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Signalling Mechanisms Underlying Congenital Malformation: The Gatekeepers, Glypicans

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1. Introduction

Congenital malformations contribute to a significant proportion of infant morbidity and remain a leading cause of death in both the neonatal and postneonatal periods (Brunner and van Driel, 2004). Despite the high frequency of these disorders, their underlying causes remain often obscure due to the complexity of human development. The human genome contains approximately 25,000 genes and most of them become active to build both tissue and body structures (Brunner and van Driel, 2004). Moreover, growth and morphogenesis of the human embryo relies on the precise orchestration and intercalation of multiple cellular functions, and the spatio/temporal control of the inherent molecular and biological processes. Therefore, modern human genetics regards the detailed understanding of genes and molecular strategies involved as "an indispensable investment" that can foster greater progress in the diagnosis and treatment of these human disorders.

From the beginning of the last century, going back to the Spemann and Mangold experiments, cell to cell signalling has been recognized as one fundamental principle of animal development (Freeman and Gurdon, 2002). At almost every developmental stage cells communicate with each other and such processes permit the generation of cell-type differences during development and the coordination of cell functions during tissue/organ morphogenesis or tissue/organ homeostasis (Freeman and Gurdon, 2002; Pires-daSilva and Sommer, 2003).

Chemical communication is by far the major form of information transfer between cells. Following release by instructive cells, signals move towards target cells through either direct contact or by short and long diffusion (Freeman and Gurdon, 2002; Papin et al., 2005). On target cells signals are captured by distinct cellular receptors that upon integrating and interpreting them activate appropriate intracellular signalling pathways and effectors to modify cell fate, metabolism or function (Freeman and Gurdon, 2002; Pires-daSilva and Sommer, 2003).

Research in the past two decades has yielded important advances towards the identification of the signal proteins, receptors, and intracellular proteins involved in signalling processes. For example the human genome contains more than 1500 genes that encode receptor

proteins, and the number of different receptor proteins is further increased by alternative RNA splicing and post-translational modifications. Surprisingly, genetic and biochemical studies revealed that only a few different classes of signalling pathways mediate patterning of a wide variety of cells, tissues and organs. For example, Fibroblast growth factors (FGF) Hedgehog (Hh), Wingless related (Wnt), Transforming growth factor-b (TGF-b) and Notch are used reiteratively during development to mediate very different biological processes in different animals (Freeman and Gurdon, 2002; Pires-daSilva and Sommer, 2003). In other words, a signal that in one instance will cause a cell to differentiate terminally will elsewhere lead another cell type to undergo mitosis and in a third context will trigger cell death.

These findings have raised the question of how generic signals can trigger tissue-specific responses. As a general principle specificity relies on the repertoire of receptors and intracellular mediators that are active in a given cell at a given time (Freeman and Gurdon, 2002). Nevertheless, there is now clear evidence that specificity of signal outcome is also the product of biological strategies ensuring signal level, strength, duration and its spatio-temporal distribution (Freeman and Gurdon, 2002). For example, several of these signalling molecules function as morphogens that form concentration gradients across developmental fields and specify different cell fates in a concentration dependent fashion during pattern formation (Freeman and Gurdon, 2002). Other studies have also shown that differences in the kinetics of the ligand or receptor binding mode, and changes in the temporal and quantitative supply of active ligand can contribute to increases in the heterogeneity of biological responses to incoming signals, but without losing the cell specific effects that ensure reproducibility of developmental processes (Freeman and Gurdon, 2002).

In the pursuit of molecular mechanisms that underlie these further layers of regulation attention has progressively shifted towards components of the extracellular matrix. Besides being structural scaffolds, these proteins are now evaluated as vital elements of the cell signalling machinery that provide processing and bioavalaibility of instructive signals (Bernfield et al., 1999; Bulow and Hobert, 2006).

In particular, cell surface proteoglycans such as Glypicans (Gpcs) interact with chemokines, growth factors/morphogens and their receptors (Bulow and Hobert, 2006; Hacker et al., 2005; Nybakken and Perrimon, 2002). Disruption of Gpc functions in *Drosophila*, Zebrafish, *Xenopus Laevis* and mouse results in phenotypes reminiscent of defects in cellular responses to regulatory signalling molecules (Hacker et al., 2005; Lin, 2004). Yet, genetic and embryological studies link Gpcs to the regulation of cell signalling events during morphogenesis and adult physiology (Bishop et al., 2007; DeBaun et al., 2001; Hacker et al., 2005; Lin et al., 1999). In humans, mutations in the *Gpc-3* gene are associated with several diseases. For example, mutations in the *Gpc-3* gene underlie a condition called Simpson-Golabi-Behmel syndrome, which is characterized by overgrowth of the body and other birth defects (DeBaun et al., 2001). Homozygosity for null mutations in the *Gpc-6* gene cause autosomal recessive omodysplasia, a genetic condition characterized by severe short stature and congenital heart defects (Campos-Xavier et al., 2009). Additionally, increased and decreased activity of some *Gpcs* (including *Gpc*-1, -3, -4 and -6) occurs in certain forms of cancer (Filmus, 2001). Here, we review our current knowledge on the implication of these proteoglycans in congenital malformations, and discuss our understanding of their mechanism of action.

2. Molecular design of cell surface glypicans

The name "glypicans" identifies a family of heparan sulphate proteoglycans (HSPGs) that are linked to the exocytoplasmic surface of the plasma membrane through a covalent glycosyl-phosphatidylinositol (GPI) linkage (Bulow and Hobert, 2006). Together with Syndecans, *gpc* gene products are the major cell surface HSPGs (Bulow and Hobert, 2006). Gpcs have been highly conserved throughout evolution and most likely arose early during metazoan evolution (Filmus et al., 2008). In this section, we describe their major structural features that have been crucial to elucidate their in vivo roles.

2.1 Glypican assembly

Gpcs are proteins of around 60–70 kDa with a characteristic pattern of 14-conserved cysteine residues mainly located to the central domain (De Cat and David, 2001). Gpcs also share an N-terminal signal sequence and a hydrophobic C-terminal sequence involved in the formation of the GPI anchor structure (Fig. 1; De Cat and David, 2001). Heparan sulphate glycosaminoglycan (HSGAG) polysaccharide side chains can be attached to serine residues in consensus sequences, such as XGlyXGlySerX, that are located between the central domain and the C-terminal GPI-anchor (De Cat and David, 2001). The HSGAG of proteoglycans can undergo complex patterns of modification consisting of sulphations of hydroxyl groups in individual sugar molecules, epimerizations of specific carbon atoms and changes in length of the individual sugar residues (Bulow and Hobert, 2006; Nybakken and Perrimon, 2002). Such modifications are thought to generate a large structural diversity that might encode information for the selective binding of protein ligands (Bulow and Hobert, 2006; Nybakken and Perrimon, 2002). In general, Gpcs carry these HS chains, but Gpc5 also displays chondroitin sulfate modifications (Saunders et al., 1997).

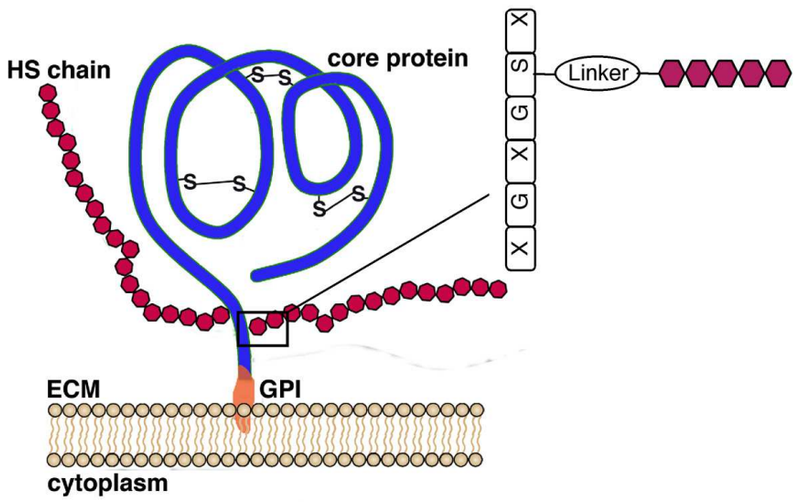

Fig. 1. Schematic representation of the Gpc structure.

The glypican core protein spans the extracellular space. Disulfide bridge (S-S) are thought to organize the core protein as a compact globular domain. HS chains are covalently bound to serine residues that are part of X-Gly-X-Gly-Ser-X motifs in the polypeptide chain close to the plasma membrane. The carboxyterminus of Gpcs is covalently linked to the plasma

membrane via a GPI anchor. Red filled hexagon chains represent HS chains. S = serine; G = glycine; X = aminoacid (adapted from De Cat, B. and David, G., 2001).

Proteolytic cleavages of the core proteins can also contribute to generate distinct Gpc forms. As shown for several vertebrate and invertebrate proteins, the N-terminal cysteine-rich domain of Gpcs can be splitted from the HS-modified and GPI-anchored C-terminal domain following endo-proteolytic processing (Song and Filmus, 2002). To which extent this event occurs in physiological condition is matter of investigation because the ratio between uncleaved and cleaved Gpcs varies according to the *gpc* family member and the tissue-specific context. The N-terminal Gpc fragment is not membrane-associated and, once generated, it can remains linked to its C-terminal half through one or more disulfide bridges (De Cat et al., 2003; Eugster et al., 2007). Thus, proteolytic processing can provide a molecular strategy to rapidly make available a secreted Gpc product, if needed, as such a form could be released from producing cells simply following redox changes of the extra-cellular environment.

2.2 Evolutionary origins

Gpcs are evolutionarily conserved proteins found in organisms as distinct as nematodes, fruit flies and mammals (De Cat and David, 2001; Fico et al., 2007; Filmus et al., 2008). The human and mouse genome contain six Gpc family members, *Gpc-1* to *Gpc-6* in humans and *gpc-1* to *gpc-6* in mice (De Cat and David, 2001; Fico et al., 2007; Filmus et al., 2008) whereas five *gpc*-like genes have been identified in zebrafish (Filmus et al., 2008; Topczewski et al., 2001), two in *Drosophila* (*dally* and *dally-like*; Baeg et al., 2001; Nakato et al., 1995) and two in *C. elegans* (*gpn-1* and *lon-2*; Gumienny et al., 2007; Hudson et al., 2006). The amino acid sequences of mammalian Gpc vary from being 17% to 63% identical. However, sequence relationships and exon organizations suggest that mammalian *gpcs* consist of two distinct subfamilies (De Cat and David, 2001; Fico et al., 2007; Filmus et al., 2008). The first subfamily includes *gpc*-1, -2, -4 and -6 genes with amino acid sequence homology ranging from 40–60% and composed of nine exons. The other subfamily incorporates *gpc*-3 and -5, with amino acid sequences 40% identical and containing eight exons. Of note, *gpc*-6 maps near to *gpc*-5 on mouse chromosome 14 and on human chromosome 13. *Gpc*-4, which is most homologous to *gpc*-6 maps to chromosome Xq26 near to *gpc*-3, which is highly related to *gpc*-5 (De Cat and David, 2001; Fico et al., 2007; Filmus et al., 2008). Therefore, members of the different subfamilies are inclined to cluster on the same chromosome. Whether *gpc* subfamilies and the genomic linkage of different members have evolved from a series of gene and genome duplications is still a matter of debate. In support of this possibility there are studies in organisms such as *Drosophila* and zebrafish showing the existence of distinct orthologs for each mammalian subfamily and their genomic linkage (Filmus and Capurro, 2008). It will be interesting to examine to what extent the appearance of *gpc* subfamilies might underlie the evolution of functional similarities in members of the same subfamily and functional differences among those more divergent. In this context, studies on *Dally* and *Dally-like* aimed at distinguish their activity on Hh have shown that *Dally-like* but not *Dally* is required for Hh response in a *Drosophila* cultured cell assay (Williams et al., 2010). Intriguingly, Gpc-4 and -6, which are the mammalian Gpcs most closely related to *Dally-like*, complement *Dally-like* function in this biological system (Williams et al., 2010). In contrast, *Dally* and its ortholog Gpc-3 exhibit trans-dominant negative activities (Williams et al., 2010). These

studies suggest a large trend in which related Gpc members may have evolved similar activities in distinct cellular contexts, but further understanding will come from studies on other signalling activities.

3. Glypicans: From structural compounds to signalling molecules

Once considered as acting merely as structural components of the extra cellular matrix, Gpcs are now widely recognized as essential modulator of many biological processes. These include their role as carriers in cellular uptake of growth promoting polyamines such as spermine (Casero and Marton, 2007; Fransson et al., 2004). It has been proposed that the binding of Gpcs to polyamines is mediated by electrostatic interactions occurring between Gpc HS side chains and polyamine residues. After transport to endosomes, HS moieties are degraded by nitric oxide. This is expected to weaken HS interaction with polyamines and results in their unloading and possibly exit from endosomes to elicit functions. The mechanisms underlying polyamines uptake has been analyzed in several systems and discussed in previous reports (Belting, 2003; Fransson et al., 2004).

To date, Gpcs are also considered as potential carriers of cell-penetrating peptides. Cell-penetrating peptides are short cationic peptides extensively studied in medicine as drug delivery agents for the treatment of different diseases including cancer and virus infection (Rajendran et al. , 2010). Their entry into cells is typically initiated through interaction with cell-surface HS proteoglycans (HSPGs) via electrostatic interactions, followed by endocytosis (Poon and Gariepy, 2007). Studies on the intracellular delivery of cell-penetrating peptides have shown that the migration of these peptides into cells as well as their final destination could depend on the nature of the HSPGs expressed at the cell surface (Poon and Gariepy, 2007). The GPI-anchor typical of Gpc proteins provides them with specific membrane-trafficking properties distinct from those of transmembrane HS molecules such as Syndencans (Chatterjee and Mayor, 2001; Payne et al., 2007). Therefore, Gpcs mediated uptake of cell-penetrating peptide is currently evaluated as a new strategy to enhance target-specific delivery of a large variety of entrapped therapeutic drugs.

Research on Gpcs has further increased due to the discovery that they act at the interface between the extra cellular environment and the inner cellular domain to fine tune inputs triggered by key secreted regulatory proteins. Although Gpcs have important physiological roles (Bishop et al., 2007), we concentrate here on their developmental functions and on the molecular mechanisms by which Gpcs trigger cell fate and tissue pattern.

3.1 Glypicans as modulator of regulatory extra-cellular signals

Our knowledge of Gpc biology has significantly expanded over the past decade with the discovery that Gpcs are not simply structural proteins. Being mostly extracellular, Gpcs are involved in the regulation of various signalling pathways triggered by secreted peptides including that of Wnt, Fgf, Hh, bone morphogenic protein (Bmp), insulin-like growth factor and hepatocyte growth factor (Fico et al., 2007; Filmus and Capurro, 2008).

The functional relevance of Gpcs as signalling modulators has come from the genetic analysis and embryological manipulation of Gpcs in different species and in cultured cells (Table 1).

Core protein	Species	Major defect	Affected Signals	References
Lon-2	*C elegans*	Body length	Bmp	Gumienny TL, Curr Biol 2007
Dally	Drosophila mutant	Embryogenic epidermis	Hg, Wg	Nybakken K, Biochim Biophys Acta 2002 Han C, Development 2004
		Wing imaginal discs	Hg, Wg	Nybakken K, Biochim Biophys Acta 2002 Han C, Development 2004
		Eye-antennal discs	Dpp	Hacker U, Nat Rev Mol Cell Biol 2005 Lin X, Development 2004
		Germline stem cells	Bmp	Guo Z, Development 2009
Dally-like	Drosophila mutant	Wing imaginal disc	Wg	Nybakken K, Biochim Biophys Acta 2002 Kreuger J, Dev Cell 2004
		Tracheal morphogenesis	Fgf	Kirkpatrick CA, Dev Cell 2004 Yan D, Dev Biol 2007
Knypek	Zebrafish mutant	Gastrulation	Wnt	Topczewski J, Dev Cell 2010; Caneparo L, Genes Dev 2007; Sepich DS, Development 2011
		Cartilage/bone morphogenesis	Wnt	LeClair EE, Dev Dyn 2009
gpc4	Xenopus morpholino	Gastrulation Dorsal forebrain	Wnt Fgf	Song HH, J Biol Chem 2005 Galli A, Development 2003
gpc1	Mouse null allele	Early neurogenesis	Fgf	Jen YH, Neural Dev 2009
gpc3	Mouse null allele	Body size	Wnt	Song HH, J Biol Chem 2005
		Limb mesenchyme	Bmp	Grisaru S, Dev Biol 2001
		Ureteric mesenchyme	Bmp, Fgf	Paine-Saunders S, Dev Biol 2000

Table 1. Glypicans function in model organisms. This table reports the major phenotypes observed by genetic and embryological studies on *glypican* genes and the main involved signals

For example, in vitro studies have shown that Gpc4 positively modulated hepatocyte growth factor activity during renal epithelial branching morphogenesis (Karihaloo et al., 2004). Mice lacking Gpc3 are affected by overgrowth, renal cystic dysplasia and limb defects. Some of these phenotypes are consistent with defects in Wnt and Bmp signalling pathways, respectively (Grisaru et al., 2001; Paine-Saunders et al., 2000; Song et al., 2005). Additional studies have also shown that the developmental overgrowth observed in *gpc3*-null mice is, at least in part, a consequence of the hyperactivation of the Hh pathway indicating that Gpc3 inhibits Hh (Gallet et al., 2008; Capurro et al., 2009). Interestingly, Gpc5 stimulates the proliferation of rhabdomyosarcoma cells by eliciting a positive action on Hh signalling (Li et al., 2011), in contrast to the Gpc3-mediated negative control of Hh.

These findings reveal that members of the Gpc family can display opposite roles in the regulation of a given signalling protein. The *C. elegans* Gpc Lon-2 also controls body size length (Gumienny et al., 2007). It has been proposed that Lon-2 negatively regulates Bmp signalling as *lon-2* mutants recapitulate phenotypes caused by Bmp over-expression (Gumienny et al., 2007). Another example is the Zebrafish *knypek*, which encodes the *gpc* homolog to mammalian Gpc4/Gpc6 (Topczewski et al., 2001). *knypek* controls convergent-extension movements during zebrafish gastrulation by positively modulating Wnt11 activity (Topczewski et al., 2001).

Modulation of extra-cellular signals by Gpcs has also been reported in *Xenopus*. In particular, reducing Gpc4 (Xgly4) disrupts cell movements during gastrularion (Ohkawara et al., 2003). We have also shown that loss-of Gpc4 function in *Xenopus* embryos impairs forebrain patterning and cell survival from early neural plate stages onwards, and that these early developmental defects result in brains affected by microcephaly at later stages (Fig. 2; Galli et al., 2003). Xgly4 physically interacts with Wnt11, and might enhance function in the Wnt/PCP pathway during gastrulation (Ohkawara et al., 2003). In addition to Wnt11, we have demonstrated that Xgly4 also binds Fgf2. Inhibition of Fgf signalling results in dorsal forebrain phenotypes similar to those of Xgly4 depleted embryos, indicating that establishment and patterning of the dorsal forebrain territory may require modulation of Fgf signalling by Xgly4 (Galli et al., 2003).

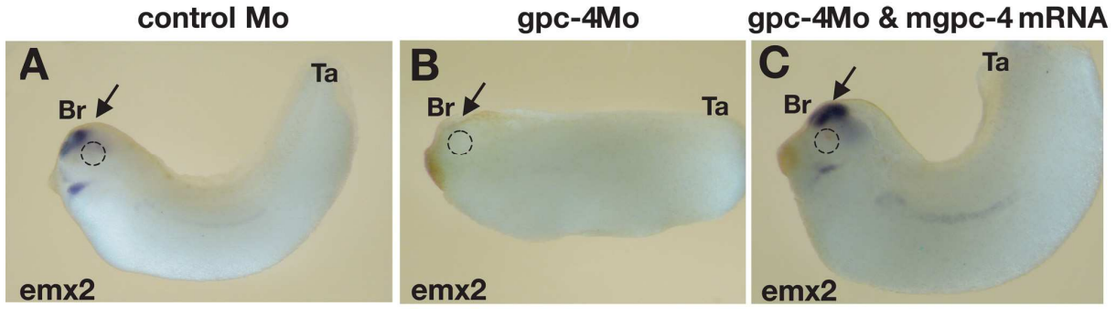

Fig. 2. Forebrain defects in GPC-4 depleted embryos. Side view of Xenopus embryos at tail bud stage showing expression of the dorsal forebrain marker *emx-2* as detected by whole mount in situ hybridization (arrow in all panels). Xenopus embryos were injected at 2 cell stage with morpholino oligos to interfere with Gpc-4 activity. Injections were done by using control morpholino (*control*Mo), or morpholino targeting *gpc-4* (*gpc-4*Mo). Embryos were also co-injected with morpholino targeting *gpc4* (*gpc-4*Mo) and mouse *gpc-4* mRNA (*mgpc-4* mRNA) for rescue experiments. (**A**, **B**) *emx-2* expression in the dorsal forebrain of tailbud embryos; note the loss of *emx-2* expression and the forebrain microcephalic morphology in GPC-4 depleted embryos (**B**). (**C**) Rescue of *emx-2* expression and forebrain morphology in a tail bud embryo co-injected with Gpc-4Mo and *mGpc-4* mRNA. Br: brain; Ta: Tail.

In *Drosophila* the Gpc *Dally-like* is required for Hh signalling in the embryonic ectoderm whereas both Gpcs *Dally* and *Dally-like* are required and redundant in Hh movement in developing wing imaginal discs (Han et al., 2004; Yan and Lin, 2009). Additional studies on the wing disc patterning have also demonstrated that in *Dally* and *Dally-like* mutants the distribution and signalling of Wnt and Bmp family members, Wingless (Wg) and Decapentaplegic (Dpp) respectively, are altered (Nybakken and Perrimon, 2002).

Furthermore, *Dally* and *Dally-like* also act on Wg during segment polarity determination and on Dpp in the developing eye and antennal discs (Hacker et al., 2005; Lin, 2004). Overall, these and other studies reveal that different cell types can take advantage of Gpc-mediated regulation to control signal supply during distinct developmental processes. In addition, they show that vertebrate and invertebrate Gpcs have diverse and specialized functions towards a given signalling protein including their capability of enhancing or suppressing its activity in a stage- and/or tissue-specific regulated manner.

3.2 Heparan sulphate chains and core proteins for signal control

The findings that Gpcs enable cells controlling activity of a wide range of extracellular effectors with greater selectivity towards biological outcomes, suggest that Gpcs are rather dynamic proteins capable of employing various mechanisms to exert their regulatory effects in biological processes. One question that has arisen is whether these properties are conferred by their unique structural motifs. As discussed above, Gpcs are most likely globular proteins with HS chains at the carboxyl terminus. Of note, Chen and Lander have identified that the Gpc1 globular domain is a structural motif that potently influences HS substitutions (Chen and Lander, 2001). Moreover, it has been proposed that the physical constraint of HS attachment sites at the carboxyl terminus could result in proteins with HS chains in the proximity of the cell surface. These basic structural features, together with the known versatile conformation and orientation of HS functional groups, could prime Gpc HS modifications to a degree that facilitate their contacts with cell-membrane proteins (e.g. signalling receptors) while retaining specificity in binding modes (Tumova et al., 2000). The GPI-anchorage is yet another feature that makes Gpc proteins subject to distinct subcellular localization and intracellular trafficking processes as well as to release into the extracellular environment through shedding mechanisms involving distinct extracellular lipases (Chatterjee and Mayor, 2001; Payne et al., 2007). Gpcs trafficking and shedding can both lead to a gain of signal, down-regulation properties and cell non-autonomous activities (Yan and Lin, 2009). These issues are the subjects of intense investigation, and a growing body of data is being published on Gpc mechanism of action. In this paragraph we briefly summarize the current state of our knowledge related to HS-mediated Gpc activity. We focus more on studies showing that the core protein and its GPI anchor confer on Gpcs additional functional versatility.

3.2.1 The heparan sulphate chains

The hypothesis that functional Gpc properties are mediated by HS modifications was first evaluated for the Fgf/Fgf receptor axis as it is well established that Fgfs rely on the co-receptor role of HSPGs for receptor binding and activation. As demonstrated by experiments performed mainly in cultured cells and in cell-free systems, Gpc HS modifications can catalyze binding of Fgfs to their receptors and boost receptor activation and its biological functions. For example, purified HS chains derived from Gpc-1, following protease-mediated digestion, augment the binding of Fgf-2 and Fgf-1 to Fgf receptor 1 to an extent that lower concentrations of ligands are needed for activation (Bonneh-Barkay et al., 1997b). Also, the Gpc-dependant Fgf binding and activation of receptors is nearly abolished when cells are treated with chlorate to inhibit Gpc sulfatation (Steinfeld et al., 1996). In this context, covalent cross-linking of Fgf-2 to cells

expressing its receptor demonstrates a putative Fgf-2/Fgf receptor complex when HS modified Gpcs are present in the same cell (Steinfeld et al., 1996). A conclusion that can be drawn from these studies is that cell-surface Gpcs by means of their HS modification can function as essential partners for the Fgf tyrosine kinase receptor. Potential mechanisms of action may include immobilizing of the ligand, increasing its local concentration, presenting it to a signalling receptor, or otherwise modifying the molecular encounters between ligands and receptors. The expected overall effect is thus enhancing receptor activation at low ligand concentrations. Interestingly, whereas Gpc-1 HS chains potentiate the biological activity of Fgf-1 they strongly inhibit Fgf-7 function (Berman et al., 1999; Bonneh-Barkay et al., 1997a). This suggests that HS chains can also act as a dual modulator of biological activities exerting both stimulatory and inhibitory effects depending on factors involved. More recently, experimental settings involving depletion of these sulphate groups both in vivo and in cultured cells have demonstrated that Gpcs HSs mediate interaction with additional HS binding proteins and impact their activity. This includes the binding and stimulation of Wg (the drosophila homolog of Wnt) signalling in the wing imaginal disc by *Dally* as well as the ability of human Gpc-5 to interact with Hh and enhance its growth promoting activity in rhabdomyosarcoma cells (Yan and Lin, 2009; Li et al. , 2011,). Interestingly, Dally HS chains are required in vivo to activate high-threshold but not low-threshold target genes of Dpp (Kirkpatrick et al., 2006), suggesting that HSPG core proteins could serve distinct functions in low- versus high-threshold morphogenetic signalling.

Recent studies have revealed that the co-receptor function of Gpcs can also provide a new paradigm of cell-cell communication. In the stem cell niche associated with germ cells the Gpc Dally is critical for making and maintaining the female germ cells (Hayashi et al., 2009). However, in this stem cell niche, *dally* is expressed by the cap cells, which also produce the Dpp signalling molecule, but not in the receiving cells (germ cells), which instead express Dpp receptor (Hayashi et al., 2009). These findings have raised questions and interest about the underlying molecular mechanisms. Studies in cultured cells have provided evidence that Dally enhances Dpp signalling in *trans* through a contact-dependent mechanism allowing the complementation of co-receptor-receptor complexes in adjacent cells (Dejima et al., 2011). Therefore, unlike typical co-receptor functions, Dally can serves as a *trans* co-receptor for Dpp when it has to enhance its signalling on neighboring cells. So far the mechanism for contact-dependent signalling has been mainly attributed to membrane-bound ligands and receptors such as Delta-Notch and Ephrins and their receptor tyrosine kinases (Hainaud et al., 2006). The fact that Gpcs act as *trans* activator partners establishes new strategies for crosstalk between adjacent cells during tissue assembly and maintenance.

In conclusion, a common theme throughout all studies is that HS chains are responsible for different aspects of Gpc biology. By means of HS chains Gpcs sequester secreted soluble ligands and modulates their activity. As co-receptors and *trans* co-receptor, Gpcs modulate ligand-receptor encounters that can activate and inhibit cell proliferation, motility, and differentiation. Also HS side chains are not uniform and changes in the distribution of sulphate groups may affect ligand-binding properties and biological outcomes in a cell type-specific manner.

3.2.2 The glypican core proteins

The GPI anchor and the core protein are two additional structural motifs that impinge on functional versatility of Gpcs at different levels. Most insights have come from cell biological approaches undertaken to investigate how Gpcs affect Hh and Wg signalling and gradient formation.

Concerning Wg, genetic analysis of Dally-like in the wing imaginal discs has highlighted a role for this Gpc in polarizing the Wg morphogenetic gradient. In the wing imaginal disc, Wg is secreted by a narrow strip of cells located at the dorsal–ventral boundary and spreads over a distance of up to 20 cell diameters. Wg first accumulates on the cell surface apical side in expressing cells to be then re-distributed to the basolateral membrane of receiving cells, where it is released in association with lipoprotein particles (Panakova et al., 2005; Strigini and Cohen, 2000). It has been proposed that polarizing Wg on the cell membrane allows the subsequent polarization of morphogen distribution within an epithelium, thus resulting in distinct tissue patterns (Marois et al., 2006). Therefore, one major question in the field is how Wg reaches the basolateral cell surface when it is secreted apically. Gallet and colleagues have investigated the subcellular localization of Dally-like in this cellular system and shown that Dally-like, which is apically targeted by means of its GPI anchor, undergoes internalization and redistribution to the basolateral membrane through a dynamin-dependent endocytosis (transcytosis; Fig. 3; Gallet et al., 2008). Interestingly, Wg is no longer detected at the basolateral surface of cells away from the Wg source in mutant cells lacking Dally-like protein. Moreover, tethering Dally-like at the cell membrane (by replacing the GPI anchor with a trans-membrane domain) strongly stabilized Wg at the apical surface while decreasing the amount of Wg at the basolateral compartment (Gallet et al., 2008). Altogether, these findings support the view that Wg is secreted apically and it is then endocytosed with the help of Dally-like (Fig. 3). Once internalized, Dally-like targets Wg by transcytosis to the basolateral compartment, where it is stabilized and can then spread farther away in a polarized manner (Fig. 3); Gallet et al., 2008). These findings also open the intriguing possibility that Dally-like-mediated basolateral polarization of Wg accounts for Wg activity in long-range signalling (Gallet et al., 2008). However, whether this mechanism underlies distinct Wg signalling activity remains a matter of debate (Williams et al.; Yan et al., 2009).

In contrast to the Wg situation, GPI-mediated endocytosis of Gpcs appears directly implicated in modulating Hh signalling in a positive and negative manner. For example, Dally-like endocytosis from the cell surface catalyzes the internalization of Hh in flies. In this context, internalization of Hh occurs together with its receptor Patched (Fig. 4; Gallet et al., 2008). Removing Patched from the membrane alleviates the inhibition of the transmembrane protein Smoothened by Patched and enables Smoothened to activate Hh target genes (Fig. 4; Gallet et al., 2008). Complementary studies performed in mice have revealed that the mammalian Gpc-3, via its GPI-anchor, also mediates internalization of Shh (the vertebrate homolog of Hedgehog) and regulates its signalling, with however opposing outcomes. Indeed, through endocytosis Gpc-3 inhibits Shh activity rather than activating it as in flies (Fig. 4; Capurro et al., 2008; Gallet et al., 2008). It has been proposed that Gpc-3 has high affinity for Shh and can, therefore, compete with Patched for Shh binding (Capurro et al., 2008). Upon binding, Gpc-3 targets Shh to endocytic vesicles for degradation, thus leaving the unliganded Ptc at the cell surface, and free to inhibit Smoothened (Fig. 4; Capurro et al., 2008). This possibility is also

consistent with results showing that hyperactivation of Shh can in part explain the Simpson-Golabi-Behmel overgrowth syndrome caused by loss-of-function mutations in Gpc-3, and with other experiments revealing an increased expression of Shh target genes in Gpc-3 deficient mice and mouse embryonic fibroblasts (Capurro et al., 2008).

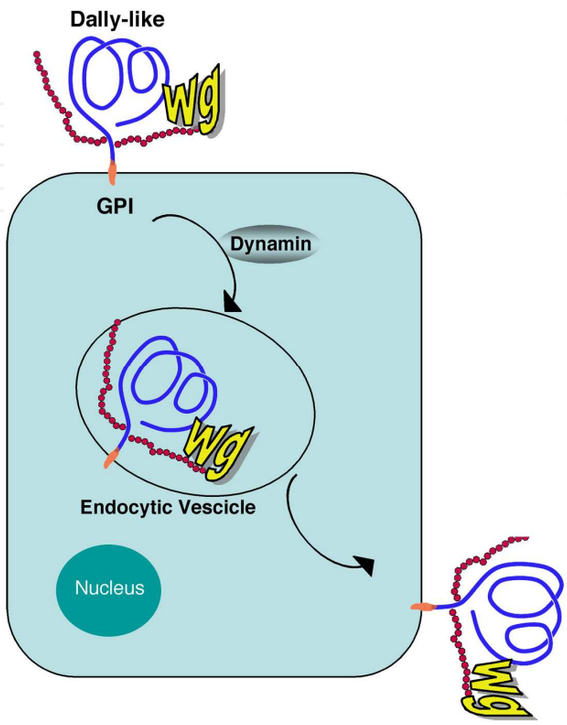

Fig. 3. The GPI anchor of Dally-like triggers Wg transcytosis. The GPI anchor of Dally-like is required for its apical targeting, subsequent internalization through dynamin-dependent endocytosis and relocalization to the basolateral compartment. It has been proposed that Wg is secreted apically and is then endocytosed with the help of Dally-like. Once internalized, Dally-like targets by transcytosis Wg to the basolateral compartment , where it is stabilized and can then spread farther away in a polarized manner (adapted from Dong Yan and Xinhua Lin 2008).

Interestingly, the Gpc3 core protein (without HS chains) binds with high affinity to Shh in cultured cells independently of its HS chains, while it does not interact with Patched (Capurro et al., 2008). These findings raise the possibility that the Gpc core protein cooperate with the GPI motif to establish differences in Gpc binding properties of signalling molecules, which will in turn affect biological readout. Of note, Williams and colleagues found that the Dally-like core protein without HS chains substantially rescues lack of Hh signalling in Dally-like mutant embryos, demonstrating specific activity for this structural domain (Williams et al., 2010). Similarly, the core proteins of the mammalian Gpc-4 and -6, which are the closest relatives of Dally-like, allow full dose-dependent re-activation of Hh, in contrast to Gpc-2, -3 and -5 that have no activity (Williams et al., 2010). This configuration of sequence homology and functional conservation suggests that the two major Gpc subfamilies have evolved similar roles in Hh signalling control (see also above). Therefore, Gpc agonistic and antagonistic signalling activities should also be identifiable in the Gpc core protein.

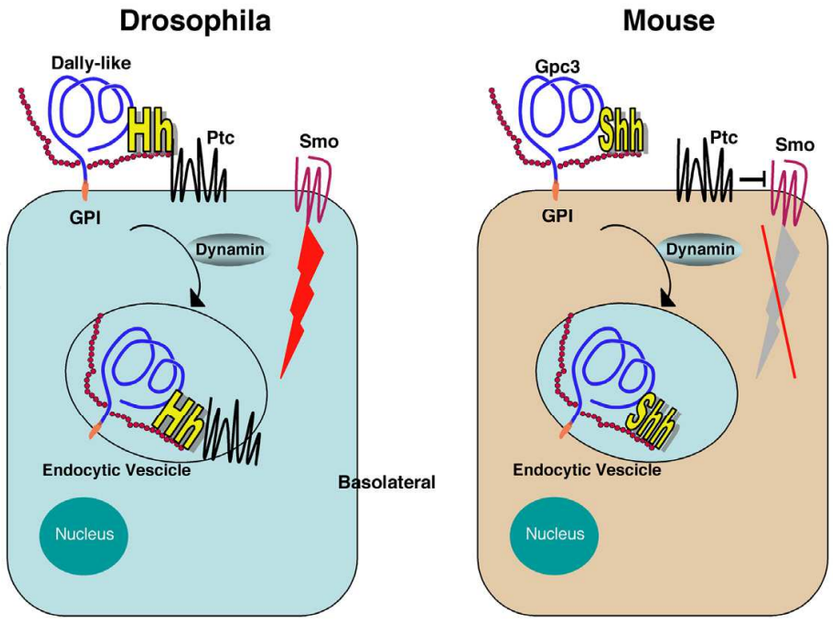

Fig. 4. Opposing roles for Gpcs in Hh signalling. (**A**) In *Drosophila* wing discs, Dally-like promotes Hh signalling. The GPI anchor of Dally-like is required for its apical targeting and subsequent internalization through dynamin-dependent endocytosis. Dally-like endocytosis from the cell surface catalyzes the internalization of Hh in flies that occurs together with Patched (Ptc). Removing Ptc from the cell membrane alleviates the inhibition of the transmembrane protein Smoothened (Smo) by Patched and enables Smoothened to activate Hh target genes. (**B**) In mouse development, Gpc3 acts as an inhibitor of Hh signalling. The Gpc3 core binds Shh on the cell surface and compete with Patched for Shh binding. Upon binding, Gpc-3 targets Shh to endocytic vesicles for degradation, thus leaving the unliganded Ptc at the cell surface, and free to inhibit Smoothened (adapted from Dong Yan and Xinhua Lin 2008).

To date, additional studies support the idea that the protein cores selectively impact on functions of distinct Gpcs. For example, as opposed to the positive role of Dally in Wg signalling (Lin and Perrimon, 1999), Dally-like shows biphasic activities: as repressor for Wg short-range signalling and as activator for long-range responsiveness. It has been proposed that the Dally-like core protein has high binding affinity for Wg (Yan et al., 2009), a property that allows Dally-like to bind and retain Wingless on the cell surface. Interestingly, ectopic expression of Dally-like inhibits activation of Wg targets. In contrast, increasing the expression of the Wg receptor Frizzled leads to their activation (Yan et al., 2009). These and other results suggest that the physiological role of Wg is linked to the cellular ratio between Dally-like and Frizzled (Yan et al., 2009). In other words, Dally-like binds and retains Wingless on the cell surface away from its receptor Frizzled. However, Dally-like can also facilitate Wg binding to Frizzled depending on the ratio of ligand, receptor and Dally-like. Although intriguing, these results arise additional question that need to be answered. For example, how different is the affinity of Dally and Dally-like core proteins for Wg? Do Dally-like related mammalian Gpcs show dual roles in Wg modulation? Is there any specific protein domain required for Wg binding? Concerning the latter question, it has been proposed that Wg binding could occur via the N-terminal domain of Dally-like (Yan et al., 2009).

Moreover, structural analysis combined with structure-guided mutagenesis also suggests that this domain could guide Dally-like/Shh interaction (Kim et al., 2011). Further studies will address whether and in which manner the N-terminal domain impacts Gpc activity.

In conclusion, the studies above discussed begin to unravel how Gpcs fulfil diverse functions in signalling pathways during development. In particular, they highlight the importance of GPI-mediated Gpc endocytosis in participating at the control of Wingless intracellular trafficking and possibly gradient formation, and in the modulation of Hh signalling in different biological context. Other important findings are the demonstration that Gpcs core proteins show binding affinity for certain signals independent of HS-side chains, and that they can modulate events as opposed as signal activation and inhibition. Thus, core proteins of Gpcs appear to ensure on its own an additional degree of signal modulation that increases specificity of biological readouts.

4. Glypicans in human diseases

Genome-wide linkage scan and mutation analysis have revealed that alteration in GPC functions can underlie human congenital malformation compromising developmental events such as bone growth and heart pattern formation. This discovery has permitted a better comprehension of pathophysiology of these disorders, their diagnosis and management. The generation of animal models has significantly broadened the understanding of these distinct developmental processes and their molecular bases.

4.1 The Simpson-Golabi-Behmel Syndrome

The Simpson-Golabi-Behmel Syndrome is an X-linked overgrowth disorder characterized by pre- and postnatal overgrowth, minor facial anomalies, skeletal defects, polydactyly and fingernail hypoplasia, small bulge in the small intestine, umbilical or inguinal hernia, genitourinary abnormalities, heart defects, supernumerary nipples and an increased risk of neonatal death (Gurrieri et al. , 2011). In patients there is also an increased risk of embryonal tumour development, mainly Wilms' tumour. Mental retardation is not constantly found and is usually mild (Gurrieri et al. , 2011).

Pilia and colleagues uncovered the genetic bases of this disorder in 1996 with the demonstration that mutations in the *Gpc-3* gene are responsible for a large proportion of Simpson-Golabi-Behmel Syndrome cases. Since then, different *Gpc-3* mutations have been identified in patients and these were found to be rather heterogeneous ranging from large chromosomal rearrangements to micro deletions and point mutations in different exons (Gurrieri et al. , 2011; Hughes-Benzie et al., 1996; Pilia et al., 1996; Sakazume et al., 2007; Xuan et al., 1999). Sequence analysis of mutated loci, led to the proposal that Simpson-Golabi-Behmel Syndrome is caused by a non-functional GPC-3 protein while additional unknown genetic factors were possibly responsible for the phenotypic variations among patients. The role of GPC-3 in this disorder was then confirmed by the generation of *Gpc-3*-deficient mice, since these mice recapitulate several phenotypes of the Simpson-Golabi-Behmel Syndrome patients including developmental outgrowth and dysplastic kidneys (Cano-Gauci et al., 1999; Paine-Saunders et al., 2000). Moreover, recent findings showing that GPC3 polymorphisms have a significant impact in the body size of mice have provided additional support for a role of GPC3 in the regulation of body size (Oliver et al., 2005).

At a cellular level the tissue overgrowth syndrome of *Gpc-3*-deficient mice appears to be a consequence of an increased proliferation rate, which is consistent with the possibility that Gpc-3 acts as a negative regulator of cell proliferation in the mouse embryo (Hartwig et al., 2005). However, Gpc-3 can also induce apoptosis in a cell-type specific manner suggesting that enhanced cell survival may also contribute to the overgrowth defects (Filmus, 2001), Interestingly, Simpson-Golabi-Behmel Syndrome patients develop embryonal tumours (see above). Moreover, *Gpc-3* expression is markedly decreased in human gastric cancer. Therefore, it is likely that in humans Gpc-3 functions also as a tumour suppressor gene (Gonzalez et al., 1998).

One of the current major challenges is to identify the GPC-3 targets relevant for the pathogenesis of this complex disease. It has been proposed that GPC-3 inhibits embryonic growth by negatively regulating Insulin-like growth factor-II (Pilia et al., 1996). However, studies in cultured cells have failed to detect any biochemical or genetic interaction between Gpc-3 and the Insulin-like growth factor-II signalling pathway (Cano-Gauci et al., 1999; Chiao et al., 2002; Song et al., 2005). Capurro and colleagues explored the possibility that Gpc-3 acts as a negative regulator of body size by inhibiting two mammalian Hh proteins: Shh and Indian Hh (see also above; Capurro et al., 2008; Capurro et al., 2009). The rational behind this approach is linked to findings revealing that 1) these two Hh family members are both present in the developing embryo, with Shh more widely expressed and Indian Hh restricted to the developing bones; 2) hyperactivation of the Hh signalling pathway in mice causes overgrowth phenotypes (Makino et al., 2001; Milenkovic et al., 1999); 3) in humans, Patched mutations are linked to Gorlin's Syndrome, a disorder causing multiple basocellular carcinomas accompanied by large head size, longer-larger bones, and polydactyly (Hahn et al., 1996). As discussed above, secreted Hh proteins binds and antagonizes the function of the Patched receptor known to block the activity of the signalling effector Smoothened. Binding of Hhs to Patched thus results in the activation of Smoothened, which in turn transduces the Hh signal intracellularly leading to the activation of Hh target genes such as Gli and Patched (Hooper and Scott, 2005). In a first study, Capurro and colleagues compared the degree of activation of the Hh signalling pathway in *gpc-3* null mouse embryos and culture cells as well as potential Gpc-3/Shh protein interaction (Capurro et al., 2008). Results showed that the levels of Shh protein and of its targets increases in structures affected by Simpson-Golabi-Behmel Syndrome (e.g. gut and digits). As discussed above, they also uncovered that Gpc-3 can bind Shh and activate its signalling pathway, and acts as a competitive inhibitor of the Shh-Patched interaction, and triggers Shh endocytosis and degradation (Capurro et al., 2008). Therefore, a reasonable picture that can be drawn from these studies is that Gpc-3 normally restrains Hh signalling to control body size. Lack of Gpc-3 leads to the hyperactivation of this pathway causing the overgrowth phenotype of the Simpson-Golabi-Behmel Syndrome patients (Capurro et al., 2008).

To provide further genetic evidence that the Hh signalling pathway mediates, at least in part, the regulatory activity of Gpc-3 on embryonic growth, Capurro and colleagues performed a second study where they attempted to rescue the overgrowth phenotype of Gpc-3 deficient embryos by crossing them with mice carrying an Indian Hh null allele (Capurro et al., 2009). Indian Hh was chosen because its activity is more confined to endochondral skeleton. Indeed, Indian Hh deficient mice show a severe growth deficiency

in the endochondral skeleton as a result of a reduced chondrocyte proliferation and maturation, as well as osteoblast formation (St-Jacques et al., 1999). In contrast, Viviano and colleagues reported an abnormal persistence of hypertrophic chondrocytes in Gpc-3-deficient embryonic bones and a delay in endochondral ossification (Viviano et al., 2005). As for Shh, Gpc-3 deficient mice show more Indian Hh and Patched protein levels in the developing long bones (Capurro et al., 2009). Moreover the overgrowth syndrome of Gpc-3 deficient mice is partially rescued in the Indian Hh null background (Capurro et al., 2009). Therefore, the author proposed that the accumulation of Indian Hh as a result of the lack of Gpc-3 might be the cause of an unbalance rate of chondrocyte proliferation versus differentiation, which ultimately causes the longer bone overgrowth found in mutant mice (Capurro et al., 2009; Viviano et al., 2005). Although further investigations will elucidate how lack of Gpc-3 affects development at the cellular level, these genetic studies provide important clues on the molecular basis of Simpson-Golabi-Behmel Syndrome in humans by beginning to unravel the aberrant signalling mechanisms. Moreover, they have also told us that more broad approaches such as tissue micro-arrays need to be taken into account to understand this complex disorder. In this context, the *gpc-3*-deficient mouse model will be instrumental to identify and evaluate the involvement of other signalling pathways as well as to determine whether Gpc-3 has tissue specific effects in this disease. Of note, there are the studies on the *gpc-3*-deficient mice indicating that an impairment of the Fgf/Shh signalling axis in the embryonic hearts could underlie the congenital cardiac malformations in Simpson-Golabi-Behmel syndrome (St-Jacques et al., 1999) while the renal dysplasia could be linked to an inbalance of stimulatory and inhibitory signals (e.g. Fgf-7 and Bmp-2 respectively) during tissue morphogenesis (Grisaru et al., 2001).

Further help in understanding the involvement of *Gpc-3* in this human disorder could come from attempts to define structural-functional relationships associated with specific *Gpc-3* mutations in humans. Interestingly, one Simpson-Golabi-Behmel Syndrome patient has a deletion affecting both *Gpc-3* and *Gpc-4,* which is found immediately centromeric to *Gpc-3* at Xq26 (Veugelers et al., 1998). Recently, a wide screening has identified patients carrying mutations in the *Gpc-4* but not in the *Gpc-3* gene (Waterson et al., 2010). We anticipate that future research will extensively evaluate whether connections between GPC-4 functions and the clinical features of this syndrome exist.

4.2 Autosomal-recessive omodysplasia

Autosomal recessive omodysplasia is a genetic condition characterized by skeletal and craniofacial defects (Maroteaux et al., 1989). Skeletal abnormalities include shortening and distal tapering of the humerus and femur, proximal radioulnar diastasis, and anterolateral radial head dislocation. In patients with autosomal recessive omodysplasia both upper and lower limbs are affected in contrast to the dominant form of the disorder in which the lower limbs are normal. Facial defects comprise frontal bossing, a flat nasal bridge, low set ears, a long philtrum, anteverted nostrils, and frontal capillary hemangiomas. Variable findings are hernias, congenital heart defects, mental retardation and delayed motor development. Being recessive, autosomal recessive omodysplasia is a rare disorder with an incidence of <1 / 1000000 and, to date, around 22 cases of recessive omodysplasia have been described. Recent advances in understanding its pathophysiology have come from Campos-Xavier and collaborators reporting that the autosomal-recessive omodysplasia maps to chromosome 13

(13q31.1-q32.2; (Campos-Xavier et al., 2009). By performing fine analysis of candidate genes, Campos-Xavier and collaborators have further linked autosomal recessive omodysplasia to point mutations or to larger genomic rearrangements in the *Gpc-6* gene (Campos-Xavier et al., 2009).

All mutations found in the individuals affected by omodysplasia predict absence of a functional protein. Hypothetical mutant proteins would be truncated and thereby lose both the GPI and the HS-binding sites, essential for the putative GPC-6 functions (Campos-Xavier et al., 2009). Similarly to *Gpc-3*, *Gpc-6* mutations are also found in the entire coding region without any mutational hotspot and include one or more exons. Recently, *Gpc-5* haploinsufficiency has been proposed as the molecular cause of upper limb anomalies and growth retardation in 13q deletion syndrome because of its expression in the developing limb (Quelin et al., 2009). *Gpc-5* colocalises with *Gpc-6* on 13q31.2–q31.3, and the two genes are clustered, similarly to *Gpc-3* and *Gpc-4* on chromosome X, suggesting that these members of the *Gpc* family share an evolutionary link (see also above; Filmus, 2001) that might reflect a common function (De Cat and David, 2001; Paine-Saunders et al., 2002; see also above). However, because GPC-5 does not compensate for loss of GPC-6 in omodysplasia patients, their functional relationship is not supported.

Axial bone growth occurs through growth plates in which chondrocytes undergo proliferation, hypertrophy, cell death, and osteoblastic replacement (Ornitz and Marie, 2002). The immature chondrocytes are rapidly proliferating cells characterized by a small size and irregular shape. In the hypertrophic cartilage zone, chondrocytes make matrix and enlarged lacunae. The pathological characteristics of the omodysplastic growth plates are an expanded zone of proliferating cartilage and an increased number of closely packed, small chondrocytes suggesting that omodysplasia is due to an impaired endochondral ossification (Borochowitz et al., 1998). During endochondral ossification, *Gpc-6* is predominanlty expressed in the proliferative zone decreasing dramatically in the prehypertrophic and hypertrophic zones (Campos-Xavier et al., 2009). These expression data correlate with the morphologic findings in the human omodysplasia. They also correlate with the distribution of Indian Hh, Fgf, Bmp and Wnt proteins, which are known to regulate endochondral ossification (Ornitz and Marie, 2002) and to have the potential of functionally interacting with Gpc-6. Moreover, the etiology of many other human skeletal dysplasias with defects in endochondral ossification has been attributed to specific mutations in the gene encoding FGF receptor 3 (Ornitz and Marie, 2002). The international mouse strain resource (IMSR ; http://www.findmice.org/) has recently generated different mouse strains carrying loss of function mutations in the *gpc-6* gene but to our knowledge, no studies have been yet reported. The functional analysis of *gpc-6* mutant mice will be crucial to establish the involvement of Gpc-6 functions in this disorder and to uncover the cellular and molecular basis of all associated clinical features.

5. Conclusion and future direction

Research over the past years has advanced our understanding of Gpc functions during mammalian development and the list of human syndromes associated with their aberrant function is likely to grow. Indeed, recent studies have described *Gpc-5* and *Gpc-6* as candidate genes for postaxial polydactyly type A, an inherited human condition causing

digit duplications (van der Zwaag et al. , 2010). Gpc genes have also been linked to other less defined human diseases, such bipolar disorder and Sudden Cardiac Arrest (Arking et al. 2011; Maheshwari et al., 2002) and further studies will clarify their involvement. We and others have shown that Gpcs are among the most abundant HSPGs in the developing nervous system and are expressed in embryonic and adult neural stem cells (Bandtlow and Zimmermann, 2000; Hagihara et al., 2000; Luxardi et al., 2007). Our embryological manipulations in *Xenopus* embryos have begun to provide insight into their role in brain size as abrogation of Gpc4 activity in *Xenopus* embryos disrupts forebrain patterning and cell survival, and causes microcephaly (see also above and Ficure 2; Galli et al., 2003). Therefore, our findings raise the possibility that some of the congenital microcephalies may arise as a consequence of disrupting *Glpc-4* gene function during brain development.

As discussed above, Gpc genes are inclined to cluster on the same chromosome. Simpson-Golabi-Behmel patients can carry deletions that affect not only the *Gpc-3* but also the *Gpc-4* gene. In mouse, *gpc-3* shows distinct expression patterns compared to *gpc-4* and the latter is highly expressed in the developing brain and kidney (Luxardi et al., 2007). Therefore, it is possible that mutation in the *Gpc-4* gene also contribute to aspects of the Simpson-Golabi-Behmel syndrome. Further studies will require the analysis of *gpc* compound mutant embryos and mice as they could recapitulates additional clinical features of this syndrome.

A second major area of research will concern the identification of the pathological signalling events underlying the clinical features of disorders associated with abnormal GPC functions. When analysing pathologies involving GPC, it is important to take into account that these diseases might be also linked to GPC gain-of-functions rather then loss-of-functions. The gene targeting approach in mice has begun to clarify this issue. As described above, Gpcs control different signalling proteins in a cell-type and developmental-stage specific manner. Therefore, further studies will require tissue- and stage-specific loss-of-function mutations of *gpc* genes. We think that a better understanding of Gpc involvement in normal and pathological processes, as well as the identification of the associated signals can hopefully provide a wider clinical spectrum for the development of targeted therapies.

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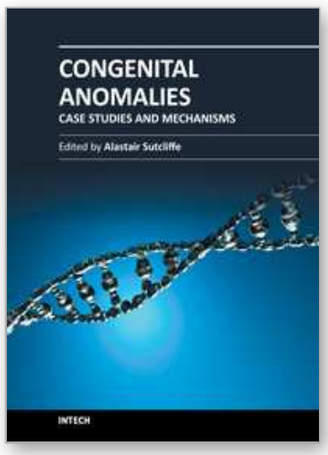

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This book is in essence a collection of essays which are state of the art in their respective areas of knowledge. They inform the reader of all sorts of mechanistic considerations when developing understanding of issues surrounding the origins of congenital abnormalities. These chapters are written by world renown authorities in this area of science and represent a wide range of expertise from a clinician perspective, through to genetic mechanisms. Unlike some books which take a formal textual, somewhat plodding way through pathophysiology here instead we have cut through chapters in which the student , or scientist or medic is lead to understand just how complex the origins can be via examples from different parts of the body. With the erudite chapters are relevant tables and other diagrams to help clarify the text . These chapters represent a starter text for the stimulus for further knowledge of what is an increasingly important area of human health.

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