Golgi proteomics: Identification of a novel cartilage-specific Golgi protein GoPro49

Maarit Takatalo

Department of Biological and Environmental Sciences
Division of Biochemistry
Faculty of Biosciences
and
Viikki Graduate School in Molecular Biosciences
University of Helsinki, Finland

ACADEMIC DISSERTATION

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Supervisor: PhD Ragna Rönnholm
Department of the Biological and Environmental Sciences
Division of Biochemistry
University of Helsinki
Helsinki
Finland

Reviewers: Docent Marjo Salminen
Faculty of Veterinary Medicine
University of Helsinki
Helsinki
Finland

Docent Jussi Jäntti
Institute of Biotechnology
University of Helsinki
Helsinki
Finland

Opponent: Professor Elina Ikonen
Institute of Biomedicine/Anatomy
University of Helsinki
Helsinki
Finland

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred in the text by their Roman numerals, and on unpublished results presented in the text.

I. Takatalo MS, Kouvonen P, Corthals G, Nyman TA, Rönnholm RH.
Identification of new Golgi complex specific proteins by direct organelle proteomic analysis.
Proteomics. 2006, 6(12):3502-8.

II. Mathivanan S, Ahmed M, 157 coauthors including Rönnholm R and Takatalo MS, and Pandey A.
Human Proteinpedia enables sharing of human protein data.
Nature Biotechnology. 2008, 26(2):164-7.

III. Takatalo MS, Järvinen E, Laitinen S, Thesleff I, Rönnholm RH.
Expression of the novel Golgi protein GoPro49 is developmentally regulated during mesenchymal differentiation.
Developmental Dynamics. 2008, 237(8):2243-2255.

IV. Takatalo MS, Tummers M, Thesleff I, Rönnholm RH.
GoPro49 is a novel, specific marker for the dental follicle.
Journal of Dental Research, in press.

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ABBREVIATIONS

Abbreviations used only once are not included in this list.

ACBD3	acyl-Coenzyme A binding domain containing 3
AER	apical ectodermal ridge
Agc	*aggrecan*
Arf	ADP-ribosylation factor
BMP	bone morphogenetic protein
bp	base pair
Bsp1	bone sialoprotein 1
CASP	CDP/cut alternatively spliced product
Col	Collagen
COPI	Coat protein type I
COPII	Coat protein type II
CtBP/BARS	C-terminal binding protein 1, short form/brefeldin-A dependent ADP ribosylation substrate
dpn	days postnatal
E	embryonic day
ECM	Extracellular matrix
En-1	*Engrailed*
ER	Endoplasmic reticulum
ERGIC	ER-Golgi-intermediate-compartment
EXT	Exostoses (multiple)
FGF	fibroblast growth factor
GAP	GTPase activating protein
GC	Golgi complex
GEF	Guanine nucleotide exchange factor
Gli	glioma-associated oncogene homolog (zinc finger protein)
GlcNac	*N*-acetylglucosamine
GM130	Golgi matrix protein, 130 kDa
GoPro49	Golgi protein 49 kDa, NP_775823
GRASP65	Golgi Reassembly Stacking Protein 65 kDa
HERS	The Hertwig's epithelial root sheath
Ihh	Indian hedgehog
k.d.	knockdown
LC	liquid chromatography
MALDI	matrix-assisted laser desorption/ionisation
MEF2C	myocyte enhancer factor 2C
MMP	matrix metalloproteinase
MS	mass spectrometry
MSX	msh homeobox
Myt1	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase
Nir2	phosphatidylinositol transfer protein, membrane-associated 1
NMRI	Naval medical research institute, an inbred mouse strain
Pax9	*paired box gene 9*
PCR	polymerase chain reaction
PDL	periodontal ligament
pf	post fertilisation
PM	plasma membrane
PTHrP	Parathyroid hormone-related protein
qPCR	quantitative PCR
Rab	Ras-related protein
RT-PCR	reverse transcriptase PCR
Runx2	runt related transcription factor 2, also known as Cbfa1
Shh	Sonic hedgehog
Smad	mothers against decapentaplegic homolog
SNAP	soluble NSF attachment proteins
SNARE	soluble NEM-sensitive factor attachment protein receptors
Sox	*SRY-box containing gene*
Tbx	T-box
Tbp	TATA-box binding protein
Tcf/Lef	T-cell factor/Lymphoid-enhancing factor transcription factor
TGFβ	transforming growth factor beta
TGN	Trans Golgi network
TOF	time-of-flight
TM	transmembrane
TRAPP	transport protein particle
VEGF	vascular endothelial growth factor
VSV-G	vesicular stomatitis virus G-protein
Wnt	Wingless-related MMTV integration site
wt	wild type
ZPA	zone of polarising activity

SUMMARY

The Golgi complex is a central organelle of the secretory pathway, responsible for a range of post-translational modifications, as well as for membrane traffic to the plasma membrane and to the endosomal-lysosomal pathway. In addition, this organelle has roles in cell migration, in the regulation of traffic, and as a mitotic check point. The structure of the Golgi complex is highly dynamic and able to respond to the amount of cargo being transported and the stage of the cell cycle. The Golgi proteome reflects the functions and structure of this organelle, and can be divided into three major groups: the Golgi resident proteins (e.g. modification enzymes), the Golgi matrix proteins (involved in structure and tethering events), and trafficking proteins (e.g. vesicle coat proteins and Rabs). The Golgi proteome has been studied on several occasions, from both rat liver and mammary gland Golgi membranes using proteomic approaches, but still little more than half of the estimated Golgi proteome is known. Nevertheless, methodological improvements and introduction of shotgun proteomics have increased the number of identified proteins, and especially the number of identified transmembrane proteins.

Cartilage, even though not a typical tissue in which to study membrane traffic, secretes large amounts of extracellular matrix proteins that are extensively modified, especially by amino acid hydroxylation, glycosylation and sulfation. Furthermore, the cartilage ECM contains several, large oligomeric proteins (such as collagen II) that are difficult to assemble and transport. Indeed, cartilage has been shown to be susceptible to changes both in secretory pathway (e.g. the COPII coat assembly) and in post-translational modifications (e.g. heparan sulfate formation). Dental follicle, and the periodontal ligament (PDL) that it forms, are another type of connective tissue, and they have a role in anchoring teeth to bone. This anchorage is achieved by numerous matrix fibres that connect the bone matrix with the cementum. These tissues have in common the secretion of large matrix molecules.

In this study the Golgi proteome was analysed from purified, stacked Golgi membranes isolated from rat liver. The identified, extensive proteome included a protein similar to Ab2-095, or Golgi protein 49kDa (GoPro49), which was shown to localise to the Golgi complex as an EGFP fusion protein. Surprisingly, *in situ* hybridisation showed the *GoPro49* expression to be highly restricted to different mesenchymal tissues, especially in cartilage, and this expression pattern was clearly developmentally regulated. In addition to cartilage, *GoPro49* was also expressed in the dental follicle, but was not observed in the mature PDL. Importantly, *GoPro49* is the first specific marker for the dental follicle. Endogenous GoPro49 protein co-localised with β-COP in both chondrosarcoma and primary dental follicle cell lines. The COPI staining in these cells was highly dynamic, showing a number of tubules. This may reflect the type of secretory cargo they secrete. Currently *GoPro49* is the only Golgi protein with such a restricted expression pattern.

1 REVIEW OF THE LITERATURE

1.1 Introduction to the secretory pathway

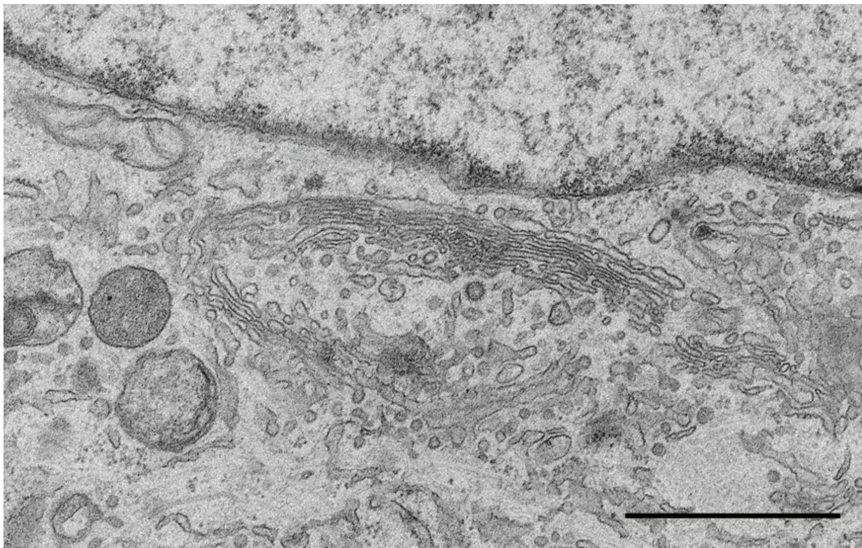
Membrane trafficking within the cell occurs by two main pathways: endocytosis, where material is engulfed and transported from outside the cell into the cytoplasm, and exocytosis, where material produced inside the cell is transported towards the plasma membrane. The endoplasmic reticulum (ER) forms the starting point of the exocytotic pathway, where membrane-associated ribosomes translate and, together with the Sec61 translocon, translocate cargo proteins across the ER membrane (reviewed in Alder and Johnson, 2004). In the lumen of the ER, heat shock family proteins function as chaperones to guide the process of protein folding, and to recognise hydrophobic patches, immature *N*-glycans and exposed thiol residues. In addition, they can assist in disulfide bond formation and oligomerisation of protein complexes. Besides the common chaperones that facilitate folding of many different proteins, several tissue and substrate-specific chaperones are known that have a limited number of target proteins. One of these is Hsp47, which is involved in collagen biosynthesis (reviewed in Anelli and Sitia, 2008).

Translocated proteins undergo first post-translational modifications as they enter the ER lumen. Precursor *N*-glycan chains are transferred from dolichol-linked precursors by a multisubunit oligosaccharyltransferase complex to the asparagine residues on growing amino acid chain (Asn-X-Ser/Thr, *N*-glycosylation) (Stanley et al., 2009). Glycosylation enzymes (α-glucosidases I and II, and α-mannosidase I) can then trim these nascent *N*-glycans, while UDP-Glc:glycoprotein glucosyltransferase adds new glycan moieties to misfolded proteins (reviewed in Anelli and Sitia, 2008). Trimming of *N*-glycan chains helps in monitoring the folding level of a protein; this process is called 'ER quality control' (Hurtley and Helenius, 1989). Other modifications that proteins may undergo in the ER are lipid modification (GPI anchors) and proline hydroxylation (Anelli and Sitia, 2008, Myllyharju, 2003).

As folded proteins leave the ER, they carry the core *N*-glycan chain, and lectins (such as ERGIC-53) can assist in forward transport of these glycosylated proteins (reviewed in Anelli and Sitia, 2008). From the ER, cargo is transported to the ER-Golgi-intermediate compartment (ERGIC) and onwards to the Golgi complex (GC) in coated vesicles (see Figure 1). In the GC, escaped ER resident proteins are captured and recycled back to the ER, leaving cargo proteins to traffic through the GC stack. Membrane traffic to and through the GC is discussed in more detail in Chapter 1.3, Functions of the Golgi complex. Different model proteins used to study anterograde membrane traffic to the plasma membrane (PM) include vesicular stomatitis virus G-protein (VSV-G) and procollagen type I. In particular, procollagen has been used to demonstrate the transport of large, soluble cargo (300nm bundles) not fitting into normal vesicles, and it has been suggested to traverse the GC without use of vesicles (Bonfanti et al., 1998).

When a cargo molecule reaches the *trans* Golgi network (TGN), it is sorted and transported by clathrin coated vesicles to its final destination: the apical and basolateral PM or the endosomal-lysosomal pathway. Sorting signals that are recognised in the basolateral PM cargo proteins include tyrosine, leucine, and dileucine-based motifs, while the apical PM targeting motifs include GPI-anchors, *N*- and *O*-glycans, lipid rafts, and proteinaceous targeting motifs (reviewed in Rodriguez-Boulan and Musch, 2005). Endosomal proteins can be sorted based on recognition of DXXLL motifs, or through interactions with the mannose-6-phosphate receptor (reviewed in Bonifacino and Traub, 2003).

While exocytosis is responsible for membrane traffic towards the PM, endocytosis is important for regulation of the number and activity of membrane proteins, for the recycling of synaptic vesicles, and for



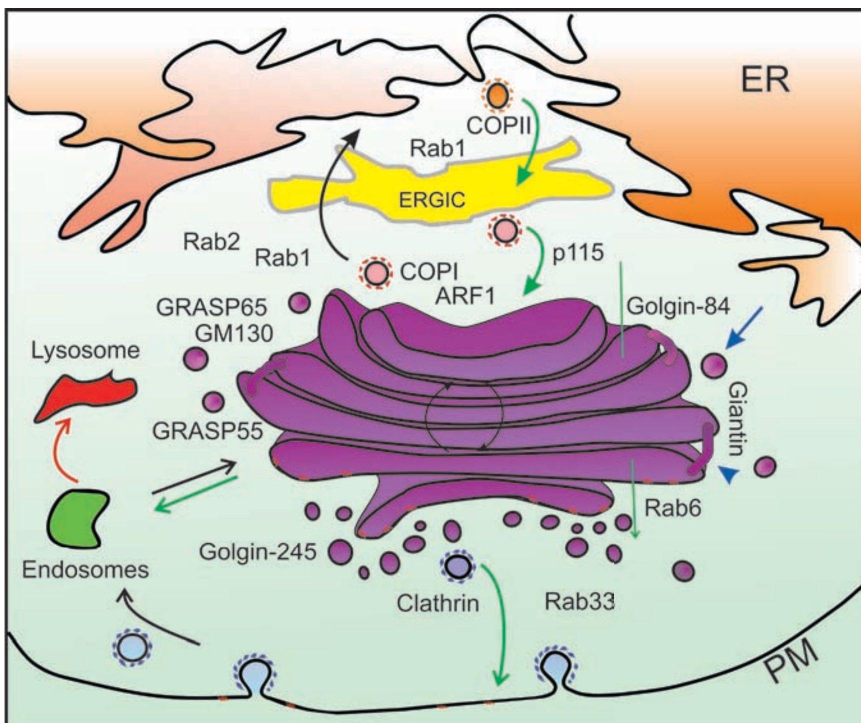


Figure 1. Electron micrograph of the GC, and a schematic view of the membrane traffic machinery of a cell

A) An electron micrograph of the NRK cell GC. The structure of this diverse membrane-bound organelle is best described as ribbon-like in interphase cells. The cisternal stack has polarity from *cis* to *trans* both in protein and lipid composition and in pH. Image courtesy of Eija Jokitalo. Scale bar 1 µm.

B) A schematic image of the secretory pathway of a cell. Proteins translated in the ER are packed into COPII coated vesicles (orange with orange coat) for transport towards the ERGIC (yellow), from where COPI vesicles (pink with red coat) are needed for further transport to the GC. Rab GTPases, together with Golgins as tethers, mediate targeting of these vesicles. Cargo is then transported through the GC stack by vesicles (blue arrow), maturation, or cisternal membrane continuities (blue arrowhead). As cargo passes through the *cisternae* from *cis* to *trans* it undergoes further post-translational modifications.

In the *trans* Golgi network, cargo is sorted and packed into a third set of vesicles: clathrin coated vesicles (light purple or light blue at the PM, with blue coat). Different recognition motifs are utilised (e.g. partition to rafts, red areas on the two *trans*-most *cisternae*). From the PM, escaped Golgi enzymes and PM receptors can be transported back to endosomes (green), from where they can be recycled to the TGN or to the PM, or directed to degradation in the lysosomes (red). Some proteins that participate in transport or that are required for Golgi structure are marked. The GC structure also includes closely associated vesicles (purple). Green arrows mark transport of cargo, black arrows transport and recycling of resident proteins, and the red arrow transport of proteins to lysosomes for degradation.

nutrient intake. The uptake can occur through receptor-mediated endocytosis (clathrin coated vesicles), or through phagocytosis and pinocytosis (without vesicle coat formation) (reviewed in Nichols and Lippincott-Schwartz, 2001). The endocytosed material is transported to the early endosomes that mature first into late endosomes and then gradually into lysosomes, with transport of hydrolysing enzymes from the GC and lowering of the pH. From early and late endosomes, proteins can either be recycled back to the TGN or to the PM, or they can be directed to degradation in the lysosomes (reviewed in Bonifacino and Rojas, 2006).

Along with proteins, lipids are central components of the endomembranes, the most abundant ones

being cholesterol, glycerophospholipids, sphingophospholipids and glycosphingolipids or ceramides. In mammalian cells, glycerophospholipids and sterols are produced in the ER, peroxisomes and mitochondria, while the majority of sphingolipids are produced in the GC (reviewed in van Meer et al., 2008). The lipid composition forms a gradient between the ER and the PM, with the lowest amounts of cholesterol and glycolipids in the ER and the greatest in the PM. The lipid composition of the GC is between these two, as is its position in the secretory pathway (van Meer et al., 2008).

Besides these changes in the total membrane lipid composition across the pathway, there are also differences in the composition of the inner (cytoplasmic) and outer (luminal) membrane leaflet. Membranes of the ER have a uniform composition, with comparable amounts of the different phospholipids in both leaflets, while the GC and the PM bilayers have phosphatidylcholine and glycolipids mainly in the outer leaflet, and phosphatidylserine and phosphatidylethanolamine in the inner leaflet (reviewed in Daleke, 2007). This organised lipid composition may affect the fluidity of the membrane, as well as the localisation of proteins, as is observed for lipid rafts (Harder et al., 1998). Lipid rafts, enriched in glycosphingolipids and cholesterol, are dynamic membrane domains that may sort and concentrate proteins into distinct domains (e.g. apical sorting).

1.2 Structure and dynamics of the Golgi complex

The GC is a central organelle of the secretory pathway and the intracellular membrane trafficking route. In interphase cells, the GC structure is ribbon-like, in juxtanuclear position, and it is composed of stacked flattened *cisternae* that have a polarisation from *cis* (input from the ER) to *trans* (export to the PM, Figure 1). The *cis* and *trans* Golgi networks form recycling compartments from which vesicles bud for both retrograde and anterograde transport. The enzyme composition of the *cisternae* is also polarised from *cis* to *trans*, with the transferase distribution roughly following the order of the glycosylation events (Rabouille et al., 1995). Moreover, the pH of the secretory compartments decreases gradually beginning from the ER (~7.4), towards the Golgi (~6.2), and the secretory granules (~5.5) (Wu et al., 2001). In addition to the *cisternae*, the GC structure encompasses a number of closely associated small vesicles or tubules that have been observed in, for example 3D reconstruction electron tomography studies (Figure 1B, Marsh et al., 2001, Marsh et al., 2004, Trucco et al., 2004).

To accomplish all its functions, the Golgi complex has to be a highly dynamic organelle. Depending on the cell type, the amount of secretory cargo and the stage of the cell cycle, it is able to change shape from the stacked morphology adjacent to the nucleus to vesicular-tubular structures (Kreis el al., 1997). Furthermore, the GC structure is intimately linked with the cytoskeleton, and thus is highly susceptible to changes in cytoskeleton organisation (Ho et al., 1989, Kreis el al., 1997). Indeed, the perinuclear GC localisation is dependent on a functional cytoskeleton and cytoskeletal motor proteins that interlink membranes with the cytoskeleton (Turner and Tartakoff, 1989, Xu et al., 2002). In addition to the cytoskeleton, several other factors contribute to the GC structure, including the Golgi matrix proteins, GTPases such as ADP-ribosylation factors (Arfs), kinases, and membrane input from the ER (Short et al., 2005, Gillingham and Munro, 2007, Feinstein and Linstedt, 2007, Shaul and Seger, 2006, Marra et al., 2007).

The morphology of the GC is linked to the cell cycle, and changes rapidly depending on the stage of the cell cycle. As a mammalian cell enters mitosis, the typical interphase *cisternae* organisation is progressively lost, and by metaphase numerous small punctuated dispersed structures of the GC fragments can be observed. In addition, traffic from the ER to the GC comes to a halt when cells are

near the end of the G2 phase, which is caused by the disruption of the ER exit sites (ERES) (Warren et al., 1983, Kano et al., 2004). These changes in the Golgi morphology may be linked to the need for even distribution of the GC between cells during cytokinesis. When the cell cycle reaches telophase, the GC is reassembled and membrane traffic resumes to provide the membranes required for the completion of cell division (Goss and Toomre, 2008).

There are two different models that attempt to explain GC inheritance (reviewed in Colanzi and Corda, 2007). The first one regards the GC as dependent on, and in a dynamic equilibrium with the ER, and considers the Golgi as an inherited part of the ER. The key step in this model is the blockage of membrane transport from the ER, causing redistribution of Golgi proteins to the ER (Kano et al., 2004, Zaal et al., 1999). The second model considers the GC as an independent organelle, which requires the Golgi remnants for reassembly. In this model, the key step is disruption of membrane tethering complexes, followed by equal distribution of Golgi fragments between daughter cells (Seemann et al., 2002, Pecot and Malhotra, 2004).

Regardless of the model, fragmentation of the GC is a necessary step for the progression of the mammalian cell cycle, but it can be bypassed by artificial GC fragmentation (Feinstein and Linstedt, 2007, Colanzi et al., 2007). The fragmentation is controlled by numerous phosphorylation events and several kinases, including Cell division cycle 2 kinase (Cdc2 or CDK1), the RAF/MEK1/ERKc1 pathway, Polo-like kinase 1 (Plk1) and Plk3 (reviewed in Lowe and Barr, 2007). Furthermore, inhibition of phosphorylation by MEK1/ERKc1 significantly delays the G2/M transition (Feinstein and Linstedt, 2007, Shaul and Seger, 2006). One of the phosphorylation targets is Golgi matrix protein Golgi reassembly stacking protein 65kDa (GRASP65), which is required, together with the fission protein CtBP1/BARS (C-terminal binding protein 1, short form/brefeldin-A dependent ADP ribosylation substrate), to sever non-compact zones during early GC fragmentation (Feinstein and Linstedt, 2007, Carcedo et al., 2004, Preisinger et al., 2005). The GC reassembly at telophase requires membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase (Myt1), which phosphorylates Cdc2 and inhibits mitotic entry (Nakajima et al., 2008). However, Myt1 function is not required for maintenance of the interphase GC structure.

In addition to the need for even distribution of the Golgi membranes between cells, the mitotic GC fragmentation has also been found to be associated with other aspects of cell duplication. Several proteins are released into the cytosol by the fragmentation, including clathrin, phosphatidylinositol transporter Nir2, and acyl-Coenzyme A binding domain-containing protein 3 (ACBD3). During mitosis, clathrin plays a role in the regulation of chromosome segregation, and in the stabilisation of the connections between chromosomes and the mitotic spindle (Royle et al., 2005, Okamoto et al., 2000). Phosphorylated Nir2 protein relocates during mitosis to the cleavage furrow, where it is required for Plk1 docking and normal cytokinesis (Litvak et al., 2004). The third released protein, ACBD3, regulates signalling of Numb in asymmetric cell division and is an essential Numb partner in cell-fate specification (e.g. in neural progenitor cells) (Zhou et al., 2007).

In addition to mitosis, Golgi fragmentation is observed during apoptosis. However, the apoptotic GC fragmentation is not controlled by phosphorylation, but instead by irreversible caspase cleavage of Golgi matrix proteins, and is needed for the progression of apoptosis (Mancini et al., 2000, Lowe et al., 2004, Maag et al., 2005). This GC fragmentation occurs early in apoptosis, and is thought to be required for inhibition of membrane traffic (Lowe et al., 2004, Mukherjee et al., 2007). Furthermore, proapoptotic caspase-2 has been directly localised to the GC, where it can cleave golgin-160 and other substrates (Mancini et al., 2000).

1.3 Functions of the Golgi complex

The GC is responsible for a range of post-translational modifications of proteins and lipids on the secretory pathway, and for the transport and sorting of cargo proteins to their appropriate locations (Varki, 1998, Honke and Taniguchi, 2002, van Vliet et al., 2003). In addition, recent reports have described Golgi complex proteins instrumental in the process of cell migration and signalling cascades (e.g. mitotic Golgi check point, cell proliferation and regulation of traffic within the GC) (Preisinger et al., 2005, Preisinger et al., 2004, Sheen et al., 2004, Pulvirenti et al., 2008).

The main modifications for a protein in the lumen of the GC are glycosylation, modification of the glycans, and sulfation. While *N*-glycosylation is initiated in the ER during protein translocation, *O*-glycosylation can begin in the GC by addition of an *N*-acetylgalactosamine to serine or threonine residues by polypeptide-*N*-acetyl-galactosaminyltransferases (Figure 2, Elhammer and Kornfeld, 1986). Modification of both *N*- and *O*-glycans continues in the GC (Li et al., 1978, Brockhausen et al., 2009, Stanley et al., 2009). Several different *O*-glycan core structures can be formed, of which core 1 and core 2 types are the most common (Figure 2B, Brockhausen et al., 2009). *O*-glycan core structures can be further branched and elongated, and *N*-glycans can be trimmed and modified to obtain complex and hybrid-type structures. The oligo- or high-mannose type *N*-glycans, found in mature proteins (e.g. lysosomal proteins) of multicellular animals, result from incomplete trimming of the precursor glycan chain attached in the ER, as further mannose residues are not added to *N*-glycans in the GC (Stanley et al., 2009).

Enzymes that are needed to produce the complex and hybrid-type *N*-glycans include mannosidases (e.g. endo- and type II) that trim the nascent chain in the GC, and *N*-acetylglucosaminyltransferases that add *N*-acetylglucosamine (GlcNAc) residues to α3- and α6-mannose residues of the glycan core structure in the medial-Golgi (Figure 2A, Tabas and Kornfeld, 1978, Roth et al., 2003, Stanley et al., 2009). Further glycan modifications take place in the *trans*-Golgi, where several monosaccharides, including fucose, *N*-acetylglucosamine, galactose, sialic acid, and *N*-acetylgalactosamine can be added to the core glycan chain (Stanley et al., 2009). Modified glycans such as phosphorylated GlcNac, can also be added to the terminal mannose on *N*-glycans of lysosomal proteins. This GlcNac is then trimmed away to yield mannose-6-phosphate, which is recognised by Man-6-P receptors (Reitman and Kornfeld, 1981, Varki and Kornfeld, 1980).

Finally, a third type of glycosylation in the GC is attachment of glycosaminoglycan (repeated disaccharide units e.g. heparan sulfate) to core protein serine residues (reviewed in Gorsi and Stringer, 2007). Enzymes needed for glycosaminoglycan formation include exostoses (multiple) 1 (EXT1) and EXT2, which are involved in heparan sulfate biosynthesis and chain elongation (Busse et al., 2007). Glycolipids are the second major structure bearing glycans in cells. The lipid moiety of glycosphingolipids is produced in the ER and it is transported to the GC, where it can be further glycosylated to obtain e.g. lactosylceramide or the more complex glycan structures of gangliosides (reviewed in Degroote et al., 2004).

Each of these glycan types can be further modified by sulfate, phosphate, acetate or methyl groups. Of these, sulfate groups can be attached both to glycans (e.g. in heparan sulfate to GlcNac or glucuronic acid), and to tyrosine residues on proteins (reviewed in Gorsi and Stringer, 2007, Kehoe and Bertozzi, 2000). Even though sulfation is not as abundant a modification as glycosylation, it has been shown to be important for the development of many tissues, including cartilage, where sulfated glycosaminoglycans bind growth factors, and thus help to regulate and limit their diffusion (Kluppel et al., 2005, Chintala et al., 1995).

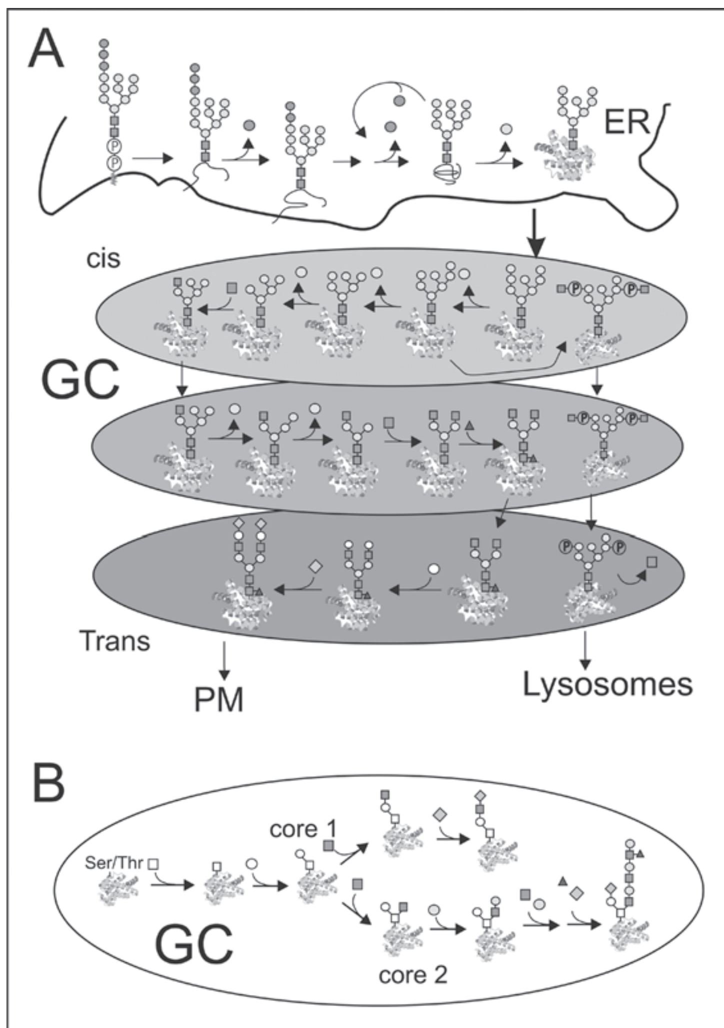


Figure 2. A schematic view of *N*- and *O*-glycosylation and glycan modification.

A) The precursor *N*-glycan chains are transferred from dolichol-linked precursors to a translocated protein (on asparagine residues) by a multisubunit oligosaccharyltransferase complex. In the ER these nascent *N*-glycans are further trimmed by removal of three glucose residues (grey circle) and a mannose residue (light gray circle). Trimming of *N*-glycan chains helps in monitoring the folding level of a protein and new glucose moieties can be added to misfolded proteins to assist further folding. Lectins can assist the transport of folded proteins to the GC.

In the GC the *N*-glycans undergo further trimming and modifications, and several of the mannose residues can be removed by specific mannosidases. The following branching of *N*-glycan begins by addition GlcNAc residues (grey square) to mannoses of the core structure. The glycan chains can be further modified and elongated by other glycan moieties, such as fucose (to core GlcNAc residue, triangle), or galactose (white circle) and *N*-acetylneuramic acid (light gray diamond) to the branches.

The lysosomal proteins are differentially modified in the GC by addition of phosphorylated GlcNac to the terminal mannose. This GlcNac is then trimmed away in later compartments yielding mannose-6-phosphate, which is recognised by Man-6-P receptors and sorted to endosomal-lysosomal pathway.

B) The mucin type *O*-glycosylation begins in the GC by addition of an *N*-acetylgalactosamine (white square) to serine or threonine residues. There are eight different *O*-glycan core structures, of which core 1 and 2 structures are the most common. In core 1 structures galactose is added to *N*-acetylgalactosamine, and this core 1 structure can be further modified e.g. by addition of *N*-acetylneuramic acid or GlcNAc residues. The core 2 structure is formed from the core 1 structure by adding GlcNAc residue to the *N*-acetylgalactosamine. Both branches of core 2 structure can be further modified (e.g. as the one shown).

Glucose grey circle, mannose light gray circle, GlcNAc grey square, galactose white circle, fucose triangle, *N*-acetylgalactosamine white square, N-acetylneuramic acid light gray diamond. Modified from Brockhausen et al. 2009 and Stanley et al. 2009.

The major task of the GC is to mediate membrane traffic. Coat protein type I (COPI) and type II (COPII) on the ER-Golgi side, and clathrin on the TGN-PM side, constitute the three major coat types of the secretory pathway. The COPII coated membranes are filled with anterograde cargo and traffic from the ER to the ERGIC, a tubulovesicular membrane cluster (Barlowe et al., 1994, Aridor et al., 1995). Further transport from the ERGIC to the GC requires COPI membranes (Aridor et al., 1995, Scales et al., 1997, Styers et al., 2008). In addition, COPI vesicles participate in retrograde transport from the GC to the ER (Letourneur et al., 1994). The morphology of these transport membranes can range from small vesicles to larger tubules. The debate on the shape and size of the membrane transport compartments is connected to the controversy surrounding the different Golgi transport models in the field.

As the protein reaches the GC in the exocytotic pathway, it needs to traverse the GC stack. Whether COPI vesicles move in a retrograde or anterograde direction, or in both directions, within the GC is still

under debate. Moreover, recent studies have suggested that besides the vesicular or tubular transport carriers, direct membrane continuities between adjacent *cisternae* may mediate intra-Golgi transport (Marsh et al., 2004, Trucco et al., 2004). There are four models that attempt to explain membrane traffic within the GC. The traditional models include the cisternal maturation model and the vesicular transport model, while more recent models include a hybrid of these two and a continuity-based or inter-cisternal connection model (reviewed in Elsner et al., 2003, Mironov et al., 2005). This debate on the correct model for transport has been going on for the past 50 years (reviewed in Elsner et al., 2003). Problems with these models are that the observed vesicles are small and unable to fit large cargo (e.g. procollagen), while tubules are not observed in many cell types and maturation of *cisternae* is a process too slow to explain rapid transport of cargo proteins (VSV-G).

In the maturation model, the *cisternae* are dynamic and move or 'mature' with the cargo as new *cisternae* are formed at the *cis* side, while at the *trans* side *cisternae* are consumed for membrane transport (see Figure 3A). In this model, the modification enzymes are recycled back to the adjacent *cisterna*, causing it to 'mature'. In yeast, direct maturation of *cisternae* has been observed supporting the maturation model (Matsuura-Tokita et al., 2006, Losev et al., 2006). However, the yeast *cisternae* are not stacked, and when COPI vesicle formation is defective, maturation is considerably slowed (Matsuura-Tokita et al., 2006). In the vesicle transport model, the *cisternae* form a stable compartment, and the cargo is transported through the GC in vesicles, from the *cis cisternae* to the *medial* and then to the *trans* side (Figure 3B). In support of this model some studies have identified anterograde cargo, such as proinsulin and VSV-G protein, enriched in vesicles (Orci et al., 1997).

As neither of these models can explain all aspects of transport, a hybrid model has been proposed. According to this model, coated vesicles move in both directions, while the *cisternae* mature (Pelham and Rothman, 2000). The most recent model, the continuity-based model, incorporates the continuities observed between the *cisternae* in 3D tomography studies. In this model *cisternae* can either be viewed as stable, with cargo flowing through continuities (the modified 'vesicle' model), or they can undergo maturation with enzymes flowing backwards (the modified maturation model) (Marsh et al., 2004, Trucco et al., 2004).

1.3.1 Golgi resident proteins

All Golgi resident proteins are thought to be membrane proteins: either peripheral membrane proteins on the cytoplasmic leaflet (e.g. Golgi matrix proteins), or integral to the membrane (Altan-Bonnet et al., 2004). The Golgi resident proteins use different methods to retain their localisation, including recycling (from the ER or PM), lipid modifications, and interactions or oligomerisation with other proteins (reviewed in van Vliet et al., 2003). Sequence features can also affect localisation, including GRIP domains and the length of the transmembrane domain, which is longer than in ER resident proteins and shorter than in PM proteins, reflecting the lipid composition of the GC (Bretscher and Munro, 1993, Munro and Nichols, 1999). Proteins that are used as examples are listed in Table I.

The wide variety of Golgi resident modification enzymes or transferases reflects one of the main functions of the GC, which is to modify proteins and lipids. This group includes enzymes needed to form and modify both *O*- and *N*-glycans (Brockhausen et al., 2009, Stanley et al., 2009). Glycosyltransferases are highly specific and catalyse the transfer of only one specific glycan to a target position. Some of the enzymes may be shared between these pathways, while some belong to just one or the other. Unlike the ER enzyme oligosaccharyltransferase, these Golgi enzymes only transfer monosaccharides from

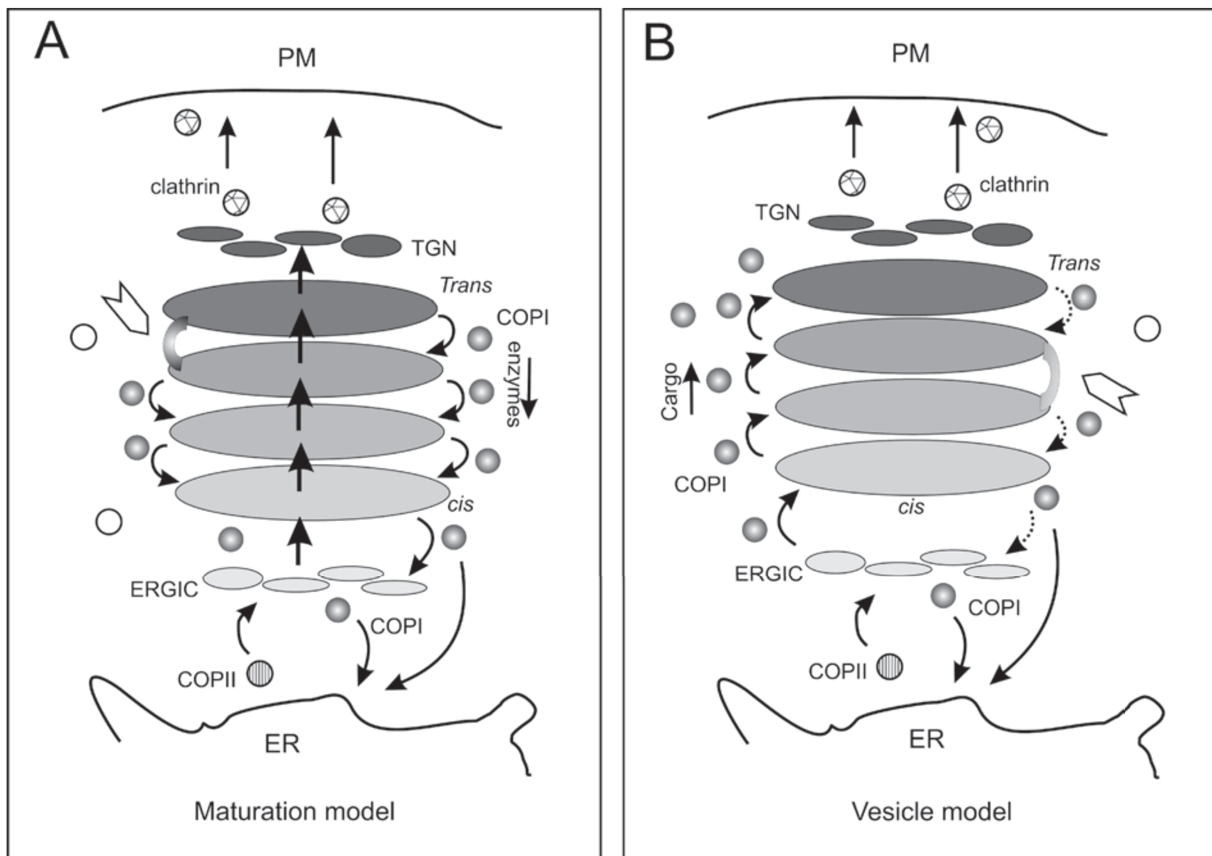


Figure 3. The Golgi transport models

Transport through the GC has been explained by two traditional models, the cisternal maturation model (A) and the vesicle transport model (B). COPII vesicles (striped vesicles) transport cargo from the ER to the ERGIC, from where COPI vesicles (shaded vesicles) are needed for further transport. The intra-Golgi transport is handled with COPI vesicles moving in as anterograde (vesicle transport model) or retrograde (maturation model) direction.

In the cisternal maturation model (A) cargo proteins transit the GC inside the *cisternae*, which move and 'mature' from the *cis* side to the *medial* and *trans* sides, pushed by formation of new *cisternae* on the *cis* side. *Cisternae* maturation is caused in this model by the backwards transport of enzymes either in vesicles or in cisternal continuities (marked with white arrow in A). In the vesicle transport model (B), *cisternae* are a more stable compartment and cargo is transported through the stack, either in vesicles or through cisternal continuities (marked with white arrow in B), while enzymes maintain their distribution in the stack. In both models, once the cargo has reached the TGN, it is sorted and packed into clathrin coated vesicles (patterned vesicles) for transport to final destinations.

nucleotide donors such as UDP-Gal, and thus several Golgi resident nucleotide-sugar transporters are also known (e.g. UDP-*N*-acetylglucosamine transporter) (Berninsone and Hirschberg, 2000, Guillen et al., 1998). Besides glycan modifications, other Golgi resident enzymes (e.g. sulfotransferases and phosphotransferases) can further modify the glycopeptide chain.

In addition to the modification enzymes, Golgi resident proteins include proteins that are important for the regulation of lumenal pH and ion concentration (e.g. the Ca^{2+} and Cl^- needed for normal protein trafficking). These proteins include ion transporters (secretory-pathway Ca^{2+}-transport ATPase 1, SPCA1) and proton pumps (Golgi pH regulator, GPHR) (Van Baelen et al., 2003, Maeda et al., 2008). Furthermore, several proteins required for signalling and regulation of membrane traffic have been localised to the GC, including kinases Plk3 and Ysk1 (Preisinger et al., 2004, Bahassi el et al., 2002). Plk3 regulates apoptosis after stress responses and the GC fragmentation in mitosis (Bahassi el et al., 2002, Ruan et al., 2004). The second Golgi kinase, Ysk1 through phosphorylation of 14-3-3ζ, is involved in the orientation of the GC towards the leading edge in migrating cells together with ERK (through

GRASP65) (Preisinger et al., 2004, Bisel et al., 2008).

The second major class of resident Golgi proteins is the Golgi matrix proteins that are believed to have a role in the structure of the GC, and many of them also have a role in membrane trafficking events. These proteins are defined according to their behaviour in cells where ER-to-Golgi transport is blocked: they do not relocate to the ER like Golgi enzymes, instead they remain in the independent Golgi-like structures (reviewed in Short et al., 2005). These Golgi matrix proteins can be integral to the membrane, lipid anchored, or recruited to the membrane by adapter proteins (such as Ras-related proteins, Rabs) (Linstedt and Hauri, 1993, Barr et al., 1997, Matanis et al., 2002).

A major part of this Golgi matrix is formed by a large protein family called Golgins that are characterised by their Golgi localisation and extensive coiled-coiled domains. The other feature shared by these Golgins is their interaction with small GTPases. These Golgins have distinct localisations within the Golgi stack. Golgins localising to the *cis*-Golgi side include Golgi matrix 130kDa (GM130), p115 and Giantin, which participate in vesicle tethering events together with Rab1 (Figure 1B, Linstedt and Hauri, 1993, Waters et al., 1992, Nakamura et al., 1995). Giantin in COPI, and Rab1 in COPII vesicles are suggested to recruit p115 into these membranes (Sonnichsen et al., 1998, Moyer et al., 2001). GM130 in the GC could then tether these vesicles to the Golgi membrane for fusion (Nakamura et al., 1997, Moyer et al., 2001). Moreover, GM130 has been shown to regulate centrosome organisation during interphase (Kodani and Sutterlin, 2008). Another *cis*-Golgi localising protein is GMAP-210 (Infante et al., 1999). Earlier studies suggested GMAP-210 to be a Golgi microtubule-associated protein, while more recent studies have shown it to function as a membrane curvature sensor in vesicle tethering events (Drin et al., 2008, and reviewed in Barr and Egerer, 2005).

A second tethering pair is formed by Golgin-84, which is localised throughout the Golgi stack, and CDP/cut alternatively spliced product (CASP) (Bascom et al., 1999, Gillingham et al., 2002). Golgin-84 binds Rab1, and has a role in reassembly and maintenance of the Golgi structure, while CASP binds the Arf guanine nucleotide exchange factor (GEF) cytohesin and yeast soluble NEM-sensitive factor attachment protein receptor (SNARE) Gos1p (Gillingham et al., 2002, Satoh et al., 2003, Mansour et al., 2002). Together, Golgin-84 and CASP tether a subpopulation of COPI vesicles different from those bound by p115 (Malsam et al., 2005).

Golgins localised to the the *trans* Golgi side include Golgin-245 and Bicaudals. Golgin-245 is required for tumor necrosis factor-α (TNF) secretion, while its shorter splice variant CrpF46 binds centrosomes (Figure 1, Fritzler et al., 1995, Lieu et al., 2008, Wei et al., 2008). Bicaudal-D1 and Bicaudal-D2 localise to the TGN by interacting with Rab6, as well as with dynein, thereby linking the microtubule network to the GC and tethering events (Matanis et al., 2002, Hoogenraad et al., 2001).

A second important Golgi matrix protein family is the GRASP family. It is composed of two members: GRASP55 and GRASP65, both of which are required for formation and maintenance of the Golgi structure (Figure 1, Barr et al., 1997, Shorter et al., 1999, Feinstein and Linstedt, 2008, Puthenveedu et al., 2006). In addition, GRASP65 may have a role in spindle dynamics and as a mitotic check point (Preisinger et al., 2005, Sutterlin et al., 2005). Furthermore, GRASP65, together with GM130, has a role in the formation of lateral cisternal connections that mediate Golgi ribbon formation (Puthenveedu et al., 2006).

Table I List of resident Golgi and transport proteins discussed in this thesis

The Golgi and transport proteins described in this thesis are listed in alphabetical order under classification of Golgi resident, transport or signalling proteins. The first column lists the short names or abbreviations of each protein or protein complex, with full name and subunits shown in the second column. The membrane status (integral and type/peripheral), localisation and protein function (in the context of this thesis) are given together with references for each entry.

Name of the protein or protein complex	Full name/subunits	Membrane protein type/localisation	Protein function	Reference
Golgi resident proteins				
Bicaudal D1 and D2	Bicaudal D1 and D2	peripheral/GC and TGN	interact with Rab6 and dynein, tethering	Matanis et al., 2002, Hoogenraad et al., 2001
CASP	CDP/cut alternatively spliced product	integral, type IV/GC	tethering with Golgin-84, binds Arf GEF cytohesin and SNARE Gos1p	Gillingham et al., 2002, Mansour et al., 2002
Ext1, Ext2	Exostoses (multiple) -1 and -2	integral, type II/ER and as dimer GC	heparan sulfate biosynthesis and chain elongation, HME disease	Busse et al., 2007, Stickens et al., 2000, Koziel et al., 2004
Giantin	Giantin	integral, type I/COPI vesicles and GC	tethering together with p115, GM130 and Rab1	Sonnichsen et al., 1998
GM130	Golgi matrix 130kDa	peripheral/GC	tethering together with p115, Giantin and Rab1	Moyer et al., 2001
GMAP-210	Golgi microtubule-associated protein 210 kDa	peripheral/GC	membrane curvature sensor in vesicle tethering events	Drin et al., 2008
Golgin-245/CrpF46	Golgin-245/ centrosome-related protein F46	peripheral/TGN or variant centrosomal	tumor necrosis factor-α secretion/ splice variant binds centrosomes	Lieu et al., 2008, Wei et al., 2008
Golgin-84	Golgin-84	integral, type I/GC	tethering with CASP, reassembly and maintenance of the Golgi structure	Satoh et al., 2003, Malsam et al., 2005
GPHR	Golgi pH regulator	integral, multi-TM/ GC	proton pump	Maeda et al., 2008
GRASP65, GRASP55	Golgi Reassembly Stacking Proteins 65 kDa and 55 kDa	peripheral, lipid anchored/GC	formation and maintenance of the Golgi structure, mitotic check point, formation of lateral cisternal connections, can directly bind COPII	Barr et al., 1997, Shorter et al., 1999, Feinstein and Linstedt, 2008, Puthenveedu et al., 2006, Sutterlin et al., 2005, Preisinger et al., 2005, Reilly et al., 2001
mannosidases e.g. endomannosidase and type II	endomannosidase mannosidase type II	integral, type II/*cis*-Golgi	trim the nascent *N*-glycan chain	Tabas and Kornfeld, 1978
N-acetylglucosaminyl-transferase	*N*-acetylglucosaminyl-transferase	integral, type II/ medial-Golgi	adds GlcNAc residues to *N*-glycan mannose residues	Roth et al., 2003

Nir2	phosphatidylinositol transfer protein, membrane-associated 1	peripheral/mainly GC	transfer of phosphatidylinositol, during mitosis required for Plk1 docking and normal cytokinesis	Litvak et al., 2004
p115	General vesicular transport factor p115	peripheral/COPI vesicles and GC	tethering together with GM130, Giantin and Rab1	Nakamura et al., 1997
polypeptide-*N*-acetyl-galactosaminyl-transferases	polypeptide-*N*-acetyl-galactosaminyltransferases	integral, type II/GC	initiation of *O*-glycosylation by addition of an *N*-acetylgalactosamine to serine or threonine residues	Elhammer and Kornfeld, 1986
SPCA1	Secretory-pathway Ca^{2+} transport ATPase	integral, multi-TM/ GC	ion transporter	Van Baelen et al., 2003
UDP-*N*-acetylglucosamine transporter	UDP-*N*-acetylglucosamine transporter	integral, multi-TM/ GC	sugar transporter of UDP-xylose and UDP-*N*-acetylglucosamine	Guillen et al., 1998
Transport proteins				
AP1 to AP4	adapter proteins 1-4	peripheral/TGN, PM, endosomal pathway	clathrin adapter proteins, cargo selection and recruitment	Benmerah and Lamaze, 2007
ARF1	ADP-ribosylation factor 1	peripheral/ERGIC-GC	in this context: COPI coatomer recruitment and binding, GTP hydrolysis and vesicle uncoating, membrane deformation	Palmer et al., 1993, Tanigawa et al., 1993, Beck et al., 2008
ArfGAP	ADP-ribosylation factor GTPase activating protein	peripheral/GC and ERGIC	activates Arf1 GTPase activity, cargo selection, membrane curvature sensor	Lanoix et al., 2001, Reinhard et al., 2003, Bigay et al., 2005
clathrin vesicle coats	subunits: clathrin heavy and light chains	peripheral/TGN, PM, endosomal pathway	coat formation, endocytic and exocytic transport on the PM	Benmerah and Lamaze, 2007
COPI vesicle coats	Coat protein type I, subunits: α, β, β', ε, $\gamma 1$, δ, $\zeta 1$, $\gamma 2$ and $\zeta 2$	peripheral/ERGIC-GC	ERGIC-Golgi transport, and Golgi biogenesis, form a heptameric protein complex coatomer, participate in cargo selection	Lowe and Kreis, 1996, Wegmann et al., 2004, Styers et al., 2008
COPII vesicle coats	Coat protein type II, subunits: Sec23, Sec24, Sec13, Sec31, Sar1	peripheral/ER-ERGIC	coat subunit, Sec23/Sec24 inner coat, Sec13/Sec31 outer coat, Sar1 GTPase	Aridor et al., 1998, Stagg et al., 2006
CtBP1/BARS	C-terminal binding protein 1, short form/Brefeldin A dependent ADP ribosylation substrate	peripheral/ER and GC	membrane fission in anterograde transport to PM, and retrograde transport of KDEL receptor in COPI vesicles	Bonazzi et al., 2005, Yang et al., 2005

dynamin	dynamin	peripheral/ cytoplasmic	required for pinching of the clathrin coated vesicle	Benmerah and Lamaze, 2007
KDEL-receptor	KDEL-receptor	integral, multi-TM/ GC-ER	capture of ER resident proteins for recycling	Wilson et al., 1993, Orci et al., 1997
Motor proteins e.g. Kinesin, Dynein	Kinesin, Dynein	peripheral/ cytoplasmic	cytoskeletal motor proteins, move vesicles to target membranes along microtubule tracks	Xu et al., 2002, Gupta et al., 2008, Watson et al., 2005
p23, p24	Transmembrane emp24 domain-containing proteins	integral, type I/ ERGIC-GC	recruitment of Arf1 and the coatomer to the membrane	Gommel et al., 2001
Rab proteins	Ras-related proteins Rab1, 2, 6, and 33b	peripheral, some lipid anchored/ in GC	vesicle budding, transport, tethering, essential for membrane fusion, effectors GM130, TRAPP, Bicaudal-D	Matanis et al., 2002, Moyer et al., 2001, Satoh et al., 2003, Grosshans et al., 2006, Wickner and Schekman, 2008, Valsdottir et al., 2001, Cai et al., 2007
SNAREs	soluble NEM-sensitive factor attachment proteins, GS15, GOS28, syntaxin5, Ykt6, Bet1, membrin, and ERS24	integral, type I/in GC	formation of a four-helix structure that precedes membrane fusion	Wickner and Schekman, 2008, Volchuk et al., 2004
TRAPPI complex, subunit Bet3	transport protein particle I	peripheral/ER and GC	binds directly to COPII coat proteins	Cai et al., 2007
	Regulatory proteins			
Caspase-2	Caspase-2	peripheral/GC	apoptotic cleavage of Golgi matrix proteins	Mancini et al., 2000
Myt1	Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase	peripheral/ER and GC	phosphorylates Cdc2 and inhibits mitotic entry	Nakajima et al., 2008
Plk3	Polo like kinase 3	peripheral/GC	regulates apoptosis after stress response, and the GC fragmentation in mitosis	Ruan et al., 2004, Bahassi el et al., 2002
Ysk1	yeast Sps1/Ste20-related kinase 1	peripheral/GC	orientation of the GC towards the leading edge in migrating cells	Bisel et al., 2008

1.3.2 Transport proteins

Throughout this thesis the term 'transport proteins' will refer to proteins with a function in any of the following processes: membrane traffic, membrane deformation and vesicle budding, recruitment of coat proteins, or recognition and tethering to target membranes. The majority of these proteins are located on the vesicles or tubules carrying proteins and lipids, at least during transport events. The coat complexes, together with adaptor proteins, form the oligomeric cage which deforms the membrane and recruits cargo. On clathrin coated vesicles, which function on both endocytic and exocytic pathways on the PM, these coats are composed of oligomers of three light and three heavy chains. Several adaptor proteins, including AP1 to AP4 participate in the cargo selection and recruitment, and others, such as dynamin, are required for pinching off the vesicle (reviewed in Benmerah and Lamaze, 2007).

The COPII coats are composed of five major subunits: the small GTPase Sar1 (Secretion associated, ras related protein), Sec23 and Sec24, which form the 'inner coat', and the 'outer coat' subunits Sec13 and Sec31 (Figure 4B and Table I). The Sec proteins were first characterised in yeast secretory mutants screens: Sec13, Sar1 and Sec23 were among the 23 mutants identified in the first screen (Novick et al., 1980). The Sec23/24-Sar1 complex participates in the cargo selection and 'outer coat' recruitment, while Sec13/31 forms the outer cage that drives membrane deformation (Aridor et al., 1998, Stagg et al., 2006). In addition, Sec23 functions as a GTPase activating protein (GAP) for Sar1, which results in vesicle coat depolymerisation after budding (Aridor et al., 1998, Yoshihisa et al., 1993).

Membrane traffic is tightly regulated, and proteins of the COPII coat have been shown to be targets for post-translational modifications. Sec24 undergoes cytoplasmic *O*-GlcNAc modification during interphase and phosphorylation during mitosis. This phosphorylation blocks membrane transport, possibly by preventing association of the protein with the membrane (Dudognon et al., 2004). Besides its role in coat formation and membrane budding, the COPII coat has also been implicated in vesicle targeting and homeostasis. Several recent studies have shown that tethering, fusion and transport proteins, including transport protein particle I (TRAPPI) subunit Bet3, and Grh1p (yeast GRASP65 homologue), can bind directly to COPII coat proteins (Cai et al., 2007, Reilly et al., 2001). In addition, $p150^{Glued}$, a component of the dynactin complex, has been shown to directly interact with Sec23A, and blockage of this interaction slows down ER export kinetics (Watson et al., 2005).

Intact membrane traffic is highly important, as illustrated by a single amino acid change, F382L, in the COPII coat protein Sec23A that causes a rare genetic disorder called cranio-leticulo-sutural-dysplasia (CLSD). CLSD is characterised by malformation of the craniofacial structure and abnormal collagen secretion, in addition to the development of cataracts (Boyadjiev et al., 2006). The defect caused by this mutation was shown to be due to the reduced affinity of Sec23A towards the outer coat complex Sec13/31, and subsequent reduced vesicle formation (Fromme et al., 2007).

The functions of the COPI coat are not as well characterised as those of the COPII and clathrin coats, but the mode of action and regulation is thought to be similar. There are nine COPI coat protein subunits known: α-, β-, β'-, ε-, γ1-, δ- and ζ1-COP, with alternative isoforms γ2- and ζ2-COP. Together they form a variable heptameric protein complex (coatomer) containing a single copy of each subunit (Figure 4A, Lowe and Kreis, 1996, Wegmann et al., 2004). The different coatomer subunits have been shown to preferentially select cargo with different cytoplasmic signals. The KKXX motif is recognised by α-, β'-, or γ-COP, while the di-phenylalanine motif is recognised by γ-COP and WXXW/Y/F by δ-COP (reviewed in Bethune et al., 2006). In β-COP depleted HeLa cells, many ERGIC, Golgi and TGN markers co-localise in fragmented compartments. In addition, secretion of soluble cargo is inhibited

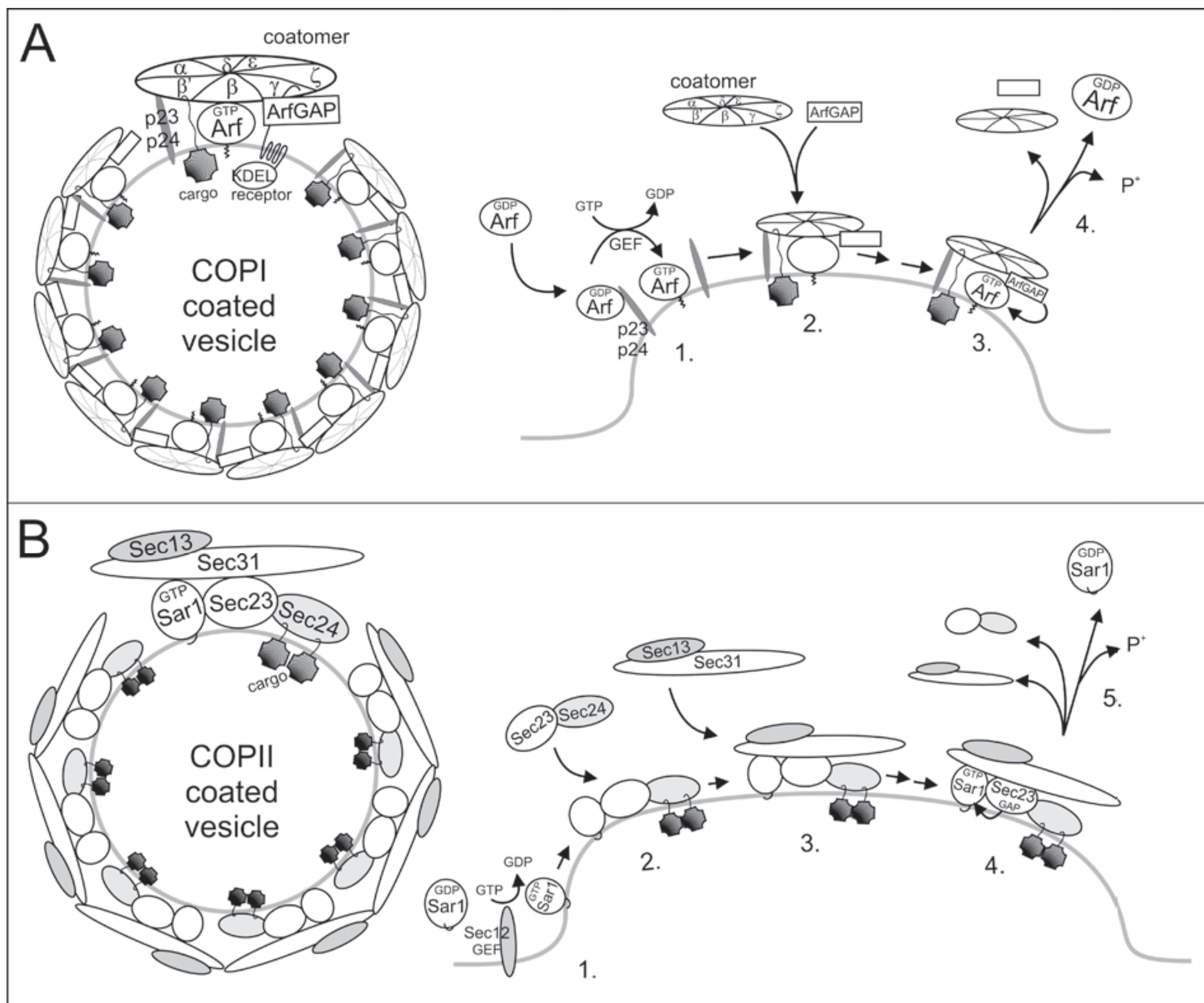


Figure 4. Schematic view of COPI and COPII vesicle coat formation

A) COPI vesicle coats are composed of coatomer (seven subunits α-ζ), Arf1, ArfGAP and p23/p24-family proteins. Coat formation begins from recruitment of Arf1 by p23/p24 proteins, and activation of Arf by nucleotide exchange (1). Both GTP-bound Arf1 and p23 will then participate in coatomer and ArfGAP recruitment (2). The coatomer, ArfGAP and KDEL receptor can then take part in cargo selection. After the membrane deformation is complete, the vesicle buds off. ArfGAP in free vesicles stimulates Arf1 GTPase activity, leading to GTP hydrolysis (3) and coat disassembly (4), leaving an uncoated vesicle to travel to the target membrane. The coat subunits are released to the cytoplasm and are then free to undergo further rounds of coat formation.

B) COPII vesicle coats include Sar1 GTPase, inner coat subunits Sec23 and Sec24, and outer coat subunits Sec13 and Sec31. COPII coat formation begins by recruitment of Sar1 to the membrane by Sec12, a Sar1 GEF (1). Sar1 then recruits the Sec23/Sec24 complex and cargo selection can begin (2). Sec24 binds cargo, while Sec23 and Sar1 recruit the outer coat subunits Sec13/Sec31 (3). The Sec13/Sec31 coat polymerisation drives the membrane deformation. Once the vesicle is formed, the GAP activity of Sec23 will stimulate the GTPase activity of Sar1, leading to coat disassembly and vesicle uncoating (4).

in these cells, suggesting that COPI vesicles are needed for both ERGIC-Golgi transport, and Golgi biogenesis (Styers et al., 2008).

In addition to the coatomer, proteins needed for COPI vesicle formation are: Arf1, p23, p24, and ArfGAP (see Figure 4A). Arf1 is a small GTPase required for coatomer recruitment and binding, GTP hydrolysis and vesicle uncoating, while the p23/24 family proteins are needed for efficient recruitment of Arf1 and the coatomer to the membrane (Palmer et al., 1993, Tanigawa et al., 1993, Gommel et al., 2001). ArfGAP might not be essential for vesicle assembly, but it is needed to activate Arf1 GTPase activity for vesicle uncoating, and may have a role in cargo selection (Lanoix et al., 2001, Reinhard et al., 2003).

Furthermore, the KDEL-receptor, a seven transmembrane helix protein, recognises KDEL-sequences of ER resident proteins in a pH dependent manner. The cargo-bound KDEL-receptor is incorporated into retrograde COPI vesicles for recycling back to the ER (Orci et al., 1997, Wilson et al., 1993).

Deformation of the membrane is a necessary step for carrier formation: in COPI vesicles the coatomer and Arf1 perform this task (Ostermann et al., 1993, Spang et al., 1998). Furthermore, Arf1 is able to induce membrane deformation as a dimer, and this dimerisation is required for COPI vesicle formation (Beck et al., 2008). In addition, ArfGAP1 might function as a membrane curvature sensor, since its GAP activity can be stimulated by increased membrane curvature (Bigay et al., 2005). Moreover, lipids, including diacylglycerol and phosphatidic acid, are required for COPI vesicle budding and retrograde transport, and for fission and recruitment of ArfGAP and BARS (Fernandez-Ulibarri et al., 2007, Yang et al., 2008).

After coat assembly and membrane deformation, the vesicle buds off from the membrane. CtBP1/BARS is a membrane fission protein required for retrograde transport of the KDEL receptor in COPI vesicles, and for anterograde transport from the GC to the basolateral PM (Bonazzi et al., 2005, Yang et al., 2005). The vesicles formed by fission can then be moved to the target membranes along the microtubule tracks by cytoskeletal motor proteins like kinesins and dyneins (Xu et al., 2002, Watson et al., 2005, Gupta et al., 2008).

Fusion to the target membrane is the last stage of membrane traffic. The current view on how vesicle fusion occurs involves three major steps: 1) recognition and tethering to the target membrane; 2) docking; and 3) fusion with the target membrane. There are several proteins and protein families involved in these steps, including SNAREs, soluble NSF attachment proteins (SNAPs), Golgins and Rab GTPases. Of these, SNAREs function in the recognition of and fusion with the target membrane (reviewed in Wickner and Schekman, 2008). Both the donor and acceptor membranes have a specific set of SNAREs, which upon vesicle docking form a four-helix coiled-coil structure that precedes membrane fusion. Of the Golgi specific SNAREs, ERS24, membrin and Bet1 are found mostly on the *cis* side, while GS15 is found mostly on the *trans* side. Syntaxin5 and GOS28 are nearly equally distributed throughout the stack, while for Ykt6 the precise Golgi localisation has not been reported (Volchuk et al., 2004).

Rab small GTPases participate in multiple steps during vesicle budding, transport, and tethering, and they are essential for membrane fusion (reviewed in Wickner and Schekman, 2008). These proteins have highly restricted subcellular localisations and cooperate with specific effectors like Golgins, and it has been speculated that they regulate targeting (Moyer et al., 2001, Satoh et al., 2003, Valsdottir et al., 2001). GTP-bound Rabs can bind to tethering complexes on the target membrane and subsequently recruit other proteins and lipids needed for assembly of the fusion microdomain, including the SNARE complex. Over 60 different Rab proteins have been identified in mammalian cells to date, a few of which are Golgi specific, including Rab1, Rab2, Rab6 and Rab33b. Rab effectors are numerous, and they have roles in tethering (GM130, TRAPP), regulation of GEF and membrane fusion (Rabex-5 and rabaptin-5), and transport (Bicaudal-D) (Matanis et al., 2002, Moyer et al., 2001, Cai et al., 2007, Valsdottir et al., 2001, Horiuchi et al., 1997).

In addition to Rabs, another family of small G proteins has been identified in the Golgi membranes: the Arf and Arf-related GTPase (Arl) family. Many of these proteins have a role in the regulation of membrane traffic and the actin cytoskeleton (reviewed in Gillingham and Munro, 2007, Myers and Casanova, 2008).

1.4 Proteomics

Proteomics is the study of the proteome or 'all proteins expressed in a given cell or tissue at specific conditions' (Wasinger et al., 1995). The most popular proteomic method consists of mass spectrometry (MS) analysis of the proteome to obtain a list of protein identifications. Suitable MS methodology for identification of proteins has been available since early 1990, but completion of the human genome sequencing project made this technique fashionable (chromosome 11 in 2006) (Chen, 2008, Taylor et al., 2006). Since then, proteomics and 'transcriptomics' have been a central area of biological research. The potential of proteomics is great, and in combination with other methods it allows for example dissection of cellular pathways or domains, and quantitative comparison of samples (such as proteins modified in cancer) (Dormeyer et al., 2008).

MS identifies charged molecules by obtaining precise mass-to-charge ratios (*m/z*) and deconvoluted masses that can then be used to identify the ionised small molecules. This technique is very powerful, as it allows identification of numerous proteins at once, and through the use of semi-automated platforms can analyse large sample sets. In addition, recent technological advances both in methods and sensitivity have facilitated the analysis of protein modifications such as phosphorylation and glycosylation, although these analyses are still not straightforward (Chen, 2008). The two most common methods used to ionise protein and peptide samples are matrix-assisted laser desorption/ionisation (MALDI) and electro-spray ionisation (ESI) (Hillenkamp and Karas, 1990, Tanaka et al., 1988, Fenn et al., 1989). Both are soft ionisation methods suitable for biomolecules. These can be coupled to different MS analysis methods, of which ion trap, quadrupol, time-of-flight (TOF), Orbitrap and Fourier-transform ion-cyclotron resonance have been used in proteomic LC-MS/MS studies (Bachi and Bonaldi, 2008, Han et al., 2008). However, many proteins are too large to be identified as such using MS, as the mass/resolution ratio limits the identification of larger molecules even with MALDI-TOF (Chen, 2008). Therefore, the usual approach relies on protein digestion by trypsin or other proteases prior to MS analysis.

By coupling two analysers (MS/MS) it is possible to first obtain the *m/z* value of the peptide, and then to sequence the peptide by gas-phase fragmentation. However, the proteomic samples are complex mixtures with varying amounts of each protein, and low abundance proteins may not be detected. In order to simplify this complex task, proteins or peptides can be separated by one or more methods prior to MS analysis (Chen and Pramanik, 2008). Traditional methods rely on the separation of proteins by gel electrophoresis based on size and/or pI (1D or 2D). 'Shotgun' proteomics, or multidimensional protein identification technology (MudPIT), was introduced in 2001 to eliminate gel electrophoresis as a separation method, and to minimise the loss of small, basic, and hydrophobic proteins (Wolters et al., 2001). In shotgun proteomics, proteins are digested and peptides are fractionated in one or two 'dimensions' in different liquid chromatography (LC) conditions such as μ-scale cation-exchange or reverse-phase, prior to analysis either off-line or on-line with the LC. The LC separation of peptides, followed by direct analysis, also helps to prevent loss of TM-proteins, which is a frequent issue in gel-based separation techniques (Tan et al., 2008, Lu et al., 2008a).

Since proteomic samples are usually complex mixtures that contain hundreds or thousands of different proteins, several types of software have been developed to assist in their identification. However, this raises other issues, as the different programmes use different methods to identify the proteome. There have been initiatives to standardise data for distribution and collection, and to aid comparison of the data from different laboratories, e.g. the Human Proteome Organisation (HUPO) Proteomics Standards Initiative (http://www.psidev.info/). Nevertheless, all these programmes are only as reliable as the

accuracy of the *m/z* data. Thus, in spite of the methodological advances, sample preparation is still a key issue, together with the purity of the sample and the extent of contamination. Furthermore, homologous proteins with highly similar sequences might not be distinguished reliably. Other factors to bear in mind are possible post-translational modifications, which can affect the mass and charge of the peptide and thus identification. Then again, the characteristic change in the peptide mass caused by the modification allows the detection of these modifications, e.g. glycomics, which studies glycans on proteins and lipids (Chen, 2008).

The cell make-up has been addressed in several organelle proteomic approaches that attempt to obtain the proteome of the ER, plasma membrane, mitochondria, and the GC, and have identified a number of novel proteins in these organelles (most recently by Stevens et al., 2008, Dormeyer et al., 2008, Zhang et al., 2008, Gilchrist et al., 2006). Each of these studies was performed using the shotgun proteomic approach, which makes the solubilisation of the sample less of an issue when compared to gel based approaches. Indeed, in each of these studies a significant fraction of membrane proteins could be identified (from 21% to 56%) (Stevens et al., 2008, Zhang et al., 2008). In addition to giving an insight into the total proteome, proteomic studies are used to compare samples of different cell types or conditions: for example, PM in healthy and cancerous cell lines, or mitochondria in diverse apoptotic conditions (Dormeyer et al., 2008, Miller et al., 2008). These studies can also provide spatial information on the relative distribution of the proteins in different compartments (Gilchrist et al., 2006).

1.4.1 The Golgi proteome

The Golgi proteome has been estimated to contain about 1000 proteins (Taylor et al., 2000). Less than 2/3 of these proteins (641 in humans) have been identified to date, based on their subcellular localisation according to the UniProt protein database. The Golgi proteome reflects the functions and structure of this membrane-bound organelle. Indeed, 35% of the Golgi proteins annotated on UniProt are different transferases that modify proteins and lipids. The second major group of GC proteins is involved in membrane traffic (23%). The major types of Golgi proteins are described in Section 1.3 and Table I.

That so many GC proteins have been identified is partially thanks to the several Golgi proteomic studies that have been performed. The Golgi proteome has been analysed from isolated Golgi membranes, from both rat liver and mammary gland, using both gel electrophoresis-based techniques (Taylor et al., 2000, Breuza et al., 2004, Bell et al., 2001, Wu et al., 2000) and shotgun proteomics (Gilchrist et al., 2006, Wu et al., 2004). The first study, by Taylor et al. (2000), identified 73 proteins using fractionation of the Golgi membranes and 2D gels, while Bell et al. (2001) used 1D gels and were able to identify 81 proteins. Another study focused on differences in the GC proteome between the two functional states of mammary epithelial cells and identified 30 proteins that were upregulated in the lactating mammary gland (Wu et al., 2000). The latest gel-based study focussed on the ERGIC proteome and identified 24 proteins (Breuza et al., 2004).

Technical advances in proteomic methods as well as progress in the genome sequencing projects resulted in a dramatic increase in the number of identified proteins, from less than a hundred in gel-based studies to several hundred using shotgun approaches. The first shotgun study of the GC reported identification of a total of 421 proteins, of which 110 were known Golgi specific proteins, and 41 were previously unknown (Wu et al., 2004). Furthermore, 64% of the Golgi specific proteins identified in this study were predicted to be integral membrane proteins. The most recent of these proteomic studies examined the whole secretory pathway and identified 1430 proteins, of which 345 were of unknown

function (Gilchrist et al., 2006). In this study, the reported distribution between organelles showed that the majority of the proteins were unique to the ER (832), and 193 unique proteins were found in the Golgi/COPI fraction.

1.5 Sequence analysis and databases

Advances in proteomics and genomics have also driven the development of bioinformatics, as the amount of data generated in a single experiment has increased greatly. Sequence analysis is a powerful method to rapidly analyse data, and automated analysis softwares can facilitate analysis of large data sets. Usually, analysis is based on sequence comparisons to identify similar domains such as transmembrane (TM) domains, or binding motifs and structures (Emanuelsson et al., 2007, Puntervoll et al., 2003). Thus, predicted patterns are based on known protein and gene sequence data. There are numerous different prediction programmes on-line for identification and characterisation of proteins (e.g. for the analysis of MS, or MS/MS data), for pattern searches (to detect specific motifs, domains or post-translational modification sites), as well as software for structure prediction (Puntervoll et al., 2003, Perkins et al., 1999, Letunic et al., 2008).

Besides prediction programmes, there is a vast amount of biological information available in on-line databases. The human genome was completed in 2006 (Taylor et al., 2006), and since then, the number of sequenced species has rapidly increased as methods have improved. Currently 358 eukaryotic genomes are available as draft genomes or are being assembled (NCBI, 3.10.2008). The largest sequence databases are the Uniprot (protein database), the NCBI (network of databases including nucleotide, genome and protein), and the Ensembl databases (genome database by EMBL-EBI and the Sanger Institute) (UniProt Consortium, 2008, Birney et al., 2004). These databases include data from genome-wide sequencing approaches, from single submissions and from automated sequence analysis.

However, none of these databases include information about large data sets, such as those from yeast-2-hybrid or proteomic approaches. Many of these large datasets are published as supplementary data that is not easily examined, if published in full at all. Some journals recommend or require deposition of full data sets for journal archives, but this data is not easily accessible and it is spread over multiple websites. The ‘Mouse Genome Informatics’ (MGI) database collects data on mouse phenotypes, gene targeted mice and gene expression, and the ‘Zebrafish Information Network’ (ZIN) collect analogous data on zebrafish (Eppig et al., 2007, Sprague et al., 2008). However, neither of these databases includes proteomic or interaction data sets. The database for yeast, ‘*Saccharomyces* Genome database’ (SGD) includes such data sets, but until now there has not been such a functional database available for vertebrates, where authors could submit large, searchable data sets of multiple techniques.

1.6 Skeleton and cartilage

Cartilage and chondrocytes are not traditional cell types used to study the secretory pathway, although they are highly active with regard to the secretion of extracellular matrix (ECM), and to the modification of matrix proteins. Furthermore, cartilage and bone are generally considered restricted to vertebrates, although invertebrates, like the purple sea urchin *Strongylocentrotus purpuratus*, may have mineralised tissues and may secrete collagen (Col) or collagen-like proteins (Livingston et al., 2006).

The vertebrate skeleton has evolved to support the body and to facilitate motility. The skeleton consists of bones as well as cartilage, which in juveniles is still present in growth plates, while in adults it can be found in joints, airways and ears. In addition to cartilage and bone, the skeleton includes the bone marrow, which fills the central regions of the bone after endochondral ossification has formed the mineralised bone collar. The bone marrow harbours the progenitors of blood cells, and also some stromal or mesenchymal stem cells that can function as progenitors for cartilage and bone (Pittenger et al., 1999).

However, during development cartilage forms the anlagen of the future skeleton. As development proceeds, chondrocytes and the cartilage anlagen are replaced by osteoblasts and bone (reviewed in Kronenberg, 2003). Bones of the trunk are formed in this way in the process called ‘endochondral ossification’. In the craniofacial area, bone formation and ossification can occur without a cartilage template, and thus craniofacial skeleton development differs markedly from that of other skeletal elements (reviewed in Helms and Schneider, 2003).

The types of vertebrate cartilage present in different parts of the body arise from different embryonic cell types. The cartilage of the body is formed by the mesoderm, while cranial cartilage and bone are derived from the cranial neural crest cells (Helms and Schneider, 2003, Goldring et al., 2006). The vertebral column and ribs develop from somites, and limb cartilage from the lateral plate mesoderm (Olsen et al., 2000). Development of all these cartilage types is tightly controlled. Furthermore, the chondroblast differentiation and proliferation rates determine the size of the future bones: cell division that occurs too rapidly can lead to gigantism, while if the process is too slow, it may lead to premature hypertrophy, terminal differentiation of chondroblast, and shorter bones (van der Eerden et al., 2003).

Cartilage-forming chondroblasts and chondrocytes, and bone-forming osteoblasts and osteocytes, are all of mesenchymal origin, while osteoclasts, which regulate bone formation by absorbing any excess bone matrix, originate from the macrophage lineage (reviewed in Olsen et al., 2000, Datta et al., 2008). The chondrocyte population can be further divided into five to seven different categories depending on the differentiation status (see Figure 5, reviewed in Goldring et al., 2006). Chondrogenic mesenchymal cells condense and commit to pre-chondroblasts, which then enter the early chondroblast stage and begin the secretion of cartilage-specific ECM. The proliferating, columnar chondroblasts are active in ECM secretion and these cells provide the main force for elongation of the cartilage anlagen. At prehypertrophy, chondroblasts exit the cell cycle and become chondrocytes. This transition is accompanied by changes in the ECM expression profile. By hypertrophy, the cell morphology changes, giving enlarged cells filled with cytoplasm. These chondrocytes will terminally differentiate, and will begin the mineralisation of the matrix. These cells are termed chondroblasts in the proliferating stages, and chondrocytes when they are fully committed and no longer divide. The early and late stages can be distinguished by differential expression of ECM proteins, while in the stages inbetween the differences in ECM are quantitative rather that qualitative.

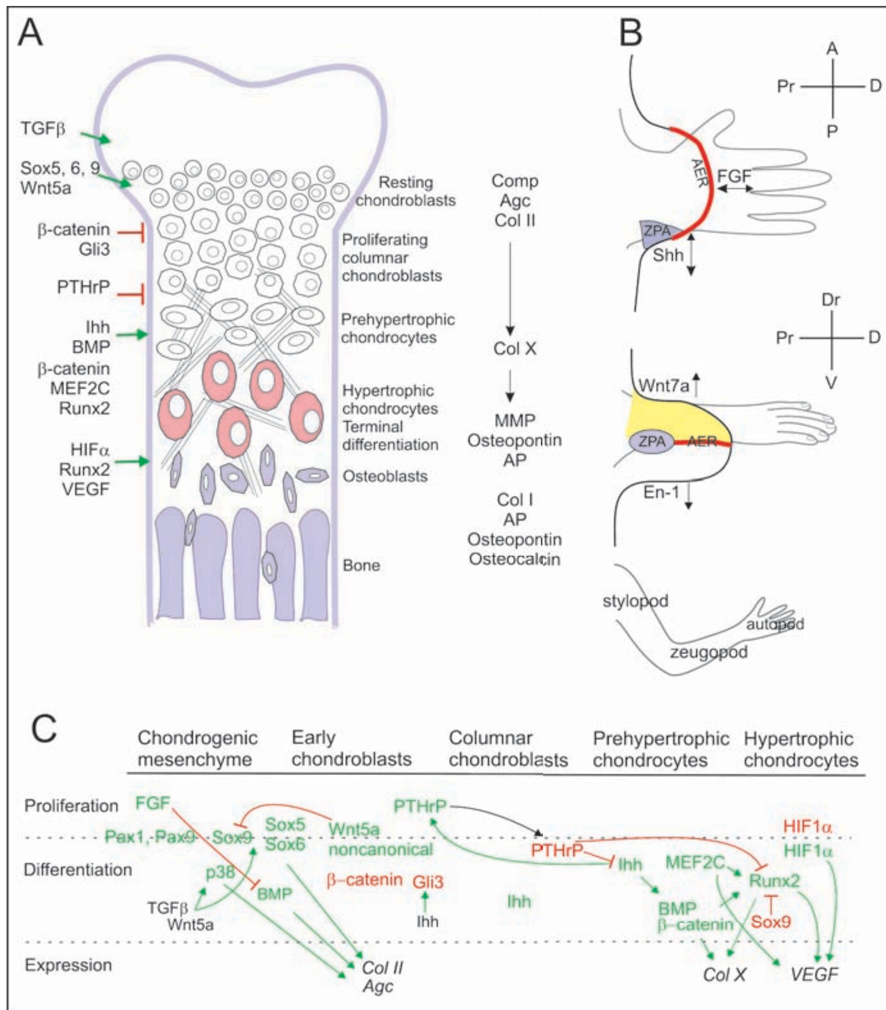


Figure 5. Schematic view of the growth plate of a long bone, the signalling zones of limb development and the network regulatory signals directing chondrocyte differentiation

A) A schematic view of the embryonic long bone growth plate with different cartilage cell stages marked. The resting chondroblasts are at the epiphyseal end of the plate and hypertrophic chondrocytes and osteoblasts at the distal end. The differential expression of ECM genes at various times is indicated along with growth factors and transcription factors promoting (green arrows) or inhibiting (red bars) each stage.

B) A schematic view of the different signalling zones during limb formation. The AER (red ridges) directs the proximal (Pr) – distal (D) patterning and mesenchyme proliferation. The ZPA (violet areas) directs the anterior (A) – posterior (P) patterning of more distal elements, and Wnt7a and En-1 of the non-ridge ectoderm direct the dorsal (Dr) – ventral (V) patterning. The three limb segments, the stylopod, zeugopod and autopod, are indicated on a schematic image of an upper limb.

C) Schematic view of the network of interactions that direct chondrocyte proliferation and differentiation. Signals are placed so as to reflect their roles in either differentiation or proliferation; signals affecting both are placed on the separating dotted line. Factors promoting each stage are marked with green, while factors inhibiting are marked with red. Green arrows mark upregulation or activation by a signalling molecule, while red bars signify downregulation or inhibition. Downstream genes and upstream regulators that have not been shown to be directly involved at that stage are marked in black. The same signals are shown multiple times, as they may have different effects at different stages.

1.6.1 Development and patterning of the limb

As the skeleton is a hallmark of vertebrates, it is not surprising that the development and maintenance of this tissue is tightly regulated by growth factors and transcription factors. Limbs form in specific, pre-patterned areas called ‘limb fields’. The position of these limb fields is determined, at least partially, by the expression of *homeobox* (*Hox*) genes that help to specify position along the anterior-posterior axis (reviewed in Hueber and Lohmann, 2008). However, ectopic expression of fibroblast growth factor 8 (FGF8) has demonstrated that the whole embryonic flank contains potency to form a limb (Vogel et al., 1996). Hindlimbs and forelimbs have different identities, and paired-like homeodomain transcription factor 1 (Pitx1) contributes to hindlimb determination, while T-box 4 (Tbx4, hindlimb) and Tbx5

(forelimb) have roles in their respective limb initiation events, but not in determination (DeLaurier et al., 2006, Naiche and Papaioannou, 2007, Minguillon et al., 2005). The subsequent limb development is regulated by signals from three different regions of the limb bud: the zone of polarising activity (ZPA), the apical ectodermal ridge (AER), and the non-ridge ectoderm (see Figure 5B). These signalling centres direct the development of the limb proximal-distal (AER), dorsal-ventral (non-ridge) and anterior-posterior (ZPA) axes in interconnected ways (reviewed in Mariani and Martin, 2003).

Limb development begins as a limb bud, a thickening of lateral plate mesoderm mesenchyme surrounded by the surface ectoderm. To initiate the limb bud, a signal from the mesoderm (FGF10) is required for limb formation to proceed (Sekine et al., 1999). FGF10 signals to the surrounding ectoderm and initiates a cascade that leads to *Fgf8* expression and AER induction (Sekine et al., 1999, Yonei-Tamura et al., 1999). Wingless-related MMTV integration site 10a (Wnt10a) and Wnt3 are also involved in the *Fgf8* upregulation, and in AER formation and maintenance (Barrow et al., 2003, Narita et al., 2005). Furthermore, bone morphogenetic proteins (BMPs) are required, as inhibition of BMP signalling in ectoderm will disrupt AER formation (Ahn et al., 2001).

The AER runs along the anterior-posterior axis of the limb bud, and separates dorsal and ventral sides. It promotes mesenchymal cell proliferation and it directs the proximal-distal patterning of the limb (reviewed in Niswander, 2003). The AER is thought to time the generation of the progenitor cells and thus influence the number of cells available for condensation (Lu et al., 2008b). AER functions are mediated through four different FGFs: FGF4, -8, -9 and -17. FGF8 is required for normal limb development, for the maintenance of limb bud outgrowth, and for normal limb patterning (Moon and Capecchi, 2000, Lewandoski et al., 2000, Boulet et al., 2004). Moreover, FGF8 alone is enough to sustain normal limb formation, and FGF9/FGF17 functions, while FGF4 might be able to compensate for some of the functions of FGF8 in gene targeted mice (Boulet et al., 2004, Mariani et al., 2008).

The anterior-posterior patterning is regulated by the ZPA. Sonic hedgehog (Shh) is expressed in the ZPA and is the morphogen directing the anterior-posterior patterning, and is required for the patterning of digits two to five (reviewed in Hill, 2007). However, the proximal limb patterning (humerus and femur) appears to be independent of Shh in ZPA (Chiang et al., 2001, Kraus et al., 2001). In addition, the patterning role of Shh is dependent on Gli3 activation and the subsequent activator:repressor ratio of Gli3 (te Welscher et al., 2002). An FGF signal from the AER is needed to induce Shh in the ZPA, which in turn maintains FGF4 expression in the AER by suppressing BMP signalling through Gremlin (Khokha et al., 2003).

The dorsal-ventral polarity of the limbs is directed by the non-ridge ectoderm. Wnt7a is expressed in the dorsal ectoderm, and regulates the expression of *LIM homeobox transcription factor 1* (*Lmx-1*) in the dorsal mesenchyme (Cygan et al., 1997). *Engrailed* (*En-1*), induced by BMPs in the ventral ectoderm, helps to restrict *Wnt7a* expression to the dorsal region (Ahn et al., 2001, Loomis et al., 1996). In addition, dorsal-ventral signalling is connected to the maintenance of the ZPA, as loss of *Wnt7a* expression in the dorsal ectoderm will reduce or abolish *Shh* expression in the ZPA (Parr and McMahon, 1995).

Mesenchymal cells of the limb bud obtain the proximal-distal specification either from the AER in the progress zone model or from signals in the early limb bud (reviewed in Tickle, 2003). Either way, as these cells proliferate and exit the AER zone, they form prechondrogenic condensations if a sufficient number of cells is available, and they will then give rise to skeletal elements through endochondral ossification (reviewed in Mariani and Martin, 2003). Development of the three segments (stylopod, zeugopod and autopod; Figure 5B) is differentially controlled from the three signalling regions. The digit identity is additionally regulated by the interdigital mesenchyme (Dahn and Fallon, 2000).

In species with free digits, after the cartilage anlagen are formed, the interdigital mesenchyme is removed by apoptosis (reviewed in Zuzarte-Luis and Hurle, 2005). The transforming growth factor beta (TGFβ) superfamily might play a role in determining the digital/interdigital fate, while BMPs have a role in promoting apoptosis in the interdigital zone (Ganan et al., 1996, Montero et al., 2008, Bandyopadhyay et al., 2006). TGFβs are expressed in the presumptive digit regions, and can induce formation of extra digits from the interdigital mesenchyme (Ganan et al., 1996).

FGFs have been implicated in blockage of BMP activity and prevention of interdigital apoptosis (Macias et al., 1996). Consistent with this protective role for interdigital mesenchyme, FGF8 has been linked to maintaining the interdigital webs in bat (Weatherbee et al., 2006). The BMP signalling in the interdigital zone downregulates the FGF expression and thus allows the interdigital areas to be removed by programmed cell death (Pajni-Underwood et al., 2007). In mouse limbs, *Fgf4* expression in the AER ceases by E11.5, while *Fgf8* expression is first downregulated over the interdigital regions, and no expression is detected after E12.5 (Lewandoski et al., 2000, Salas-Vidal et al., 2001).

1.6.2 Endochondral ossification

Endochondral ossification is the process by which most of the bones of the vertebrate skeleton are formed (with the exception of many craniofacial bones). It begins with the condensation of the mesenchyme and the formation of cartilage anlagen, which is replaced by bone and marrow as development proceeds (reviewed in Mariani and Martin, 2003). Long bones are considered the model organ for endochondral ossification. As the chondroblasts in cartilage anlagen divide, the cells in central areas (e.g. diaphysis in long bones) start to differentiate into hypertrophic chondrocytes and will eventually form the structure called the 'growth plate' (see Figure 5A). The growth plate is located between the epiphyseal and the metaphyseal bone at the distal end of long bones. The chondrocyte proliferation in the growth plate provides the driving force for bone elongation (reviewed in Horton, 2003).

The growth plate consists of chondroblasts and chondrocytes at different developmental stages as well as osteoblasts. The growth plate is highly ordered: resting chondroblasts are located at the epiphyseal end, while hypertrophic chondrocytes and ossification of the matrix is observed at the distal end of the plate (reviewed in Horton, 2003). This highly organised structure is achieved by tight control of cell proliferation and differentiation. Chondroblasts undergo several stages of differentiation before entry into hypertrophy. The whole differentiation cascade and transition into hypertrophy is crucial for endochondral bone formation. As a consequence, several genetic factors, hormones, and growth factors, together with nutrition and environment, are known to affect bone growth and chondroblast proliferation.

The four major growth factor families involved in chondrogenesis are hedgehog (Hh), FGF, TGFβ-related proteins (TGFβs, BMPs and activins), and Wnt. The FGF family has been shown to be important for cell survival, and many of the family members affect chondrocytes and bone growth. FGF1, -2, -17 and -19 have been found in human fetal cartilage at the protein level, and FGF-receptors 1-3 are expressed in mesenchyme and in chondrocytes (Krejci et al., 2007, Delezoide et al., 1998). The Wnt growth factors act through three distinct pathways, the canonical or β-catenin pathway, the non-canonical planar-cell polarity pathway and the calcium pathway (reviewed in Macsai et al., 2008). The canonical pathway leads to the activation of T-cell factor/Lymphoid enhancing factor (Tcf/Lef) transcription factor and transcription of target genes, while the non-canonical calcium pathway leads to an increase in the intracellular Ca^{2+} and to NF-AT activation and expression of e.g. retinoblastoma-like 2 protein (p130) (Yang et al., 2003).

The endochondral ossification begins with condensation of the mesenchymal cells (Figure 5C). These cells differentiate to form the chondroblasts, which start proliferation and secretion of the ECM (Lefebvre and Smits, 2005). Important transcription factors in the chondrogenic mesenchyme are Pax1 and Pax9, which together contribute to the maintenance of the chondrogenic fate of the mesenchymal progenitor cells (Peters et al., 1999). The p38 kinase pathway, an alternative pathway for the Smad signalling downstream of TGFβs, may also act as a positive regulator of the differentiation of mesenchymal progenitors to chondroblasts, and of the expression of *collagen type II* (*Col II*) and *aggrecan* (*Agc*) (Watanabe et al., 2001, Oh et al., 2000). However, the p38 pathway can additionally be regulated by other growth factors, including Wnt5a (Jin et al., 2006).

Chondroblast proliferation requires SRY (sex determining region Y)-box transcription factors (Sox5, Sox6, and Sox9), which cooperatively activate *Col II* and *Agc* expression (Akiyama et al., 2002, Smits et al., 2001). TGFβ/BMP, Rac1/N-cadherin and Wnts may regulate expression and activation of these Sox proteins (Woods et al., 2007, Dong et al., 2006, Yoon et al., 2005). Furthermore, BMP signalling is required for promoting the expression of cartilage-specific ECM components, e.g. *Col II*, *Agc* and *link protein* (Yoon et al., 2005).

Gli3, the Indian hedgehog (Ihh) effector in cartilage, has an inhibitory role in early chondroblast differentiation into columnar chondroblasts, which is independent of parathyroid hormone-related protein (PTHrP) (Koziel et al., 2005). Moreover, canonical Wnt signalling inhibits chondrogenesis, and expression of active β-catenin in early chondroblast blocks chondrocyte differentiation and matrix mineralisation (Tamamura et al., 2005, Akiyama et al., 2004). Nevertheless, the non-canonical Wnt pathway promotes chondroblast proliferation and differentiation, and gene targeting of non-canonical *Wnt5a* in mice leads to a severe skeletal phenotype with shorter limbs and missing digits (Yang et al., 2003, Topol et al., 2003).

The columnar, proliferative chondroblasts are highly active with respect to the secretion of ECM. However, changes in the gene expression profile during chondroblast differentiation are quantitative, rather than qualitative, and the majority of these cartilage ECM genes are expressed until the cells enter hypertrophy (Vornehm et al., 1996). Ihh is the main Hh controlling chondrocyte differentiation and hypertrophy, and it coordinates the ossification process (reviewed in Ehlen et al., 2006). Ihh signalling from the prehypertrophic chondrocytes up-regulates the expression of *PTHrP* in perichondrium. PTHrP in turn promotes columnar chondroblast proliferation, and suppresses chondroblast entry into hypertrophy thus establishing the distance between *PTHrP* and *Ihh* expressing cells. However, Ihh can also regulate chondroblast proliferation and columnar cell mass independently of PTHrP (Kobayashi et al., 2005).

When chondroblasts enter prehypertrophy and form chondrocytes, they exit the cell cycle. By the time the cells enter terminal differentiation, they have increased their volume up to twenty fold, and the surrounding ECM has a high degree of mineralisation. These cells will eventually undergo apoptosis and make space for the arriving osteoblasts and bone marrow (reviewed in Kronenberg, 2003). In the absence of PTHrP, Ihh can drive the chondroblast differentiation into prehypertrophic chondrocytes, as in postnatal and adult cartilages, possibly through canonical Wnt or BMP signalling (Mak et al., 2008). This Ihh/PTHrP feedback loop ensures the correct balance and speed for cell proliferation and entrance into prehypertrophy. Furthermore, FGF2 can downregulate Ihh expression in tissue culture and regulate the onset of hypertrophic differentiation (Minina et al., 2002).

In addition to Ihh, BMPs and Wnts have a role in controlling hypertrophy. BMP regulates the transition from proliferating, columnar chondroblasts to hypertrophic chondrocytes and induces *collagen X* (*Col X*)

expression and alkaline phosphatase activity (reviewed in Adams et al., 2007). Expression of active β-catenin (Wnt pathway) in mature chondrocytes also stimulates hypertrophy and mineralisation, and gene targeting or inhibition of β-catenin at this stage causes a severe delay in chondrocyte hypertrophy (Tamamura et al., 2005, Akiyama et al., 2004, Chen et al., 2008).

Transcription factors that induce chondrocyte hypertrophy and *Col X* expression include Runx2 (runt related transcription factor 2), and myocyte enhancer factor 2C (MEF2C) (Enomoto et al., 2000, Arnold et al., 2007). *Runx2* can be regulated at the expression level by BMP2 and Wnt/β-catenin (upregulation), and by PTHrP (downregulation), while FGF2 (via the ERK1/2 pathway) causes phosphorylation and activation of Runx2 at the protein level (Dong et al., 2006, Franceschi and Xiao, 2003, Li et al., 2004). In addition, Sox9 regulates *Runx2* expression and inhibits chondrocyte hypertrophy (Zhou et al., 2006). Furthermore, Runx2 provides epigenetic control by maintaining its association with chromosomes in cells undergoing mitosis, and thus influences the cell lineage fate (Young et al., 2007). MEF2C is required for the activation of *Col X* and *vascular endothelial growth factor* (*VEGF*) expression, and it may act upstream of *Runx2* (Arnold et al., 2007).

In the very last phase of endochondral ossification, the calcified growth plate cartilage is invaded by blood vessels. Angiogenesis is necessary for osteoblast infiltration and bone formation. VEGF plays an important role as an endothelial cell chemoattractant, expressed by the hypertrophic chondrocytes, and Runx2 and hypoxia-induced factor α induce the *VEGF* expression in growth plate hypertrophic chondrocytes (Zelzer et al., 2001, Schipani et al., 2001). Furthermore, hypoxia induced factor α has been implicated as a survival factor for the hypertrophic chondrocytes, and may act as a negative regulator of cell proliferation (Schipani et al., 2001).

1.6.3 Cartilage extracellular matrix

For cartilage and bone the ECM is essential, as robustness and function of these tissues depend on an ECM that is hardened by mineralisation. Indeed, major functions of chondrocytes are secretion of the different ECM proteins like collagens, as well as calcification of the matrix (Lefebvre and Smits, 2005). Col II is the most abundant protein secreted by chondroblasts. The gene encodes two splice variants that have different expression patterns during development. These alpha (A) and beta (B) types can be distinguished by differences in exon 2, which encodes a cysteine-rich von Willebrand factor C-like domain, which is missing in collagen type IIB (McAlinden et al., 2005).

For cartilage tissues, the capacity of the ECM to bind water is crucial, as it influences the ability to resist pressure and to be elastic at the same time. The water binding capacity is highly dependent on the glycosylation level of the ECM proteins, and indeed a high degree of glycosylation is typical for the cartilage extracellular matrix. The GC, along with the ER, is the major site for the generation and modification of glycosyl groups. Therefore, defects in glycosylation or sulfation of ECM proteins can disturb cartilage formation (Kluppel et al., 2005, Chintala et al., 1995) and defects in Golgi modification enzymes can cause cartilage diseases. As an example, human *EXT1* and *EXT2* genes are linked to cartilage and bone formation, and mutations in these genes were identified as a cause of the 'Hereditary multiple exostoses' (HME) disease characterised by benign bone tumors (Stickens et al., 2000, Koziel et al., 2004, and reviewed in Duncan et al., 2001).

The HME mutations have been linked to three different loci, *EXT1*, *EXT2* and *EXT3*. Of these, the *EXT1* and *EXT2* genes encode two different glycosyltransferases located in the GC as a dimer, and involved in heparan sulfate biosynthesis and chain elongation (Busse et al., 2007). The function

of the *EXT3* locus is not known. Gene targeting of either *Ext1* or *Ext2* in mice is embryonic lethal. In heterozygote *Ext1* embryos there is an increase in the area of Ihh diffusion and in chondroblast proliferation, while chondrocyte hypertrophy is delayed (Hilton et al., 2005). *Ext2* heterozygote embryos have impaired heparan sulfate synthesis and develop exostoses (Stickens et al., 2005). In addition, the typical columnar organisation of proliferating chondroblasts is slightly disturbed.

Chodrocytes are known to secrete multiple ECM proteins at different stages (Figure 5A, reviewed in Lefebvre and Smits, 2005). While Col I is only secreted by mesenchymal progenitor cells and pre-chondroblasts, Col II synthesis occurs during most of the stages of chondroblast differentiation. *Col IIA* is expressed during mesenchymal cells condensation and proliferation (Sandell et al., 1994, Sandell et al., 1991). A splice variant, type IIB, along with proteoglycans such as Agc, and Col IX and XI, are secreted by early chondroblasts (Vornehm et al., 1996, Mallein-Gerin et al., 1988). Columnar chondroblasts upregulate the expression of, for example, *Agc* and *cartilage oligomeric protein* (*Comp*). During prehypertrophy, cells secrete high levels of Col IIB, Agc, and most other early cartilage matrix proteins, and they initiate expression of *Col Xa1*. *Col X* expression continues as cells progress to hypertrophy, while expression of other early matrix proteins stops (Lefebvre and Smits, 2005). During terminal differentiation, cells secrete matrix metalloproteinase-13 (MMP-13), osteopontin and alkaline phosphatase, which regulate the extracellular matrix mineralisation (Inada et al., 2004, and reviewed in Gerstenfeld and Shapiro, 1996).

The non-cartilage mesenchymal tissues also display active secretion of ECM proteins, if not as abundantly as cartilaginous tissues. At least N-CAM, N-cadherin and Col I are expressed in non-differentiated mesenchymal cells (Lefebvre and Smits, 2005). In contrast, osteoblasts and osteocytes do not secrete Col II, despite the fact that they display active secretion. Nevertheless they retain expression of Col I, which is the major protein found in the bone ECM. Other glycoproteins, including osteopontin, osteocalcin and alkaline phosphatase, which are thought to regulate mineralisation of the bone, are also expressed (Gehron Robey, 1996).

The ECM needs to be remodelled as endochondral ossification proceeds. Several proteases with substrate specificity for collagens have been identified. Probably the best known family of proteases are the matrix metalloproteinases (MMPs), of which MMP-9, MT1-MMP and MMP-13 are critical for normal bone formation (Inada et al., 2004, Vu et al., 1998, Holmbeck et al., 1999). In addition, the ECM is rich in different growth factors that bind to the ECM (e.g. heparan sulphates) and are released when proteases process the matrix. This growth factor binding capacity of the ECM is used to control the amount, the diffusion, and the rate of release of different growth factors, and thus regulate the rate of signalling (Kluppel et al., 2005, Chintala et al., 1995).

1.7 Dental tissues and periodontium

In mammals, four types of teeth can be observed: incisors, canines, premolars, and molar teeth. The number of molars and premolars varies, as well as the number and shape of the incisors. Mice and other rodents only develop one set of teeth which are permanent, while most other mammals have two sets of teeth, deciduous or 'milk teeth', and permanent teeth, which replace the deciduous teeth as an animal reaches a larger body size. There is much variation in the vertebrate incisors. Rodent incisors grow continuously, and while human incisors are covered with enamel on the whole crown area, rodent incisors are covered with enamel only on their labial side. Furthermore, the rodent incisor labial side is considered to be reminiscent of the molar crown, while the lingual side may follow root patterning (Figure 6B). This asymmetry requires inhibition of BMP on the lingual side (by follistatin), and proliferation of ameloblasts and odontoblasts in the posterior end of the incisor (Wang et al., 2004b, Harada et al., 1999).

Teeth are composed of four markedly different layers with different functions for each layer (Figure 6A). Dental pulp is soft tissue, while enamel, dentin, and cementum are hard mineralised tissues that have a high mineral to protein ratio (reviewed in Fong et al., 2005). The function of these hard tooth layers is to withstand the forces of mastication, and to protect the vulnerable dental pulp. The enamel is secreted by ameloblasts, which are derived from the dental epithelium. Once formed, the enamel can no longer be remodelled. The three other dental layers are derived from the dental mesenchyme (see Figures 6A and C). Dentin is secreted by the odontoblasts at the surface of the dental pulp, and helps to prevent enamel fractures from the forces of mastication. Cementum covers the roots of teeth, but unlike enamel, cementum can be deposited and remodelled throughout the life of the tooth. Dental pulp has a role as a pain sensor for the tooth, and in dentin repair after damage. In addition, dental pulp harbours the dentin-forming odontoblast cells, and it contains most of the precursor cells that regenerate dentin and cementum (Fong et al., 2005).

Periodontium is defined as tissues supporting and investing the tooth, and it consists of cementum, periodontal ligament (PDL), bone lining alveolus (socket), and the gingiva facing the tooth (Ten Cate, 1997). The dental mesenchyme also contributes to the formation of the PDL. PDL is not strictly speaking one of the dental tissues, but it has an important role in anchoring teeth to the bone. It is a soft, specialised connective tissue which is situated between the cementum and the bone of the socket wall. The main constituents of the PDL matrix are collagen fibres that protrude into the cementum and the bone socket, and crosslink these tissues (Nanci and Somerman, 2003). The PDL has many functions: it supports the tooth and allows the slight movement of teeth during mastication, and it also has a role as the sensory organelle for the jaws.

Another important feature of the PDL is its ability to regulate the level of mineralisation and to prevent ankylosis (fusion of bone and tooth). Periodontal ligament-associated protein-1 (PLAP-1) has been suggested to suppress mineralisation and to bind directly to BMP2 (Yamada et al., 2007). In addition, msh homeobox 2 protein (Msx2) has been found in PDL fibroblasts, and it can repress Runx2 activity and mineralisation (Yoshizawa et al., 2004).

1.7.1 Tooth formation

Tooth development has been considered a model for epithelial-mesenchymal organ development. Teeth develop from the oral ectoderm and the neural crest cell derived ectomesenchyme (Figure 6C, reviewed in Miletich and Sharpe, 2003, Thesleff et al., 2001). This whole process is highly organised and tightly regulated. *Hox* genes are important for determining tooth identity, with *BarH-like homeobox 1* (*Barx1*) expression marking the future molar region, and *Msx1* the incisor region (Tucker et al., 1998b, Tucker et al., 1998a). The timing and combination of the different signals is crucial for correct patterning. Many of the signalling molecules (BMP, FGF, Hh, and Wnt) and transcription factors important for cartilage and bone development are also important for tooth development. These molecules are used during several stages, and their expression may fluctuate between ectoderm and mesenchyme (reviewed in Thesleff, 2006). Fine tuning of the signals by additional inhibitory signals (e.g. follistatin and Ectodin) is necessary for normal tooth formation (Wang et al., 2004a, Kassai et al., 2005).

Tooth morphogenesis begins by formation of the dental placode, a thickening of oral ectoderm (Figure 6C). The dental epithelium forms a bud and mesenchymal cells condense around it. The bud develops into a dental cap, and the ectomesenchyme beneath the epithelial cap forms the dental papilla, while the surrounding ectomesenchyme will form the dental follicle. The bell stage marks shaping of the tooth crown, and differentiation of ameloblasts and odontoblasts begins. In the differentiation stage the dental lamina that has connected the tooth to the oral epithelium breaks up, separating the developing tooth from the oral epithelium (Miletich and Sharpe, 2003, Thesleff et al., 2001).

The primary enamel knot (Figure 6C, cap state) functions as a signalling centre and directs tooth growth and shape, while secondary enamel knots direct the formation of future cusps in molars. Formation of the primary enamel knot requires Msx1 and BMP4, followed by Wnt/Lef1 to induce *FGF4* expression, leading to stimulation of enamel epithelium and dental papilla proliferation (Bei et al., 2000, Kratochwil et al., 2002). At the end of crown formation, secretion of the enamel and dentin by terminally differentiated ameloblasts and odontoblasts begins. Incisors have primary, but not secondary, enamel knots, and the signalling involved has not been studied in detail (Thesleff et al., 2001).

The root formation begins when the crown formation is almost complete. However, this process is not understood as well as crown formation, but similar principles may apply to the formation of single-rooted and multi-rooted teeth. Cessation of FGF10 signalling at the end of crown formation may regulate the transition from crown to root formation in mouse molars, while continuous expression is important for survival and maintenance of stem cell populations in mouse incisors (Yokohama-Tamaki et al., 2006, Harada et al., 2002). In addition, nuclear factor I/C (Nfic) transcription factor is essential for root formation, while BMP4 is important for root formation through regulation of Hertwig's epithelial root sheath (HERS) (Steele-Perkins et al., 2003, Hosoya et al., 2008).

At the beginning of root formation, a bilayer of cells from inner and outer dental epithelium of HERS extends apically, outlining the shape of the future root, and leaving only the basal portion of the dental papilla unenclosed (Figure 6A, reviewed in Luan et al., 2006). HERS separates the periodontal ligament from odontoblasts and dental papilla, and is thought to signal to the dental papilla to induce odontoblast differentiation. As the HERS subsequently fragments to epithelial cell rests of Malassez, the dental follicle cells come in contact with the predentin. The future cementoblasts begin differentiation and start the deposition of cementum matrix (reviewed in Nanci and Bosshardt, 2006). The precursors of cementoblasts are believed to arise either from the dental follicle cells along with precursors for periodontal ligament fibroblasts and osteoblasts, or from HERS cells undergoing epithelial-mesenchymal

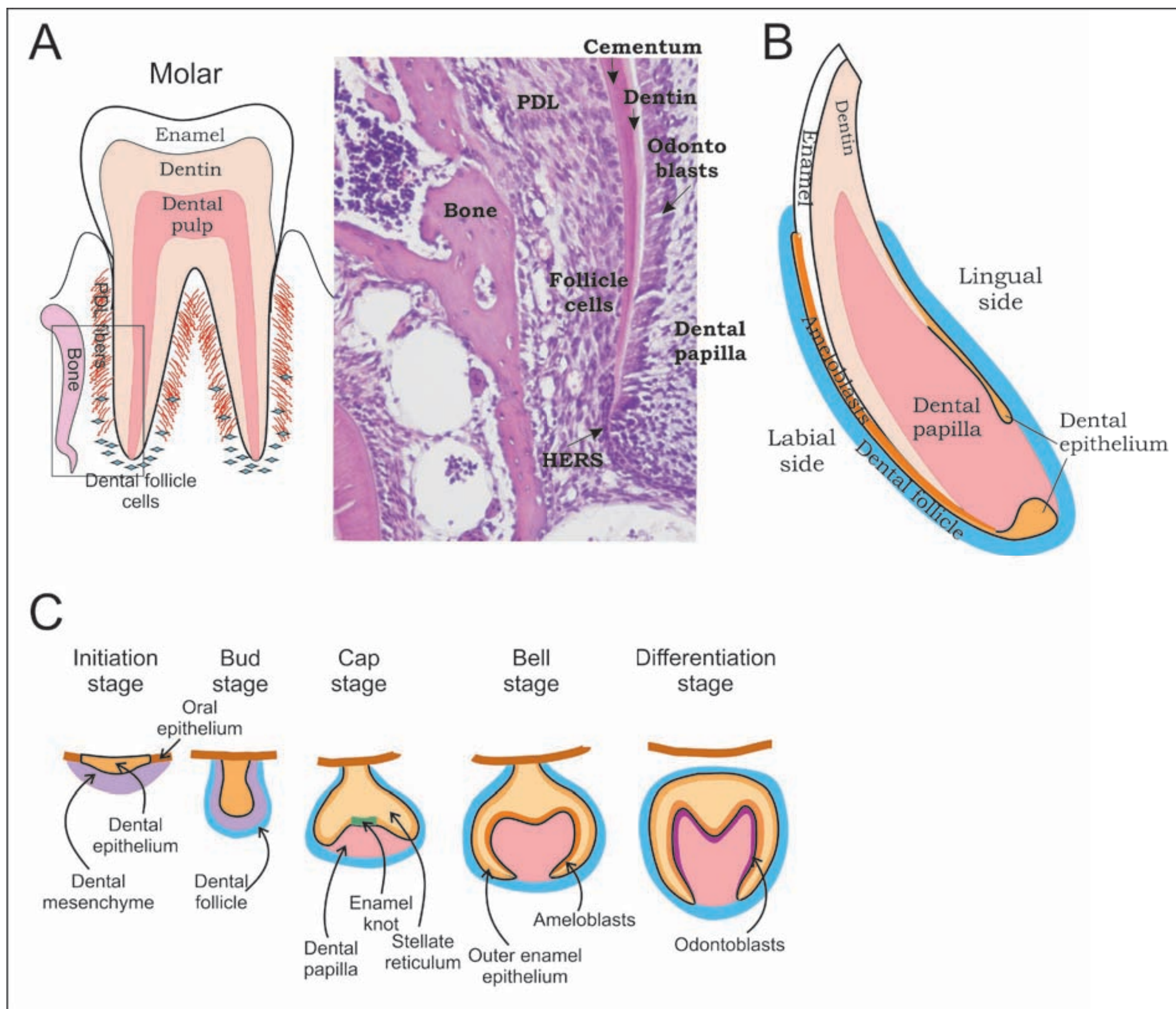


Figure 6. View of a molar tooth and the different cell types, schematic view of rodent incisor and stages of tooth development

A) Schematic view of a molar tooth, and the different cell types that can be observed in the root follicle at the root forming stage (hematoxyline-eosin staining). PDL and dental follicle cells, cementum and dentin layers, odontoblasts, bone, Hertwig's epithelial root sheath (HERS) and dental pulp are marked in the hematoxyline-eosin image.

B) Schematic view of a rodent incisor. The lingual side is reminiscent of molar roots while the labial side is comparable with the molar crown. Mesenchyme of the dental papilla is marked with pink, enamel with white, ameloblasts with orange, dentin with light rose, dental follicle with cyan and dental epithelium cells with ochre. Lingual and labial sides are indicated.

C) The main stages of tooth development. The dental epithelium thickens, forming a placode (initiation), followed by budding of the epithelium and condensation of the dental mesenchymal cells around the bud (bud stage). The future dental follicle and dental papilla are a morphologically homogenous population of mesenchymal cells at this stage. The enamel knot forms, and the bud develops into the cap stage. Mesenchymal cells beneath the dental epithelium will form the dental papilla, while the peripheral dental mesenchymal cells will give rise to the dental follicle. At the bell stage the crown shape is formed, and ameloblasts and odontoblasts differentiate. By the differentiation stage, the tooth is separated from the oral epithelium. Secretion of enamel and dentin begins from cusp sites and continues in the cervical direction. Root formation begins when the enamel front reaches the base of the crown. Epithelial cells proliferate and form the HERS, which extends and outlines the future root.

Oral epithelium is marked with dark ochre, dental epithelium with light ochre, dental mesenchyme with lilac, dental follicle with cyan, dental papilla with pink, enamel knot with green, ameloblasts with orange, and odontoblasts with purple.

transformation (Grzesik and Narayanan, 2002).

The cementum, which will cover the root in the same way as the enamel covers the crown, is deposited on top of a layer of dentin. In human teeth, dentin mineralisation starts internally, permitting intermingling of cementum matrix collagen fibrils with un-mineralised dentin fibrils (Nanci and Bosshardt, 2006). As mineralisation continues, it spreads to the cementum matrix and creates the dentin enamel junction. In rodents, dentin mineralisation happens initially, and the cementum is laid down on mineralised dentin, making the connection between dentin and cementum weaker than it is in humans (Nanci and Bosshardt, 2006). As the cementum layer reaches its final thickness, periodontal ligament fibrils become imbedded in it. *Bone sialoprotein 1* (*Bsp1*) is a marker for these terminally differentiated mineralised cells (cementoblasts lining the root surface, and osteoblasts lining the bone surface), while *Activinβ* is a marker for the dental follicle during early stages of tooth formation (Wang et al., 2004a, Yamashiro et al., 2003).

PDL is thought to be formed by the dental follicle cells, but its formation has not been characterised in detail. The mature PDL is composed of a heterogeneous population of mesenchymal cells. The principal cells of PDL are fibroblasts, which are responsible for the PDL matrix secretion that anchors teeth to the bone. Other differentiated cells identified in the PDL include osteoblasts and osteoclasts, monocytes, macrophages, cementoblasts and odontoclast. Moreover, an important class of PDL cells are the undifferentiated mesenchymal, or progenitor cells, needed for tissue repair and regeneration (reviewed in Nanci and Bosshardt, 2006). PDL is a connective tissue, and the major part of it is composed of the long fibre bundles that crosslink bone to the cementum. Collagen is a major component of this fibre network, and dental follicle cells are known to secrete at least type I, III and XII collagens (the same as in the cementum) (MacNeil et al., 1998, Hou et al., 1999, Berkovitz, 2004).

In addition to its role in PDL formation, the dental follicle is required for tooth eruption (Marks, 1995, Marks and Cahill, 1987). It has been suggested that the crown follicle regulates the resorption of bone that is needed for tooth eruption, while the root follicle may regulate the deposition of new bone at the tip of the root.

2 AIMS OF THE STUDY

When this study was started, several hundred of the estimated 1000 Golgi proteins (Taylor et al., 2000) were still unknown or uncharacterised. There was also an increasing amount of data indicating that the GC has additional functions besides the traditional transport, sorting and modification of cargo.
The specific aims of this study were:

1. To identify the Golgi proteome by an organelle proteomic approach.
2. To verify the localisation of some of the novel proteins.
3. To further characterise a novel, interesting Golgi protein.

3 EXPERIMENTAL PROCEDURES

3.1 Methods used in articles I-IV

Detailed descriptions of the materials and methods are found in the original publications.

Materials and methods	*Original publications*			
Antibody production	I		III	
Bioinformatic methods				
MS/MS data analysis	I			
***In silico* searches**	I		III	
Cell culture	I		III	IV
Cloning	I		III	
Golgi membrane isolation	I			
Imaging and confocal microscopy	I	II	III	IV
Immunofluorescence	I	II	III	IV
***In situ* hybridisation**				
Radioactive			III	IV
Whole mount			III	
LC	I			
Mass spectrometry	I			
Primary cell culture				IV
Protein isolation			III	
qPCR			III	
RNA isolation			III	
SDS-PAGE	I			
Subcellular fractionation	I			
Tissue culture			III	
Transfection	I	II		

3.2 siRNA experiments

Chondrosarcoma cells were transfected with small interfering RNAs (siRNAs) by electroporation. Both self-designed siRNAs (sense sequence for duplex 1: CGAACUGACCGACCGGCGCTT; duplex 2: GAACGUGUACUUCGCGCAGTT; duplex 3: GCGCUAUGGCAGAUUCCAGTT; and control duplex: AGCUUCAUAAGGCGCAUGCTT), and predesigned SMARTpool siRNAs from Dharmacon were used. Briefly, cells were trypsinised and suspended to a cell density of $2x10^6$ /ml in phosphate buffered saline. $2x10^5$ cells were mixed with 10 µl of siRNA (20 µM), $CaCl_2$ was added to a final concentration of 1 mM, and cells were preincubated in electroporation cuvettes on ice for 10 min. After incubation, cells were electroporated once with a BTX Electro cell manipulator 630 (300V, 100Ω, 25µF) and cooled on ice for further 10 min. Cells were then suspended into 3 ml of growth medium, and plated and grown for 72 to 96 h, after which cells were fixed and stained for GoPro49, β-COP, GM130, or other markers.

3.3 Zebrafish gene expression and morpholino injections

To obtain the time scale of gene expression in zebrafish (*Danio rerio*), the RNA was isolated from embryos at 0.5 h, 6 h, 24 h, 48 h and 72 h post fertilisation (pf), and gene expression was studied using reverse transcriptase polymerase chain reaction (RT-PCR) and gene specific primers for *zgc:101666* (CGATGCGGCAAGTTCCTCTT and GGAACCCAAGCGTTTGAGCA, product 300 bp), *LOC571492* (CTGCGCTTCCTCCCTCTCAA and TTCACGAGGCTCCCCGTACT, 300 bp), and β-*actin* (TGTGGCCCTTGACTTTGAGCAG and TAGAAGCATTTGCGGTGGACGA, 474 bp).

Translation blocking morpholino oligos were designed for the 5' UTR sequence, for both zebrafish genes (Gene-Tools, LLC, for *zgc:101666* ATTTAAGCGACAATATCCGCAG[CAT] and for *LOC571492* AAGCGCAG[CAT]GTTCGTGCACTAGC), and the corresponding five mismatch oligos were used as controls. Oligos were dissolved in sterile water to a concentration of 10 mg/ml and stored in aliquots at -70°C. For injection, oligos were further diluted in sterile water and ~30% phenol red to obtain injection dilutions.

Zebrafish embryos were collected 30 minutes pf and injected at the one-to-four cell stage with 4 nl of 0.5-2 ng/nl morpholino to the yolk sac. Fifty embryos were injected for each oligo, in each experiment. Injected and non-injected control embryos were further grown for 24 to 144 hours at the standard 28.5°C temperature, in T3-buffer supplemented with phenylthiourea to prevent pigmentation. Any changes in phenotypes were monitored daily, and samples collected at 3d (72 h) and 6d (144 h), cooled on ice for 15 min, and fixed with either 4% paraformaldehyde or methanol according to the protocol approved by the University of Helsinki Review Board for Animal Experiments.

3.4 Alcian blue staining

To analyse any cartilage phenotypes in morpholino injected zebrafish, Alcian blue staining was performed on paraformaldehyde fixed zebrafish embryos according to Schilling et al. (1996) and modifications therein. Briefly, paraformaldehyde fixed embryos were incubated overnight at +4°C in 0.1% Alcian blue in 80% ethanol/20% glacial acetic acid. Embryos were rinsed with ethanol and rehydrated stepwise in phosphate buffered saline. Tissues were cleared with 0.05% trypsin in saturated sodium tetraborate for 1-3 h, and bleached in 3% hydrogen peroxide/1% potassium hydroxide to remove pigmentation. Samples were transferred to 70% glycerol in phosphate buffered saline for imaging.

4 RESULTS

A brief description of the main results is given, together with some unpublished data.

4.1 The Golgi proteome (I, II)

Despite the several Golgi proteomic studies performed, the Golgi proteome is still not complete (Gilchrist et al., 2006, Taylor et al., 2000, Breuza et al., 2004, Bell et al., 2001, Wu et al., 2000, Wu et al., 2004, Publ. I). Of these studies three were done using LC-based fractionation of peptides coupled to direct analysis by MS/MS (Gilchrist et al., 2006, Wu et al., 2004, Publ. I). The study conducted by Wu et al. used isolated stacked Golgi membranes, using an approach which is similar to our own study. The most recent of these studies (Gilchrist et al.) involved a quantitative study of the whole secretory pathway.

Publication I describes our LC-ESI-MS/MS approach to identifying the Golgi proteome from highly purified (130-fold enrichment) stacked rat liver Golgi membranes (verified by EM), and lists the identification of 1125 proteins, of which 1031 were identified with two or more peptides. All identified proteins were grouped into 16 different categories based on Swiss-Prot database information on subcellular localisation or function (Publ. I, Fig. 2 and Supplementary table 1). The main categories were: Golgi and membrane transport, ER, cargo, cytosolic, and localisation undetermined. 201 (17.5%) of these identified proteins were previously known Golgi and transport proteins, 101 (8.8%) were known ER proteins, and 89 (7.7%) were previously unknown or uncharacterised. Counting the number of peptides in each category showed that 29.9% were from different Golgi (20.2%) and transport proteins, 13.3% were from ER proteins, and 4.6% of proteins from contaminating organelles (PM, endosomes, lysysomes, mitochondria, nucleus and peroxisomes). 22.8% of the peptides were from different cargo proteins, while cytosolic proteins and those with undetermined localisation accounted for 18.1%. This is well in line with what Gilchrist et al. also reported in their study.

Comparison of our data with the previous Golgi proteomic studies showed that overlap between the studies, especially of novel proteins, was relatively small. Wu et al. used similar rat liver Golgi membranes and thus should be the most alike. Of the 421 protein identified by Wu et al. (2004), 399 could be identified in our study. However, of the 42 unknown proteins identified in their study, only 24 were identified in our study, and of our 89 unknown proteins only 11 were found in theirs. The Gilchrist et al. study (2006) found only 122 of the Golgi, ERGIC and transport proteins identified in our study (59% overlap). Thus, to obtain a comprehensive list of all Golgi proteins, data from multiple studies need to be combined and verified.

Two of the unknown proteins identified in our study were selected for initial characterisation: the human homologue for RIKEN cDNA 2510039O18 (later called KIAA2013) and the protein similar to Ab2-095 (later called GoPro49). Both of these proteins were shown to localise to the GC as EGFP-fusion proteins in HeLa cells, with clear co-localisation with the Golgi marker, GM130. For KIAA2013, co-localisation of the endogenous protein with GM130 was also seen (Publ. I, Fig.2). KIAA2013 was one of the unknown proteins shared between Wu et al. (2004) and our study, but GoPro49 could not be identified in their data, while both of these proteins were found in the data from Gilchrist et al. (2006).

Some of the unknown proteins identified in Publication I, including the protein similar to Ab2-095, were also included in a novel protein database, Human Proteinpedia (Publ. II). This database is the first attempt to collect interaction and functional data on human proteins into one database which contains full analysis datasets that can be cross-searched. This database is open to all researchers, and after

registration everyone can contribute their datasets on proteomic, yeast-2-hybrid, and localisation studies, as well as data on post-translational modifications. In addition, the contributors' names and contact details will stay in the entry, and only the contributor can change the deposited data.

4.2 In silico analysis of the protein, GoPro49 (III)

The protein similar to Ab2-095/hypothetical protein LOC205428/c3orf58/DIA1, or from here on Golgi protein 49kDa (GoPro49), was selected for further study. Based on the *in silico* data, the gene is predicted to code for a 49kDa protein containing a signal peptide. However, the prediction programmes were not unanimous in this prediction and all gave a very low score to the cleavage site. Furthermore, these prediction programmes (such as SignalP, Emanuelsson et al., 2007) may not reliably distinguish signal peptide from signal anchors. Indeed, of the 226 different Golgi localising transferases (annotated in UniProt) that are all supposedly signal anchored, 51% were predicted not to contain a signal anchor (with over 50% probability), and 22% did not even score a 10% probability, demonstrating how unreliable this type of prediction is. Thus *in vitro* analysis is required to determine whether GoPro49 has a signal peptide or a signal anchor.

The further *in silico* analysis of GoPro49 did not reveal any known functional domains or homology to known proteins, but it did suggest a few short binding motifs that might either be related to the function or to the regulation of the protein. One of these motifs is the potential binding sites for 14-3-3 proteins. The GoPro49 sequence showed no potential *N*- or *O*-glycosylation or lipid modification sites, but two potential glycosaminoglycan attachment sites (Puntervoll et al., 2003) were identified. In addition, the sequence contained numerous potential phosphorylation sites (Figure 7A).

The orthologous GoPro49 proteins are highly homologous and the sequence alignment of twelve different species shows the middle third to be especially conserved (Publ. III, Fig. 1). Of the analysed species, zebrafish was the only one where two, highly homologous sequences could be identified. This was not unexpected, as the zebrafish genome has undergone a recent genome duplication (Woods et al., 2000). Based on the current sequence data, the human gene for *GoPro49* is predicted to code for two different splice variants: a large one used in this study (isoform A, Q8NDZ4 or NP_775823), and a smaller one that has alternative exon 1 (isoform B) that was not in the databases when this study was conducted (unpublished data). Isoform B encodes a protein (NP_001127942) that contains amino acids 220-430 of the longer isoform A and eleven unique amino acids in the N-terminus. Unlike isoform A, isoform B is not predicted to have a signal sequence or a transmembrane domain, and thus it is predicted to be cytoplasmic. Both isoforms are depicted in Figure 7A. Whether the corresponding mouse gene also encodes two similar isoforms is unclear at the moment, but it is likely that, as for some other species (e.g. dog and horse), only the corresponding shorter isoform can be found in databases. The peptides that were used to identify the protein similar to Ab2-095 in Publication I, cover 23% of the GoPro49 sequence and both N- and C-terminal halves, indicating that rat liver expressed the longer isoform.

The EST data for the gene suggested that the highest expression levels are found in leukocytes and blood vessels. However, as the EST data seldom includes samples of cartilage and bone, these tissues are easily missed in this kind of data (Figure 7B). The *in silico* analysis of the 2kb sequence 5' upstream of the *GoPro49* coding sequence showed a number of potential transcription factor binding sites. Several sites for Tcf/Lef (Wnt pathway), Runx2 (BMP-2, TGFβ and FGF pathways), a homeobox gene Msx2, Smads and Sox5 (BMP pathways) and Pax9 were among the hits conserved between human and mouse (with rVista 2.0: Tcf/Lef, Runx2, Smad and Sox5; and with MatInspector: all the previous, plus Msx2 and Pax9).

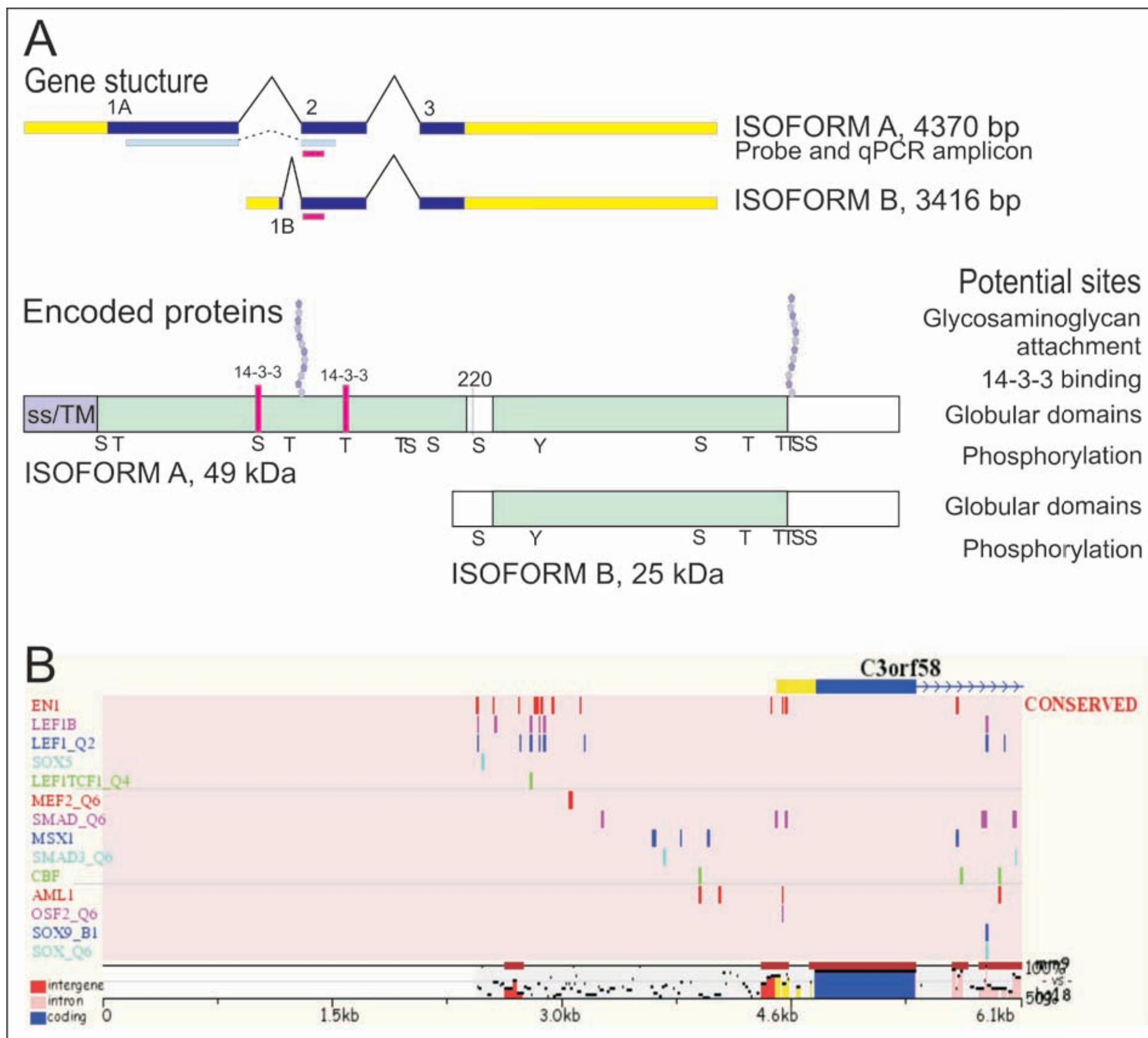


Figure 7. Schematic view of the two GoPro49 isoforms and selected transcription factor binding sites identified, using rVista, in 5kb sequence 5' upstream of the GoPro49 long isoform coding sequence

A) Schematic view of the gene structure and the two protein isoforms coded by the *GoPro49* gene. *Isoforms* differ in the alternative exon 1. Exon 1A is 1192 bp, while exon 1B is 239 bp long. The untranslated regions of both transcripts are marked in yellow, and the coding regions in blue. The binding site of probe used in Publications III and IV is indicated with light blue bar under the isoform A (not including the intronic sequence marked with dashed line), and the qPCR amplicon (108 bp) is marked with magenta.

Potential modification sites on the encoded proteins: glycosaminoglycan attachment and 14-3-3 binding sites (ELM), globular domains (green), and potential phosphorylation sites (NetPhos2.0) are shown for both isoforms. Amino acid 220 marked in the isoform A is the first homologous amino acid shared by these isoforms.

B) Schematic view of the selected potential transcription factor binding sites that are conserved between human and mouse (identified by rVista 2.0). Sites for Tcf/Lef (Lef1, Lef1B, Lef1Tcf1), Runx2 (Cbf and Osf2), Smads, Sox5, and En1 are among the ones identified in the sequence from the 3 kb marker to the beginning of the coding sequence, marked by the blue bar. The untranslated region is marked by yellow bar.

4.3 The GoPro49 tissue expression pattern (III, IV)

To identify the tissues where the *GoPro49* gene was expressed during development, the expression was studied using both quantitative PCR (qPCR) and *in situ* hybridisation. The expression was shown to increase from embryonic day 7 (E7) towards E15, after which the expression leveled off (Publ. III, and Figure 8B). The probe (680nt) used for the *in situ* hybridisation was designed for the longer isoform A, but overlaps

the first 160nt of the human isoform B sequence, which was not in the databases at the time. Thus, the probe may also recognise the shorter isoform B. Moreover, the qPCR primers used were designed for a site that overlaps in the two sequences, and thus they do not distinguish between the possible two mouse isoforms.

In situ hybridisation analysis of *GoPro49* expression during mouse embryogenesis clearly showed the highest expression levels in different mesenchymal tissues that give rise to the skeletal elements later at life (Figure 8A). Furthermore, the *GoPro49* expression pattern was both restricted and developmentally regulated in the mesenchymal and cartilaginous tissues during development. Intense expression was observed in vertebrae, ribs and limbs, and, in the craniofacial area, in the nasal septum and dental follicle. In the vertebrae and snout region, clear co-expression with *Col II* was detected. In paws, additional expression was detected in the interdigital area at E12 and E14. Expression levels in the trunk area were decreasing from E14, while in the craniofacial cartilages, expression continued to be strong in the postnatal tissues (Publ. III, Fig. 2-6). The highest expression levels were detected in the proliferating and columnar chondroblasts, but no expression was observed in the hypertrophic or terminal chondrocytes.

To compare the *GoPro49* expression levels in different stages of the limb development, the expression was analysed in E10.5-E18.5 limb bud RNA using qPCR, and the results were normalised against *TATA box binding protein* (*Tbp*), a housekeeping gene with a relatively low expression level that was comparable to the level of *GoPro49* expression (unpublished data, Figure 8B). Expression was seen already in E10.5 RNA, at which stage the limb buds have formed. By E12.5, even more distal elements (autopod condensations) have formed and *Col II* expression can be observed in *in situ* hybridisation. The *GoPro49* expression level increased throughout the examined period, and E18.5 had the highest expression, with 40% more expression than at E10.5 (Figure 8B, unpublished data). The bones at E18.5 have well-formed growth plates, and mineralisation of the matrix has begun at the diaphysis.

In addition to the cartilage, clear and restricted expression of *GoPro49* was observed during embryonic and postnatal tooth development. The expression was restricted mainly to the dental follicle from E12 to 12 d postnatal (dpn), in both molars and incisors (Publ. III, Fig. 6 and Publ. IV). No significant expression was observed in other tooth tissues at any of the time points examined. Noticeably, while *GoPro49* was expressed intensely in most of the condensed mesenchyme surrounding the bud stage tooth germ, it was absent from the presumptive dental papilla mesenchyme. During embryonic stages, the expression was detected in the whole dental follicle, while in the postnatal molars only a little expression was observed in the crown follicle of non-erupted teeth.

GoPro49 expression in the dental follicle was clearly complementary to *Bsp1* in bone and cementoblasts, and strongly restricted to the loosely located fibroblast-like cells that later become embedded in the matrix. *ActivinβA* was co-expressed with *GoPro49* in the incisor dental follicle during embryonic and postnatal tooth development, but expression appeared more intense in the peripheral cells of the follicle bordering the area of bone formation. In addition, strong *ActivinβA* expression was seen in the dental papilla, in the osteogenic mesenchyme and in ameloblasts and it was downregulated in the root follicle earlier than *GoPro49* (Publ. IV).

The *GoPro49* expression levels in the different craniofacial tissues were also analysed using qPCR (Figure 8B). As expected, the highest expression levels in the craniofacial area were detected in the nasal septum. Surprisingly, the expression levels in the molars did not follow the staining intensity observed in the *in situ* hybridisation, with higher expression levels detected in E16.5 rather than in 4 dpn molars. Unfortunately, on western blot the antibodies we raised were only able to detect transfected fusion

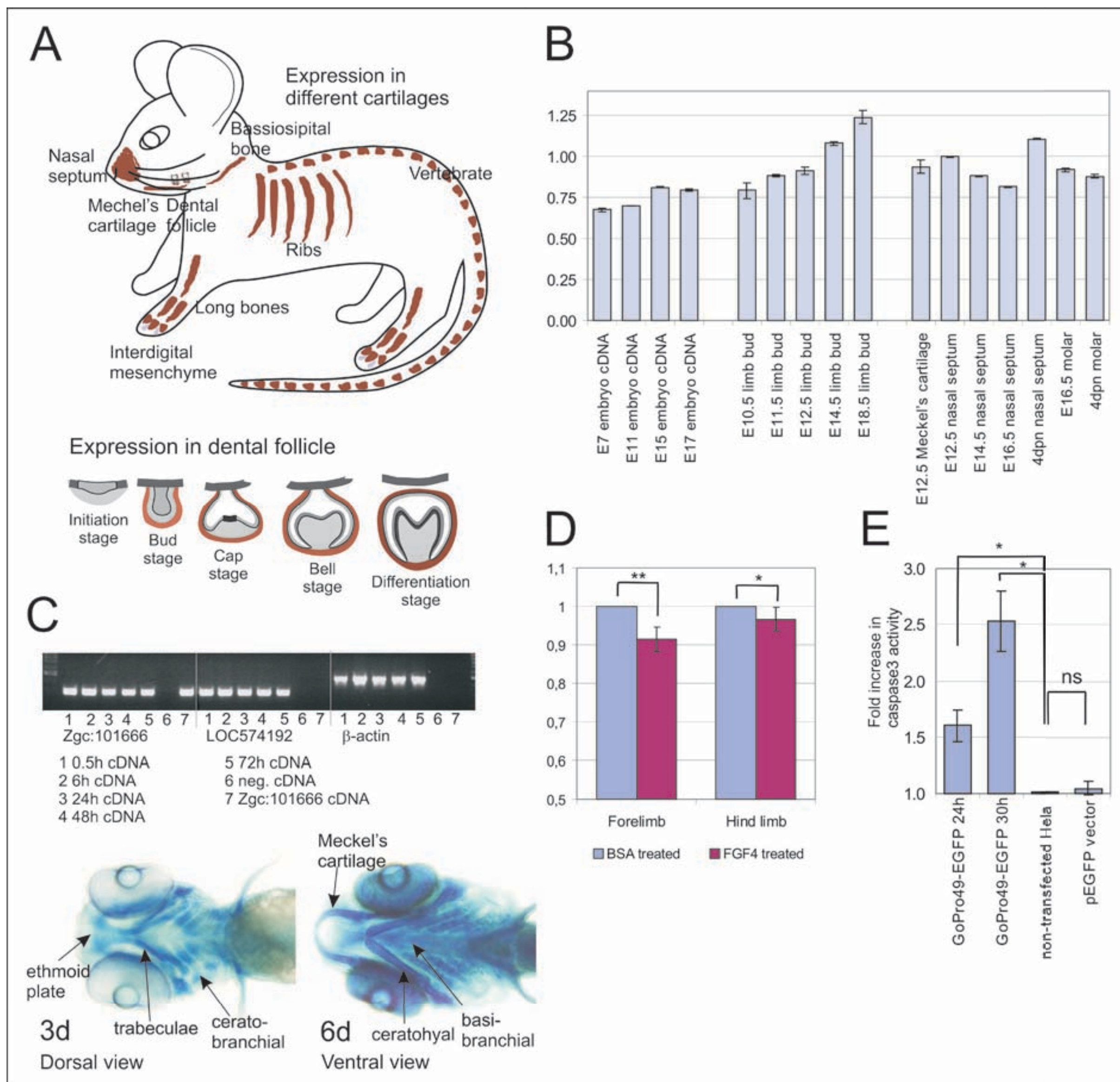


Figure 8. Pattens of GoPro49 expression

A) The schematic view of the *GoPro49* expression pattern from *in situ* hybridisation. The figure shows all the different mesenchymal tissues where expression was detected during the embryonic period. *GoPro49* expression (brown) was observed in mouse embryos using *in situ* hybridisation, in the nasal septum (from E12 to 4 dpn), in the dental follicle (from E12 to 12 dpn), in the spine and ribs (from E12 to E16), in long bones (from E12 to E16) and between the digits (blue, at E12 and E14). *GoPro49* expression observed in the dental follicle is also shown in the different stages of tooth development.

B) A comparison of the expression levels using qPCR. The *GoPro49* expression levels were analysed in whole embryo cDNA (from E7-E17), in limbs (from E10.5 to E18.5), in nasal septum (from E14.5 to 4 dpn) and in molars (at E16.5 and 4 dpn). The qPCR data was normalised against *Tbp* and compared to the reference sample (E12.5 nasal septum), which was set as 1. For the whole embryo cDNA samples the *GoPro49* expression levels increased from E7 to E15, but were considerably lower than in e.g. E14.5 limbs or nasal septum. The expression levels in limbs increased from E10.5 to E18.5, with 40% more expression in E18.5 than in E10.5 which suggests that expression in limbs increases with formation of more and larger cartilaginous elements. In the craniofacial samples there was less variation in the expression levels between stages. Of the tissues examined, the E18.5 limbs had the highest expression levels, suggesting that the protein function is linked to the level of cartilage differentiation.

C) Expression in the zebrafish embryonal RNA. The expression levels of the homologous zebrafish genes *zgc:101666* (300 bp) and *LOC571492* (300 bp), as well as *β-actin* (474 bp) from embryos at 0.5 h to 72 h post fertilisation were analysed using RT-PCR. The expression levels of both zebrafish *GoPro49* orthologs stayed relatively constant throughout the period and β-*actin* intensity shows that the RNA levels in the different samples are comparable. The strong expression that is observed at the 0.5 h time point indicates that both gene products are already present in maternal RNA. It also suggests that the gene products are required in zebrafish before formation of cartilaginous elements around 3d pf. The specificity of the primers was tested in a cDNA sample of *zgc:1010666*.

protein, but not the endogenous protein. Thus the expression could not be analysed at the protein level.

To check whether FGF, BMP or TGFβ pathways could regulate the *GoPro49* expression, tissue culture experiments were performed. Shh and BSA, were used as negative controls, as no Gli sites were identified in the 5' upstream sequence. Bead implantation experiments were tested first, but the results were inconclusive as a very weak signal was observed; thus the role of FGF was further tested using a qPCR-based approach. The qPCR results (in Publication III as RT-PCR) indicated that the *GoPro49* expression was not affected by FGF4 in these samples or that the expression was even slightly downregulated (Figure 8D).

4.4 GoPro49 subcellular localisation (I, III, IV)

To gain insight into the role of GoPro49, its subcellular localisation was studied. The EGFP-fusion protein showed clear co-localisation with the Golgi protein GM130 in HeLa cells (Publ. I, Fig. 2). However, the endogenous protein levels in HeLa cells were too low to be detected using the antibodies we raised against GoPro49. Thus, endogenous localisation was studied in the chondrosarcoma cell line HTB-94 (Publ. III), and also in primary dental follicle cells isolated from E18.5 mouse molars (Publ. IV). A clear co-localisation with β-COP was detected in both cell lines (Publ. III, Fig. 7 and Publ. IV, Fig. 3). However, the endogenous protein did not co-localise with the GM130 staining as was expected based on the EGFP-fusion protein localisation in HeLa cells (Publ. I, Fig. 2).

Furthermore, GM130 staining in chondrocytes showed a markedly different Golgi morphology, from that of hamster kidney (BHK) or HeLa cells, which are often used in membrane traffic studies. The more reticular appearance of the GC observed in both chondrosarcoma and dental follicle cells could suggest higher amounts of secretory cargo in these cells, as the GC size is known to be indicative of the membrane traffic load. Cycloheximine treatment for 3 h did not change the GoPro49 co-localisation with β-COP in chondrosarcoma cells, but it did reduce the number of tubules seen in these cells (Publ. III, Fig. 7G-I). In addition to β-COP, GoPro49 also co-localised partially with Col II (unpublished data, Figure 9F and F').

However, the antibodies used to study protein localisation could not distinguish between the isoforms A and B, as the peptide antibody was made against the C-terminus, which is identical in the isoforms, and the epitope used to raise the antibody against the full length protein also covers the whole of isoform B. However, an EGFP-tagged deletion mutant [covering amino acids 236-430 (Δ1-235, 88% of the isoform B

Representative images of alcian blue stained, morpholino-injected zebrafish at 3d and 6d. No major differences could be observed between uninjected, specific, and mismatch morpholino injected embryos. The lack of a strong phenotype might be related to the duplication of the zebrafish genome, and possible functional compensation by the homologous protein; however the presence of maternal RNA makes the success of k.d. less likely.

D) Downregulation of *GoPro49* expression by FGF4 in tissue culture. FGF4 added to the growth medium in E11.5 forelimb and hind limb tissue culture experiments caused a slight downregulation of *GoPro49* expression. This effect was more pronounced in forelimb than in hind limb. These results suggest that FGF4 could regulate *GoPro49* expression in limbs during limb development and perhaps help to determine its expression domain. The error bars present standard deviation, significant differences (t-test, $p<0.01$ *, $p<0.001$ **) are indicated.

E) Caspase-3 activity in transfected HeLa cells. Caspase-3 activity was measured from HeLa cell lysates of GoPro49-EGFP and control transfected cells, and the activity of GoPro49-EGFP samples was compared with the non-transfected and EGFP-transfected samples. A clear increase in the caspase-3 activity (1.6 fold) was seen from 24h onwards in the GoPro49-EGFP transfected samples in comparison with EGFP-transfected control sample, where no significant increase was observed. This activity was most pronounced at the 30 h time point where 2.5 fold increase in caspase-3 activity was observed. Error bars present standard deviations, significant differences ($p<0.01$, t-test) are indicated by asterisk, ns means non-significant difference.

sequence)] of GoPro49 localises to punctuated structures, that have no co-localisation with GM130 (Figure 9L) or with β-COP (unpublished results), suggesting that the β-COP co-localisation of the endogenous protein is specific to isoform A.

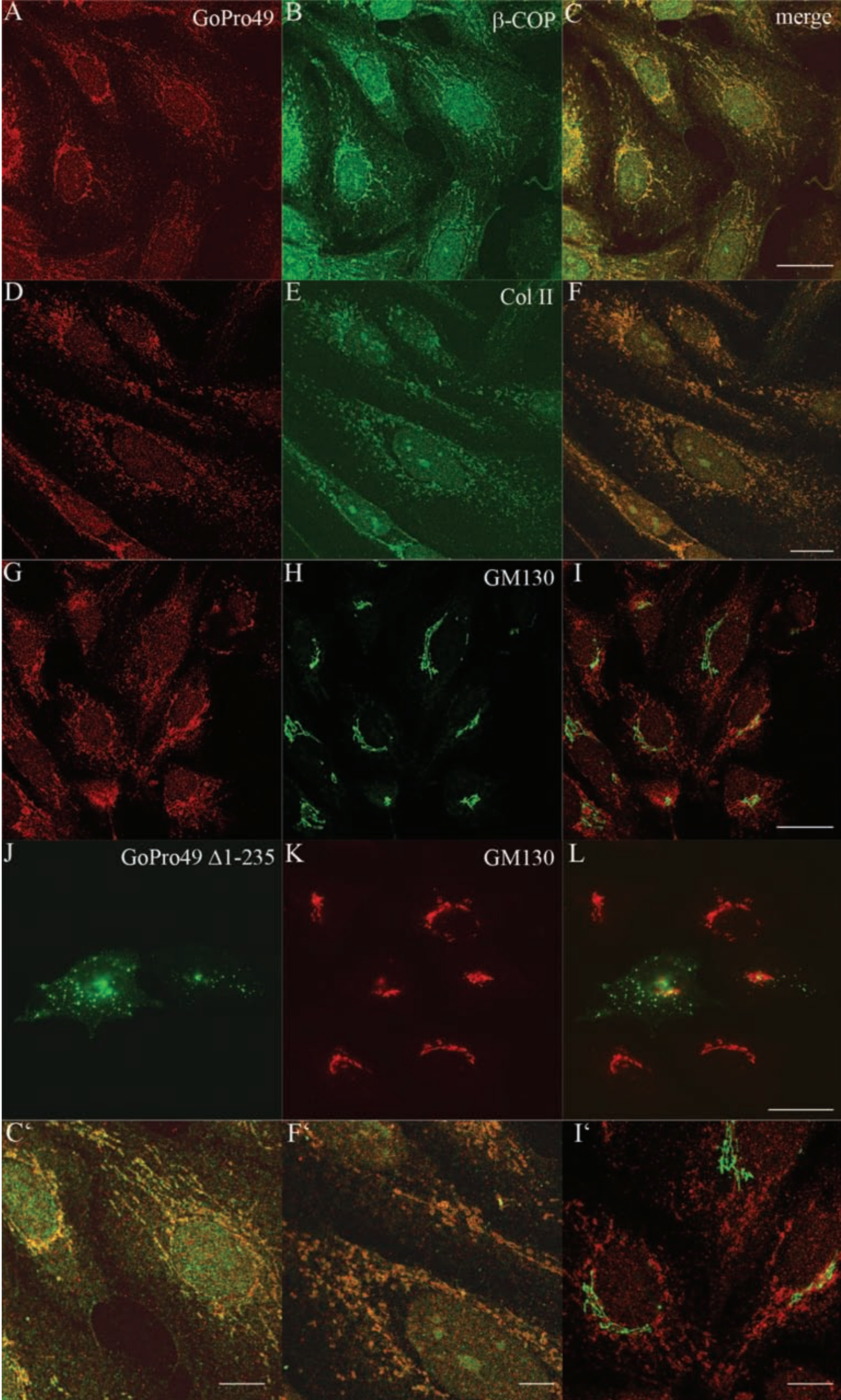


Figure 9. Localisation of GoPro49, β-COP, Col II, and GM130 in HTB-94 cells and localisation of Δ1-235 GoPro49-EGFP in HeLa cells

The GoPro49 (A, D, G) subcellular localisation was compared with that of β-COP (B), Col II (E) and GM130 (H) in a chondrosarcoma cell line using confocal microscopy (C, F and I are merged images). The endogenous protein clearly co-localised with both β-COP (C') and Col II (F'), but not with GM130 in the *cis*-Golgi (I'). The β-COP-positive structures observed in these cells were more widespread than those showing GM130 staining, perhaps indicating more active membrane traffic in these cells.

The localisation of the deletion mutant Δ1-235 GoPro49-EGFP in HeLa cells (J) was compared with GM130 (K, fluorescence microscopy). The mutant localised to punctuated structures that did not co-localise with GM130 (L). Scale bars A-L 25 µm, C', F' and I' 10 µm.

4.5 Knockdown experiments (unpublished results)

Our early observation for GoPro49-EGFP transfected HeLa cells was that more cells were dying within 24-48 h after transfection than control cells transfected with EGFP plasmid only. This increased cell death resulted from 2.5 times higher apoptotic caspase-3 activity (detected from cell lysate with a luminescent substrate peptide) than was seen in the control HeLa cells transfected with EGFP only (unpublished data, Figure 8E). One possibile explanation for this apoptotic activation is that GoPro49 somehow influences the cell cycle or division, as many Golgi proteins have been shown to be important as mitotic regulators. However, this is unlikely, as *GoPro49* was not identified in a large screen for genes affecting the cell cycle performed in *Drosophila* (M. Björklund, personal communication).

To gain insight into the protein's function, several different siRNAs were tested in a chondrosarcoma cell line. The knock down (k.d.) efficiency of each experiment was verified using indirect immunofluorescence. Unfortunately, these cells are extremely difficult to transfect, and a lentivirus vector or microinjection of siRNA would be required to perform siRNA experiments. With electroporation, k.d. efficiency varied greatly between experiments. The slight tubulation of COPI vesicles could be a significant phenomenon (seen in experiments with better GoPro49 k.d. efficacy), but more efficient transfer of siRNA is needed to verify this observation. From these experiments it can be concluded that the k.d. of GoPro49 is not lethal for chondrosarcoma cells.

Zebrafish (*Danio rerio*) expresses both homologous *GoPro49* genes from the beginning of fertilisation, and the expression levels stay relatively stable between 0.5 h and 72 h, indicating that both genes are already present early in the maternal RNA pool (see Figure 8C, unpublished data). Therefore, to analyse the role of *GoPro49* in zebrafish, a morpholino k.d. approach was tested. Morpholinos were designed against both zebrafish genes, *zgc:101666* and *LOC571492*, and were used in subsequent experiments both one at a time and in combination. However, no significant change in the phenotype was observed when injecting 8 ng of a single morpholino or a mixture of morpholinos against both genes (4+4 ng).

K.d. embryos fixed at 3d and 6d were also stained with Alcian blue, in order to study possible defects in the cartilage (Figure 8C). However, no major differences could be observed between uninjected, specific, and control morpholino injected embryos. As translation blocking morpholinos function at the level of translation and may not cause k.d. of mRNA, their functionality cannot be verified using PCR on the RNA level, and since our antibody cannot detect the endogenous zebrafish protein in western blots, the k.d. level could not be verified.

5 DISCUSSION

5.1 Diversity of the Golgi proteome and the 'proteinpedia' database

When this project was started, the Golgi proteome (estimated at 1000 proteins, Taylor et al., 2000) was far from complete. Furthermore, it had become apparent that the GC had additional functions besides the traditional roles in post-translational modifications and membrane traffic, including cell migration and signalling (Preisinger et al., 2005, Preisinger et al., 2004, Sheen et al., 2004, Bivona et al., 2003). Our aim was to discover new proteins from isolated rat liver Golgi membranes that might contribute to novel GC functions. Because proteomics is a highly powerful method to identify proteins, this approach was selected to study the Golgi proteome. Furthermore, introduction of shotgun proteomics a few years earlier had made this method better suited for the study of membranous organelles such as the GC (Wolters et al., 2001). This approach led to a significant improvement in the identification of integral membrane proteins compared to traditional approaches such as 2D gels. Indeed, 35% of the proteins identified in our study were integral to the membrane based on TM prediction (Publ. I).

The identified proteome was very extensive and the known Golgi and membrane transport proteins formed a major fraction (17.5%, 201). 89 previously unknown proteins were also identified, of which GoPro49 was selected for further study. Our study is the most extensive Golgi proteomics study performed to date and the number of novel proteins found was greater that in previous Golgi proteomic studies (Taylor et al., 2000, Breuza et al., 2004, Bell et al., 2001, Wu et al., 2000, Wu et al., 2004). The study by Gilchrist et al. did not focus on the GC, but instead it studied the proteome of the whole secretory pathway (Gilchrist et al., 2006).

One aspect of the GC is its dynamic morphology, which is related to its functions in membrane traffic. Indeed, the GC membranes and proteins are in constant flux with both the ER and the PM. On the one hand, newly synthesised Golgi proteins are transported from the ER, and escaped, Golgi resident proteins are returned from the PM (Bonifacino and Rojas, 2006, Barlowe et al., 1994, Aridor et al., 1995). On the other hand, escaped ER resident proteins are recycled from the GC in COPI vesicles, while proteins and receptors on the PM can be internalised in clathrin coated vesicles, transported through endosomes to the TGN, and re-inserted into the PM (Nichols and Lippincott-Schwartz, 2001, Bonifacino and Rojas, 2006, Orci et al., 1997, Benmerah and Lamaze, 2007, Wilson et al., 1993). Because of all this trafficking, the recycling ER and PM proteins might, at least partially, reflect proteins in transit such as the KDEL-proteins.

Furthermore, it is a matter of definition whether proteins like microtubule motors, which are required for transport and GC morphology and which function in multiple locations, should also be considered part of the Golgi proteome (Gupta et al., 2008, Lippincott-Schwartz et al., 1995, Kreis el al., 1997). Thus, are the estimated 1000 proteins the whole Golgi proteome? In my opinion the Golgi proteome cannot be considered to be a totally separate entity from the ER, the PM and the numerous cytosolic factors, and thus the number of proteins needed to 'make' the GC is likely to be greater than 1000.

In addition to the traditional Golgi proteome, the GC also includes proteins with 'double identity' or splice variants with different functions and localisations. Proteins with one isoform in the GC include, for example, BARS/Ctbp1 and Golgin-245/CrpF46 (Carcedo et al., 2004, Fritzler et al., 1995, Lieu et al., 2008, Wei et al., 2008). For these proteins, the two different isoforms have clearly separate functions. BARS is involved in fission events, while Ctbp1 acts as a transcription factor. For the second pair, Golgin-245 has a role in transport events from the TGN while CrpF46 binds centrosomes, and may

participate in their duplication (Carcedo et al., 2004, Lieu et al., 2008, Wei et al., 2008, Bonazzi et al., 2005, Yang et al., 2005). Such splice variants might explain some controversial results, such as staining in multiple subcellular locations e.g. in the GC and centrosomes, or interactions with nuclear proteins.

The human gene for *GoPro49* is also predicted to produce two splice variants that have different N-termini, according to the current databases. Our proteomic study clearly identified the longer isoform with matching peptides covering the whole sequence, while the shorter isoform is supported by similar protein isoforms identified in other species including dog and horse (Publ. I and unpublished data). Distinguishing between two or more isoforms with large identical domains might not be possible on the protein level. However, on the RNA level these genes are likely to have different 3' or 5' sequences and thus differences in expression could be studied. Furthermore, different isoforms might have different tissue expression patterns or differential expression during development as, for example, with collagen type 2 isoforms A and B (Sandell et al., 1994, Sandell et al., 1991).

Do the two GoPro49 isoforms have similar subcellular localisation and tissue expression patterns? As the shorter isoform is predicted not to be an integral membrane protein, these two proteins cannot localise to the same compartment, at the same side of the membrane. In addition to differences in the subcellular localisation, the *in situ* probe used in this study (Publ. III and Publ. IV) might recognise these two isoforms with different affinities. Thus they could also have different tissue expression patterns and the shorter isoform B might even be more ubiquitously expressed. Moreover they might have differential expression in tissues where our probe detected expression, e.g. *isoform A* mainly in cartilage and *isoform B* in the interdigital area. Based on these divergences, their functions are likely to be diverse. Thus, in my opinion, these two proteins should be considered different yet homologous proteins.

Sequence analysis of GoPro49 isoform A would indicate a cleavable signal peptide, but no transmembrane domain (SignalP, Emanuelsson et al., 2007). Based on the current view, if the signal sequence was cleaved the protein should be secreted to the ECM. However, this is not in line with the subcellular localisation of the endogenous protein in β-COP positive structures (Publ. III), or of the EGFP fusion protein in the GC (Publ. I). In addition, treatment of cells for 3h with cycloheximide did not change the co-localisation of endogenous protein with β-COP indicating that this localisation is not dependent on newly synthesised protein (Publ. III). Thus, the signal peptide prediction requires further validation.

In the proteomics study of Gilchrist et al., the protein similar to Ab2-095, or GoPro49, was identified in the COPI/Golgi fraction, and was clearly enriched in the COPI population (Gilchrist et al., 2006) supporting our notion that GoPro49 does indeed localise to COPI vesicles. Thus, the interpretation of these results is that the signal peptide is not cleaved and it rather behaves as a signal anchor retaining GoPro49 in the β-COP-positive structures. Furthermore, if the software prediction were correct and this type of signal is cleaved, then several Golgi transferases should also be secreted, as they give similar prediction results. One possibility could be that the GC and COPI vesicles also contain soluble resident proteins, but this does not agree with the current view of different types of Golgi resident proteins (Altan-Bonnet et al., 2004).

What could be the retention mechanism if GoPro49 were soluble? There appear to be no lipid modification sites in the sequence, nor KDEL or other ER recycling signals. The protein is also unlikely to undergo *N*- or *O*-glycosylation, but it may be a target for glycosaminoglycan attachment (two potential sites) (Puntervoll et al., 2003). On the one hand, lectin-based interactions are possible, but on the other hand, this seems unlikely as glycosaminoglycan modifications are mostly found on extracellular proteins. The few predicted peptide motifs (e.g. 14-3-3 binding) are all for cytoplasmic interactions, and if the

membrane orientation prediction of the C-terminus being in the lumen is correct, these sites are not accessible in the longer isoform. Therefore, there are no accessible interaction motifs identified so far in GoPro49 isoform A. This leaves interactions with unknown proteins, or oligomerisation (van Vliet et al., 2003) as possible mechanisms for retention in the COPI vesicles/GC.

The Golgi membranes have typically been isolated from rat liver, but could these membranes be isolated from cartilage? Unlike the liver, a major constituent of cartilage is the ECM, which is additionally mineralised in later stages. Furthermore, cartilage forms a major part of the skeleton only during embryogenesis. In adults, the amount of cartilage is greatest in the craniofacial area, which is not an abundant source. The number of cells could thus limit the material available, and critical mass for isolation might be hard to reach. However, based on our results for *GoPro49* expression, it would be beneficial to isolate the Golgi membranes also from other sources besides liver, and to compare the Golgi proteomes, since Golgi proteins clearly can have restricted expression patterns.

The limiting factor for proteomics and other high throughput methods is the subsequent characterisation of the identified proteins. The high sensitivity can also give false positive results. Thus, even though systems biology approaches may help to dissect connections and pathways that might not otherwise be detected, it also requires that most of the data is further analysed and validated *in vivo* and *in vitro*, for example to confirm expression or protein function. The wealth of data generated by the different '–omics' is difficult to keep up with. Thus databases are needed in which data from different approaches are integrated. The novel protein database Human Proteinpedia (http://www.humanproteinpedia.org/ Publ. II) is one such attempt to collect data on human proteins and interactions, and so far it is the only one for vertebrates where proteomic, yeast-2-hybrid interaction, localisation and modification data are all collected in the same database that can be easily explored and updated by contributors. As this database incorporates data from multiple sources and links the data to each protein, it makes the several levels of data easily accessible. The protein GoPro49 has also been included in this database.

5.2 Restricted, developmentally regulated expression of GoPro49

As the secretory pathway is essential for all cells it is not surprising that ubiquitous expression has been reported for most Golgi proteins, with perhaps increased expression levels in secretory tissues. Unexpectedly, *GoPro49* expression was observed in a clearly restricted pattern during mouse embryogenesis, with highest levels detected in developing cartilage, i.e. in columnar, proliferating chondroblasts (Publ. III).

The differences in expression levels in developing limbs were analysed using qPCR. Expression was detected already in the E10.5 limb bud, and it increased as development proceeded. This is well in line with the observed expression in columnar chondroblasts, as increased expression corresponds to the formation of more cartilaginous elements at later stages. In the E18.5 limb, where the strongest expression was detected, the diaphysis is already ossified, but cartilage is still forming in the growth plates, and chondroblasts proliferation is rapid (Lefebvre and Smits, 2005).

This expression pattern was surprising, as cartilage is not one of the 'traditional' tissues used to study the GC and secretory pathways. Liver and brain, in which many currently known membrane traffic components have been identified, are much more commonly used. What role could a Golgi protein have in this 'non-typical' secretory tissue? The cargo secreted by cartilage is very different from the cargo of liver and brain. Nevertheless, chondroblast and chondrocytes do secrete large quantities of ECM with Col II as a major component (Lefebvre and Smits, 2005). Furthermore, the membrane traffic is

clearly vital for these cells, since defects in COPII vesicle formation cause severe cartilage phenotypes (Boyadjiev et al., 2006, Lang et al., 2006, Townley et al., 2008). In addition, the extent of different post-translational modifications in the cartilage ECM is remarkable (Myllyharju, 2003, Kluppel et al., 2005, Chintala et al., 1995). The cartilage matrix modifications begin in the ER with numerous hydroxylysine and hydroxyproline modifications that are especially frequent in collagen chains (Myllyharju and Kivirikko, 2004). The extensive glycosylation and especially glycosaminoglycan modification of the matrix has been shown to be important for the regulation of chondrogenesis and for growth factor signalling (Kluppel et al., 2005, Chintala et al., 1995, Koziel et al., 2004, Stickens et al., 2005). In this respect, cartilage can indeed be viewed as a tissue where the secretory pathway has a central role.

In addition to expression in the cartilage anlagen, *GoPro49* expression was also detected in the interdigital zones that are removed by apoptosis in species with free digits (Zuzarte-Luis and Hurle, 2005). Surprisingly, at E12.5 *GoPro49* expression was limited to these interdigital areas with little expression observed in the digit condensations marked by *Col II*. At E14.5, expression could be seen in both the interdigital zones and digit cartilage. This change in expression domain from the interdigital zones (undifferentiated mesenchyme) to the digit chondroblasts could be connected with the stage of chondroblast differentiation. At E12.5, majority of the digit chondroblasts are at early chondroblasts stage, while in E14.5 digits columnar chondroblasts form a major fraction. Congruent with this the *GoPro49* expression in long bones was mainly seen in the proliferating and columnar chondroblasts, with little expression in the resting chondroblasts.

BMP has been shown to be a major growth factor that regulates apoptosis in the interdigital zone, and the changes that it directs in these zones include downregulation of FGF signalling (Ganan et al., 1996, Montero et al., 2008, Bandyopadhyay et al., 2006, Pajni-Underwood et al., 2007). The *Fgf8* expression is first downregulated in the interdigital areas, then in the digit condensations. Notably, neither *Fgf8* nor *Fgf4* expression has been observed in mouse limbs after E12.5 (Lewandoski et al., 2000, Salas-Vidal et al., 2001). These interdigital areas correspond to the areas where *GoPro49* expression is observed first (at E12). *GoPro49* expression in the digit chondroblasts follows a few days later (E14). The timing of *Fgf* downregulation coincides with upregulation of *GoPro49* expression from interdigital areas to digits and could be significant in determining the areas where *GoPro49* is expressed. Consistent with this, FGF4 caused a slight downregulation of *GoPro49* expression in the tissue culture experiments.

This interdigital expression of *GoPro49* is interesting, as overexpression of the GoPro49 fusion protein in HeLa cells caused an increase in the number of apoptotic cells and caspase-3 activity compared to vector transfected cells (Figure 8E). On one hand this could suggest that GoPro49 has some role in the apoptotic cascade in these interdigital cells. The GC itself has been implicated in the ER stress-induced apoptosis, and several Golgi matrix proteins are known to be caspase targets during early apoptotic events, which functions to stop membrane traffic (Lowe et al., 2004, Maag et al., 2005). In addition, the GC can regulate intra-Golgi transport in cells with transport loads (Pulvirenti et al., 2008). Thus, it would be interesting to see what role GoPro49 has in these cells, and if it is a target for caspase cleavage.

On the other hand, cells of the interdigital zone are undifferentiated mesenchyme capable of forming cartilage elements and ectopic digits (Ganan et al., 1996). This could suggest that GoPro49 already has a role in mesenchyme before formation of the cartilage condensations, even though expression increases in later stage chondroblasts. In line with this hypothesis, *GoPro49* expression could also be detected in E10.5 limb bud RNA, where mesenchyme is still largely undifferentiated. The interdigital cells do not express *Col II* and the amount of secreted ECM is not nearly as high as in cartilage. Furthermore, in several species like bats, and in BMP2-BMP4 double conditional mice, these areas are retained

(Bandyopadhyay et al., 2006, Weatherbee et al., 2006). If *GoPro49* is expressed in these areas in bats or in BMP2-BMP4 double conditional mice, this would indicate that the role of GoPro49 is not related to interdigital apoptosis.

In teeth *GoPro49* was expressed most intensely in the condensed mesenchyme surrounding the tooth germ, but it was absent from the dental papilla mesenchyme (Publ. III and Publ. IV). The expression was limited to the dental follicle area at all the stages examined. At the bud stage the condensed dental mesenchyme is a morphologically homogenous cell population. The fact that *GoPro49* is already differentially expressed in the presumptive dental follicle and papilla cells at the bud stage shows its usefulness as a marker for the dental follicle cells. However, at later stages the dental follicle consists of a heterogeneous population of cells that can differentiate into cementoblasts, osteoblasts and PDL fibroblasts (Nanci and Bosshardt, 2006). It could well be that *GoPro49* is only expressed in a subset of these cells.

GoPro49 expression was observed in the dental follicle of both incisors and molars, and during postnatal stages it was mainly expressed in the root follicle, but not in the crown follicle (Publ. IV). At least in dogs, the crown and root follicle appear to have different functions during tooth eruption (Marks, 1995, Marks and Cahill, 1987). The bone resorption that allows eruption of the tooth through the bone is regulated by the crown follicle, while the root follicle stimulates bone formation at the tip of the root, and this is necessary for tooth movement towards the oral cavity (Marks and Cahill, 1987). This could indicate a specific function for GoPro49 in the root follicle, perhaps related to the eruption and movement of the tooth. How GoPro49 might participate in this process remains to be seen.

During postnatal tooth development, strong *GoPro49* expression was observed in the dental follicle, but not in the mature periodontal ligament (Publ. IV). This is well in line with observation that it is expressed in columnar chondroblasts, but not in the terminally differentiated hypertrophic chondrocytes (Publ. III). However, while dental follicle cells may form several cell types, the columnar chondroblast are already committed to the chondrocyte linage and they show as almost synchronised stage of differentiation with neighbouring cells (Lefebvre and Smits, 2005). Whether protein function is linked to the differentiation level of cells requires further study. Cartilage and dental follicle have in common the formation of connective tissue, and the secretion of extensive fibrous ECM. Perhaps trafficking of specific cargo (such as Col II), or a change in the membrane transport load could trigger *GoPro49* expression.

In addition to the mesenchymal tissues where *GoPro49* expression was detected on the RNA level, our proteomic study showed GoPro49 to be expressed in liver. However, little expression was observed in embryonic liver using *in situ* hybridisation, but expression levels in adult tissues could be different. Some expression was detected in the floor plate at E13, and in the brain. As the probe used may also detect the shorter isoform, it will be interesting to study which isoform is expressed in neurons. It could be that the isoform B expression could explain some of these differences in expression in different tissues.

Besides the large scale genome sequencing approaches and our studies there is only one other publication about GoPro49, in which *GoPro49* is mapped to an autism locus (Morrow et al., 2008). These authors describe a large, 886-kbp homozygous deletion in 3q24 [including the whole coding region for GoPro49/DIA1 (deleted in autism 1)] as a possible cause for autism in one patient affected by autism and epilepsy (Morrow et al., 2008). This human deletion indicates that GoPro49 functions can be compensated for by other proteins during development; but whether it is a cause of autism remains to be seen, as the large deletion also included several other genes. Furthermore, as no growth phenotype

was reported in this patient, the role of GoPro49 in cartilage development can not be indispensable. Nevertheless, as this deletion was only detected in one patient, subtle phenotypes in growth can not be excluded. In addition, whether the longer or shorter isoform is the one expressed in the human postnatal brain was not addressed in that study.

Morrow et al. also suggest that the gene is a downstream target of the MEF2 transcription factor, as MEF2A and MEF2D lentivirus RNAi vectors caused downregulation of *GoPro49* gene expression in cultured, activated rat hippocampal neurons. In addition, after depolarisation with KCl the *GoPro49*/*DIA1* gene expression increased (~1.4x) in rat hippocampal cultures, as detected by using an Affymetrix microarray, which recognises the longer isoform. Based on *GoPro49* downregulation by MEF2 RNAi, it would be interesting to study whether the third MEF2 transcription factor (MEF2C), which activates *Col X* and *VEGF* expression (Arnold et al., 2007), could also regulate *GoPro49* expression.

5.3 GoPro49 localisation in β-COP positive structures and possible function

GoPro49 was shown to localise to the GC as an EGFP-fusion protein and to co-localise with GM130 in HeLa cells (Publ. I). However, the endogenous protein co-localised with β-COP in chondrosarcoma cells, but only slightly with the *cis*-Golgi marker GM130 (Publ. III and Publ. IV). The observed difference in the localisation of the endogenous GoPro49 and the EGFP-fusion protein could be due to various factors. Firstly, chondrosarcoma cells have a higher amount of secretory cargo than HeLa cells. Secondly, the tag could interfere with some protein interactions required for correct cellular localisation. Thirdly, overexpression might alter the localisation. The latter hypothesis is not surprising if we consider that the protein is in the secretory pathway. Membrane trafficking is a very sensitive system, and overexpression of any of its components might disturb the natural balance and alter the localisation (Cosson et al., 2005).

The endogenous protein localisation suggests that the protein might have a role in the secretory pathway, maybe in membrane transport events between the ER and the GC. The β-COP staining observed in the chondrosarcoma cells was broader and more tubular than GM130 in the *cis*-Golgi, and it could indicate COPI vesicles in the ERGIC. Furthermore, the atypically long vesicles/tubules seen in these cells could reflect the differential requirements of cartilage matrix transport (such as Col II). Indeed, when the amount of newly synthesised proteins transported from the ER was reduced by treatment with cycloheximine, both GoPro49 and β-COP were observed in more juxtanuclear structures (Publ. III, Fig 7). In addition, the vesicular/tubular morphology of these β-COP positive structures was considerably reduced. However, cycloheximine treatment might also affect the cytoskeleton and thus reduce tubule formation from the GC.

What role could GoPro49 have in the β-COP positive membranes, especially in cartilage? The co-localisation of GoPro49 and Col II in these β-COP-positive structures could suggest a role in ECM secretion, for example as a specific chaperone for ECM protein or as a receptor in recycling or transport of Col II. Proteins that have been identified in COPI vesicles include proteins needed for recruitment and formation of the coat: p23/24, coatomer subunits, Arf and ArfGAP; and cargo receptors like the KDEL-receptor and cargo (Lowe and Kreis, 1996, Palmer et al., 1993, Tanigawa et al., 1993, Gommel et al., 2001, Lanoix et al., 2001, Reinhard et al., 2003). In addition, proteins that participate in the tethering event, such as Giantin and p115, have been found in these vesicles (Sonnichsen et al., 1998).

Whether these potential COPI vesicles are retrograde or possible anterograde transport vesicles was not addressed in this study. However, if these membranes participate in retrograde transport towards the ER, what could be the role of GoPro49? Could it behave as a specific chaperone for Col II and assist in its folding? Or could it act as a receptor that detects unfolded or unassembled collagen and relocates it to the ER? The GoPro49 sequence does not contain any typical heat shock protein domains, but this is also true for the collagen-specific chaperon Hsp47 (SMART domains, Letunic et al., 2008). Furthermore, Hsp47 and GoPro49 proteins are roughly the same size, and both are predicted to have two large globular domains separated by a short linker (Puntervoll et al., 2003).

If these membranes are moving in an anterograde direction, they are probably involved in transport from the ERGIC to the GC. According to the current view, procollagen I is too large to be accommodated in small vesicles and is transported through the GC without leaving the lumen (Bonfanti et al., 1998), which is also likely the case for Col II. Thus if collagen does not utilise vesicles for intra-Golgi transport, it should not cause such tubulation in the morphology of the β-COP positive structure, unless the pre-Golgi stage is the one affected.

Orthologous genes for *GoPro49* have been identified in several species, and the sequence identity is high. However, as orthologous sequences were also found in non-vertebrate species, it is unlikely that the protein has a role only in cartilage ECM or collagen secretion. Perhaps GoPro49 activity is related to the transport of large or difficult cargo. Nevertheless, some non-vertebrates do express collagen-like proteins (Livingston et al., 2006), so there could be some similarities in their cargo too. While there was a clear increase in *GoPro49* expression levels from E7 to E15 in mice (Publ. III and Figure 8B), the orthologous zebrafish genes were detected already in the maternal RNA pool, and with relatively constant levels from 0.5 h to 72 h pf. During this time (by 72 h post fertilisation) the first cartilaginous elements of the zebrafish craniofacial area have already formed (Kimmel et al., 1995). This suggests that in zebrafish these genes may be required at an earlier stage than the corresponding gene is in mouse.

The only siRNA phenotype that was observed was characterised by a slight tubulation of β-COP-stained structures. This suggests that GoPro49 might have a role in the assembly or packing of these membranes. Whether the lack of phenotype in morpholino k.d. zebrafish was due to inefficient k.d. by morpholinos or to paralogous gene functions is unclear. Unfortunately, the dose of morpholinos in the double k.d. experiments could not be increased as the concentration used was close to that reported for non-sequence specific effects. In addition, the early presence of maternal RNA makes the morpholino k.d. less efficient. Regardless, the gene deletion in the autism patient would suggest that GoPro49 functions can be compensated for during development and formation of cartilage (Morrow et al., 2008). However, as this deletion has been found in only a single patient, the presence of a subtle phenotype in cartilage might be possible.

5.4 Potential regulation mechanisms of GoPro49 expression

Based on the *GoPro49* expression in columnar chondroblasts and dental follicle, and known signalling in these tissues, what growth factors could regulate *GoPro49* expression? Which of them should be studied further? FGF did not upregulate *GoPro49* in our tissue culture experiment; instead it seemed to slightly downregulate its expression. The role of FGFs is to promote proliferation of chondroblast progenitors, and to inhibit differentiation during limb bud formation (Lu et al., 2008b, Moon and Capecchi, 2000, Lewandoski et al., 2000). Furthermore, the expression of *Fgf*s in AER is reduced by E11.5-12.5, when *GoPro49* expression is still prominent (Lewandoski et al., 2000, Salas-Vidal et al., 2001). Thus, the

role of FGF might be to downregulate *GoPro49* expression at earlier stages. The FGF receptors are expressed throughout the limb mesenchyme at early stages, but then become restricted to condensations and cartilage (Delezoide et al., 1998). Thus FGF signalling is active in these cells.

The Wnt pathway is a good candidate to be a regulatory pathway for *GoPro49* expression, because several Tcf/Lef sites (Wnt/β-catenin) were identified in the 5’ upstream sequence. However, canonical Wnt signalling inhibits chondrogenesis, and expression of active β-catenin in early chondrocytes blocks differentiation and mineralisation (Tamamura et al., 2005, Akiyama et al., 2004), which is the opposite of the *GoPro49* expression pattern in columnar chondroblasts. In contrast, non-canonical Wnt signalling promotes proliferation and differentiation of chondroblasts, but in this regulation it does not use the Tcf/Lef transcription factors (Yang et al., 2003, Topol et al., 2003).

BMPs (Smads and Sox5 sites) are expressed between digits and have a role in interdigital apoptosis (Ganan et al., 1996, Montero et al., 2008, Bandyopadhyay et al., 2006). BMPs also regulate the transition from proliferating, columnar chondroblasts to hypertrophic chondrocytes (Adams et al., 2007). Furthermore, the balance between FGF and BMP signalling is believed to determine the pace of proliferation versus differentiation (Minina et al., 2002). All these correlate well with *GoPro49* expression in columnar chondroblasts, and thus BMP is a good candidate to mediate regulation. In addition, TGFβ family signalling positively regulates chondroblast differentiation from progenitor mesenchyme via p38 kinase pathway (Watanabe et al., 2001, Oh et al., 2000). Thus TGFβs could also be likely candidates for regulation. The reason why the bead induction assay did not reveal these pathways could be related to the fact that the probe for *GoPro49* was not suited to whole mount *in situ* hybridisation, and therefore other methods of detection such as RT-PCR should be tested. Furthermore, the fact that at the time point studied (E11.5 to E12.5) BMP promotes apoptosis in limb mesenchyme, which decreases the number of cells possibly especially those where *GoPro49* is expressed, could make the detection more difficult.

The fourth major regulator of chondrogenesis, Ihh, promotes chondrocyte hypertrophy and chondroblast differentiation in a regulatory loop with PTHrP and also independently (Kobayashi et al., 2005, Lanske et al., 1996, Vortkamp et al., 1996). This does not coincide well with *GoPro49* expression in columnar chondroblasts. In addition, no Gli sites were observed in the 2kb upstream sequence. However, the other half of this regulatory loop, PTHrP, could be a possible regulator. PTHrP promotes columnar chondroblast proliferation and it suppresses entry into hypertrophy (Lanske et al., 1996, Vortkamp et al., 1996). The target cells for PTHrP signalling match exactly with the cells where *GoPro49* expression was observed.

Growth factors that regulate dental follicle and root formation are not as well characterised as those for cartilage. Enamel knot is known to signal to the dental papilla and to promote proliferation. Downregulation of FGF10 is important for root formation, and BMP4 regulates formation of HERS (Thesleff, 2006, Yokohama-Tamaki et al., 2006, Hosoya et al., 2008). However, none of these growth factors have been directly linked to dental follicle development, which might or might not be regulated in a manner similar to the dental papilla. In conclusion, at present there is not sufficient information to provide rationalisation for the regulation of *GoPro49* in the dental follicle.

6 CONCLUDING REMARKS

Although much is known about transport and cell biological aspects of the Golgi complex, the difference in the secretory pathway between different tissues has not been thoroughly explored, with the focus being on the cellular level (and on a few cell lines). However, different tissues have different requirements and cargo, and many aspects of transport may depend on the cell type, organism or developmental stage.

This thesis describes identification and characterisation of a novel protein, GoPro49 that localises to the β-COP positive structures based on immunofluorescence, and is expressed mainly in cartilage during embryogenesis. *GoPro49* expression levels seem to be linked with the differentiation stage of cartilage and dental follicle cells, and may be connected to transport of specific types of cargo, such as Col II. This restricted expression pattern also raises the question of whether there are other Golgi proteins with tissue specific expression patterns. Based on the localisation in the β-COP positive structures, GoPro49 is likely to have a role in membrane traffic. However, what exact role this protein or its shorter isoform has will require further study. Furthermore, whether the two splice variants have similar expression patterns remains to be seen.

Retrospectively, it is surprising how few studies there are on cell biological aspects of both cartilage and dental follicle. Both of these connective tissues have a large impact on the individual if their functions, including secretion of the matrix, are impaired. The morphology and ECM are well known, but molecular mechanisms, for example of Col transport, are still not known in detail. The differential, large cargo of chondroblasts, mainly Col II, might also explain why these cells would require a different set of Golgi proteins, especially during development.

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