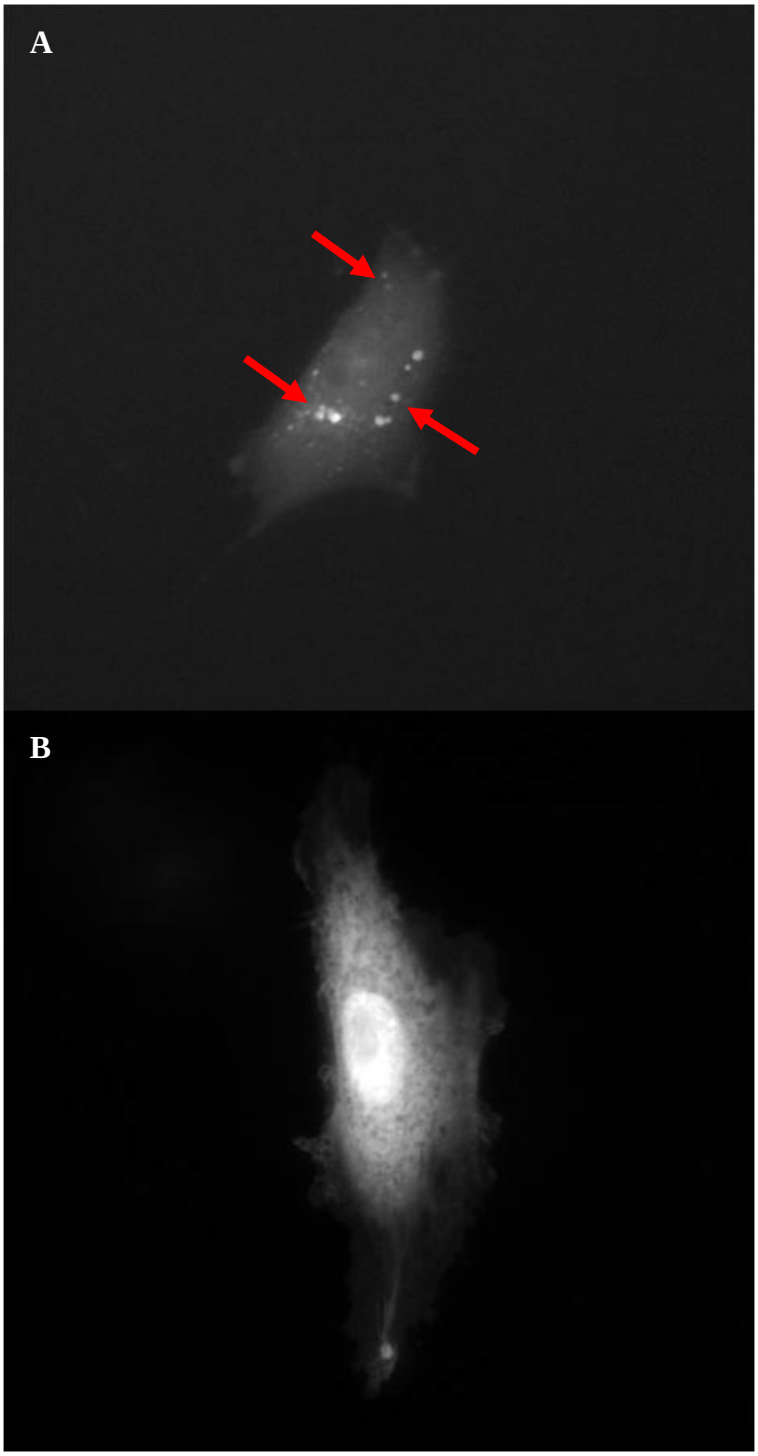


Appendix C

Figure C.1 Localization of XGIPC in *Xenopus* A6 cells. Different patterns of XGIPC staining were observed 48 hours after A6 cell transfection with XGIPC plasmid DNA depending on the amount of protein expressed. Cells expressing low levels of XGIPC showed a punctuate patterns (A, red arrows), suggestive of association with endocytic vesicles. Cells expressing high levels of XGIPC exhibited a diffuse cytosolic staining pattern and dense nuclear staining (B).

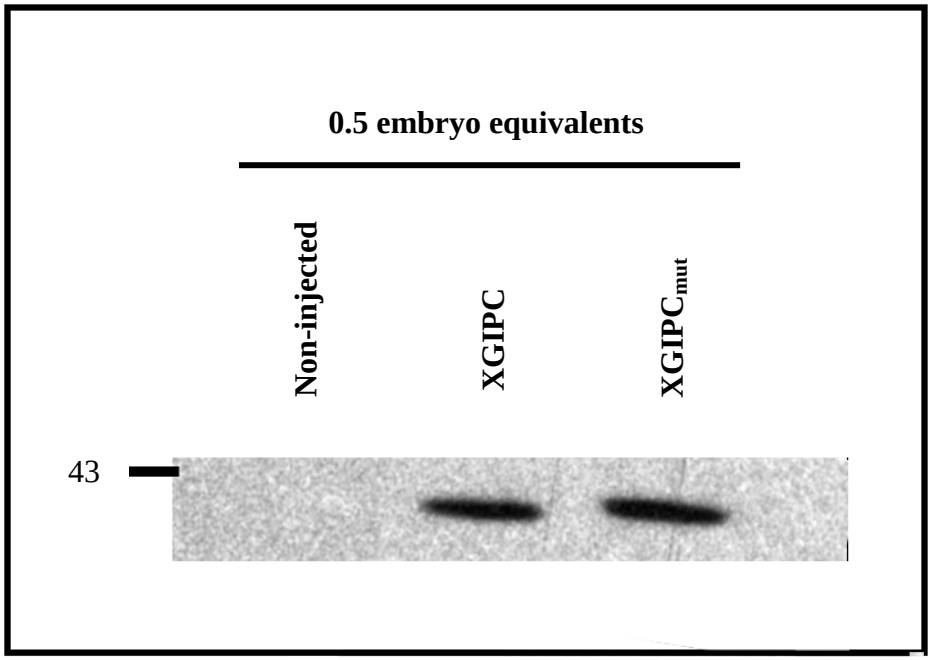
Low XGIPC expression

High XGIPC expression



Appendix D

Figure D.1 Expression of XGIPC and XGIPC$_{mut}$. Whole embryo lysates were analysed on Western blots to verify protein expression. HA-tagged XGIPC constructs were detected using anti-HA antibody (12CA5, Roche). XGIPC and XGIPC$_{mut}$ were expressed at approximately equal levels (*XGIPC and XGIPCmut*). Protein expression was not detected using anti-HA antibody in control embryo lysate (*Non-injected*). Each lane represents approximately 0.5 embryo equivalent.


0.5 embryo equivalents
Non-injected
XGIPC
XGIPCmut
43

References

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002). *Molecular Biology of the Cell, 4th Ed.* New York, NY: Garland Science. Pp 737-766, 1113.

Awan, A., Lucic, M.R., Shaw, D.M., Sheppard, F., Westwater, C., Lyons, S.A., and Stern, P.L. (2002). 5T4 interacts with TIP-2/GIPC, a PDZ-protein, with implications for metastasis. *Biochem Biophys Res Commun* **290**, 1030-1036.

Bischof, P., Haenggeli, L., and Campana, A. (1995). Integrins and adhesion molecules: Gelatinase and oncofetal fibronectin secretion is dependent on integrin expression on human cytotrophoblasts. *Hum Repro* **10**, 734-742.

Blobe, G.C., Liu, X., Fang, S.J., How, T., and Lodish, H.F. (2001). A novel mechanism for regulating transforming growth factor beta (TGF-β) signaling. Functional modulation of type III TGF-β receptor expression through interaction with the PDZ domain protein, GIPC. *J Biol chem.* **276**, 39608-39617.

Bokel, C., and Brown, N.H. (2002). Integrins in development: moving on, responding to, and sticking to the extracellular matrix. *Dev Cell* **3**, 311-321.

Booth, R.A., Cummings, C., Tiberi, M., and Liu, X.J. (2002). GIPC partcipates in G protein signaling downstream of insulin-like growth factor 1 receptor. *J Biol Chem* **277**, 6719-6725.

Boucaut, J.C., Darribere, T., Boulekbache, H., and Thiery, J.P. (1984). Prevention of gastrulation, but not neurulation by antibodies to fibronectin in amphibian embryos. *Nat* **307**, 364-367.

Bretscher, M.S. (1992). Circulating integrins: alpha 5 beta 1, alpha 6 beta 4, and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. *EMBO J* **11**, 405-410.

Bretscher, M.S. (1989). Endocytosis and recycling of the fibronectin receptor in CHO cells. *EMBO* **8**, 1341-1348.

Bretscher, M.S. (1996). Moving membrane up to the front of migrating cells. *Cell* **85**, 465-467.

Brower, D.L., Brower, S.M., Haywood, D.C., and Ball, E.E. (1997). Molecular evolution of integrins: Genes encoding integrin β subunits from a coral and a sponge. *Proc Nat Acad Sci USA* **94**, 9182-9187.

Burke, D., Dawson, D., and Stearns, T. (2000). *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Buss, f., Luzio, J.P., and Kendrick-Jones, J. (2002). Myosin VI, an actin motor for membrane traffic and cell migration. *Traffic* **3**, 851-858.

Cai, H., and Reed, R.R. (1999). Cloning and characterization of neuropilin-1-interacting protein, a PSD-95/DlgZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. *J Neurosci* **19**, 6519-6527.

Calderwood, D.A. (2004). Integrin activation. *J of Cell Sci* **117**, 657-666.

Caswell, P. (2007). Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. *Dev Cell* **13**, 496-510.

Caswell, P., and Norman, J. (2008). Endocytic transport of integrins during cell migration and invasion. *Trends Cell Biol* **18**, 257-263.

Caswell, P., and Norman, J. (2006). Integrin trafficking and the control of cell migration. *Traffic* **7**, 14-21.

Chan, B.M., Wong, J.G., Rao, A., and Hemler, M.E. (1991). T cell receptor-dependent antigen-specific stimulation of a murine T cell induces a transient, VLA protein-mediated binding to extracellular matrix. *J Immunol* **147**, 398-404.

Coppolino, M.G. and Dedhar, S. (2000). Bi-directional signal transduction by integrin receptors. *The Int J of Biochem & Cell Biol* **32**, 171-188.

Cousin, H., DeSimone, D.W., and Alfandari, D. (2008). PACSIN2 regulates cell adhesion during gastrulation in *Xenopus laevis*. *Dev Biol* **319**, 86-99.

Cox, E.A., and Huttenlocher, A. (1998). Regulation of integrin-mediated adhesion during cell migration. *Micro Res Tech* **43**, 412-419.

Critchley, D.R. (2000). Focal adhesions – the cytoskeletal connection. *Curr Opin Cell Biol* **12**, 133-139.

Dance, A.L., Miller, M., Seragaki, S., Aryal, P., White, B., Aschenbrenner, L., and Hasson, T. (2004). Regulation of myosin-VI targeting to endocytic compartments. *Traffic* **5**, 798-813.

Davidson, L.A., Hoffstrom, B.G., Keller, R., and DeSimone, D.W. (2002). Mesendoderm extension and mantle closure in *Xenopus laevis* gastrulation: combined roles for integrin alpha(5)bet(1), fibronectin, and tissue geometry. *Dev Biol* **242**, 109-129.

Davidson, L.A., Keller, R., and DeSimone, D.W. (2004). Assembly and remodelling of the fibrillar fibronectin extracellular matrix during gastrulation and neurulation in *Xenopus laevis*. *Dev Dyn* **231**, 888-895.

Davidson, L.A., Marsden, M., B.G., Keller, R., and DeSimone, D.W. (2006). Integrin alpha5beta1 and fibronectin regulate polarized cell protrusions required for *Xenopus* convergence and extension. *Curr Biol* **16**, 833-844.

De Vries, L., Elenko, E., McCaffery, J.M., Fischer, T., Hubler, L., McQuistan, T., Watson, N., and Farquhar, M.G. (1998a). RGS-GAIP, a GTPase-activating protein for Galphai heterotrimeric G proteins, is located on clathrin-coated vesicles. *Mol Biol Cell* **9**, 1123-1134.

De Vries, L., Lou, X., Zhao, G., Zheng, B., and Farquhar, M.G. (1998b). GIPC, a PDZ domain containing protein, interacts specifically with the C terminus of RGS-GAIP. *Proc Natl Acad Sci USA* **95**, 12340-12345.

Duncker, B., Shimada, K., Tsai-Pflugfelder, M., Pasero, P., and Gasser, S. (2002). An N-terminal domain of Dbf4p mediates interaction with both origin recognition complex (ORC) and Rad53p and can deregulate late origin firing. *Proc Natl Acad Sci USA* **99**, 16087-16092.

Dzamba, B.D., Bolton, M.A., and DeSimone, D.W. (2002). Cell Adhesion: Frontiers in Molecular Biology; The integrin family of cell adhesion molecules. Oxford, UK: Oxford University Press, pp 100-154.

El Mourabit, H., Poinat, P., Koster, J., Sondermann, H., Wixler, v., Wegener, E., Laplantine, E., Geerts, D., Georges-Labouesse, E., Sonnenberg, A, and Aumailley, M. (2002). The PDZ domain of TIP-2/GIPC interacts with the C-terminus of the integrin alpha5 and ahpha6 subunits. *Matrix Biol* **21**, 207-214.

Fernandez-Valle, Wood, P.M., and Bunge, M.D. (1998). Localization of focal adhesion kinase in differentiating Schwann celléneuron cultures. *Microsci Res Tech* **41**, 416-430.

Gao, Y., Li, M., Chen, W., and Simons, M. (2000). Synectin, syndecan-4 cytoplasmic domain binding PDZ protein, inhibits cell migration, *J Cell Physiol* **184**, 373-379.

Gilbert, S.F. (2006). Early development and axis formation in Amphibians. *Developmental Biology*. Pp 751. Sunderland, MA: Sinauer Associates, Inc., Publishers.

Giancotti, F.G., and Ruoslahti, E. (1999). Integrin signaling. *Sci* **285**, 1028-1032.

Ginsberg, M.H., Du, X., and Plow, E.F. (1992). Inside-out integrin signaling. *Curr Opin Cell Biol* **4**, 766-771.

Goto, T., Davidson, L., Asashima, M., and Keller, R. (2005). Planar cell polarity genes regulate polarized extracellular deposition in Frog gastrulation. *Curr Biol* **15**, 787-793.

Gyuris, J., Golemis, E., Chertkov, H., and Brent, R. (1993). Cdi1, a human GI and S phase protein phosphatise that associates with Cdk2. *Cell* **75**, 791-803.

Hasson, T. (2003). Myosin VI: two distinct roles in endocytosis. *J Cell Sci* **116**, 3453-3461.

Hemler, M.E., Kassner, P.D., and Chan, B.M.C. (1992). Functional roles for integrin alpha subunit cytoplasmic domains. *Cold Spring Harb Symp Quant Biol* **57**, 213-220.

Hirakawa, T., Galet, C., Kishi, M., and Ascoli, M. (2003). GIPC binds to the human lutropin receptor (hLHR) through an unusual PDZ domain binding motif, and it regulates the sorting of the internalized human choriohonadotropin and the density of cell surface hLHR. *J Biol Chem* **278**, 49348-49357.

Hu, L.A., Chen, W., Martin, N.P., Whalen, E.J., Premont, R.T., and Lefkowitc, R.J. (2003). GIPC interacts with the beta1-adrenergic receptor and regulates beta1-adrenergic receptor-mediated ERK activation. *J Biol Chem* **278**, 26295-26301

Hynes, R.O. (1987). Integrins: a family of cell surface receptors. *Cell* **19**, 549-554.

Hynes, R.O. (1992). Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* **69**, 11-25.

Johnson, K.E., Darribere, T., and Boucaut, J.C. (1992). Ambystoma maculatum gastrulae have an oriented, FN-containing extracellular matrix. *J Exp Zool* **261**, 458-471

Joos, T.O., Reintsch, W.E., Brinker, A., Klein, C., and Hausen, P. (1998). Cloning of the *Xenopus* integrin alpha(v) subunit and analysis of its distruption during early development. *Int J Dev Biol* **42**, 171-179.

Joos, T.O., Whittaker, C.A., Meng, F., DeSimone, D.W., Gnau, V., and Hausen, P. (1995). Integrin alpha 5 during early development of *Xenopus laevis*. *Mech Dev* **50**, 187-199.

Kabir-Salmani, K., Shiokawa, S., akimoto, Y., and Iwashita, M. (2004). The role of alpha(5)beta(1)-integrin in the IGF-1-induced migration of extravillus trophoblast cells during the process of implantation. *Mol Hum Reprod* **10**, 91-97.

Katoh, M. (2002). GIPC gene family (Review). *Int J Mol Med* **9**, 585-589.

Keller, R. (2002). Shaping the vertebrate body plan by polarized embryonic cell movements. *Sci* **298**, 1950-1954.

Keller, R. and Gerhart, J. (1986). Region-specific cell activities in amphibian gastrulation. *Ann Rev* **2**, 201-229.

Keller, R., Shih, J., and Sater, A. (1992). The cellular basis of the convergent and extension of the *Xenopus* neural plate. *Dev Dyn* **193**, 199-217.

Lallier, T.E., Whittaker, C.A., and DeSimone, D.W. (1996). Integrin alpha 6 expression is required for early nervous system development in *Xenopus laevis*. *Dev* **122**, 2539-2554.

Lee, G., Hynes, R.O., and Kirschner, M. (1984). Temporal and spatial regulation of fibronectin in early *Xenopus* development. *Cell* **36**, 729-740.

Li, R., Mitra, N., Gratkowski, H., Vilaire, J.W., Lear, J.D., DeGrado, W.F., and Bennett, J.S. (2003). Activation of integrin αIIbβ3 by modulation of transmembrane helix associations. *Sci* **300** 795-798.

Ligensa, T., Krauss, S., Demuth, D., Schumacher, R., Camonis, J., Jaques, G, and Weider, K.M. (2001). A PDZ domain protein interacts with the C-terminal tail of the insulin-like growth factor-1 receptor but not with the insulin receptor. *J Biol Chem* **276**, 33419-33427.

Liu, Y., Calderwood, D.A., and Ginsberg, M.H. (2000). Integrin cytoplasmic domain-binding proteins. *J Cell Sci* **113**, 3563-3571.

Liu, Y., Pan, D., Bellis, S., and Song, Y. (2008). Effect of altered glycosylation on the structure of the I-like domain of β_1 integrin: A molecular dynamics study. *Accepted to Proteins: Structure, Function and Bioinformatics*. August 5 2008.

Lou, X., McQuistan, T., Orlando, R.A., and Farquhar, M.G. (2002). GAIP, GIPC, and Galphai3 are concentrated in endocytic compartments of proximal tubule cells: putative role in regulating megalin's function. *J Am Scho Nephrol* **13**, 918-927.

Lou, X., Yano, H., Lee, F., Chao, M.V., and Farquhar, M.G. (2001). GIPC and GAIP form a complex with TrkA: a putative link between G protein and receptor tyrosine kinase pathways. *Mol Biol Cell* **12**, 615-627.

Marsden, M., and DeSimone, D.W. (2003). Integrin-ECM interactions regulate cadherin-dependent cell adhesion and are required for convergent extension in *Xenopus*. *Curr Biol* **13**, 1182-1191.

Marsden, M., and DeSimone, D.W. (2001). Regulation of cell polarity, radial intercalation and epiboly in *Xenopus*: novel roles for integrin and fibronectin. *Dev* **128**, 3635-3647.

Meng, F., Whittaker, C.A., Ransom, D.G., and DeSimone, D.W. (1997). Cloning and characterization of cDNAs encoding the integrin alpha2 and alpha3 subunits from *Xenopus laevis*. *Mech of Dev* **67**, 141-155.

Na, J., Marsden, M., and DeSimone, D.W. (2003). Differential regulation of cell adhesive functions by integrin alpha subunit cytoplasmic tails in vivo. *J Cell Sci* **116**, 2333-2343.

Nakatsuji, N., and Johnson, K.E. (1983). Conditioning of a culture substratum by the ectodermal layer promotes attachment and oriented locomotion by amphibian gastrula mesodermal cells. *J Cell Sci* **59**, 43-60.

Pancer, Z., Kruse, M., Muller, I., and Muller, W.E. (1997). On the origin of Metazoan adhesion receptors: cloning of alpha subunit from the sponge *Geodia cydonuim*. *Mol Biol Evol* **14**, 391-398.

Pellinen, T., and Ivaska, J. (2006). Integrin Traffic. *J Cell Sci* **119**, 3723-3731.

Pera, E.M., Wessley, 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.

Pera, E.M., Ikeda, A., Eivers, E., and De Robertis, E.M. (2003). Integration of IGF, FF, and anti-BMP signals via Smad1 phosphorylation in neural induction. *Genes Dev* **244**, 3023-3028.

Pierschbacher, M.D., and Ruoslahti, E. (1984). Variants of the cell recognition of fibronectin that retain attachment-promoting activity. *Proc Natl acad Sci USA* **81**, 5985-5988.

Ramos, J.W., and DeSimone, D.W. (1996). Xenopus embryonic cell adhesion to fibronectin: position-specific activation of RGD-synergy site-dependent migratory behaviour at gastrulation. *J Cell Biol* **134**, 227-240.

Ramos, J.W., Whittaker, C.A., and DeSimone, D.W. (1996). Integrin-dependent adhesive activity is spatially controlled by inductive signals at gastrulation. *Dev* **122**, 2873-2883.

Ransom, D.G., Hens, M.D., and DeSimone, D.W. (1993). Integrin expression in early amphibian embryos: cDNA cloning and characterization of *Xenopus* beta 1, beta 2, beta 3, and beta 6 subunits. *Dev Biol* **160**, 265-275.

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.

Schwartz, M.A., and Assoian, R.K. (2001). Integrins and cell proliferation: regulation of cyclin-dependent kinases via cytoplasmic signaling pathways.

Schwartz, M.A., Schaller, M.D., and Ginsberg, M.H. (1995). Integrins: emerging paradigms of signal transduction. *Ann rev Cell Dev Biol* **11**, 549-599.

Sczekan, M.M., and Juliano, R.L. (1990). Internalization of the fibronectin receptor is a constitutive process. *J Cell Physiol* **142**, 574-580.

Semple, J.W., Da-Silva, L.F., Jervis, E.J., Ah-Kee, J., Al-Attar, H., Kummer, L., Heikkila, J.J., Pasero, P., and Duncker, B.P. (2006). An essential role for Orc6 in Dna replication through maintenance of pre-replicative complexes. *EMBO J* **25**, 5150-5158.

Shi, D.L., Darribere, T., Johnson, K.E., and Boucaut, J.C. (1989). Initiation of mesodermal cell migration and spreading relative to gastrulation in the urodele amphibian Pleurodeles waltl. *Dev* **105**, 351-363.

Shih, J. And Keller, R. (1992). Cell motility driving mediolateral intercalation in explants of *Xenopus laevis*. *Dev* **116**, 901-914.

Sive, H. (1996). *Early Development of Xenopus laevis; Course Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Smith, J.C., and Howard, J.E. (1992). Meso-inducing factors and the control of gastrulation. *Dev Suppl* 127-136.

Smith, J.C., Symes, K., Hynes, R.O., and DeSimone, D.W. (1990). Mesoderm induction and the control of gastrulation of *Xenopus laevis*: the roles of fibronectin and integrins. *Dev* **108**, 229-238.

Tan, C., Deardoff, M.A., Saint-Jeannet, J.P., Yang, J., Arzoumanian, A., and Klein, P.S. (2001). Kermit, a frizzled interacting protein, regulates frizzled 3 signaling in neural crest development. *Dev* **128**, 3665-3674.

Tani, T.T. and Mercurio, A.M. (2001). PDZ interaction sites in integrin alpha subunits. T14835, TP/GIPC binds to a type 1 recognition sequence in alpha 6A/alpha 5 and a novel sequence in alpha 6B. *J Biol Chem* **276**, 36535-36542.

Varrin, A.E., Prasad, A.A., Scholz, R., Ramer, M.D., and Duncker, B.P. (2005). A mutation in Dbf4 motif M impairs interactions with DNA replication factors and confers increased resistance to genotoxic agents. *Mol Cell Biol* **25**, 7494-7504.

Varsano, T., Dong, M., Niesman, I., Gacula, H., Lou, X., Ma, T., Testa, J.R., Yates, J.R. III, and Farquhar, M.G. (2006). GIPC is recruited by APPL to peripheral TrkA endosomes and regulates TrkA trafficking and signaling. *Mol Cell Biol* **26**, 8942-8952.

Wang, L., Kalb, R.G., and Strittmatter, S.M. (1999). A PDZ Protein Regulates the Distribution of the Transmembrane Semaphorin, M-SemF. *J Biol Chem* **274**, 14137-14146.

Whittaker, C.A. and DeSimone, D.W. (1998). Molecular cloning and developmental expression of the *Xenopus* homolog of integrin alpha 4. *Ann N Y Acad Sci* **857**, 56-73.

Winklbauer, R. and Keller, R. (1996). Fibronectin, mesoderm migration and gastrulation in *Xenopus*. *Dev Biol* **177**, 413-426.

Winklbauer, R. and Nagel, M. (1991). Directional mesoderm cell migration in the *Xenopus* gastrula. *Dev Biol* **148**, 573-589.

Wolpert, L., Jessell, T., Lawrence, P., Meyerowitz, E., Robertson, E., and Smith, J. (2007). Patterning the vertebtate body plan I: axes and germ layers; Morphogenesis: changes in form in the early embryo. *Principles of Development, 3rd Ed.* Pp 94; 280. New York, New York: Oxford University Press.

Wu, C. (1997). Roles of integrins in fibronectin matrix assembly. *Histol Histopathol* **12**, 233-240.

Wu, J., O'Donnell, M., Gitler, A.D., and Klein, P.S. (2006). Kermit 2/XGIPC, an IGF-1 receptor interacting protein, is required for IGF signaling in *Xenopus* eye development. *Dev* **133**, 3651-3660.

Zhong, Y., Briehr, W.M., and Gumbiner, B.M. (1999). Analysis of C-cadherin regulation during tissue morphogenesis with an activating antibody. *J Cell Biol* **144**, 351-359.