CHAPTER 3

RESULTS
3. Results

Earlier results from our lab has shown that expression of β1,6 branched N-oligosaccharides correlates with metastatic potential of B16 murine melanoma cells (low metastastic, B16F1 and high metastastic, B16F10). β1,6 branched N-oligosaccharides is the preferred site for terminal substitutions such as sialic acids, Lewis antigens and poly-N-acetyllactosamine (polyLacNAc) which may serve as ligands for several endogenous lectins. It was found that levels of polyLacNAc substituted β1,6 branched N-glycans in these cells correlate with their metastatic potential. PolyLacNAc is a high affinity ligand for endogenous lectin, galectin-3 which is expressed in highest amounts in lungs and constitutively on its vascular endothelium. LAMP1 and β1 Integrin which were identified as polyLacNAc carriers could mediate adhesion of melanoma cells on lungs via galectin-3 and thus facilitate their homing (5).

Adhesion of tumor cells to target organ endothelium is the primary step of organ homing which is followed by extravasation events to establish a metastastic colony. It is reported that galectin-3 is secreted out in a non classical pathway and interact with cell membrane or become a part of BM/ECM. In lung tissues, expression of galectin-3 was not only restricted to vascular endothelium, but it was shown to be expressed on all the other major compartments. On these grounds, participation of galectin-3 in post adhesion events was investigated. It was shown that apart from mediating arrest of melanoma cells onto lung endothelium, galectin-3 could also facilitate cell spreading, matrix degradation and cell motility. The role of galectin-3 in mediating these post adhesion events clearly suggests that it can play an important role in extravasation of melanoma cells (6).

Swainsonine (SW), N-glycosylation inhibitor, inhibits α-mannosidase II, an enzyme involved in initiating the formation of complex type N-glycans. Treatment with SW resulted in inhibition of expression of complex type, including β1,6 branched N-glycans,
and significant loss in the metastatic potential of melanoma cells. Although, β1,6 branched of N-oligosaccharides is one of the most preferred sites for polyLacNAc substitutions, even O-oligosaccharides often carry them. However, inhibition of O-glycan synthesis in F10 cells using Benzyl-α-N-acetylgalactosamine (BG) did not affect the galectin-3 mediated processes including their lung metastatic potential. Further, participation of T/Tn antigen, a weak affinity ligand for galectin-3 in these processes, was also ruled out (162). Although, these results highlighted the importance of expression of β1,6 branched N-glycans on melanoma cells in metastasis associated processes, they did not conclusively establish the specific involvement of polyLacNAc in metastasis. Thus, their role was confirmed by specifically targeting the genes which code for enzymes involved in the synthesis of polyLacNAc.

Objective I: To understand the role of galectin-3/polyLacNAc pair in mediating metastasis associated events by inhibiting the expression of polyLacNAc on melanoma cells and its effect on galectin-3 mediated processes.

Poly-N-acetyllactosamine (polyLacNAc) consists of repeating units of N-acetylglucosamine and galactose. The synthesis of polyLacNAc is mediated by concerted action of β1,3 N-acetylglucosaminyltransferases (GnTs) and β1,4 galactosyltransferases (GalTs). Inhibition of either of GnTs or GalTs can affect polyLacNAc synthesis. We chose GalTs for shRNA mediated downregulation as their participation in synthesis of polyLacNAc on β1,6 branched N-glycans has been reported. Further, expression of some of the GalTs including GalT-V has been shown to correlate with metastatic potential of tumor cell lines (178).

Previous work in the lab has shown that out of six GalTs, GalT-I and –V are expressed in melanoma cells in a metastatic potential dependent manner (6). The levels of GalT-VII
was not analysed as it is reported to be involved in polyLacNAc synthesis on proteoglycans. We chose a unique 22 nucleotide sequence for targeting transcripts of GalT-I and –V. The shRNA was cloned in an inducible lentiviral vector, pTRIPZ. The first major advantage of using inducible system is that it allows tight temporal control of expression for shRNA and second, the shRNA mediated effects can be rescued in absence of inducer.

3.1 Cloning of GalTshRNAmir in pTRIPZ vector, transduction and selection of positive clones

The primers containing GalTshRNA sequences along with mir, loop and restriction enzyme recognition sequences were amplified by PCR. The amplified PCR product of 120 base pairs (bp) and empty pTRIPZ vector were double digested with XhoI and EcoRI restriction enzymes, ligated and transformed into *E.Coli* DH5α cells (Figure 1A). Positive clones were confirmed by EcoRI and AgeI digestion, shRNA containing pTRIPZ vector showed insert release of 958 bp, whereas empty vector without shRNA gave release of 838 bp of vector DNA (Figure 1B).

Lentiviral particles were then generated in HEK293FT cells by co-transfecting GalT pTRIPZ or Non targeting pTRIPZ (NT) with packaging vectors (pMD2.G and psPAX2) which were further transduced in B16F10 cells (Figure 1C). One NT and two GalT pTRIPZ clones I and II were selected after puromycin treatment. Expression of NTshRNA and GalTshRNA was confirmed by observing for RFP fluorescence after induction by doxycycline for 96 hours (Figure 1D).
Figure 1: Cloning of GalTshRNA in pTRIPZ lentiviral vector. (A) Agarose gel electrophoresis for XhoI and EcoRI double digested GalTshRNA and pTRIPZ empty vector. (B) Screening of positive clones using double digestion with AgeI and EcoRI. Empty pTRIPZ vector served as control. (C) Illustration describing the procedure for preparation of lentiviral particles and transduction of F10 melanoma cells. (D) Red fluorescent protein (RFP) expressed in pTRIPZ clones on treatment with doxycycline.

3.2 Downregulation of GalT-I and -V genes in F10 cells leads to significant reduction in the expression of polyLacNAc and galectin-3 binding

Since a common shRNA was used for targeting two genes, it was necessary to analyze if mRNA levels of both GalT-I and –V genes were indeed affected in clones I and II. Using real time quantitative reverse transcriptase PCR (qRT-PCR), it was observed that upon
doxycycline induction, GalT downregulated clones I and II express reduced levels of both GalT-I and V transcripts as compared to NT cells (Figure 2A).

Western blotting of total cell lysates of the B16F10 and clones expressing NT and GalT shRNAs showed reduction in expression of polyLacNAc only in cells expressing shRNA specific for GalT-I and –V and not in cells expressing NTshRNA (Figure 2B). Flow cytometry experiments using LEA showed that GalT shRNA expression also resulted in reduced levels of polyLacNAc on the cell surface and thus possibly resulting in reduced galectin-3 binding, as evident in Figure 2C.

Reduction in binding of galectin-3 to GalT downregulated clones should inhibit their participation in galectin-3 mediated processes such as cell adhesion, spreading, matrix degradation and motility which aid organ colonization.
Figure 2: Validation of down regulation of GalT-I and –V transcripts, its effect on polyLacNAc expression and galectin-3 binding. (A) Analysis of transcript levels of beta 1,4 GalT-I and GalT-V in clones I and II, after doxycycline induction by real time PCR. NT (Non Targeting) shRNA served as the vector control. (B) Comparison of expression of polyLacNAc in untransduced F10 cells, doxycycline treated NT, Clones I and II by western blotting using biotinylated LEA. β actin served as loading control. (C) and (D) Surface levels of polyLacNAc and galectin-3 binding of NT and GalT downregulated clones was compared under doxycycline treated and non treated conditions by flow
cytometry using biotinylated LEA and galectin-3. Cells treated with only Extravidin FITC served as control.

3.3 Galectin-3 mediated cell adhesion and spreading is affected in polyLacNAc downregulated clones

Adhesion to vascular endothelium is the first major event involved in organ colonization. Cell surface molecules on tumor cells interact with their receptors expressed on organ endothelium to regulate host-tumor adhesive interactions. Our earlier results clearly suggest that galectin-3 expressed on lungs can act as a receptor for polyLacNAc expressed on melanoma cells in regulating adhesion on lung endothelium. If participation of polyLacNAc is crucial in this process then downregulation of GalTs which resulted in reduced polyLacNAc and galectin-3 binding (Figure 2C), should in turn, affect the adhesion of melanoma cells on immobilized galectin-3.

Initially, we confirmed the adhesive potential of untransduced F10 melanoma cells on immobilized galectin-3 (IM Gal-3). In comparison to cells incubated in uncoated wells, there was twofold increase in adhesion of cells on coated galectin-3 wells, confirming that galectin-3 promotes adhesion of melanoma cells (Figure 3A).

In case of polyLacNAc downregulated clones, upon induction with doxycycline, adhesion of clones I and II was affected as compared to uninduced cells. However, percent adhesion of NT cells remained unaltered in induced or uninduced state (Figure 3B). These results confirm that polyLacNAc plays a vital role in galectin-3 mediated adhesion of melanoma cells.

Cell spreading is the subsequent post adhesion event which facilitates firm anchorage of tumor cells on vascular endothelium. It not only helps in counteracting the hemodynamic forces, but also aids extravasation of tumor cells. At molecular level, in cell spreading process, cytoskeletal network is reorganized leading to formation of membrane protrusions.
like lamellipodia and filopodia which initiate assembly of adhesion complexes involving integrins and associated proteins (179,180).

Cell spreading assay was performed using Phalloidin FITC staining. Phalloidin binds to F-actin which is the key player involved in regulation of cytoskeletal reorganization. F10 melanoma cells incubated on galectin-3 coated coverslips typically have flattened morphology and they show lamellipodial like projections (Figure 4A, IM gal-3). Cells incubated on uncoated coverslips do not show such membrane protrusions and these cells have rounded morphology (Figure 4A, Un). Changes in cellular morphology were quantitated by calculating the ratio of cytoplasmic area to nuclear area (C/N ratio) of spreaded cells. C/N ratio of F10 cells spread on galectin-3 was significantly higher (approximately three fold) than that of cells spread on uncoated coverslips (Figure 4B).

Effect of downregulation of polyLacNAc on galectin-3 mediated cell spreading was analyzed in GalT downregulated clones. Results demonstrate that on doxycycline induction, clones I and II show absence of typical galectin-3 induced membrane protrusions, which are clearly visible in uninduced cells. NT cells did not show any change in morphology in induced or uninduced state (Figure 4C). Comparison of C/N ratio between NT and GalT downregulated clones spread on galectin-3 confirm that reduction of polyLacNAc levels affects galectin-3 mediated spreading of melanoma cells (Figure 4D).
Figure 3: Reduced polyLacNAc levels in GalT downregulated clones affect galectin-3 mediated adhesion and spreading. (A) Bar graph representing adhesion of F10 cells on 96-well plates coated with 50 µg/ml of galectin-3 (Immobilized galectin-3-IM Gal-3). Adhesion on galectin-3 was taken as 100%. Cells incubated in uncoated wells served as control. (B) Adhesion of clones I, II and NT in presence or absence of doxycycline on galectin-3 coated wells. Values are mean ± standard error of three independent experiments. Unpaired student’s t-test was applied for statistical analysis and p value > 0.05 was considered significant.
Figure 4: Downregulation of polyLacNAc affected spreading of F10 cells on immobilized galectin-3.

(A) Comparison of cell spreading of F10 cells on uncoated or galectin-3 coated coverslips by F actin staining (green) with Phalloidin FITC using laser confocal microscopy at 63x magnification. DAPI was used to stain nuclei (blue). Scale bar = 10 μm. (B) Bar graph represents quantitation of cell spreading by determining the mean ratio of cytoplasmic to nuclear area of around 100 cells from three different experiments. (C) Spreading of NT, Clones I and II on galectin-3 coated coverslips in presence or absence of doxycycline. (D) Bar graph represents ratio of cytoplasmic to nuclear (C/N) area for around 100 cells of
NT, Clone and II from three independent experiments. Unpaired students t-test was employed for statistical analysis and p value < 0.05 was considered significant.

3.4 Reduced levels of polyLacNAc in GalT downregulated clones affect galectin-3 induced matrix degradation, cell motility as well as the metastatic potential

For gaining access into the organ parenchyma, tumor cells need to displace the organ endothelium, degrade the underlying basement membrane (BM) and create space for movement. The key players involved in matrix degradation are matrix metalloproteinases (MMPs) especially members of gelatinases family, MMP2 and MMP9. These MMPs are involved in degradation of different types of collagens (I, IV, V, VII, etc.), non collagenous proteins including fibronectin, vitronectin, laminin and protein part of proteoglycans (43,46). Previous work in the lab has shown that extracellular galectin-3 in both immobilized as well as soluble form induces secretion of MMP9 in melanoma cells. Further, the levels of secretion of MMP9 correlated with metastatic potential of melanoma cells which also differ in expression of polyLacNAc on β1,6 branched N-oligosaccharides (6). The role of polyLacNAc in induction of galectin-3 mediated MMP9 secretion can be confirmed using polyLacNAc downregulated melanoma cells.

Before analyzing the levels of MMP9 in polyLacNAc downregulated cells, we first confirmed the induction of MMP9 in culture supernatant of F10 cells by both zymography as well as Western blotting. As compared to cells grown on uncoated plates, higher levels of MMP9 were secreted in culture supernatant collected from cells grown on coated galectin-3 plates (Figure 5A and B, F10). Densitometric analysis further confirmed this observation. Gelatin zymography was performed to compare the levels of MMP9 in culture supernatants of induced and uninduced clones grown on immobilized galectin-3. The levels of MMP9 in NT clone was unaltered in either doxycycline treated or untreated
condition. In case of GalT downregulated clones I and II, expression of shRNA significantly affected the secretion of MMP9 (Figure 5A). This was also confirmed by immunoblotting using anti MMP9 antibody (Figure 5B). These results emphasize that polyLacNAc expression on melanoma cells is crucial for galectin-3 induced MMP9 expression.

After degradation of vascular basement membrane and the matrix for extravasation, cells move into the organ parenchyma where they can proliferate and form metastatic colony. Tumor cells can use matrix proteins as traction for motility. Galectin-3 has been known to be incorporated in ECM/BM (155,181). Immobilized galectin-3, as a component of ECM, can facilitate movement of tumor cells.

Wound healing assays using time lapse video microscopy showed that motility of F10 cells on immobilized galectin-3 was significantly higher than that on BSA (Figure 6A). Reduced expression of polyLacNAc in GalT downregulated clones affected the percent wound closure after induction with doxycycline (Figure 6C and D). The motility of NT cells showed no alteration either in presence or absence of doxycycline (Figure 6B). The effect of downregulation of polyLacNAc on metastasis associated events including cell adhesion, spreading, degradation of matrix and motility should ultimately affect the metastatic potential. Experimental metastasis assay was performed to compare the metastastic ability of untreated F10 cells and doxycycline treated NT, Clones I and II. The number of colonies on lungs of mice injected with F10 cells or NT cells were comparable while those injected with GaIT downregulated clones showed significantly reduced number of metastatic colonies (Figure 7A and B).

These results altogether conclude that polyLacNAc substituted β1,6 branched N-glycans on melanoma cells not only aid arrest, but also mediate other events of extravasation including spreading, matrix degradation, motility and ultimately metastasis.
Figure 5: PolyLacNAc downregulation affects galectin-3 mediated secretion of MMP9 (A) Levels of MMP9 in culture supernatants of F10 cells grown on uncoated or
coated galectin-3 plates, NT, Clone I and II grown in presence and absence of doxycycline on coated galectin-3 as detected by zymography and (B) Western blotting. The adjacent right panel of (A) and (B) represent densitometry analysis for bands observed in zymography and western blotting.
Figure 6: Reduction in expression of polyLacNAc results in reduced motility of GalT downregulated clones on galectin-3. (A) Represent time lapse video microscopy images at 0 and 20 h of wound closure of F10 cells grown on BSA or galectin-3 coated plates. (B) Represent time lapse images of NT, Clone I and II under doxycycline treated and untreated conditions. Adjacent right panel represents mean percent wound closure at 5 h interval. Area of wound closure was measured by Image J software and each image from two different experiments, was analyzed at three different positions. For comparison of wound closure between the groups two way ANOVA test with the Bonferroni posttest was conducted * indicates p value < 0.05 which was considered significant.

Figure 7: Reduced levels of polyLacNAc in GalT downregulated clones affect their metastastic potential. Experimental metastasis assay for NT and GalT downregulated clones I and II. Untreated F10 cells served as control. The left panel shows the lungs images while right panel is the graphical representation of the mean +/- standard error of mean number of lung colonies from three independent experiments. For statistical
analysis, unpaired students t-test was employed, mean numbers of colonies were compared between two groups and p value < 0.05 was considered significant.

The importance of polyLacNAc galectin-3 interaction in metastasis of melanoma cells has also been underscored by other experiments in the lab. For e.g. competitive inhibition of endogenous galectin-3 by incubating melanoma cells with truncated galectin-3 (containing only carbohydrate recognition domain of galectin-3) or feeding mice with modified citrus pectin (MCP) not only affected the galectin-3 mediated cellular processes but also affected metastasis (6).

Overall these findings strongly point towards the role of both galectin-3 on the target organ and polyLacNAc expressed on melanoma cells in organ homing. Further, it is plausible that galectin-3 binding/polyLacNAc carrying proteins would play a major role in mediating events associated with metastasis of melanoma cells. These proteins would act as upstream regulators to initiate the downstream signalling events which would then modulate metastasis associated processes. It is thus important to identify the galectin-3 binding proteins that carry polyLacNAc on N-glycans. Previously, only LAMP1 and β1 integrin on melanoma cells have been identified as polyLacNAc carriers, but other proteins carrying such modifications should be identified and their role in metastasis associated events should be addressed.
Objective II: To identify the galectin-3 binding proteins carrying polyLacNAc on β1,6 branched N-oligosaccharides and to study their involvement in regulation of galectin-3 mediated processes.

For identification of glycoproteins, lectin affinity enrichment is the primary step. Lectins not only recognize carbohydrate structures but also differentiate them with respect to subtle variations in their composition. Sepharose conjugated lectins are generally used for enrichment of glycoproteins from cell or tissue lysates. Enriched proteins can then be identified using mass spectrometry [182,183].

Galectin-3 is a member of family of β-galactoside binding proteins. Through carbohydrate recognition domain (CRD), it recognizes galactose containing glycans with differential affinity. PolyLacNAc consisting of repeats of galactose and N-acetylglucosamine residues is the preferred ligand for galectin-3. Depending on the number of polyLacNAc units, its affinity for galectin-3 varies, longer units bind more strongly as compared to shorter ones [149] (Illustration 6A). PolyLacNAc expressed on both N- as well as O-glycans can serve as ligands for galectin-3 [184]. Apart from recognizing polyLacNAc, galectin-3 also binds to ligands known as Thomsen Friedenreich (T/Tn) antigens with weak affinity [185].

Galectin-3 affinity chromatography can be used for purification of polyLacNAc carrying proteins, but considering the broader affinity of galectin-3, it would be practically difficult to selectively purify proteins carrying polyLacNAc only on β1,6 branched N-glycans using this approach. Alternatively, Leuco-Phyto haemagglutinin (L-PHA), a lectin which specifically recognizes β1,6 branched N-glycans (Illustration 6B), also can be used for such purification. β1,6 branch on N-glycans is the preferred site for addition of terminal substitutions such as polyLacNAc, sialic acids, Lewis antigens and fucose residues. Since purification by this approach would also lead to copurification of other proteins which carry terminal substitutions apart from polyLacNAc, this method would also be...
insufficient to selectively enrich proteins carrying polyLacNAc on β1,6 branched N-glycans. Therefore using the above two approaches in tandem appears to be the best possible strategy to purify such proteins.

Illustration 6: (A) Galectin-3 specific ligands expressed on N- and O- glycans and (B) L-PHA and galectin-3 binding sites on β1,6 branched N- glycans modified from (186).

3.5 Strategy to purify and identify galectin-3 binding proteins carrying polyLacNAc substitutions on β1,6 branched N-oligosaccharides

Considering the above facts, a serial lectin affinity chromatography approach was employed. In the first step, galectin-3 binding proteins were purified and in the subsequent step, proteins carrying β1,6 branched N-glycans were enriched. Though this strategy had advantage of enriching proteins with glycan modifications of our interest, the total yield of proteins obtained after purification was insufficient for analysis by mass spectrometry. Hence, galectin-3 and L-PHA binding proteins were independently purified and subjected to mass spectrometry.

To enrich membrane associated glycoproteins, F10 cell lysates were prepared in buffer containing N-octyl β-D galactopyranoside (30 mM) which as a non ionic mild detergent
not only aided extraction of membrane proteins but was also compatible with lectin affinity purification. For glycoprotein enrichment, F10 melanoma cell lysate (25 mg) was incubated overnight with galectin-3 or L-PHA sepharose beads. Unbound protein fraction was collected and non specifically bound proteins were removed by repeated washes with Tris buffer containing 1 M NaCl. Proteins bound to galectin-3 and L-PHA sepharose beads were eluted with buffer containing either lactose (150 mM) or N-acetylgalactosamine (300 mM), respectively. After volume normalisation with input/original, 200 μg equivalent protein from eluted fractions was resolved by 1-D gel electrophoresis and protein profile was visualised by coomassie blue staining (Figure 9). Observed bands were cut and processed for sample preparation required for mass spectrometry analysis. The workflow in Figure 8 briefly summarises the overall methodology used for purification and identification of glycoproteins.

3.6 Deglycosylation of glycopeptides and identification using nano LC-ESI-Q-TOF MS/MS

Identification of glycoproteins is difficult due to several reasons including low abundance, high heterogeneity and poor ionisation of glycopeptides. Therefore, to avoid interference of glycans in glycoprotein identification, deglycosylation strategies are preferred. Enzymatic deglycosylation methods are widely used as compared to chemical deglycosylation as former are more selective and can preserve peptide backbone more efficiently than the later.

N-glycosidase F also called as PNGase F deglycosylates proteins or peptides by cleaving the glycosidic bond between Asparagine and innermost N-acetylglucosamine on hybrid, oligomannose and complex type N-glycans. However, it cannot cleave a subset of N-glycans modified with α1,3 fucose at the innermost N-acetylglucosamine (187). This modification is majorly found in plants and insects and therefore this would not serve as a
barrier in deglycosylation of proteins from mammalian sources (105). After PNGase F treatment, N-glycans are released and asparagine is converted to aspartic acid (Figure 8B) with shift in monoisotopic mass by +1 Da. This shift in the molecular mass is readily detected by mass spectrometry and is used for annotating N-glycosylation sites (8).

Earlier, an attempt was made to standardise deglycosylation conditions at protein level by in-gel as well as in-solution PNGase F treatment. We found that deglycosylation by in-solution PNGase F treatment was incomplete, probably due to the heterogeneity as well as complexity of the sample; also steric hindrance by large glycoproteins can restrict the access of PNGase F to N-glycosylation sites. In-gel deglycosylation methods also did not help in identification of proteins.

Therefore, protocol for deglycosylation at peptide level was standardised. Peptides generated after trypsin digestion were subjected to PNGase F treatment. Deglycosylated peptides were passed through C-18 columns and then subjected for analysis using nano LC-ESI-Q-TOF MS/MS by Synapt, Waters. Protein Lynx Global Server (PLGS) software was used for post acquisition analysis.
Figure 8: Workflow for purification of galectin-3 binding proteins and deglycosylation of peptides by PNGase F treatment. (A) Summary of steps involved in purification of galectin-3 and L-PHA binding proteins. (B) Illustration representing action of PNGase F on glycopeptides carrying N-glycans.
Table 5 and 6 briefly summarises the results obtained by mass spectrometry analysis. Protein identities for all the processed bands were obtained with p value > 0.05. In total, 97 glycoproteins were identified, out of which 51 proteins were identified as polyLacNAc carrying proteins (galectin-3 binding proteins) and 79 proteins were identified as β1,6 branched N-glycans carrying proteins (L-PHA binding proteins). Representative fragmentation data for some of the annotated glycopeptides in Table 5 and 6 is shown in Figure 11 and 12.
After comparative analysis, 33 proteins were found to have affinity for both galectin-3 as well as L-PHA. Therefore, it is possible that these proteins might carry both polyLacNAc as well as β1,6 branched N-glycans. Some of the identified proteins such as integrins (α5 and β1) and LAMPs (LAMP1, -2 and -3) are reported to be substituted with β1,6 branched N-glycans.

Further, in many cases as shown in Table 7, even the annotated N-glycosylation sites for individual proteins were common which strongly point towards the possibility that these sites most likely carry polyLacNAc substituted β1,6 branched N-glycans (For e.g. LAMP1 identified by both the approaches shared 9 modified N-glycosylation sites). Basigin, a protein belonging to immunoglobulin family, has been identified as L-PHA binding protein. Further, it has been recently reported that addition of β1,6 branch on basigin modulates its cell surface localization and function (188,189). Thus, Asn-160 on basigin which is reported to carry by N-glycans (190,191) and identified as a common glycosylation site in our study (Table 7) is highly likely to be modified with polyLacNAc substituted β1,6 branched N-glycans. Embigin, another member of immunoglobulin family which has been reported to be N-glycosylated, was also identified and the reported glycosylation sites (N-55, 216 and 221) also matched with our data (190,191). We believe that even for rest of the identified proteins, it is highly likely that detected glycosylation sites could be modified with polyLacNAc on β1,6 branched N-glycans.
Table 5: Summary of identified galectin-3 binding proteins

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<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Molecular weight</th>
<th>Sequence Coverage (%)</th>
<th>Number of glycosylated peptides</th>
<th>Amino acids modified</th>
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<tr>
<td>Integrin alpha 5</td>
<td>P43406</td>
<td>114971</td>
<td>15.57</td>
<td>3</td>
<td>472,581,715, 727, 185, 678</td>
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<td>Integrin beta 1</td>
<td>P09055</td>
<td>88173</td>
<td>19.17</td>
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<td>43837</td>
<td>47</td>
<td>20</td>
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<td>LAMP2</td>
<td>P17047</td>
<td>45652</td>
<td>47</td>
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<td>L-dopachrome tautomerase</td>
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<td>58473</td>
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Figure 10: Representation of glycopeptide fragmentation pattern of identified galectin-3 binding proteins

Lysosome associated membrane protein-1 (LAMP1)

Basigin

Embigin
Integrum alpha 5  

Integrum beta 1  

Transmembrane glycoprotein NMB  

Sulfated glycoprotein-1
Table 6: Summary of identified L-PHA binding proteins

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Accession number</th>
<th>Molecular weight</th>
<th>Sequence Coverage (%)</th>
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<td>Cell surface glycoprotein MUC18</td>
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<td>71500</td>
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<td>Nicastrin</td>
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<td>78442</td>
<td>40.4</td>
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<td>529, 530, 611, 434</td>
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<td>Transmembrane glycoprotein NMB</td>
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<td>63635</td>
<td>37.8</td>
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<tr>
<td>Cell adhesion molecule 1</td>
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<td>Basigin</td>
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<td>Embigin</td>
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<tr>
<td>5, 6 dihydroxyindole 2 carboxylic acid</td>
<td>P07147</td>
<td>60722</td>
<td>62.2</td>
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<tr>
<td>L dopachrome tautomerase</td>
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<td>58473</td>
<td>50.0967</td>
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<td>15.6</td>
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<td>51578</td>
<td>37</td>
<td>1</td>
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<tr>
<td>Sulfated glycoprotein -1</td>
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<td>61381</td>
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</table>
Figure 11: Representation of glycopeptide fragmentation pattern of identified L-PHA binding proteins

**LAMP1**  
LNMTLPDALVPTFSISNHSLK

**LAMP2**  
CNSVLTYNLTPVVQK

**Integrin beta 1**  
SAVGTLSDGNSSNVQILIDAYNLSSEVILENSK
**Results**

**Cell adhesion molecule 1**

**Transmembrane glycoprotein NMB**

**Cell surface glycoprotein MUC18**

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**FQLLNFSSSELK**

**DLPVFVDVLIHDPSHFLNDSAISYK**

**ASGNFSQVDWFLIHK**
Table 7: Comparison of modified N-glycosylation sites of identified proteins

<table>
<thead>
<tr>
<th>Glycoproteins carrying β1,6 branched N-glycans and polyLacNAc</th>
<th>N-glycosylation sites modified only with polyLacNAc (blue) or β1,6 branched N-glycans (green) or both (red)</th>
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</thead>
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<tr>
<td>Integrin β1</td>
<td>212, 571, 669, 363</td>
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<tr>
<td>Integrin α5</td>
<td>472, 581, 185, 678, 715, 727</td>
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<tr>
<td>Basigin</td>
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<tr>
<td>Embigin</td>
<td>55, 118, 216, 221, 253, 270</td>
</tr>
<tr>
<td>Cell adhesion molecule 1</td>
<td>104, 116</td>
</tr>
<tr>
<td>LAMP1</td>
<td>70, 78, 97, 101, 159, 177, 252, 296, 311</td>
</tr>
<tr>
<td>LAMP2</td>
<td>115, 156, 161, 175, 265, 312, 322, 361</td>
</tr>
<tr>
<td>LAMP3</td>
<td>130, 143, 172</td>
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<tr>
<td>Transmembrane protein NMB</td>
<td>94, 134, 197, 200, 246, 249, 275</td>
</tr>
<tr>
<td>L dopachrome tautomerase</td>
<td>230, 237, 242, 246, 300, 342</td>
</tr>
<tr>
<td>5, 6 dihydroxyindole 2 carboxylic acid</td>
<td>132, 175, 181, 256, 280, 304, 350</td>
</tr>
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<td>MUC 18</td>
<td>58, 167, 304</td>
</tr>
<tr>
<td>Nicastrin</td>
<td>44, 434, 529, 530, 461, 611</td>
</tr>
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</table>
3.7 Validation of identified proteins by immunoblotting and their functional involvement in one of the galectin-3 mediated processes

Identities of some of the proteins including LAMP1, LAMP3, α5 and β1 integrin, Basigin and Embigin were confirmed by immunoblotting. Galectin-3 affinity enriched fraction indeed showed presence of these proteins. Proteins from F10 lysate probed with respective antibodies served as positive control (Figure 13).

![Figure 12: Validation of identified galectin-3 binding proteins using immunoblotting](image)

Detection of integrins (α5 and β1), LAMPs (LAMP1 and 3), Basigin and Embigin in F10 cell lysate (100 μg) and galectin-3 enriched fraction (100 μg equivalent protein) using western blotting.

Earlier, LAMP1 and β1 integrin were identified as galectin-3 binding proteins and it was also shown that blocking these proteins using specific antibodies, affected galectin-3 mediated adhesion of melanoma cells. Here, using a similar strategy, we have looked at the effect of blocking galectin-3 binding proteins on cell spreading which is another event associated with metastasis. Apart from using antibodies to block LAMP1 and β1 integrin,
we have used antibody against another protein, basigin, which has been reported to be associated with galectin-3 and integrins at the cell surface. Blocking LAMP1 and β1 integrin affected formation of typical membrane protrusions which were seen when cells were incubated with coverslips coated with galectin-3. However, quantitatively the maximum inhibition was seen in cells treated with basigin antibody (Figure 13A and B). Cells treated with non specific Rat IgG antibody served as a control.

Figure 13: Role of galectin-3 binding proteins in cell spreading of melanoma cells on galectin-3 (A) Cell spreading assay of F10 cells incubated on galectin-3 coated coverslips in presence of antibodies to LAMP1, β1 integrin and Basigin. Cells incubated with Rat
IgG served as control. All antibodies were used at 10 μg/ml concentration. (B) Quantitation of cell spreading results by calculating the ratio of cytoplasmic to nuclear (C/N) area for around 100 cells from two different experiments. Unpaired students t-test was employed for statistical analysis and p value < 0.05 was considered significant.

In summary, we have successfully identified and validated galectin-3 binding proteins which carry polyLacNAc on β1,6 branched N-glycans. Inhibition of some of the identified proteins including LAMP1, β1 Integrin and Basigin affected galectin-3 mediated cell spreading, which is an important event in extravasation of tumor cells. The role of these proteins in regulating galectin-3 mediated processes should be studied at cellular signalling level which would be the final confirmation for function of these proteins in metastatic events.
Objective III: To elucidate the downstream signalling events of galectin-3 induced MMP9 secretion and involvement of identified proteins in this process.

Galectin-3 is a nucleo-cytoplasmic protein that also gets secreted in a non-classical manner. The secreted galectin-3 can get associated with the cell surface or can become a part of the ECM and BM. Earlier; we have shown that both forms of extracellular galectin-3 i.e. soluble and immobilized galectin-3 induce MMP9 secretion. Induction of MMP9 expression was a dose dependent effect, maximum induction observed when F10 melanoma cells were grown in presence of 50 μg/ml of immobilized galectin-3. Cells grown on plastic alone served as negative control while cells grown on immobilized fibronectin served as positive control (Figure 14).

![Zymography gel](image)

Figure 14: Zymography gel showing levels of MMP9 in culture supernatants of F10 cells grown in presence of different concentrations of immobilized galectin-3 (10-50 μg/ml). Mice blood mixed with 1X non reduced sample buffer served as control for gelatin gel.

Though it is known that intracellular galectin-3 can regulate the expression of MMP9, role of extracellular galectin-3 in regulating MMP9 expression has not been explored. Thus
exact mechanism of galectin-3 induced MMP9 secretion including the role of galetin-3 binding proteins and downstream signalling pathways was explored.

Matrix metalloproteinases (MMPs), especially gelatinases, MMP2 and MMP9 are the key molecules involved in cancer cell invasion and tissue remodelling. MMPs are tightly regulated at multiple levels as it may impact tissue integrity. Multiple mechanisms have evolved to regulate the action of MMPs. It can be at the level of its transcription, expression, secretion, activation of pro form to active form, localization at the invading front and interaction with its tissue inhibitors (192). The primary regulation is at the mRNA level and under normal physiological conditions; only basal levels of MMP transcripts are produced. In response to external stimuli such as growth factors, cytokines and extracellular matrix (ECM) components, the expression of MMPs is upregulated (193).

3.8 Extracellular galectin-3 induces MMP9 expression at mRNA level through p38 MAPK pathway

The levels of transcripts for MMP9 in cells grown on uncoated plastic plates were compared to those grown on fibronectin or galectin-3 coated plates by real time PCR. Cells grown on immobilized galectin-3 expressed significantly increased levels of MMP9 transcripts as compared to cells grown on plastic alone. Fibronectin, a well studied ECM protein and a known inducer of MMP9 secretion (9,194), served as a positive control (Figure 15A). These results suggest that galectin-3 in extracellular form can indeed induce the transcription of MMP9.

ECM proteins regulate the cellular signalling pathways involved in matrix remodelling by interacting with their receptors on the cell surface to initiate outside in signalling and vice versa (195). In response to external stimuli, cellular signalling pathways are activated which then modulate the transcription of MMPs. ECM proteins such as fibronectin and
osteopontin are known to activate MMP9 expression through either ERK, PI3K or NF-κB pathways. To understand which downstream pathways are activated by extracellular galectin-3, inhibitors for PI3K and MAPK (ERK, p38 MAPK and JNK) pathways were used.

Comparison of transcript levels of MMP9 in cells treated with different signalling inhibitors showed that inhibition p38 MAPK pathway had major effect on transcript levels of MMP9 (Figure 15B). Cells grown on galectin-3 coated plates treated with vehicle alone served as control. These results were further confirmed upon evaluation of the conditioned medium collected from cells grown on galectin-3 coated plates in absence or presence of different signalling inhibitors for the presence of MMP9. Both zymography and immunoblotting confirmed that in comparison to cells grown in presence of ERK, PI3K and JNK inhibitors, cells treated with p38 MAPK inhibitor (SB) showed maximum reduction in MMP9 levels (Figure 16).

![Figure 15](image)

**Figure 15:** Immobilized galectin-3 induces expression of MMP9 at transcript level and inhibition of p38 MAPK affects induction of MMP9 expression.

(A) Bar graph represents comparison of levels of MMP9 transcripts by real time PCR in F10 cells grown on immobilized galectin-3 or on plastic alone. Error bars indicate standard errors of mean from three independent experiments performed in duplicates. (B)
Detection of MMP9 transcript levels by real time PCR in F10 cells grown on immobilized galectin-3 in presence of signalling inhibitors, PI3K (Wortmannin-WM, 100 nM and 500 nM), ERK (PD, 10 µM and 50 µM), p38 MAPK (SB, 10 µM and 50 µM) and JNK (5 µM and 25 µM). Cells grown on immobilized galectin-3 in presence of vehicle served as control. For statistical analysis, unpaired student’s t-test was employed and p value < 0.05 was considered significant.

Figure 16: Inhibition of p38 MAPK affects galectin-3 mediated MMP9 secretion at protein levels.

(A) Western blotting of culture supernatants collected from F10 cells grown in presence of signalling inhibitors probed with anti MMP9 antibody. (B) Densitometric analysis of bands in (A) by Image J analysis. Each bar represents intensity of bands in respective lanes and error bars represent standard errors of mean from three different experiments.
(C) Zymography of culture supernatants collected from F10 cells grown in presence of signalling inhibitors. (D) Densitometric analysis of bands in (C) by Image J software using Each bar represents intensity of bands in respective lanes and error bars represent standard errors of mean from three different experiments. Unpaired student’s t-test was employed for statistical analysis and p value < 0.05 was considered significant.

3.9 Downregulation of polyLacNAc affects galectin-3 induced MMP9 secretion through p38 MAPK pathway

Down regulating the enzymes involved in polyLacNAc synthesis not only affected binding of galectin-3 (Figure 2C) but also affected galectin-3 induced MMP9 induction as assessed by gelatin zymography and immunoblotting (Figure 5). Since galectin-3 induces MMP9 at transcript level (Figure 16A) therefore it was important to understand if high affinity galectin-3 ligand, polyLacNAc is involved in regulating MMP9 expression at transcript level.

We have used inducible lentiviral shRNA clones of F10 cells in which genes for the enzymes involved in polyLacNAc synthesis (GalT-I and -V) were downregulated (Figure 2). Induction of GalT shRNA lead to reduction in levels of MMP9 transcripts which clearly suggests that polyLacNAc is indeed involved in mediating induction of MMP9 at mRNA level (Figure 17A).

If inhibition of p38 MAPK pathway affected MMP9 induction (Figure 17), then it would be interesting to investigate if the activation of the same pathway is hampered in polyLacNAc downregulated clones. Cell lysates of these clones (untreated or treated with doxycycline for inducing shRNA expression) were probed with phospho-specific antibody for p38 MAPK. Lysates probed with antibody for detecting total levels of p38 MAPK served as loading control. Both the clones upon doxycycline induction showed
significantly reduced levels of phospho-p38 MAPK (Figure 17B and C), suggesting that polyLacNAc also signals galectin-3 mediated processes via this pathway.

Figure 17: Downregulation of polyLacNAc affects MMP9 induction via activation of p38 MAPK pathway (A) Bar graph represents analysis of MMP9 transcript levels in polyLacNAc downregulated clones I and II grown on immobilized galectin-3 in doxycycline treated and untreated conditions (-D and +D respectively). Non targeting clones in presence or absence of doxycycline (NT-D and NT+D) served as controls served as vector controls. Error bars indicate standard errors of mean from three independent experiments performed in duplicates. (B) Western blotting for detection of phospho-specific forms of p38 in NT, clone I and clone II (-D and +D) grown on immobilized galectin-3. Total levels of p38 served as loading control. (C) Densitometric analysis of
bands in (B) by Image J software. Each bar represents ratio of intensity of phospho-p38 to total p38 levels in respective lanes and error bars represent standard errors of mean from three different experiments. Unpaired student’s t-test was employed for statistical analysis and p value <0.05 is considered to be statistically significant.

3.10 LAMP1 identified as a major carrier of polyLacNAc can regulate galectin-3 induced MMP9 secretion

LAMP1 and β1 integrin are among the major proteins identified to carry polyLacNAc substituted β1,6 branched N-oligosaccharides on these melanoma cells. LAMP1 is a highly glycosylated protein that lines the lysosomes (196). More than 60% of its weight is contributed by carbohydrates and each molecule carries about 17-20 N-glycans that are highly substituted (196,197). In metastatic cells, LAMP1 is known to get translocated to the cell surface. The extent of its surface expression and the levels of polyLacNAc on N-glycans have been shown to correlate with the metastatic potential of melanoma cells. In addition, glycosylation in these cells has been shown to modulate the surface expression of LAMP1(198). LAMP1 is reportedly a known ligand for galectin-3 (199,200). Recently, downregulation of LAMP1 expression has been shown to significantly affect its surface expression, as well as the spreading and motility on galectin-3 and experimental metastasis of F10 cells (198,201). It is thus possible that LAMP1 can be involved in galectin-3 mediated signalling that induces MMP9 transcription and secretion.

To investigate the role of LAMP1 in MMP9 expression, melanoma cells expressing shRNAs for LAMP1 were used. Real time PCR results show that downregulation of LAMP1 in F10 cells (Sh1 and Sh2) severely affects galectin-3 induced transcription of MMP9 (Figure 18A). This is further reflected in significantly reduced secretion of MMP9 by LAMP1 downregulated clones grown on galectin-3, as analyzed by both
immunoblotting and zymography (Figure 18B-D) suggesting that LAMP1 can be an upstream regulator in MMP9 induction. This was corroborated by decreased levels of activated (phospho) p38 MAPK in the lysates of LAMP1 downregulated clones (Figure 19E and F).

Figure 18: Downregulation of LAMP1 affects galectin-3 induced MMP9 expression via p38 MAPK pathway. (A) Bar graph represents analysis of levels of MMP9 transcripts by real time PCR for NT and LAMP1 downregulated clones Sh1 and Sh2 (-D and +D). Error bars indicate standard errors of mean from three independent experiments performed in duplicates. (B) Western blotted culture supernatants of NT, Sh1 and Sh2 clones probed with anti-MMP9 antibody. (C) Levels of MMP9 in culture supernatants of NT, Sh1 and Sh2 cells grown in presence and absence of doxycycline on immobilized
galectin-3 as detected by zymography. (D) Densitometric analysis of bands in (C) by Image J software. Each bar represents intensity of bands in respective lanes and error bars represent standard errors of mean from three different experiments. (E) Detection of phospho-specific forms of p38 in NT, Sh1 and Sh2 clones (-D and +D) grown on immobilized galectin-3. (F) Densitometric analysis of bands in (E) by Image J software. Each bar represents ratio of intensity of phospho-p38 to total p38 levels in respective lanes and error bars represent standard errors of mean from three different experiments. Unpaired student’s t-test was employed for statistical analysis and p value <0.05 is considered to be statistically significant.