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# **Chapter 1:**

## **INTRODUCTION**



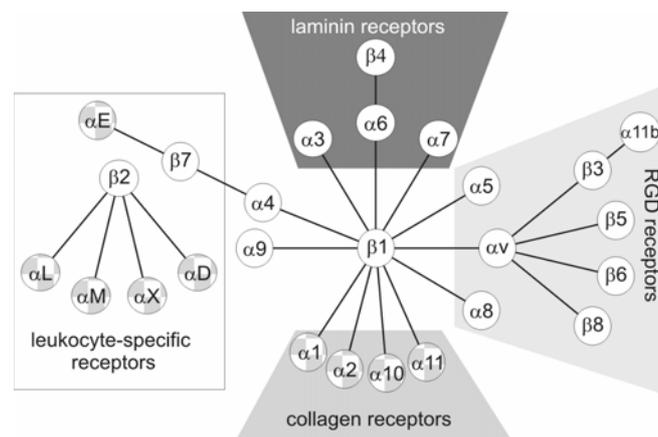
## Introduction

Anchorage-dependent cell growth and survival is a hallmark of most normal cells. The ability of cells to adhere to an extracellular matrix (ECM) is mediated by a family of proteins called the integrins. Integrins are transmembrane proteins that form the integral membrane complex that links the ECM to the intracellular cytoskeleton [1]. Except for anchorage-dependent growth, integrins also maintain tissue integrity and cell polarity within the organism and regulate differentiation. The integrins are only expressed in the metazoan and homologues are not present in prokaryotes, plants or fungi [2].

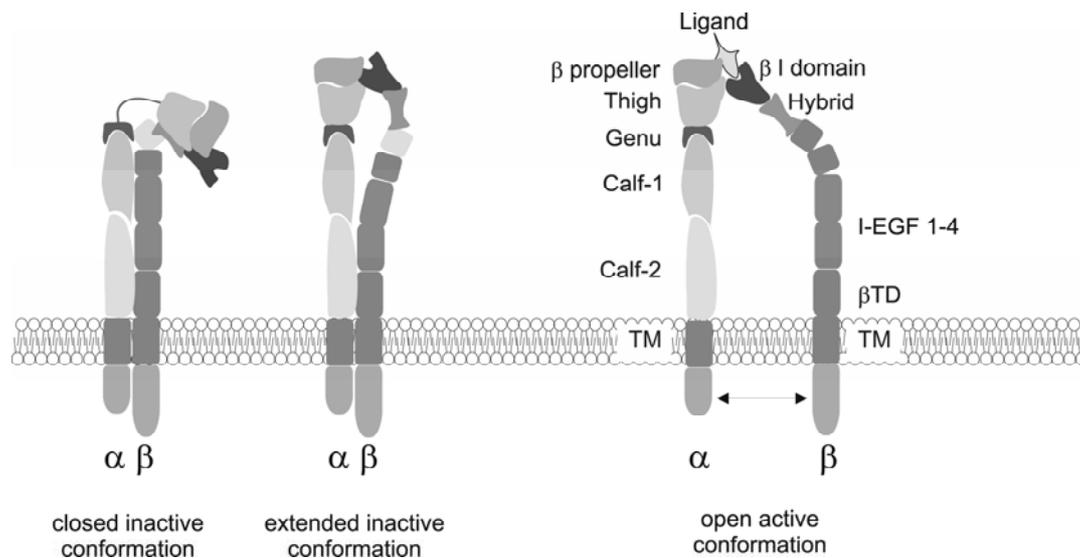
## The integrins

Integrins form a heterodimeric complex consisting of two subunits that are non-covalently linked in a  $\alpha\beta$  configuration. Presently, there are 18  $\alpha$  subunits and 8  $\beta$  subunits known that can form 24 different heterodimers [3]. The  $\beta 1$  subunit is promiscuous and binds to 12 different  $\alpha$  subunits but other  $\beta$  subunits are more restricted and only bind to a single  $\alpha$  subunit [3] (Figure 1). Heterodimerization occurs in the extracellular domain where continuous intersubunit contact exists between the  $\beta A$  region of the  $\beta$  subunit and the  $\beta$ -propeller of the  $\alpha$  subunit [4]. An arginine residue that protrudes from the  $\beta A$  region is locked in the core of the central channel of the  $\beta$ -propeller by two rings of mostly aromatic amino acids [5]. Interestingly, it has also been shown that the heterodimerization of  $\alpha 5\beta 1$  integrins depend on the glycosylation of three sites on the  $\beta$ -propeller of  $\alpha 5$  [6].

Organisms such as *Caenorhabditis elegans* have one  $\beta$  and two  $\alpha$  subunits while *Drosophila melanogaster* has two  $\beta$  and two  $\alpha$  subunits [7]. One of the two heterodimers recognizes the tripeptide RGD sequence present in ECM components such as fibronectin (FN) and vitronectin (VN) while the second binds to basement membrane laminin [3]. In vertebrates these classes are expanded to include collagen receptors containing an inserted I/A domain like  $\alpha 1$  or  $\alpha 2$  and a pair of related integrins that recognize ECM proteins like fibronectin and Ig-superfamily cell surface counterreceptors like VCAM-1 (Figure 1) [3]. The ability of integrins to adhere to their ligand needs to be regulated in order to promote dynamic processes such as migration and cell adhesion. In the last years, structural biology has provided illuminating new insights into regulation of integrin activation.



**Figure 1.** The integrin heterodimers present in mammalian cells are divided into categories by their ability to bind to laminin, collagen or RGD-containing protein-containing proteins. Integrins containing an inserted I/A domain are represented as blocked.



**Figure 2.** A schematic diagram of an integrin and its activation after the opening of its cytoplasmic legs making the extracellular ligand-binding site accessible.

Integrins are regulated by the affinity for their ligand that in turn is regulated by the conformation of the  $\alpha\beta$  heterodimer [3]. The extracellular domains of the  $\alpha$  and  $\beta$  subunits are in a bend, closed conformation when the integrin is inactive, only allowing low affinity binding of ligand. Different queues can initiate a conformational change resulting in an active, open extended position of the integrin. For example, activation occurs when talin binds to the cytoplasmic tail of the integrin and separates the cytoplasmic legs from one another which in turn opens the extracellular domains allowing high affinity binding of the integrin to ligand (Figure 2) [3]. Therefore, adhesive properties of the integrins can be regulated from inside the cell in a process called inside-out signaling but ligand binding outside the cell also influences processes like cell growth and gene expression, through outside-in signaling.

At the site of integrin-ligand interactions a protein complex, called the focal adhesion (FA), forms that contains more than fifty known interacting proteins and is connected to the intracellular actin cytoskeleton [8]. Focal adhesions were first identified as electron dense regions of the plasma membrane (PM) at the cell-substratum interface in cultured cells [9]. As integrins themselves do not have inherent enzymatic or actin-binding abilities, proteins associated with the integrins must mediate these activities. These proteins can be divided into three classes according to their localization as shown in table 1 and include proteins in the extracellular space, at the cell membrane and in the cytoplasm [8]. FAs not only form the link between the ECM and the actin cytoskeleton but also actively govern this linkage and the organization of the cytoskeleton. This regulatory activity is essential for processes like adhesion and migration but also influences transcription and protein expression. The study of FA dynamics was greatly accelerated with the advent of fluorescence technology. GFP-tagged proteins are now used to visualize the localization of FAs and to calculate their dynamics and turnover [10]. Several important features have been discovered using this technology. Firstly, using GFP-tagged integrins, FAs can be divided into low-density and high-density adhesions. Secondly, the density of FAs can change dramatically over time. Thirdly, high- and low-density adhesions are located in different compartments within the PM and fourthly, only high-density adhesions have mobility or are sliding [10]. Furthermore, we now know that low-density adhesions are formed in response to the activity of the small GTPases Rac1 and cdc42 and transform to high-density adhesion formation when RhoA GTPase activity increases and acto-myosin contraction occurs [11].

### The Src protein family

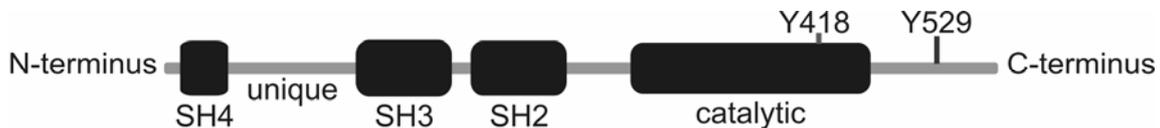
As mentioned above signaling from the FA regulates the dynamics of the actin cytoskeleton and its own turnover and these processes are mediated by protein tyrosine kinases such as Src.

Location	Focal adhesion protein	
Extracellular	Collagen, fibronectin, heparan sulphate, laminin, proteoglycan, vitronectin	
Transmembrane	Integrins, LAR-PTP receptor, layilin, syndecan-4	
Cytoplasmic:	structural	Actin, $\alpha$ -actinin, EAST, ezrin, filamin, fimbrin, kindling, lasp-1, LIM nebullette, MENA, moesin, nexilin, paladin, parvin, profilin, ponsin, radixin, talin, tensin, tenuin, VASP, vinculin, vinexin, MARCKS
	enzymatic	Tyrosine kinases: Abl, Csk, FAK, Pyk2, Src, prot. Serine/threonin kinases: ILK, PAK, PKC, Protein phosphatases: SHP-2, PTP-1B, ILKAP, Small GTPase modulators: ASAP1, DLC-1, Graf, PKL, PSGAP, RC-GAP72
	adaptors	p130Cas, caveolin-1, Crk, CRP, cten, DOCK180, DRAL, FRNK, Grb7, Hic-5, LIP.1, LPP, Mig-2, migfilin, paxillin, PINCH, syndesmos, syntenin, tes, Trip 6, zyxin

**Table 1.** Proteins present in the FA ordered by localization and general function [8].

v-Src is the prototype of the Src protein tyrosine kinase family and was discovered as the transforming protein in the oncogenic retrovirus, Rous sarcoma virus (RSV) [12]. The Src family contains ten proteins with significant homology to Src. They are Fyn, Yes, Yrk, Blk, Fgr, Hck, Lck, Lyn and the Frk subfamily proteins Frk/Rak and Iyk/Bsk (reviewed in: [13]).

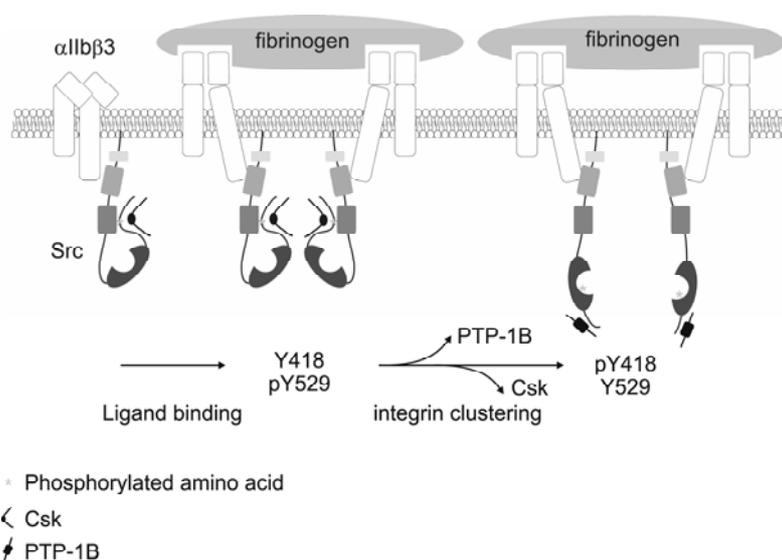
Src proteins contain six functional domains as depicted in Figure 3. The domains are a Src homology 4 domain (SH4), the unique region, the SH3 domain, the SH2 domain, the catalytic domain and a short negative regulatory tail. The proteins are targeted to the membrane by the myristoylation moiety on the N-terminal glycine residue [14]. Src is kept in an inactive state by several intramolecular connections that are released in a stepwise process when it is activated. The SH2 and SH3 domain bind to the catalytic domain preventing it from phosphorylating its substrates [15]. More specifically, the SH3 domain binds to the catalytic domain as well as the linker region that lies between the SH2 and the catalytic domain [16] while the SH2 domain interacts with the Tyr529 residue in the negative regulatory tail when it is phosphorylated by the tyrosine protein kinase Csk [17,18].



**Figure 3.** Diagram of the structure of Src showing the six different domains and the two tyrosine residues involved in regulating the proteins activity.

In a recent review Shattil [19] proposes that the first constraint, the interaction of SH3 with the catalytic domain, is removed when Src directly binds to the cytoplasmic C-terminal 4 amino acids of integrin  $\beta 3$  resulting in a primed Src molecule [20]. When  $\alpha$ IIB $\beta 3$  binds to the multivalent ligand fibrinogen the integrins are clustered and their associated Src molecules come in close contact to one another (Figure 4) [21]. This allows the autophosphorylation of Src in *trans* on Tyr418 that is essential for full activation of Src. Simultaneously, clustering would lead to the displacement of Csk [22] and the dephosphorylation of Tyr529 by the protein tyrosine phosphatase, PTP-1B [23] making the catalytic cleft accessible.

Within the FA, active Src can bind to the scaffold protein, focal adhesion kinase (FAK) and subsequently phosphorylate a number of sites on FAK that enable the docking of other FA proteins [24]. For example, Src-mediated phosphorylation of Tyr925 on FAK allows the recruitment of Grb2 [25] while the Crk-associated tyrosine kinase substrate p130Cas binds to FAK when the residues Tyr576 and Tyr577 are phosphorylated [26]. Src-mediated FAK binding of p130Cas initiates a major signaling route to Rac GTPase and lamellipodia formation (Figure 5) [27]. Phosphorylated p130Cas recruits Crk, that in turn recruits the Rac1 activating protein, DOCK180 to FAs [28]. This complex then activates RacGTPase that induces lamellipodia formation at the leading edge of the cell. On the other hand, Src-mediated binding of Grb2 to FAK initiates a pathway via Sos, Ras and Raf to Mek leading to ERK activation [29]. While these are two examples of signaling within the FA, many signaling pathways are initiated from the FA (Figure 5).



**Figure 4.** The steps leading to the activation of Src as proposed by S.H. Shattil is a multi-step process dependent on integrin binding to fibrinogen and subsequent clustering of the integrins.

### Integrins and gene regulation

As depicted in Figure 5 signaling pathways initiated from the integrins via FA proteins can also regulate gene expression. For instance, in cultured mammary epithelial cells it was shown that upon integrin binding to ligand, casein transcription is upregulated [30]. In *Drosophila*, expression of mutants of the integrin βPS were used to identify genes whose transcription is compromised indicating that the integrin is important for gene expression [31]. Moreover, fibroblasts adhering to a mixed matrix of FN and tenascin had increased collagenase, stromelysin, gelatinase and c-fos expression [32] while the addition of antibodies against the integrin subunits α1 or α2 to cells inhibited stromelysin expression [33]. In another study it was shown that α5β1 binding to FN or αvβ3 binding to VN resulted in increased Bcl-2 expression mediated by the PI3K-Akt pathway [34]. Lastly, re-expression of αvβ3 expression in a β3-knockout cell line resulted in increased cdc2 expression along with increased migration [35] while β3 overexpression in CHO cells led to decreased uPAR expression [36].

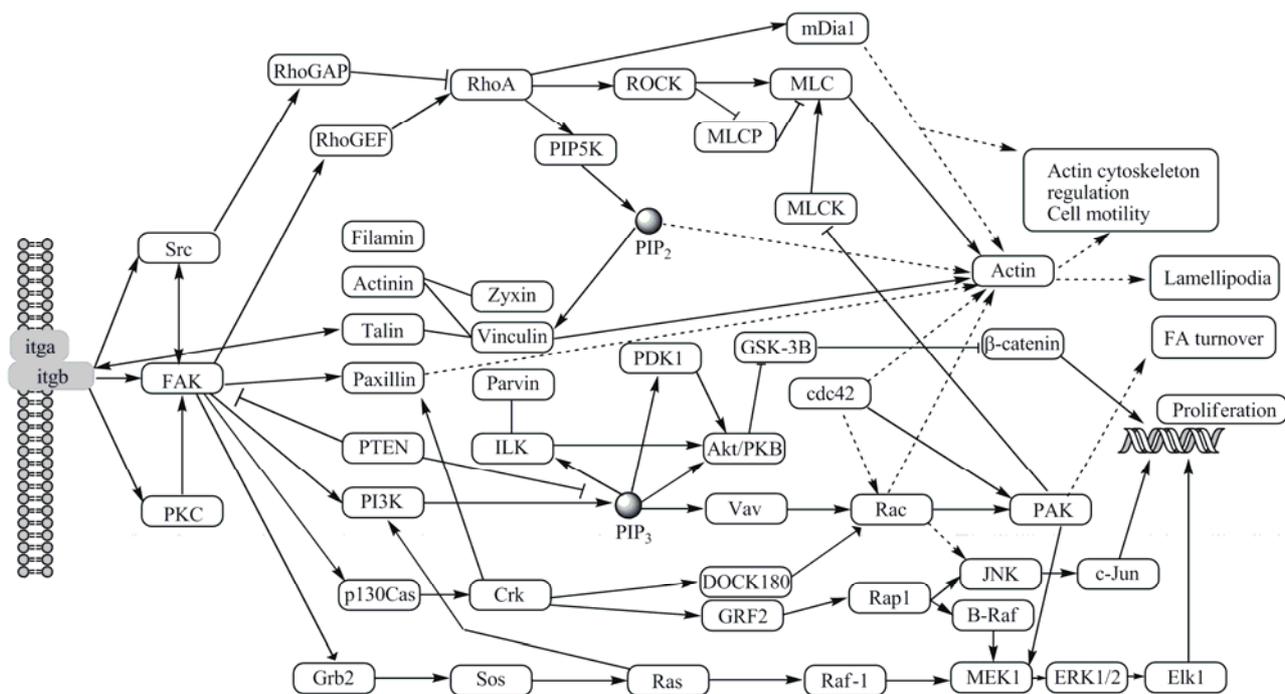
Integrins not only regulate transcription but can also influence other checkpoints of expression. For example, protein expression is altered in platelets from patients with Glanzmann's thrombasthenia during the platelet activation response [37] through the regulation of pre-mRNA splicing even though platelets are anucleate [38].

FAs contain proteins that can shuttle between the FA and the nucleus and are involved in transcription or mRNA localization. Treating cells with Leptomycin B to inhibit CRM-1-dependent nuclear export promotes the nuclear accumulation of a number of FA proteins such as zyxin family proteins including zyxin, LPP and TRIP6 and paxillin family proteins including paxillin and Hic-5 (for review: [39]). In the cytoplasm zyxin regulates the assembly and organization of actin [40] but it accumulates in the nucleus when cells are mechanically stimulated while zyxin knockdown influences stretch-induced gene expression [41]. Zyxin simultaneously binds to p130Cas and ZNF384 (zinc finger protein 384) linking these two proteins together in the nucleus where they influence transcription [39].

Paxillin is another protein that shuttles between the FA and the nucleus [39]. At FAs it recruits proteins such as Crk and influences cell migration [42]. Paxillin binds to the mRNA polyA binding protein, PABP1, in the nucleus and assist in the nuclear export of mRNA to sites of translation in the ER but also at the leading edge of cells [43]. Localized translation at the leading edge regulated by paxillin and PABP1 was subsequently shown to be important for migration and FA turnover [44]. Therefore, integrins can signal through focal adhesion proteins to regulate gene expression.

### N-glycosylation and its role in integrin-mediated cell adhesion.

As mentioned above some integrins like α5β1 are highly glycosylated and these N-glycan chains are important for the heterodimerization of the integrin [6].



**Figure 5.** A diagram showing a number of pathways originating from the focal adhesion and ultimately regulating processes like motility, proliferation and focal adhesion turnover.

More specifically, the N-glycosylation of residues on the extracellular  $\beta$ -propeller domain of the  $\alpha$  subunit are essential for the dimerization of the integrin [6]. Moreover, inhibition of glycosylation was shown to inhibit adhesion of cells to the ECM [6].

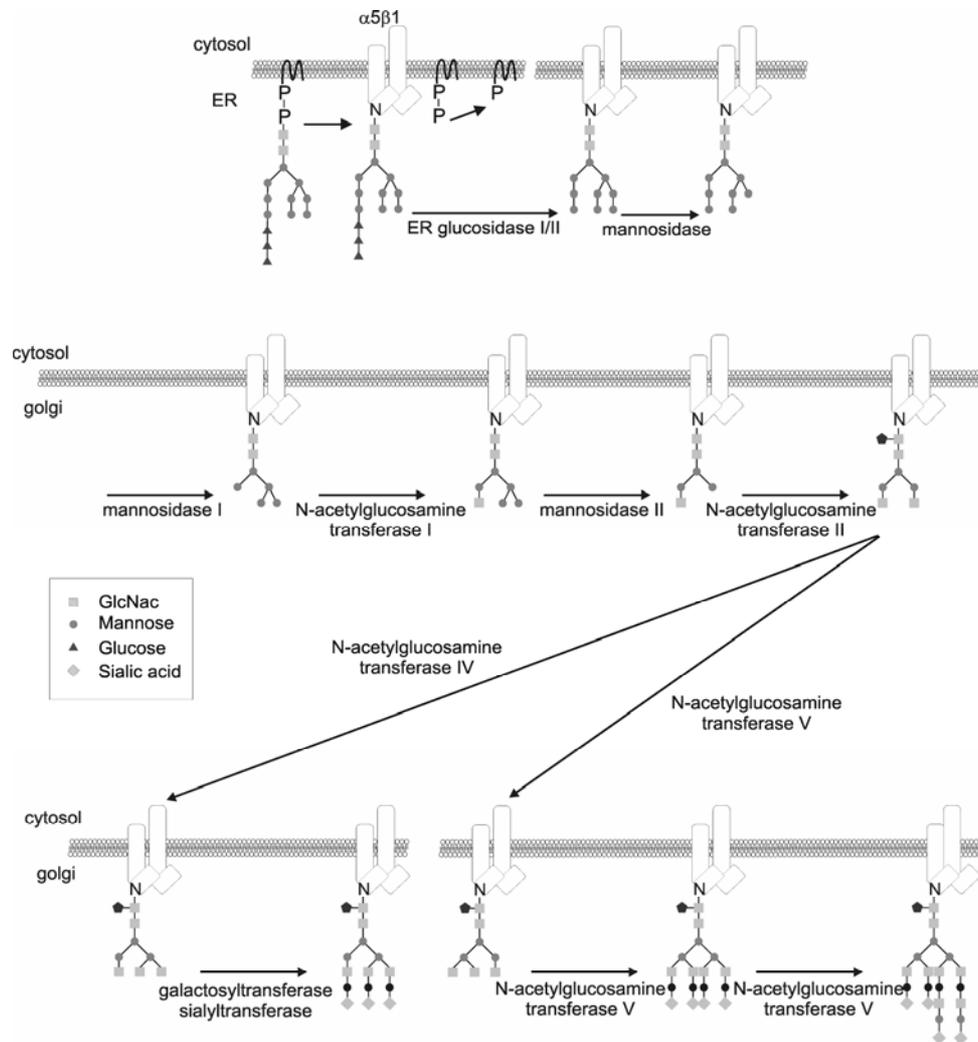
What are N-glycan chains? They are carbohydrate chains consisting of  $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$  that are co-translationally added from the dolichol carrier onto the Asn residue of almost 70% of the Asn-X-Ser/Thr ( $X \neq \text{Pro}$ ) motifs exposed to the lumen of the endoplasmic reticulum (ER)(Figure 6) [45]. These chains are subsequently trimmed and remodeled upon passage through the Golgi resulting in heterogeneous chains [46]. Remodeling and addition of N-glycan chains is dependent on the availability of the substrate UDP-N-acetylglucosamine (UDP-GlcNAc) and determines the heterogeneous mix of modified N-glycan chains on receptors [46].

A class of proteins called lectins is characterized by their ability to bind to carbohydrate chains. Within this class one family of proteins, called galectins, binds specifically but with low affinity to  $\beta$ -galactose. However, when  $\beta$ -galactose is attached to other saccharides like acetylglucosamine to form N-acetyllactosamine, galectins can bind with high affinity [47]. Branching of the N-glycan chains into tri- or tetra-antennary forms in the trans-golgi creates the high affinity ligand galectins preferentially binds to [46]. Galectins are defined by the conserved, 130 amino acid long carbohydrate-recognition-binding domain (CRD) [48]. Thus far 14 mammalian galectins have been identified but homologues are also present in plants, fungi, sponges, nematodes, insects and viruses, but not in yeast [49]. Based on the number and organization of the CRD, galectins can be divided into three subtypes. These are (1) the prototype group consisting of galectin-1, -2, -5, -7, -10, -11, -13, and -14 and characterized by the presence of a single CRD, (2) the chimera group consisting only of galectin-3 that contains a single CRD connected to a long N-terminal proline- and glycine-rich region and (3) the tandem repeat group consisting of galectin-4, -6, -8, -9, and -12 characterized by the presence of two CRD domains [48].

The CRD is responsible for the binding to N-glycan side chains. While all galectins bind to the basic disaccharide unit,  $\text{Gal}\beta 1-3/4\text{GlcNAc}$ , some specificity has been observed due to different affinity for branched, repeated or substituted glycan chains [47].

Of all the galectins, galectin-3 has been studied most since it has been recognized as a prognostic marker in different cancers [50]. Galectin-3 expression is a poor prognostic marker in colorectal cancer [51] while overexpression of galectin-3 in gastric cancer is correlated with metastasis formation [52]. Expression of

galectin-3 has been observed in non-small-cell lung cancer [53] and medullary thyroid carcinoma [54]. Interestingly, it was reported that galectin-3 is expressed in normal mammary ducts and lobules of the breast but this expression is downregulated in cancers of these tissues, correlating with increasing histologic grade [55].



**Figure 6.** The addition and modification of N-glycan chains to integrins as it passes through the endoplasmic reticulum and the Golgi.

Galectin-3 was first identified as a cell surface antigen of peritoneal macrophages called Mac-2. Synonyms for galectin-3 include CBP-35, εBP, RL-29, HI-29 and L-34, LBP or non-integrin laminin binding protein [56]. Galectin-3 is expressed in a number of tissues including macrophages, neutrophils, mast cells, gastrointestinal epithelia, kidneys and neurons [56].

Structure	Group	Galectin
	prototype	1,2,5,7,10,11,13,14
	chimera	3
	Tandem repeat	4,6,8,9,12

**Table 2.** The galectin family is organized into three groups according to their domain structure.

Galectin-3 contains one CRD attached to a long, flexible, 100-150 amino acid long N-terminal region made up of homologous repeats each including the consensus region Pro-Gly-Ala-Tyr-pro-Gly [49], [56]. The N-terminus does not bind to carbohydrates itself but is essential for the activity of the protein and capable of protein-protein interactions [49]. Moreover, the N-terminus is important for the secretion of galectin-3 through a novel but unknown pathway [57].

The CRD of galectin-3 is responsible for the preferential binding to N-acetylglucosamine [49]. Within the protein sequence of the CRD the NWGR motif is of interest since it is homologous to the highly conserved motif within the BH1 domain of Bcl-2 proteins that is responsible for the anti-apoptotic activity of both Bcl-2 and galectin-3 [58]. The structure of the CRD with two anti-parallel  $\beta$ -sheets composed of five and six  $\beta$ -strands is typical for all galectins [59].

Although galectin-3 has a single CRD it has multivalent binding properties [60]. It was shown that galectin-3 precipitates as a pentamer in the presence of ligand [61], while FRET was used to show that galectin-3 can form pentamers on the extracellular surface of cells [62]. Therefore, while galectin-3 exists in solution as a monomer it is assumed that ligand binding induces its oligomerization.

Galectin-3 is found at the plasma membrane, but has also been observed in the extracellular space, in the nucleus and in the cytoplasm [49] where it can bind to a large number of proteins (Figure 7). As mentioned above, galectin-3 binds to Bcl-2 and inhibits apoptosis [58,63]. Galectin-3 also binds other proteins involved in apoptosis and cell survival such as CD95, a member of the death receptor family [64] and nucling, a regulator of apoptosis and this interaction induces apoptosis. Nucling also regulates the expression of galectin-3 by interfering with NF- $\kappa$ B activation [65]. Moreover, it was shown that galectin-3 interacts with K-Ras. Interestingly, it has a higher affinity for activated K-Ras-GTP than for inactive K-Ras-GDP [66]. By binding to K-Ras-GTP, galectin-3 increases ERK activation and induces apoptotic resistance and anchorage-independent cell growth [67].

Some controversy still exists about the mechanism responsible for the import of galectin-3 into the nucleus. It is known that galectin-3 is present in the nucleus in some cells types but this is not a general characteristic [49]. Galectin-3 associates with ribonucleoprotein complexes [68] and assists in the assembly of spliceosomes [69]. Nuclear galectin-3 is also implicated in the regulation of transcription by stabilizing transcription factors to CRE and SP1 sites present in the cyclin D1 promoter region [70]. Other genes such as cyclin A, cyclin E, p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> also contain these sites and are regulated by galectin-3 expression (Figure 7) [71].

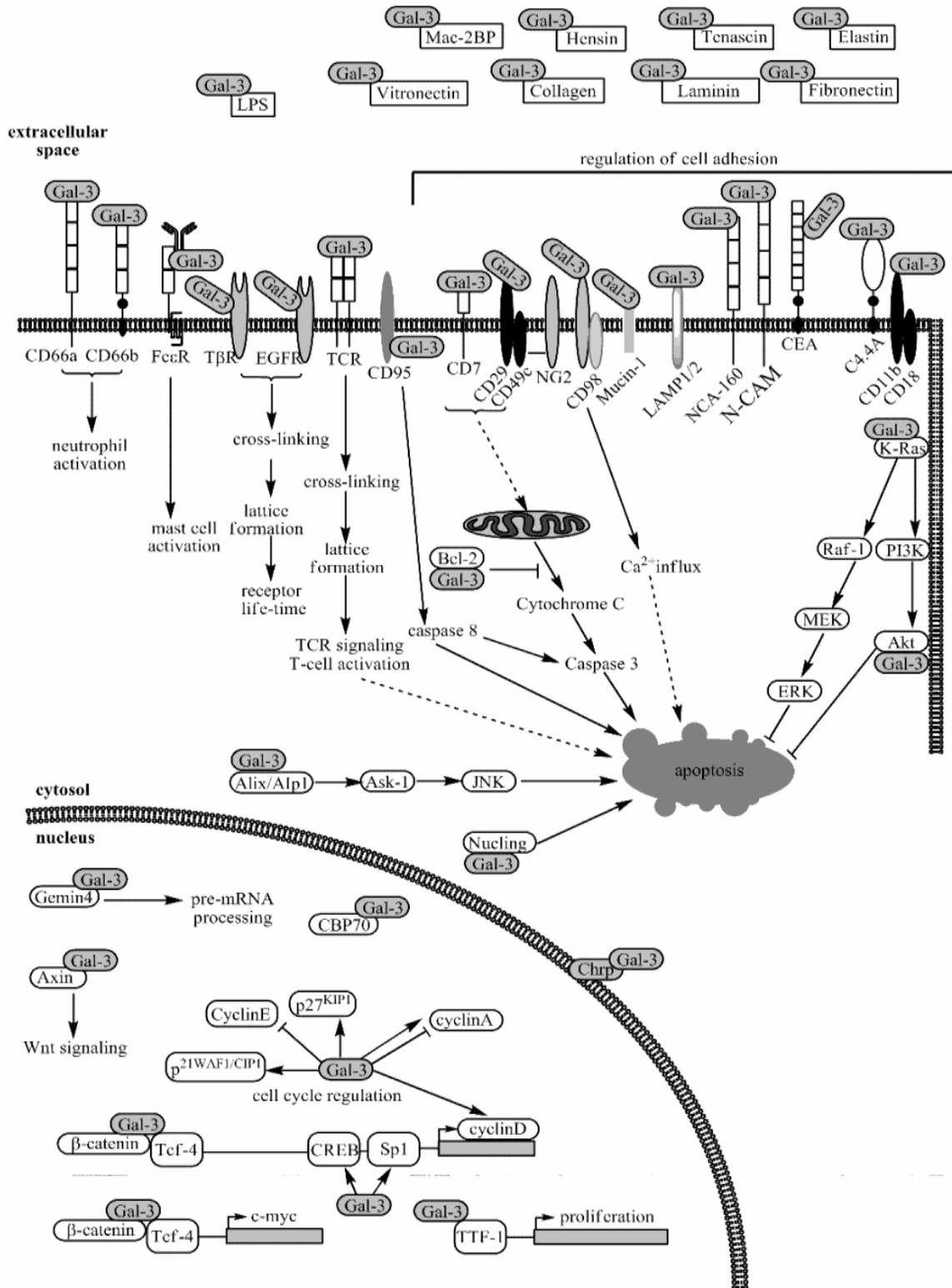
Galectin-3 is secreted into the extracellular space through an unknown mechanism since the protein contains no signal motif. In the extracellular space it can bind to a myriad of receptors that influence various processes. Except for binding to cell surface receptors, galectin-3 can also bind to a variety of extracellular proteins (Figure 7). ECM proteins that carry the tri- and tetra-antennary complex-type glycans such as laminin [72], fetal-derived fibronectin [72] and tenascin [73] are excellent galectin-3 ligands. Cell surface receptors like integrins have also been shown to directly interact with galectins. For instance, it has been shown that galectin-1 binds to  $\alpha$ 7 $\beta$ 1 in myoblasts [74], galectin-3 to  $\alpha$ 1 $\beta$ 1 [75] and galectin-8 to  $\alpha$ 3 $\beta$ 1 [76]. Furthermore, galectin-1 and galectin-3 can bind to the  $\alpha$  subunit of  $\alpha$ M $\beta$ 2 on macrophages [77,78].

There is direct evidence for a role of galectin-3 in cell adhesion. Addition of exogenous galectin-3 at low concentrations blocks adhesion of fibroblasts to laminin [75]. On the other hand, addition of increasing amounts of galectin-3 to neutrophils strengthened cell adhesion to fibronectin [79]. Moreover, overexpression of galectin-3 in the breast carcinoma cell line, BT549, leads to increased adhesion to laminin and collagen IV [80]. The role of galectin-3 in adhesion has been highlighted by studies in mice lacking the enzyme N-acetylglucosaminyl transferase (Mgat5) that catalyzes the formation of the tri- and tetra-antennary N-glycans that galectin-3 recognize. In Mgat5 knockout mouse, cells adhere poorly to FN and fibronectin fibrillogenesis is strongly inhibited, while knockdown of galectin-3 also inhibited fibrillogenesis suggesting that the proper glycosylation of integrins such as  $\alpha$ 5 $\beta$ 1 is important for the binding of galectin-3. The interaction between galectin-3 and integrins mediates cell adhesion and fibrillogenesis [81].

### **An overview of the structure and function of the MARCKS protein family.**

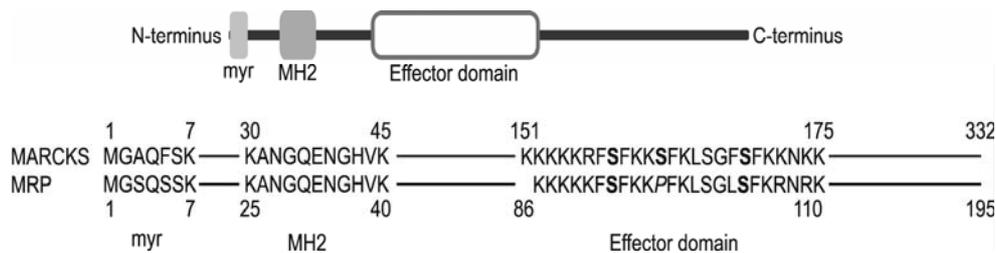
The actin cytoskeleton is essential for the ability of cells to maintain their shape and needs to be actively regulated during cellular processes like migration and spreading. The integrins regulate the actin cytoskeleton through their effect on the localization and activity of downstream proteins. There are a large number of actin-interacting proteins of which the family of MARCKS proteins is one.

The MARCKS family consists of two related proteins, MARCKS and MacMARCKS. MARCKS, which stands for myristoylated alanine rich C-kinase substrate is a 32kD protein that is ubiquitously expressed while MacMARCKS or macrophage related MARCKS, also called MRP, MLP or F52 is a 20kD protein mainly expressed in the brain, the reproductive tissue and macrophages [82], [83]. Both proteins are highly acidic which results in a higher apparent molecular weight when analyzed by SDS-PAGE. The proteins in this family have three characteristic motifs (Figure 8a). The N-terminus is myristoylated on the glycine residue present directly after the initial methionine residue. Myristoylation occurs when a C<sub>14</sub> saturated fatty acid is added to the amino group of a glycine residue via an amide bond [84].



**Figure 7.** A schematic representation of the interactions between galectin-3 and cellular proteins at different locations.

The myristoylation moiety can penetrate into the hydrophobic core of lipid bilayers and in so doing can anchor proteins to lipid membranes. A MH2 domain, or MARCKS homology 2 domain (GQENGHV) in the N-terminal half of MARCKS and MRP is homologous to a region within the cytoplasmic tail of the cation-independent mannose-6-phosphate receptor [84]. While no function for this motif has been identified, it is part of a region of the mannose-6-phosphate receptor that contains many sorting motifs regulating the localization of the receptor. Lastly, a central region called the effector domain (ED) is present in both proteins [85]. This region is basic due to the lysine residues but the region also contains several serine residues that can be phosphorylated by PKC (Figure 8b). Moreover, the ED interacts electrostatically with negatively charged phospholipids present in membranes [86]. Except for the features the ED of MARCKS and MRP have in common there are also some differences. Within the MARCKS ED there are four serine residues while in the MRP ED one central serine residue is replaced by a proline residue (Figure 8b) [84]. Of the four serine residues in the MARCKS ED three are in a consensus site for PKC phosphorylation while in the MRP ED only two serines are phosphorylated by PKC [87]. Other phosphorylation sites have been identified that are outside of the ED. Within the C-terminus of MARCKS two serine residues (serine-291 and serine-299 in rat) followed by proline residues are phosphorylated by proline-directed kinases [88]. A third site for proline-directed kinases in the N-terminus (serine-25 in rat) is specifically phosphorylated in differentiating chick neurons [89,90]. Two proline-directed kinases were identified that can phosphorylate MARCKS, namely cdc42 kinase and tau protein kinase II (TPKII) [91]. A potentially important finding is that mitogen-activated kinase (MAPK) phosphorylates MARCKS at serine-113 [92]. MAPK is responsible for the long-term phosphorylation of MARCKS in glutamate-stimulated rat hippocampal neurons [93] but the function of this phosphorylation is still not known.



**Figure 8.** (a) The structure of MARCKS proteins showing the different domains. (b) Sequence comparison of different domains of MARCKS and MRP. Within the effector domain phosphorylated serine residues are in bold and the proline residue replacing a serine residue in MRP is represented in italics.

Proteins involved in transformation regulate MARCKS expression. For instance, cells transformed by the expression of v-Jun [94] or NIH3T3 cells transformed by p21-Ras or pp60-V-Src have reduced MARCKS levels [95]. Another factor that regulates not only MARCKS but also MRP expression is lipopolysaccharide (LPS). In two reports it was shown that LPS induces a rapid increase in the expression of both proteins in microglia [96], [97].

A number of proteins have been identified that associate with MARCKS and MRP. The  $Ca^{2+}$  regulated protein calmodulin can bind to the ED of MARCKS when it is activated by increased intracellular  $Ca^{2+}$  levels [98]. This association between calmodulin and MARCKS prevents the phosphorylation of the serine residues within the ED [99]. However, in smooth-muscle cells PKC activation triggers CaM translocation from the plasma membrane to the cytoplasm before MARCKS is translocated. The authors suggest that the function of MARCKS is to target CaM to the plasma membrane and release it in a PKC-dependent manner to contribute to CaM-mediated signaling in differentiating smooth-muscle cells [100]. Different reports show that MARCKS and MRP can associate with F-actin [101], [102]. Moreover, full-length MARCKS can induce the aggregation of filamentous F-actin in vitro and this ability is regulated by PKC phosphorylation of the ED [101]. The ED of MARCKS is responsible for the aggregation of F-actin as an isolated ED is still able to induce aggregation [101]. However, it was also shown that myristoylation of the full-length protein enhances its actin polymerizing activity probably by changing the proteins conformation [103]. Subsequently, it was shown that the MARCKS ED has two distinct actin-binding sites; one in the N-terminal

part (KKKKK) and one in the C-terminal part (KKNKK) [104]. The ED of MARCKS and MRP are 96% identical and it was assumed that it would have the same actin aggregation ability. However, while MRP can bind to F-actin and the isolated ED is very efficient in F-actin aggregation [105], the full-length protein does not induce F-actin aggregation [106].

Diverse functions have been suggested for MARCKS and MRP but most of these can be related to their ability to regulate the cortical actin network. The cortical actin network is a mesh of actin filaments present just below the plasma membrane that acts as a barrier for vesicle movement in and out of the cell and is involved in the regulation of cell shape, cell spreading and the formation of cell extensions [107].

After egg activation, cortical granule exocytosis (CGE) is initiated to block polyspermy [108]. MARCKS was shown to be important during GCE because inhibition of MARCKS phosphorylation reduced the relaxation of the cortical actin network thereby preventing CGE [109]. Later, during embryogenesis MARCKS regulates cortical actin formation, cell adhesion, formation of protrusions and cell polarity during gastrulation [110].

In different respiratory diseases such as asthma and chronic bronchitis mucin hypersecretion has been observed [111]. MARCKS is required for mucin secretion from bronchial epithelial cells in vitro [111]. Subsequently, in a mouse model of asthma it was shown that a peptide corresponding to the N-terminus of MARCKS was able to inhibit mucin hypersecretion from goblet cells providing a possible therapy for these respiratory diseases [112]. Chromaffin cells store their secretory products in vesicles close to the PM. MARCKS phosphorylation results in F-actin disassembly and potentiation of secretion of these vesicles [113]. Together, these data show that MARCKS is important for exocytosis by regulating the rigidity of the actin cortical network. Dendritic spine formation and maintenance is an F-actin dependent process and knockdown of MARCKS expression or expression of a pseudophosphorylated MARCKS results in reduced spine density and size [114]. The reduction in size is accompanied by a reduction of F-actin content in the spines [114].

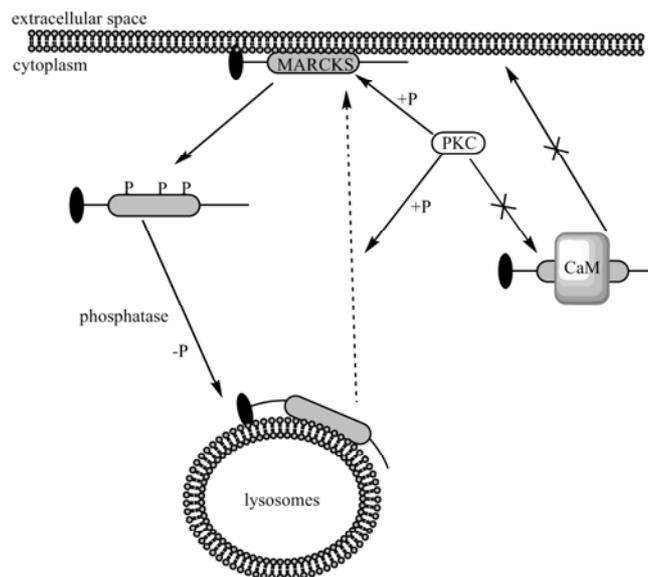
Another process that involves actin reorganization is cell spreading. Multiple reports suggest that both MARCKS and MRP are involved in cell spreading [115-119]. First, it was shown that expression of a MARCKS mutant containing two palmitoyl residues results in the permanent anchorage of the protein to the plasma membrane and inhibits fibroblast spreading on fibronectin [119]. Conversely, when MARCKS is expressed in tumor-derived choroidal melanoma cells that do not express MARCKS, spreading, tyrosine phosphorylation of paxillin and focal contact formation is increased [118]. A detailed study of cell spreading showed that during initial adhesion integrin-mediated activation of PKC causes the translocation of MARCKS. This translocation is important as mutants of MARCKS that can not be phosphorylated inhibit cell adhesion [116]. Dephosphorylation and relocation back to the plasma membrane of MARCKS was shown to be important during later stages of cell spreading [116].

The group of Jianxun Li implicates MRP in integrin-mediated cell adhesion and cell spreading in a series of papers. Firstly, they show that the expression of an ED-deletion mutant of MRP inhibits PMA-stimulated macrophage spreading [117]. Next, using single particle tracking they implicates MRP in the diffusion of  $\beta 2$  integrins on the cell surface and they argue that the PKC-mediated relaxation of the cortical actin network by the phosphorylation of MRP is important for the diffusion of the integrin [120]. Intriguingly, MRP binds directly to dynamitin [121]. Dynamitin is a subunit of dynactin, the regulator of the minus-end motor protein dynein [121]. It was shown that dynamitin and MRP associate with one another at the plasma membrane but that after phosphorylation of MRP by PKC, the interaction is lost while both proteins translocate away from the plasma membrane [121]. Disruption of the microtubule network has long been known to inhibit cell spreading, especially in macrophages [122]. Now the interaction between dynamitin and MRP is implicated in cell spreading as overexpression of dynamitin abrogates the necessity for an external PKC activation signal leading to MRP phosphorylation to initiate of cell spreading [123].

MARCKS and MRP not only bind to proteins but also associate with lipids. More specifically, both proteins can associate with negatively charged phospholipids because of an electrostatic interaction with the positively charged ED [86]. The ED of MARCKS binds to phosphatidylserine and phosphatidylglycerol resulting in its own phosphorylation while the binding of the ED to phosphatidic acid, phosphatidylinositol (PI), phosphatidylinositol-4-phosphate, and phosphatidylinositol-4, 5-biphosphate (PIP<sub>2</sub>) results in the inhibition of its own phosphorylation [124].

The binding and sequestration of PIP2 is seen as a very important function of MARCKS [125]. In this paper it is argued that cellular MARCKS sequesters the majority of PIP2 in the plasma membrane and protects it degradation [125]. This pool of PIP2 can be released when MARCKS is phosphorylated.

The association of the ED and the myristoylation moiety with membranes is important for MARCKS and MRP to associate with the plasma membrane. A model for the binding of MARCKS and MRP to the plasma membrane and the regulation of this binding has been proposed and is called the myristoyl-electrostatic switch model (Figure 9) [126]. According to this model, MARCKS and MRP will associate with the plasma membrane by inserting the myristoyl moiety into the lipid bilayer, bringing the ED to in close proximity to the membrane, allowing the electrostatic interaction between the positively charged ED and the negatively charged phospholipids and thus generating a stable association between the protein and the PM [126]. Upon phosphorylation of the serine residues within the ED by PKC, the positive charge of the ED is reduced leading to the dissociation of the ED from the lipid membrane, weakening the association between the protein and the plasma membrane and subsequently resulting in the translocation of the protein away



**Figure 9.** The myristoyl electrostatic switch model predicts that the phosphorylation of the effector domain results in the translocation of MARCKS and upon subsequent dephosphorylation it associates with lysosomes. Through an unknown pathway MARCKS returns to the plasma membrane. Binding of calmodulin inhibits phosphorylation and plasma membrane binding of MARCKS.

from the plasma membrane to the cytoplasm. It has also been shown that after several hours cytoplasmic MARCKS is dephosphorylated and re-associates with the membranes of internal vesicles like lysosomes [116].

While this model has been experimentally tested for MARCKS it is still unknown if MRP behaves in the same way. Moreover, some differences between MARCKS and MRP suggest that it is possible that MRP does not behave exactly the same as MARCKS. For instance, it was shown that MRP has a 100-fold lower affinity than MARCKS for vesicles containing negatively charged phospholipids [87]. Moreover, the phosphorylation of MARCKS inhibited its affinity while phosphorylation of MRP did not have an effect on its affinity for these vesicles [127]. Also, MARCKS does become cytoplasmic when phosphorylated but no data is available that MRP becomes cytoplasmic although it does translocate away from the plasma membrane after PKC activation [121].

### Scope of this thesis

It has been reported that the re-expression of the integrin  $\beta 1$  in the mouse embryonic  $\beta 1$  knockout cell line, GE11, causes dramatic changes in morphology and influences the activity of an important signaling molecule, RhoA [128]. Later studies showed that the overexpression of another integrin subunit,  $\beta 3$ , induces a different morphology. This is partially related to the fact that  $\beta 3$ , in contrast to  $\beta 1$ , does not increase the activity of the small GTPase, RhoA [129]. Moreover, FA turnover and migration of the cell lines expressing  $\beta 1$  or  $\beta 3$  are different suggesting that the integrins initiate distinct signaling pathways [130].

As discussed in this introduction, integrins can mediate signaling pathways leading to the regulation of gene expression. Since dramatic changes were observed in the morphology of GE11 cells when either  $\beta 1$  or  $\beta 3$  integrins were introduced we hypothesized that gene expression would also be altered. In **chapter 2**, we analyzed the gene expression of cells expressing  $\beta 1$  and identify three genes. Thymosin  $\beta 10$  and IGFBP-4 are both downregulated when  $\beta 1$  is introduced in cells while galectin-3 is upregulated. We further analyze the role of galectin-3 and its relation to the integrin and suggest that galectin-3 assists in integrin-mediated adhesion. In **chapter 3**, we analyze the effect of  $\beta 3$  overexpression on gene expression and identify one gene coding for MRP that is downregulated. We demonstrate that while the Ras/MAPK pathway regulates the basal level of MRP expression,  $\beta 3$  overexpression bypasses this to downregulate MRP. In **chapter 4**, we continued to investigate MRP and test if the myristoyl-electrostatic switch model applies to the regulation of MRP membrane binding just like it does for MARCKS. Using different techniques we show that MRP is targeted to the plasma membrane by the association of the ED to PIP2 but that phosphorylation of the protein leads to a rapid relocation to vesicles we identify as late endosomes and lysosomes. We further show that the ED is not essential while the myristoylation motif is sufficient for membrane binding. We conclude that the model is only partially supported by the behavior of MRP because there is no cooperation needed between the myristoyl moiety and the ED for membrane binding. However, the ED is important for the proper targeting of the protein and the phosphorylation of the ED results in the rapid relocalization of MRP. Lastly, the cooperation between integrins and oncogenes is analyzed in **chapter 5** by investigating the role of the integrin in the oncogenic transformation by primed Src. We show that primed Src transforms cells in the presence of  $\beta 3$  but not of  $\beta 1$ . The cytoplasmic tail of  $\beta 3$  is essential for this transformation confirming that Src and  $\beta 3$  have a functional connection.

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