PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
Facultad de Ciencias Biologicas
Programa de Doctorado en Ciencias Biologicas
Mencion Biologia Celular y Molecular

DETERMINANTS OF DIFFERENTIAL GALECTIN-8 FUNCTION IN T CELLS AND TUMORAL CELLS: INTERPLAY OF GALECTIN-8 ISOFORMS, INTEGRIN AND GROWTH FACTOR SIGNALING PATHWAYS

Por

REMZIYE DÖGER

Director de Tesis : ALFONSO GONZALEZ DE LA ROSA
Co-director de Tesis : ANDREA MORENA SOZA GAJARDO

Comision de Tesis : Dr. Enrique Brandan
Dra. Silvana Zanlungo
Dr. Hugo Olguin

JULIO 2014

TABLE OF CONTENTS

INDEX OF FIGURES

LIST OF ABBREVIATIONS

AG1478: 4 - (3-chloroanilino) -6,7-dimethoxyquinazoline

BSA: Bovine Serum Albumin

CRD: Carbohydrate recognition domain

DMEM: Dulbecco Modified Essential Medium

DMSO: Dimethyl sulfoxide

ECM: Extracellular Matrix

EDTA: Ethylene diamine tetra-acetic acid

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ERK: Extracellular signal-Regulated Kinase

ER: Endoplasmic reticulum

FACS: Fluorescence-Activated Cell Sorting

FBS: Fetal Bovine Serum

Gal: Galectin

Gal-8S: Galectin-8 standard (medium) isoform

Gal-8L: Galectin-8 long isoform

Gal-8N: N terminal carbohydrate recognition domain of Gal-8

Gal-8C: C terminal carbohydrate recognition domain of Gal-8

GPCR: G Protein-Coupled Receptor

HRP: Horseradish peroxidase

IF: Immunofluorescence

kDa: kilo Dalton

MAPK: Mitogen-Activated Protein Kinase

MDCK: Madin-Darby Canine Kidney

Lac: Lactose

μM: micromolar

mM: milimolar

nM: nanomolar

ºC: Celsius Degree

PAGE: Polyacrylamide Gel Electrophoresis

Ptch: Patched

PBS: Phosphate Buffered Saline

PBMN cells: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

PCTA-1: Prostate Carcinoma Tumor Antigen-1

PS: Phosphatidyl serine

PI3K: Phosphoinositide-3-Kinase

PKC: Protein Kinase C

PMSF: Phenyl-methyl-sulphonyl-fluoride

PP2: 4-Amino-5-(4-chlorofenil) -7 - (t-butyl) pyrazolo [3,4-d] pyrimidine

Ro318220 3 - [1 - [3 - (Amidinotio) propyl-1H-indole-3-yl] -3 - (1-methyl-1H-indole-3-yl) maleimide

rpm: Revolutions Per Minute

SDS: Sodium Dodecyl Sulfate

Shh: Sonic Hedgehog

SLE: Systemic Lupus Erythamatosus

Smo: Smoothened

TEMED: N, N, N ', N'-tetramethyl ethylene diamine

TGF-alpha: Transforming Growth Factor Alpha

Tris-HCl: Hydrochloride tris (hydroxymethyl) aminomethane

WB: Western blot

ABSTRACT

Galectins are a subfamily of lectins involved in a variety of cellular processes mainly related with the immune system and cancer. Galectins are secreted through unconventional mechanisms, because they lack a signal peptide, and their carbohydrate recognition domains (CRD) bind to N-acetyllactosamine residues present on cell surface N- and O-glycoconjugates. Each of the 15 galectin family members can have redundant as well as particular functions depending on the structural organization of their CRDs, which is classified in three major groups: Monomeric chimera-type design (Gal-3); Homodimeric prototype (Gal-1, -2, -5, -7, -10, -11, -13, and -15); Tandem-repeat type with two different CRDs connected by a linker peptide (Gal-4, -6, -8, -9 and -12). The function of each particular member can also vary in different cellular contexts through mechanisms still not well understood, but likely dependent on differential subsets of glycoproteins and signaling pathways engaged. Therefore, understanding the role of a particular galectin requires to define the determinants of its variable functions in different cellular contexts.

This thesis studies the function of Gal-8, one of the most widely expressed galectins, both in normal tissues and cancerous cells. Gal-8 has been shown to induce apoptosis in activated T cells from freshly isolated peripheral blood mononuclear cells (PBMC), an effect suggesting a suppressive role in the immune system. In addition, unpublished data from our laboratory shows that Gal-8 increases proliferation, migration and invasion of normal epithelial MDCK cells, activating the Epidermal Growth Factor Receptor (EGFR) (PhD thesis of Claudia Oyanadel). Such an effect might be important in the pathogenesis of tumoral cells, as many kinds of cancers

overexpress this lectin. The determinants of such a distinct Gal-8 effects are unknown but likely depends o differential engagement of signaling pathways.

An additional level of control of Gal-8 function might depend on particular isoforms. As a tandem-repeat type, Gal-8 has two distinct CRD linked by a peptide that varies in size giving rise to different isoforms. The linker of Gal-8 medium/standard isoform (Gal-8S) has 34 amino acids while Gal-8 long isoform (Gal-8L) has 42 additional residues that include a thrombin cleavage site. Most of the studies, including the effects already mentioned, have focused on Gal-8S and it is currently unknown whether Gal-8L has a redundant or distinct function.

Here we asked two main related questions: 1) whether the proliferation pathway induced by Gal-8 always depend on EGFR activation, or can involve another signaling pathway, and; 2) whether the linker size of Gal-8 isoforms has functional implications. To answer these questions we initially compared the effects of Gal-8S and Gal-8L on the pathways leading to apoptosis in Jurkat T cells and to proliferation in tumoral HeLa cells overexpressing the EGFR. The results lead to include studies on the effects of separate N- and C-terminal CRDs, which can be proteolitically released from Gal-8L, on apoptosis, migration and proliferation.

The hypothesis is: “Gal-8 function varies accordingly to its operating isoform and the potential to engage different signaling pathways depending on the cell context”.

We show that:

(i) Gal-8S apoptotic effect of Jurkat T cells depends on the activation of of β1-integrin pathway leading to ERK1/2 activation. Gal-8L, as well as separate carbohydrate recognition domains (CRDs), counteracts the pro-apoptotic activity of Gal-8S in Jurkat T cells. Gal-8L

diminished the Gal-8S-induced ERK1/2 activation and activated the anti-apoptotic AKT pathway. Although both isoforms interacted with and induced endocytosis of β1-integrin only Gal-8S induced its activation. Gal-8L and CRD pre-treatments also inhibited β1-integrin activation induced by Gal-8S.

(ii) Gal-8 isoforms have differential effects on the proliferation and similar effects on migration of HeLa and U87 cells. While Gal-8S induced cell proliferation in both cell types, Gal-8L and CRDs diminished the proliferation in HeLa cells and had no effect on U87 cells. Both Gal-8S and Gal-8L either inhibits or increase HeLa and U87 cell migration depending on the lectin presentation. Pre-binding of Gal-8 to cells in suspension inhibits cell migration while adding Gal-8 after cell attachment to substrate increases migration.

(iii) Gal-8S binds and activates the EGFR and induces its endocytosis. Although it is very suggestive we do not known whether these effects are causally related.

(iv) Gal-8S induced HeLa cell proliferation involves activation of Sonic Hedgehog but not EGFR activation, even though both pathways become activated. Activation of Shh was demonstrated by Gal-8S induced expression of Shh target genes such as Gli-1 and Patched. These effects contrast with the previous observations on MDCK cells proliferation that does require EGFR activation.

In conclusion, both length-linker isoforms and differential engagement of signaling pathways determines distinct cell responses to Gal-8.

1. INTRODUCTION

1.1 Problem to be resolved

Galectins are a family of β-galactoside binding lectins characterized by carbohydrate recognition domains (CRDs) that recognize β-galactoside sugars such as N-acetyllactosamine sequences (Galβ1-3GlcNAc or Galβ1-4GlcNAc) in proteins bearing N-linked or O-linked glycans (Gabius et al., 2002). Through their interactions with β-galactosides on the cell surface glycoproteins, galectins modulate processes as diverse as cell adhesion, migration, invasion, proliferation, differentiation and apoptosis, influencing in various manners the corresponding signaling pathways (Arbel-Goren et al., 2005; Carcamo et al., 2006; Hadari et al., 2000b; Lahm et al., 2004; Levy et al., 2001; Perillo et al., 1998; Yang and Liu, 2003; Zick et al., 2004).

Different galectins play redundant and non-redundant functions based on the glycan selectivity of their CRDs, the subsets of cell surface receptors they recognize and the consequential modulation of the corresponding signaling networks (Bi et al., 2008; Boscher et al., 2011; Di Lella et al., 2011; Kaltner and Gabius, 2012; Lajoie et al., 2009). The CRDs of different galectins display variations that determine the subsets of functionally distinct glycoproteins they engage (Carlsson et al., 2007b; Hirabayashi et al., 2002; Ideo et al., 2011; Ideo et al., 2003; Patnaik et al., 2006; Stillman et al., 2006; Stowell et al., 2008). Because the glycosylation patterns differ in different cellular contexts the ligands to which galectins bind also change and, therefore, the functions of galectins vary depending on the cell context, being not possible to generalize or predict definitive roles

for any galectin. Understanding the function of a particular galectin requires defining its range of interacting elements in different cellular contexts.

According to the organization of their CRDs, three different groups of galectins are currently distinguished: 1) Prototype galectins (Gal-1, -2, -5, -7, -10, -11, -13, and -15) that have one CRD and may form homodimeric complexes; 2) Tandem repeat galectins (Gal-4, -6, -8, -9 and -12) that have two different CRDs separated by a linker peptide, conforming heterodimer equivalents that can also dimerize leading to tetravalent complexes; 3) A unique chimera type member, Gal-3, that has one CRD and a non-lectin domain through which multimers are usually assembled.

The two different CRDs of tandem-repeat type galectins (Gal-4, -6, -8, -9, and -12) are joined by a functional linker peptide of varying sizes and therefore various isoforms are present in this subgroup of galectins. It is suggested that presence of a linker peptide makes these tandem-repeat type galectins more susceptible to proteolysis (Nishi et al., 2005). However whether this property has a regulatory role upon the function of this type of galectins has been little explored.

Gal-8 is a ubiquiotously expressed protein and one of the most widely expressed galectins in normal human tissues (Bidon et al., 2001a; Hadari et al., 1995), as well as in cancerous cells (Bidon-Wagner and Le Pennec, 2004; Lahm et al., 2004). In different cellular contexts, Gal-8 has been shown to modulate cell adhesion, spreading, migration, growth and apoptosis (Arbel-Goren et al., 2005; Bidon et al., 2001a; Carcamo et al., 2006; Hadari et al., 2000a; Levy et al., 2001; Zick et al., 2004). Although Gal-8 is overexpressed or downregulated in various kinds of tumors, its function in relation with tumor malignancy is yet not understood and little explored (Bidon-Wagner and Le Pennec, 2004; Lahm et al., 2004). A previous PhD Thesis in our laboratory has shown that Gal-8 induces cancerigenic properties such as increased cell proliferation and migration when

overexpressed in MDCK cells (PhD thesis of Claudia Oyanadel). Our laboratory also has reported evidence that involve Gal-8 in the immune system, particularly in immune suppression, as it induces apoptosis in Jurkat T cells and in activated peripheral blood mononuclear cells (Norambuena et al., 2009). Here we study some aspects that have not been clarified, regarding the role of isoforms and the N- and C-terminal CRDs of Gal-8.

Gal-8 belongs to tandem-repeat type galectins. N- and C-terminal CRDs of Gal-8 have different sugar-binding specificities. As many as 14 different transcripts of Gal-8 have been described, resulting from alternative mRNA splicing of the 11 exons present in the single Gal-8 gene (Bidon et al., 2001b; Bidon-Wagner and Le Pennec, 2004). These transcripts encode 6 different isoforms. Three of the isoforms belong to the tandem-repeat type, containing the two CRDs (Bidon et al., 2001a; Bidon et al., 2001b; Bidon-Wagner and Le Pennec, 2004; Satelli et al., 2008) that only differ in the length of linker peptides and are so-called as short, medium (standard; Gal-8S) and long Gal-8 (Gal-8L) isoforms. However, up to now only Gal-8S and Gal-L have been detected at the protein level. Most functional studies have been restricted to Gal-8S, while only a couple of reports include Gal-8L. Whether Gal-8S and Gal-8L play redundant or distinct functions remain unknown. An interesting feature of the Gal-8L is that its linker peptide includes a thrombin cleavage site, which may cause separation of the CRDs in the presence of thrombin (Nishi et al., 2005; Nishi et al., 2006). This cleavage may affect the functionality of Gal-8L, but this has not been explored before. In addition, an alternative splicing of Gal-8 gene also generates mRNAs coding for isoforms with proto-type structures (Bidon et al., 2001b) that have only single CRD, but their biological functions have never been studied.

This thesis studies the function of Gal-8S and Gal-8L, as well as the separated Gal-8 N-CRD (Gal-8N) and C-CRD (Gal-8C) domains, using three model systems: Jurkat (human T lymphocytes), HeLa (derived from human cervical cancer) and U87 (derived from

human glioblastoma) cells, that address the potential roles of this lectin in immune cells and in tumoral cells. The results led us to consider the functional relationship with the epidermal growth factor receptor (EGFR) and the pathway of the morphogen Sonic Hedgehog, both important in cancer pathogenesis.

1.2.1 Hypothesis

Gal-8S exerts different functions according to variations in the signaling pathways and a distinct role of Gal-8L, which is based on the proteolytic release of the carbohydrate recognition domains.

1.2.2 General Objective

To understand how Galectin-8 isoforms differentially regulate different cellular processes and to analyze some of the factors that may determine their different functional outcomes.

1.2.3 Specific Objectives

1. To determine whether Gal-8S and Gal-8L have differential functions.

2. To determine whether Gal-8S and Gal-8L have differential functions, including the contribution of each CRDs domain.

3. To elucidate factors and pathway/s involved in these differential effects.

1.3 Bibliographic Antecedents

1.3.1 Galectins and their classification

Galectins are a family of evolutionary conserved carbohydrate binding proteins whereby members of this family are present in organisms from nematodes to mammals (Houzelstein et al., 2004). Since the discovery of first galectin in 1970s, 15 galectins have been described in mammalia and are numbered from 1 to 15 in the chronological order of

their discovery. They are classified in three groups in the base of the organization of their CRDs: 1) Prototype galectins have one CRD and can be biologically active either as monomers (Gal-5, -7, -10) or as homodimers (Gal-1, -2, -11, -13, -14, -15); 2) Tandem repeat galectins that have two non-homologous CRDs with different sugar-binding specificities joined by a linker peptide (Gal-4, -6, -8, -9, 12) (Hirabayashi et al., 2002); 3) Gal-3 also has single CRD and contains a short proline- glycine- and tyrosine-rich N-terminal domain through which forms oligomers (Leffler, 2001; Leffler et al., 2004).

1.3.2 Cellular localization of galectins

Galectins are synthesized on cytoplasmic ribosomes. Since galectins lack the signal sequence that is required to enter classical secretory pathway they are secreted through an endoplasmic reticulum (ER) /Golgi-independent/ non-classical (unconventional) pathway (Hughes, 1999; Nickel, 2003; Seelenmeyer et al., 2008; Seelenmeyer et al., 2005). After being synthesized galectins are either secreted outside of the cells, stay in the cytosol and some may be targetted to distinct subcellular organelles such as nucleus in the case of Gal-1, 3, 7, 8, 10, 11, 12 (Liu et al., 2002), mitochondria in the case of Gal-3, 7 and 12 (Hotta et al., 2001; Villeneuve et al., 2011; Yu et al., 2002). Yet what determines where a specific galectin localizes is very little known.

It was shown that Gal-1, Gal-3 and Gal-9 are secreted outside of the cells by exosomes (Keryer-Bibens et al., 2006; Perone et al., 2006; Thery et al., 2001). Some factors associated with celular stress such as inflammation and malignization are suggested to induce destruction of these vesicles releasing their galectin content into the intercellular space (Hughes, 1999; Nickel, 2003). However exact mechanism of the subsequent destruction of the vesicles are unknown (Hughes, 1999). Galectins with point mutations making them incapable of binding to saccharides are not encountered on the cell surface and are not secreted into the intercellular space; therefore, glycolipids and glycoproteins of

the cell membrane are suggested to act as chemoattractants for galectins (Delacour et al., 2005; Seelenmeyer et al., 2005).

Eventhough galectins have N-glycosylation sites in their aminoacid sequence since they do not enter to ER and Golgi and thus they do not get glycosylated (Delacour et al., 2009). Thus these proteins can be produced as recombinant proteins using recombinant DNA and an expression system such as bacteria. Galectins are encountered as soluble proteins and as part of the extracellular matrix (ECM). In order to study their function recombinantly produced galectins can either be exogenously added into the media of the cells to act as soluble proteins or can be used like a matrix protein by simply treating the plates or coverslips upon where the cells are seeded. These are established methods for studying galectin functions *in vitro*.

1.3.3 Functions of galectins

Eventhough all galectins contain conserved amino acid sequences in their CRDs (Lu et al., 2007; Stillman et al., 2006; Tribulatti et al., 2007) they differ in their sugar binding selectivities and affinities due to the presence of unconserved amino acid sequences in the CRDs. Upon the glycoconjugates that they are bound to the function they modulate may differ. Since there are many possible combinations of glycosyl structures galectins have many unredundant functions.

Galectins modulate various cellular functions both from outside and inside the cells. Outside of the cells, galectins bind to the cell surface and ECM through their specific interactions with the sugars that are on the cell surface and ECM glycoconjugates. Thus the functions that are modulated from outside are generally thought to be carbohydrate dependent. Carbohydrate dependence of galectin function can be shown by inhibitory effect of lactose (the cognate sugar that galectins are highly affined to) treatment.

Intracellular functions that they exert are generally through carbohydrate independent interactions with either cytosolic, mitochondrial or nuclear proteins (Liu et al., 2002). Through these carbohydrate dependent and independent interactions galectins trigger or modulate signaling pathways involved in a wide spectrum of biological events such as: mRNA splicing, cell adhesion, cell migration, apoptosis, cell proliferation, cell growth, cell differentiation and cytokine production, also can be either and pro- or anti-apoptotic, depending on the type of galectin and the cellular context (Arbel-Goren et al., 2005; Carcamo et al., 2006; Dagher et al., 1995; Hadari et al., 2000a; Levy et al., 2001; Perillo et al., 1998; Rabinovich et al., 1999; Vyakarnam et al., 1997; Yang and Liu, 2003).

The major galectin ligands on the surface of mammalian cells are branched N-glycans found on transmembrane proteins (Patnaik et al., 2006). However O-glycans are also their ligands. The affinity of galectins toward different glycoconjugates depend on: i) the number of N and O-glycans present in their sequence, ii) glycan branching and iii) terminal end modifications of the glycan structures (Lau et al., 2007).

Galectin function considerably varies according to the cell context. The main reason for these context dependent functions is that different cells present different glycosylation patterns. A protein´s pattern of glycosylation depends on many factors, including the type of cell producing the glycoprotein, nutrient concentrations, pH, cell density and cellular proliferation state. The glycosylation patterns of the cell surface proteins can also change in autoimmune diseases, as well as in different types of cancer, due to changes in the expression of some glycosidase enzymes and/or glyco-transferases of Golgi that modify the number of branches and glycosyl compositions (Cazet et al., 2010; Li et al., 2010).

Furthermore mode of presentation of galectins also affects their interactions with

glycans leading to different functional outputs. For instance, soluble Gal-8 inhibits cell adhesion while immobilized Gal-8 acts as a matrix molecule promoting cell adhesion and spreading in various types of cells (Levy et al., 2001).

1.3.4 Altered expression of galectins in cancer

Differential galectin expression has been observed in tumoral tissues relative to their normal counterparts (Balan et al., 2010; Danguy et al., 2002). Moreover there seems to be correlations between the expression of galectins and tumorigenesis (Bresalier et al., 1998; Chiariotti et al., 2004; Lahm et al., 2004; Schoeppner et al., 1995; Su et al., 1996). For instance increased expression of Gal-1, -2, -3, -4, -7, -8 and -9 in tumor cells was shown to correlate with the degree of invasiveness and metastasis of certain cancers such as astrocytoma, thyroid carcinoma, carcinoma of head and neck and colon tumors (Barondes et al., 1994; Barrow et al., 2011; Bidon-Wagner and Le Pennec, 2004; Camby et al., 2001). It is suggested that the increased expression of these galectins in tumor cells could control cellular functions such as cell adhesion, proliferation, migration, invasion and metastasis and induction of infiltrating T cells apoptosis to help tumor cells escape from immune system (Barrow et al., 2011; Boscher et al., 2011; Lajoie et al., 2009; Le Mercier et al., 2010; Li et al., 2010; Liu and Rabinovich, 2005). However the mechanisms through which galectins exert their pro-tumorigenic functions remain largely unknown.

1.3.5 Apoptotic function of galectins in immune system

Apoptosis also named asprogrammed cell death is a normal celular process that is generally characterized by certain cellular changes and energy-dependent biochemical mechanisms. Morphological changes that occur during apoptosis include blebbing, cell shrinkage (cytoplasm gets dense and the organelles are more tightly packed), chromatin condensation, and chromosomal DNA fragmentation. This process occurs via activation of

a group of cysteine proteases called “caspases”. Apoptosis is crucial for processes such as normal cell turnover, embryonic development and aging. It is also a defense mechanism against immune reactions (Norbury and Hickson, 2001).

Several galectins have been implicated in T cell homeostasis due to their capacity to kill thymocytes, activated T cells and T cell lines (Lu et al., 2007; Perillo et al., 1995; Stillman et al., 2006; Sturm et al., 2004; Tribulatti et al., 2007; Yang et al., 1996) while others have anti-apoptotic functions (Clark et al., 2012). This particular pro-apoptotic property of galectins is suggested to restrict the immune response by eliminating activated T cells at the periphery (Rabinovich et al., 2007). Some galectins secreted by tumoral cells are also thought to be involved in tumor immune escape by eliminating cancer-infiltrating T cells (Liu and Rabinovich, 2005). Moreover galectins have a promising potential to eliminate abnormally activated T cells and inflammatory cells and thus are suggested to be used as therapeutic tools (Rabinovich et al., 2007).

Comparative studies on the most explored Gal-1, -3, and -9 indicate that different galectins most likely play complementary roles, coordinately controlling and finely regulating the critical events leading to apoptosis (Bi et al., 2008; Harjacek et al., 2001; Nishi et al., 2003; Stillman et al., 2006). Therefore, complete understanding of their immunological functions and therapeutic potentials require extensive definition of the pro-apoptotic properties and mechanisms of different members of this lectin family.

1.3.6 Galectins as modulators of cell proliferation

Cell proliferation is a normal cellular process whereby there is an increase in the number of cells as a result of cell growth and cell division. Growth factors that are found in the extracellular enviroment activate cell proliferation. Many of these growth factors bind

to tyrosine kinase receptors leading their activation and further activation of signaling cascades that ultimately change gene expression causing cell proliferation (Liu et al., 2013; Ordener et al., 1994; Wu et al., 1999). Among these tyrosine kinase receptors one of the most studied and understood is Epidermal Growth Factor Receptor (EGFR). Like other cell surface receptors EGFR is also glycosylated. Infact EGFR contains 12 typical N-glycosylation consensus sites (Carpenter and Cohen, 1990) that may be regulated by galectins.

1.3.6.1 Epidermal Growth Factor Receptor

EGFR is a member of four closely related transmembrane tyrosine-kinase receptors of ErbB-family (Jorissen et al., 2003). Upon ligand binding EGFR becomes auto-phosphorylated in several tyrosine residues, which are recruiting sites for the elements engaged in downstream signaling (Schlessinger, 2000; Schlessinger, 2002; Ullrich and Schlessinger, 1990; Yarden and Sliwkowski, 2001). The activated EGFR also phosphorylates several intracellular substrates that have signaling or endocytic trafficking functions (Sorkin and Goh, 2008; Wiley, 2003). The transduction system of the EGFR includes the signaling pathways of p42/44 Extracellular signal regulated kinase (ERK1/2), PI3K/AKT, PLC-γ, and STAT-3 (Schlessinger, 2000; Schlessinger, 2002; Yarden and Sliwkowski, 2001). The combination of all these pathways within different cellular contexts and environmental cues generate a variety of responses, such as proliferation, differentiation, migration, and survival to apoptotic stimulus.

The main consequence of EGFR dysfunction is cell transformation and tumorigenesis, frequently involving expression of exaggerated levels and oncogenic mutants of the receptor, with subsequent dysregulation of downstream signaling networks (Mizoguchi et al., 2006; Yarden, 2001). Indeed, the EGFR has been proved to be a suitable

target for directed anti-tumoral agents (Klein and Levitzki, 2009; Sawyers, 2003; Yarden, 2001). Thus the function of the EGFR has to be tightly regulated and therefore it is a great interest to discover new mechanisms of EGFR regulation.

At multiple cellular levels a great variety of mechanisms have been shown to converge upon regulating EGFR function, such as regulation of expression levels, intrinsic tyrosine kinase activity, ligand-affinity, ligand-availability, trans-activation and endocytic trafficking (Buvinic et al., 2007; Ullrich and Schlessinger, 1990; Wiley and Burke, 2001; Yarden and Sliwkowski, 2001).

The EGFR displays high (2-5 % of total) and low (95-98 %) affinity binding receptors and the high affinity receptors are thought to be the ones that are pre-formed dimers before ligand binding (Lax et al., 1989; Ullrich and Schlessinger, 1990). Thus stimuli that will change the number of high or low affinity receptors can also modulate the receptor function.

EGFR regulates its own expression by ligand binding (Clark et al., 1985). There are at least six identified EGFR ligands, which include EGF, HB-EGF and TGF-α, all of them synthesized as transmembrane protein precursors (Dong et al., 2005) and proteolytically cleaved by metalloproteases to yield mature growth factors (Massague and Pandiella, 1993). A variety of other stimuli, specific for other receptors, also promote the release of the ligands and provoke trans-activation of the EGFR (Carpenter, 2000). Indeed, EGFR has been identified as a key element in the complex signaling network that is utilized by various classes of cell surface receptors such as tyrosine kinase receptors, cytokine receptors, ion channels, G-protein coupled receptors (GPCRs) and integrins (Prenzel et al., 2000).

It is now clear that the mechanisms that govern receptor signaling are tightly linked to its endocytic trafficking (Miaczynska et al., 2004; Roepstorff et al., 2008). Upon ligand binding and activation, the EGFR undergoes rapid endocytosis and then either recycles or enters into the lysosomal-degradation route remaining active during variable periods, depending on the bound ligand and dimerization partner (Ceresa and Schmid, 2000; Polo and Di Fiore, 2006; Sorkin and Goh, 2008; Sorkin and Von Zastrow, 2002; Wiley, 2003). The endocytic route then provides wide opportunities to transmit and modify signals at different cellular locations in addition to desensitize or gradually attenuate receptor signaling. In this way, ligand-induced receptor trafficking expands the range of signaling modulation and response variability along alternative endocytic pathways (Sigismund et al., 2008; Sigismund et al., 2005).

EGFR contains 12 typical N-glycosylation consensus sites (Carpenter and Cohen, 1990), containing both complex-type and high mannose-type N-glycans (Cummings et al., 1985; Stroop et al., 2000). The roles of these N-glycans in EGFR functions have been extensively investigated and properties like ligand binding, dimerization, activation, endocytic trafficking has been shown to highly relate with glycosylation of the receptor (Gamou and Shimizu, 1988; Slieker and Lane, 1985; Slieker et al., 1986; Soderquist and Carpenter, 1984). N- glycans are also required for the proper sorting of EGFR to the membrane (Gamou and Shimizu, 1988; Slieker and Lane, 1985; Slieker et al., 1986; Soderquist and Carpenter, 1984). It has also been suggested that the interaction of certain lectins with receptor oligosaccharides leads to an alteration in ligand binding capacity as well as in tyrosine kinase activity (Carpenter and Cohen, 1977; Hazan et al., 1995; Rebbaa et al., 1996; Zeng et al., 1995). Recently, complex N-glycans of EGFR were shown to be required for maintenance of EGFR on the cell surface as clusters and for EGFR signaling (Partridge et al., 2004). Complex types N-glycans of the receptor are recognized by

galectins. And the absence of the interaction of EGFR with the galectins is thought to a decrease in cell surface expression of EGFR accompanied with decreased EGFR signaling (Partridge et al., 2004). In our laboratory overexpression of Gal-8S in normal epithelial MDCK cells was shown to increase proliferation and migration of these cells in Epidermal Growth Factor Receptor (EGFR) dependent manner (PhD thesis of Claudia Oyanadel, manuscript in preparation). However yet very little is known about galectin modulation of EGFR.

1.3.6.2 Sonic Hedgehog Pathway

In this thesis, Sonic Hedgehog (Shh) pathway has been the second most closely investigated cell proliferation inducing pathway.

Hedgehog (Hh) pathway is an evolutionarily conserved signaling pathway that regulates fundamental and diverse cellular processes during embryogenesis and adult life. Hedgehog (Hh) pathway was originally identified in Drosophila melanogaster (Nusslein-Volhard and Wieschaus, 1980), where it was shown to control segmental pattern formation. Hh family of proteins was also shown to control processes like cell proliferation, survival, and fate, organogenesis and maintenance of tissue homeostasis.

In mammals there are three Hh genes: Desert Hedgehog, Indian Hedgehog, Sonic Hedgehog (Shh). They are the ligands for the 12-pass transmembrane proteins Patched1 and Patched2 (Ptch1 and Ptch2). In vertebrates Shh is the most studied one. Shh is translated as a 45-kDa-precursor protein that goes through multiple steps to produce active ligands. Shh undergoes autocatalytic processing, which yields a 19-kDa, N-terminal, Shh signaling domain (Shh-N) and a 25-kDa C-terminal domain (Shh-C). Shh-C has no known signaling activity but is responsible for the intramolecular precursor processing, acting as a cholesterol transferase, which is essential to restrict Shh-N to the cell surface and prevent it

from freely diffusing throughout the developing embryo. Along with the cleavage, ShhC covalently binds a cholesterol moiety to the carboxy-terminus of the ShhN. ShhN is further modified by a membrane bound O-acyltransferase which covalently links a molecule of palmitate to the 19-kDa fragment. When modified Shh-N reaches its target cells, it binds to the 12-pass transmembrane protein Patched (Ptch) with high affinity at the cell surface.

In the absence of ligand, Ptch, which localizes to the primary cilium, inhibits pathway activation by preventing the seven transmembrane protein Smoothened (Smo) to enter the cilium (Rohatgi et al., 2009; Rohatgi et al., 2007). This results in the formation of repressor forms of the Gli zinc finger transcription factor Gli3 and Gli2, which repress Shh target gene expression (Wong et al., 2009). In the presence of ligand, Ptch is internalized and degraded, thus its ciliary localization is lost (Rohatgi et al., 2007), thereby releasing Smo to enter the primary cilia where it promotes dissociation of a Suppressor-of-fused (Sufu)–glioma-associated oncogen homolog (Gli) complex. Smo becomes enriched in primary cilia (Corbit et al., 2005) and promotes nuclear translocation and activation of the Gli1 and Gli2 transcription factors, and degradation of the repressor forms of Gli (primarily Gli3). Subsequently Gli proteins induce transcription of Shh patway target genes including Gli1, Gli2 and Ptch1 (Bale and Yu, 2001; Ingham and McMahon, 2001).

Abnormalities in Hh signaling pathway might result in an aberrant cell growth, cell differentiation, and migration and may cause severe disorders (Bak et al., 2003; Beachy et al., 2004; Cohen, 2003; Kasper et al., 2006; Liu et al., 2006). Inappropriate activity of this pathway can also lead to the development of tumors. Infact Hh signaling pathway activity is encountered enhanced in a wide variety of cancers: leukemia, multiple myeloma, and brain, skin, head and neck, lung, liver, gastrointestinal, colorectal, pancreatic, prostate, mammary, ovarian, and renal carcinomas (as rewieved in (Mimeault and Batra, 2010)).

Analyses of human tumors have revealed mutations in various components of the Shh signaling pathway that cause sustained activation of the pathway and thus increased expression of the transcription factor, Gli1 and Gli2, which has been shown to account for the initiation and growth of Hh-associated tumors (Ruiz i Altaba et al., 2007; Wetmore, 2003). Up to now loss of Ptch1 or Sufu, activating mutations in Smo, mutations in Sufu have been detected in Shh-associated tumors. There are also some tumors that lack these mutations but they are sensitive to Smo inhibitors (Ng and Curran, 2011; Teglund and Toftgard, 2010). Overall it is estimated that 25 % of human tumors may depend on Hh pathway activity for growth (Lum and Beachy, 2004) and, as a consequence, a broad range of tumors was included in the early clinical trials of Smo inhibitors.

Up to now, there is no precedent regarding how Shh pathway can be modulated by any galectins. Here we unexpectedly found a modulatory link between Galectin-8 and Shh pathway.

1.3.6 Galectins as modulators of cell migration

Cell migration is very essential in regulating various biological processes under normal and pathological conditions such as proper organization of multicellular organisms, wound healing and cancer metastasis (Trepat et al., 2012; Vedula et al., 2013).

Cell migration occurs through multiple adhesion and spreading events. First of all cells have to adhere to ECM components. Then cells have to mobilize reorganizing the actin cytoskeleton, maninly through modifications of interactions between integrins and ECM (Friedl, 2004).

Galectins have been shown to have both anti-adhesive and pro-adhesive functions (Elola et al., 2007). Although galectins do not contribute to structural roles in the ECM, they act as cross-linkers between ECM proteins and cell surface receptors once they are found as insoluble substrate in the ECM. However as galectins also function as soluble

proteins by binding to integrins they may inhibit the binding of these receptors to the ECM molecules. Thus cells can no longer adhere to the matrix.

Effect of various galectins have been analyzed either immobilized (plates, wells or covers were pre-treated with the gal) or as soluble substrates (simply by adding to the media of the cells) on cell adhesion and migration (Elola et al., 2007). While in most of the cases immobilized galectins induce cell adhesion, soluble galectin treatments have differential effects depending on the cell type (as reviewed in (Elola et al., 2007)). For instance while soluble Gal-3 treatment of XK4-A3 human adenocarcinoma cells increases cell binding to laminin treated plates (Le Marer and Hughes, 1996), soluble Gal-8 treatment of HeLa cells diminishes cell binding to laminin and fibronectin treated plates (Levy et al., 2001).

1.3.7 Galectin-8

Galectin-8 (Gal-8) was isolated for the first time in 1995 from a rat liver cDNA library using a monoclonal antibody directed against the Insulin Receptor Substrate-1 (Hadari et al., 1995). Due to its structural and sequence similarities to the previously discovered Gal-4 and Gal-6 it was categorized into the tandem repeat type galectins. In 1996, a human cDNA with 83 % nucleotide identitiy to rat Gal-8 was isolated from a prostate cancer expression library. After this discovery Gal-8 was also named as Prostate Carcinoma Tumor Antigen-1 (PCTA-1) (Su et al., 1996).

Gal-8 gene has 11 exons, multiple stop codons and polyadenylation signals. Up to now 14 different Gal-8 transcripts have been identified due to alternative splicing and due to the presence of multiple stop codons (Bidon et al., 2001a; Bidon et al., 2001b; Bidon-Wagner and Le Pennec, 2004). In theory these transcripts encode 6 different isoforms. 3 of the

isoforms have 2 CRDs that are linked by a linker peptide. Sole function of only the linker peptide of Gal-8 has not been determined yet. The linker peptide of these 3 isoforms differs in length giving rise to short, medium (standard) and long isoforms. Gal-8 seems to be unique in the galectin family with isoforms belonging to both the prototype and tandem-repeat groups (Bidon et al., 2001a; Bidon et al., 2001b; Su et al., 1996). However, the prototype Gal-8 isoforms have only been detected as isolated transcripts. Up to now at protein level only the standard (Gal-8S) and long (Gal-8L) isoforms have been detected. The linker peptide of Gal-8S has 26 aminoacids whereas Gal-8L has 41 extra aminoacids in its linker peptide. In these 41 extra aminoacids a thrombin cleavage site is encountered. The presence of this cleavage site may cause liberation of the two CRDs from being linked and may have an impact on the functionality of Gal-8L.

N- and C-terminal CRDs of Gal-8 display sequence differences thus they present distinct glycan selectivities (Carlsson et al., 2007b; Patnaik et al., 2006). Gal-8N shows strong affinity for sulfated and α2-3-sialylated glycans whereas Gal-8C recognizes blood group antigens and poly-LacNAc glycans (Stowell et al., 2008). As these CRDs differ in their glycosyl binding specificities they may crosslink different type of ligands.

Gal-8 is one of the most widely expressed galectin in human tissues (Bidon et al., 2001a; Hadari et al., 1995), as well as in cancerous cells (Bidon-Wagner and Le Pennec, 2004; Lahm et al., 2004). Depending on the cell context and mode of presentation (either as soluble stimulus or extracellular matrix) Gal-8 has been involved in cell adhesion, spreading, growth and apoptosis (Arbel-Goren et al., 2005; Carcamo et al., 2006; Hadari et al., 2000b; Lahm et al., 2004; Levy et al., 2001; Perillo et al., 1998; Yang and Liu, 2003; Zick et al., 2004). To execute most of its functions, Gal-8 binds to membrane glycoproteins, especially those containing sialic acid (Ideo et al., 2011). Gal-8S interacts with integrins, especially with β1 type (Carcamo et al., 2006), αM-integrin (Nishi et al.,

2003), platelet coagulation factor V (Zappelli et al., 2012), CD4 (Tribulatti et al., 2009), CD45 (Tribulatti et al., 2009)and LFA-1 (Vicuna et al., 2013), podoplanin (Cueni and Detmar, 2009) and IgA (Cederfur et al., 2008). It can also bind to glycosphingolipids such as GM3 and GD1α (Hirabayashi et al., 2002; Ideo et al., 2003). However it is important to note that as glycosylation patterns on these glycoconjugates vary in different cellular context, binding studies in a cell line should not be generalized for all other cell types. Gal-8 regulates signaling pathways mediated by Rho, ERK1/2, pZAP70 and PI3K (Diskin et al., 2012; Levy et al., 2006; Tribulatti et al., 2009). Gal-8 is encountered on the plasma membrane, inside the endosomes, in the cytosol and in the nucleus (Carlsson et al., 2007a). Gal-8 is endocytosed by an clathrin and cholesterol independent mechanism (Carlsson et al., 2007a). Its role in the endosomal compartments and in the nucleus has not been studied.

So far, Gal-8S has been the most studied isoform. It is mainly studied in the immune system. There is some evidence suggesting that Gal-8S could play important roles in T cell homeostasis and rheumatic autoimmune or inflammatory disorders. Anti-inflammatory properties of Gal-8S have been implicated in rheumatoid arthritis (RA), promoting apoptosis in synovial fluid cells (Eshkar Sebban et al., 2007). A role in shaping the T cell repertoire has been suggested by its intrathymic expression and caspase-mediated pro-apoptotic effect upon $CD4^{high}CD8^{high}$ thymocytes (Tribulatti et al., 2007). Recently a proliferative effect of Gal-8S and Gal-8L upon CD4 (+) T cells isolated from mouse spleen has been reported (Tribulatti et al., 2009).

In Jurkat T cells, our laboratory has previously reported that Gal-8 interacts with specific integrins, such as α1β1-, α3β1-, α5β1-integrins but not α4β1-integrin, and as a matrix protein promotes cell adhesion and asymmetric spreading through activation of the ERK1/2 (Carcamo et al., 2006). Recently our laboratory have published that Gal-8S

induces apoptosis in Jurkat T cells, as well as in anti-CD3/CD28 activated human T cells from freshly isolated PBMC, increasing the expression of pro-apoptotic FasL receptor (Norambuena et al., 2009). Gal-8S induced Jurkat T cell apoptosis is inhibited by β1-integrin function blocking antibodies (Norambuena et al., 2009).

ERK1/2 signaling was shown to be involved in long-term processes such as T cell survival or death, depending on the moment of the immune response. During T cell activation, ERK1/2 contributes to enhance the expression of interleukin-2 required for T cell clonal expansion (Cantrell, 1996). It also supports T cell survival against pro-apoptotic Fas ligand produced by them and by other previously activated T cells (Gendron et al., 2003; Holmstrom et al., 2000). Later, ERK1/2 is required for activation-induced cell death (AICD), which eliminates recently activated T cells when they become restimulated through the T cell receptor (van den Brink et al., 1999; Zhu et al., 1999), thus controlling the extension of the immune response (Janssen et al., 2000). ERK1/2 contributes to enhance the expression of FasL required for AICD (Janssen et al., 2000; van den Brink et al., 1999; Zhu et al., 1999). Our laboratory has previously shown that Gal-8S induced apoptosis of Jurkat T cells depends on ERK1/2 pathway activation (Norambuena et al., 2009).

The function of the Gal-8L isoform has recently started to be investigated and a significant difference between Gal-8S and Gal-8L in neutrophil adhesion-inducing activity has been reported (Nishi et al., 2006). However so far this article has been the only study that illustrated functional difference between these isoforms. The main goal of this thesis project is to study the effect of the Gal-8L in comparison to Gal-8S on different models and to get insight into the importance of the linker length variation in the functionality of Gal-8 isoforms.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Bacteria

Top -10 of *Escherichia coli* (*E. coli*) bacteria were used.

2.1.2 Eukaryotic cells

Jurkat T (immortalized human T lymphocyte cell line) cells and HeLa (immortalized human cervical cancer cell line) cells and U87 (human primary glioblastoma cell line) were used.

2.1.3 Plasmids

Prokaryotic expression vector pGEX-4T3-GST-Gal-8S containing the sequences of human Gal-8S was used (it was previously cloned in the laboratory). In this thesis Gal-8L was subcloned into the same expression vector.

2.1.4 Antibodies

For immunoprecipitating EGFR HB8506 hybridoma obtained from the ATCC was grown in RPMI with 10 % serum and its supernatant was used. Commercially available antibodies that were used in this thesis are: anti-phosphotyrosine 4G10 (Millipore), polyclonal anti- Cleaved Caspase-3 (Asp175) (Cell Signaling Technology, Beverly, MA, USA), monoclonal anti-phospho-ERK1/2 and polyclonal anti-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-AKT antibody (BD Transduction Lab, San Jose, CA, USA), rabbit monoclonal anti-phospho-AKT (Ser473) (Cell Signaling Technology, Danvers, MA, USA), mouse monoclonal anti-β1-integrin (12G10) (Abcam, Cambridge, UK), polyclonal anti-β_1-integrin (Chemicon International Inc. Temecula, CA, USA), monoclonal anti-β-actin antibody (Abcam) and anti-human

CD3 and CD28 (BD Pharmingen, BD Biosciences, San Jose, CA, USA). Rabbit anti-Sonic Hedgehog, goat anti-Patched, goat anti-Smoothened, goat anti-Gli-1 antibodies were from Santa Cruz Biotechnology. Secondary antibodies (anti-human, mouse, goat, rabbit) were from Jackson Immuno Research Laboratories (West Grove, PA, USA). Alexa-488, Alexa-555 secondary antibodies were from Molecular Probes (Eugene, OR, USA).

2.1.5 Radioactive material

NaI^{125} used to label EGF was obtained from Perkin Elmer (Wellesley, MA, USA) .

2.1.6 Material for eukaryotic cell culture

T-75 and T-25 flasks, 35, 65 and 100 cm plates and 6, 24 and 96 well plates were obtained from NUNC (Karmstrup, Denmark).

15 and 50 ml Falcon tubes were obtained from BD Biosciences (Bedford, MA, USA).

6.5 mm in diameter, 8.0 μm pore Transwell polycarbonate filters were obtained from Corning Corp. (NY, USA)

2.1.7 Reagents

Pig skin gelatin, leupeptin, pepstatin, antipain, PMSF, HEPES, NaOH, NaCl, EDTA, $MgCl_2$, $CaCl_2$, KCl, DMSO, trizma base, glycine, SDS, DTT, TEMED, ammonium persulphate, Triton X-100, Tween 20, acrylamide and bisacrylamide, bromophenol blue, xylene cyanol FF, Coomassie Brilliant Blue R-250, ethidium bromide, ampicillin, FAK inhibitor (PF-573228), PI3K inhibitor (LY-294,002), EGFR inhibitor Tryphostin (AG1296 and AG1478), cyclopamine hydrate were obtained from Sigma Chemical Co. (St. Louis, USA).

Agarose, trypsin, DMEM media, RPMI-1640 media, FBS, penicillin, streptomycin, tryptone, yeast agar extract were obtained from GIBCO Invitrogen Corporation (Grand Island, N, USA).

DNA Plasmid Purification Kits (mini and midiprep) and gel extraction kits were obtained from QIAGEN (Duesseldorf, Germany).

Hyperfilm ™ ECL plates and ECL Chemiluminescence detection system was obtained from Amersham-Pharmacia Biotech (Uppsala, Sweden).

Ultraviolet Products were revealed by ChemiDoc-It Imaging System and analyzed by VisionWorks LS Image Acquisition and Analysis Software (Upland, CA, USA).

Affi-Gel 15 resin and Bio-Rad protein assay that is used to determine protein concentration were obtained from Bio- Rad (Hercules, CA , USA).

Sucrose, lactose, acetone, ethanol, methanol, isopropanol, glacial acetic acid, HCl, DMSO and glycerol were obtained from Merck (Darmstadt, Germany).

ERK inhibitor (PD98059) and Purmorphamine, Ro inhibitor (Ro-31-8220), PKC inhibitor (PP2) are from Calbiochem.

General Metalloprotease inhibitor (Ilomastat-GM6001) is from Milipore (Temecula, CA).

2.2 Methods

2.2.1 Molecular biology techniques

2.2.1.1 Sub-cloning of Gal-8L into pGEX vector:

The amplified DNA encoding Gal-8L obtained by RT-PCR was inserted in frame into the *SalI/NotI* sites of the pGEX-4T-3 vector (Amersham-Pharmacia Biotechnology, Piscataway, NJ, USA) that harbors the sequence of the GST tag at its 5' end.

2.2.1.2 Digestion and ligation

2.2.1.1 Digesting DNA with restriction enzymes

All restriction enzymes were used as recommended by the manufacturer (Invitrogen or Promega), adding <10 % of final volume (10 to 20 µl) of enzyme reaction in the corresponding buffer. In the case of double digestion, in order to make both digestions simultaneously a common compatible for both enzymes activity was selected following restriction enzymes table. Incubations were performed at 37 °C overnight. After linearization of the vectors DNA was purified from agarose using Qiagen kit.

2.2.1.2 Ligation of DNA fragments

Ligations were performed with T4 phage ligase following manufacturer's recommendations. The ratio of vector: insert mass was 1:5. Ligations were incubated for 16 hours at 16 °C and immediately transformed into competent bacteria.

2.2.1.3 Bacterial transformation

100 µl of *E. coli* TOP10 competent bacteria were slowly thawn on ice. 0.1 a 1 µg DNA of interest was added on the bacteria, gently mixed and incubated at 4 °C for 10 min and then a pulse of heat shock is given (42 °C for 1 min), then the mixture is returned to ice for 2min. The mixture was suspended in 900µl of fresh and sterile Luria Broth (LB) (10 g/L

tryptone, 5 g/L NaCl and 5 g/L yeast extract). Transformation mixture was vigorously shaken for 1 h at 37 °C on shaker. 500 µl of resulting culture was spread on agar plates containing the corresponding selection antibiotic (Ampicilin in this case) and plates were incubated in an oven at 37 °C facing upside down. The next day plates were stored at 4 °C until the selection of the colonies. Isolated colonies were selected and grown in 3 ml LB with Ampicilin to analyze the presence and identity of transformed plasmid.

2.2.1.4 Electrophoresis of DNA on agarose gels

For purification of DNA Qiagen mini and midiprep kits were used. To visualize DNA 0.7 to 1 % agarose gels were used depending on the size of the DNA (ranging from 1kb to 10kb). Agarose was dissolved in TAE buffer (40 mM Tris, 20 mM CH_3COONa, 2 mM EDTA, pH 8 adjusted with glacial acetic acid). Ethidium bromide was added to the gel (final concentration 1 µg/ml). DNA samples were mixed with 6X Orange loading buffer (Invitrogen) and electrophoresis was performed using TAE as running buffer by applying 100 V. The DNA was visualized on a trans-illuminator under UV light. Gels were pictured in Gel Doc 2000 using Quantity One Software, both from Bio-Rad (Hercules, CA, USA).

2.2.1.6 Maintenance of bacterial strains and growth

The bacteria were kept at -80 °C in 50 % glycerol. To grow bacteria to purify recombinant proteins or plasmids, an aliquot of the bacteria from -80 °C will be inoculated into 3 ml of LB medium with selection antibiotics and grown overnight at 37 °C with vigorous shaking. Of these saturated cultures aliquots were taken to inoculate for mini, midi plasmid DNA preparations or for recombinant protein preparations.

2.2.2 Cell Biology Techniques

2.2.2.1 Maintenance and freezing of cell lines

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM- 4,5g/L glucose) supplemented with 10 % fetal bovine serum (FBS), 100 Unit/ml penicillin and 0.1 mg/ml streptomycin (Sigma). Jurkat T cells were maintained in suspension in T25 or T75 flasks at $5x10^6$ -$1x10^7$ cells/ml in Roswell Park Memorial Institute medium-1640 (RPMI-1640, Gibco) containing 10 % FBS supplemented with 100 Unit/ml penicillin and 0.1 mg/ml streptomycin. To avoid function-blocking interactions of serum glycoproteins, cells were serum starved for 2 h or overnight depending on the experiment before addition of the lectin. Both cell lines were always maintaned in an incubator at 37 °C with 5 % CO_2 until 80-90 % confluency.

2.2.2.2. Trypsinization and seeding cells for the experiments

Media of semi-confluent HeLa cells were aspirated and then cells were washed twice with 1X Phosphate Buffered Saline (PBS). PBS was fully aspirated and then cells were incubated with 1X Trypsin (diluted from 10X Trypsin with PBS) and cells were placed back into the 37 °C incubator for about 3 min (depending on the cell type). (Amount of trypsin depends on the size of the flask, for 10cm diamenter plate 1 ml of 1X trypsin, for 6cm diameter plate 0.5 ml of 1X trypsin was used). After detachment, media with FBS (complete media) was added on the cells to deactivate the trypsin and pipetted gently to disperse the cells. Then cells were centrifuged at 1000 rpm at room temperature for 5 min in a tabletop centrifuge Kubota KS-5400 (Kubota Corporation, Bunkyo-ku, Japan). Cells were resuspended in a small volume of complete media and counted using a haemocytometer or a cell counter (Invitrogen). Cells were cultured in different size flasks or plates at required density depending on the experiment. For immunoprecipitation experiments 6 well plates were used, for crystal violet experiments 96 well plates were

used, for immunofluorescence 12 mm coverslips and 24 well plates were used. Cells were frozen at -80 °C in cryotubes in 90 % FBS and 10 % dimethyl sulfoxide (DMSO).

Since Jurkat T cells were cultured in suspension no trypsinization was required for their maintanence. Every 2 to 3 days fresh medium was added to keep the cell density at $5x10^6$ - $1x10^7$ cells/ml. For analysis of apoptosis by immunofluorescence, cells were centrifuged at 1000 rpm for 5 min and diluted in RPMI media without serum at a cell density of $3x10^6$ cells/ml and serum starved for 2 h. Incubation without serum was done to diminish the inhibitory effect of glycosylated proteins that were in the serum upon the effect of galectin. For each treatment point 100 μl ($3x10^5$ cells) was used. For biochemical analysis cells were overnight serum starved and 1 ml ($5x10^6$ cells/ml) was used for each treatment.

2.2.2.3 Protein analysis

2.2.2.3.1 Dilution of recombinant Galectin-8 isoforms and CRDs:

In this thesis we worked in collaboration with Dr. Joachim Gabius, Ludwig-Maximilians-University Munich, Germany who provided us Gal-8S, Gal-8L and Gal-8N and Gal-8C recombinant proteins. These proteins were produced as in (Cludts et al., 2009; Habermann et al., 2011). Recombinant proteins were lyophilized and sent to Chile by mail. According to the amount needed lyophilized proteins were diluted in 2 % glycerol in PBS and freshly used for the treatments. If more than a month passed after the dilution, the diluted proteins were no longer used for functional studies. We also have collaborated with Dr. Gonzalo Mardones from Universidad Austral, Valdivia, Chile, who had provided us Gal-8S.

2.2.2.3.2 Preparation of total cell extracts

Cells were washed twice with PBS at 4 °C and lysed for 30-45 min at 4 °C with gentle shaking in either classic lysis buffer (50 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 % Triton X-100; 10 % glycerol, pH 7.4) or denaturing RIPA buffer (in the case

of EGFR immunoprecipitation experiments only) (50 mM Tris, 150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.1 % SDS, pH 8.0). Both solutions were supplemented with protease inhibitors (2 mM PMSF, 2 μg/ ml leupeptin and 2 μg/ ml pepstatin A). Additionally, in the case of analysis of tyrosine phosphorylation of the EGFR were made in the lysis buffer supplemented with 1.5 mM $MnCl_2$, 1 mM $MgCl_2$, and 1 mM sodium orthovanadate. Analysis of p-ERK and p-AKT was done adding 1mM sodium orthovanadate into the lysis buffer in additiom to the protease inhibitors. The extract was centrifuged at 20,000 × g for 10 min at 4 °C and the supernatant was measured by the amount of proteins using the spectrophotometric method of Bradford.

2.2.2.3.3. Protein electrophoresis in denaturing conditions

Electrophoretic separation of proteins was carried out according to procedures described by Laemmli, using 10 % or 7.5 % (for EGFR) SDS-polyacrylamide gels. Electrophoresis was done in running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS), between 80-120 V, at room temperature. Protein samples (50-100 μg per lane) were diluted with 5X protein loading buffer (150 mM Tris-HCl, 6 % SDS, 0.3 % bromophenol blue, 30 % glycerol, 100 mM DTT, pH 6.8) and then heated for 5 min at 95 °C prior to loading on gels. Finally, gels were processed for immunoblotting, fluorography or Coomassie blue staining.

2.2.2.3.4. Staining with Coomassie Brilliant Blue R-250

Coomassie Staining was performed by overnight incubation of the polyacrylamide gel in a staining solution (50 % methanol, 10 % acetic acid, 0.1 % Coomassie Brilliant Blue R-250) at room temperature with gentle shaking. Then the gel was washed several times with a washing solution (50 % methanol, 10 % acetic acid) until the background staining was removed and dried on Whatman paper in a Slab Gel Dryer SGD 4050 coupled to a GP100pump (both from Savant Instruments Inc., Farmingdale, NY) incubating at 65 °C for 2 h.

2.2.2.3.5. Immunoprecipitation of EGFR

To analyze activity of EGFR signaling, first EGFR was immunoprecipitated with the monoclonal antibody HB8506. For this HB8506 antibody against EGFR (2-5 mg per immunoprecipitation) was attached to protein A-Sepharose beads (30 ml for each point) in the RIPA lysis solution (50 mM Tris, 150 mM NaCl, Triton X-100 1 %, 0.5 % sodium deoxycholate, 0.1 % SDS, pH 8.0) with protease inhibitors (2 mM PMSF, 2 μg/ ml leupeptin and pepstatin) for 16 h at 4 °C. Protein A-Sepharose beads bound to HB8506 antibody were washed twice with the same lysis solution to remove unbound antibody, by short steps of centrifugation at 425 xg for 5 min. Then cell lysates were added onto the beads and incubated for 2 h at 4 °C in a rotary shaker. Then antigen-antibody complex was washed with RIPA buffer. Immunoprecipitated proteins bound to beads were released by boiling in 30 μl 5X protein loading buffer. Samples were analyzed using anti-phospho tyrosine antibody. For analysis of total EGFR total cell lysates were analyzed for EGFR using anti-EGFR antibody.

2.2.2.3.6. Quantification of cell surface EGFR by radioactive EGF binding.

The ^{125}I-EGF was prepared using chloramine T method with modifications described to avoid excessive oxidation of EGF (Carpenter and Cohen, 1976) using 10 μg of EGF and 1 mCi of NaI^{125}. Activities were obtained in the order of 50,000 to 70,000 cpm per ng of protein. After the corresponding treatments are done at 37 °C incubators, cells are moved to 4 °C cold room to perform binding assays of I^{125}-EGF using all the solutions pre-cooled. 24-well plates were washed 2 times PBS with supplemented with 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS-CM) and incubated for 2 h with agitation in binding medium (0.1 % BSA, 20 mM HEPES in DMEM Hanks medium) containing 20 ng of I^{125}-EGF/ ml. After the incubation, the unbound ligand was removed by washing 3 times with PBS-CM. Bound ligand was determined by lysing the cells with 500 μl 1 N NaOH for 1 h at room

temperature and counting the lysate in a γ counter. Nonspecific binding is obtained by adding excess amount of unlabeled EGF (500-1000X) in one of the wells.

2.2.2.3.7. GST Pull-down assay

Jurkat T cells ($1x10^6$ cells) were lysed in 1 % NP-40 buffer containing 25 mM Tris–HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin for 1 h at 4 ºC. The lysates were centrifuged at 4 ºC for 10 min at 10,000 g. The supernatants were first incubated with glutathione S-transferase (GST) attached to glutathione–sepharose for 1 h at 4 ºC and then with GST-Gal-8 linked to glutathione–sepharose beads. Competence experiments were performed pre-incubating GST-Gal-8S/L beads with 100 mM lactose. The beads were washed with PBS, suspended and boiled in loading buffer. Bound β1-integrins were resolved by 10 % SDS-PAGE, immunoblotted onto nitrocellulose membranes and visualized by ECL. When HeLa cell extracts were analyzed for GST-pull down assay cells were lysed in classic lysis buffer. Extracts were prepared as described for Jurkat T cells. Gal-8S intereaction with EGFR, β1-integrin and Patched were analyzed by immunoblotting.

2.2.2.3.8. Cell surface biotinylation

The cells were washed twice in ice-cold PBS-CM, and then sulfo-NHS-biotin was added to a final concentration of 0.5 mg/ml twice for 20 min. After three washes in ice-cold PBS-CM, the cells were incubated with 50 mMNH_4Cl for 10 min and then lysed in 20 mM Tris-HCl buffer, pH 8, 0.150 mM NaCl, 5 mM EDTA, 1 % Triton X-100, 0.2 % bovine serum albumin, and anti-protease mixture (2 mM PMSF, 2 μg/ml leupeptin and 2 μg/ml pepstatin). Cell extract was incubated with neutroavidin-Sepharose for 2 h at 4 °C. The beads were washed six times in ice-cold lysis buffer containing 500 mM NaCl, 0.1 % (v/v) Triton X-100, resuspended in sampler buffer, and boiled for 5 min. Proteins were resolved

by SDS-PAGE, electrotransferred to nitrocellulose membranes, and biotinylated β1-integrins were detected with specific antibodies and ECL detection kit.

2.2.2.3.9 Indirect immunofluorescence

After the treatments of Jurkat T cells were concluded, cells were seeded on polylysine treated coverslips and cultured at 37 °C for 15min to adhere them onto the covers. HeLa cells were already cultured on coverslips. Then the cells on coverslips were washed with PBS-CM and fixed in 4 % paraformaldehyde in PBS-CM for 30 min at room temperature. After fixation the cells were washed three times with PBS-CM. For detection of intracellular antigens cells were permeabilized with 0.2 % Triton X-100 in PBS-CM for 10 min at room temperature. Then the cells were blocked with 0.2 % gelatin in PBS-CM for 30 min and incubated with primary antibody diluted in blocking solution for 30 min at 37 °C. The coverslips were washed three times with PBS-CM for 5 min. Cells were then incubated with Alexa-488-conjugated second antibody (green) or Alexa-555 (red) antibody. Secondary antibody was washed 3 times with PBS, and then with deionized water just prior to mounting the coverslip on a drop of Fluoromount G (Washington, USA). The preparation was dried in an oven for 30-45 min at 65 °C. The preparations were stored in darkness at room room temperature until examined in the fluorescence microscope.

2.2.2.4 Cell proliferation assays

2.2.2.4.1. Crystal violet assay

For this assay cells were seeded on 96 well plates. After the treatments were done, the cells washed two times with PBS and incubated with 100 µl of 0.2 % crystal violet solution in 50 % ethanol for 5 min. Then crystal violet was washed with excess amount of water until the background staining is removed. Stained cells were lysed with 100 µl of a 0.1 M Na_2HPO_4 pH 7.4 in 50 % ethanol; incubating for 10 min. 570 nm absorbance was measured using ELISA reader.

2.2.2.5 Apoptosis assessments

2.2.2.5.1 Apoptosis analysis by immunofluorescence

Jurkat T cells incubated with 0.15μM of soluble Gal-8S for 4h in the presence or absence of Gal-8L or separate CRDs were then adhered to polylysine-coated coverslips and apoptosis was assessed by indirect immunofluorescence as in (Soza et al., 2004), using anti-cleaved Caspase-3 antibody (1:100) and Hoescht 33342 nucleus staining to detect nuclear condensation or fragmentation. Digital images for quantification of apoptotic cells (1300 X 1030 pixels) were acquired on a Zeiss Axiophoto microscope with either 63X/1.4 oil immersion objective or 40Xobjective (for quantification) and the 16-bit Zeiss Axiocam camera and transferred to a computer workstation running Axiovision imaging software (Zeiss). Apoptotic cells were counted in a cell counter plugin of Image J. Immunofluorescence images represented in figures were acquired by an inverted epifluorescence microscope (Leica DMI6000b) using a 63x glycerin immersion objective. Data were transferred to a computer workstation running the Leica LAS AF 7000 software and images were deconvolved using Huygens software.

2.2.2.5.2 Phosphatidyl serine exposure analysis

After the treatments were completed, cells were washed twice with cold PBS and then resuspended in 1X Binding Buffer (10X Binding Buffer content: 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) at a concentration of $1x10^6$ cells/ml. 100 µl of the solutions were transfered (1×10^5 cells) to a 5 ml culture tube. 5 µl of FITC Annexin V and 5 µl PI were added onto the solutions containing the cells, gently vortexed and incubated for 15 min at RT (25°C) in the dark. 400 µl of 1X Binding Buffer was added to each tube and samples were analyzed by flow cytometry within 1 h.

2.2.2.5.3 Analysis of apoptosis by flow cytometry

Once the treatments were completed, cells were washed with cold PBS and then resuspended in 100 μl PBS and 2 μl FC Block and incubated for 10 min on ice. Then cells were washed with 2 % FBS in PBS and centrifuged at 600 g for 5 min. Cells were then resuspended in 250 μl Cytofix/ Cytoperm (BD Pharmingen) to fix and permeabilize the cells and incubated on ice for 15 min in the dark. Cells were twice washed in 1 % FBS 0.1 % Saponin in PBS (wash buffer). Cells were then resuspended in 80 μl wash buffer and 20 μl anti-cleaved caspase 3 antibody conjugated with FITC and incubated for 20 min on ice in the dark. After washing twice with wash buffer, cells were resuspended in 500 μl of 2 % FBS.

2.2.2.6 Migration assays in transwell filters

Transwell migration chambers were hydrated with media. Cells deprived of serum for 16 to 20 h were trypsinized, counted and 5 x 10^4 cells seeded into the upper chamber of polycarbonate filters with pores of 8.0 m at 100μL of DMEM without serum (in the presence or absence of treatments). In some assays lower part of the filters were treated with galectins for 15min prior to seeding the cells on upper part of the filter. In the lower chamber 450 mL of 5 % FBS medium was added and incubated filters in oven at 37 ° C for 16h. In some treatments galectins were added into the media. The cells were fixed and stained with 0.2 % crystal violet/ 50 % ethanol for 10 min. After washing the chambers with dH2O, the cells at the top of the chamber are removed with Q-tips. The cells migrated to the bottom side of the membrane were photographed by the lower face Motic BA310 microscope and Moticam 2500 4X camera and plotted as the percentage of migrated cells per field.

2.3. Statistical analysis:

Statistical analysis of the results was performed with Excel using the Student`s T test of statistical formulas.

3. RESULTS

Results of this thesis are divided into two chapters. Chapter 1 includes the results of the function of Gal-8 isoforms on Jurkat T cell apoptosis. Chapter 2 includes the results that show the function of Gal-8 isoforms on HeLa and U87 cell proliferation, migration and the link between Gal-8S and Sonic Hedgehog Pathway.

3.1 Effect of Gal-8 isoforms on Jurkat T cell apoptosis

In a previous work our laboratory showed that Gal-8S induces apoptosis in Jurkat T cells, as well as in anti-CD3/CD28 activated human T cells from freshly isolated PBMC, increasing the expression of pro-apoptotic FasL receptor (Norambuena et al., 2009). Human recombinant Gal-8S and chimeric GST-Gal-8S triggers apoptosis at concentrations as low as 0.03-0.08 μM (Norambuena et al., 2009). Jurkat T cell apoptosis induced by Gal-8S involves phospholipase D/phosphatidic acid and ERK1/2 activation (Norambuena et al., 2009). Although the pro-apoptotic effect in Jurkat T cells was not found in other studies, likely reflecting variations in this cell line, the apoptotic effect of Gal-8S on activated T cells has been corroborated (Cattaneo et al., 2011). Thus, our Jurkat T cells display features of activated T cells and provide a convenient model system to approach the functional significance of Gal-8 linker-length variants. Here we analyzed the effect of Gal-8L and separate CRDs, Gal-8N and Gal-8C on Jurkat T cell apoptosis in comparison to Gal-8S that was studied previously in our and other laboratories.

3.1.1 Structural difference between Gal-8S and Gal-8L isoforms

Gal-8S and Gal-8L isoforms both contain exactly the same CRDs but differ in the length of their linker peptide. The 42 extra aminoacids of the Gal-8L linker peptide includes a thrombin cleavage site which may cause separation of the CRDs in the presence of thrombin (Nishi et al., 2005; Nishi et al., 2006). Single CRD can be generated by alternative splicing of the Gal-8 gene (Bidon et al., 2001b). Figure 1 shows the schematic structure of Gal-8S and Gal-8L as well as coomassie staining of these two isoforms along with N and C terminal CRDs (Fig. 1).

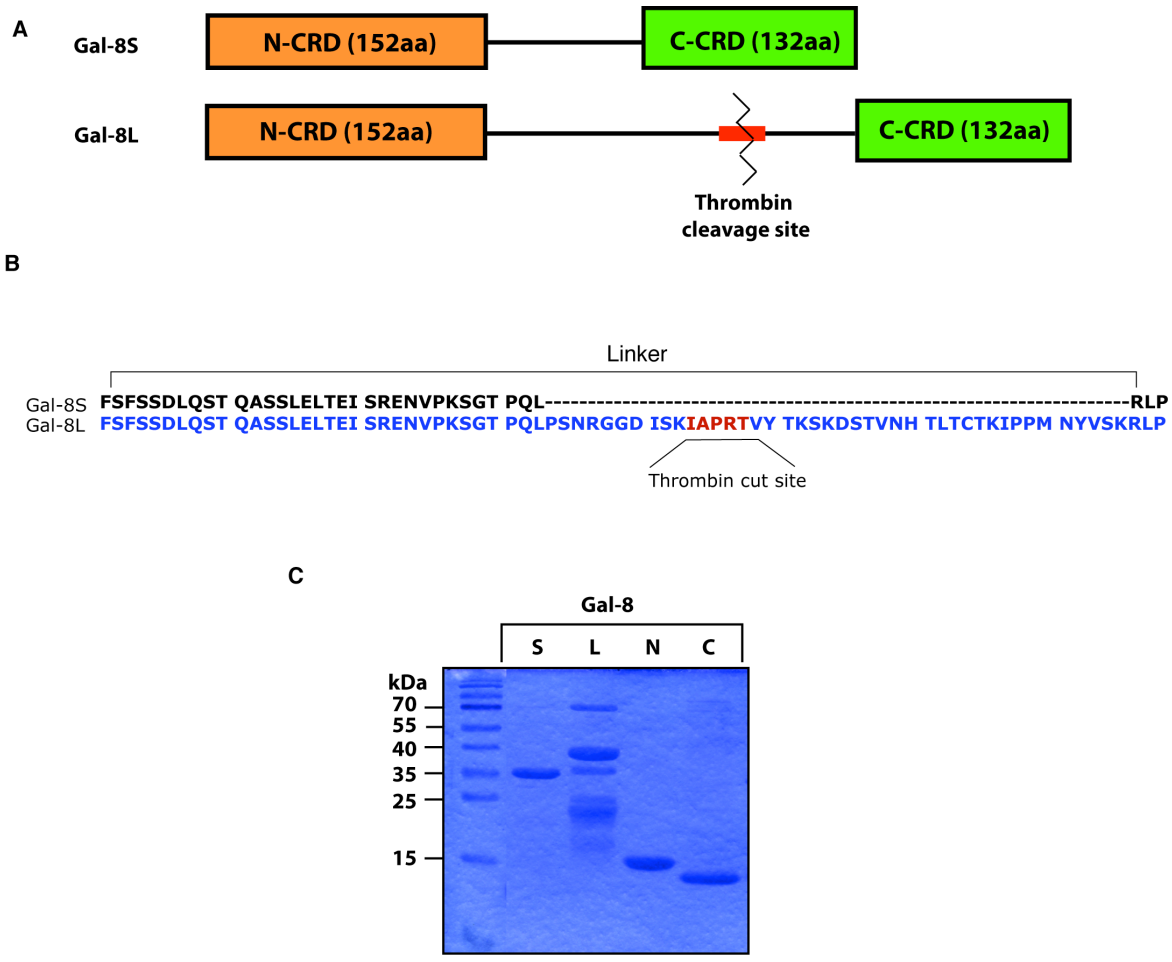

Figure 1: Structural difference between Gal-8 isoforms: Gal-8L has 42 additional aminoacids in its linker peptide in which resides a thrombin cleavage site.
A) Amino terminal carbohydrate recognition domain (N-CRD) of both Gal-8 isoforms contain 152 aminoacids(aa). Carboxy terminal (C-CRD) contains 132aa. Gal-8S has a 32aa of linker peptide whereas Gal-8L has additional 42 aa in its linker peptide in which a thrombin cleavage side resides. B) Aminoacid sequence of the linker peptide of both Gal-8 isoforms C) Coomassie staining of recombinant N-CRD, C-CRD and Gal-8 isoforms.

3.1.2 Different binding affinities of Gal-8L and Gal-8S on Jurkat T cells

Before comparing the effects of Gal-8S and Gal-8L on Jurkat cells we first characterized their cell surface binding in order to decide the amount of recombinant galectins to be used for comparative analysis. Jurkat T cells were incubated at 4 ºC with increasing concentrations of biotinylated Gal-8 isoforms for 30 min. FACS analysis of the percentage of galectin-positive cells and the mean fluorescence intensities (MFI) showed that Gal-8S is 10-fold more effective than Gal-8L in binding activity, as shown by similar binding at 2 μg/ml (0.06 μM) Gal-8S and 20 μg/ml (0.5 μM) Gal-8L (Fig. 2A and C). In order to validate whether cell surface binding of these galectins dependent on carbohydrate dependent interactions, cells were treated with lactose along with the galectins. Lactose competition indicated that such binding activity relays on interactions with cell surface glycans (Fig. 2B and D). For most of the ensuing experiments we used 5 μg/ml Gal-8S and 20 μg/ml of Gal-8L, Gal-8N and Gal-8C which allow comparisons in similar percentage of cells bearing similar binding levels of each isoform.

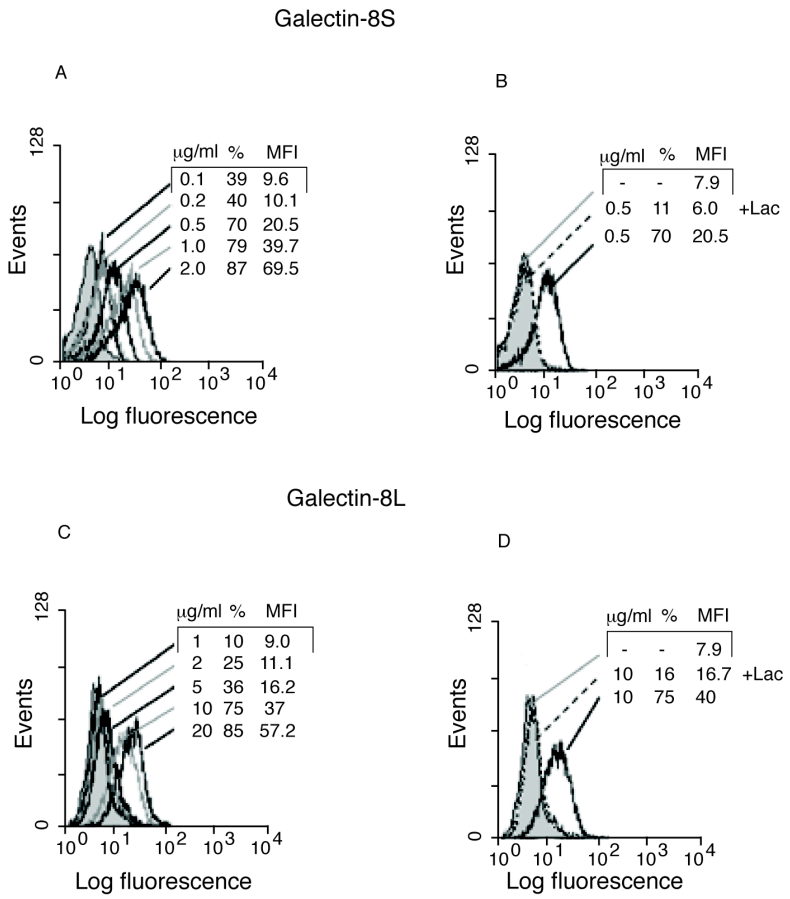

Figure 2: Gal-8S has higher avidity than Gal-8L for Jurkat T cell surface binding.
Jurkat T cells were incubated at 4 ºC for 30 min with increasing concentrations of biotinylated Gal-8S (A; 0.1, 0.2, 0.5, 1 and 2 µg/ml) or Gal-8L (C; 1, 2, 5, 10 and 20 µg/ml). The percentage of galectin positive cells was analyzed by Fluorescence Activated Cell Sorting (FACS), quantified by mean channel fluorescence and represented semi-logarithmically. Carbohydrate dependence is shown by the inhibition exerted by 100 mM lactose upon Gal-8S (0.5 µg/ml) and Gal-8L (10 µg/ml) binding (B and D).

3.1.3 Gal-8L lacks the capability to induce Jurkat T cell apoptosis and counteracts the pro-apoptotic effect of Gal-8S.

Serum has glycoproteins that hide the effect of added galectins since they also bind to some of these glycoproteins that diminishes the effect. Therefore, we serum deprived the cells for 2 h in RPMI medium and then incubated them with 5 μg/ml Gal-8S or with 20 μg/ml of Gal-8L for 4 h (long enough to observe about 25 % apoptosis in the presence of 5 μg/ml Gal-8S). We then evaluated their effect on apoptosis by analysis of Caspase-3 activation and phosphatidylserine (PS) cell surface exposure. Gal-8S and Gal-8L showed contrasting results in the analyzed parameters. While Gal-8S treatment led to Caspase-3 activation (Fig. 3A) and PS exposure (Fig. 3B), Gal-8L did not elicit these effects (Fig. 3A and 3B) even though at the used concentration (20 μg/ml) it bound to 85 % of the cells with similar intensity as Gal-8S did (see Fig. 2C). In order to analyze whether Gal-8L was inhibitory upon the apoptotic effect of Gal-8S, we first pre-treated the cells with Gal-8L at 37 ºC before addition of Gal-8S. Infact Gal-8L pre-treatments decreased Caspase-3 activation almost to the level of control (Fig. 3A). Similar results were obtained on anti-CD3/CD28 activated PBMCs isolated from healthy people (Fig. 3C). These results show for the first time that Gal-8L counteracts the pro-apoptotic effect of Gal-8S on activated T cells.

Cell surface exposure of PS is an early sign of apoptosis that can be analyzed by the binding of fluorescently conjugated Annexin V (Callahan et al., 2000). As expected FACS analysis showed that Gal-8S induced PS exposure whereas Gal-8L did not. However, unexpectedly PS exposure induced by Gal-8S was not inhibited upon Gal-8L pre-treatment (Fig. 3B). Thus PS exposure may not be enough for triggering apoptosis in the Gal-8S induced pathway, as apoptosis was inhbited by Gal-8L pre-treatment while PS exposure was not.

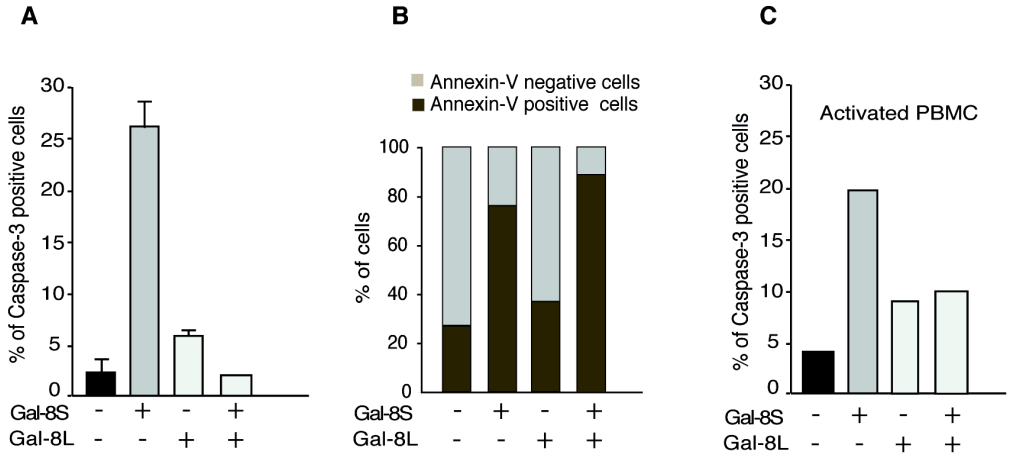

Figure 3: Gal-8L inhibits Caspase-3 activation induced by Gal-8S but not PS exposure.
Jurkat T cells were maintained for 2 h in serum free media and were then incubated for 4 h in suspension in the presence of 5 µg/ml Gal-8S or 20 µg/ml Gal-8L at 37 ºC, or pre-treated with Gal-8L at 37 ºC for 30 min prior to addition of Gal-8S. Percentage of apoptotic cells was analyzed by Caspase-3 activation by IF (A) and PS exposure detected by Annexin V staining was analyzed by FACS (B). C) PBMCs activated with anti-CD3/anti-28 antibodies for 48 h were treated with galectins and analyzed as described in 2A.

3.1.4 Differential effects of Gal-8L and Gal-8S on β1-integrin and ERK1/2 activation correlate with their opposite actions on apoptosis

β1-integrins can coordinate survival or death responses depending on the ligand (Stupack and Cheresh, 2004), mediating apoptosis in cells as distinct as smooth muscle, rheumatoid synovial, endothelial and T cells (Arencibia et al., 2002; Damle et al., 1993b; Mary et al., 1999; Nakayamada et al., 2003; Wernig et al., 2003). ERK1/2 activation is required for the cell death that restricts the extension of the immune response eliminating recently activated and re-stimulated T cells (van den Brink et al., 1999; Zhu et al., 1999). Our laboratory has previously shown in Jurkat T cells that Gal-8S binds to certain β1-integrins, such as α1β1, α3β1 and α5β1 but not α2β1 or α4β1 integrins (Carcamo et al., 2006) and triggers an ERK1/2 pro-apoptotic pathway (Norambuena et al., 2009). To count on more detailed assays for testing the Gal-8L counteracting mechanism here we first assessed whether the ERK1/2 pro-apoptotic pathway does require activation of β1-integrins. We evaluated ERK1/2 activation induced by Gal-8S in the presence of an inhibitor of Focal Adhesion Kinase (FAKi) that is known to inhibit β1-integrin signaling (Slack-Davis et al., 2007). Jurkat T cells incubated for 2 h with 5 μg/ml of Gal-8S in the presence of 3 μM FAKi, showed reduced ERK1/2 activation (Fig. 4A). Therefore, β1-integrin activation induced by Gal-8S seems to be a crucial step to trigger the previously reported pro-apoptotic ERK1/2 pathway in Jurkat T cells (Norambuena et al., 2009).

This pathway from β1-integrins to ERK1/2 signaling engaged by Gal-8S (Carcamo et al., 2006; Norambuena et al., 2009) provides then a possible target for the anti-apoptotic effects of Gal-8L. To test this possibility we analyzed the effect of the isoforms on β1-integrin activation status. Using an antibody that only detects β1-integrin in its active form

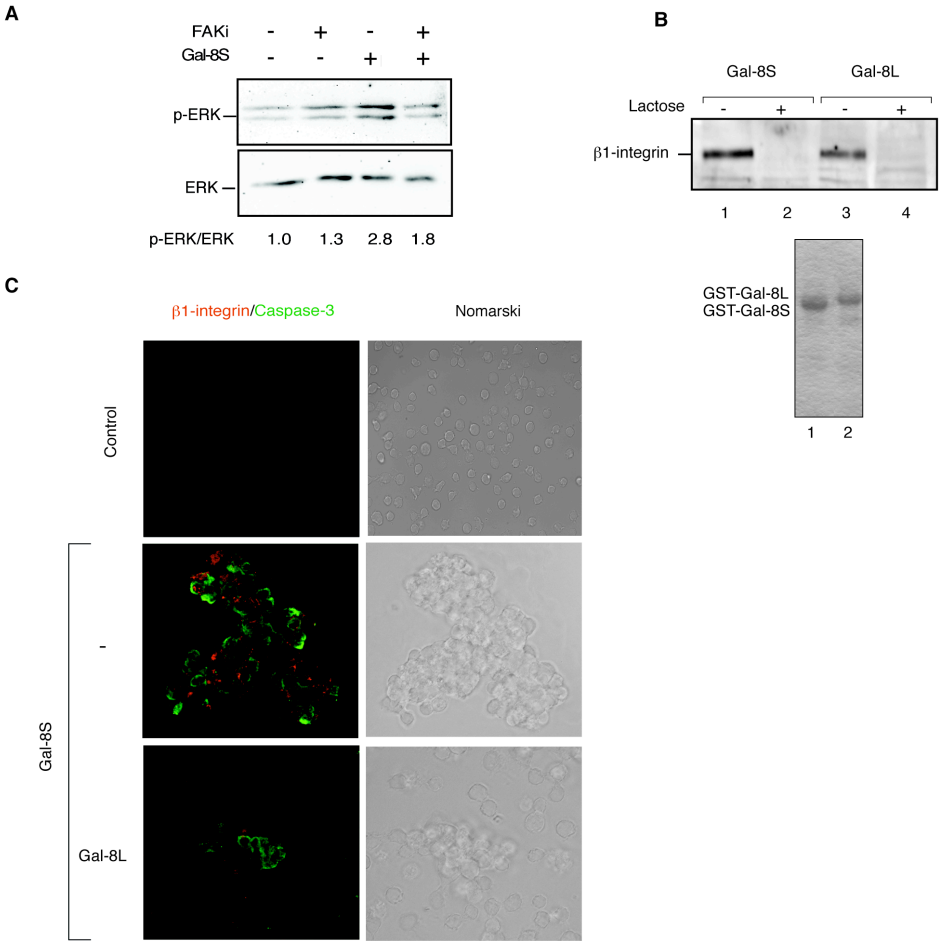

Figure 4: Gal-8S induces ERK1/2 activation via β1-integrin pathway.
A) Jurkat T cells co-treated with 3 µM FAK inhibitor (PF-573228) and 5 µg/ml Gal-8S for 2 h at 37 ºC were analyzed for phospho-ERK1/2 (P-ERK) and total-ERK (T-ERK). The numbers below the blot represents the densitometric ratio of P-ERK and T-ERK normalized according to the control. B) Affinity chromatography assay was performed by incubating Jurkat T cell extracts with GST-Gal-8S or GST-Gal-8L linked to Glutathione–Sepharose in the absence or presence of 100 mM lactose and analyzed by immunoblot using β1-integrin antibody. C) Jurkat T cells were maintained for 2 h in serum free media and were then incubated for 4 h in suspension in the presence of 5 µg/ml Gal-8S at 37 ºC, or pre-treated with 0.5 µM of Gal-8L at 37 ºC for 30 min prior to addition of Gal-8S. Cells were analyzed for Cleaved- Caspase-3 and activated β1-integrin by IF.

we found that only Gal-8S induces both β1-integrin (in red) and Caspase-3 (in green) activation. Furthermore, pre-incubation with Gal-8L prevented Gal-8S induced β1-integrin activation (Fig. 4C). We then analyzed binding of Gal-8 isoforms to the β1-integrins by GST pull-down experiments. Both Gal-8S and Gal-8L bound to β1-integrin (Fig. 4B). Thus the reason why Gal-8L does not induce activation of β1-integrin is not due that it cannot bind to β1-integrin.

We then compared the effects of Gal-8 isoforms alone and in combination on ERK1/2 activation. As we have previously showed, Gal-8S induced strong ERK1/2 activation during the first hour of treatment with Gal-8S (Fig. 5A, lane 2), which could still be detected with a decreased intensity for at least 3 h (Fig. 5A, lane 6). In contrast, no ERK1/2 activation was detected under Gal-8L treatment (Fig. 5A, lanes 3 and 7). Pre-incubation with Gal-8L for 30 min reduced the effect of Gal-8S on ERK1/2 activation by 20 % at the first hour (Fig. 5A, lane 5) and to the basal levels after 3 h (Fig. 5B, lane 8).

All these results indicate that Gal-8L counteracting effects may at least partially be due to inhibition of β1-integrin activation that constitutes at least one of the first steps in the ERK1/2 pro-apoptotic pathway triggered by Gal-8S. Reduced ERK1/2 activation is then insufficient for triggering Caspase-3-mediated apoptotic process.

3.1.5 Activation of AKT pathway also contributes to the anti-apoptotic effect of Gal-8L

Because it seems unlikely that just a single mechanism accounts for the dramatic Gal-8L counteraction of the pro-apoptotic action of Gal-8S, we asked whether AKT pathway known to exert anti-apoptotic effects in many cellular systems (Vivanco and Sawyers, 2002) is also involved. Strikingly, both Gal-8S and Gal-8L activated AKT but with different timing (Fig. 4B). Gal-8S activates AKT activation with higher intensity

within the first hour, returning to basal levels after 3 h (Fig. 5B, lanes 2 and 6). In contrast, Gal-8L induced a more robust AKT activation that extended for at least 3 h even in the presence of Gal-8S (Fig. 5B, lanes 7 and 8). These results indicate that Gal-8L may block apoptosis induction by Gal-8S through a dual complementary mechanism: decreasing the activity of β1-integrin-to-ERK1/2 pro-apoptotic pathway while increasing the activity of an anti-apoptotic AKT pathway.

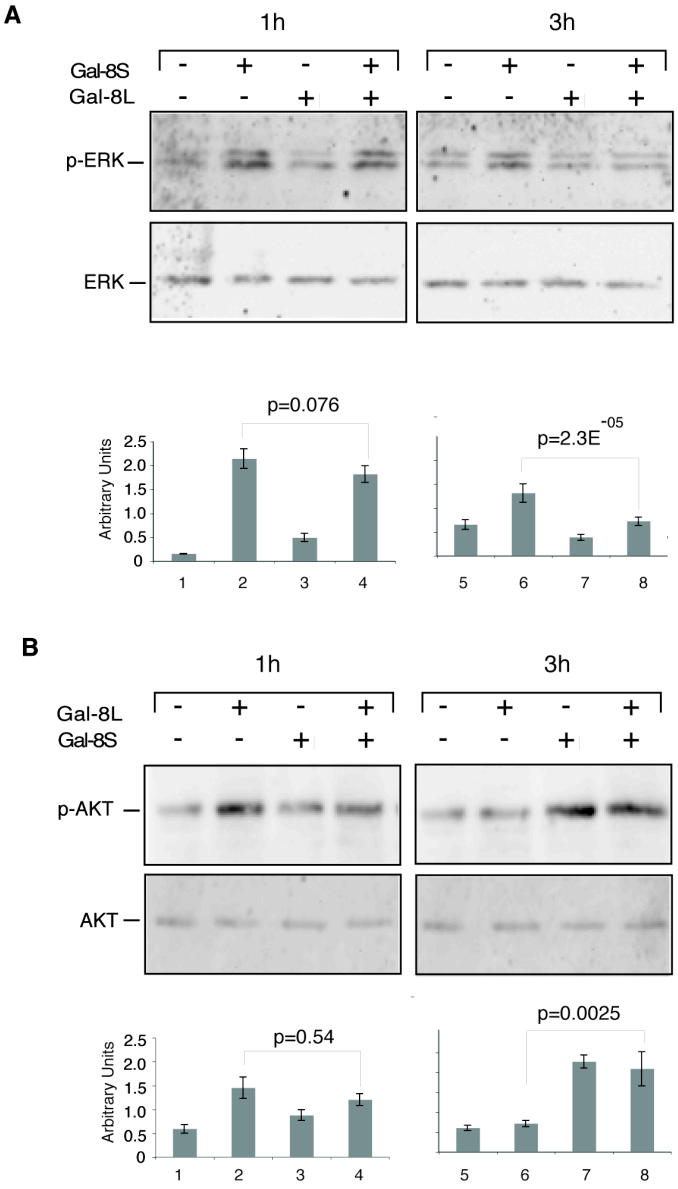

Figure 5: Effect of Gal-8S and Gal-8L on status of phosphorylation of ERK1/2 and AKT. Jurkat cell extracts were lyzed and analyzed by WB for P-ERK1/2 / T-ERK (A) and P-AKT / T-AKT (B). Graphs represent densitometric quantifications of three independent experiments including the standard error.

3.1.6 Gal-8S and Gal-8L are endocytosed and induce endocytosis of β1-integrin

One possibility for how Gal-8L could be counteracting the effects of Gal-8S is that during 30 min pre-treatments Gal-8L may be endocytosed along with its bound glycoconjugates. To analyze this possibility we analyzed endocytosis of the recombinant proteins by Jurkat T cells. We fluorescently labeled Gal-8S with Alexa-488 and Gal-8L with Alexa-594. This way we can differentiate recombinant proteins from the endogenously expressed ones. We pre-bound these fluorescently labeled proteins to the cells at 4 ºC for 30 min. At this temperature endocytosis does not occur thus the proteins were only observed on the cell surface (Fig. 6a, 6d). This surface bound galectins could be removed by 15 min lactose wash with 100 mM lactose treatment (Fig. 6b, 6d), confirming the sugar dependence for binding to the surface of the cells. After 30 min binding at 4 ºC, temperature was shifted to 37 ºC and cells were cultured for 30 more min and cells were washed for 15 min with 100 mM lactose to only visualize endocytosed galectins. Localization of both isoforms changed from cell surface to the intracellular compartments (Fig. 6c, 6f).

Once galectins are endocytosed, their bound glycoconjugates may also be endocytosed along with them, including the one (s) responsable for induction of apoptosis.

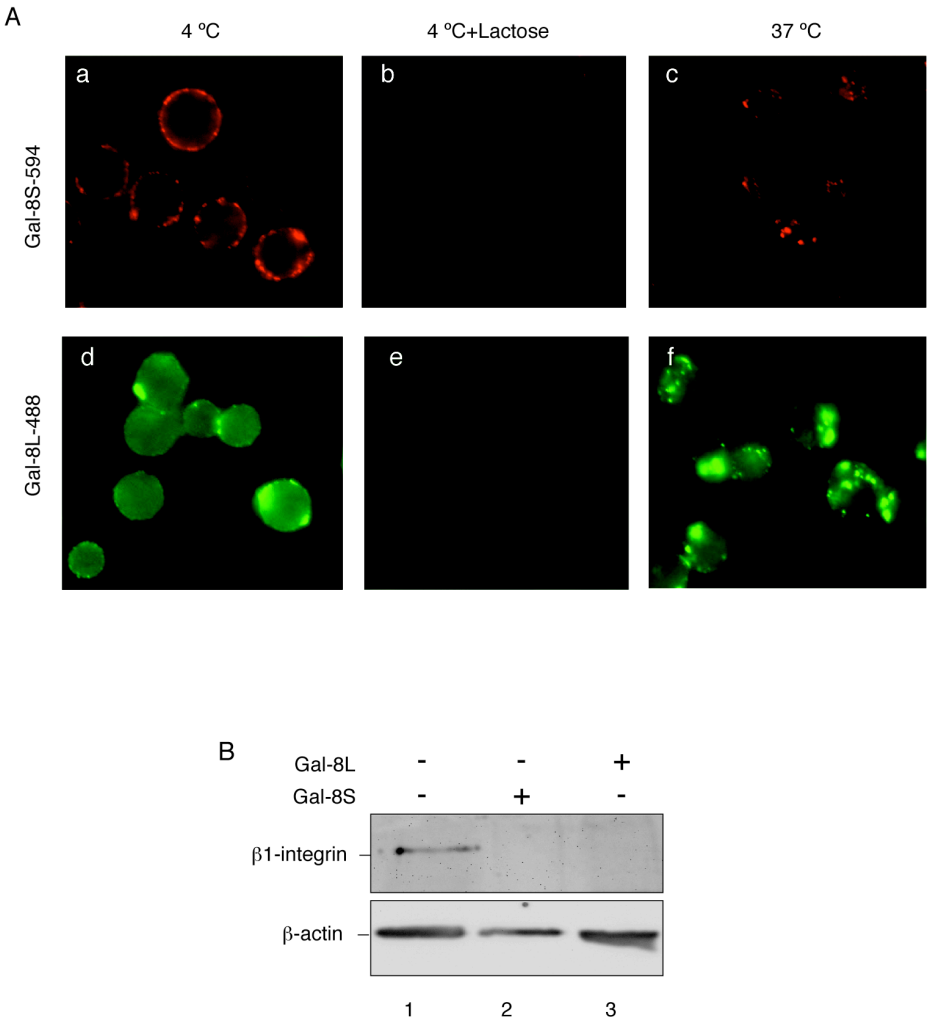

Figure 6: Gal-8S and Gal-8L get endocytosed and induces endocytosis of β1-integrin. 2 h serum starved Jurkat T cells were incubated with either 5 µg/ml Gal-8S conjugated with Alexa-594 or 20 µg/ml Gal-8L conjugated with Alexa-488 at 4 ºC for 30 min, washed by 200 mM lactose (5b, 5e) or not (5a, 5d) and then temperature was shifted to 37 ºC and cells were incubated for 30 min (5c, 5f). B) Jurkat T cells were treated with either 5 µg/ml Gal-8S or 20 µg/ml Gal-8L for 30 min at 37 ºC. Then the surface of the cells was biotinylated at 4 ºC for 1 h. Biotinylated proteins were precipitated with streptavidin beads and then analyzed by western blot for β1-integrin. In the total cell lysates actin was analyzed as loading control.

So far the only receptor that was shown to be involved in Gal-8S induced apoptosis is β1-integrin. Thus, we analyzed endocytosis of β1-integrin after Gal-8S and Gal-8L treatments. Cells were treated with Gal-8S or Gal-8L and cell surface removal of β1-integrin was analyzed by biotinylation assay. In this assay cell surface is biotinylated and biotinylated proteins were captured on neutravidin-sepharose beads. Then the bound proteins were resolved by SDS-PAGE and analyzed for β1-integrin. 30 min treatment with Gal-8S or Gal-8L induced endocytosis of β1-integrin (Fig. 6B). This suggests that Gal-8L pre-treatment could counteract apoptotic effect of Gal-8S by removing β1-integrin from cell surface.

3.1.7 Effect of separate carbohydrate recognition domains (CRDs)

Because Gal-8L can be cleaved by thrombin, its functions might be not only exerted by the entire molecule but also by proteolytically released N- and C-terminal fragments. To assess whether the tandem-repeat structure is required for the counteracting effects of Gal-8L on Gal-8S, we analyzed the effect of separate CRDs (Gal-8N and Gal-8C) on the same parameters that we analyzed for Gal-8L.

3.1.7a Gal-8N and Gal-8C of Gal-8 mimic the binding properties of Gal-8L

We first analyzed the binding of Gal-8N and Gal-8C to the surface of Jurkat T cells. Similar to Gal-8L, Gal-8N and Gal-8C showed lower binding avidities than Gal-8S (Fig. 7a, 7c), involving sensitivity to lactose inhibition for cell surface binding (Fig. 7b, 7d). Thus we used 20 μg/ml of Gal-8N and Gal-8C to test their effect on apoptosis.

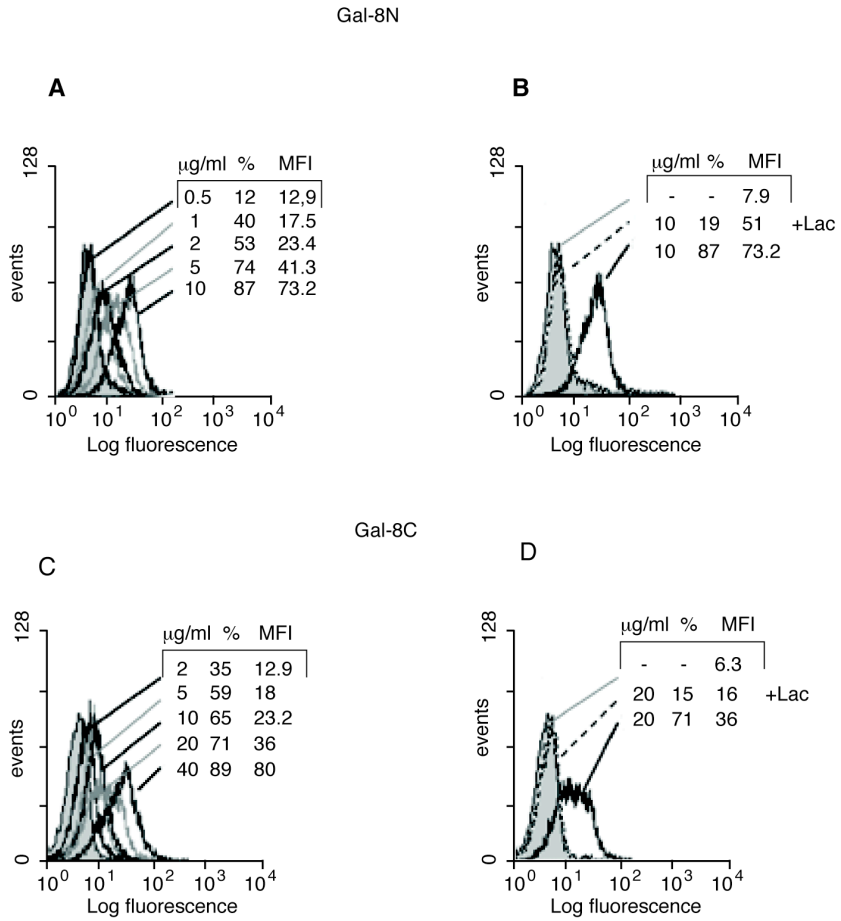

Figure 7: Binding properties of separate Gal-8N and Gal-8C are similar to Gal-8L.
Jurkat T cells were incubated at 4 ºC for 1 h with biotinylated Gal-8N or Gal-8C at the following concentrations: A) Gal-8N (0.5, 1, 2, 5 and 10 µg/ml); C) Gal-8C (2, 5, 10, 20 and 40 µg/ml). Percentage of galectin positive cells was analyzed by FACS, quantified by mean channel fluorescence and represented semi-logarithmically. Incubation with biotinylated Gal-8N (0.5 µg/ml), or Gal-8C (10 µg/ml) in the absence or presence of 100 mM lactose (B and D) shows carbohydrate dependency of the binding.

3.1.7b Gal-8N and Gal-8C of Gal-8 mimic the counteracting effects of Gal-8L

Also similar to Gal-8L, neither Gal-8N nor Gal-8C induced Caspase-3 activation, while both abolished Gal-8S triggered Caspase-3 and β1-integrin (Fig. 8A, 8C) activation. CRDs alone did not induce PS exposure and pre-treatment with CRDs did not diminish Gal-8S induced PS exposure (Fig. 8B). These results open the possibility that Gal-8L may exert counteracting effects upon Gal-8S function due to the generation of CRD-containing fragments generated by thrombin cleavage rather than full length Gal-8L.

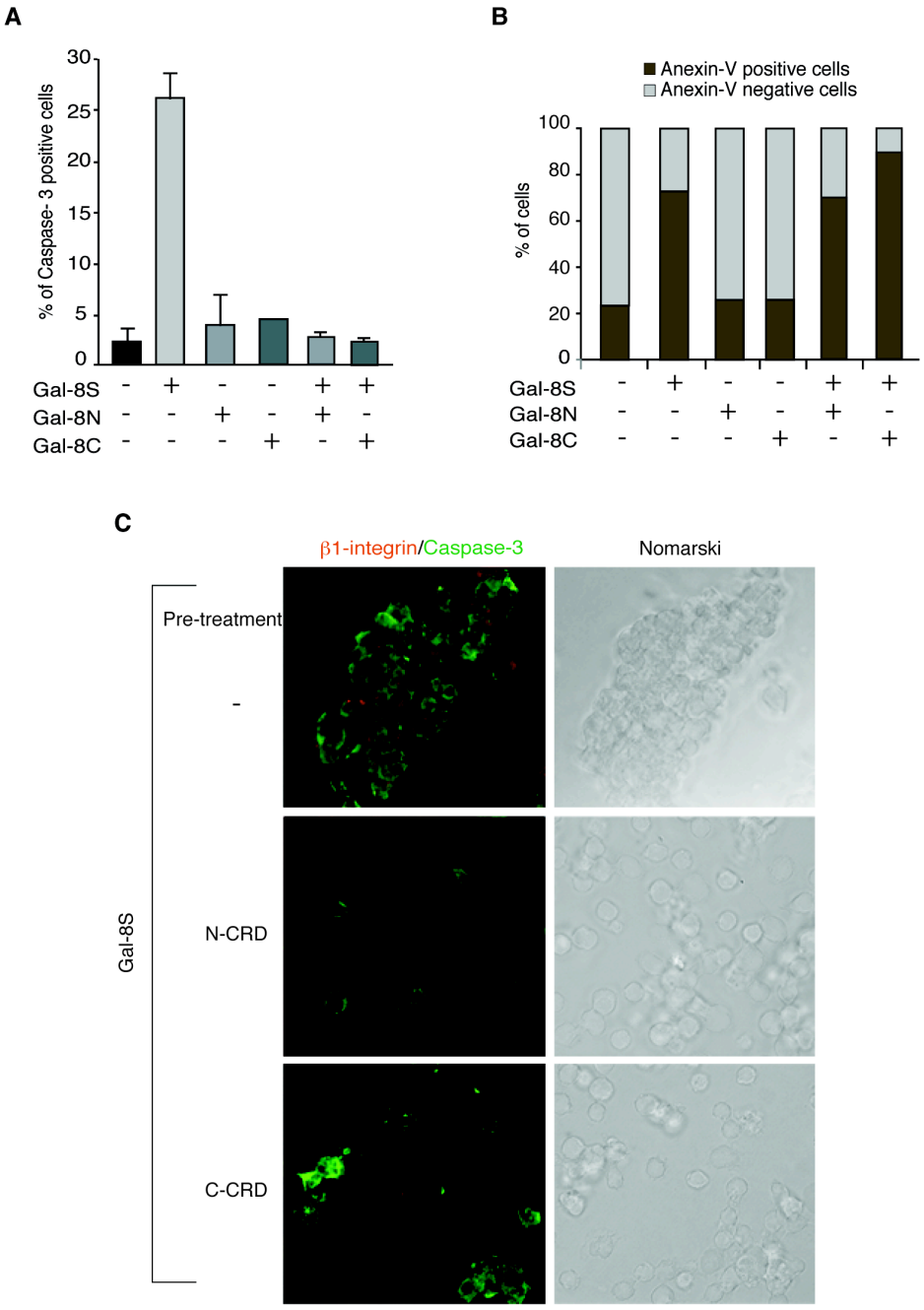

Figure 8: Gal-8N and Gal-8C inhibit Caspase-3 and β1-integrin activation induced by Gal-8S but not PS exposure.

Jurkat T cells were incubated at 37 ºC for 4 h in suspension with of 5 µg/ml Gal-8S or 20 µg/ml Gal-8N or Gal-8C, either alone or together with 0.5 mM of Gal-8L pre-incubated for 30 min. Percentage of apoptotic cells was analyzed by IF staining of activated caspase-3 and quantified (A) and PS exposure was analyzed by annexin V staining and FACS analysis (B). Activation of β1-integrin and Caspase-3 is shown by IF (C).

3.1.8 Gal-8L inhibitory effects upon Gal-8S involves cleavage of the linker

Because we noticed that not all the generated batches of Gal-8L presented the smaller fragments indicative of proteolytic cleavage, we used length Gal-8L to test whether its cleavage is essential for its inhibitory efects upon the apoptotic effect of Gal-8S. Gal-8L was digested with thrombin at 37 ºC for 4 h, with an enzyme to substrate weight ration of 1:300. Cells were treated with 5 μg/ml Gal-8S, 20 μg/ml Gal-8L or 20 μg/ml Gal-8L previously incubated with thrombin for 4 h. On the contrary to what we have observed with the batches that contains cleaved Gal-8L, full length Gal-8L (Gal-8L-FL) induced apoptosis, although at a lower percentage (23.4%) than Gal-8S (36%) (Fig. 9). Control cells presented 16% basal level apoptosis. Thrombin digestion of Gal-8L-FL completely abolished pro-apoptotic effect of Gal-8L-FL (Fig. 9).

As shown in Fig. 2A, pre-treatment with fragmented Gal-8L inhibit Gal-8S induced apoptosis. We then analyzed whether thrombin digested Gal-8L-FL also inhibits Gal-8S induced apoptosis. Effectively thrombin digested Gal-8L-FL diminished Gal-8S induced apoptosis from 36% to 21.5% (*** p=0.0007). In the absence of thrombin treatment Gal-8L pre-treatment diminished Gal-8S induced apoptosis from from 36% to 29% (* p=0.02). These results show that thrombin cleaved Gal-8L-FL is much more effective than Gal-8L in counteracting the effect of Gal-8S.

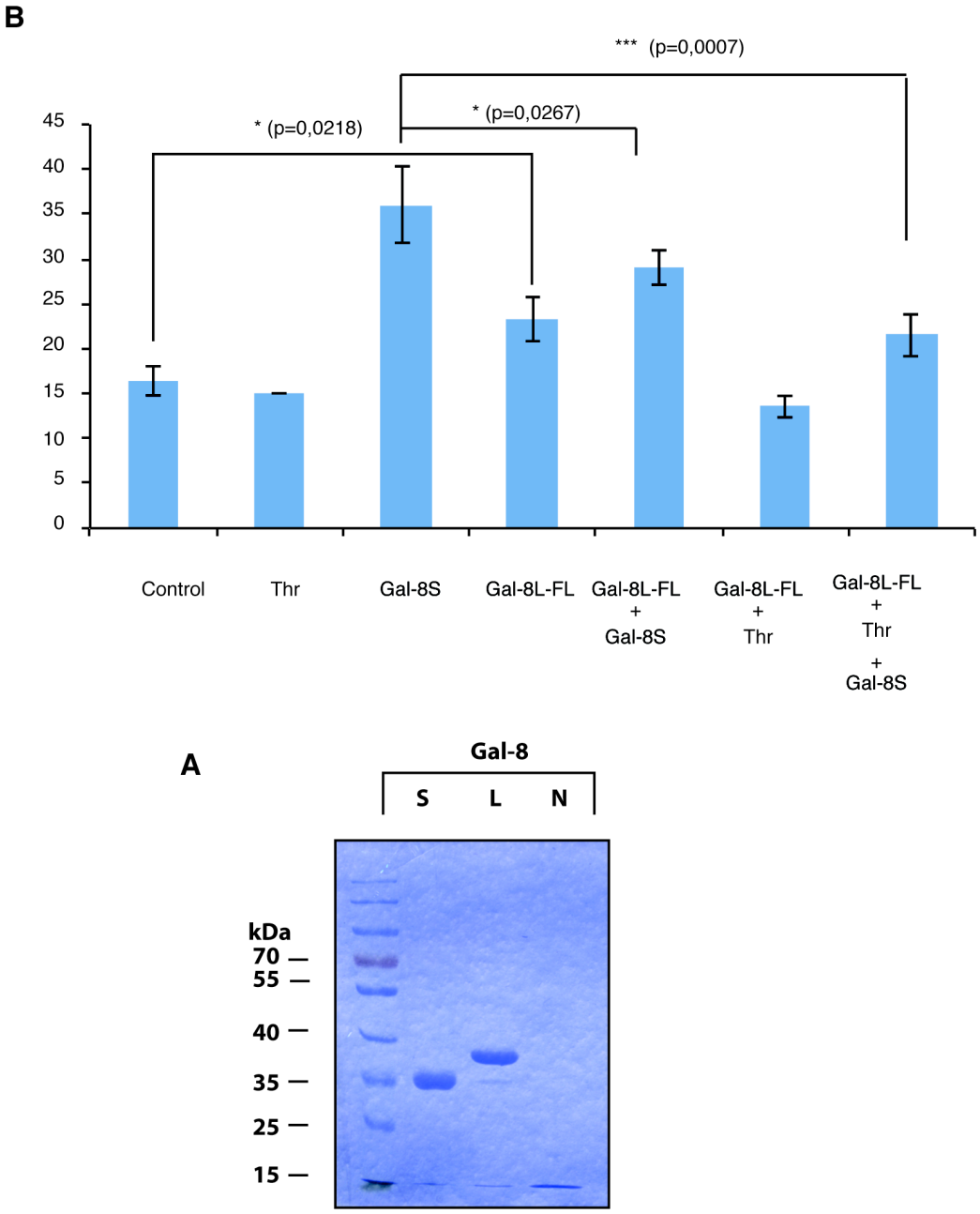

Figure 9: Thrombin cleavage of full length Gal-8L is essential for it to show its inhibitory effect upon proapoptotic function of Gal-8S

A) Jurkat T cells were maintained for 2 h in serum free media and were then incubated for 4 h in suspension in the presence of 3units/ml Thrombin (Thr), 5 µg/ml Gal-8S or 20 µg/ml full length Gal-8L (Gal-8L-FL), or pre-treated with Gal-8L or Thr digested Gal-8L for 30 min prior to addition of Gal-8S. Percentage of apoptotic cells was analyzed by Caspase-3 activation by flow cytometry using Caspase-FITC antibody. B) Galectins used in these experiments.

3.2 Effect of Gal-8 isoforms on HeLa and U87 cells

3.2.1 Gal-8S induces proliferation of Hela and U87 cells while Gal-8L, -8N and -8C do not.

Our findings on the differential effect of Gal-8 isoforms on Jurkat T cell apoptosis led us to analyze their function in model systems related to cancer. We analyzed cell proliferation by crystal violet (CV) assay on two very widely used cancer cell lines: HeLa (derived from human cervical cancer) and U87 (derived human glioblastoma cancer) cells. CV is a triphenylmethane dye (4-[(4-dimethylaminophenyl)-phenyl-methyl]-N,N-dimethyl-aniline) used to quantify the relative density of adhered cells as a function of the absorbance of the dye taken up by the cells (Gillies et al., 1986). CV stains DNA and upon solubilization the amount of dye taken-up by the monolayer and the intensity of the color produced are proportional to cell number .

Serum starved HeLa cells were treated with 20 μg/ml Gal-8S, Gal-8L, Gal-8N, Gal-8C for 3 consequitive days in 0.5% serum. Gal-8S induced (Fig. 10A), whereas Gal-8L and separate CRDs diminished HeLa cell proliferation, which may be due to apoptosis, cell cycle arrest or inhibition of cell adhesion.

In U87 cells Gal-8S also induced proliferation whereas Gal-8L, Gal-8N and Gal-8C did not have any effect (Fig. 10B).

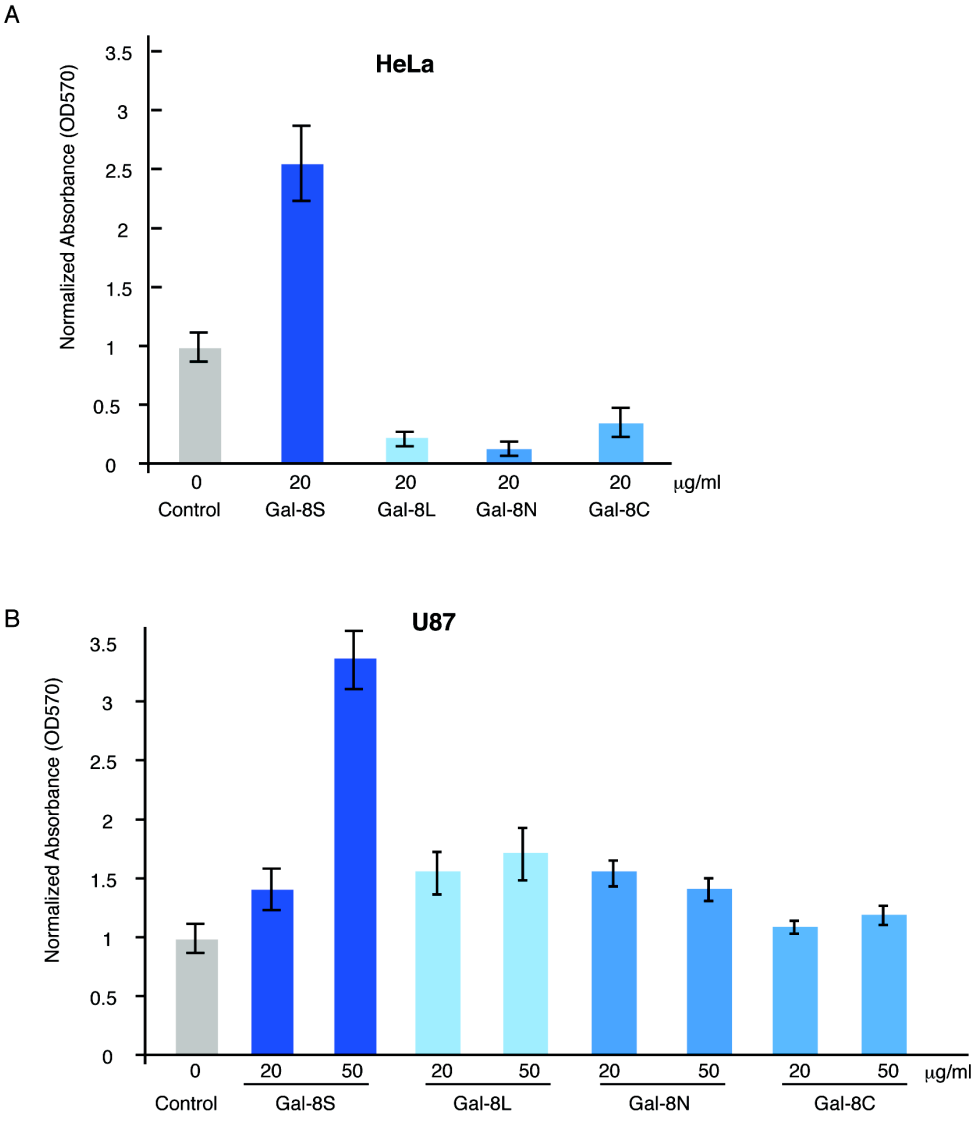

Figure 10: Gal-8S increases HeLa and U87 cell proliferation whereas Gal-8L, Gal-8N and Gal-8C inhibit HeLa cell proliferation and have no effect on U87 cells.
A) 1500 overnight serum starved Hela cells were seeded on 96 well plates on day 1 and the next 3 days cells were treated with 20 µg/ml of each galectin in 0.3% serum for three consequitive days. On the fifth day, cells were analyzed by crystal violet assay in order to assess cell proliferation. B) 2000 U87 cells were seeded on 96 well plates on day 1, overnight serum starved on day 2, treated with 20 or 50 µg/ml of each galectin in 0.3% serum on day 3 and analyzed by crystal violet assay on day 4.

3.2.2 Effect of Gal-8S on EGFR activity

In non-tumorigenic dog kidney epithelial (MDCK) cells, Gal-8S overexpression has been found to increase proliferation, migration and invasion in an EGFR dependent manner (PhD thesis of Claudia Oyanadel). MDCK cells overexpressing Gal-8S also had higher levels of EGFR activity. Here we found that Gal-8S also induced cell proliferation in HeLa cells but independently of EGFR function.

3.2.3 Gal-8S induces activation of EGFR and ERK pathways

Overnight serum depleted HeLa cells were treated with increasing concentrations of recombinant Gal-8S (5, 10, 20 μg/ml) for 30 min and the effect of the treatment was analyzed on activation of EGFR by immunoprecipitating the receptor and analyzing its tyrosine phosporylation. As shown in Fig. 11A recombinant Gal-8S treatment of HeLa cells induced concentration dependent activation of EGFR and its downstream ERK1/2 pathways even in the absence of serum or addition of any ligands. ERK1/2 activated by Gal-8S is at least partially via EGFR, as ERK1/2 phosphorylation is diminished in the presence of AG1478 (Fig. 11A). Gal-8S induced a transient activation of ERK1/2 and AKT, showing a peak at 5 min and gradually decreasing with time (Fig. 11B).

EGFR is endocytosed during ligand-induced activation. Therefore, we analyzed endocytosis of EGFR under Gal-8S treatment using a radioactive ligand-binding assay. In this assay cells were overnight serum starved to accumulate EGFR on the cell surface, then were treated with Gal-8S for different periods of time (0, 15, 30, 45, 60 min) at 37 ºC. A mild but statistically significant endocytosis of EGFR was observed for all the treatments, but maximum endocytosis (25 %) was observed with 30 min Gal-8S treatment.

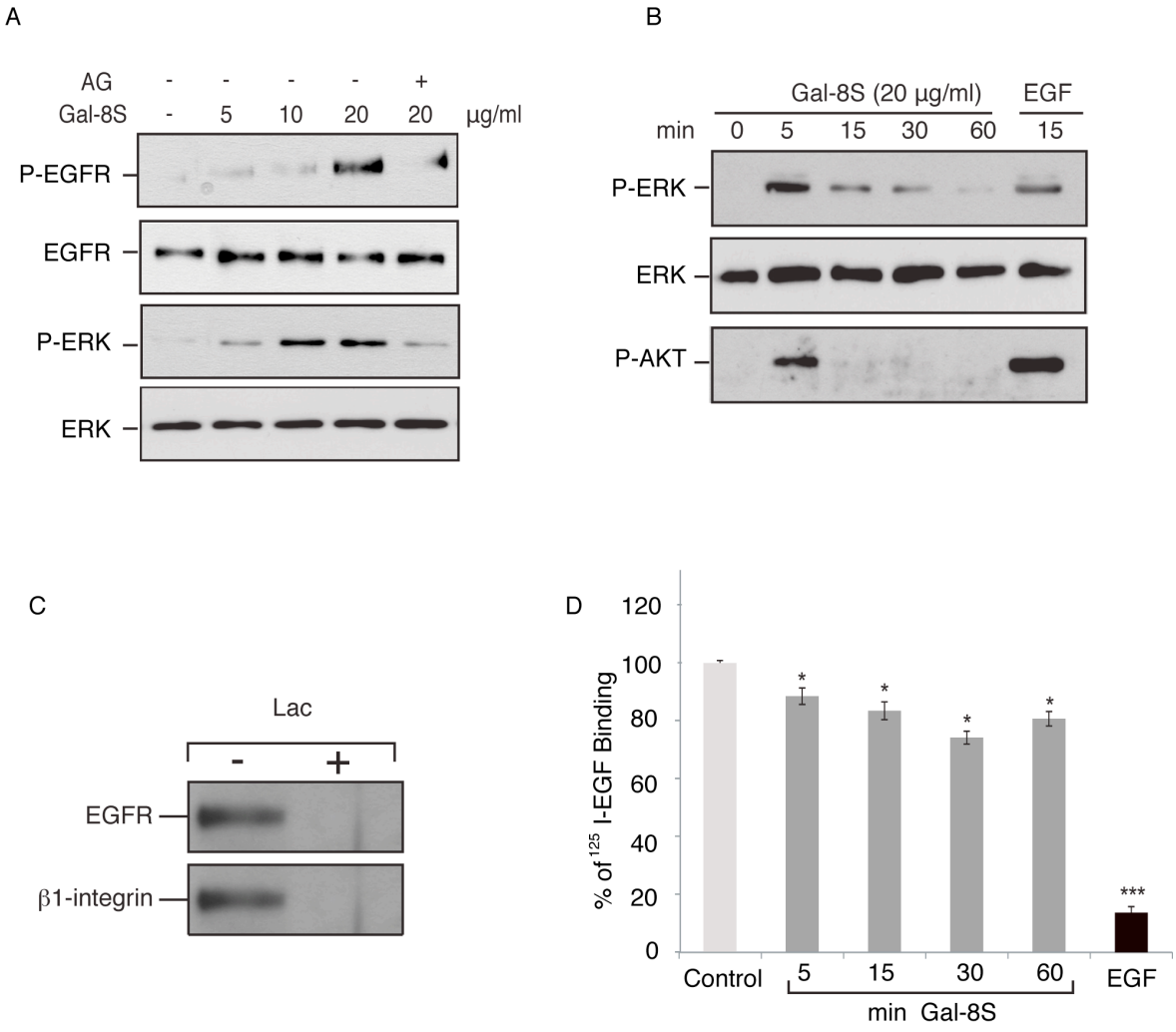

Figure 11: Gal-8S interacts with EGFR, transiently activates it and induces its endocytosis. A) Quiescent Hela cells were treated with 5, 10 or 20 µg/ml of Gal-8S as a soluble stimulus, and Gal-8 in the presence of AG1478, EGFR tyrosine kinase inhibitor. EGFR was immunoprecipitated and elutions were loaded on SDS-PAGE gel and analyzed by phosphotyrosine antibody. 50 µg cell lysates were also analyzed for EGFR, P-ERK and ERK. B) Cells were treated with 20 µg/ml Gal-8S for 0, 5, 15, 30 and 60 min or with 50 ng/ml EGF for 15 min.Total cell lysates were analyzed for P-ERK, P-AKT and ERK. C) HeLa cell extracts were incubated with GST-Gal-8 columns in the presence or absence of lactose. Gal-8 interacted with EGFR in a carbohydrate dependent manner. β1-integrin interaction was used as the positive control. D) HeLa cells were treated at 37 ºC with 20 µg/ml Gal-8 for 0, 5, 15, 30 and 60 min or 50 ng/ml EGF for 15min as positive control. Cell surface EGFR levels were evaluated by radiolabeled ligand binding assay.

By GST-Gal-8S pull down experiment we also found that Gal-8S interacts with EGFR (Fig. 11C). Having found these effects of Gal-8S on EGFR activity we next analyzed whether Gal-8S induced HeLa cell proliferation was dependent on EGFR signaling.

3.2.4 Gal-8 increases HeLa cell proliferation in an EGFR independent manner

Previously our lab has shown that HeLa cell proliferation is dependent on EGFR activity (Buvinic et al., 2007). Here we confirmed this finding by analyzing HeLa cell proliferation in the presence of tyrosine kinase inhibitor of EGFR (AG1478). Proliferation of HeLa cells cultured in the absence of Gal-8S diminished by AG1478 treatment (Fig 10A), as previously observed (Buvinic et al., 2007). However, in the presence of Gal-8 the cells no longer responded to this inhibitor (Fig 12B).

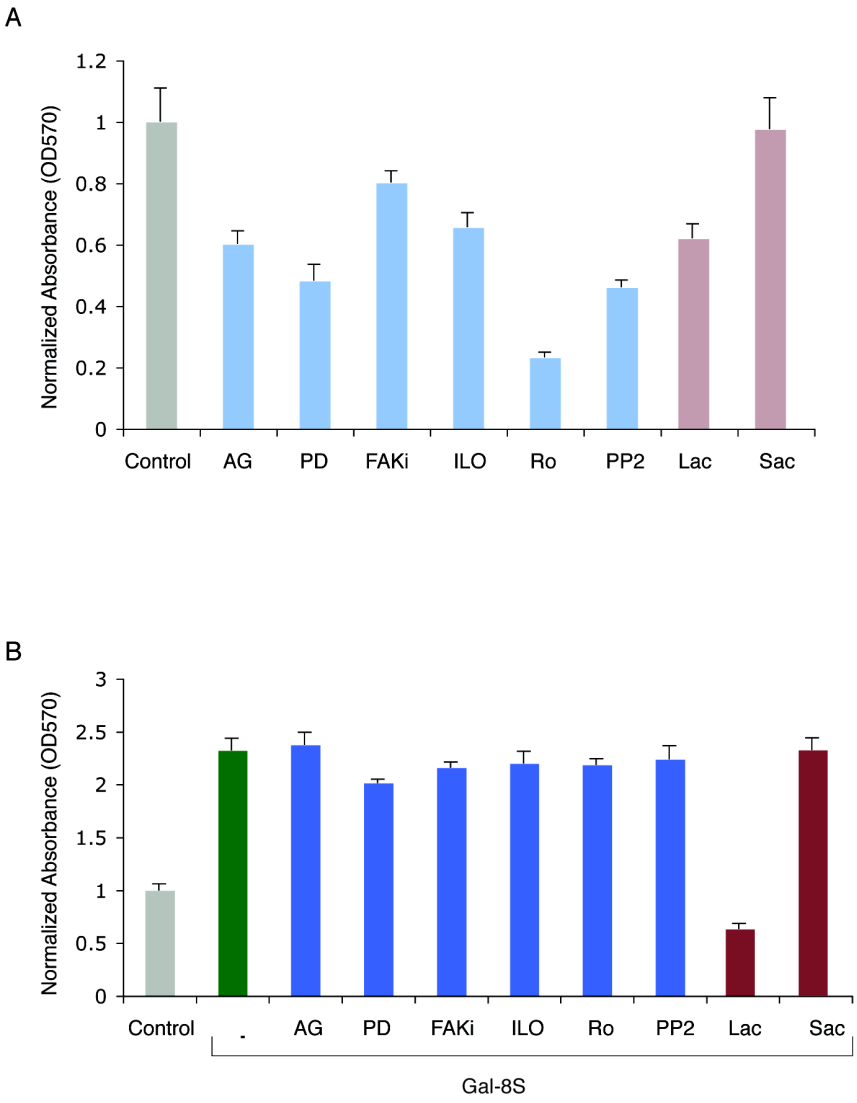

Figure 12: Gal-8S induced Hela cell proliferation is not via EGFR pathway
1500 Hela cells were seeded on 96 well plates on day1 and the next three days were pre-treated for 30min by the inhibitors as well as lactose and saccarose as controls.After the pre-treatments 20mg/ml of Gal-8S was added (B) or not (A) and cells were cultured for 24h. The media was changed every 24h for 3 days.On the fifth day, cells were analyzed by crystal violet assay. The graph shows absorbance normalized to the absorbance of the control without treatment.AG is EGFR tyrosine kinase inhibitor, PD 98059 is MAP kinase 1 inhibitor, FAK is Focal Adhesion kinase inhibitor, ilomastat (ILO) is a general metalloprotease inhibitor, Ro 318220 is inhibitor of PKC, PP2 is Src tyrosike kinase inhibitor, Lac is Lactose and is competitive inhibitor of binding for galectins, Sac is sacarose and is not a competitor for binding to galectin and is used as negative control.

Activation of EGFR is highly regulated not only by its ligands but also by other receptors and signal transduction pathways, through a process known as "transactivation" (Chaturvedi et al., 2009; Filardo, 2002; Fischer et al., 2003; Hobbs et al., 2011; Kasina et al., 2009). EGFR transactivation pathways involve Src, PKC and cell-surface metalloproteases (Carpenter, 1999; Daub et al., 1997; Gschwind et al., 2001; Prenzel et al., 1999; Soltoff et al., 1998; Tsai et al., 1997). Thus we also analyzed effects of inhibitors of PKC (Ro318220), Src (PP2) and general metalloproteases (Ilomastat) on Gal-8 induced cell proliferation. Eventhough in the absence of Gal-8S the cells were sensitive to all of these inhibitors, none of these inhibitors diminished Gal-8 induced cell proliferation (Fig 12A).

To test whether Gal-8S induced cell proliferation is carbohydrate dependent and thus modulated through extracellular interactions, we tested the effect of Gal-8S in the presence of lactose, which competes, and saccarose, which does not compete, for binding sites in the galectins. Lactose but not saccarose inhibited Gal-8S induced HeLa cell proliferation (Fig 12B). Thus, Gal-8S induced cell proliferation is dependent on carbohydrate-based interactions. Lactose treatment also diminished proliferation of HeLa cells cultured in the absence of Gal-8S (Fig. 12A), suggesting inhibition of endogenously expressed galectins.

3.2.5 Gal-8 also independizes HeLa cells from some other proliferation inducing pathways:

In order to elucidate pathways involved in Gal-8S induced cell proliferation we used inhibitors of several other pathways involved in cell proliferation, including Phospatidyl Inositol 3 Kinase (PI3K), Transforming Growth Factor beta Receptor 1 (TGFβR1),

Glycogen Synthase Kinase 3 (GSK3) and Sonic Hedgehog (Shh) pathways. HeLa cell proliferation was sensitive to all of these inhibitors (Fig 13A). However, the incubation with Gal-8S provided resistance to most of these inhibitors excepting for PI3K inhibitor (LY-294002) and Shh pathway inhibitor (cylopamine) (Fig 13B). These results indicate that PI3K and Shh pathways are stimulated by Gal-8S leading to cell proliferation.

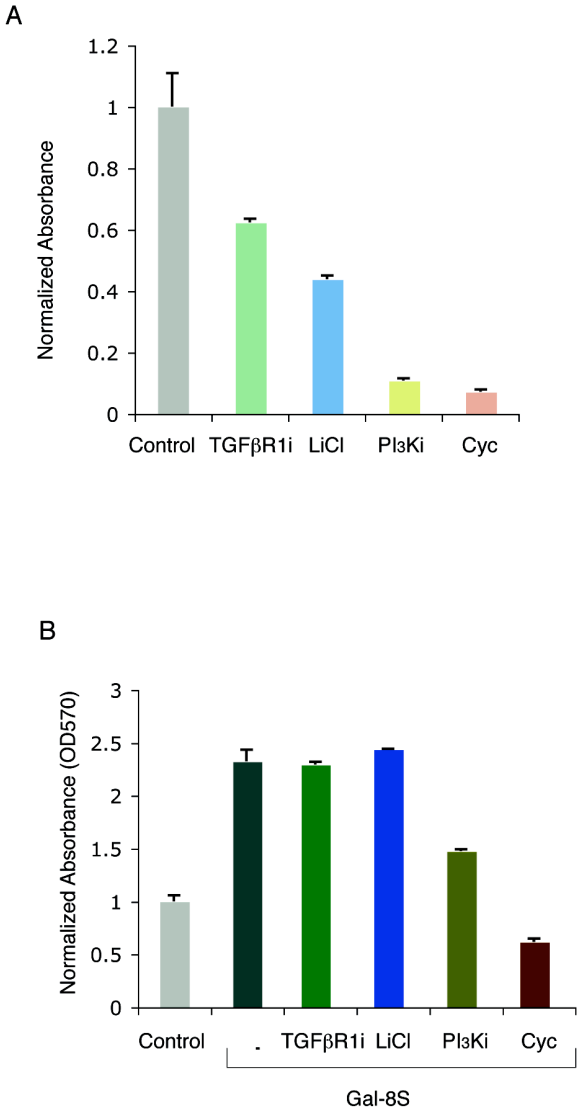

Figure 13: Gal-8S induced Hela cell proliferation is only inhibited by inhibitors of PI3K and Sonic Hedgehog signaling.

1500 Hela cells were seeded on 96 well plates on day1 and the next three days were pre-treated for 30 min by the inhibitors. After the pre-treatments 20 µg/ml of Gal-8S was added (B) or not (A) and cells were cultured for 24 h. The media was changed every 24 h for 3 days.On the fifth day, cells were analyzed by crystal violet assay. The graph shows absorbance normalized to the absorbance of the control without treatment. TGFβR1i is SB525334 and inhibits TGFβ receptor 1, LiCl inhibits Wnt pathway, PI3Ki is LY294002 and inhibits PI3kinase, Cyc is cyclopamine and inhibits Shh pathway.

3.3 Sonic hedgehog and Gal-8S

3.3.1 Gal-8S interacts with Sonic Hedgehog

Shh signaling is activated when the ligand of the pathway (Shh) binds to the Shh receptors, Ptch 1 and 2. This binding relieves the repression of Ptch on Smo, which is a membrane protein that is related to G protein-coupled receptors. Upon activation, Smo promotes nuclear translocation of a family of transcription factors named as Glis (Gli1, 2, 3) and subsequently activates target genes through Glis (Bale and Yu, 2001; Ingham and McMahon, 2001). Since we observed that Gal-8S induced HeLa cell proliferation was inhibited by Shh pathway inhibitor we next evaluated whether Gal-8S interacted with Shh pathway receptor, Patched (Ptch) and Smoothened (Smo). No interaction was observed betweeen GST-Gal-8S and Patched or Smoothened (Smo) in GST pull down assays, although in total cell lysates both proteins were detected (Fig. 14A).

3.3.2 Gal-8 increases activity of Shh pathway

In order to study the Shh pathway in HeLa cells and the effect of Gal-8S, we first treated the cells with the agonist of Shh pathway, Purmorphamine as a positive control. Overnight serum starved HeLa cells treated with 10 μM Purmorphamine for 4, 16 or 24 hours showed increased levels of Ptch and Gli-1 proteins (Fig. 14B and 14D), as for activation of Shh pathway. Then we evaluated the effect of Gal-8S treatments and found a similar increament in the expression of Ptch (Fig. 14C) and Gli-1 (Fig. 14D). These results indicate that Gal-8S activates the Shh pathway.

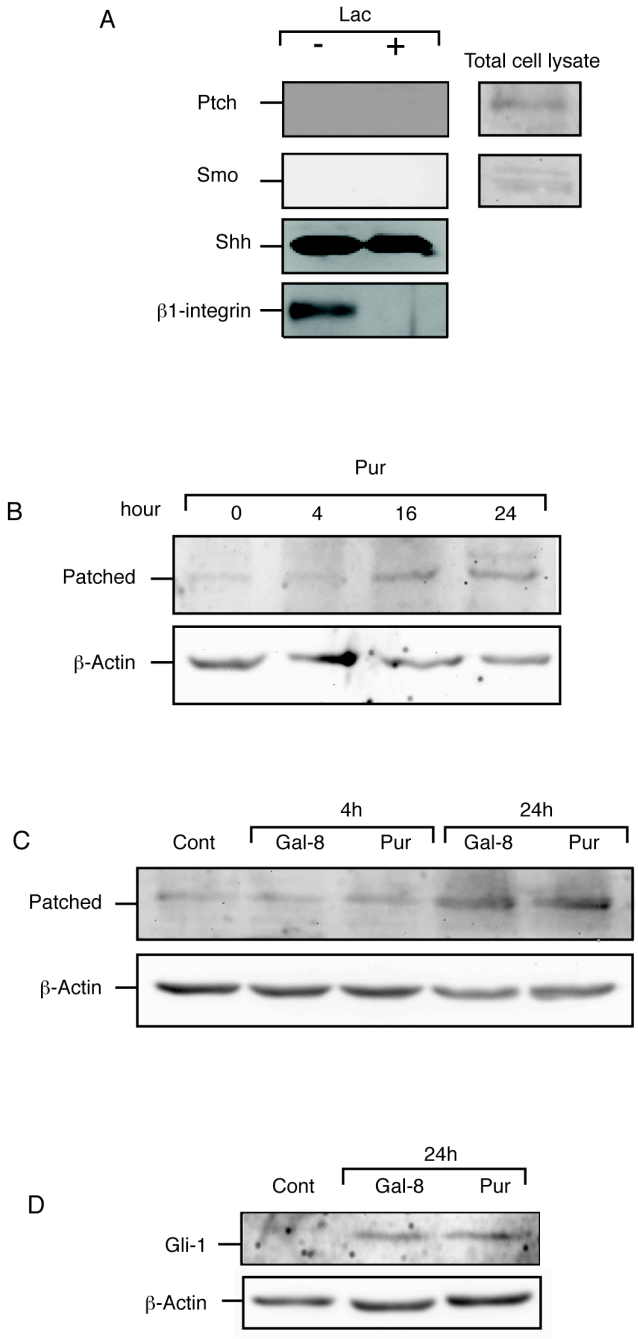

Figure 14: Gal-8S induces activation of Sonic Hedgehog Pathway.
(A) HeLa cells were treated by an agonist of Sonic Hedgehog pathway (Purmorphamine (Pur), for 4, 6, 8, 16 y 24h and analyzed by WB using anti-Patched antibody (C and D) Hela cells were treated either with Gal-8 and Pur for 4 or 24h and analyzed by WB for Patched and Gli-1 (B,C). D)HeLa cell extracts were incubated with GST-Gal-8 columns in the presence or absence of lactose. Gal-8 interacts with Shh (N terminal fragment) in a carbohydrate independent manner. β1-integrin interaction is the positive control.

3.4 Effect of Gal-8 isoforms pre-treatments on HeLa and U87 cell migration

Gal-8S was shown to induce cell adhesion and migration in various cellular contexts (Bi et al., 2011; Camby et al., 2002; Delgado et al., 2011; Elola et al., 2007). Here we analyzed the effect of Gal-8S, Gal-8L and Gal-8N on cell migration of HeLa and U87 cells. The cells were induced to migrate through 8 μm pores of a polycarbonate membrane (transwell) that separates an upper from a lower chamber. The cells were previously serum starved for about 1 h and then seeded (50,000 cells) on the upper chamber in the absence of serum, while placing medium with serum as a chemoattractant stimulus on the lower chamber. After 18 h at 37 °C, the filters were stained with crystal violet to count the cells that moved from upper part to the lower side of the filter.

In HeLa cells, both Gal-8S and Gal-8L diminished cell migration in a concentration dependent manner (Fig. 15). Gal-8N showed inhibitory effects on cell migration only at higher concentrations (50 and100 μg/ml) (Fig. 15). Gal-8S and Gal-8L also diminished cell migration of U87 cells (Fig. 16), but much less than HeLa cells. No inhibitory effect of Gal-8N was observed on U87 cells (Fig. 16).

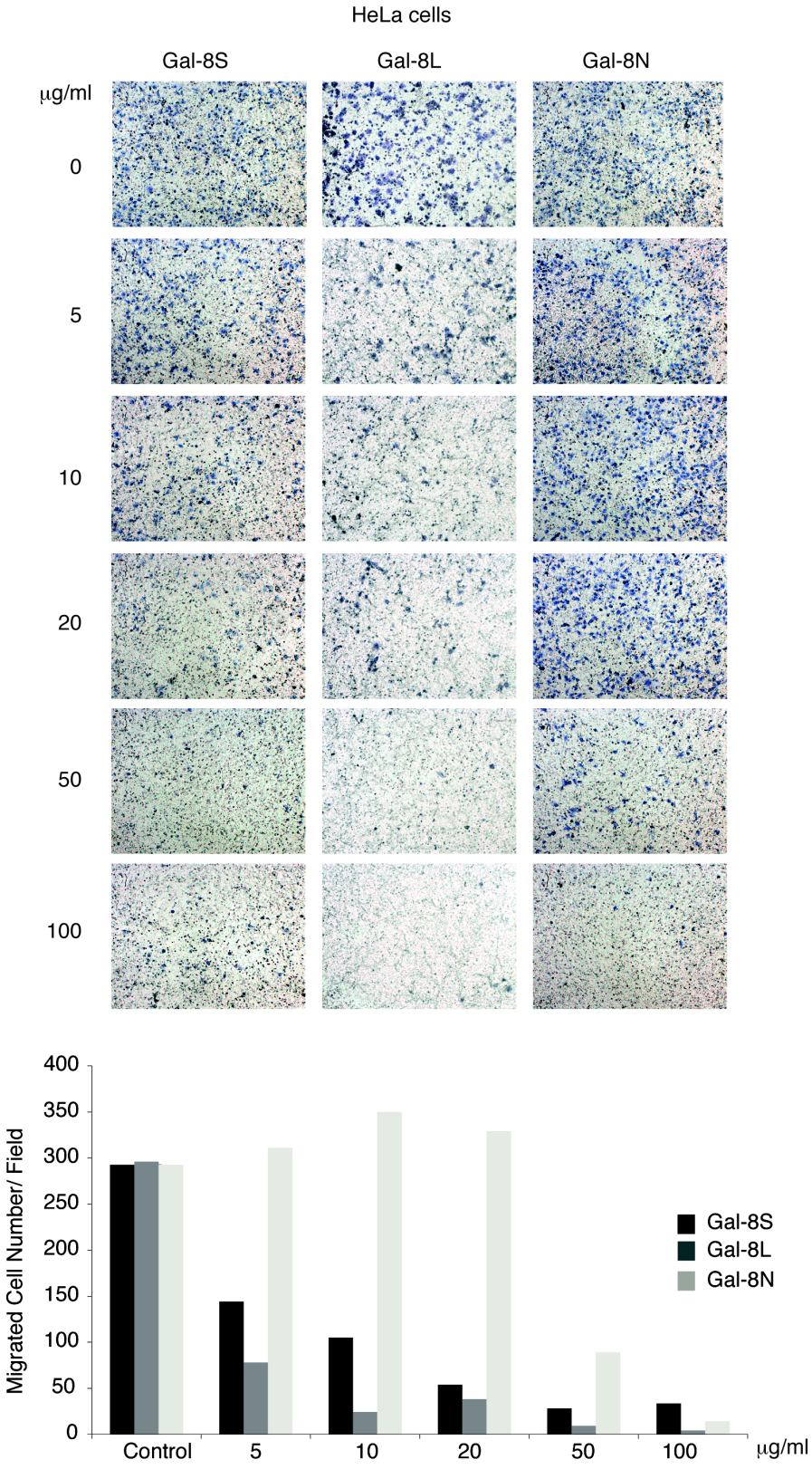

Figure 15: Pre-treatments with soluble Gal-8S, Gal-8L and Gal-8N inhibit HeLa cell migration. 50.000 HeLa cells were treated with galectins and then seeded onto 8mm transwell filters in the absence of serum and cultured for 20 h. Cell migration toward the lower side of the filter was analyzed by using 10 % serum as chemoattractant in the lower chamber via crystal violet assay.

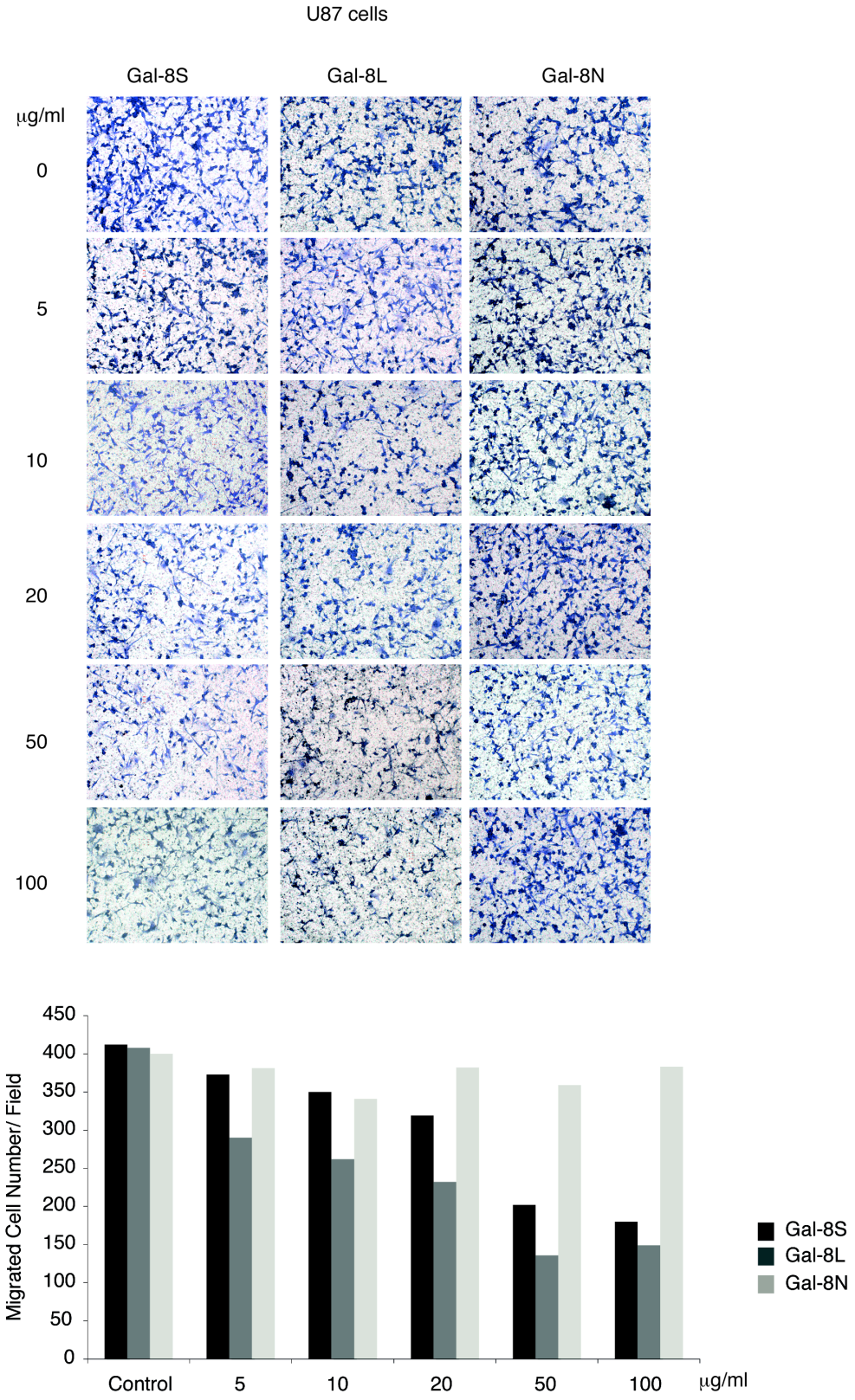

Figure 16: Pretreatments with soluble Gal-8S and Gal-8L inhibit U87 cell migration whereas Gal-8N does not.

50.000 U87 cells were treated with galectins in the absence of serum for 30 min and then seeded onto 8µm transwell filters and cultured for 20 h in the absence of serum. Cell migration toward the lower side of the filter was analyzed by using 10 % serum as chemoattractant in the lower chamber via crystal violet assay.

3.5 Effect of Gal-8 isoforms as chemoattractant on HeLa cells

In this experiment HeLa cells were were previously serum starved for about 1 h and then seeded on the upper chamber in the absence of serum, while placing medium with serum with or without different Gal-8 isoforms as a chemoattractant stimulus on the lower chamber. After 18 h at 37 °C, the filters were stained with crystal violet to count the cells that moved from upper part to the lower side of the filter.

When used as chemoattractant all the analyzed Gal-8 isoforms increased cell migration (Fig. 17). Whether cell migration induced by these proteins depend on EGFR is one of the pending experiments.

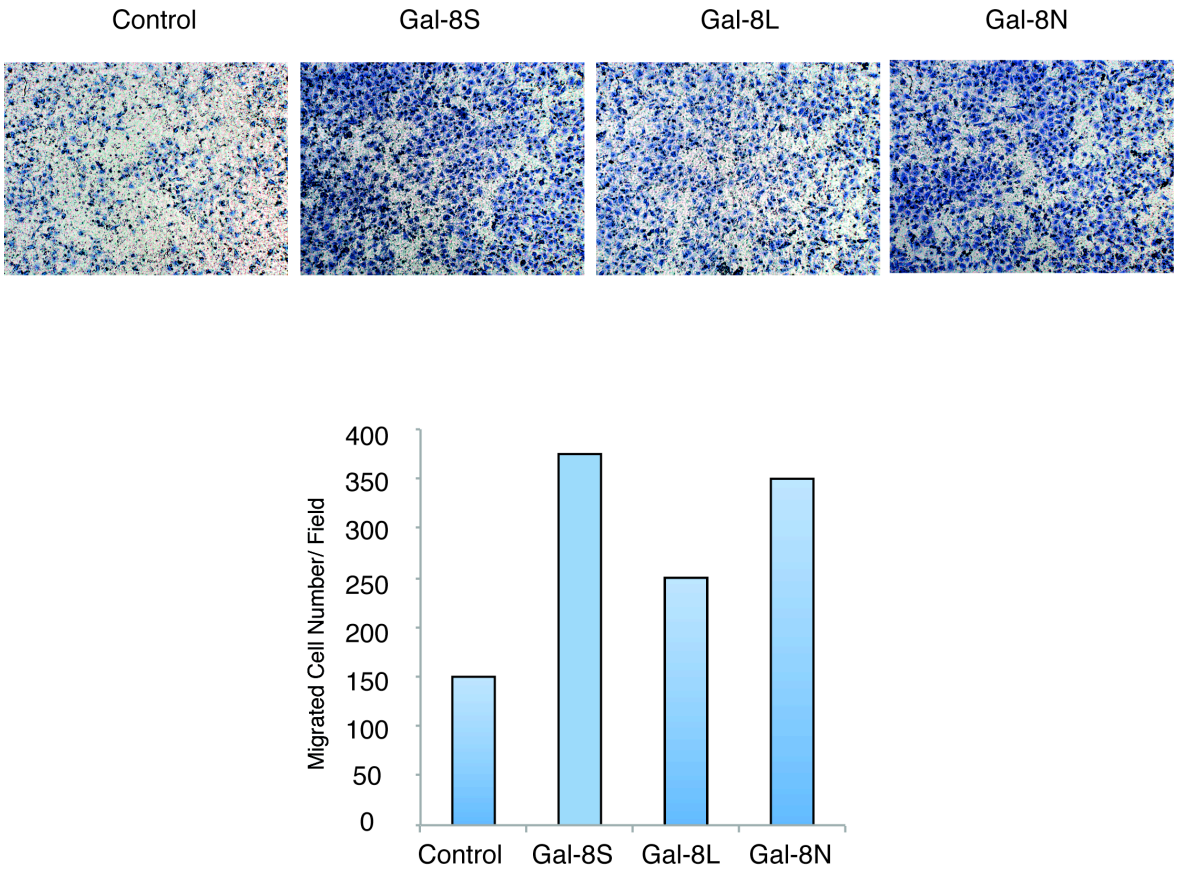

Figure 17: When used as chemoattractant Gal-8S, Gal-8L and Gal-8N induce migration of HeLa cells. 50.000 U87 cells were treated with galectins in the absence of serum for 30 min and then seeded onto 8μm transwell filters and cultured for 20 h in the absence of serum. Cell migration toward the lower side of the filter was analyzed by using 10 % serum and 50 μg/ml galectins as chemoattractant in the lower chamber via crystal violet assay.

4. DISCUSSION

This thesis contributes to understand a new regulatory aspect in the still uncompletely known function of Gal-8, one of the most widely expressed members of the galectin family. We provide evidence that Gal-8 isoforms have distinct and even counteracting functions depending on whether CRDs act as linked tandem-repeat type structure or separated molecular entities, which can be generated by both alternative splicing or proteolytic cleavage of the linker peptide. Our results constitute the first evidence that linker length variation of standard and long Gal-8 isoforms determine differential functions.

Since the discovery of first galectin in 1970s very diverse cellular functions were attributed to the members of galectin family of proteins. Most cellular functions of galectins are dependent on carbohydrate based interactions. Different cells or the same cells under different physiological condition may possess different glycosylation patterns, thus ligands of galectins on the cell surface may differ in different cellular contexts. For instance during tumor progression expression of some glycosidase enzymes and/or glyco-transferases change thus both the number of branches and glycosyl composition of the cell surface molecules change as well (Burchell et al., 2001; Seales et al., 2005; Shimodaira et al., 1997; Solatycka et al., 2012; Yang et al., 1994). Galectin expression also varies in different types of tumors. For instance Gal-8 expression decreases in malignant or benign tissues from colon, pancreas, liver, skin and larynx tumors versus normal tissue whereas its expression increases in breast and prostate tumoral tissues (Bidon-Wagner and Le Pennec, 2004).

Since galectin ligands vary in different cell types or under different conditions, distinct intracellular signaling pathways are activated by galectins through which they may exert

differential functions. Therefore functions of galectins cannot be generalized for every cell type and so called to be `context dependent`. This illustrates the importance of studying many different cell types to have a better understanding of functions of galectins in normal as well as in cancer cells.

4.1 Effect of Gal-8 isoforms on Jurkat T cells

We first showed that Gal-8L counteracts the pro-apoptotic effect of Gal-8S on Jurkat T cells and anti-CD3/CD28 activated human T cells. The mechanism analyzed in Jurkat T cells involves inhibition of a β1-integrin to ERK1/2 pro-apoptotic pathway and stimulation of an anti-apoptotic AKT pathway. N- and C-CRDs have similar counteracting effects on apoptosis, suggesting that Gal-8L linker cleavage by thrombin can generate two opponents of Gal-8S. Interestingly, Gal-8L does not inhibit PS exposure induced by Gal-8, thus dissociating this effect from apoptosis.

These observations acquire more relevance in the light of unpublished data from our laboratory, in which the analysis of PBMCs from patients with autoimmune disorders such as SLE and MS reveals a shift towards higher Gal-8S expression. Therefore, changes in the relative expression of Gal-8L and Gal-8S can occur in the organism and constitute a mechanism for modulating Gal-8 function in the immune system.

4.1.1 Different binding affinites of Gal-8S and Gal-8L on Jurkat T cells

Even though a previous study in CHO cells have shown similar binding properties of Gal-8S and Gal-8L isoforms (Patnaik et al., 2006), our FACS analysis showed that Gal-8S has higher avidity than Gal-8L for Jurkat cell surface glycans. About 10-fold more concentration of Gal-8L (20 μg/ml-0.5 μM) over Gal-8S (2 μg/ml-0.06 μM) is required to achieve similar binding levels and compromising more than 80 % of the cells. As lactose

treatments inhibit binding of these galectins, we can conclude that carbohydrate recognition domains are responsible for the cell surface binding with distinct avidity. Such a difference between these two isoforms can be explained by the loss of tandem-repeat type structure occurring by proteolytic cleavage of the linker peptide. Supporting this possibility are the lower binding avidity than Gal-8S shown by the separate CRDs, Gal-8N and Gal-8C, which were similar to the Gal-8L.

4.1.2 Gal-8 induced apoptosis involves β1-integrin induced ERK1/2 activation

β1-integrin has been shown to mediate apoptosis of smooth muscle cells, rheumatoid synovial cells, endothelial cells, and T cell activation induced T cell death (Arencibia et al., 2002; Damle et al., 1993a; Nakayamada et al., 2003; Vivinus-Nebot et al., 1999; Wernig et al., 2003). Our laboratory has previously reported that Gal-8S binds to β1-integrin (Carcamo et al., 2006) and that β1-integrin blocking antibodies inhibit about 20 % of the Gal-8S induced apoptosis in Jurkat T cells (Norambuena et al., 2009). Here we detected binding of both isoforms to β1-integrin. This was expected as both isoforms have exactly the same CRDs. However only Gal-8S induced Jurkat T cell aggregation along with activation of β1-integrin. Interestingly activation of β1-integrin was inhibited when cells were pretreated with Gal-8L, Gal-8N or Gal-8C. β1-integrin mediated signaling promotes or inhibits apoptosis depending on the ligands and apoptotic insult. Both blocking antibodies for β1-integrin and ERK1/2 inhibitor inhibit Gal-8S induced Jurkat T cell apoptosis (Norambuena et al., 2009). Our results with FAK inhibitor show that ERK1/2 activation induced by Gal-8S is via β1-integrin. These evidence points to β1-integrin-ERK1/2 signaling pathway as one of the main target for differential effects of Gal-8 isoforms.

4.1.3 Regulation of opposite effects of Gal-8S and Gal-8L via differential activation of ERK1/2 and AKT pathways

It is well established that ERK1/2 activation is required for activation-induced cell death, which controls the extension of the immune response by eliminating recently activated and restimulated T cells (van den Brink et al., 1999; Zhu et al., 1999). The pro-apoptotic mechanism of ERK1/2 pathway likely involves molecular and biochemical interventions at different levels, including inhibition of AKT pathway (Sinha et al., 2004). ERK1/2 may act upstream of mitochondrial Cytochrome *c* release and Caspase-3 activation up-regulating Bax and p53 and suppressing AKT-mediated survival signaling (Zhuang and Schnellmann, 2006). ERK1/2 and AKT have been reported to coexist in a multimolecular complex in which ERK1/2 exerts inhibitory actions upon AKT (Sinha et al., 2004). A release of this block by inhibitors of the ERK1/2 pathway may lead to AKT activation and apoptosis prevention, as previously suggested (Sinha et al., 2004). Such interrelated effects of ERK1/2 and AKT might be regulated by Gal-8S and Gal-8L in T cells. We found that both Gal-8S and Gal-8L activate the PI3K/AKT pathway but with different duration patterns. Gal-8S exerts a relatively transient AKT activating effect compared with that of Gal-8L, which prolongs at least for 3 h even in the presence of Gal-8S. The difference can be explained by the strong ERK1/2 activation occurring when cells are treated with Gal-8S alone, which is expected to counteract the AKT activation as reported (Sinha et al., 2004). Instead, Gal-8L abrogation of the β1-integrin-ERK1/2 signaling would release its inhibitory effect on the anti-apoptotic PI3K/AKT pathway. Thus, Gal-8L and Gal-8S seem to regulate cell death in activated T cells exerting opposite actions on pro-apoptotic ERK1/2 and anti-apoptotic AKT signaling.

4.1.4 Gal-8L inhibits Gal-8S induced apoptosis but not PS exposure

In a non-apoptotic cell, PS is mainly distributed to the internal leaflet of the plasma membrane as a result of ATP-dependent inward-directed aminophospholipid translocases (flippases) that continuosly transfer PS from the external to internal leaflet against a concentration gradient (Friebe et al., 2011; Leventis and Grinstein, 2010; Lyles et al., 2003; Pomorski et al., 2004; Smriti et al., 2007). Translocation of PS to the external plasma membrane leaflet is an early sign of apoptosis and is recognized by phagocytic cells that then remove such marked dying cells (Fadok and Chimini, 2001; Williamson and Schlegel, 2002). Apoptotic Ag-specific T cells are this way removed during the contraction phase of the immune response (Albacker et al., 2010). However, PS exposure also occurs dissociated from apoptosis in a variety of cells including neutrophils (Stowell et al., 2007) and T cells (Elliott et al., 2005). Activated neutrophils treated with Gal-1, Gal-2, and Gal-4 (Stowell et al., 2007), expose PS as a signature for phagocytic engulfment (preaparesis) (Dias-Baruffi et al., 2003; Stowell et al., 2008). The role of PS exposure in T cells seems to extend to different processes, such as signaling modulation in T lymphocytes that express low levels of the transmembrane tyrosine phosphatase CD45RB (Elliott et al., 2005) and immunologic synapse formation in Ag-stimulated $CD8^+$ cytotoxic T cells.

Our results indicate that Jurkat T cells possess a mechanism of PS exposure without apoptosis that is kept operative while Gal-8L counteracts certain Gal-8S actions. Thus, further investigation is required to understand the functional consequences of the PS exposure induced by Gal-8S that occurs in these T cells.

4.1.5 Size of linker peptide and presence of thrombin cleavage site

The influence of linker length in the function of tandem-repeat galectins has been previously illustrated in several studies. In colon carcinoma LoVo cells, Gal-9L plays different roles in comparison to short and medium Gal-9 isoforms regulating E-selectin

expression (Zhang et al., 2009) although no apparent differential effect was observed on eosinophil chemoattractant activity. Artificial linker shortening in Gal-4 alters cell surface affinity and ligand selection (Kopitz et al., 2012), while in shortening of rat Gal-8 reduces cell adhesion and PKB and S6 kinase signaling (Levy et al., 2006). Altogether this information underlines the arising concept for a translation of natural linker-length variation for such signaling routes.

Gal-8L has a thrombin cleavage site in its linker peptide (Nishi et al., 2006), providing at least one possible regulatory pathway through which Gal-8S and Gal-8L may present differential functions. The proteolytic cleavage of Gal-8L creates a proto-type galectin like structure due to the separation of CRDs. Several studies show that tandem-repeat galectins are more potent than prototype galectins in triggering many cell responses, including cell death (Earl et al., 2011). This was suggested to be due to the ability of the linker domain to permit intermolecular CRD interactions, resulting in the formation of higher-order multimers with increased valency, rather than differences in CRD specificities (Earl et al., 2011). Moreover proper orientation of CRDs by a linker peptide might be required for certain cellular functions. For instance, Gal-8 separate CRDs do not promote cell adhesion and are poorly effective in triggering MAPK and PI3K signaling in CHO cells (Levy et al., 2006), displaying weaker binding than entire Gal-8 (Patnaik et al., 2006). Other studies have shown that the tandem-repeat structure is crucial for inducing proliferation of naïve T cells, but not for co-stimulating antigen-specific responses, which can be independently elicited by either N- or C-CRDs despite their lower binding properties (Cattaneo et al., 2011). It was also shown that loss of tandem repeat structure of Gal-8L by thrombin digestion decreased its capacity to induce neutrophil adhesion when used as a substrate. Similarly, thrombin treatment almost completely abolished eosinophil

chemoattractant activity of Gal-9L (Nishi et al., 2006). Here we also showed that thrombin treatment also completely diminishes apoptotic function of full length Gal-8L on Jurkat T cells but different from Nishi et al., we also showed that thrombin digested Gal-8L inhibits at least the pro-apoptotic function of Gal-8S. Inactivation of Gal-8L and obtaining a new inhibitory role upon its shorter isoform may be an important regulatory system under certain conditions where thrombin is generated.

Thrombin is a serine protease that plays a pivotal role in the coagulation cascade. However, it also plays a significant pro-inflammatory role in inflammatory diseases. In Multiple Sclerosis thrombin activity precedes onset of neurological signs and increased at disease peak (Davalos et al., 2014). Thrombin generation was suggested as marker to estimate thrombosis risk in patients with lupus (Boeer et al., 2013). Also during tumorigenesis thrombosis may also occur and thrombin is generated. Thrombin activates tumor cell adhesion to platelets, endothelial cells, and subendothelial matrix proteins; enhances tumor cell growth; increases tumor cell seeding and spontaneous metastasis; stimulates tumor cell angiogenesis and thus can augment the malignant phenotype (Nierodzik and Karpatkin, 2006). Moreover thrombin generation found elevated in other diseases such as coronary heart disease, inflammatory pulmonary diseases, inflammatory bowel disease and sickle cell disease (Deutschmann et al., 2013; Noubouossie et al., 2012; Opstad et al., 2010; Undas et al., 2011). Under such pathological conditions generation of thrombin may produce digestion of thrombin substrates such as Gal-8L that may cause unknown biological functions in these different types of tissues.

Here we also found that N- and C-CRDs of Gal-8 reproduce the properties of Gal-8L on T cells, including a weaker binding and inhibition of Gal-8S induced apoptotis. The similarity of the counteracting effects of Gal-8N and Gal-8C on Gal-8S is unexpected

considering that they are not functionally equivalent and even recognize distinct glycans (Carlsson et al., 2007a; Carlsson et al., 2007b; Cattaneo et al., 2011; Stowell et al., 2008). This suggests that Gal-8S actions require the interaction of both N- and C-terminal CRDs and, therefore, blocking any of them by the separate CRDs is enough to abrogate the entire effect of Gal-8S.

In tandem-repeat, Gal-8 accounts for induction of proliferation in naïve T cells (Cattaneo et al., 2011) and PS exposure in promyelocytic leukemia HL60 cells (Stowell et al., 2008), while N-CRD mediates Gal-8 dimerization (Stowell et al., 2008). Gal-8N recognizes sulfated and sialylated glycans with similar relative affinity as that of entire Gal-8, whereas Gal-8C recognizes polyLacNac glycans or blood group antigens with a lower affinity than Gal-8 (Stowell et al., 2008). However, in Jurkat cells we found that recombinant Gal-8N and Gal-8C display similar binding properties to that of Gal-8L, and all present lower affinities than Gal-8S. This suggests that different modes of cell surface interactions exist depending on both the cell context and Gal-8 isoform. Gal-8L functional equivalence with separate N-and C-CRD fragments suggests its effects might derive from proteolytic cleavage of its linker peptide.

These data thus direct attention to the potential of linker-length variation for functional modulation. A special factor for this aspect can be the cell type, nature of the counter receptor and type of application.

4.2. Effect of Gal-8 isoforms on HeLa cells

4.2.1 Gal-8S induces cell proliferation via distinct signaling pathways

Gal-8S induces cancerigenic properties such as increased cell proliferation and migration when overexpressed in MDCK cells that are both dependent on EGFR activity (PhD thesis of Claudia Oyanadel). Taking this previously discovered link between Gal-8S and EGFR as a start point, we first analyzed the effect of Gal-8S on EGFR activity. Gal-8S transiently activated EGFR, induced a mild endocytosis of the receptor and interacted with EGFR. Gal-8S treatments increased proliferation of the HeLa cells, similar to what was observed in MDCK cells (PhD thesis of Claudia Oyanadel). However, different from MDCK cells Gal-8S induced HeLa cell proliferation was not dependent of EGFR but dependent on Shh Pathway. Until now, there has not been any precedent for an effect of any galectin on Shh signaling.

The data from this thesis along with the PhD thesis of Claudia Oyanadel clearly illustrates that the proliferative functions of Gal-8 are not exerted through a unique regulatory system.

4.2.2 Gal-8L, Gal-8N and Gal-8C do not induce cell proliferation

Gal-8L, Gal-8N or Gal-8C did not induce HeLa or U87 cell proliferation. This again raises the question whether Gal-8L acts as separate CRDs. If so, to induce cell proliferation in HeLa and U87 cells intact tandem repeat structure of Gal-8 is required. Interestingly Gal-8L, Gal-8N and Gal-8C seems to diminish HeLa cell number, while no such an effect was observed in U87 cells. Such an effect might be due to inhibition of cell cycle progresión, apoptosis induction or cell adhesion decrease. Further investigation is required to understand the mechanisms behind this effect.

4.2.3 Gal-8S independizes HeLa cells from various proliferation inducing pathways

HeLa cells are dependent on EGFR for their proliferation (Buvinic et al., 2007). Here we tested the effect of various inhibitors of proliferation inducing pathways on HeLa cell proliferation including inhibitors of EGFR, GSK3β, TGFβR1, PI3K, Shh pathway etc. In the presence of Gal-8S, HeLa cells became insensitive to the inhibitory effect of most of these inhibitors, excepting the inhibitors of the Shh and PI3K pathways.

Possible explanations of the Gal-8S effects against inhibitors other than Shh and PI3K inhibitors incluye: 1) Gal-8S may change or reinform certain signaling pathways responsible for cell proliferation, reducing the importance of other pathways; 2) Inhibitors are no longer able to get into the cells or are exported out of the cells under Gal-8S influence.

4.2.4 How may Gal-8S induce activation of Shh pathway?

Here we showed that Gal-8S increases Shh pathway signaling increasing Gli-1 and Patched expression. We are the first to show such an effect of a galectin on Shh signaling.

We discovered an unknown modulatory link between Gal-8S and Shh, being Gal-8S upstream of Shh in induction of a proliferative signal. Whether this has a general implicance or specific to the cell type that we have analyzed remain an open question. As both Gal-8S and Shh are highly related to cancer it is essential to further study this modulatory link in different model systems.

In principle, the mechanisms by which Gal-8S may stimulate Shh signaling include an increment in the expression and/or secretion of Shh ligand, increment in the expression of Gli1 or stimulation of Smo trafficking to the cell surface.

4.2.5 Gal-8 isoforms not always present differential function

Here we used the migration process of these cells as a model to test whether there is another differential of Gal-8 isoforms. Gal-8S was previously shown to inhibit cell migration when used as a soluble stimulus and induce cell migration when used as a matrix protein, through inhibition of integrin interaction with ECM or through activation of integrins (Zick et al., 2004). Here we analyzed the effect of isoforms as a soluble stimulus and as a chemoattractant.

As we observed in the HeLa and U87 cell migration assays, Gal-8S and Gal-8L had very similar effects on cell migration. Both isoforms when added on cells as a soluble stimulus inhibited the cell migration of HeLa cells at a similar percentage. On the other hand Gal-8N pre-treatment at lower concentrations had no effect on HeLa cell migration. Though at 50 and 100 μg/ml concentrations Gal-8N also induced an inhibitory effect on the migration of these cells.

With Gal-8S and Gal-8L pre-treatments of U87 cells an inhibitory effect on migration was also observed although with a lesser extent except that at the concentrations used Gal-8N had no effect on U87 cell migration, perhaps when used at higher concentrations an inhibitory effect could be observed. This difference between concentrations could be that binding of Gal-8N in comparison to Gal-8S and Gal-8L could be much lower in HeLa and U87 cells.

U87 cells might have much more integrins to be blocked in comparison to HeLa cells. This might be the reason for why inhibitory effect of Gal-8S and Gal-8L are much lesser on U87 cells.

As explained above Gal-8 interacts with various β1-integrins and pre-treatment of HeLa and U87 cells with Gal-8 may inhibit the interaction of β1-integrins with the ECM through which inhibition of cell migration may occur. We are interested in analyzing mechanism behind this inhibition as well analyzing the mechanism behind the inducing effect of Gal-8 as chemoattractant on these cells. However, these objectives are above the scope of this thesis.

In conclusion, Gal-8 isoforms may have differential or redundant functions over certain processes. Not all the processes requires the intact tandem repeat structure of Gal-8 nor the presence of both CRDs as observed for inhibition of migration. In different cells Gal-8 may induce different signaling pathways according to the participating isoforms. On the other hand, a same cellular process stimulated by Gal-8 can proceed through recruiting different signaling pathways. The cellular context dependency of the galectin function is indeed a matter of the actual operative glycan code.

5. CONCLUSIONS

1. Gal-8S induces apoptosis of Jurkat T cells whereas Gal-8L, Gal-8N and Gal-8C pre-treatments inhibit pro-apoptotic effect of Gal-8S on Jurkat T cells at least via inhibition of β1-integrin-ERK1/2 pathway.
2. Gal-8L requires to be cleaved from linker in order to inhibit apoptosis induced by Gal-8S.
3. Gal-8S induces proliferation of HeLa cells whereas Gal-8L, Gal-8N and Gal-8C diminish it.
4. PI3K and Shh pathways inhibitors inhibit Gal-8S induced cell proliferation.
5. Gal-8S increases expression of Gli-1 and Patched, two targets of the Shh pathway.
6. Gal-8S does not interact with Shh pathway receptors but interacts with Shh ligand.
7. Soluble Gal-8S and Gal-8L pre-treatment of HeLa and U87 cells diminish their cell migration in a similar manner.
8. Gal-8N diminishes HeLa cell migration at higher concentrations while does not have an effect on U87 cell migration at the analyzed concentrations.

6. REFERENCES

Albacker, L.A., P. Karisola, Y.J. Chang, S.E. Umetsu, M. Zhou, O. Akbari, N. Kobayashi, N. Baumgarth, G.J. Freeman, D.T. Umetsu, and R.H. DeKruyff. 2010. TIM-4, a receptor for phosphatidylserine, controls adaptive immunity by regulating the removal of antigen-specific T cells. *J Immunol*. 185:6839-6849.

Arbel-Goren, R., Y. Levy, D. Ronen, and Y. Zick. 2005. Cyclin-dependent kinase inhibitors and JNK act as molecular switches, regulating the choice between growth arrest and apoptosis induced by galectin-8. *J Biol Chem*. 280:19105-19114.

Arencibia, I., G. Frankel, and K.G. Sundqvist. 2002. Induction of cell death in T lymphocytes by invasin via beta1-integrin. *Eur J Immunol*. 32:1129-1138.

Bak, M., C. Hansen, N. Tommerup, and L.A. Larsen. 2003. The Hedgehog signaling pathway--implications for drug targets in cancer and neurodegenerative disorders. *Pharmacogenomics*. 4:411-429.

Balan, V., P. Nangia-Makker, and A. Raz. 2010. Galectins as Cancer Biomarkers. *Cancers (Basel)*. 2:592-610.

Bale, A.E., and K.P. Yu. 2001. The hedgehog pathway and basal cell carcinomas. *Hum Mol Genet*. 10:757-762.

Barondes, S.H., V. Castronovo, D.N. Cooper, R.D. Cummings, K. Drickamer, T. Feizi, M.A. Gitt, J. Hirabayashi, C. Hughes, K. Kasai, and et al. 1994. Galectins: a family of animal beta-galactoside-binding lectins. *Cell*. 76:597-598.

Barrow, H., J.M. Rhodes, and L.G. Yu. 2011. The role of galectins in colorectal cancer progression. *Int J Cancer*. 129:1-8.

Beachy, P.A., S.S. Karhadkar, and D.M. Berman. 2004. Tissue repair and stem cell renewal in carcinogenesis. *Nature*. 432:324-331.

Bi, S., L.A. Earl, L. Jacobs, and L.G. Baum. 2008. Structural features of galectin-9 and galectin-1 that determine distinct T cell death pathways. *J Biol Chem*. 283:12248-12258.

Bi, S., P.W. Hong, B. Lee, and L.G. Baum. 2011. Galectin-9 binding to cell surface protein disulfide isomerase regulates the redox environment to enhance T-cell migration and HIV entry. *Proc Natl Acad Sci U S A*. 108:10650-10655.

Bidon, N., F. Brichory, P. Bourguet, J.P. Le Pennec, and L. Dazord. 2001a. Galectin-8: a complex sub-family of galectins (Review). *Int J Mol Med*. 8:245-250.

Bidon, N., F. Brichory, S. Hanash, P. Bourguet, L. Dazord, and J.P. Le Pennec. 2001b. Two messenger RNAs and five isoforms for Po66-CBP, a galectin-8 homolog in a human lung carcinoma cell line. *Gene*. 274:253-262.

Bidon-Wagner, N., and J.P. Le Pennec. 2004. Human galectin-8 isoforms and cancer. *Glycoconj J*. 19:557-563.

Boeer, K., L. Cuznetov, and W. Loesche. 2013. Thrombin generation as marker to estimate thrombosis risk in patients with abnormal test results in lupus anticoagulant routine diagnostics. *Thromb J*. 11:24.

Boscher, C., J.W. Dennis, and I.R. Nabi. 2011. Glycosylation, galectins and cellular signaling. *Curr Opin Cell Biol*. 23:383-392.

Bresalier, R.S., N. Mazurek, L.R. Sternberg, J.C. Byrd, C.K. Yunker, P. Nangia-Makker, and A. Raz. 1998. Metastasis of human colon cancer is altered by modifying

expression of the beta-galactoside-binding protein galectin 3. *Gastroenterology*. 115:287-296.

Burchell, J.M., A. Mungul, and J. Taylor-Papadimitriou. 2001. O-linked glycosylation in the mammary gland: changes that occur during malignancy. *J Mammary Gland Biol Neoplasia*. 6:355-364.

Buvinic, S., M. Bravo-Zehnder, J.L. Boyer, J.P. Huidobro-Toro, and A. Gonzalez. 2007. Nucleotide P2Y1 receptor regulates EGF receptor mitogenic signaling and expression in epithelial cells. *J Cell Sci*. 120:4289-4301.

Callahan, M.K., P. Williamson, and R.A. Schlegel. 2000. Surface expression of phosphatidylserine on macrophages is required for phagocytosis of apoptotic thymocytes. *Cell Death Differ*. 7:645-653.

Camby, I., N. Belot, F. Lefranc, N. Sadeghi, Y. de Launoit, H. Kaltner, S. Musette, F. Darro, A. Danguy, I. Salmon, H.J. Gabius, and R. Kiss. 2002. Galectin-1 modulates human glioblastoma cell migration into the brain through modifications to the actin cytoskeleton and levels of expression of small GTPases. *J Neuropathol Exp Neurol*. 61:585-596.

Camby, I., N. Belot, S. Rorive, F. Lefranc, C.A. Maurage, H. Lahm, H. Kaltner, Y. Hadari, M.M. Ruchoux, J. Brotchi, Y. Zick, I. Salmon, H.J. Gabius, and R. Kiss. 2001. Galectins are differentially expressed in supratentorial pilocytic astrocytomas, astrocytomas, anaplastic astrocytomas and glioblastomas, and significantly modulate tumor astrocyte migration. *Brain Pathol*. 11:12-26.

Cantrell, D. 1996. T cell antigen receptor signal transduction pathways. *Annu Rev Immunol*. 14:259-274.

Carcamo, C., E. Pardo, C. Oyanadel, M. Bravo-Zehnder, P. Bull, M. Caceres, J. Martinez, L. Massardo, S. Jacobelli, A. Gonzalez, and A. Soza. 2006. Galectin-8 binds specific beta1 integrins and induces polarized spreading highlighted by asymmetric lamellipodia in Jurkat T cells. *Exp Cell Res*. 312:374-386.

Carlsson, S., M.C. Carlsson, and H. Leffler. 2007a. Intracellular sorting of galectin-8 based on carbohydrate fine specificity. *Glycobiology*.

Carlsson, S., C.T. Oberg, M.C. Carlsson, A. Sundin, U.J. Nilsson, D. Smith, R.D. Cummings, J. Almkvist, A. Karlsson, and H. Leffler. 2007b. Affinity of galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. *Glycobiology*. 17:663-676.

Carpenter, G. 1999. Employment of the epidermal growth factor receptor in growth factor-independent signaling pathways. *J Cell Biol*. 146:697-702.

Carpenter, G. 2000. The EGF receptor: a nexus for trafficking and signaling. *Bioessays*. 22:697-707.

Carpenter, G., and S. Cohen. 1977. Influence of lectins on the binding of 125I-labeled EGF to human fibroblasts. *Biochem Biophys Res Commun*. 79:545-552.

Carpenter, G., and S. Cohen. 1990. Epidermal growth factor. *J Biol Chem*. 265:7709-7712.

Cattaneo, V., M.V. Tribulatti, and O. Campetella. 2011. Galectin-8 tandem-repeat structure is essential for T-cell proliferation but not for co-stimulation. *Biochem J*. 434:153-160.

Cazet, A., S. Julien, M. Bobowski, M.A. Krzewinski-Recchi, A. Harduin-Lepers, S. Groux-Degroote, and P. Delannoy. 2010. Consequences of the expression of sialylated antigens in breast cancer. *Carbohydr Res*. 345:1377-1383.

Cederfur, C., E. Salomonsson, J. Nilsson, A. Halim, C.T. Oberg, G. Larson, U.J. Nilsson, and H. Leffler. 2008. Different affinity of galectins for human serum glycoproteins:

galectin-3 binds many protease inhibitors and acute phase proteins. *Glycobiology*. 18:384-394.

Ceresa, B.P., and S.L. Schmid. 2000. Regulation of signal transduction by endocytosis. *Curr Opin Cell Biol*. 12:204-210.

Chaturvedi, D., X. Gao, M.S. Cohen, J. Taunton, and T.B. Patel. 2009. Rapamycin induces transactivation of the EGFR and increases cell survival. *Oncogene*. 28:1187-1196.

Chiariotti, L., P. Salvatore, R. Frunzio, and C.B. Bruni. 2004. Galectin genes: regulation of expression. *Glycoconj J*. 19:441-449.

Clark, A.J., S. Ishii, N. Richert, G.T. Merlino, and I. Pastan. 1985. Epidermal growth factor regulates the expression of its own receptor. *Proc Natl Acad Sci U S A*. 82:8374-8378.

Clark, M.C., M. Pang, D.K. Hsu, F.T. Liu, S. de Vos, R.D. Gascoyne, J. Said, and L.G. Baum. 2012. Galectin-3 binds to CD45 on diffuse large B-cell lymphoma cells to regulate susceptibility to cell death. *Blood*. 120:4635-4644.

Cludts, S., C. Decaestecker, V. Mahillon, D. Chevalier, H. Kaltner, S. Andre, M. Remmelink, X. Leroy, H.J. Gabius, and S. Saussez. 2009. Galectin-8 up-regulation during hypopharyngeal and laryngeal tumor progression and comparison with galectin-1, -3 and -7. *Anticancer Res*. 29:4933-4940.

Cohen, M.M., Jr. 2003. Craniofacial anomalies: Clinical and molecular perspectives. *Ann Acad Med Singapore*. 32:244-251.

Corbit, K.C., P. Aanstad, V. Singla, A.R. Norman, D.Y. Stainier, and J.F. Reiter. 2005. Vertebrate Smoothened functions at the primary cilium. *Nature*. 437:1018-1021.

Cueni, L.N., and M. Detmar. 2009. Galectin-8 interacts with podoplanin and modulates lymphatic endothelial cell functions. *Exp Cell Res*. 315:1715-1723.

Cummings, R.D., A.M. Soderquist, and G. Carpenter. 1985. The oligosaccharide moieties of the epidermal growth factor receptor in A-431 cells. Presence of complex-type N-linked chains that contain terminal N-acetylgalactosamine residues. *J Biol Chem*. 260:11944-11952.

Dagher, S.F., J.L. Wang, and R.J. Patterson. 1995. Identification of galectin-3 as a factor in pre-mRNA splicing. *Proc Natl Acad Sci U S A*. 92:1213-1217.

Damle, N.K., K. Klussman, G. Leytze, A. Aruffo, P.S. Linsley, and J.A. Ledbetter. 1993a. Costimulation with integrin ligands intercellular adhesion molecule-1 or vascular cell adhesion molecule-1 augments activation-induced death of antigen-specific CD4+ T lymphocytes. *J Immunol*. 151:2368-2379.

Damle, N.K., G. Leytze, K. Klussman, and J.A. Ledbetter. 1993b. Activation with superantigens induces programmed death in antigen-primed CD4+ class II+ major histocompatibility complex T lymphocytes via a CD11a/CD18-dependent mechanism. *Eur J Immunol*. 23:1513-1522.

Danguy, A., I. Camby, and R. Kiss. 2002. Galectins and cancer. *Biochim Biophys Acta*. 1572:285-293.

Daub, H., C. Wallasch, A. Lankenau, A. Herrlich, and A. Ullrich. 1997. Signal characteristics of G protein-transactivated EGF receptor. *Embo J*. 16:7032-7044.

Davalos, D., K.M. Baeten, M.A. Whitney, E.S. Mullins, B. Friedman, E.S. Olson, J.K. Ryu, D.S. Smirnoff, M.A. Petersen, C. Bedard, J.L. Degen, R.Y. Tsien, and K. Akassoglou. 2014. Early detection of thrombin activity in neuroinflammatory disease. *Ann Neurol*. 75:303-308.

Delacour, D., V. Gouyer, J.P. Zanetta, H. Drobecq, E. Leteurtre, G. Grard, O. Moreau-Hannedouche, E. Maes, A. Pons, S. Andre, A. Le Bivic, H.J. Gabius, A. Manninen,

K. Simons, and G. Huet. 2005. Galectin-4 and sulfatides in apical membrane trafficking in enterocyte-like cells. *J Cell Biol*. 169:491-501.

Delacour, D., A. Koch, and R. Jacob. 2009. The Role of Galectins in Protein Trafficking. *Traffic*.

Delgado, V.M., L.G. Nugnes, L.L. Colombo, M.F. Troncoso, M.M. Fernandez, E.L. Malchiodi, I. Frahm, D.O. Croci, D. Compagno, G.A. Rabinovich, C. Wolfenstein-Todel, and M.T. Elola. 2011. Modulation of endothelial cell migration and angiogenesis: a novel function for the "tandem-repeat" lectin galectin-8. *FASEB J*. 25:242-254.

Deutschmann, A., A. Schlagenhauf, B. Leschnik, K.M. Hoffmann, A. Hauer, and W. Muntean. 2013. Increased procoagulant function of microparticles in pediatric inflammatory bowel disease: role in increased thrombin generation. *J Pediatr Gastroenterol Nutr*. 56:401-407.

Di Lella, S., V. Sundblad, J.P. Cerliani, C.M. Guardia, D.A. Estrin, G.R. Vasta, and G.A. Rabinovich. 2011. When galectins recognize glycans: from biochemistry to physiology and back again. *Biochemistry*. 50:7842-7857.

Dias-Baruffi, M., H. Zhu, M. Cho, S. Karmakar, R.P. McEver, and R.D. Cummings. 2003. Dimeric galectin-1 induces surface exposure of phosphatidylserine and phagocytic recognition of leukocytes without inducing apoptosis. *J Biol Chem*. 278:41282-41293.

Diskin, S., W.S. Chen, Z. Cao, S. Gyawali, H. Gong, A. Soza, A. Gonzalez, and N. Panjwani. 2012. Galectin-8 promotes cytoskeletal rearrangement in trabecular meshwork cells through activation of Rho signaling. *PLoS One*. 7:e44400.

Dong, S., C.L. Nutt, R.A. Betensky, A.O. Stemmer-Rachamimov, N.C. Denko, K.L. Ligon, D.H. Rowitch, and D.N. Louis. 2005. Histology-based expression profiling yields novel prognostic markers in human glioblastoma. *J Neuropathol Exp Neurol*. 64:948-955.

Earl, L.A., S. Bi, and L.G. Baum. 2011. Galectin multimerization and lattice formation are regulated by linker region structure. *Glycobiology*. 21:6-12.

Elliott, J.I., A. Surprenant, F.M. Marelli-Berg, J.C. Cooper, R.L. Cassady-Cain, C. Wooding, K. Linton, D.R. Alexander, and C.F. Higgins. 2005. Membrane phosphatidylserine distribution as a non-apoptotic signalling mechanism in lymphocytes. *Nat Cell Biol*. 7:808-816.

Elola, M.T., C. Wolfenstein-Todel, M.F. Troncoso, G.R. Vasta, and G.A. Rabinovich. 2007. Galectins: matricellular glycan-binding proteins linking cell adhesion, migration, and survival. *Cell Mol Life Sci*. 64:1679-1700.

Eshkar Sebban, L., D. Ronen, D. Levartovsky, O. Elkayam, D. Caspi, S. Aamar, H. Amital, A. Rubinow, I. Golan, D. Naor, Y. Zick, and I. Golan. 2007. The involvement of CD44 and its novel ligand galectin-8 in apoptotic regulation of autoimmune inflammation. *J Immunol*. 179:1225-1235.

Fadok, V.A., and G. Chimini. 2001. The phagocytosis of apoptotic cells. *Semin Immunol*. 13:365-372.

Filardo, E.J. 2002. Epidermal growth factor receptor (EGFR) transactivation by estrogen via the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer. *J Steroid Biochem Mol Biol*. 80:231-238.

Fischer, O.M., S. Hart, A. Gschwind, and A. Ullrich. 2003. EGFR signal transactivation in cancer cells. *Biochem Soc Trans*. 31:1203-1208.

Friebe, A., A.J. Douglas, E. Solano, S.M. Blois, E. Hagen, B.F. Klapp, D.A. Clark, and P.C. Arck. 2011. Neutralization of LPS or blockage of TLR4 signaling prevents stress-triggered fetal loss in murine pregnancy. *J Mol Med (Berl)*. 89:689-699.

Friedl, P. 2004. Prespecification and plasticity: shifting mechanisms of cell migration. *Curr Opin Cell Biol*. 16:14-23.

Gabius, H.J., S. Andre, I. Gunsenhauser, H. Kaltner, G. Kayser, J. Kopitz, H. Lahm, D. Harms, J. Szymas, and K. Kayser. 2002. Association of galectin-1- but not galectin-3-dependent parameters with proliferation activity in human neuroblastomas and small cell lung carcinomas. *Anticancer Res*. 22:405-410.

Gamou, S., and N. Shimizu. 1988. Glycosylation of the epidermal growth factor receptor and its relationship to membrane transport and ligand binding. *J Biochem*. 104:388-396.

Gendron, S., J. Couture, and F. Aoudjit. 2003. Integrin alpha2beta1 inhibits Fas-mediated apoptosis in T lymphocytes by protein phosphatase 2A-dependent activation of the MAPK/ERK pathway. *J Biol Chem*. 278:48633-48643.

Gillies, R.J., N. Didier, and M. Denton. 1986. Determination of cell number in monolayer cultures. *Anal Biochem*. 159:109-113.

Gschwind, A., E. Zwick, N. Prenzel, M. Leserer, and A. Ullrich. 2001. Cell communication networks: epidermal growth factor receptor transactivation as the paradigm for interreceptor signal transmission. *Oncogene*. 20:1594-1600.

Habermann, F.A., S. Andre, H. Kaltner, D. Kubler, F. Sinowatz, and H.J. Gabius. 2011. Galectins as tools for glycan mapping in histology: comparison of their binding profiles to the bovine zona pellucida by confocal laser scanning microscopy. *Histochem Cell Biol*. 135:539-552.

Hadari, Y.R., R. Arbel-Goren, Y. Levy, A. Amsterdam, R. Alon, R. Zakut, and Y. Zick. 2000a. Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis. *J Cell Sci*. 113 (Pt 13):2385-2397.

Hadari, Y.R., R. Arbel-Goren, Y. Levy, A. Amsterdam, R. Alon, R. Zakut, and Y. Zick. 2000b. Galectin-8 binding to integrins inhibits cell adhesion and induces apoptosis. *J Cell Sci*. 113:2385-2397.

Hadari, Y.R., K. Paz, R. Dekel, T. Mestrovic, D. Accili, and Y. Zick. 1995. Galectin-8. A new rat lectin, related to galectin-4. *J Biol Chem*. 270:3447-3453.

Harjacek, M., S. Diaz-Cano, M. De Miguel, H. Wolfe, C.A. Maldonado, and G.A. Rabinovich. 2001. Expression of galectins-1 and -3 correlates with defective mononuclear cell apoptosis in patients with juvenile idiopathic arthritis. *J Rheumatol*. 28:1914-1922.

Hazan, R., L. Krushel, and K.L. Crossin. 1995. EGF receptor-mediated signals are differentially modulated by concanavalin A. *J Cell Physiol*. 162:74-85.

Hirabayashi, J., T. Hashidate, Y. Arata, N. Nishi, T. Nakamura, M. Hirashima, T. Urashima, T. Oka, M. Futai, W.E. Muller, F. Yagi, and K. Kasai. 2002. Oligosaccharide specificity of galectins: a search by frontal affinity chromatography. *Biochim Biophys Acta*. 1572:232-254.

Hobbs, S.S., J.A. Goettel, D. Liang, F. Yan, K.L. Edelblum, M.R. Frey, M.T. Mullane, and D.B. Polk. 2011. TNF transactivation of EGFR stimulates cytoprotective COX-2 expression in gastrointestinal epithelial cells. *Am J Physiol Gastrointest Liver Physiol*. 301:G220-229.

Holmstrom, T.H., I. Schmitz, T.S. Soderstrom, M. Poukkula, V.L. Johnson, S.C. Chow, P.H. Krammer, and J.E. Eriksson. 2000. MAPK/ERK signaling in activated T cells

inhibits CD95/Fas-mediated apoptosis downstream of DISC assembly. *Embo J*. 19:5418-5428.

Hotta, K., T. Funahashi, Y. Matsukawa, M. Takahashi, H. Nishizawa, K. Kishida, M. Matsuda, H. Kuriyama, S. Kihara, T. Nakamura, Y. Tochino, N.L. Bodkin, B.C. Hansen, and Y. Matsuzawa. 2001. Galectin-12, an Adipose-expressed Galectin-like Molecule Possessing Apoptosis-inducing Activity. *J Biol Chem*. 276:34089-34097.

Houzelstein, D., I.R. Goncalves, A.J. Fadden, S.S. Sidhu, D.N. Cooper, K. Drickamer, H. Leffler, and F. Poirier. 2004. Phylogenetic Analysis of the Vertebrate Galectin Family. *Mol Biol Evol*.

Hughes, R.C. 1999. Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim Biophys Acta*. 1473:172-185.

Ideo, H., T. Matsuzaka, T. Nonaka, A. Seko, and K. Yamashita. 2011. Galectin-8-N-domain recognition mechanism for sialylated and sulfated glycans. *J Biol Chem*. 286:11346-11355.

Ideo, H., A. Seko, I. Ishizuka, and K. Yamashita. 2003. The N-terminal carbohydrate recognition domain of galectin-8 recognizes specific glycosphingolipids with high affinity. *Glycobiology*. 13:713-723.

Ingham, P.W., and A.P. McMahon. 2001. Hedgehog signaling in animal development: paradigms and principles. *Genes Dev*. 15:3059-3087.

Janssen, O., R. Sanzenbacher, and D. Kabelitz. 2000. Regulation of activation-induced cell death of mature T-lymphocyte populations. *Cell Tissue Res*. 301:85-99.

Jorissen, R.N., F. Walker, N. Pouliot, T.P. Garrett, C.W. Ward, and A.W. Burgess. 2003. Epidermal growth factor receptor: mechanisms of activation and signalling. *Exp Cell Res*. 284:31-53.

Kaltner, H., and H.J. Gabius. 2012. A toolbox of lectins for translating the sugar code: the galectin network in phylogenesis and tumors. *Histol Histopathol*. 27:397-416.

Kasina, S., P.A. Scherle, C.L. Hall, and J.A. Macoska. 2009. ADAM-mediated amphiregulin shedding and EGFR transactivation. *Cell Prolif*. 42:799-812.

Kasper, M., G. Regl, A.M. Frischauf, and F. Aberger. 2006. GLI transcription factors: mediators of oncogenic Hedgehog signalling. *Eur J Cancer*. 42:437-445.

Keryer-Bibens, C., C. Pioche-Durieu, C. Villemant, S. Souquere, N. Nishi, M. Hirashima, J. Middeldorp, and P. Busson. 2006. Exosomes released by EBV-infected nasopharyngeal carcinoma cells convey the viral latent membrane protein 1 and the immunomodulatory protein galectin 9. *BMC Cancer*. 6:283.

Klein, S., and A. Levitzki. 2009. Targeting the EGFR and the PKB pathway in cancer. *Curr Opin Cell Biol*.

Kopitz, J., S. Ballikaya, S. Andre, and H.J. Gabius. 2012. Ganglioside GM1/Galectin-Dependent Growth Regulation in Human Neuroblastoma Cells: Special Properties of Bivalent Galectin-4 and Significance of Linker Length for Ligand Selection. *Neurochem Res*.

Lahm, H., S. Andre, A. Hoeflich, H. Kaltner, H.C. Siebert, B. Sordat, C.W. von der Lieth, E. Wolf, and H.J. Gabius. 2004. Tumor galectinology: insights into the complex network of a family of endogenous lectins. *Glycoconj J*. 20:227-238.

Lajoie, P., J.G. Goetz, J.W. Dennis, and I.R. Nabi. 2009. Lattices, rafts, and scaffolds: domain regulation of receptor signaling at the plasma membrane. *J Cell Biol*. 185:381-385.

Lau, K.S., E.A. Partridge, A. Grigorian, C.I. Silvescu, V.N. Reinhold, M. Demetriou, and J.W. Dennis. 2007. Complex N-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. *Cell*. 129:123-134.

Lax, I., F. Bellot, R. Howk, A. Ullrich, D. Givol, and J. Schlessinger. 1989. Functional analysis of the ligand binding site of EGF-receptor utilizing chimeric chicken/human receptor molecules. *Embo J*. 8:421-427.

Le Marer, N., and R.C. Hughes. 1996. Effects of the carbohydrate-binding protein galectin-3 on the invasiveness of human breast carcinoma cells. *J Cell Physiol*. 168:51-58.

Le Mercier, M., S. Fortin, V. Mathieu, R. Kiss, and F. Lefranc. 2010. Galectins and gliomas. *Brain Pathol*. 20:17-27.

Leffler, H. 2001. Galectins structure and function--a synopsis. *Results Probl Cell Differ*. 33:57-83.

Leffler, H., S. Carlsson, M. Hedlund, Y. Qian, and F. Poirier. 2004. Introduction to galectins. *Glycoconj J*. 19:433-440.

Leventis, P.A., and S. Grinstein. 2010. The distribution and function of phosphatidylserine in cellular membranes. *Annu Rev Biophys*. 39:407-427.

Levy, Y., R. Arbel-Goren, Y.R. Hadari, S. Eshhar, D. Ronen, E. Elhanany, B. Geiger, and Y. Zick. 2001. Galectin-8 functions as a matricellular modulator of cell adhesion. *J Biol Chem*. 276:31285-31295.

Levy, Y., S. Auslender, M. Eisenstein, R.R. Vidavski, D. Ronen, A.D. Bershadsky, and Y. Zick. 2006. It depends on the hinge: a structure-functional analysis of galectin-8, a tandem-repeat type lectin. *Glycobiology*. 16:463-476.

Li, M., L. Song, and X. Qin. 2010. Glycan changes: cancer metastasis and anti-cancer vaccines. *J Biosci*. 35:665-673.

Liu, F.T., R.J. Patterson, and J.L. Wang. 2002. Intracellular functions of galectins. *Biochim Biophys Acta*. 1572:263-273.

Liu, F.T., and G.A. Rabinovich. 2005. Galectins as modulators of tumour progression. *Nat Rev Cancer*. 5:29-41.

Liu, J., L. Qian, R.J. Wessells, Y. Bidet, K. Jagla, and R. Bodmer. 2006. Hedgehog and RAS pathways cooperate in the anterior-posterior specification and positioning of cardiac progenitor cells. *Dev Biol*. 290:373-385.

Liu, S., W.R. Kam, J. Ding, M.P. Hatton, and D.A. Sullivan. 2013. Effect of growth factors on the proliferation and gene expression of human meibomian gland epithelial cells. *Invest Ophthalmol Vis Sci*. 54:2541-2550.

Lu, L.H., R. Nakagawa, Y. Kashio, A. Ito, H. Shoji, N. Nishi, M. Hirashima, A. Yamauchi, and T. Nakamura. 2007. Characterization of galectin-9-induced death of Jurkat T cells. *J Biochem*. 141:157-172.

Lum, L., and P.A. Beachy. 2004. The Hedgehog response network: sensors, switches, and routers. *Science*. 304:1755-1759.

Lyles, J.V., K. Cornely-Moss, C.M. Smith, and D.L. Daleke. 2003. Purification of the human erythrocyte PS-stimulated Mg2+-ATPase: a putative PS flippase. *Methods Mol Biol*. 228:257-269.

Mary, F., C. Moon, T. Venaille, M.L. Thomas, D. Mary, and A. Bernard. 1999. Modulation of TCR signaling by beta1 integrins: role of the tyrosine phosphatase SHP-1. *Eur J Immunol*. 29:3887-3897.

Massague, J., and A. Pandiella. 1993. Membrane-anchored growth factors. *Annu Rev Biochem*. 62:515-541.

Miaczynska, M., L. Pelkmans, and M. Zerial. 2004. Not just a sink: endosomes in control of signal transduction. *Curr Opin Cell Biol*. 16:400-406.

Mimeault, M., and S.K. Batra. 2010. Frequent deregulations in the hedgehog signaling network and cross-talks with the epidermal growth factor receptor pathway involved in cancer progression and targeted therapies. *Pharmacol Rev*. 62:497-524.

Mizoguchi, M., R.A. Betensky, T.T. Batchelor, D.C. Bernay, D.N. Louis, and C.L. Nutt. 2006. Activation of STAT3, MAPK, and AKT in malignant astrocytic gliomas: correlation with EGFR status, tumor grade, and survival. *J Neuropathol Exp Neurol*. 65:1181-1188.

Nakayamada, S., K. Saito, K. Fujii, M. Yasuda, M. Tamura, and Y. Tanaka. 2003. beta1 integrin-mediated signaling induces intercellular adhesion molecule 1 and Fas on rheumatoid synovial cells and Fas-mediated apoptosis. *Arthritis Rheum*. 48:1239-1248.

Ng, J.M., and T. Curran. 2011. The Hedgehog's tale: developing strategies for targeting cancer. *Nat Rev Cancer*. 11:493-501.

Nickel, W. 2003. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem*. 270:2109-2119.

Nierodzik, M.L., and S. Karpatkin. 2006. Thrombin induces tumor growth, metastasis, and angiogenesis: Evidence for a thrombin-regulated dormant tumor phenotype. *Cancer Cell*. 10:355-362.

Nishi, N., A. Itoh, A. Fujiyama, N. Yoshida, S. Araya, M. Hirashima, H. Shoji, and T. Nakamura. 2005. Development of highly stable galectins: truncation of the linker peptide confers protease-resistance on tandem-repeat type galectins. *FEBS Lett*. 579:2058-2064.

Nishi, N., A. Itoh, H. Shoji, H. Miyanaka, and T. Nakamura. 2006. Galectin-8 and galectin-9 are novel substrates for thrombin. *Glycobiology*. 16:15C-20C.

Nishi, N., H. Shoji, M. Seki, A. Itoh, H. Miyanaka, K. Yuube, M. Hirashima, and T. Nakamura. 2003. Galectin-8 modulates neutrophil function via interaction with integrin alphaM. *Glycobiology*. 13:755-763.

Norambuena, A., C. Metz, L. Vicuna, A. Silva, E. Pardo, C. Oyanadel, L. Massardo, A. Gonzalez, and A. Soza. 2009. Galectin-8 induces apoptosis in Jurkat T cells by phosphatidic acid-mediated ERK1/2 activation supported by protein kinase A down-regulation. *J Biol Chem*. 284:12670-12679.

Norbury, C.J., and I.D. Hickson. 2001. Cellular responses to DNA damage. *Annu Rev Pharmacol Toxicol*. 41:367-401.

Noubouossie, D.F., P.Q. Le, F. Corazza, F. Debaugnies, L. Rozen, A. Ferster, and A. Demulder. 2012. Thrombin generation reveals high procoagulant potential in the plasma of sickle cell disease children. *Am J Hematol*. 87:145-149.

Nusslein-Volhard, C., and E. Wieschaus. 1980. Mutations affecting segment number and polarity in Drosophila. *Nature*. 287:795-801.

Opstad, T.B., A.A. Pettersen, V. Bratseth, H. Arnesen, and I. Seljeflot. 2010. The influence of tissue factor and tissue factor pathway inhibitor polymorphisms on thrombin generation in stable coronary artery disease. *Pathophysiol Haemost Thromb*. 37:98-103.

Ordener, C., I. Lascombe, M. Sallot, B. Cypriani, G.L. Adessi, and M. Jouvenot. 1994. [Effects of 17 beta-estradiol and growth factors on cell proliferation and c-fos gene expression, in vitro, in endometrial cells in normal growth]. *C R Seances Soc Biol Fil*. 188:387-396.

Partridge, E.A., C. Le Roy, G.M. Di Guglielmo, J. Pawling, P. Cheung, M. Granovsky, I.R. Nabi, J.L. Wrana, and J.W. Dennis. 2004. Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis. *Science*. 306:120-124.

Patnaik, S.K., B. Potvin, S. Carlsson, D. Sturm, H. Leffler, and P. Stanley. 2006. Complex N-glycans are the major ligands for galectin-1, -3, and -8 on Chinese hamster ovary cells. *Glycobiology*. 16:305-317.

Perillo, N.L., M.E. Marcus, and L.G. Baum. 1998. Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J Mol Med*. 76:402-412.

Perillo, N.L., K.E. Pace, J.J. Seilhamer, and L.G. Baum. 1995. Apoptosis of T cells mediated by galectin-1. *Nature*. 378:736-739.

Perone, M.J., A.T. Larregina, W.J. Shufesky, G.D. Papworth, M.L. Sullivan, A.F. Zahorchak, D.B. Stolz, L.G. Baum, S.C. Watkins, A.W. Thomson, and A.E. Morelli. 2006. Transgenic galectin-1 induces maturation of dendritic cells that elicit contrasting responses in naive and activated T cells. *J Immunol*. 176:7207-7220.

Polo, S., and P.P. Di Fiore. 2006. Endocytosis conducts the cell signaling orchestra. *Cell*. 124:897-900.

Pomorski, T., J.C. Holthuis, A. Herrmann, and G. van Meer. 2004. Tracking down lipid flippases and their biological functions. *J Cell Sci*. 117:805-813.

Prenzel, N., E. Zwick, H. Daub, M. Leserer, R. Abraham, C. Wallasch, and A. Ullrich. 1999. EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature*. 402:884-888.

Prenzel, N., E. Zwick, M. Leserer, and A. Ullrich. 2000. Tyrosine kinase signalling in breast cancer. Epidermal growth factor receptor: convergence point for signal integration and diversification. *Breast Cancer Res*. 2:184-190.

Rabinovich, G.A., A. Ariel, R. Hershkoviz, J. Hirabayashi, K.I. Kasai, and O. Lider. 1999. Specific inhibition of T-cell adhesion to extracellular matrix and proinflammatory cytokine secretion by human recombinant galectin-1. *Immunology*. 97:100-106.

Rabinovich, G.A., F.T. Liu, M. Hirashima, and A. Anderson. 2007. An emerging role for galectins in tuning the immune response: lessons from experimental models of inflammatory disease, autoimmunity and cancer. *Scand J Immunol*. 66:143-158.

Rebbaa, A., H. Yamamoto, J.R. Moskal, and E.G. Bremer. 1996. Binding of erythroagglutinating phytohemagglutinin lectin from Phaseolus vulgaris to the epidermal growth factor receptor inhibits receptor function in the human glioma cell line, U373 MG. *J Neurochem*. 67:2265-2272.

Roepstorff, K., L. Grovdal, M. Grandal, M. Lerdrup, and B. van Deurs. 2008. Endocytic downregulation of ErbB receptors: mechanisms and relevance in cancer. *Histochem Cell Biol*. 129:563-578.

Rohatgi, R., L. Milenkovic, R.B. Corcoran, and M.P. Scott. 2009. Hedgehog signal transduction by Smoothened: pharmacologic evidence for a 2-step activation process. *Proc Natl Acad Sci U S A*. 106:3196-3201.

Rohatgi, R., L. Milenkovic, and M.P. Scott. 2007. Patched1 regulates hedgehog signaling at the primary cilium. *Science*. 317:372-376.

Ruiz i Altaba, A., C. Mas, and B. Stecca. 2007. The Gli code: an information nexus regulating cell fate, stemness and cancer. *Trends Cell Biol*. 17:438-447.

Satelli, A., P.S. Rao, P.K. Gupta, P.R. Lockman, K.S. Srivenugopal, and U.S. Rao. 2008. Varied expression and localization of multiple galectins in different cancer cell lines. *Oncol Rep*. 19:587-594.

Sawyers, C.L. 2003. Opportunities and challenges in the development of kinase inhibitor therapy for cancer. *Genes Dev*. 17:2998-3010.

Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. *Cell*. 103:211-225.

Schlessinger, J. 2002. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. *Cell*. 110:669-672.

Schoeppner, H.L., A. Raz, S.B. Ho, and R.S. Bresalier. 1995. Expression of an endogenous galactose-binding lectin correlates with neoplastic progression in the colon. *Cancer*. 75:2818-2826.

Seales, E.C., G.A. Jurado, B.A. Brunson, J.K. Wakefield, A.R. Frost, and S.L. Bellis. 2005. Hypersialylation of beta1 integrins, observed in colon adenocarcinoma, may contribute to cancer progression by up-regulating cell motility. *Cancer Res*. 65:4645-4652.

Seelenmeyer, C., C. Stegmayer, and W. Nickel. 2008. Unconventional secretion of fibroblast growth factor 2 and galectin-1 does not require shedding of plasma membrane-derived vesicles. *FEBS Lett*. 582:1362-1368.

Seelenmeyer, C., S. Wegehingel, I. Tews, M. Kunzler, M. Aebi, and W. Nickel. 2005. Cell surface counter receptors are essential components of the unconventional export machinery of galectin-1. *J Cell Biol*. 171:373-381.

Shimodaira, K., J. Nakayama, N. Nakamura, O. Hasebe, T. Katsuyama, and M. Fukuda. 1997. Carcinoma-associated expression of core 2 beta-1,6-N-acetylglucosaminyltransferase gene in human colorectal cancer: role of O-glycans in tumor progression. *Cancer Res*. 57:5201-5206.

Sigismund, S., E. Argenzio, D. Tosoni, E. Cavallaro, S. Polo, and P.P. Di Fiore. 2008. Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation. *Dev Cell*. 15:209-219.

Sigismund, S., T. Woelk, C. Puri, E. Maspero, C. Tacchetti, P. Transidico, P.P. Di Fiore, and S. Polo. 2005. Clathrin-independent endocytosis of ubiquitinated cargos. *Proc Natl Acad Sci U S A*. 102:2760-2765.

Sinha, D., S. Bannergee, J.H. Schwartz, W. Lieberthal, and J.S. Levine. 2004. Inhibition of ligand-independent ERK1/2 activity in kidney proximal tubular cells deprived of soluble survival factors up-regulates Akt and prevents apoptosis. *J Biol Chem*. 279:10962-10972.

Slack-Davis, J.K., K.H. Martin, R.W. Tilghman, M. Iwanicki, E.J. Ung, C. Autry, M.J. Luzzio, B. Cooper, J.C. Kath, W.G. Roberts, and J.T. Parsons. 2007. Cellular characterization of a novel focal adhesion kinase inhibitor. *J Biol Chem*. 282:14845-14852.

Slieker, L.J., and M.D. Lane. 1985. Post-translational processing of the epidermal growth factor receptor. Glycosylation-dependent acquisition of ligand-binding capacity. *J Biol Chem*. 260:687-690.

Slieker, L.J., T.M. Martensen, and M.D. Lane. 1986. Synthesis of epidermal growth factor receptor in human A431 cells. Glycosylation-dependent acquisition of ligand binding activity occurs post-translationally in the endoplasmic reticulum. *J Biol Chem*. 261:15233-15241.

Smriti, E.C. Nemergut, and D.L. Daleke. 2007. ATP-dependent transport of phosphatidylserine analogues in human erythrocytes. *Biochemistry*. 46:2249-2259.

Soderquist, A.M., and G. Carpenter. 1984. Glycosylation of the epidermal growth factor receptor in A-431 cells. The contribution of carbohydrate to receptor function. *J Biol Chem*. 259:12586-12594.

Solatycka, A., T. Owczarek, F. Piller, V. Piller, B. Pula, L. Wojciech, M. Podhorska-Okolow, P. Dziegiel, and M. Ugorski. 2012. MUC1 in human and murine mammary carcinoma cells decreases the expression of core 2 beta1,6-N-acetylglucosaminyltransferase and beta-galactoside alpha2,3-sialyltransferase. *Glycobiology*. 22:1042-1054.

Soltoff, S.P., H. Avraham, S. Avraham, and L.C. Cantley. 1998. Activation of P2Y2 receptors by UTP and ATP stimulates mitogen-activated kinase activity through a pathway that involves related adhesion focal tyrosine kinase and protein kinase C. *J Biol Chem*. 273:2653-2660.

Sorkin, A., and L.K. Goh. 2008. Endocytosis and intracellular trafficking of ErbBs. *Exp Cell Res*. 314:3093-3106.

Sorkin, A., and M. Von Zastrow. 2002. Signal transduction and endocytosis: close encounters of many kinds. *Nat Rev Mol Cell Biol*. 3:600-614.

Soza, A., A. Norambuena, J. Cancino, E. de la Fuente, P. Henklein, and A. Gonzalez. 2004. Sorting competition with membrane-permeable peptides in intact epithelial cells revealed discrimination of transmembrane proteins not only at the trans-Golgi network but also at pre-Golgi stages. *J Biol Chem*. 279:17376-17383.

Stillman, B.N., D.K. Hsu, M. Pang, C.F. Brewer, P. Johnson, F.T. Liu, and L.G. Baum. 2006. Galectin-3 and galectin-1 bind distinct cell surface glycoprotein receptors to induce T cell death. *J Immunol*. 176:778-789.

Stowell, S.R., C.M. Arthur, K.A. Slanina, J.R. Horton, D.F. Smith, and R.D. Cummings. 2008. Dimeric Galectin-8 induces phosphatidylserine exposure in leukocytes through polylactosamine recognition by the C-terminal domain. *J Biol Chem*. 283:20547-20559.

Stowell, S.R., S. Karmakar, C.J. Stowell, M. Dias-Baruffi, R.P. McEver, and R.D. Cummings. 2007. Human galectin-1, -2, and -4 induce surface exposure of phosphatidylserine in activated human neutrophils but not in activated T cells. *Blood*. 109:219-227.

Stroop, C.J., W. Weber, G.J. Gerwig, M. Nimtz, J.P. Kamerling, and J.F. Vliegenthart. 2000. Characterization of the carbohydrate chains of the secreted form of the human epidermal growth factor receptor. *Glycobiology*. 10:901-917.

Stupack, D.G., and D.A. Cheresh. 2004. A Bit-role for integrins in apoptosis. *Nat Cell Biol*. 6:388-389.

Sturm, A., M. Lensch, S. Andre, H. Kaltner, B. Wiedenmann, S. Rosewicz, A.U. Dignass, and H.J. Gabius. 2004. Human galectin-2: novel inducer of T cell apoptosis with distinct profile of caspase activation. *J Immunol*. 173:3825-3837.

Su, Z.Z., J. Lin, R. Shen, P.E. Fisher, N.I. Goldstein, and P.B. Fisher. 1996. Surface-epitope masking and expression cloning identifies the human prostate carcinoma tumor antigen gene PCTA-1 a member of the galectin gene family. *Proc Natl Acad Sci U S A*. 93:7252-7257.

Teglund, S., and R. Toftgard. 2010. Hedgehog beyond medulloblastoma and basal cell carcinoma. *Biochim Biophys Acta*. 1805:181-208.

Thery, C., M. Boussac, P. Veron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, and S. Amigorena. 2001. Proteomic analysis of dendritic cell-derived exosomes: a secreted subcellular compartment distinct from apoptotic vesicles. *J Immunol*. 166:7309-7318.

Trepat, X., Z. Chen, and K. Jacobson. 2012. Cell migration. *Compr Physiol*. 2:2369-2392.

Tribulatti, M.V., V. Cattaneo, U. Hellman, J. Mucci, and O. Campetella. 2009. Galectin-8 provides costimulatory and proliferative signals to T lymphocytes. *J Leukoc Biol*. 86:371-380.

Tribulatti, M.V., J. Mucci, V. Cattaneo, F. Aguero, T. Gilmartin, S.R. Head, and O. Campetella. 2007. Galectin-8 induces apoptosis in the CD4(high)CD8(high) thymocyte subpopulation. *Glycobiology*. 17:1404-1412.

Tsai, W., A.D. Morielli, and E.G. Peralta. 1997. The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity. *Embo J*. 16:4597-4605.

Ullrich, A., and J. Schlessinger. 1990. Signal transduction by receptors with tyrosine kinase activity. *Cell*. 61:203-212.

Undas, A., M. Jankowski, P. Kaczmarek, K. Sladek, and K. Brummel-Ziedins. 2011. Thrombin generation in chronic obstructive pulmonary disease: dependence on plasma factor composition. *Thromb Res*. 128:e24-28.

van den Brink, M.R., R. Kapeller, J.C. Pratt, J.H. Chang, and S.J. Burakoff. 1999. The extracellular signal-regulated kinase pathway is required for activation-induced cell death of T cells. *J Biol Chem*. 274:11178-11185.

Vedula, S.R., A. Ravasio, C.T. Lim, and B. Ladoux. 2013. Collective cell migration: a mechanistic perspective. *Physiology (Bethesda)*. 28:370-379.

Vicuna, L., E. Pardo, C. Curkovic, R. Doger, C. Oyanadel, C. Metz, L. Massardo, A. Gonzalez, and A. Soza. 2013. Galectin-8 binds to LFA-1, blocks its interaction with ICAM-1 and is counteracted by anti-Gal-8 autoantibodies isolated from lupus patients. *Biol Res*. 46:275-280.

Villeneuve, C., L. Baricault, L. Canelle, N. Barboule, C. Racca, B. Monsarrat, T. Magnaldo, and F. Larminat. 2011. Mitochondrial proteomic approach reveals galectin-7 as a novel BCL-2 binding protein in human cells. *Mol Biol Cell*. 22:999-1013.

Vivanco, I., and C.L. Sawyers. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat Rev Cancer*. 2:489-501.

Vivinus-Nebot, M., M. Ticchioni, F. Mary, P. Hofman, V. Quaranta, P. Rousselle, and A. Bernard. 1999. Laminin 5 in the human thymus: control of T cell proliferation via alpha6beta4 integrins. *J Cell Biol*. 144:563-574.

Vyakarnam, A., S.F. Dagher, J.L. Wang, and R.J. Patterson. 1997. Evidence for a role for galectin-1 in pre-mRNA splicing. *Mol Cell Biol*. 17:4730-4737.

Wernig, F., M. Mayr, and Q. Xu. 2003. Mechanical stretch-induced apoptosis in smooth muscle cells is mediated by beta1-integrin signaling pathways. *Hypertension*. 41:903-911.

Wetmore, C. 2003. Sonic hedgehog in normal and neoplastic proliferation: insight gained from human tumors and animal models. *Curr Opin Genet Dev*. 13:34-42.

Wiley, H.S. 2003. Trafficking of the ErbB receptors and its influence on signaling. *Exp Cell Res*. 284:78-88.

Wiley, H.S., and P.M. Burke. 2001. Regulation of receptor tyrosine kinase signaling by endocytic trafficking. *Traffic*. 2:12-18.

Williamson, P., and R.A. Schlegel. 2002. Transbilayer phospholipid movement and the clearance of apoptotic cells. *Biochim Biophys Acta*. 1585:53-63.

Wong, S.Y., A.D. Seol, P.L. So, A.N. Ermilov, C.K. Bichakjian, E.H. Epstein, Jr., A.A. Dlugosz, and J.F. Reiter. 2009. Primary cilia can both mediate and suppress Hedgehog pathway-dependent tumorigenesis. *Nat Med*. 15:1055-1061.

Wu, X., R. Zhang, A. Di, H. Shan, and R. Xu. 1999. [Effects of growth factors and estrogen on the proliferation and prolactin gene expression in anterior pituitary cells of rats]. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao*. 21:331-337.

Yang, J.M., J.C. Byrd, B.B. Siddiki, Y.S. Chung, M. Okuno, M. Sowa, Y.S. Kim, K.L. Matta, and I. Brockhausen. 1994. Alterations of O-glycan biosynthesis in human colon cancer tissues. *Glycobiology*. 4:873-884.

Yang, R.Y., D.K. Hsu, and F.T. Liu. 1996. Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc Natl Acad Sci U S A*. 93:6737-6742.

Yang, R.Y., and F.T. Liu. 2003. Galectins in cell growth and apoptosis. *Cell Mol Life Sci*. 60:267-276.

Yarden, Y. 2001. The EGFR family and its ligands in human cancer. signalling mechanisms and therapeutic opportunities. *Eur J Cancer*. 37 Suppl 4:S3-8.

Yarden, Y., and M.X. Sliwkowski. 2001. Untangling the ErbB signalling network. *Nat Rev Mol Cell Biol*. 2:127-137.

Yu, F., R.L. Finley, Jr., A. Raz, and H.R. Kim. 2002. Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation. *J Biol Chem*. 277:15819-15827.

Zappelli, C., C. van der Zwaan, D.C. Thijssen-Timmer, K. Mertens, and A.B. Meijer. 2012. Novel role for galectin-8 protein as mediator of coagulation factor V endocytosis by megakaryocytes. *J Biol Chem*. 287:8327-8335.

Zeng, F.Y., A. Benguria, S. Kafert, S. Andre, H.J. Gabius, and A. Villalobo. 1995. Differential response of the epidermal growth factor receptor tyrosine kinase activity to several plant and mammalian lectins. *Mol Cell Biochem*. 142:117-124.

Zhang, F., M. Zheng, Y. Qu, J. Li, J. Ji, B. Feng, A. Lu, M. Wang, and B. Liu. 2009. Different roles of galectin-9 isoforms in modulating E-selectin expression and adhesion function in LoVo colon carcinoma cells. *Mol Biol Rep*. 36:823-830.

Zhu, L., X. Yu, Y. Akatsuka, J.A. Cooper, and C. Anasetti. 1999. Role of mitogen-activated protein kinases in activation-induced apoptosis of T cells. *Immunology*. 97:26-35.

Zhuang, S., and R.G. Schnellmann. 2006. A death-promoting role for extracellular signal-regulated kinase. *J Pharmacol Exp Ther*. 319:991-997.

Zick, Y., M. Eisenstein, R.A. Goren, Y.R. Hadari, Y. Levy, and D. Ronen. 2004. Role of galectin-8 as a modulator of cell adhesion and cell growth. *Glycoconj J*. 19:517-526.