FUNCTIONAL ROLE FOR THE MANNOSE PHOSPHORYLATION ON TWO NON-LYSOSOMAL PROTEINS

by

JARROD WESLEY BARNES

(Under the Direction of Richard Steet)

ABSTRACT

The mannose-6-phosphate (Man-6-P) targeting pathway represents one of the best-studied examples of the ability of carbohydrates to carry information. Acid hydrolases that are modified with Man-6-P residues on their N-glycans are targeted to the lysosomes by Man-6-P receptors. In addition to these enzymes, many non-lysosomal proteins have been shown to bear phosphomannosyl residues. The functional relevance of the mannose phosphorylation on non-lysosomal proteins, however, remains poorly defined. More importantly, the pathogenic consequences associated with the impaired mannose phosphorylation of these proteins in the lysosomal storage disease mucolipidosis II (ML-II) have not been determined. In the present work, the extent and functional role of mannose phosphorylation on two non-lysosomal proteins, leukemia inhibitory factor (LIF) and transforming growth factor beta 1 (TGF-β1), was investigated using biochemical approaches. Secreted LIF was demonstrated to be highly Man-6-P modified by cation-independent Man-6-P receptor (CI-MPR) affinity chromatography. Removal of specific N-glycan sites on LIF resulted in decreased mannose phosphorylation and a corresponding increase in its steady-state extracellular levels. Subsequent experiments showed that LIF

mannose phosphorylation controls its extracellular levels by means of Man-6-P-dependent internalization and direct intracellular targeting of this molecule to the lysosomes. Loss of mannose phosphorylation in ML-II likely leads to excessive extracellular LIF, providing a potential mechanism for the bone phenotypes associated with this disease. Contrary to our findings with LIF, latent TGF-β1 was demonstrated to be poorly mannose phosphorylated and its activation was not altered by free Man-6-P addition when latent forms were present. These results indicate that latent TGF-β1 activation is not mediated by direct Man-6-P modification of its latency-associated peptide. Although loss of mannose phosphorylation in ML-II was not predicted to alter TGF-β1 activation, decreased signaling of this growth factor, and a corresponding reduction in wound closure, was surprisingly noted in human and feline ML-II fibroblasts. Diminished activation of latent TGF-β1 was further associated with a striking decrease in its detergent solubility, possibly due to upregulation of tissue transglutaminase. Collectively, these results indicate that the loss of Man-6-P biosynthesis on non-lysosomal proteins may directly (LIF) or indirectly (TGF-β1) affect their function and contribute to the pathogenesis of ML-II.

INDEX WORDS: N-linked glycosylation, mannose-6-phosphate, non-lysosomal protein, leukemia inhibitory factor, transforming growth factor beta, transglutaminase 2, mucolipidosis II

FUNCTIONAL ROLE FOR THE MANNOSE PHOSPHORYLATION ON TWO NON-LYSOSOMAL PROTEINS

by

JARROD WESLEY BARNES

B.S., University of Georgia, 2001

M.S., University of Georgia, 2004

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2011

FUNCTIONAL ROLE FOR THE MANNOSE PHOSPHORYLATION OF TWO NON-LYSOSOMAL PROTEINS

by

JARROD WESLEY BARNES

Major Professor: Richard Steet

Committee: Kelley Moremen
Michael Tiemeyer
Walter Schmidt

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2011

DEDICATION

First, I would like to dedicate this thesis as well as my entire graduate studies to everyone who wears the label "SMALL TOWN," especially those individuals residing in Armuchee, GA. I realize now more than ever how hard it is to achieve great things. You have to put your heart and soul into every aspect of your goals and aspirations. Not to mention, fight the stigma associated with being from the rural south. I just want to say, "FINALLY!!!!!"

Secondly, I would like to dedicate this present body of work and any future work to my baby girls, both of whom are my pride and joy. My goal for coming back to finish my PhD after obtaining my Master's degree was to create a better life for the both of you. As I dedicate this thesis to you (which is a symbol of great things to come), I promise to continue to give you the best opportunity, the best support and the greatest guidance I know how. Lastly, I dedicate this thesis to the future scientists that I may have the pleasure of training, working with, or mentoring. I promise that I will give to you, what my mentors have given me – PASSION and OBSESSION!!!!

ACKNOWLEDGEMENTS

The pathway to achieving any goal is always marked with many who mentor and teach you along the journey. I would like to start by thanking those who initialized my journey and end with those special people who have defined my career as a scientist. I would first like to thank Dr. Carl Bergman for all the guidance and support throughout the years. You saw the scientific potential in me that I did not even understand or know that I had. Thanks for all you have done for my career and my family. Secondly, I would like to extend my gratitude my committee members Dr. Michael Tiemeyer, Dr. Kelley Moremen, and Dr. Schmidt for all the guidance and advice on my projects. All of you have contributed significantly to my maturation and scientific development. In addition, I would like to thank Dr. Lance Wells for being an honorary committee member. You have been a big influence in my scientific development as well.

I would like to thank all the Steet lab members for the fruitful discussions and fun times. You guys have become like family to me. To Aaron, I leave you my cubby. It's all yours. No, no, seriously you can have it all to yourself. To Abigail, I leave you my Western blot capabilities. In all seriousness, I appreciate the lab environment that we created. Good luck in all your future accomplishments.

To Rich and Heather, thanks so much for giving me a chance to work in your lab. You guys took a real chance on me when I came back here to finish my PhD. Remember, it all started with exposing a whole box of developer's film and developing a piece of cardboard!!! Now, I laugh at those who do such things. Sincerely, though, I would say my stint here has been

very productive. In general, you guys have contributed so much to my scientific career and I will be forever grateful to you. Both of you have given me confidence to proceed forward into other scientific endeavors.

To Heather, thanks for all the advice, personal and scientifically. It has been great working with you. I will be forever grateful to you for your guidance and willingness to help me advance my own career. To Rich, thanks for always having your door open. I have gained invaluable experience and knowledge from our scientific discussions. Never lose your passion and enthusiasm. It has helped shape into the scientist I am today. I am indebted to you for all your guidance.

Lastly, I would like to thank my family for everything. Without you guys I would not be here today. To my mom and dad, I owe you so much. You guys raised a man with integrity, character, and work ethic, all of which have been important in my pathway to obtaining this degree. I love you guys!!! To my In-laws, I owe you much gratitude for all the financial support of my family through these times. There is a purpose for all this I PROMISE!!! Thanks again for everything… I appreciate the trips to airport and all our conversations to get me mentally prepared for my interviews. I love you guys!!!

To my wife, Jennifer, who has been very supportive and patient over the years. It's not over yet… It has really just started. Thanks for everything!!! You have been such a blessing in my life. Thanks for helping me keep my sanity. They say that, "behind every great man, is a good woman," but I firmly believe that "behind every great man, is an even greater woman." Because of you, all this has come to fruition. While I was here at the CCRC working my a$# off, you were at home taking care of our wonderful daughters. Thanks for taking care of all those house duties, so I could work on getting finished. This thesis marks another step in our

accomplishments. We both know that there will be many more to come. I could not (or would not) do this anyone else. I love you!!!

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS v

LIST OF TABLES xi

LIST OF FIGURES xii

CHAPTER

1 LITERATURE REVIEW AND INTRODUCTION 1

N-linked Glycosylation – Biosynthesis and Biological Function 1

Mannose-6-Phosphate Targeting Pathway 6

Mannose-6-Phosphate Receptors – Function, Structure and Multiple Ligands 9

Man-6-P Independent and Alternative Pathways to the Lysosome 12

Impaired Man-6-P Biosynthesis Results in the Lysosomal Storage Disease Mucolipidosis II 14

Mannose Phosphorylation of Secreted Glycoproteins and their Potential Contribution to ML-II Pathogenesis 20

Thesis Overview 27

References 29

2 EXTENSIVE MANNOSE PHOSPHORYLATION ON LEUKEMIA INHIBITORY FACTOR (LIF) CONTROLS ITS EXTRACELLULAR LEVELS BY MULTIPLE MECHANISMS 50

Abstract 51

Background 52
Materials and Methods 54
Results 61
Discussion 67
Acknowledgements 71
References 72
3 THE LATENCY-ASSOCIATED PEPTIDE OF TRANSFORMING GROWTH FACTOR-β1 IS NOT SUBJECT TO PHYSIOLOGICAL MANNOSE PHOSPHORYLATION 84
Abstract 85
Background 86
Materials and Methods 88
Results 91
Discussion 96
Acknowledgements 99
References 100
4 UPREGULATION OF TRANSGLUTAMINASE ALTERS TRANSFORMING GROWTH FACTOR-β1 IN MUCOLIPIDOSIS TYPE II CELLS 107
Abstract 108
Background 109
Materials and Methods 112
Results 117
Discussion 124

Acknowledgements.....129

References.....130

5 DISCUSSION AND FUTURE PERSPECTIVES 145

The Role of Non-Lysosomal Man-6-P-Modified Proteins in the Pathogenesis of ML-II 145

The Impact of Other Non-Lysosomal Man-6-P-Modified Proteins in ML-II Disease 150

TGF-β1 – Reduced Signaling, Increased Insolubility and Possible Mechanisms 150

References.....156

APPENDIX

A SUPPLEMENTAL FIGURES.....158

LIST OF TABLES

Page

Table 1.1: Lysosomal Storage Disorders 47

Table 1.2: Nomenclature for ML-II and ML-III disease 48

Table 1.3: Bone and Cartilage Clinical Features in ML-II disease 48

Table 1.4: Bone and Connective Tissue Diseases 49

Table 2.1: Tandem Mass spectrometry identification of modified peptides following CI-MPR chromatography enrichment 83

LIST OF FIGURES

Page

Figure 1.1: Biosynthesis and maturation of an N-glycan on a newly synthesized protein 39

Figure 1.2: ML-III alpha/beta patient skin fibroblasts viewed by a contrast light microscope 40

Figure 1.3: Man-6-P biosynthesis is catalyzed by GlcNAc-1-phosphostransferase 41

Figure 1.4: Structural illustration of the Mannose-6-phosphate receptors, CD-MPR (left) and CI-MPR (right) 42

Figure 1.5 Man-6-P-dependent and alternative sorting pathways to the lysosome 43

Figure 1.6 Clinical profiles of children with ML-II and ML-III 44

Figure 1.7 The processing and secretion of latent TGF-β 45

Figure 1.8 Two modes of activation of latent TGF-β1 in the extracellular matrix 46

Figure 2.1 Secreted mouse and human LIF are modified with Man-6-P residues 75

Figure 2.2 Increasing the high mannsoe glycoforms on LIF does not potentiate further mannose phosphorylation 76

Figure 2.3 Amino acid sequence, tryptic peptides, and N-glycan sites of human LIF 76

Figure 2.4 Loss of mannose phosphorylation results in the extracellular accumulation of LIF 77

Figure 2.5 Activity of Man-6-P-modified LIF in ESC self-renewal is reduced due to internalization by cell surface Man-6-P receptor 78

Figure 2.6 Man-6-P-modified LIF is subject to intracellular targeting and degradation 79

Figure 2.7 Mannose phosphorylation controls LIF extracellular levels by multiple mechanisms 80

S. Figure 2.1 Single glycan-deleted LIF mutants bind to the CI-MPR column to a similar degree as the wild type protein 81

S. Figure 2.2 Mouse LIF is targeted to the lysosome and readily degraded 82

Figure 3.1 The mannose phosphorylation of secreted latent TGF-β1 from PMA-stimulated human erythroleukemia cultures is undetectable 102

Figure 3.2 Unmasking of the N-glycans on latent TGF-β1 from PMA-stimulated HEL cultures does not increase CI-MPR column binding 103

Figure 3.3 Latent TGF-β1 overexpressed in HeLa and CHO cells minimally binds the immobilized CI-MPR 104

Figure 3.4 The majority of N-glycans on media-derived latent TGF-β1 from PMA-stimulated HEL and transfected CHO and HeLa cells are processed to complex-type 105

Figure 3.5 Addition of active TGF-β1 ligand, but not latent TGF-β1 or LAP, stimulates differentiation of corneal fibroblasts to myofibroblasts in a Man-6-P independent manner 106

Figure 4.1 TGF-β1signaling is altered in human ML-II fibroblasts 134

Figure 4.2 A decrease in transcription or steady-state levels of TGF-β1 does not account for the altered TGF-β1 signaling 135

Figure 4.3 Media isoforms of latent TGF-β1 are reduced in human ML-II fibroblasts as determined by ^{35}S metabolic labeling 136

Figure 4.4 Latent TGF-β1 is abnormally deposited into the DOC-insoluble ECM fractions as a storage reservoir in ML-II fibroblasts 137

Figure 4.5 Latent TGF-β1 and fibrillin-1 are more rapidly deposited into the fibrillar network of human ML-II fibroblasts 138

Figure 4.6 The transamidase enzyme, transglutaminase 2, is upregulated and observed in high quantities in the insoluble ECM of human ML-II fibroblasts.....139
Figure 4.7 The increased insoluble ECM forms of latent TGF-β1 and TG2 are reproducible in feline ML-II synovial fibroblasts.....140
Figure 4.8 Latent TGF-β1 solubility is not altered in 4 day chondrocytes and corresponds to normal levels of TG2.....141
Figure 4.9 Cystamine and MDC (not shown) do not shift the solubility of latent TGF-β1 in human or feline ML-II fibroblasts.....142
Figure 4.10 Addition of RA increases the insoluble ECM quantities and transamidation activity of TG2, resulting in greater amounts of latent TGF-β1 insolubility in human WT fibroblasts.....143
Figure 4.11 Model of potential mechanisms that may account for altered ECM deposition of latent TGF-β1 and impaired bioavailability.....144
Appendix Figure 1 Fibronectin is abnormally fragmented in human and feline ML-II cells.....159
Appendix Figure 2 Fibronectin fragments generated by various recombinant cathepsin proteases and analyzed by Western blot.....160
Appendix Figure 3 LIF is a bona fide substrate for GlcNAc-1-phosphotransferase.....161

CHAPTER 1: LITERATURE REVIEW AND INTRODUCTION

N-Linked Glycosylation – Biosynthesis and Biological Function

Overview of Glycosylation

Glycosylation is one of the most prevalent post-translational modifications. It is hypothesized that approximately 50% of all eukaryotic proteins obtain some form of glycosylation during protein synthesis. The presence of an oligosaccharide moiety on soluble and membrane-bound proteins is biologically important for solubility in water, protease protection, and protein stability. In addition, glycans mediate molecular and cellular recognition events, including those critical for intracellular transport of glycoproteins. Protein bound glycosylation encompasses two major classes - O-linked glycosylation and N-linked glycosylation. Of these two classes, asparagine (N)-linked glycosylation will be highlighted here. N-linked glycosylation is important throughout biology, since N-glycans affect the function of the proteins to which they are attached, and defects in N-glycan biosynthesis result in a variety of human diseases. Here, I will briefly discuss the biosynthesis of N-glycans and the specific function of these oligosaccharides in biology.

Biosynthesis of N-linked Glycosylation

The early biosynthetic steps of N-linked glycosylation are highly conserved in eukaryotic cells, including the synthesis of the 14-sugar containing oligosaccharide on dolichol and the *en bloc* transfer of the pre-formed oligosaccharide onto emergent polypeptide chains catalyzed by the membrane-bound enzyme complex called the oligosaccharyl transferase or OST (1,2). Upon transfer, the newly synthesized glycoproteins must undergo rapid trimming and re-addition of glucose residues, which is an important sensor for improperly folded proteins within the ER. Glycoproteins have limited time to fold appropriately before they are deglucosylated and targeted for ER associated Degradation (ERAD) (*see* Figure 1.1 for more detail).

Following the removal of glucose and an additional mannose, glycoproteins are transported from the ER to the *cis*-Golgi (1,2). Within the Golgi, these protein-bound oligosaccharides are either subject to limited processing giving rise to high-mannose type structures, or they undergo more extensive processing resulting in the formation of hybrid and complex-type structures, that typically terminate with sialic acids (Figure 1.1). Upon transit through the Golgi, these glycoproteins are subsequently sorted to the intracellular organelles (i.e., lysosome), secreted from the cell or positioned at the cell surface.

N-Glycans Have Diverse Functions

The functional roles of N-glycans in biology are diverse - influencing cell-matrix or cell-cell adhesion, cell migration, conformation, folding and stability of proteins, and glycoprotein sorting. Our understanding of the function of N-glycans has been greatly assisted by the use of specific inhibitors that prevent N-glycan biosynthesis and processing (2,3). In addition, the use of site-directed mutagenesis to prevent specific-site glycosylation and the use of animal and

disease models (i.e., congenital disorders of glycosylation) has been beneficial in studying functional aspects of N-glycans. Protein N-linked glycosylation has been demonstrated to be important for the function of glycoproteins across biology and some of these examples are discussed below.

Biological Function of N-Glycans: Protein Folding, Half-Life and Biological Activity

It has been shown that N-linked glycosylation is important for ensuring the correct folding of glycoproteins, and the half-life and biological activity of these proteins. For instance, inhibition of lipid-linked oligosaccharide assembly by the bacterial metabolite tunicamycin demonstrated that N-linked glycosylation is important for protein folding in the ER. In particular, chemical inhibition of oligosaccharide assembly or N-glycan processing was shown to elicit an unfolded protein response (UPR), due to underglycosylation of proteins, resulting in their turnover prior to ER exit (4,5). In a similar manner, detailed studies of viral glycoprotein using mutations of N-linked sites determined that N-glycans are needed for proper folding, as underglycosylated proteins form intracellular aggregates and are retained in the ER (6-8).

One of the best-described functions of N-linked glycosylation is in regulation of protein half-life and biological activity. Altered N-glycan processing on the secreted protein, erythropoietin, was shown to drastically reduce its biological activity (at least 500 fold less effective) and increase protein clearance (9). Other studies have shown that specific receptors such as the asialoglycoprotein receptor (ASGP-R) facilitate the removal of serum glycoproteins that bear terminal α2,6-linked sialic acid (10). In fact, it is clear that binding affinities and biological activities of glycoproteins can be regulated by their degree of N-glycan modifications, such as sulfation (11).

Biological Function of N-Glycans: Stability of Proteins.

It has been shown that protein N-linked glycosylation is important in transporting membrane-bound proteins to the cell-surface (12). In addition, these N-linked oligosaccharides have been demonstrated to be necessary for the stability and function of proteins at the cell surface. Studies have shown that defects in the N-linked glycosylation of FGF receptor-3 or the cystic fibrosis transmembrane conductance regulator (CFTR) result in abnormal or abrupt turnover of these proteins, resulting in the diseases hypochondroplasia and cystic fibrosis, respectively (13,14). Differential glycosylation of cell surface proteins, like glucose transporters (i.e., Glut-2) (15) and insulin receptors (16), can result in loss of binding and turnover. Interestingly, proteins such as MHC class II molecules lose antigen presenting capabilities if not properly glycosylated (17).

Biological Function of N-Glycans: Adhesion and Migration

To date, one of the most remarkable functions for N-linked glycosylation is its role in cell adhesion and migration, specifically in cancer metastasis. Controlling the degree of N-glycan branching has been demonstrated to regulate both adhesion and migration of tumor cells. For instance, inhibition of the glycosyltransferase GnT-V (a glycosyltransferase that initiates the β6 "branching" of N-glycans) is important in the regulation of cancer progression (18,19). In addition, fucosylation of sialylated N-glycan structures has been documented to increase E- and L-selectin binding thereby increasing adhesion of leukocytes to inflamed endothelium (20). Indeed, the impact of core fucosylation has been shown to facilitate cancer metastasis (21) as well as regulate growth and development (22). These reports highlight just a few of the monosaccharide modifications that govern cell adhesion and migration.

Biological Function of N-Glycans: Lysosomal Enzyme Targeting

Another essential function of N-linked glycosylation is to mediate the intracellular sorting of proteins. Modification of N-glycans with phosphomannosyl residues serves to sort most lysosomal acid hydrolases to the lysosome. These residues allow the hydrolases to bind specific receptors in the *trans*-Golgi that carry to them to late endosomes and ultimately lysosomes. The discovery of this carbohydrate-based sorting signal, how the enzymes involved in its biosynthesis recognize and act on their substrates, and how the receptors mediate sorting to lysosomes is discussed in detail below.

In 1967, Leroy and Demar described a disorder that clinically resembled Hurler Syndrome (MPS-I, see Table 1.1), but lacked the hallmark mucopolysaccharidurıa (23,24). They discovered large vacuoles or inclusions in the cytoplasm of the patient fibroblasts and therefore named the disorder, inclusion-cell (I-cell) disease (see Figure 1.2). Similar inclusions were seen in pseudo-Hurler polydystrophy, a clinically milder form of I-cell disease (25). Later, these diseases were renamed mucolipidosis (Table 1.1) due to the combined abnormal levels of mucopolysaccharides and sphingolipides. The more severe I-cell disease and the milder pseudo-Hurler polydystrophy are classified as mucolipidosis II (ML-II) and III (ML-III), respectively, based on the nature of the respective mutations in the *GNPTAB* gene (Table 1.2) (24).

The notion that a specific recognition marker was responsible for the uptake and transport of lysosomal enzymes was first suggested by the laboratory of Elizabeth Neufeld, who demonstrated that co-culture of Hunter and Hurler fibroblasts resulted in cross-correction of their respective defect (26). Subsequently, it was shown that I-cell patient fibroblasts were capable of endocytosis of acid hydrolases secreted by normal fibroblasts. In contrast, normal fibroblasts could not rescue the large amounts of secreted acid hydrolases from I-cell fibroblasts. This

suggested that the acid hydrolases synthesized by normal cells must bear a recognition marker that facilitate their transport to the I-cell lysosomes. More importantly, these findings suggested that missorted lysosomal enzymes from ML-II fibroblasts lacked this marker (27). The recognition marker was later identified as mannose-6-phosphate (28,29). Below, I will discuss the Man-6-P biosynthetic pathway whereby newly synthesized hydrolases receive the lysosomal recognition marker (30,31).

Mannose-6-phosphate Targeting Pathway

Mannose-6-phosphate (Man-6-P) is one of the best-characterized carbohydrate based sorting signals in biology. As stated above, lysosomal hydrolases and selected glycoproteins receive mannose-6-phosphate during their biosynthesis and are directly sorted to the lysosome (Figure 1.1). Upon modification with Man-6-P by the enzyme UDP-N acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) in the *cis*-Golgi, further processing of the N-glycans on these lysosomal enzymes is blocked and they remain high mannose type. The addition of the Man-6-P modification to lysosomal proteins is a two-step enzymatic process (Figure 1.3A). The first step in the process is transfer of a GlcNAc-1-phosphate to the carbon-6 position of α1,2-linked mannose residues on high mannose oligosaccharide chains of lysosomal proteins by GlcNAc-1-phosphotransferase, generating a phosphodiester linkage (32). The second step occurs in the *trans*-Golgi network (TGN) where the GlcNAc residues that cover the Man-6-P monoesters are removed by a subsequent enzyme N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (Figure 1.3A) or "uncovering enzyme" (UCE) (33,34). The uncovered Man-6-P monoesters serve as recognition markers that are essential for segregating lysosomal

proteins from the secretory pathway, ultimately sorting them to the lysosome through high affinity interactions with two receptors, the cation-dependent and the cation-independent mannose-6-phosphate receptors CD-MPR and CI-MPR, respectively (35,36).

Molecular Structure and Function of GlcNAc-1-Phosphotransferase α/β-Subunits

The GlcNAc-1-phosphotransferase enzyme is composed of an α/β subunit and a γ-subunit encoded from two different genes (37). The precursor α/β protein is encoded on a single gene *GNPTAB* (38,39), while γ is encoded from a different gene, GNPTG (40,41). The cDNA sequence of the α/β gene is highly conserved in human, cow, mouse, rat, chicken and zebrafish (42). In fact, GlcNAc-1-phosphotransferase enzyme was purified from a bovine source as a 540 kDa heterohexameric (α2β2γ2) protein complex (shown in Figure 1.3B) (37). In addition, it has been determined that the precursor α/β subunit contains the catalytic activity (43,44) as well as the ability to specifically recognize lysosomal proteins (45,46). It has long been proposed that cleavage of the α/β subunit precursor is a prerequisite for catalytic function. Recently, it was determined that the Golgi resident protein, site-1 protease (a protease involved in lipid metabolism), cleaves the precursor α/β subunit rendering it catalytically active (47). This cleavage event gives rise to two subunits of 166 kDa (α-subunit) and 51 kDa (β-subunit), containing 19 potential N-linked glycosylation sites (16 for α-subunit and 3 for β-subunit).

Molecular Structure and Function of GlcNAc-1-Phosphotransferase γ-Subunit

As stated above, the γ-subunit is derived from a single gene that encodes a 56 kDa protein. Individual γ-subunits form disulfide-linked homodimers containing two potential N-linked glycosylation sites (Figure 1.3B) (42). Unlike the α/β subunit, the function of γ-subunit is

less clear. It has been demonstrated that γ-subunit can enhance phosphorylation of lysosomal proteins. In fact, the γ-subunit contains a mannose receptor homology (MRH) domain and is thought to facilitate additional mannose phosphorylation by orienting high mannose glycans on lysosomal proteins to the α/β catalytic site in a favorable fashion (46). Other reports have suggested that coordinated proteolysis of the γ-subunit functions in the regulation of activity and expression of the α/β subunits (48,49).

Molecular Structure and Function of Uncovering Enzyme

Uncovering enzyme (UCE) is the second enzyme in the two-step Man-6-P biosynthetic pathway (described above). It is a 515 amino acid Type I transmembrane protein that is synthesized as an inactive precursor and resides in the TGN. There, in the TGN, it is processed to an active protein by furin convertase (33). Recently, it was shown that mutations in the gene that encodes UCE is associated with persistent stuttering (50). However, no other phenotypes have been documented, even though it has been determined mutagenic disruption of the gene encoding UCE leads to hypersecretion of lysosomal enzymes similar to loss of GlcNAc-1-phosphotransferase (51).

GlcNAc-1-Phosphotransferase Recognizes Acid Hydrolases by a Protein Recognition Motif

GlcNAc-1-phosphotransferase has a 100-fold higher transfer efficiency for lysosomal enzymes compared to secretory proteins, suggesting that lysosomal proteins contain a specific recognition determinant that is absent in most secretory proteins (30). The exact nature of the protein recognition domain, however, has remained enigmatic. A role for specific lysine residues has been suggested by several studies (52-56). In one instance, manipulation of lysine

and arginine residues on the Man-6-P modified secretory protein DNase I was shown to lower its phosphorylation amounts (52,57). Addition of lysines adjacent to N-linked oligosaccharides of DNase I, however, was shown to induce phosphorylation of these proteins. Similar studies carried out in Dr. Kornfeld's lab, comparing cathepsin D (a known phosphorylated acid hydrolase) with a highly homologous secretory protein, pepsinogen (a non-phosphorylated molecule), determined that substitution of two lysines on pepsinogen stimulated mannose phosphorylation 116-fold, suggesting that substitution of two lysines in the correct surface patch is the minimal requirement for interaction with the GlcNAc-1-phosphotransferase (58-61). While these results indicate that lysine residues are principal determinant elements on the surface of the protein for GlcNAc-1-phosphotransferase recognition, it is clear from these studies that other residues likely contribute to recognition.

Mannose-6-Phosphate Receptors – Function, Structure and Multiple Ligands

The Primary Function of the Mannose-6-Phosphate Receptors

Mannose-6-phosphate receptors (M6PRs) bind to lysosomal proteins via the Man-6-P recognition marker and play an important role in sorting these enzymes to the lysosome. The two M6PRs are similar but have several key differences. Some of these distinct features are highlighted here. The M6P receptors differ in their size (the CI-MPR ~300 kDa and the CD-MPR ~46 kDa) as well as their binding affinity for lysosomal enzymes. The CI-MPR has a higher binding affinity for the acid hydrolases than the CD-MPR. For this reason, the CI-MPR is more efficient in targeting acid hydrolases to the lysosome compared to the CD-MPR. Still, certain hydrolases show a binding preference for an individual receptor (62,63). Both receptors

show optimal binding of lysosomal proteins at a slightly acidic pH of 6.5 (*trans*-Golgi network; TGN). Upon delivery to the endosomes, the M6PRs release the enzymes due to the moderately acidic pH conditions (pH ~5) (35). Then, M6PRs recycle back to the TGN to retrieve more M6P-modified proteins or move to the plasma membrane. Roughly 10% of the M6PRs are present at the cell surface while the majority of the receptors are found in endosomal compartments and the TGN (63). Since it is known that 5-20% of the newly synthesized acid hydrolases are missorted and secreted extracellularly (36,64), the plasma membrane associated CI-MPR, not the CD-MPR, has the important task of scavenging and internalizing missorted lysosomal enzymes—redirecting their paths to the lysosomes (35,36,64).

The Structure of the CD- and CI-MPR

The MPRs are type I transmembrane oligomeric glycoproteins (Figure 1.4). A single CD-MPR monomer is composed of a 159 amino acid extracytoplasmic domain, a 25 amino acid transmembrane region and a 67-residue carboxyl terminal cytoplasmic domain (35). The CI-MPR contains a large 2270 amino acid extracytoplasmic region, a 23 residue transmembrane region and a 163 residue carboxyl-terminal cytoplasmic domain. The large extracytoplasmic region is composed of 15 homologous and contiguous domains (Figure 1.4) that are similar in size (~150 residues). Between the two receptors, the domains share a similar sequence identity (14-38%). Recently, it was determined that the CD-MPR exhibits a similar fold to the extracytoplasmic domains 1-3, and 11-14 of the CI-MPR (65-67). Interestingly, domain 13 of the CI-MPR contains a type II fibronectin repeat (Figure 1.4), which may function to interact with collagen. However, the function of the type II fibronectin repeat in the CI-MPR remains unknown.

Mannose-6-Phosphate Ligand Binding Sites

The M6PRs differ in the number of Man-6-P binding sites and not all domains of the CI-MPR bind Man-6-P containing ligands. Only domains 3, 5 and 9 of the CI-MPR and one site on the CD-MPR have been shown to bind Man-6-P moieties (68-70). Interestingly, domain 9 exhibits a higher preference for phosphomonoesters while domain 5 has a ~15-fold higher affinity for phosphodiesters (71). In fact, experiments with multiple domains adjacent to domain 5 can increase the affinity ~60-fold (72).

The CI-MPR has been shown to bind other ligands, including mannose-6-sulfate and mannose-6-P-OCH3, with comparable affinity (10 to 20-fold lower) to Man-6-P containing ligands (73). In addition, the CI-MPR appears to bind to serglycin, a lysosomal proteoglycan, through chondroitin sulfate (74,75). Based on the observation that domains 1-3 bind to mannose-6-sulfate, it was postulated that serglycin interacts with these regions of the CI-MPR. This interaction may facilitate protein transport utilizing serglycin as a vehicle for other proteins to make their way to the lysosome (75).

The CI-MPR – A Multifunctional Receptor

The M6PRs have various functions other than binding and sorting lysosomal enzymes to the lysosome and scavenging missorted enzymes at the cell surface. In particular, the CI-MPR is involved in the regulation of cell growth, adhesion and fibrinolysis (76). Its functions in cell growth regulation are associated with the binding and internalization of insulin-like growth factor (IGF-II) through its association with domain 11 (Figure 1.4) (77). In addition, the CI-MPR binds retinoic acid, which can enhance the primary function of the receptor and aid in cell proliferation as well as apoptosis (78,79). Recently, it was determined that cellular repressor of

E1A-stimulated genes (CREG), a mannose phosphorylated protein, can interact through protein-protein interactions and regulate cell proliferation (80). Likewise, urokinase-type plasminogen activator receptor (uPAR), a receptor involved in fibrinolysis, adhesion and migration, has been shown to bind to the CI-MPR via disparate mechanisms. The soluble form of uPAR binds in a Man-6-P dependent fashion, while the full-length membrane associated form binds to domain 1 of the CI-MPR via protein-protein interactions (Figure 1.4) (81). Further studies are warranted to address the role of uPAR's interaction with CI-MPR.

Man-6-P Independent and Alternative Sorting Pathways to the Lysosome

Some hydrolases are targeted to the lysosomes by mechanisms that appear to be independent of the canonical Man-6-P-mediated pathway. The alternative trafficking pathways that contribute to the delivery of lysosomal enzymes to normal and diseased tissues and cells are discussed below.

The Basis for Exploring Alternative Pathways to the Lysosome

The initial characterization of ML-II patients (described above) determined that some cells (hepatocytes, Kupffer cells, and leukocytes) as well as tissues (kidney, liver, spleen and brain) from ML-II patients had relatively normal levels of some intracellular lysosomal enzyme activity compared to patient fibroblasts (82,83), suggesting that the Man-6-P mediated transport system is cell- and tissue-type specific. From these studies, it was hypothesized that hypersecreted acid hydrolases from ML-II patient tissues and cells may be taken up by mannose receptors and/or galactose receptors, implicating alternative modes of lysosomal enzyme

trafficking (84-87). In fact, I-cell diseased lymphoblasts have been shown to sort the acid hydrolase cathepsin D 45% efficiently to the lysosome in absence of the Man-6-P recognition marker (88). In other cases, it has been observed in cells that contain very low (less than 3%) M6PR levels accumulate high intracellular acid hydrolase activity (89). These findings (among others) proposed that some enzymes utilize alternative transport pathways to the lysosomes.

Acid Hydrolase Sorting by Membrane-Association or Receptor Mediated Trafficking

Certain acid hydrolases are now known to utilize Man-6-P independent pathways to the lysosome such as membrane association or receptor mediated trafficking. Lysosomal acid phosphatase is targeted to the lysosomes efficiently by membrane association (90-92), while glucocerebrosidase (acid β-glucosidase) traffics to the lysosome via interaction with the lysosomal integral membrane proteins, LIMP-2 (93). Similarly, the sphingolipid activator protein, prosaposin, and lysosomal hydrolase cathepsin H have been suggested substrates of sortillin receptor sorting to the lysosome (94,95).

Acid Hydrolase Sorting by Both Man-6-P Recognition and Alternative Pathways

Some hydrolases utilize both the Man-6-P recognition system as well as alternate pathways. For instance, the known M6P-modified acid hydrolases, cathepsin D and α-glucosidase, have been determined to directly transport to the lysosomes by membrane association in rabbit alveolar macrophages and HepG2 cells (cathepsin D) and human skin fibroblast (α-glucosidase) in an Man-6-P independent manner (96-99). Also, it has been determined that Man-6-P modified cathepsin D can utilize the sortilin receptor for trafficking to the lysosome (94). Still other enzymes, like β-galactosidase and neuraminidase, form a multi-

enzyme complex and "piggy-back" to the lysosome with the cathepsin A in a Man-6-P independent manner (100-103). Recently, Gonzalez-Noriega and colleagues proposed a Man-6-P independent endocytosis mechanism of β-glucuronidase by the endocytic receptor annexin VI (104), further exemplifying the complexity of independent trafficking mechanisms (Figure 1.5).

Impaired Man-6-P Biosynthesis Results in the Lysosomal Storage Disease Mucolipidosis II

Brief Overview of Lysosomal Storage Diseases

Lysosomal Storage Diseases (LSDs) (Table 1.1) are caused by defects in the degradation of macromolecules including the metabolism of lipids, glycoproteins, and mucopolysaccharides. Typically, individual LSDs are the result of loss or defects in a single catabolic lysosomal enzyme. The frequency of individual LSD is approximately 1:100,000; while grouped together the incidence is closer to 1:5000. In general, LSDs encompass over 50 inherited diseases including defects in integral membrane proteins (i.e., LAMPs).

The LSDs are categorized based on a combination of enzyme/protein characterization and specific substrate accumulation (Table 1.1). These LSDs range from shingolipidoses and mucopolysaccharidoses to integral membrane defects. In particular, glycosaminoglycan (GAG) degrading enzyme disorders, the mucopolysaccharidoses (MPS), are some of the most studied disorders. These disorders lead to an accumulation of undigested GAG chains such as heparan sulfate, dermatan sulfate, and/or keratan sulfate. The main site of pathology for these disorders is the skeleton, heart valves and other areas associated with connective tissues (105,106). Clinical features include coarse facial features, organomegaly, joint stiffness, skeletal dysplasia called dysostosis multiplex and in some cases mental retardation (107,108). In general, the MPS

disorders associated with accumulation of dermatan, keratan and chondroitn sulfate are present with the visceral manifestations, while MPS diseases that accumulate undigested heparan sulfate material tend to also have neurological issues (Table 1.1).

Genetic Lesions in GlcNAc-1-Phosphotransferase Lead to Impaired Man-6-P Biosynthesis

Mutations in the genes that encode the GlcNAc-1-phosphotransferase enzyme lead to impaired Man-6-P biosynthesis and the autosomal recessive disorders ML-II and ML-III (30,36,41,109). ML-II, a lysosomal storage disease that affects more than a single lysosomal enzyme, is caused by a frameshift mutation in the *GNPTAB* gene that prevents catalytic function (activity of the enzyme is less than 1%) (43,44). ML-III can occur from point mutations in the *GNPTAB* gene as well as mutations in the gamma subunit gene *GNPTG* (*for genetics and nomenclature see* Table 1.2) (110). The GlcNAc-1-phosphotransferase activity in these patients is between 2-20%, which correlates with the milder form of the disease (44,110). In the absence of GlcNAc-1-phosphotransferase enzyme activity, hydrolases do not receive the Man-6-P recognition marker and are not trafficked to the lysosomes. Instead, they are hypersecreted out of the cell. As a result, serum acid hydrolase activity levels increase, resulting in lysosomal storage accumulation. This phenomenon gives rise to dense inclusions that have been shown in fibroblasts and mesenchymal cells (111).

Clinically, I-cell disease is characterized by radiographic and clinical features that is similar to those observed in MPS-I (Hurler's syndrome; see Table 1.1) with earlier presentation as well as a rapid progressive course and no visible mucopolysaccharidurıa (111). Neonates with ML-II show severe psychomotor retardation and organomegaly with a failure to thrive by developmental delay. In addition, patients have craniofacial abnormalities, dysostosis multiplex,

thick and waxy skin as well as tissue overgrowth (i.e., gingival hyperplasia) (112) as shown in Figure 1.6A, B and C. These patients rarely survive with occurrence of death by cardiorespiratory complications in the first decade of life (113). In some cases, cardiac involvement is most commonly facilitated by the thickening and insufficiency of the mitral valve or the aortic valve (112). On the other hand, respiratory issues may be a consequence of progressive mucosal thickening of airways and gradual stiffening of the thoracic cage (112). Symptoms are similar in the ML-III patients (Figure 1.6C and D), however the progressive nature of the disease is milder and is not usually diagnosed until 3-5 years of age with sustainable life expectancy into adulthood (114). The pathology in bone and cartilage is perhaps the most prominent feature in ML-II patients and will be discussed in detail below.

Cellular Pathology of ML-II

Several cells and tissues from ML-II patients maintain normal acid hydrolase levels and activity, even though genetic lesions result in complete loss of GlcNAc-1-phosphotransferase activity in all cells. Nevertheless, other cells and tissues are grossly affected by loss of GlcNAc-1-phosphotransferase activity and hypersecretion of lysosomal enzymes. For instance, phase-contrast and electron microscopic (EM) analysis have determined that certain ML-II cells of mesenchymal origin contain large dense cytoplasmic membranous LAMP-2 positive vacuoles, suggesting that these organelles are lysosomal in nature (111,112). Other studies have demonstrated that secondary lysosomes contain not only lysosomal membrane proteins, but also various kinds of storage glycoconjugates with terminal sialic acid, N-acetyl-glucosamine, and mannose residues that accumulate in large quantities (115). In addition, phospholipids and cholesterol levels within ML-II skin fibroblasts have been shown to be elevated (116). The

presence of autolysosomes has also been reported in ML-II cultured fibroblasts (117) and exocrine glands of ML-II models of the disease (118). In addition, the elevation of autolysosomes and autophagosomes was reported to be associated with fragmented mitochondria and increased ubiquitinated proteins, suggesting that stress responses may be associated with lysosomal dysfunction (117).

Ultimately, the affects of lysosomal dysfunction, increased storage material and hypersecretion of acid hydrolases may contribute to the secondary pathogenic phenotypes in ML-II. For example, thickening of the heart valve may be caused by presence of enlarged vacuoles within the tissue fibroblast (119,120). It has also been shown from autopsies of ML-III patients that several tissues such as brain and myocardium as well as liver fibroblasts have cytoplasmic lysosomal granules, which may contribute to the abnormalities reported (121,122). Other reports have documented uncharacteristic de-differentiation of vacuolated chondrocytes into fibrocartilage, an affect that may result in loss of cartilage architecture and abnormal bone formation in ML-II patients (123).

Investigation of ML-II Animal Models Has Yielded New Insight Into the Pathogenesis

To date, three animal models for ML-II have been described, including feline (124,125), mouse (45,126) and zebrafish (127) models. GlcNAc-1-phosphotransferase deficient cats have strikingly similar clinical and radiographic features compared to human ML-II patients. However, working with the feline model has proven difficult since its DNA sequence has not been fully annotated. The *GNPTAB*$^{-/-}$ mouse model for the disease displays a few features similar to human ML-II such as reduced size and elevated plasma lysosomal enzyme levels, but differs in that these mice develop retinal degeneration (a characteristic shared with the feline

model (124)), secretory cell lesions of several exocrine glands, and lack cytoplasmic vacuolar inclusions in fibrocytes/mesenchymal cells (128). The zebrafish model of ML-II disease has been used to determine how the impact of impaired Man-6-P biosynthesis affects ECM production and homeostasis. This ML-II model was shown to develop craniofacial defects and display deficient intracellular acid hydrolase activity indicative of hypersecretion of lysosomal enzymes, both of which are characteristics of the human disease (127). Strikingly, the ML-II zebrafish model was observed to exhibit altered ECM production and homeostasis of matrix components, including early mistiming and expression of type II collagen resulting in accumulation of this protein at later stages of development (127). Recently, abnormal processing and activation of the lysosomal protease cathepsin K activity was shown to be the basis for abnormal cartilage morphogenesis phenotypes and increased type II collagen expression in the zebrafish ML-II model, further emphasizing the importance of the zebrafish model in exploring the secondary phenotypes associated with the disease (129).

Bone and Cartilage Represent the Major Site of Pathology in ML-II/ML-III

Cartilage and bone abnormalities found in both ML-II/ML-III represent the main site of pathology in the disease (see Table 1.3). Specifically, ML-II has been characterized by the malformation of skeletal bones and impaired cartilage homeostasis. In fact, some patients with the milder variant ML-III have been misdiagnosed with rheumatoid arthritis (RA) due to the severity of bone and joint immobility (see Figure 1.6C and D) (130,131). It has been observed that cartilage precursor chondrocytes show lysosomal storage—mainly comprising proteoglycans (132). Impaired production or turnover of ECM components such as fibronectin and glycosaminoglycans (GAGs) by chondrocytes could affect the integrity of the extracellular

matrix. Similarly, loss of ECM homeostasis directed by acid hydrolase hyperactivity could lead to proteolysis of ECM components, in particular generating fibronectin or collagen fragments, which have been implicated in cartilage destruction (133-138).

It has been hypothesized that the bone defects are "metabolic" in nature and further suggested that hyperparathyroidism may be the primary cause of the bone phenotypes, since elevated parathyroid hormone as well as the observed disorderly calcium deposition are known to cause subperiosteal bone resorption and osteopenia—characteristics seen in these patients (113,139-141). However, it is not clear if hyperparathyroidism is the primary cause of the bone disease or a secondary effect to other manifestions in I-cell disease such as osteoclast hyperactivity. Osteoclasts from *GNPTAB*$^{-/-}$ mice have alterations in secretory lyososome formation, with an increase in cathepsin K and tartrate resistant alkaline phosphotase (TRAP) secretion (142). In fact, Pacman dysplasia, a lethal skeletal dysplasia caused by epiphyseal stippling and osteoclastic hypersplasia, is a prenatal manifestation in ML-II (143,144). Similarly, osteoblast matrix deposition and organization appears to be impaired. Indeed, the disorganized production of ECM by osteoblasts combined with osteoclast hyperactivity may result in low bone density fractures as well as disorderly calcium deposition. Likewise, abnormal vacuolization of chondrocytes and osteoblasts due to the loss of Man-6-P biosynthesis and impaired lysosomal enzyme trafficking may facilitate bone and cartilage malformation (132,145). A striking accumulation of type II collagen and aberrant matrix formation along with altered calcium deposition was noted in ML-II zebrafish, a finding consistent with the notion that abnormal ECM homeostasis and production are central to the pathology of the disease (127).

Mannose Phosphorylation of Secreted Glycoproteins and their Potential Contribution to ML-II Pathogenesis

In addition to the lysosomal hydrolases, several non-lysosomal proteins have been shown to receive the Man-6-P modification. These include, but are not limited to, latent transforming growth factor beta (TGF-β) (146), leukemia inhibitory factor (LIF) (147,148), macrophage colony stimulating factor (CSF1) (, proliferin (149), renin precursor (150), and T-cell activation antigen CD26 (151). The role of mannose phosphorylation on these non-lysosomal Man-6-P modified proteins has been shown to have important consequences. In particular, it has been proposed that TGF-β1 and renin precursor may interact with the M6PRs at the cell surface to initiate activation (152-155). Others have shown that mannose phosphorylated LIF and T-cell activation antigen CD26 may be cleared from plasma and internalized for degradation in the lysosomes (147). Biological regulation of growth factors, (latent TGF-β1 and LIF) by mannose phosphorylation could have an important impact in ECM production and homeostasis as well as bone and connective tissue development. Little is known about impact of impaired mannose phosphorylation on latent TGF-β1 and LIF relative to ML-II disease. The role of these growth factors in other bone and connective tissue disorders and their concerted roles in bone remodeling will be highlighted below and discussed in greater detail within later chapters.

TGF-β1: Processing and Biological Function

TGF-β1 is an important regulator of responses in biology. It functions to regulate effects in differentiation, cell proliferation, as well as adaptive immunity (156). Some of the best-highlighted functions of TGF-β1 described in the literature are its essential role in the synthesis

of ECM components in mesenchymal cells (i.e., skin, bone and cartilage) as well as regulation of differentiation in these cells. This pleiotropic molecule is synthesized as a pro-precursor that is processed by furin in the TGN (Figure 1.7) (157,158). Upon post-translational modification and processing, the mature TGF-β1 growth factor dimerizes and non-covalently re-associates with its homodimeric prodomain known as the latency-associated peptide (LAP). This complex, termed the small latent complex (SLC or latent TGF-B), is either retained in the Golgi (159,160), secreted, or binds to the latent TGF-β1 binding protein (LTBP) via a disulfide cross-link (161,162). It has been shown that latent TGF-β1 can interact with three of four possible LTBPs: LTBP-1, 3, and 4 (163-166). The interaction of LTBP with the SLC (termed the large latent complex; LLC) induces rapid secretion of latent TGF-β1 and is targeted to the ECM for sequestration, storage, and/or activation (167,168).

TGF-β1: Activation Via ECM Cross-Linking By Tissue Transglutaminase

Tissue transglutaminase 2 (TG2), an enzyme that generates extensive insoluble isopeptide bonds between proteins, is responsible for the cross-linking of LLC to the ECM (168-170). Increased expression of TG2 has been documented to result in a greater deposition rate of LTBP-1 and latent TGF-β1 into the ECM (171). The cross-linking of latent TGFB1 to the ECM has been proposed as a crucial event for the initial steps of activation of latent TGF-B1 (172,173). In addition to its importance in cross-linking latent TGFB1 to the ECM, TG2 has been shown to have multiple functions and will be discussed in more detail.

Tissue Transglutaminase: A Multi-Functional Enzyme

Tissue transglutaminase 2 (TG2, EC 2.3.2.13) belongs to a family of transglutaminases that catalyze the formation of covalent bonds between glutamines (γ-carboxamide group) and lysines (ε-amino group). In addition, TG2 is the most studied member of its family, exhibiting a broad distribution within tissues and cell types. Besides its calcium-dependent transamidase activity, TG2 has been documented to have multiple calcium-independent functions. TG2 can act as a GTPase (174), protein disulfide isomerase (175), protein kinase (176), and DNA hydrolase. It has even been documented that TG2 bears non-catalytic functions in protein scaffolding within the ECM (177). Interestingly, the localization of TG2 is predominantly cytoplasmic (greater than 80%), while it has been observed in the nucleus and associated with the ER (178-181). Due to the low amounts of calcium and the high amounts of GTP (a known inhibitor of TG2 transamidase activity), it has been suggested that cytoplasmic localization in healthy cells is important for regulating the calcium-dependent transamidase function of TG2 (178). For transamidase activity, TG2 must translocate to areas rich in calcium, such as the extracellular matrix and endoplasmic reticulum (177,179,180). It is conceivable that under stress conditions, such as lysosomal or autophagic dysfunction TG2 may be aberrantly activated due to impaired calcium flux into the cytoplasm (182-184).

As stated above, TG2 can function in the ECM, however, it is not clear how this molecule is secreted since it bears no signal peptide or N-linked glycosylation (despite the presence of potential N-glycan sites) (185). Studies from Alexey Belkin's group determined that newly synthesized cytoplasmic TG2 in fibroblasts can be targeted to recycling endosomes and delivered to the cell surface (186). Conversely, Ed VanBavel's group demonstrated that TG2 may be secreted through microparticle release in smooth muscle cells, which has been

hypothesized to be dependent on TG2 transamidase activity (187). Others have suggested that this molecule may cross-link itself with extracellular components such as fibronectin and heparan sulfate within the cell and "piggyback" to the cell surface or ECM (188,189). The phenomenon of TG2 self cross-linking to extracellular proteins may be more appropriate once TG2 has arrived to the ECM or cell surface. Still, much work needs to be done to determine the non-canonical secretion mechanisms of TG2.

TGF-β1: *In vitro* and *In vivo* Mechanisms of Activation

Concurrent with its cross-linking, latent TGF-β1 interacts with many known ECM proteins (i.e., fibronectin and fibrillin-1) and glycosaminoglycans (GAGs) (Figure 1.8) (166,190,191). These interactions contribute to the complex mechanisms of liberation and activation (168,192). Activation of TGF-β1 occurs by (1) dissociation from its LAP and subsequent binding to TGF-β1 receptors or (2) an induced conformational change of LAP that leads to constitutive presentation of the TGF-β1 ligand to the TGF-β1 receptors. Liberation, on the other hand, refers to release of TFG-β from the ECM or LAP by one of several different mechanisms (168). In terms of activation, the best-described mechanisms have been studied *in vitro,* whereby TGF-β1 can be liberated from its LAP by heat, acid, and proteases (168,193). More importantly, modes of activation *in vivo* are best characterized by non-proteolytic mechanisms whereby the majority of TGF-β1 activation is integrin-mediated (194,195) or governed by interaction with thrombospondins (196-198). Finally, proteolytic mechanisms of activation (by serine proteases such as plasmin and MMPs) are essential for the liberation and activation of latent TGF-β1 from ECM in bone, cartilage and other matrix-producing cells (Figure 1.8) (199,200).

Mutations in Latent Forms of TGF-β1 Result in Bone and Connective Tissue Defects

Several studies have determined that TGF-β1 is involved in several bone and connective tissue-related diseases (*see* Table 1.4). These connective-tissue diseases are rare in that, mutations in LAP or LTBP alter the activation or storage/ECM assembly of latent TGF-β1. For instance, in Camurati-Engelmann disease point mutations in LAP cause long bone abnormalities due to the hyperactivation of TGF-β1 (201-203). Conspicuously, *Ltbp3* $^{-/-}$ mice (which show decreased LLC deposition and activation in the ECM) demonstrate an osteopetrosis-like phenotype due to accumulation of trabecular bone in all bone elements (204). Likewise, mutations in *LTBP4* have been reported to cause severe craniofacial, dermal and musculoskeletal development in four unrelated patients, validating the coupled importance of ECM assembly and TGF-β1 signaling in development (205).

Defects in ECM Assembly Result in Altered TGF-β1 Bioavailability and Disease

Alterations in TGF-β1 activation and signaling can be facilitated by dysfunction or loss of ECM components that are involved in sequestration of this growth factor. In the case of Marfan syndrome, TGF-β1 is rendered constitutively active due to mutations in fibrilin-1 causing disorders of connective tissue that affect the skeletal, ocular and cardiovascular systems (206). Not to mention, mutations of the Arg-Gly-Asp (RGD) region of fibrilin-1 lead to defective integrin interaction and increased TGF-β1 signaling in congenital scleroderma or stiff skin syndrome (207). Indeed, altered TGF-β1 signaling is associated with mutations in fibrillin-1 and ADAMTSL2 (a disintegrin and metalloproteinase with thrombospondin motifs) with geleophysic and acromicric dysplasias (208,209), both of which share strikingly similar phenotypes to ML-II. The connective-tissue disorder, Loeys-Dietz syndrome, does not fit into these categories, but is

worth mentioning since it shares similar phenotypes with Marfan Syndrome (*see* Table 1.4). It is caused by mutations in the TGF-β1 receptor (TGFβR1 or TGFβR2) (210).

LIF: Overview of Biological Function

LIF is a secretory protein that, like TGF-β1, has multiple biological functions. Its role in cancer progression, inflammation, and differentiation has been clearly demonstrated (211). Classified in the IL-6 family, LIF shares a similar structure and fold with IL-6, IL-11, oncostatin M (OSM) ciliary neurotrophic factor (CNTF) and cardiotropin-1 (212). LIF is a highly glycosylated protein, divergent from its family members in that it contains six potential N-linked glycosylation sites—all of which are utilized (213,214). Initially, LIF was characterized by its ability to induce the differentiation of murine myeloid leukemia cultures (215,216) and has been used in the maintenance of mouse embryonic stem cells (217). Previous literature has focused on contribution of LIF in the pathogenesis of arthritis, demonstrating high production in arthritic joint tissues, including synovium and synovial fluid (212,218-220). Other reports have highlighted the role of LIF in connective tissue metabolism, where the addition of LIF resulted in the accumulation of excess osteoblasts and new bone formation (221). In a similar fashion, LIF can regulate the activity of alkaline phosphatase (222-224), a regulator of osteoblast maturation (calcification) and govern the expression of RANKL in bone remodeling (225). LIF is controlled spatially and temporally as its expression has been documented during osteogenesis in hypertrophic chondrocytes and vascular sprouts of cartilage of newborn rats, but not detected in newborn osteoclasts, adult articular chondrocytes or bone-marrow mesenchymal stem cells (221).

Altered LIF Signaling Results in Bone Defects

Due to the role of LIF in osteogenesis and connective tissue metabolism, several studies have linked LIF to bone- and connective tissue-related diseases (Table 1.4). In particular, altered LIF signaling results in the shortened long bones and flared metaphyses in Stuve-Wiedemann syndrome (226). Likewise, disruption of the LIF receptor results in bone volume reduction and osteopenia of the perinatal bone (227,228). Recently, Katherine Ponder and colleagues determined altered LIF signaling caused by accumulation of chondroitin sulfate may contribute to shortened bones in MPS VII (*see* Table 1.1), an LSD presenting with dysostosis multiplex (229). The possibility of secondary activation or suppression of LIF transcription and its dysregulation following biosynthesis has yet to be explored for other lysosomal diseases.

Concerted Roles of LIF and TGF-β1 in Bone Growth and Homeostasis

Interplay between growth factor signaling pathways in skeletal and connective tissue diseases is commonly noted. It is well established that TGF-β1 acts in concert with BMP, Wnt, Hedgehog, and parathyroid hormone-related protein (PTHrP), playing an important role in chondrocyte differentiation and homeostasis (159). Dysregulation of any of these growth factors can contribute or cause aberrant growth plate homeostasis and skeletal abnormalities. The concerted role of LIF and TGF-β1 in bone growth and homeostasis and documents evidence for the interplay between these growth factors is discussed below.

The maturation of bone and mineralization of matrix in osteoblasts is under strict control of both systemic hormones and growth factors including TGF-β, LIF, osteoclacin, PTHrP and alkaline phosphatase (193,194). Of course, mature bone formation does not solely rely on the synthesis and apposition of matrix by osteoblasts, but also maintenance and resorption by

osteoclasts (230). The concerted affect of osteoblast bone matrix formation and osteoclast resorption regulates the homeostasis of bone production. It has been suggested that TGF-β1 and LIF (described above) regulate RANKL (Receptor Activator for Nuclear Factor κ B Ligand) differentially. For example, TGF-β1 inhibition of osteoblasts (downregulation of osteoblast formation) and activation of more osteoclast production is controlled through regulation of RANKL expression (231). Osteoblasts can also inhibit osteoclastogenesis by producing a signal ligand osteoprotegrin (a decoy receptor that binds and inhibits RANKL interaction with RANK), which is enhanced in osteoblasts by high concentrations of TGF-β1 (197) and LIF (225). As one might expect, the level of osteoclast production and activation is proportional to the rate of matrix degradation. When osteoblast/clast homeostasis is disrupted (i.e. impaired matrix integrity leading to dysregulation of growth factors, improper RANKL/osteoprotegrin signaling, etc), imbalance occurs causing osteoclast hyperactivity (leading to osteoporosis and milder forms of osteopenia) or hypoactivity contributing to osteopetrosis (232). In an osteoclast cell culture model, it has been shown that activation of TGF-β1 increases LIF expression thereby reducing osteoclast apoptosis—another report demonstrating the interplay between growth factors. This finding enforces the critical necessity of strict regulation on growth factor interplay and ECM homeostasis.

Thesis Overview

Much progress has been made over the forty years since the discovery of ML-II with regard the genetics of the disease and the enzymes and proteins that constitute the Man-6-P targeting pathway. The molecular and cellular pathogenesis of ML-II, however, is still poorly

understood. Moreover, the specific Man-6-P modified proteins (lysosomal or non-lysosomal) that are affected in different tissues are not known. These factors remain an obstacle to the development of therapies for this disease. Based on their roles in bone and cartilage homeostasis, we hypothesized that loss of mannose phosphorylation on LIF and TGF-β1 contributes to ML-II pathogenesis. This thesis explores this hypothesis by first investigating the extent of mannose phosphorylation on both LIF and latent TGF-β1 and defining the mechanisms whereby this modification controls their biological activity. In the case of LIF, evidence is presented that this secreted molecule is a highly mannose phosphorylated protein and a bona-fide substrate for GlcNAc-1-phosphotransferase. Mannose phosphorylation on LIF is shown to be important for controlling its extracellular levels by multiple mechanisms - recapture at the cell surface and direct targeting to lysosomes, leading to its extracellular accumulation when the extent of Man-6-P modification is reduced. Latent TGF-β1, however, receives only a marginal amount of mannose phosphorylation. This negligible amount of mannose phosphorylation is not an indicator of its impact in ML-II, as the present data suggests that this growth factor is dysregulated as a result of altered solubility and reduced activation and signaling. The mechanisms that underlie the impaired latent TGF-β1 bioavailability as well as how the loss of LIF mannose phosphorylation contributes to ML-II pathogenesis are discussed.

References

1. Kornfeld, R., and Kornfeld, S. (1985) *Annu Rev Biochem* **54**, 631-664
2. Varki, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., and Etzler, M.E. (2009) *Essentials In Glycobiology*, 2nd Edition ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
3. Brooks, S. A., Dwek, M.V., and Schumacher, U. (2002) *Functional and Molecular Glycobiology*, Bios Scientific Publishers: Oxford
4. Kim, P. S., and Arvan, P. (1991) *J Biol Chem* **266**, 12412-12418
5. de Virgilio, M., Kitzmuller, C., Schwaiger, E., Klein, M., Kreibich, G., and Ivessa, N. E. (1999) *Mol Biol Cell* **10**, 4059-4073
6. Machamer, C. E., Florkiewicz, R. Z., and Rose, J. K. (1985) *Mol Cell Biol* **5**, 3074-3083
7. Gallagher, P. J., Henneberry, J. M., Sambrook, J. F., and Gething, M. J. (1992) *J Virol* **66**, 7136-7145
8. Helenius, A., and Aebi, M. (2004) *Annu Rev Biochem* **73**, 1019-1049
9. Wasley, L. C., Timony, G., Murtha, P., Stoudemire, J., Dorner, A. J., Caro, J., Krieger, M., and Kaufman, R. J. (1991) *Blood* **77**, 2624-2632
10. Park, E. I., Mi, Y., Unverzagt, C., Gabius, H. J., and Baenziger, J. U. (2005) *Proc Natl Acad Sci U S A* **102**, 17125-17129
11. Roseman, D. S., and Baenziger, J. U. (2000) *Proc Natl Acad Sci U S A* **97**, 9949-9954
12. Guan, J. L., Machamer, C. E., and Rose, J. K. (1985) *Cell* **42**, 489-496
13. Winterpacht, A., Hilbert, K., Stelzer, C., Schweikardt, T., Decker, H., Segerer, H., Spranger, J., and Zabel, B. (2000) *Physiol Genomics* **2**, 9-12
14. Glozman, R., Okiyoneda, T., Mulvihill, C. M., Rini, J. M., Barriere, H., and Lukacs, G. L. (2009) *J Cell Biol* **184**, 847-862
15. Ohtsubo, K., Takamatsu, S., Minowa, M. T., Yoshida, A., Takeuchi, M., and Marth, J. D. (2005) *Cell* **123**, 1307-1321
16. Rouiller, D. G., Sharon, N., McElduff, A., Podskalny, J. M., and Gorden, P. (1986) *Endocrinology* **118**, 1159-1165
17. Ryan, S. O., Bonomo, J. A., Zhao, F., and Cobb, B. A. (2011) *J Exp Med* **208**, 1041-1053
18. Guo, H. B., Johnson, H., Randolph, M., Nagy, T., Blalock, R., and Pierce, M. (2010) *Proc Natl Acad Sci U S A* **107**, 21116-21121
19. Gu, J., and Taniguchi, N. (2008) *Cell Adh Migr* **2**, 243-245
20. Dagia, N. M., Gadhoum, S. Z., Knoblauch, C. A., Spencer, J. A., Zamiri, P., Lin, C. P., and Sackstein, R. (2006) *Nat Med* **12**, 1185-1190
21. Geng, F., Shi, B. Z., Yuan, Y. F., and Wu, X. Z. (2004) *Cell Res* **14**, 423-433
22. Wang, X., Inoue, S., Gu, J., Miyoshi, E., Noda, K., Li, W., Mizuno-Horikawa, Y., Nakano, M., Asahi, M., Takahashi, M., Uozumi, N., Ihara, S., Lee, S. H., Ikeda, Y., Yamaguchi, Y., Aze, Y., Tomiyama, Y., Fujii, J., Suzuki, K., Kondo, A., Shapiro, S. D., Lopez-Otin, C., Kuwaki, T., Okabe, M., Honke, K., and Taniguchi, N. (2005) *Proc Natl Acad Sci U S A* **102**, 15791-15796
23. Leroy, J. G., and Demars, R. I. (1967) *Science* **157**, 804-806
24. Sly, W. S. (2000) *J Clin Invest* **105**, 563-564

25. Kelly, T. E., Thomas, G. H., Taylor, H. A., Jr., McKusick, V. A., Sly, W. S., Glaser, J. H., Robinow, M., Luzzatti, L., Espiritu, C., Feingold, M., Bull, M. J., Ashenhurst, E. M., and Ives, E. J. (1975) *Johns Hopkins Med J* **137**, 156-175
26. Fratantoni, J. C., Hall, C. W., and Neufeld, E. F. (1968) *Science* **162**, 570-572
27. Hickman, S., and Neufeld, E. F. (1972) *Biochem Biophys Res Commun* **49**, 992-999
28. Natowicz, M. R., Chi, M. M., Lowry, O. H., and Sly, W. S. (1979) *Proc Natl Acad Sci U S A* **76**, 4322-4326
29. Hickman, S., Shapiro, L. J., and Neufeld, E. F. (1974) *Biochem Biophys Res Commun* **57**, 55-61
30. Reitman, M. L., and Kornfeld, S. (1981) *J Biol Chem* **256**, 11977-11980
31. Waheed, A., Hasilik, A., and von Figura, K. (1981) *J Biol Chem* **256**, 5717-5721
32. Couso, R., Lang, L., Roberts, R. M., and Kornfeld, S. (1986) *J Biol Chem* **261**, 6326-6331
33. Do, H., Lee, W. S., Ghosh, P., Hollowell, T., Canfield, W., and Kornfeld, S. (2002) *J Biol Chem* **277**, 29737-29744
34. Rohrer, J., and Kornfeld, R. (2001) *Mol Biol Cell* **12**, 1623-1631
35. Dahms, N. M., Olson, L. J., and Kim, J. J. (2008) *Glycobiology* **18**, 664-678
36. Kornfeld, S. (1987) *Faseb J* **1**, 462-468
37. Bao, M., Booth, J. L., Elmendorf, B. J., and Canfield, W. M. (1996) *J Biol Chem* **271**, 31437-31445
38. Paik, K. H., Song, S. M., Ki, C. S., Yu, H. W., Kim, J. S., Min, K. H., Chang, S. H., Yoo, E. J., Lee, I. J., Kwan, E. K., Han, S. J., and Jin, D. K. (2005) *Hum Mutat* **26**, 308-314
39. Tiede, S., Storch, S., Lubke, T., Henrissat, B., Bargal, R., Raas-Rothschild, A., and Braulke, T. (2005) *Nat Med* **11**, 1109-1112
40. Raas-Rothschild, A., Bargal, R., Goldman, O., Ben-Asher, E., Groener, J. E., Toutain, A., Stemmer, E., Ben-Neriah, Z., Flusser, H., Beemer, F. A., Penttinen, M., Olender, T., Rein, A. J., Bach, G., and Zeigler, M. (2004) *J Med Genet* **41**, e52
41. Raas-Rothschild, A., Cormier-Daire, V., Bao, M., Genin, E., Salomon, R., Brewer, K., Zeigler, M., Mandel, H., Toth, S., Roe, B., Munnich, A., and Canfield, W. M. (2000) *J Clin Invest* **105**, 673-681
42. Kollmann, K., Pohl, S., Marschner, K., Encarnacao, M., Sakwa, I., Tiede, S., Poorthuis, B. J., Lubke, T., Muller-Loennies, S., Storch, S., and Braulke, T. (2010) *Eur J Cell Biol* **89**, 117-123
43. Kudo, M., Bao, M., D'Souza, A., Ying, F., Pan, H., Roe, B. A., and Canfield, W. M. (2005) *J Biol Chem* **280**, 36141-36149
44. Kudo, M., Brem, M. S., and Canfield, W. M. (2006) *Am J Hum Genet* **78**, 451-463
45. Lee, W. S., Payne, B. J., Gelfman, C. M., Vogel, P., and Kornfeld, S. (2007) *J Biol Chem* **282**, 27198-27203
46. Qian, Y., Lee, I., Lee, W. S., Qian, M., Kudo, M., Canfield, W. M., Lobel, P., and Kornfeld, S. (2010) *J Biol Chem* **285**, 3360-3370
47. Marschner, K., Kollmann, K., Schweizer, M., Braulke, T., and Pohl, S. (2011) *Science* **333**, 87-90
48. Pohl, S., Tiede, S., Marschner, K., Encarnacao, M., Castrichini, M., Kollmann, K., Muschol, N., Ullrich, K., Muller-Loennies, S., and Braulke, T. (2010) *J Biol Chem* **285**, 23936-23944

49. Encarnacao, M., Kollmann, K., Trusch, M., Braulke, T., and Pohl, S. (2011) *J Biol Chem* **286**, 5311-5318
50. Lee, W. S., Kang, C., Drayna, D., and Kornfeld, S. (2011) *J Biol Chem*
51. Boonen, M., Vogel, P., Platt, K. A., Dahms, N., and Kornfeld, S. (2009) *Mol Biol Cell* **20**, 4381-4389
52. Nishikawa, A., Gregory, W., Frenz, J., Cacia, J., and Kornfeld, S. (1997) *J Biol Chem* **272**, 19408-19412
53. Cuozzo, J. W., and Sahagian, G. G. (1994) *J Biol Chem* **269**, 14490-14496
54. Cuozzo, J. W., Tao, K., Cygler, M., Mort, J. S., and Sahagian, G. G. (1998) *J Biol Chem* **273**, 21067-21076
55. Cuozzo, J. W., Tao, K., Wu, Q. L., Young, W., and Sahagian, G. G. (1995) *J Biol Chem* **270**, 15611-15619
56. Warner, J. B., Thalhauser, C., Tao, K., and Sahagian, G. G. (2002) *J Biol Chem* **277**, 41897-41905
57. Nishikawa, A., Nanda, A., Gregory, W., Frenz, J., and Kornfeld, S. (1999) *J Biol Chem* **274**, 19309-19315
58. Steet, R., Lee, W. S., and Kornfeld, S. (2005) *J Biol Chem* **280**, 33318-33323
59. Baranski, T. J., Cantor, A. B., and Kornfeld, S. (1992) *J Biol Chem* **267**, 23342-23348
60. Cantor, A. B., Baranski, T. J., and Kornfeld, S. (1992) *J Biol Chem* **267**, 23349-23356
61. Cantor, A. B., and Kornfeld, S. (1992) *J Biol Chem* **267**, 23357-23363
62. Qian, M., Sleat, D. E., Zheng, H., Moore, D., and Lobel, P. (2008) *Mol Cell Proteomics* **7**, 58-70
63. Ghosh, P., Dahms, N. M., and Kornfeld, S. (2003) *Nat Rev Mol Cell Biol* **4**, 202-212
64. Kornfeld, S. (1986) *J Clin Invest* **77**, 1-6
65. Olson, L. J., Yammani, R. D., Dahms, N. M., and Kim, J. J. (2004) *EMBO J* **23**, 2019-2028
66. Brown, J., Delaine, C., Zaccheo, O. J., Siebold, C., Gilbert, R. J., van Boxel, G., Denley, A., Wallace, J. C., Hassan, A. B., Forbes, B. E., and Jones, E. Y. (2008) *EMBO J* **27**, 265-276
67. Brown, J., Esnouf, R. M., Jones, M. A., Linnell, J., Harlos, K., Hassan, A. B., and Jones, E. Y. (2002) *EMBO J* **21**, 1054-1062
68. Kim, J. J., Olson, L. J., and Dahms, N. M. (2009) *Curr Opin Struct Biol* **19**, 534-542
69. Hancock, M. K., Yammani, R. D., and Dahms, N. M. (2002) *J Biol Chem* **277**, 47205-47212
70. Reddy, S. T., Chai, W., Childs, R. A., Page, J. D., Feizi, T., and Dahms, N. M. (2004) *J Biol Chem* **279**, 38658-38667
71. Chavez, C. A., Bohnsack, R. N., Kudo, M., Gotschall, R. R., Canfield, W. M., and Dahms, N. M. (2007) *Biochemistry* **46**, 12604-12617
72. Bohnsack, R. N., Song, X., Olson, L. J., Kudo, M., Gotschall, R. R., Canfield, W. M., Cummings, R. D., Smith, D. F., and Dahms, N. M. (2009) *J Biol Chem* **284**, 35215-35226
73. Marron-Terada, P. G., Hancock, M. K., Haskins, D. J., and Dahms, N. M. (2000) *Biochemistry* **39**, 2243-2253
74. Lemansky, P., Fester, I., Smolenova, E., Uhlander, C., and Hasilik, A. (2007) *J Leukoc Biol* **81**, 1149-1158

75. Lemansky, P., Gerecitano-Schmidek, M., Das, R. C., Schmidt, B., and Hasilik, A. (2003) *J Leukoc Biol* **74**, 542-550
76. Leksa, V., Godar, S., Cebecauer, M., Hilgert, I., Breuss, J., Weidle, U. H., Horejsi, V., Binder, B. R., and Stockinger, H. (2002) *J Biol Chem* **277**, 40575-40582
77. Dahms, N. M., Wick, D. A., and Brzycki-Wessell, M. A. (1994) *J Biol Chem* **269**, 3802-3809
78. Kang, J. X., Li, Y., and Leaf, A. (1997) *Proc Natl Acad Sci U S A* **94**, 13671-13676
79. Kang, J. X., Bell, J., Beard, R. L., and Chandraratna, R. A. (1999) *Cell Growth Differ* **10**, 591-600
80. Han, Y., Luan, B., Sun, M., Guo, L., Guo, P., Tao, J., Deng, J., Wu, G., Liu, S., Yan, C., and Li, S. (2011) *J Mol Cell Cardiol* **50**, 723-730
81. Kreiling, J. L., Byrd, J. C., Deisz, R. J., Mizukami, I. F., Todd, R. F., 3rd, and MacDonald, R. G. (2003) *J Biol Chem* **278**, 20628-20637
82. Owada, M., and Neufeld, E. F. (1982) *Biochem Biophys Res Commun* **105**, 814-820
83. Waheed, A., Pohlmann, R., Hasilik, A., von Figura, K., van Elsen, A., and Leroy, J. G. (1982) *Biochem Biophys Res Commun* **105**, 1052-1058
84. Furbish, F. S., Steer, C. J., Barranger, J. A., Jones, E. A., and Brady, R. O. (1978) *Biochem Biophys Res Commun* **81**, 1047-1053
85. Ullrich, K., Basner, R., Gieselmann, V., and Von Figura, K. (1979) *Biochem J* **180**, 413-419
86. Ullrich, K., and von Figura, K. (1979) *Biochem J* **182**, 245-247
87. Furbish, F. S., Krett, N. L., Barranger, J. A., and Brady, R. O. (1980) *Biochem Biophys Res Commun* **95**, 1768-1774
88. Glickman, J. N., and Kornfeld, S. (1993) *J Cell Biol* **123**, 99-108
89. Gabel, C. A., Goldberg, D. E., and Kornfeld, S. (1983) *Proc Natl Acad Sci U S A* **80**, 775-779
90. Braun, M., Waheed, A., and von Figura, K. (1989) *Embo J* **8**, 3633-3640
91. Waheed, A., Gottschalk, S., Hille, A., Krentler, C., Pohlmann, R., Braulke, T., Hauser, H., Geuze, H., and von Figura, K. (1988) *Embo J* **7**, 2351-2358
92. Peters, C., Braun, M., Weber, B., Wendland, M., Schmidt, B., Pohlmann, R., Waheed, A., and von Figura, K. (1990) *Embo J* **9**, 3497-3506
93. Reczek, D., Schwake, M., Schroder, J., Hughes, H., Blanz, J., Jin, X., Brondyk, W., Van Patten, S., Edmunds, T., and Saftig, P. (2007) *Cell* **131**, 770-783
94. Canuel, M., Korkidakis, A., Konnyu, K., and Morales, C. R. (2008) *Biochem Biophys Res Commun* **373**, 292-297
95. Canuel, M., Bhattacharyya, N., Balbis, A., Yuan, L., and Morales, C. R. (2009) *Exp Cell Res* **315**, 240-247
96. Tsuji, A., and Suzuki, Y. (1987) *Biochem Int* **15**, 945-952
97. Diment, S., Leech, M. S., and Stahl, P. D. (1988) *J Biol Chem* **263**, 6901-6907
98. Rijnboutt, S., Aerts, H. M., Geuze, H. J., Tager, J. M., and Strous, G. J. (1991) *J Biol Chem* **266**, 4862-4868
99. Zhu, Y., and Conner, G. E. (1994) *J Biol Chem* **269**, 3846-3851
100. Hiraiwa, M. (1999) *Cell Mol Life Sci* **56**, 894-907
101. Lukong, K. E., Landry, K., Elsliger, M. A., Chang, Y., Lefrancois, S., Morales, C. R., and Pshezhetsky, A. V. (2001) *J Biol Chem* **276**, 17286-17290

102. Morreau, H., Galjart, N. J., Willemsen, R., Gillemans, N., Zhou, X. Y., and d'Azzo, A. (1992) *J Biol Chem* **267**, 17949-17956
103. van der Spoel, A., Bonten, E., and d'Azzo, A. (1998) *Embo J* **17**, 1588-1597
104. Ramirez-Mata, A., Michalak, C., Mendoza-Hernandez, G., Leon-Del-Rio, A., and Gonzalez-Noriega, A. (2011) *Exp Cell Res* **317**, 2364-2373
105. Spranger, J. (2001) *J Inherit Metab Dis* **24**, 117-126
106. Neufeld, E. F. a. M. (2001) The Mucopolysaccharidoses. in *The Metabolic and Molecular Bases of Inherited Disease*, 8th Ed., McGraw Hill. pp 3421-3452
107. Muenzer, J. (1986) *Adv Pediatr* **33**, 269-302
108. Muenzer, J. (2004) *J Pediatr* **144**, S27-34
109. Cathey, S. S., Leroy, J. G., Wood, T., Eaves, K., Simensen, R. J., Kudo, M., Stevenson, R. E., and Friez, M. J. (2010) *J Med Genet* **47**, 38-48
110. Cathey, S. S., Kudo, M., Tiede, S., Raas-Rothschild, A., Braulke, T., Beck, M., Taylor, H. A., Canfield, W. M., Leroy, J. G., Neufeld, E. F., and McKusick, V. A. (2008) *Am J Med Genet A* **146A**, 512-513
111. Kornfeld, S., and Sly, W. S. (2001) I-Cell Disease and Pseudo-Hurler Polydystrophy: Disorders of Lysosomal Enzyme Phosphorylation and Localization. in *The Metabolic and Molecular Bases of Inherited Disease*, 8th Ed., McGraw Hill. pp 3469-3482
112. Leroy, J., Cathey, S., and Friez M.J. (2008) Mucolipidosis II. in *GeneReviews [Internet]* (Pagon, R. A., Bird, T.D., Dolan, C.R., and Stephens, K. ed., Seatle
113. Patriquin, H. B., Kaplan, P., Kind, H. P., and Giedion, A. (1977) *AJR Am J Roentgenol* **129**, 37-43
114. Kabra, M., Gulati, S., Kaur, M., Sharma, J., Singh, A., Chopra, V., Menon, P. S., and Kalra, V. (2000) *Indian J Pediatr* **67**, 683-687
115. Kawashima, I., Ohsawa, M., Fukushige, T., Nagayama, Y., Niida, Y., Kotani, M., Tajima, Y., Kanekura, T., Kanzaki, T., and Sakuraba, H. (2007) *Clin Chim Acta* **378**, 142-146
116. Otomo, T., Higaki, K., Nanba, E., Ozono, K., and Sakai, N. (2011) *J Biol Chem* **286**, 35283-35290
117. Otomo, T., Higaki, K., Nanba, E., Ozono, K., and Sakai, N. (2009) *Mol Genet Metab* **98**, 393-399
118. Boonen, M., van Meel, E., Oorschot, V., Klumperman, J., and Kornfeld, S. (2011) *Mol Biol Cell* **22**, 1135-1147
119. Nogami, H., Oohira, A., Suzuki, F., and Tsuda, K. (1981) *Pediatr Res* **15**, 330-334
120. Martin, J. J., Leroy, J. G., Farriaux, J. P., Fontaine, G., Desnick, R. J., and Cabello, A. (1975) *Acta Neuropathol* **33**, 285-305
121. Kobayashi, H., Takahashi-Fujigasaki, J., Fukuda, T., Sakurai, K., Shimada, Y., Nomura, K., Ariga, M., Ohashi, T., Eto, Y., Otomo, T., Sakai, N., and Ida, H. (2011) *Mol Genet Metab* **102**, 170-175
122. Kerr, D. A., Memoli, V. A., Cathey, S. S., and Harris, B. T. (2011) *Arch Pathol Lab Med* **135**, 503-510
123. Pazzaglia, U. E., Beluffi, G., Castello, A., Coci, A., and Zatti, G. (1992) *Clin Orthop Relat Res*, 283-290
124. Mazrier, H., Van Hoeven, M., Wang, P., Knox, V. W., Aguirre, G. D., Holt, E., Wiemelt, S. P., Sleeper, M. M., Hubler, M., Haskins, M. E., and Giger, U. (2003) *J Hered* **94**, 363-373

125. Hubler, M., Haskins, M. E., Arnold, S., Kaser-Hotz, B., Bosshard, N. U., Briner, J., Spycher, M. A., Gitzelmann, R., Sommerlade, H. J., and von Figura, K. (1996) *J Small Anim Pract* **37**, 435-441
126. Gelfman, C. M., Vogel, P., Issa, T. M., Turner, C. A., Lee, W. S., Kornfeld, S., and Rice, D. S. (2007) *Invest Ophthalmol Vis Sci* **48**, 5221-5228
127. Flanagan-Steet, H., Sias, C., and Steet, R. (2009) *Am J Pathol* **175**, 2063-2075
128. Vogel, P., Payne, B. J., Read, R., Lee, W. S., Gelfman, C. M., and Kornfeld, S. (2009) *Vet Pathol* **46**, 313-324
129. Petrey, A. C., Flanagan-Steet, F., Johnson,S., Fan, X., De la Rosa, M., Haskins, M., Nairn, A.V., Moremen, K.W., and Steet, R. (2011) *Disease Models and Mechanisms*
130. Chen, H. H., Lan, J. L., Shu, S. G., Chen, D. Y., and Lan, H. H. (2004) *J Formos Med Assoc* **103**, 715-720
131. Brik, R., Mandel, H., Aizin, A., Goldscher, D., Ziegler, M., Bialik, V., and Berant, M. (1993) *J Rheumatol* **20**, 133-136
132. Terashima, Y., Tsuda, K., Isomura, S., Sugiura, Y., and Nogami, H. (1975) *Am J Dis Child* **129**, 1083-1090
133. Homandberg, G. A. (1999) *Front Biosci* **4**, D713-730
134. Homandberg, G. A., Wen, C., and Hui, F. (1998) *Osteoarthritis Cartilage* **6**, 231-244
135. Li, Z., Yasuda, Y., Li, W., Bogyo, M., Katz, N., Gordon, R. E., Fields, G. B., and Bromme, D. (2004) *J Biol Chem* **279**, 5470-5479
136. Lorenzo, P., Bayliss, M. T., and Heinegard, D. (2004) *Matrix Biol* **23**, 381-391
137. Poole, A. R., Nelson, F., Dahlberg, L., Tchetina, E., Kobayashi, M., Yasuda, T., Laverty, S., Squires, G., Kojima, T., Wu, W., and Billinghurst, R. C. (2003) *Biochem Soc Symp*, 115-123
138. Squires, G. R., Okouneff, S., Ionescu, M., and Poole, A. R. (2003) *Arthritis Rheum* **48**, 1261-1270
139. Turker, G., Hatun, S., Gulleroglu, K., Cizmecioglu, F., Gokalp, A. S., and Cokun, T. (2005) *Turk J Pediatr* **47**, 376-378
140. Unger, S., Paul, D. A., Nino, M. C., McKay, C. P., Miller, S., Sochett, E., Braverman, N., Clarke, J. T., Cole, D. E., and Superti-Furga, A. (2005) *Eur J Pediatr* **164**, 236-243
141. Robinson, C., Baker, N., Noble, J., King, A., David, G., Sillence, D., Hofman, P., and Cundy, T. (2002) *J Inherit Metab Dis* **25**, 681-693
142. van Meel, E., Boonen, M., Zhao, H., Oorschot, V., Ross, F. P., Kornfeld, S., and Klumperman, J. (2011) *Traffic* **12**, 912-924
143. Saul, R. A., Proud, V., Taylor, H. A., Leroy, J. G., and Spranger, J. (2005) *Am J Med Genet A* **135**, 328-332
144. Wilcox, W. R., Wenger, D. A., Lachman, R. S., and Rimoin, D. L. (2005) *Am J Med Genet A* **135**, 333
145. Babcock, D. S., Bove, K. E., Hug, G., Dignan, P. S., Soukup, S., and Warren, N. S. (1986) *Pediatr Radiol* **16**, 32-39
146. Purchio, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L. E., Kovacina, K. S., Roth, R. A., and Marquardt, H. (1988) *J Biol Chem* **263**, 14211-14215
147. Blanchard, F., Duplomb, L., Raher, S., Vusio, P., Hoflack, B., Jacques, Y., and Godard, A. (1999) *J Biol Chem* **274**, 24685-24693

148. Blanchard, F., Raher, S., Duplomb, L., Vusio, P., Pitard, V., Taupin, J. L., Moreau, J. F., Hoflack, B., Minvielle, S., Jacques, Y., and Godard, A. (1998) *J Biol Chem* **273**, 20886-20893
149. Lee, S. J., and Nathans, D. (1988) *J Biol Chem* **263**, 3521-3527
150. Faust, P. L., Chirgwin, J. M., and Kornfeld, S. (1987) *J Cell Biol* **105**, 1947-1955
151. Ikushima, H., Munakata, Y., Ishii, T., Iwata, S., Terashima, M., Tanaka, H., Schlossman, S. F., and Morimoto, C. (2000) *Proc Natl Acad Sci U S A* **97**, 8439-8444
152. Dennis, P. A., and Rifkin, D. B. (1991) *Proc Natl Acad Sci U S A* **88**, 580-584
153. Saris, J. J., Derkx, F. H., Lamers, J. M., Saxena, P. R., Schalekamp, M. A., and Danser, A. H. (2001) *Hypertension* **37**, 710-715
154. van Kesteren, C. A., Danser, A. H., Derkx, F. H., Dekkers, D. H., Lamers, J. M., Saxena, P. R., and Schalekamp, M. A. (1997) *Hypertension* **30**, 1389-1396
155. Ghahary, A., Tredget, E. E., and Shen, Q. (1999) *J Cell Physiol* **180**, 61-70
156. Massague, J. (1998) *Annu Rev Biochem* **67**, 753-791
157. Kusakabe, M., Cheong, P. L., Nikfar, R., McLennan, I. S., and Koishi, K. (2008) *J Cell Biochem* **103**, 311-320
158. Dubois, C. M., Laprise, M. H., Blanchette, F., Gentry, L. E., and Leduc, R. (1995) *J Biol Chem* **270**, 10618-10624
159. Yang, T., Mendoza-Londono, R., Lu, H., Tao, J., Li, K., Keller, B., Jiang, M. M., Shah, R., Chen, Y., Bertin, T. K., Engin, F., Dabovic, B., Rifkin, D. B., Hicks, J., Jamrich, M., Beaudet, A. L., and Lee, B. (2010) *J Clin Invest* **120**, 2474-2485
160. Miyazono, K., Thyberg, J., and Heldin, C. H. (1992) *J Biol Chem* **267**, 5668-5675
161. Gleizes, P. E., Beavis, R. C., Mazzieri, R., Shen, B., and Rifkin, D. B. (1996) *J Biol Chem* **271**, 29891-29896
162. Saharinen, J., Taipale, J., and Keski-Oja, J. (1996) *EMBO J* **15**, 245-253
163. Chen, Y., Dabovic, B., Annes, J. P., and Rifkin, D. B. (2002) *FEBS Lett* **517**, 277-280
164. Gibson, M. A., Hatzinikolas, G., Davis, E. C., Baker, E., Sutherland, G. R., and Mecham, R. P. (1995) *Mol Cell Biol* **15**, 6932-6942
165. Miyazono, K., Olofsson, A., Colosetti, P., and Heldin, C. H. (1991) *EMBO J* **10**, 1091-1101
166. Kantola, A. K., Keski-Oja, J., and Koli, K. (2008) *Exp Cell Res* **314**, 2488-2500
167. Rifkin, D. B. (2005) *J Biol Chem* **280**, 7409-7412
168. Annes, J. P., Munger, J. S., and Rifkin, D. B. (2003) *J Cell Sci* **116**, 217-224
169. Nunes, I., Gleizes, P. E., Metz, C. N., and Rifkin, D. B. (1997) *J Cell Biol* **136**, 1151-1163
170. Kojima, S., Nara, K., and Rifkin, D. B. (1993) *J Cell Biol* **121**, 439-448
171. Verderio, E., Gaudry, C., Gross, S., Smith, C., Downes, S., and Griffin, M. (1999) *J Histochem Cytochem* **47**, 1417-1432
172. Shweke, N., Boulos, N., Jouanneau, C., Vandermeersch, S., Melino, G., Dussaule, J. C., Chatziantoniou, C., Ronco, P., and Boffa, J. J. (2008) *Am J Pathol* **173**, 631-642
173. Huang, L., Haylor, J. L., Fisher, M., Hau, Z., El Nahas, A. M., Griffin, M., and Johnson, T. S. (2010) *Nephrol Dial Transplant* **25**, 3897-3910
174. Fesus, L., and Piacentini, M. (2002) *Trends Biochem Sci* **27**, 534-539
175. Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y., and Saito, Y. (2003) *Biochem J* **373**, 793-803
176. Mishra, S., and Murphy, L. J. (2004) *J Biol Chem* **279**, 23863-23868

177. Belkin, A. M. (2011) *FEBS J*
178. Kiraly, R., Demeny, M., and Fesus, L. (2011) *FEBS J*
179. Wilhelmus, M. M., Verhaar, R., Andringa, G., Bol, J. G., Cras, P., Shan, L., Hoozemans, J. J., and Drukarch, B. (2011) *Brain Pathol* **21**, 130-139
180. Verhaar, R., Drukarch, B., Bol, J. G., Jongenelen, C. A., Musters, R. J., and Wilhelmus, M. M. (2011) *Neurobiol Dis*
181. Kuo, T. F., Tatsukawa, H., and Kojima, S. (2011) *FEBS J*
182. D'Eletto, M., Farrace, M. G., Falasca, L., Reali, V., Oliverio, S., Melino, G., Griffin, M., Fimia, G. M., and Piacentini, M. (2009) *Autophagy* **5**, 1145-1154
183. Akar, U., Ozpolat, B., Mehta, K., Fok, J., Kondo, Y., and Lopez-Berestein, G. (2007) *Mol Cancer Res* **5**, 241-249
184. Rossin, F., D'Eletto, M., Macdonald, D., Farrace, M. G., and Piacentini, M. (2011) *Amino Acids*
185. Ientile, R., Caccamo, D., and Griffin, M. (2007) *Amino Acids* **33**, 385-394
186. Zemskov, E. A., Mikhailenko, I., Hsia, R. C., Zaritskaya, L., and Belkin, A. M. (2011) *PLoS One* **6**, e19414
187. van den Akker, J., van Weert, A., Afink, G., Bakker, E. N., van der Pol, E., Boing, A. N., Nieuwland, R., and Vanbavel, E. (2011) *Amino Acids*
188. Scarpellini, A., Germack, R., Lortat-Jacob, H., Muramatsu, T., Billett, E., Johnson, T., and Verderio, E. A. (2009) *J Biol Chem* **284**, 18411-18423
189. Balklava, Z., Verderio, E., Collighan, R., Gross, S., Adams, J., and Griffin, M. (2002) *J Biol Chem* **277**, 16567-16575
190. Dallas, S. L., Keene, D. R., Bruder, S. P., Saharinen, J., Sakai, L. Y., Mundy, G. R., and Bonewald, L. F. (2000) *J Bone Miner Res* **15**, 68-81
191. Massam-Wu, T., Chiu, M., Choudhury, R., Chaudhry, S. S., Baldwin, A. K., McGovern, A., Baldock, C., Shuttleworth, C. A., and Kielty, C. M. (2010) *J Cell Sci* **123**, 3006-3018
192. Chen, Q., Sivakumar, P., Barley, C., Peters, D. M., Gomes, R. R., Farach-Carson, M. C., and Dallas, S. L. (2007) *J Biol Chem* **282**, 26418-26430
193. Lawrence, D. A., Pircher, R., and Jullien, P. (1985) *Biochem Biophys Res Commun* **133**, 1026-1034
194. Jenkins, G. (2008) *Int J Biochem Cell Biol* **40**, 1068-1078
195. Yang, Z., Mu, Z., Dabovic, B., Jurukovski, V., Yu, D., Sung, J., Xiong, X., and Munger, J. S. (2007) *J Cell Biol* **176**, 787-793
196. Schultz-Cherry, S., Chen, H., Mosher, D. F., Misenheimer, T. M., Krutzsch, H. C., Roberts, D. D., and Murphy-Ullrich, J. E. (1995) *J Biol Chem* **270**, 7304-7310
197. Schultz-Cherry, S., Lawler, J., and Murphy-Ullrich, J. E. (1994) *J Biol Chem* **269**, 26783-26788
198. Schultz-Cherry, S., Ribeiro, S., Gentry, L., and Murphy-Ullrich, J. E. (1994) *J Biol Chem* **269**, 26775-26782
199. Hyytiainen, M., Penttinen, C., and Keski-Oja, J. (2004) *Crit Rev Clin Lab Sci* **41**, 233-264
200. Hornebeck, W., Emonard, H., Maquart, F. X., and Bellon, G. (2003) *J Soc Biol* **197**, 25-30
201. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T. A. (2011) *Nature* **474**, 343-349

202. Saito, T., Kinoshita, A., Yoshiura, K., Makita, Y., Wakui, K., Honke, K., Niikawa, N., and Taniguchi, N. (2001) *J Biol Chem* **276**, 11469-11472
203. Kinoshita, A., Saito, T., Tomita, H., Makita, Y., Yoshida, K., Ghadami, M., Yamada, K., Kondo, S., Ikegawa, S., Nishimura, G., Fukushima, Y., Nakagomi, T., Saito, H., Sugimoto, T., Kamegaya, M., Hisa, K., Murray, J. C., Taniguchi, N., Niikawa, N., and Yoshiura, K. (2000) *Nat Genet* **26**, 19-20
204. Dabovic, B., Levasseur, R., Zambuto, L., Chen, Y., Karsenty, G., and Rifkin, D. B. (2005) *Bone* **37**, 25-31
205. Urban, Z., Hucthagowder, V., Schurmann, N., Todorovic, V., Zilberberg, L., Choi, J., Sens, C., Brown, C. W., Clark, R. D., Holland, K. E., Marble, M., Sakai, L. Y., Dabovic, B., Rifkin, D. B., and Davis, E. C. (2009) *Am J Hum Genet* **85**, 593-605
206. Ramirez, F., and Dietz, H. C. (2007) *Curr Opin Genet Dev* **17**, 252-258
207. Loeys, B. L., Gerber, E. E., Riegert-Johnson, D., Iqbal, S., Whiteman, P., McConnell, V., Chillakuri, C. R., Macaya, D., Coucke, P. J., De Paepe, A., Judge, D. P., Wigley, F., Davis, E. C., Mardon, H. J., Handford, P., Keene, D. R., Sakai, L. Y., and Dietz, H. C. (2010) *Sci Transl Med* **2**, 23ra20
208. Le Goff, C., Mahaut, C., Wang, L. W., Allali, S., Abhyankar, A., Jensen, S., Zylberberg, L., Collod-Beroud, G., Bonnet, D., Alanay, Y., Brady, A. F., Cordier, M. P., Devriendt, K., Genevieve, D., Kiper, P. O., Kitoh, H., Krakow, D., Lynch, S. A., Le Merrer, M., Megarbane, A., Mortier, G., Odent, S., Polak, M., Rohrbach, M., Sillence, D., Stolte-Dijkstra, I., Superti-Furga, A., Rimoin, D. L., Topouchian, V., Unger, S., Zabel, B., Bole-Feysot, C., Nitschke, P., Handford, P., Casanova, J. L., Boileau, C., Apte, S. S., Munnich, A., and Cormier-Daire, V. (2011) *Am J Hum Genet* **89**, 7-14
209. Le Goff, C., and Cormier-Daire, V. (2009) *Pediatr Endocrinol Rev* **6**, 418-423
210. Van Hemelrijk, C., Renard, M., and Loeys, B. (2010) *Curr Opin Cardiol* **25**, 546-551
211. Metcalf, D. (2003) *Stem Cells* **21**, 5-14
212. Sims, N. A., and Walsh, N. C. (2010) *BMB Rep* **43**, 513-523
213. Sasai, K., Aikawa, J., Saburi, S., Tojo, H., Tanaka, S., Ogawa, T., and Shiota, K. (1998) *J Biochem* **124**, 999-1003
214. Aikawa, J., Sato, E., Kyuwa, S., Sasai, K., Shiota, K., and Ogawa, T. (1998) *Biosci Biotechnol Biochem* **62**, 1318-1325
215. Gough, N. M., Gearing, D. P., King, J. A., Willson, T. A., Hilton, D. J., Nicola, N. A., and Metcalf, D. (1988) *Proc Natl Acad Sci U S A* **85**, 2623-2627
216. Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A., and Metcalf, D. (1987) *EMBO J* **6**, 3995-4002
217. Williams, R. L., Hilton, D. J., Pease, S., Willson, T. A., Stewart, C. L., Gearing, D. P., Wagner, E. F., Metcalf, D., Nicola, N. A., and Gough, N. M. (1988) *Nature* **336**, 684-687
218. Lotz, M., Moats, T., and Villiger, P. M. (1992) *J Clin Invest* **90**, 888-896
219. Kapoor, M., Martel-Pelletier, J., Lajeunesse, D., Pelletier, J. P., and Fahmi, H. (2011) *Nat Rev Rheumatol* **7**, 33-42
220. Upadhyay, A., Sharma, G., Kivivuori, S., Raye, W. S., Zabihi, E., Carroll, G. J., and Jazayeri, J. A. (2009) *Cytokine* **46**, 332-338
221. Grimaud, E., Blanchard, F., Charrier, C., Gouin, F., Redini, F., and Heymann, D. (2002) *Cytokine* **20**, 224-230
222. Van Beek, E., Van der Wee-Pals, L., van de Ruit, M., Nijweide, P., Papapoulos, S., and Lowik, C. (1993) *J Bone Miner Res* **8**, 191-198

223. Rodan, S. B., Wesolowski, G., Hilton, D. J., Nicola, N. A., and Rodan, G. A. (1990) *Endocrinology* **127**, 1602-1608
224. Noda, M., Vogel, R. L., Hasson, D. M., and Rodan, G. A. (1990) *Endocrinology* **127**, 185-190
225. Palmqvist, P., Persson, E., Conaway, H. H., and Lerner, U. H. (2002) *J Immunol* **169**, 3353-3362
226. Dagoneau, N., Scheffer, D., Huber, C., Al-Gazali, L. I., Di Rocco, M., Godard, A., Martinovic, J., Raas-Rothschild, A., Sigaudy, S., Unger, S., Nicole, S., Fontaine, B., Taupin, J. L., Moreau, J. F., Superti-Furga, A., Le Merrer, M., Bonaventure, J., Munnich, A., Legeai-Mallet, L., and Cormier-Daire, V. (2004) *Am J Hum Genet* **74**, 298-305
227. Ware, C. B., Horowitz, M. C., Renshaw, B. R., Hunt, J. S., Liggitt, D., Koblar, S. A., Gliniak, B. C., McKenna, H. J., Papayannopoulou, T., Thoma, B., and et al. (1995) *Development* **121**, 1283-1299
228. Sims, N. A., Jenkins, B. J., Quinn, J. M., Nakamura, A., Glatt, M., Gillespie, M. T., Ernst, M., and Martin, T. J. (2004) *J Clin Invest* **113**, 379-389
229. Metcalf, J. A., Zhang, Y., Hilton, M. J., Long, F., and Ponder, K. P. (2009) *Mol Genet Metab* **97**, 202-211
230. Yavropoulou, M. P., and Yovos, J. G. (2008) *J Musculoskelet Neuronal Interact* **8**, 204-216
231. Janssens, K., ten Dijke, P., Janssens, S., and Van Hul, W. (2005) *Endocr Rev* **26**, 743-774
232. Bartl, R., Frisch, B., von Tresckow, E., and Bartel, C. (2007) Disorders of Bone. in *Bisphosphonates in Medical Practice*, Springer Berlin Hiedelberg. pp 23-32
233. Leroy, J., Cathey, S., and Friez M.J. (2008) Mucolipidosis III Alpha/Beta in *GeneReviews [Internet]* (Pagon, R. A., Bird, T.D., Dolan, C.R., and Stephens, K. ed., Seattle
234. Wells, R. G., and Discher, D. E. (2008) *Sci Signal* **1**, pe13
235. ten Dijke, P., and Arthur, H. M. (2007) *Nat Rev Mol Cell Biol* **8**, 857-869

Figures

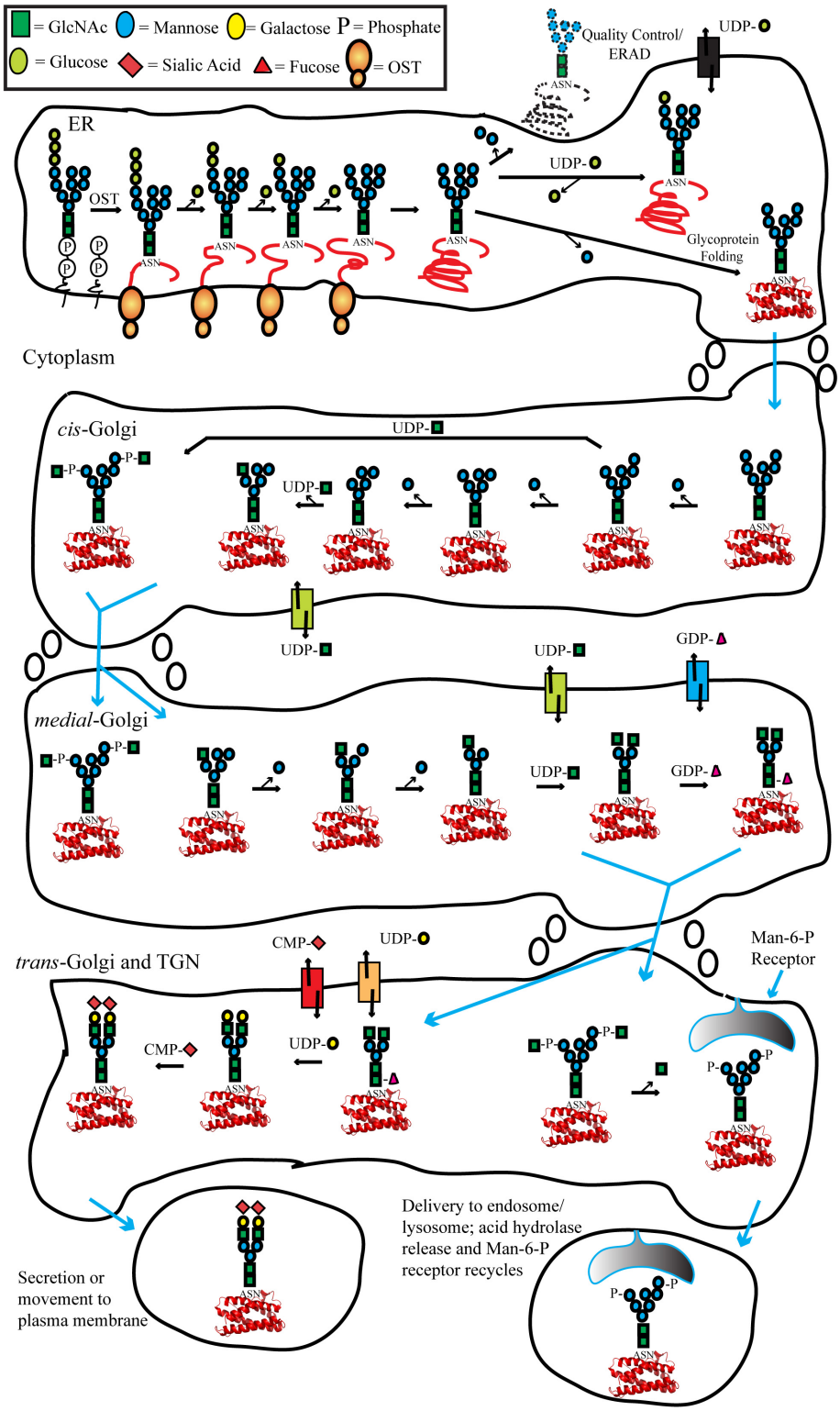

= GlcNAc
= Mannose
= Galactose
P = Phosphate
= Glucose
= Sialic Acid
= Fucose
= OST
Quality Control/
ERAD
UDP-
ASN
ER
OST
P
Glycoprotein
Folding
Cytoplasm
cis-Golgi
UDP-
GDP-
medial-Golgi
trans-Golgi and TGN
CMP-
Man-6-P
Receptor
Secretion or
movement to
plasma membrane
Delivery to endosome/
lysosome; acid hydrolase
release and Man-6-P
receptor recycles

Figure 1.1. Biosynthesis and maturation of an N-glycan on a newly synthesized protein. Protein N-linked glycosylation is initiated with the addition of a chitobiose core on a dolichol phosphate intermediate followed by the addition of nine mannose and three glucose residues. The 14 sugar-containing oligosaccharide is subsequently transferred *en bloc* onto growing polypeptide chains. The transfer is catalyzed by the membrane-bound enzyme complex called the oligosaccharyl transferase or OST. After the new addition of the branched oligosaccharide, glycoproteins undergo rapid modification and trimming including removal of two glucose residues. The remaining glucose plays an important role in protein folding—binding to the ER chaperone lectins, calnexin and calreticulin. Glycoproteins have limited time to fold appropriately before they are targeted for ER associated Degradation (ERAD). The ER quality control event marks one on the many functions of N-linked glycosylation. Once the third glucose and a mannose residue are removed, these proteins are packaged and transported to the *cis*-Golgi. In the Golgi, proteins undergo a variety of post-translational modifications and are sorted for targeting to their appropriate destination. Proteins that are designated for secretory granules and the plasma membrane receive additional mannose removal and reconstruction of their oligosaccharide chains to more complex carbohydrate structures—containing sialic acid. Lysosomal proteins, however, receive a GlcNAc-1-Phosphate at the C-6 position of mannose on their oligosaccharides in the *cis*-Golgi and elude further trimming by the Golgi-mannosidases. The GlcNAc-1-Phosphate diester linkage is further processed in the *trans*-Golgi, ultimately leading to a phosphomonoester called mannose-6-phosphate that is important for sorting lysosomal proteins to the lysosome. Adapted from *Essentials in Glycobiology* (2).

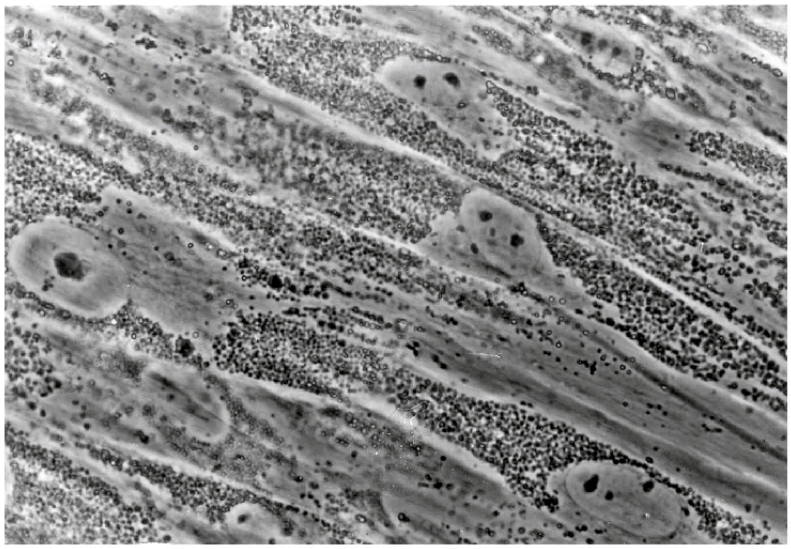


Figure 1.2. ML-III alpha/beta patient skin fibroblasts viewed by a contrast light microscope. The picture shows cytoplasmic dense granular inclusions that are very similar to that seen in ML-II fibroblasts. These dense cytoplasmic inclusions have been determined by electron microscope to be swollen lysosomal organelles. Taken from Leroy J. et al. (112)

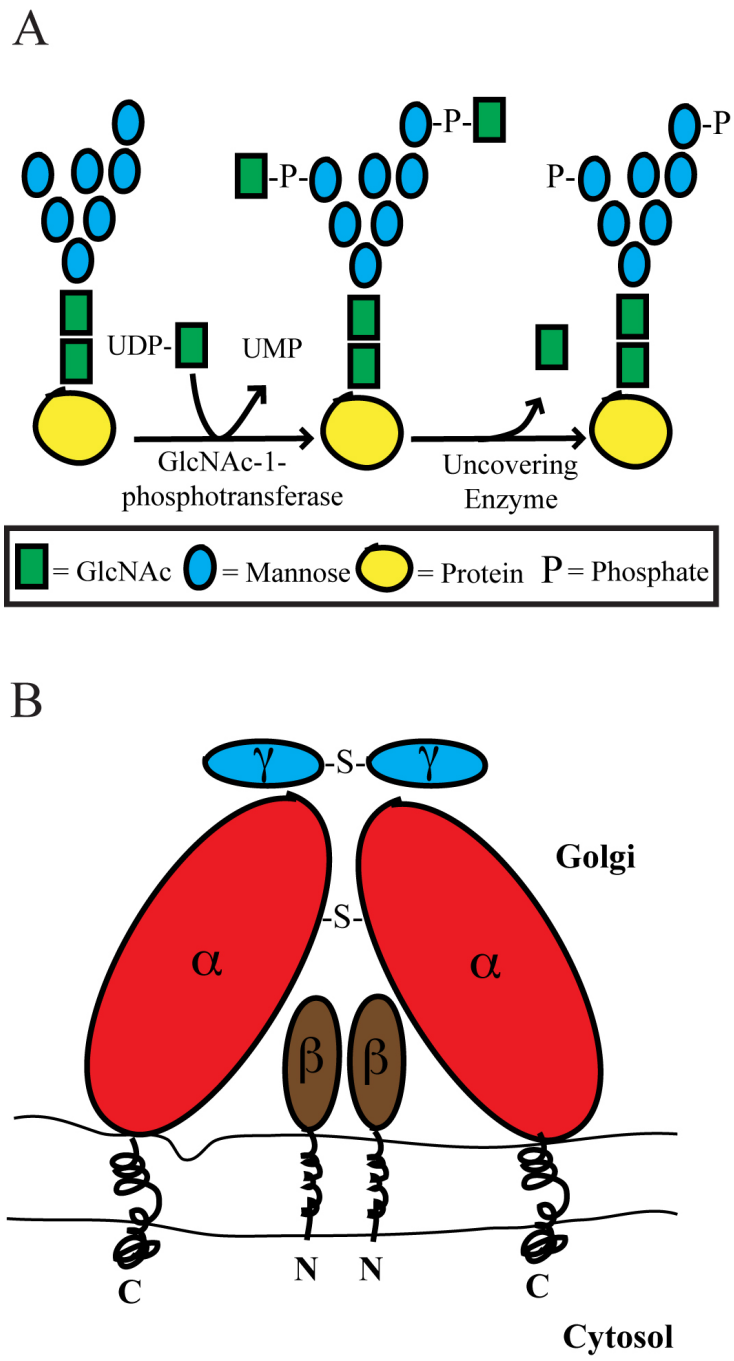


Figure 1.3. Man-6-P biosynthesis is catalyzed by GlcNAc-1-phosphostransferase.
A schematic illustration of mannose-6-phosphate biosynthesis (**A**) catalyzed by the GlcNAc-1-phosphotransferase (**B**). The structure of the GlcNAc-1-phosphotransferase consists of α2β2 catalytic subunits and a γ2, the phosphorylation enhancing subunit (**B**). The α-subunits are type I transmembrane proteins while the β-subunits are type II transmembrane. The "-S-" denotes a disulphide bridge.

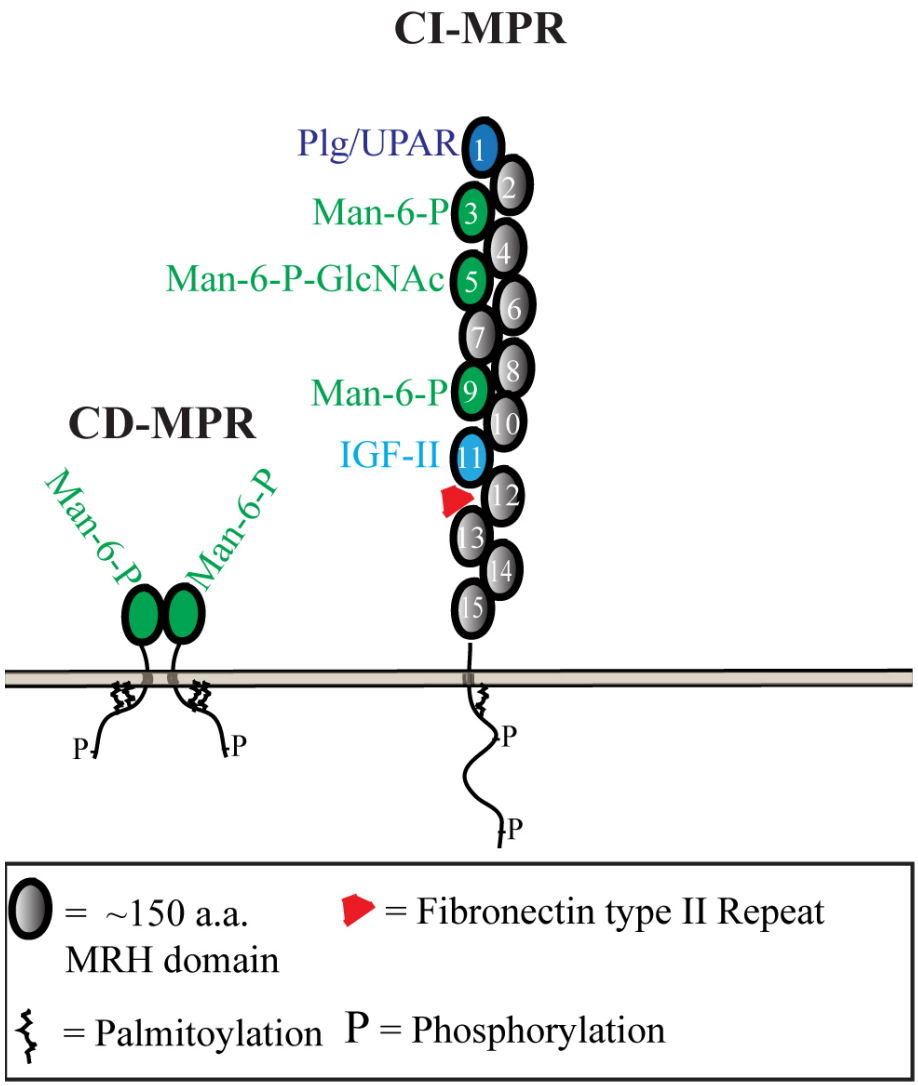


Figure 1.4. Structural illustration of the Mannose-6-phosphate receptors, CD-MPR (left) and CI-MPR (right). The M6PRs are transmembrane glycoproteins that are further anchored to the cell membrane by palmitoylation. In addition, these proteins are phosphorylated on their cytoplasmic tail. The CD-MPR is a dimer with single Man-6-P binding sites within each domain. The CI-MPR, however, is a multi-subunit complex consisting of 15 highly homologous domains. Domains 3, 5, and 9 (shown in green) all bind Man-6-P, but domain 5 has a binding preference for phosphodiesters. The CI-MPR is a multi-functional and multi-ligand binding receptor. It is known to bind to urokinase plasminogen activator receptor (uPAR) and plasminogen (Plg) through domain 1 (dark blue), while IGF-II associates with domain 11 (light blue). Interestingly, domain 13 has a fibronectin II insert (red block) that may interact with collagen, however this has not been determined. Adapted from Dahms, N. et al (35).

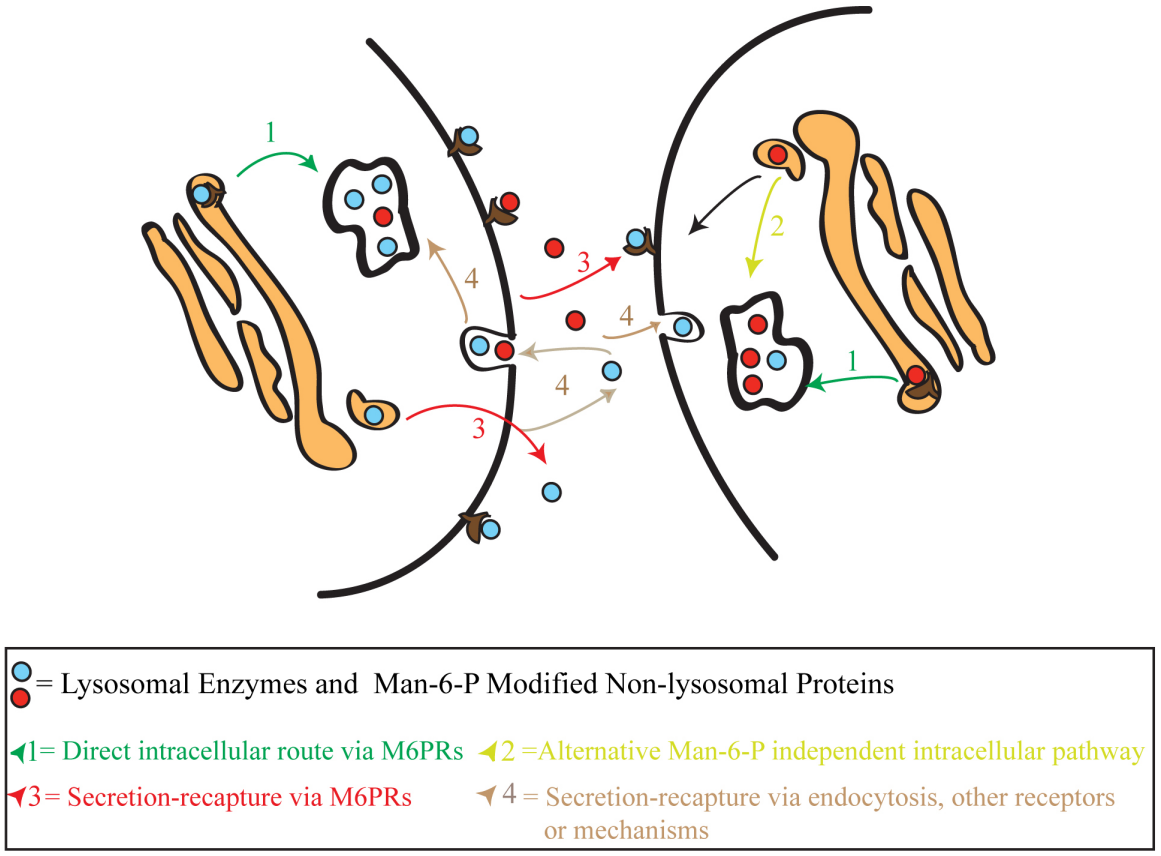


Figure 1.5. Man-6-P-dependent and alternative sorting pathways to the lysosome. Lysosomal proteins are targeted to the lysosome by multiple mechanisms. They can traffic to the lysosome via a direct intracellular route using the mannose-6-phosphate pathway (green) or by other means such as alternative sorting receptors (i.e., LIMP-2) or membrane tethering (yellow). If these lysosomal proteins end up outside the cell, they can be recaptured and internalized by the cell surface M6PRs (red) or brought into the lysosome via endocytosis and other mechanisms (brown).

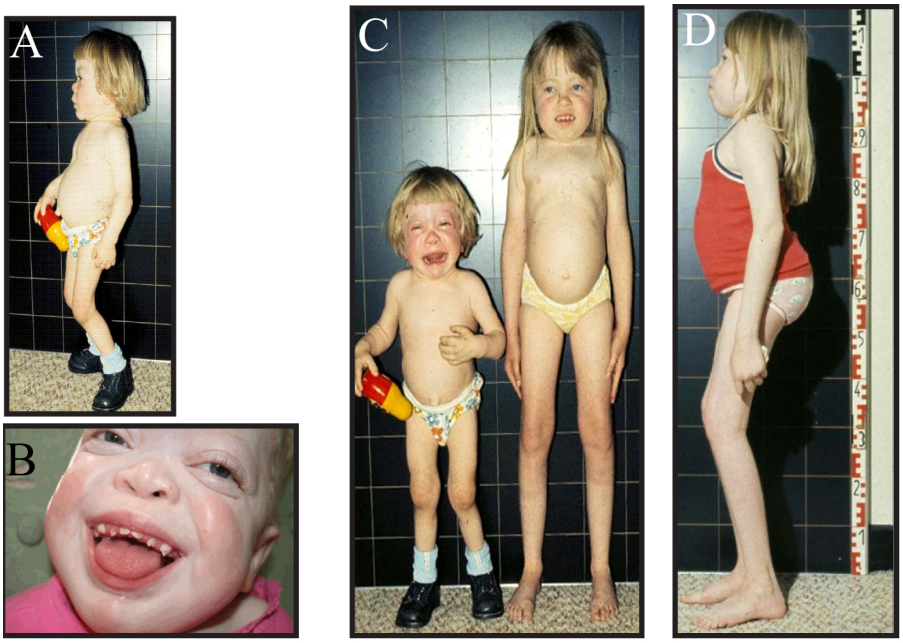


Figure 1.6. Clinical profiles of children with ML-II and ML-III. (**A**) A 3-year old child with ML-II presenting with severe bone dysplasia, developmental delay, joint stiffness and umbilical hernia. (**B**) A close-up view of the coarse facial features and gingival hypertrophy from a 5-year old child with ML-II. Notice the thick and waxy skin of the cheeks and around the eyes. (**C**) A 3-year old (same as (**A**)) and a 9.5-year old child with ML-II and ML-III α/β, respectively. The ML-III α/β disease is much less severe. Note the claw-like hands of the ML-II child and the longer hand of the ML-III patient. The ML-III α/β patient is not dwarfed like that observed in ML-II, however, the linear growth is substantially stunted with severe lumbar and spine abnormalities (**D**), similar to the bone dysplasias associated with ML-II (*see* Table 1.3). Taken from Leroy J. et al. (112,233).

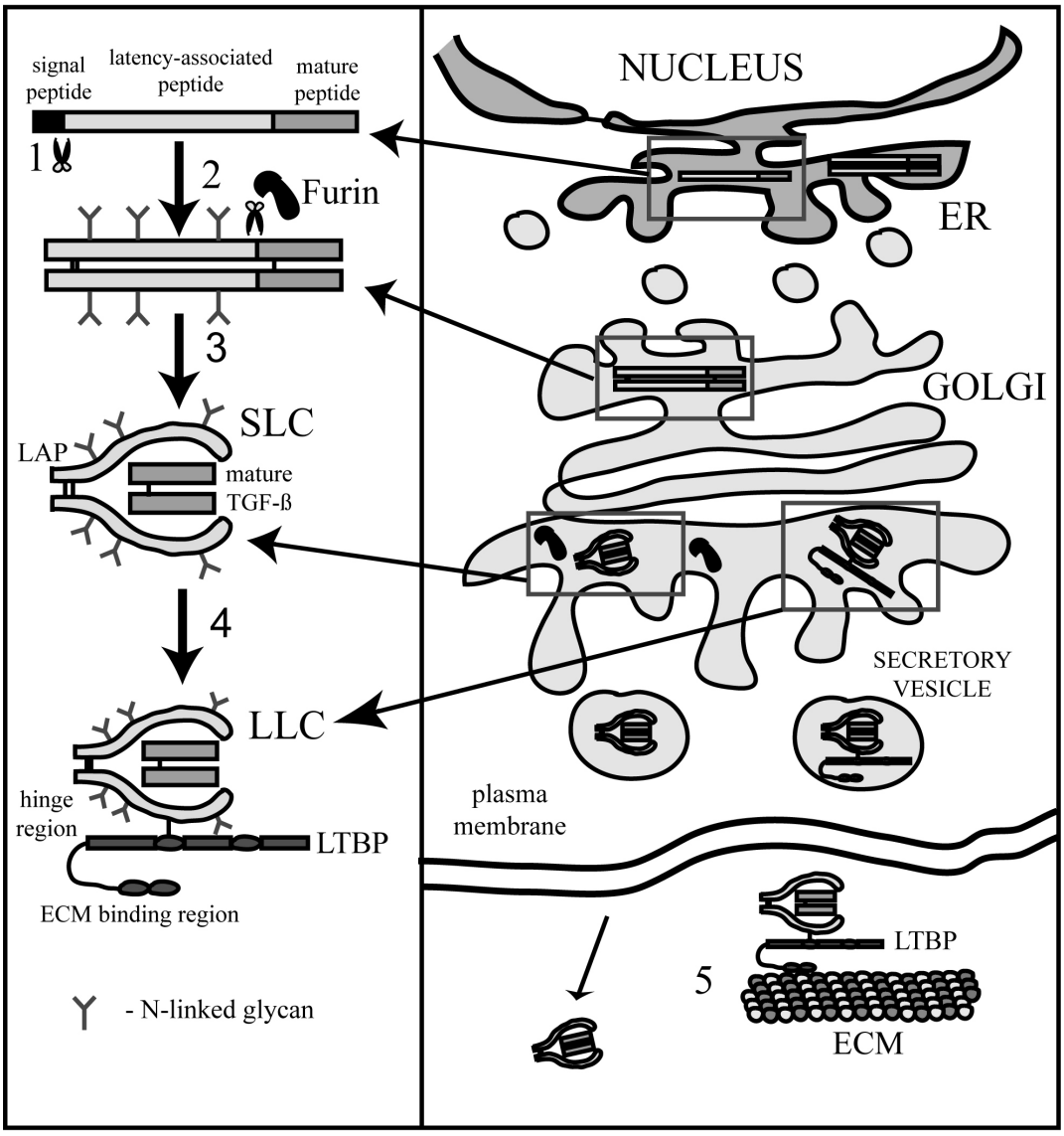


Figure 1.7. The processing and secretion of latent TGF-β. TGF-β is synthesized as a 390 amino acid precursor protein in the ER, where it undergoes co-translational modification, removal of its signal peptide and dimerization with another precursor molecule. In the Golgi, the precursor TGF-β growth factor is processed by furin convertase and mature TGF-β ligand re-associates non-covalently with its latency associated peptide (LAP) to form a latent TGF-β complex (small latent complex; SLC). This complex has been shown to be retained in the Golgi, slowly secreted, or more rapidly secreted by binding to its latent TGF-β binding protein (LTBP) forming the large latent complex (LLC). After it is secreted, it is either activated by one of several different mechanisms or sequestered in the ECM. Adapted from Janssens, et al. (231).

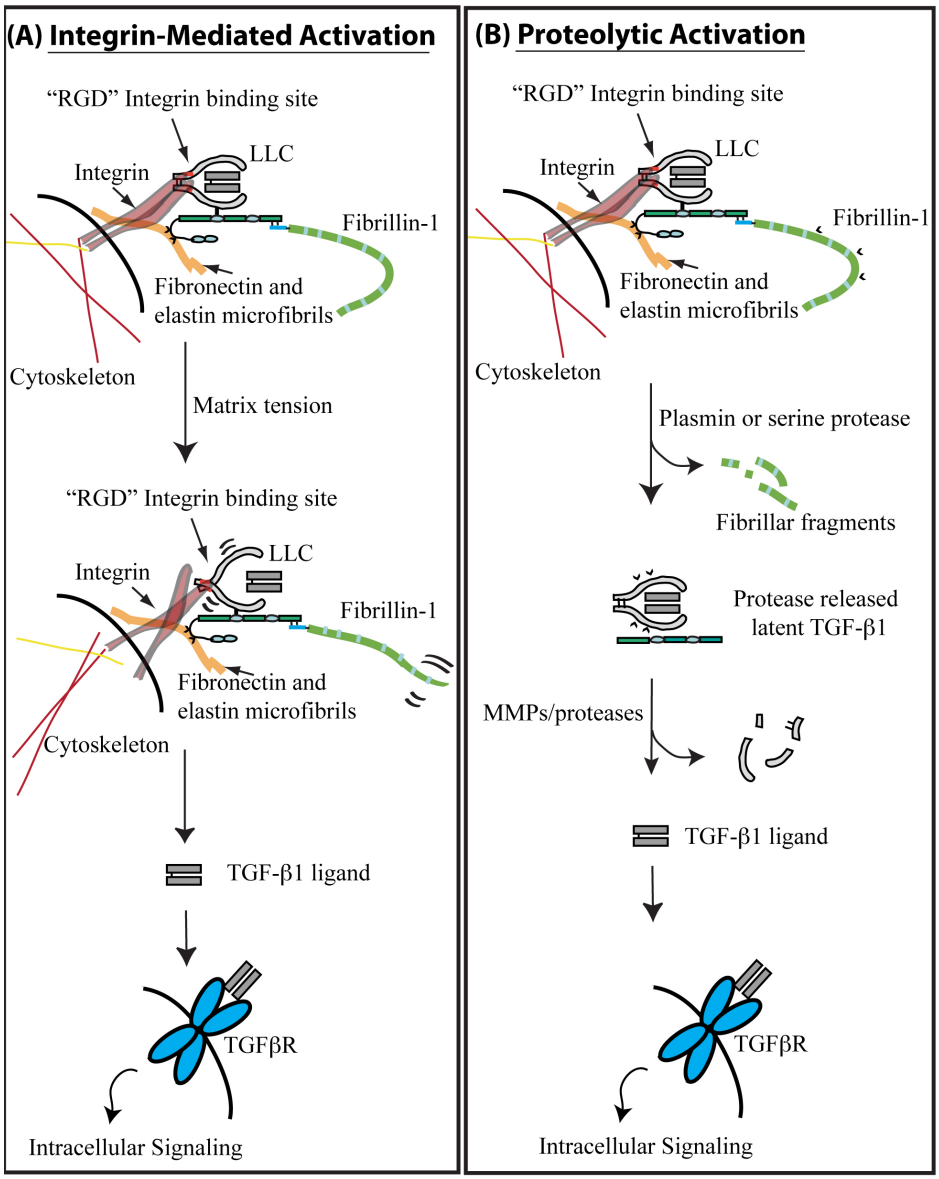


Figure 1.8. Two modes of activation of latent TGF-β1 in the extracellular matrix. A schematic illustration of latent TGF-β1 cross-linking, deposition and interaction with the several matrix components such as fibrillin-1 and fibronectin/elastin microfibrils via LTBP and integrins through an "RGD" site on LAP. (**A**) TGF-β1 can be activated by integrin-mediated mechanisms, which includes matrix tension and mechanical stretching of the ECM in association with cytoskeletal restructuring (234). The mechanical stress on the ECM results in a conformational change of LAP and liberation of the mature ligand. (**B**) TGF-β1 can be activated by proteolytic release from the ECM. Proteases such as plasmin, cathepsins, or elastases can release the LLC from the ECM by proteolyzing the "hinge region" of the LLC and by degradation of fibrillin-1 microfibrils. Once removed from the ECM, the truncated LLC is susceptible to more protease degradation and liberation of the mature ligand. Adapted and modified from ten Dijke, et al. (235).

Table 1.1 Lysosomal Storage Disorders

Disease	Defective protein	Main Storage materials
Mucopolysaccharidoses (MPS)		
MPS-I (Hurler, Sheie, H/S)*	α-Iduronidase	Dermatan & heparan sulfate
MPS-II (Hunter's Syndrome)	Iduronate-2-sulfatase	Dermatan & heparan sulfate
MPS-IIIA (Sanfillipo)	Heparan-N-sulfatase	Heparan Sulfate
MPS-IIIB (Sanfillipo)	N-Acetyl-a-glucosamindase	Heparan Sulfate
MPS-IIIC (Sanfillipo)	Acetyl-CoA:a-glucosamide N-acetyltransferase	Heparan Sulfate
MPS-IIID (Sanfillipo)	GlcNAc-6-sulfatase	Heparan Sulfate
Morquio-A Disease	GalNAc-6-sulfate sulfatase	Keratan & chondroitin-6-sulfate
Morquio-B Disease	β-Galactosidase*	Keratan sulfate
MPS VI (Maroteaux-Lamy)	GalNAc-4-sulfatase* (arylsulfatase B)	Dermatan sulfate
MPS VII (Sly) *	β-Glucoronidase*	Heparan, dermatan, chondriotin-4- & -6-sulfates
MPS IX	Hyaluronidase	Hyaluronan
Oligosaccharidosis and glycoproteinosi		
Pompe (glycogen storage disease type II)	α-Glucosidase*	Glycogen
Defective integral membrane proteins		
Cystinosis	Cystinosin	Cystine
Danon Disease	LAMP-2*	Cytoplasmic debri and glycogen
Infantile sialic-acid storage disease & Salla Disease	Sialin	Sialic acid
Mucolipidosis IV (ML-IV)	Mucolipin-1	Lipids and acid mucopolysaccharides
Niemann-Pick C (NPC)	NPC1 and 2	Cholesterol and sphingolipids
Sphingolipidoses		
Fabry	α-Galactosidase A	Globotriasylceramide & blood-group-B substances
Gaucher	β-Glucosidase*	Glucosylceramide
	Saposin-C activator	Glucosylceramide
Neimann-Pick A & B	Sphingomyelinase	Sphingomyelin
Sphingolipid-activator deficiency	Sphingolipid activator*	Glycolipids
GM1 gangliosidosis	β-Galactosidase*	GM1 ganglioside
GM2 gangliosidosis		
Tay-Sachs Disease	β-Hexosaminidase A	GM2 ganglioside & glycolipid
Sandhoff Disease	β-Hexosamindase A & B	GM2 ganglioside & glycolipid
GM-2 activator deficiency	GM2-activator protein	GM2 ganglioside & glycolipid
Other		
Galactosialidosis	Cathepsin A*	Sialyloligosaccharides
Mucolipidosis II/III*	GlcNAc-1-phosphotransferase*	Oligosaccharides, muco-polysaccharides & lipids
Multiple sulfatase deficiency	Ca-formylglycine-generating enzyme	Sulfatides
Neuronal ceroid lipofuscinosis	CLN1 (protein palmitoyl-	Lipidated thioesters
NCL1 (Batten Disease)	thioesterase-1)	
NCL2 (Batten Disease)	CLN2 (tripeptidyl amino-peptidase-1)	Subunit c of the mitochondrial ATP synthase
NCL3 (Batten Disease)	Arginine transporter	Subunit c of the mitochondrial ATP synthase

* Denotes diseases or enzymes discussed in text

Adapted from Futerman, A.H., et al., Nature Reviews, (2004), vol. (5), 554-565

Table 1.2 Nomenclature for ML-II and ML-III disease

Disorder	Affected Gene	Previous Nomenclature
ML-II α/β	*GNPTAB*	ML-II, I-cell disease
ML-III α/β	*GNPTAB*	ML-IIIA, Pseudo-Hurler polydystrophy
ML-III γ	*GNPTG*	ML-IIIC, ML-III variant

Adapted from Kerr, D.A., et al., Arch Pathol Lab Med. (April 2011). Vol.(135), 5-3-510.

Table 1.3 Bone and Cartilage Clinical Features in ML-II disease

Kyphoscoliosis
Wedging of vertal bodies
Proximal pointing of metacarpals

Restricted joint movement - progressive immobility

Craniofacial abnormalities

Osteopenia - loss of bone density and mineralization
Fractures - rare but likely due as a result of severe osteopenia and disorganized bone formation

Periosteal new bone formation / subperiosteal bone resorption

Tapering of proximal metaphyses / metaphyseal fragmentation

Femoral erosions

Prenatal Stage A:
Pacman dysplasia

Newborn Stage B:
Diffuse demineralization and irregular trabeculation without differentiaion between cortical and medullary zones

Skeletal abnormalities resemble those os rickets, osteomyelitis and hyperthyroidism

Late Stage C:
Dysostosis Multiplex

Adapted from Robinson, C., et al., J. Inherit. Metab. Dis. (2002). 25, 681-693.

Table 1.4 Bone and Connective Tissue Diseases

Disease	Major Defect	Major Clinical Manifestations
TGF-β1 related		
Marfan Syndrome	Mutations in Fibrillin-1; loss leads to hyperactivation of TGF-β1	Skeletal dysplasia, Scoliosis, ocular defects, heart defects,
Loeys-Dietz Syndrome (Marfan Type II)	Mutations in TGFβR1 and R2; results in constitutive activation	Skeletal dysplasia, osteoporosis, craniosyntosis, aortic aneurysms
Camurati-Engelmann	Point mutations in LAP; results in constitutive activation	Skeletal dysplasia, organomegaly
Stiff Skin Syndrome	Mutations in Fibrillin-1 (RGD); concentrated TGF-β1 activation	Thickening of skin and tissues, joint immobility, hypertrichosis
Geleophysic Dysplasia	Mutations in ADAMTSL2 and Fibrillin-1 (Domain 5); increased activation of TGF-β1	Skeletal dysplasia (dwarfism), thickened heart valves, skin thinkening and coarse facial features, and joint immobility
Acromicric Dysplasia	Mutations in *ADAMTSL2 ?* and Fibrillin-1 (Domain 5); increased activation of TGF-β1	*same as Geleophysic Dysplasia*
Arterial Tortuosity Syndrome	Defect in in GLUT10; affects glucose-dependent expression of decorin, resulting in activation of TGF-β1	Elongated and tortuous arteries and aorta, cutis laxa, hyperextensile joints
Undefined	Mutations in LTBP-4; impaired ECM deposition and increased TGF-β1 activation	Craniofacial, cutis laxa, impaired pulmonary development and pulmonary arterial stenosis
LIF related		
Stuve-Wiedemann Syndrome	Frame-shift insertion in LIFR; result in altered LIF signalling	dysplasia of long bones, scoliosis, respiratory complications, and temperature instability
MPS-VII	(Table 1.1); accumulation of chondroitin-sulfate thought to alter LIF signalling	Dysostosis multiplex, heptato-splenomegaly, thickened skin, joint immobility

CHAPTER 2: EXTENSIVE MANNOSE PHOSPHORYLATION ON LEUKEMIA INHIBITORY FACTOR (LIF) CONTROLS ITS EXTRACELLULAR LEVELS BY MULTIPLE MECHANISMS[1]

[1] Jarrod Barnes, Jae-Min Lim, Anne Godard, Frédéric Blanchard, Lance Wells and Richard Steet. 2011, *Journal of Biological Chemistry*, August 19; 28(8) 1891-16. Reprinted here with permission of the publisher.

Abstract

In addition to soluble acid hydrolases, many non-lysosomal proteins have been shown to bear mannose 6-phosphate (Man-6-P) residues. Quantification of the extent of mannose phosphorylation and the relevance to physiological function, however, remains poorly defined. In this report, we investigated the mannose phosphorylation status of leukemia inhibitory factor (LIF), a previously identified high affinity ligand for the cation-independent mannose 6-phosphate receptor (CI-MPR), and analyzed the effects of this modification on its secretion and uptake in cultured cells. When media from LIF-overexpressing cells was fractionated using a CI-MPR affinity column, 35-45% of the total LIF molecules were bound and specifically eluted with free Man-6-P confirming LIF as a bona fide Man-6-P-modified protein. Surprisingly, mass spectrometric analysis of LIF glycopeptides enriched on the CI-MPR column revealed that all six N-glycan sites could be Man-6-P-modified. The relative utilization of these sites, however, was not uniform. Analysis of glycan-deleted LIF mutants demonstrated that loss of glycans bearing the majority of Man-6-P residues lead to higher steady-state levels of secreted LIF. Using mouse embryonic stem cells (ESCs), we showed that the mannose phosphorylation of LIF mediates its internalization thereby reducing extracellular levels and stimulating ESC differentiation. Lastly, immunofluorescence experiments indicate that LIF is targeted directly to lysosomes following its biosynthesis, providing another mechanism whereby mannose phosphorylation serves to control extracellular levels of LIF. Failure to modify LIF in the context of mucolipidosis II and its subsequent accumulation in the extracellular space may have important implications for disease pathogenesis.

Background

Mannose 6-phosphate (Man-6-P)-dependent targeting of acid hydrolases to the lysosome remains one of the best-studied functions for protein-bound glycans. The biosynthesis of these residues on lysosomal hydrolases and other glycoproteins proceeds via a two-step enzymatic process. The initiating step, catalyzed by the heterohexameric enzyme UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase), involves the transfer of GlcNAc-1-phosphate to mannose residues on selected high mannose glycans. The second step requires the removal of the GlcNAc residues by the enzyme GlcNAc-1-phosphodiester α-N-acetylglucosaminidase or "uncovering enzyme" (UCE) to expose mannose 6-phosphate (Man-6-P) monoesters (1,2). These uncovered Man-6-P monoesters serve as recognition markers for the sorting of newly synthesized lysosomal proteins by the cation-dependent and the cation-independent mannose-6-phosphate receptors (CD-MPR and CI-MPR, respectively) (3,4). Recently, it has been demonstrated that covered Man-6-P residues can also bind to the CI-MPR via a distinct domain on this receptor (5,6).

The importance of Man-6-P biosynthesis to human health is highlighted by the fact that mutations in the genes encoding subunits of the GlcNAc-1-phosphotransferase enzyme (GNPTAB and GNPTG) result in the lysosomal disorders, mucolipidosis II (MLII) and mucolipidosis III (ML-III) (4,7,8). The clinical manifestations of these diseases are diverse, encompassing skeletal and craniofacial defects, impaired speech and cognitive function and recurrent lung infections (9). In the absence of GlcNAc-1-phosphotransferase activity, lysosomal hydrolases are not Man-6-P-modified and most are instead secreted from the cell. The molecular and cellular mechanisms that drive pathology in the various affected tissues of ML-II

patients and the specific Man-6-P modified proteins implicated in individual tissues are incompletely understood. Elucidating such mechanisms will provide a starting point for the development of much needed therapies for these disorders.

In addition to acid hydrolases, several non-lysosomal proteins - including latent transforming growth factor beta 1 (TGF-β1) (10), leukemia inhibitory factor (LIF) (11,12), proliferin (13), renin precursor (14), and T-cell activation antigen CD26 (15) – have been identified as Man-6-P-modified glycoproteins. However, the manner by which mannose phosphorylation mediates the normal function of these proteins and the extent to which they are modified in a physiological context is not well defined. Previous studies on the mannose phosphorylation of secreted proteins have suggested a wide range of possible functions. Man-6-P residues on latent TGF-ß are thought to facilitate its interaction with the CI-MPR at the plasma membrane and subsequent activation via the uPAR/plasminogen cascade (16-18). In the case of LIF, Man-6-P-mediated internalization by the CI-MPR limits its extracellular levels, in a manner analogous to the downregulation of IGF-II by this same receptor (11). The biological functions of LIF signaling are diverse - encompassing the coordination of cytokine expression in immune cells, maintenance and modulation of stem cells (19,20) and bone homeostasis (21,22). Moreover, LIF has been implicated in several disease states including multiple sclerosis (23), cancer (24,25) and arthritis (26). Since LIF has been shown to play a role in the homeostasis of bone and cartilage (two tissues strongly affected in ML-II), we have hypothesized that loss of Man-6-P modification on this cytokine in the context of ML-II might initiate or propagate the disease process.

To begin to address this hypothesis, we undertook a detailed biochemical analysis of the glycosylation status of human LIF (hLIF) and mouse LIF (mLIF) expressed in multiple cell lines

and interrogated the role of mannose phosphorylation on LIF in cultured cells. Our results confirmed the status of LIF as a bona fide Man-6-P-modified glycoprotein and unexpectedly revealed that any of the six N-glycans on this protein can bear Man-6-P residues. Our data further demonstrated that the mannose phosphorylation of LIF serves as a mechanism to control its extracellular levels through direct intracellular degradation of Man-6-P-modified LIF within the lysosome as well as rapid Man-6-P-mediated uptake following secretion. The implications of these novel findings in the pathogenesis of ML-II and relevance of mannose phosphorylation on other non-lysosomal proteins are discussed.

Materials and Methods

Cell lines, plasmids and reagents - CHO and HeLa cells were obtained from ATCC, while CHO with stable expression of mLIF (CHO-mLIF) was a generous gift from Dr. Steven Dalton (University of Georgia, Athens). All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) supplemented with 100 μg/mL penicillin in addition to streptomycin and maintained in a humidified 5% CO_2 atmosphere. All site-directed mutagenesis experiments were performed using the manufacturer (Agilent/Stratagene, Santa Clara, CA) protocol. Cell cultures were metabolically labeled with TRAN35S-label (MP Biomedicals, Solon, OH) in methionine- and cysteine-free DMEM (Invitrogen, USA). The CI-MPR lectin affinity column was a generous gift from Dr. Peter Lobel (Robert Wood Johnson Medical School, NJ). LIF polyclonal antiserum was obtained from Anne Godard while the anti-DsRed (mCherry) antibody was purchased from Clonetech (Mountain View, CA). Free mannose 6-phosphate and glucose 6-phosphate, alkaline phosphatase (*E.coli*

recombinant and bovine intestine), naphthyl phosphate disodium salt and Fast Red TR salt were obtained from Sigma (St. Louis, MO). Kifunensine was obtained from Cayman Chemical, while endoglycosidase H_f (Endo H_f) and N-glycosidase F (PNGase F) were purchased from New England Biolabs (Ipswich, MA). The transfection reagents Lipofectamine PLUS and Opti-MEM were obtained from Invitrogen and ESGRO® LIF (Millipore, Billerica, MA) was a kind gift from Dr. Lianchun Wang (University of Georgia, Athens).

Cell transfection and preparation of media samples - Cells were plated in a 35-mm (6-well) dish four hours before transfection at a cell density of 2.5 x 10^5 cells/mL. Transfection of cell monolayers with 1 μg of DNA from each construct was performed using the Lipofectamine PLUS system. This transfection media was replaced the next day with 1 mL of serum-free media. Samples were collected 48 hours after the initial transfection (unless otherwise indicated) and concentrated using Centricon 10 tubes to a volume of 250 μL. For the kifunensine experiments, transfection media was replaced with 1 mL of serum free media supplemented with 2μg/mL kifunensine and cells were incubated overnight at 37˚C prior to analysis.

CI-MPR Affinity Chromatography and Western blot analysis - Concentrated media samples (100 μL) and purified LIF (1μg) were diluted into 1 mL of CI-MPR column buffer (50 mM imidazole pH 6.0, 150 mM NaCl, 0.05% Triton-X 100, and 5 mM EDTA) and then applied to a 0.6 mL CI-MPR affinity column. Fractions (1 mL each) were eluted first with column buffer, then 5 mM glucose 6-phosphate and finally with 5 mM mannose 6-phosphate to elute Man-6-P-modified LIF. Protein within the column fractions was precipitated using nine volumes of 100% cold ethanol overnight at 20°C followed by centrifugation at 1,450 x g for 30 minutes.

Precipitates were washed with 90% ethanol and centrifuged for 10 minutes at 4 C and 19,400xg. Samples were solubilized by boiling in 3% SDS and analyzed by SDS-PAGE on a 12% gel. Transferred proteins were probed with an anti-LIF polyclonal antibody overnight at 4 °C followed by incubation with an anti-rabbit HRP secondary antibody and reactive bands visualized using ECL detection reagents (GE Healthcare, USA). In all cases, western blot analysis was performed multiple times to verify reproducibility. In some cases, media samples (40 µL) were first treated for 1.5 h with either 50 U endoglycosidase H (Endo H) or 15 U PNGase F at 37°C, or no enzyme (control) prior to SDS-PAGE and Western blot analysis. For alkaline phosphatase (AP) experiments, purified human LIF was treated in combination with 5U of bovine intestine and 1U of *E. coli* recombinant alkaline phosphatase in AP buffer (5 mM Tris pH 7.5, 1 mM $MgCl_2$ and 1 mM $CaCl_2$) overnight at 37 °C then analyzed as described above. For densitometry analysis, Image J 1.43u (27) was used to quantify the Western blot bands.

Purification of recombinant hLIF and isolation of unmodified and Man-6-P-modified LIF glycoforms - Recombinant human LIF was purified as previously described (28). For the isolation of the two distinct LIF glycoform pools, 20 µg of purified hLIF was fractionated over the CI-MPR column and unmodified (unbound) LIF and Man-6-P-modified LIF (5 mM Man-6-P eluted) were collected. Buffer was exchanged twice with PBS and concentrated to 50 µL using Centricon 10 (Millipore, Billerica, MA) tubes, followed by BCA protein assay (Pierce, Rockford, IL) to determined protein concentration.

CI-MPR chromatography enrichment of LIF and MS analysis of glycopeptides - Enrichment of Man-6-P-modified LIF glycopeptides was performed as described (29) with

modifications. Briefly, tryptic peptides generated following reduction and alkylation of the protein were transferred to a tube containing a 50 µL bed volume of CI-MPR and rocked for one hour at 4°C. The mixture was then placed in a microcolumn containing an additional 50 µL bed volume of CI-MPR and centrifuged for 30s at 100 x g in a bench-top microcentrifuge to remove flow-through but careful not to completely dry the resin. The flow-through was reloaded 10 times and repeated as described. Then the column was washed with a 10% EtOH in water twice and subsequently washed three times with 500 µL of PBS, once with 500 µl of PBS containing 10 mM glucose 6-phosphate and finally with 500 µL of 20 mM sodium phosphate buffer, pH 6.9, and then centrifuged again to remove excess buffer. The beads were resuspended in 100 µl of 20 mM sodium phosphate buffer, pH 6.9, containing 10 mM Man-6-P and incubated on ice for 5 min. The column was then centrifuged briefly, and the first fraction was collected. Another 100 µL of Man-6-P containing buffer was added and the column was then centrifuged to remove all water and reapplied. Finally, the column was washed with 50 µl of water to remove any remaining glycopeptides, and centrifuged again. Man-6-P fractions were dried and resuspended to a final concentration of 20 mM sodium phosphate pH 6.9 in ^{18}O-H_2O. Likewise flow through or peptide samples before column enrichment were similarly resuspended. All peptide samples were digested with PNGase F overnight at 37 °C as previously described (30). Resulting deglycosylated peptides were desalted and then analyzed on a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ-Orbitrap-XL; ThermoFisher) via nLC-MS/MS essentially as previously described (Lim et al). All full MS spectra (300-2000 m/z) were captured in the Orbitrap at 60,000 resolution and the top five peaks were analyzed in the linear ion trap following CID (dynamic exclusion of 2 for 30 seconds). MS/MS data was analyzed using Bioworks (Sequest algorithm) against the SWISS-PROT human/mouse/rat database allowing for dynamic

modifications +16 for methionine (oxidation), +57 for cysteine (carboxyamidomethylation) and +3 daltons for Asn (glycosylated Asn to Asp (^{18}O) conversion) with a parent mass tolerance of 20 ppm and stringently filtered for 2+/3+ ions having X_{corr} greater than 3.0/4.0 respectively. This filtering resulted in a false-discovery rate of less than 1% for all modified peptides. Furthermore, the position of 3 dalton addition was manually confirmed to be at the site of the formerly glycosylated Asn in the MS/MS spectra.

Metabolic ^{35}S labeling - Metabolic labeling was carried out as described previously (Steet et al., 2006).

Transfection of triple glycan-deleted LIF mutants and secretion assay - N-sequon sites were mutated using the QuickChange XL site-directed mutagenesis kit (Agilent Technology, Santa Clara, CA, USA). Triple glycan-deleted mutants (TDMs) were transfected into CHO cells (as described above) and media aliquots were either subjected to CI-MPR lectin chromatography to assess levels of mannose phosphorylation or secretion assays were performed. For secretion assays, transiently transfected TDM LIF mutants were incubated for various culture times in serum-free media, collected and concentrated, and subjected to Western blot analysis or Coomassie staining (to determine equivalent amounts of secreted protein). In addition, media samples were normalized to total secreted protein using micro BCA protein assay.

Self-renewal assay using embryonic stem cells (ESCs) - The self-renewal assay was performed as described (31) with modifications. Briefly, ESCs were seeded at clonal density and cultured for five days in ES medium (without LIF) supplemented with 10% FBS in equal

concentrations of ESGRO® (non-glycosylated) LIF (control), unmodified LIF, or isolated Man-6-P-modified LIF. Equivalent cultures were treated with 5 mM Man-6-P. The amount of LIF used was based on the ESGRO® LIF concentration needed to maintain the cells in self-renewal. For the alkaline phosphatase experiments, cells were washed and fixed in 4% PFA and AP staining was determined by incubation with staining buffer containing 8 mM $MgCl_2$, 25 mM Tris-HCl pH 9.0, 0.4 mg/mL naphthyl phosphate and 1 mg/mL Fast Red TR. In each independent experiment, one hundred fifty colonies in each well were scored and the percentage of AP positive colonies was calculated. AP positive stained colonies with tightly packed dome-shaped morphology were scored as undifferentiated while colonies with a flattened, non-uniform morphology and variable staining were scored as differentiated.

LIF, LIF-mCherry, sp-mCherry and plasmid preparation - Human LIF (full sequence including signal peptide) I.M.A.G.E. clone (clone I.D. 7939578) was obtained from Open Biosystems (Thermo Scientific, Waltham, MA, USA) and was sub-cloned, using PCR, with end primers including flanking BamHI and XbaI restriction sites. The PCR product was ligated into the pcDNA 3.1 expression vector. For LIF- mCherry, the mCherry plasmid was obtained from Clonetech (Mountain View, CA) and PCR sub-cloned with flanking EcoRI and XbaI restriction sites concurrently with LIF, adding BamHI and EcoRI restriction sites to the ends of its sequence. Both PCR products were subjected to triple ligation with pcDNA 3.1 to create the LIF-mCherry fusion. Generation of mCherry plasmid with a signal peptide (sp-mCherry) was synthesized by GeneArt (Invitrogen, USA) with flanking restriction sites (BamHI and EcoRI) and subsequently sub-cloned into pcDNA 3.1.

Immunofluorescence and Confocal Microscopy - For immunofluorescence experiments, cells were seeded on coverslips and cultured overnight prior to analyses. Cells were washed with phosphate-buffered saline, fixed in 3.7% formaldehyde in PBS and stained as described previously (32). The standard dilution buffer for both primary and secondary antibody incubations contained PBS, pH 7.4, 0.1% Triton X-100 and 1mg/mL bovine serum albumin. Confocal images were acquired using an Olympus FV1000 laser scanning microscope equipped a 60x oil immersion (numerical aperture 1.4) objective. Stacks of 0.25 μm (based on calculated optimums) optical sections were collected in the z-dimension, and subsequently collapsed into a single image (maximum intensity or z-projection) unless otherwise noted.

Results

Overexpression in CHO and HeLa cells results in the secretion of LIF that is highly Man-6-P modified. In an effort to quantify the relative amount of secreted LIF protein that contains mannose phosphorylated glycans, we subjected purified human LIF (hLIF) and culture media from CHO cells stably expressing mouse LIF (CHO-mLIF) to affinity chromatography using an immobilized CI-MPR column. Protein from each column fraction was precipitated, resolved by SDS-PAGE and LIF levels were analyzed by Western blot. As shown in Figure 2.1A, a substantial fraction of purified hLIF and CHO-mLIF bound to the CI-MPR column, indicating that one or more of its glycans contained Man-6-P residues. We noted in all cases, that the bound LIF had a consistently slightly faster electrophoretic mobility, likely due to differences in glycan processing upon modification with Man-6-P residues. Similar results were obtained when hLIF was expressed in HeLa and CHO cells by transient transfection (Figure 2.1B).

Interaction with the CI-MPR column was Man-6-P-mediated since treatment of CHO-mLIF with alkaline phosphatase prior to analysis effectively abolished column binding (Figure 2.1C). Quantitative densitometry of the Western blots showed that 35-45% of the total LIF molecules were decorated with Man-6-P-containing glycans. In general, these values were higher than earlier estimates of the mannose phosphorylation on endogenous LIF (5%) or transfected CHO cells (15%) (11). These discrepancies may reflect the different techniques utilized to gauge the level of mannose phosphorylation in the respective studies. Nevertheless, the extent of mannose phosphorylation of secreted LIF in CHO and HeLa cells is comparable to the level seen on overexpressed cathepsin D in these cell lines indicating that LIF is a good substrate for the GlcNAc-1-phosphotransferase enzyme (33).

Changes in N-glycan processing do not result in substantial differences in the extent of LIF mannose phosphorylation. We treated CHO cells that were transiently transfected with hLIF with the Golgi mannosidase I inhibitor, kifunensine to determine whether changes in N-glycan processing would alter the overall level of mannose phosphorylation. Inhibition of Golgi mannosidase I action is known to prevent the processing of Man8GlcNAc2 to Man5GlcNAc2 structures, resulting in failure of these glycans to be converted into complex-type (34). As shown in Figure 2.2A, kifunensine treatment caused all of the N-glycans on LIF to remain as high mannose type. Despite the lack of further glycan processing, no significant difference in the amount of LIF that bound to the CI-MPR column was noted (Figure 2.2B). These data indicate that the mannose phosphorylation of LIF does not appear to be influenced by changes in the processing of its N-glycans. Furthermore, it is also consistent with the observations that

GlcNAc-1-phosphotransferase is localized earlier within the Golgi than Golgi mannosidase I (35).

Site mapping of CI-MPR-enriched glycopeptides on purified hLIF reveals that all six glycans can bear Man-6-P residues. While the experiments above clearly demonstrated that LIF expressed in multiple cell culture systems is mannose phosphorylated, they do not provide any information regarding which N-glycans bear Man-6-P residues. To address this question, we undertook a mass spectrometric analysis of glycopeptides generated from purified human LIF. Our initial analysis demonstrated complete occupancy of the six potential N-glycan sites on human LIF (data not shown). The purified hLIF was next subjected to proteolysis with trypsin and the resulting glycopeptides were enriched using a CI-MPR affinity microcolumn. The expected profile of LIF glycopeptides following tryptic digestion is shown in Figure 2.3. Following collection of unbound peptides and the elution of Man-6-P bearing glycopeptides with free Man-6-P, these two pools were deglycosylated via PNGase F in O^{18}-H_20 and then analyzed by tandem mass spectrometry. Surprisingly, we found that all five glycopeptides could be enriched on the CI-MPR column, likely indicating that any of the six N-glycans on LIF can be modified with Man-6-P residues (Table 2.1). It should be noted that one of the glycopeptides contains two occupied Asn residues and thus it is possible that only one of these N-linked sites contains a Man-6-P modified glycan. Importantly, the non-glycosylated peptides were only recovered in the unbound pool demonstrating the specificity of the column (data not shown).

Decreased levels of LIF mannose phosphorylation correlate with greater extracellular accumulation. We next generated several glycan-deleted LIF mutants in order to investigate the

relative utilization of the various N-glycan sites for Man-6-P addition and determine the impact of reduced mannose phosphorylation of extracellular LIF levels. These mutants were expressed in CHO cells and secreted LIF was subjected to analysis by CI-MPR affinity chromatography and Western blot. Interestingly, we found that all of the single glycan deletion mutants exhibited the same overall level of column binding compared to wild type LIF, suggesting that loss of no single glycan substantially altered the mannose phosphorylation of the protein (S. Figure 2.1). In contrast to the single deletion mutants, the triple deletion mutants (TDMs) that were generated displayed a wide variability in the level of mannose phosphorylation, depending on which sites were mutated (Figure 2.4A). One such mutant, TDM31, failed to bind the column, suggesting a complete lack of Man-6-P-modified glycans on secreted LIF whereas another mutant TDM95 showed substantially increased CI-MPR column binding compared to fully glycosylated LIF. The column binding of a third mutant TDM56 was similar to wild type LIF. Thus, despite the fact that all glycopeptides could be identified as containing Man-6-P residues, their relative utilization was not uniform.

In order to determine the relationship between mannose phosphorylation and extracellular levels, CHO cells were transfected with the various TDM constructs and culture media was collected over a several day period. Aliquots of media, normalized to total secreted protein, were subjected to SDS-PAGE and Western blot analysis to gauge the extracellular levels of LIF (Figure 2.4B). Parallel experiments revealed no major differences in the amount of secreted proteins in the media samples as detected by Coomassie staining. Furthermore, metabolic labeling and analysis of newly synthesized cellular LIF from transfected cultures showed that equal amounts of two of these mutants TDM31 and TDM56 were being made (Figure 2.4C). Although levels of LIF were comparable for the three constructs tested at 48 h post-transfection,

clear differences in the amount of extracellular LIF can be noted at 96 h post-transfection. Analysis of the Western blots with the PNGase F-treated samples by densitometry (Figure 2.4D) revealed that, on average, TDM 31 and 56 were present at higher levels compared to TDM 95 (2.25 ± 0.12 and 1.44 ± 0.61 fold, respectively). Although the mechanism is unclear, we consistently observed a PNGase F-resistant form of TDM95 in these experiments. The reduced levels of TDM95 and TDM56 compared to TDM31 correlated well with the increased mannose phosphorylation on these mutants (Figure 2.4A).

Extracellular levels of Man-6-P-modified LIF and its self-renewal capacity can be controlled through internalization by the plasma membrane associated CI-MPR. Increased extracellular levels of some LIF mutants at steady state could result from either decreased intracellular sorting to lysosomes or reduced uptake and degradation in lysosomes. To address a role of mannose phosphorylation in the control of extracellular LIF levels via its uptake by cell surface CI-MPR, we performed a self-renewal assay in embryonic stem cells (ESCs). It is well documented that the presence of LIF is sufficient to maintain mouse ESCs in self-renewal (36). Removal of LIF from the culture media (or decreases in its concentration) causes mouse ESCs to spontaneously differentiate, a process that is accompanied by a decrease in alkaline phosphatase activity. Mouse ES cells were treated with various concentrations of unmodified LIF, Man-6-P-modified LIF and non-glycosylated LIF for five days. The extent of self-renewal in these cultures was then determined by quantifying the number of colonies that were positive for alkaline phosphatase staining. As shown in Figure 2.5, there was no significant difference in the ability of unmodified LIF and non-glycosylated LIF to maintain ESCs in self-renewal at all concentrations tested. In contrast, we observed a clear difference in the self-renewal capacity of

Man-6-P-modified LIF and unmodified LIF. These results suggested that Man-6-P-dependent uptake of modified LIF reduced its concentration in the media, resulting in less self-renewal. To directly test this possibility, free Man-6-P was added to ES cultures that were treated with Man-6-P-modified LIF. As shown in Figure 2.5, ES cell self-renewal was significantly increased upon addition of free Man-6-P. Addition of Man-6-P to cultures treated with unmodified LIF had no effect on the percent of AP-positive colonies, indicating that free Man-6-P alone does not influence the extent of self-renewal (data not shown). Since the presence of free Man-6-P has been shown to block the uptake of Man-6-P-modified proteins by cell surface CI-MPR, we believe that the recovery of ESC self-renewal in these cultures (to levels comparable to unmodified LIF) strongly supports the fact that mannose phosphorylated LIF is rapidly internalized, ultimately leading to reduced extracellular LIF activity and spontaneous ESC differentiation.

Man-6-P-modified LIF is subject to intracellular lysosomal targeting and degradation. We next performed experiments to explore whether LIF was being directly targeted to the lysosome following its biosynthesis and mannose phosphorylation in the Golgi. Intracellular LIF was undetectable by Western blotting with an anti-LIF polyclonal antibody (Figure 2.6A). However, it is possible that LIF (or the epitope recognized by the antibody) is subject to rapid degradation upon arrival in the lysosome. To determine whether LIF is in fact targeted to lysosomes, we generated a chimera with WT LIF fused to the fluorophore mCherry at the C-terminal end. Unlike GFP, mCherry has increased stability in the lysosomal compartment (37) and can therefore serve as a marker for the Man-6-P mediated transport of LIF to the lysosome. HeLa cells were transfected with this chimeric construct, and intracellular localization was

monitored by immunofluorescence. As shown in Figure 2.6B, mCherry staining was clearly detected in the lysosomal compartment as assessed by co-localization with LAMP-1. As a control, we next expressed mCherry with a signal peptide attached (Figure 2.6C). Although some intracellular Cherry staining was noted, there was no co-localization with LAMP-1, indicating that the presence of mCherry staining in the lysosomes in the earlier experiments was a result of the Man-6-P modified LIF portion of the chimeric protein and not the ability of mCherry itself to traffic to this compartment. Addition of Man-6-P to the culture media of transfected cells did not appear to alter overall mCherry staining, suggesting that the lysosomal staining we observed is most likely due to direct targeting of the LIF chimera to the lysosome and not its secretion and uptake via the CI-MPR (Figure 2.6D). Some intracellular staining might also be attributed to uptake of LIF by gp130+LIF receptor. We also generated and tested a TDM31 LIF-mCherry construct (Figure 2.6E). Although M6P-modified forms of this LIF mutant cannot be detected in the media, we clearly observed lysosomal staining. Therefore, it is likely that any M6P-modified forms of this LIF mutant are not secreted but instead being targeted to the lysosome. In support of its lysosomal degradation, we further showed that intracellular LIF could be readily detected in the lysosomal compartment using the anti-LIF polyclonal antibody in the CHO-LIF cells in the presence (but not absence) of protease inhibitors (S. Figure 2.2).

Culture media from transfected HeLa cells and cellular lysates were subjected to Western blot with both anti-LIF and anti-mCherry antibodies to assess the status of the chimeric protein in these cultures (see Figure 2.6A). LIF was primarily detected as two distinct molecular weight bands, likely representing forms of the molecule with variable glycosylation since PNGase F treatment reduces these forms to a single band (data not shown). Importantly, we only observed

the 29 kDa mCherry protein by Western blot in the WT LIF-mCherry transfected cells, again suggesting that LIF itself is rapidly degraded upon arrival in the lysosome. Fully intact chimeric protein could be readily detected in the culture media of WT LIF-mCherry transfectants using either antibody. Moreover, we noted only the mCherry band by Western blot in lysates of transfected cultures treated with free Man-6-P. These results suggest that direct intracellular targeting may indeed be the primary means by which the LIF-mCherry chimera reaches the lysosome. Collectively, these data indicate that LIF is normally targeted to lysosomes, providing a mechanism to limit its secretion via Man-6-P targeting.

Discussion

The observation that some non-lysosomal and secreted proteins have been demonstrated to bear Man-6-P residues on their N-glycans has raised questions regarding the biological relevance of this modification. Despite the fact that Man-6-P residues can be detected on several non-lysosomal proteins when overexpressed, it is unclear whether these proteins are modified in a physiological context or whether the residues mediate some aspect of their biological activity. Since current enrichment and mass spectrometric methodologies are capable of detecting very small amounts of Man-6-P modified glycopeptides, these analyses may identify glycoproteins in which the Man-6-P modification represents a low frequency or rare event. Additional experimentation is necessary, however, to determine whether the degree of mannose phosphorylation is comparable to that found on lysosomal hydrolases and whether the interaction of these proteins with the GlcNAc-1-phosphotransferase enzyme has been a process driven by their molecular evolution (29,38,39). Earlier studies on the cytokine LIF identified this protein as

a high affinity ligand for the CI-MPR and revealed that exogenous LIF is subject to Man-6-P mediated internalization and degradation (11,12). In this paper, we extend these findings by demonstrating extensive Man-6-P modification on secreted LIF and an unexpectedly broad distribution of the Man-6-P tag on its glycans. Furthermore, our data show that the mannose phosphorylation of LIF is important for controlling its extracellular levels by multiple mechanisms (Figure 2.7). Most significantly, we show that LIF is targeted to the lysosome and degraded, limiting the amount that can be secreted from the cell. This mechanism represents yet another example whereby glycosylation controls the half-life and activity of proteins and bears similarities to the regulation of hormone clearance by sulfated and sialic acid-containing glycans (40) (41,42) as well as the control of cell surface half-life of the glucose transporter Glut-2 by the N-acetylglucosaminyltransferase, GlcNAcTIVa (43).

In comparison to other IL-6 subfamily members, mammalian LIF contains an unusually high number of N-glycans, with six consensus sites in both the human and mouse sequences. The demonstration that these glycans do not play a role in LIF's ability to bind its receptor suggests that their presence serves another purpose (11). Enrichment of LIF glycopeptides on a CI-MPR affinity column in conjunction with MS analysis revealed that any of the six N-glycans on LIF are capable of bearing Man-6-P residues, indicating a high degree of flexibility in the manner by which GlcNAc-1-phosphotransferase can access LIF. It is important to note that despite the fact that each enriched glycopeptide is detectable, the relative utilization at each site in the purified protein (% M6P-modified peptide vs. total peptide) cannot be accurately determined with this method. Our results with the triple deletion mutants, however, clearly showed that some sites harbor glycans with a higher relative utilization for mannose phosphorylation than others. Nonetheless, the detection of multiple Man-6-P bearing

glycopeptides on LIF is consistent with the analysis of lysosomal hydrolases by several groups, suggesting that LIF has evolved to be a highly mannose phosphorylated protein despite the fact that it does not have any known function within lysosomes. Interestingly, we found that deletion of any of the six N-glycan sites on LIF failed to significantly alter the overall level of mannose phosphorylation, even though obvious differences in the ability of the triple deletion mutants to bind the CI-MPR affinity column were demonstrated. One interpretation for this finding is that loss of certain glycan sites reduces the competition for the active site of the GlcNAc-1-phosphotransferase enzyme, allowing glycans with less favorable orientations to be modified. Investigation into the protein determinants that mediate binding of LIF to GlcNAc-1-phosphotransferase is currently underway and should shed light on whether the presence of specific lysine residues aid in its mannose phosphorylation as noted with multiple lysosomal hydrolases (33,44-48) and secreted proteins such as DNase I (49,50).

We believe the inability to control extracellular levels of LIF via mannose phosphorylation of its glycans may have important implications for the initiation and progression of disease symptoms in ML-II/ML-III. Based on this mechanism, failure to modify endogenous LIF due to a reduction or loss of GlcNAc-1-phosphotransferase activity would be expected to increase its extracellular levels in tissues where LIF is expressed including the lung (51-53), growth plate chondrocytes (54) and osteoblasts (19,21). Increased extracellular LIF could subsequently lead to inappropriate signaling and altered tissue homeostasis, in particular in bone and cartilage, where LIF plays important roles in coordinating the function of several types. Indeed, increased levels of LIF in the synovial fluid have been associated with rheumatoid arthritis, a condition that is clinically similar to ML-II/ML-III in its characteristic loss of articular cartilage (55-57). LIF has also been shown to negatively regulate osteoclast apoptosis (22) and

can induce osteoclast differentiation (58). Therefore, excess LIF signaling may result in overactive osteoclast activity, another hallmark of these disorders. It is worthwhile to note that the novel "gain-of-function" mechanism described here is clearly distinct from the consequences expected from a complete lack of LIF, as evidenced by the fact that LIF knockout mice do not exhibit any phenotypes consistent with ML-II.

Our experiments using the glycan-deleted LIF mutants demonstrated that an increase in the level of mannose phosphorylation correlated with decreased accumulation in the culture media upon transfection in CHO cells. The fact that low levels of mannose phosphorylation on LIF are detected in certain tissues may indicate that most of the LIF that is made is actually targeted to the lysosome for degradation. Such a mechanism would provide an additional measure of control, beyond regulation of LIF expression at the transcriptional level, by creating a critical threshold of protein expression that would need to be exceeded in order to obtain LIF secretion in quantities necessary for a sustained signaling response. Under pathological or certain physiological scenarios where LIF expression is greatly upregulated (i.e. inflammation, placental formation or tumorigenesis), the loss of post-translational M6P-dependent regulation may compound the increase in extracellular levels and exacerbate disease pathology. In addition, certain cell types may downregulate the GlcNAc-1-phosphotransferase expression as a means of stimulating LIF secretion. In support of this, Braulke and colleagues recently demonstrated the GlcNAc-1-phosphotransferase enzyme itself might be subjected to regulation through proteolysis of its gamma subunit in macrophages (59)

In summary, our data has revealed that mannose phosphorylation can serve as a novel post-translational mechanism to control the secretion and extracellular levels of certain non-lysosomal proteins including LIF. It will be of interest to determine whether other non-

lysosomal proteins are regulated in this manner. As demonstrated by earlier studies, other cytokines such as M-CSF also bear Man-6-P residues on their N-glycans (11). Thus, the mechanism we propose for LIF may help explain the relationship between glycosylation and activity that was observed by other investigators (60,61). Although the extent of mannose phosphorylation on secreted LIF was significant (30-40%), these residues can be detected on proteins at much lower frequencies than what was observed for LIF. Indeed, our current evidence suggests that other cytokines previously reported to be Man-6-P modified such as latent TGF-ß exhibited much lower levels of these residues (J. Barnes and R. Steet, unpublished results). Thus, a determination of this extent remains an important consideration for assessing the potential function of mannose phosphorylation on this class of proteins.

Acknowledgements

We would like to acknowledge Dr. Peter Lobel for his gift of the CI-MPR affinity matrix and Dr. Steve Dalton for providing key reagents. We would like to thank Dr. Daniel Kraushaar and Dr. Lianchun Wang for their help with ESC self-renewal assays. In addition, we acknowledge Dr. Heather Flanagan-Steet for her assistance with the confocal imaging. This work was funded by grants from NIGMS and NCRR (1R01GM086524-01 to RS and 5P41RR018502, LW senior investigator) and a graduate fellowship from the Cousins Foundation (to JB).

References

1. Do, H., Lee, W. S., Ghosh, P., Hollowell, T., Canfield, W., and Kornfeld, S. (2002) *J Biol Chem* **277**, 29737-29744
2. Rohrer, J., and Kornfeld, R. (2001) *Mol Biol Cell* **12**, 1623-1631
3. Dahms, N. M., Olson, L. J., and Kim, J. J. (2008) *Glycobiology* **18**, 664-678
4. Kornfeld, S. (1987) *Faseb J* **1**, 462-468
5. Chavez, C. A., Bohnsack, R. N., Kudo, M., Gotschall, R. R., Canfield, W. M., and Dahms, N. M. (2007) *Biochemistry* **46**, 12604-12617
6. Olson, L. J., Peterson, F. C., Castonguay, A., Bohnsack, R. N., Kudo, M., Gotschall, R. R., Canfield, W. M., Volkman, B. F., and Dahms, N. M. (2010) *Proc Natl Acad Sci U S A* **107**, 12493-12498
7. Raas-Rothschild, A., Cormier-Daire, V., Bao, M., Genin, E., Salomon, R., Brewer, K., Zeigler, M., Mandel, H., Toth, S., Roe, B., Munnich, A., and Canfield, W. M. (2000) *J Clin Invest* **105**, 673-681
8. Reitman, M. L., and Kornfeld, S. (1981) *J Biol Chem* **256**, 11977-11980
9. Cathey, S. S., Leroy, J. G., Wood, T., Eaves, K., Simensen, R. J., Kudo, M., Stevenson, R. E., and Friez, M. J. (2010) *J Med Genet* **47**, 38-48
10. Purchio, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L. E., Kovacina, K. S., Roth, R. A., and Marquardt, H. (1988) *J Biol Chem* **263**, 14211-14215
11. Blanchard, F., Duplomb, L., Raher, S., Vusio, P., Hoflack, B., Jacques, Y., and Godard, A. (1999) *J Biol Chem* **274**, 24685-24693
12. Blanchard, F., Raher, S., Duplomb, L., Vusio, P., Pitard, V., Taupin, J. L., Moreau, J. F., Hoflack, B., Minvielle, S., Jacques, Y., and Godard, A. (1998) *J Biol Chem* **273**, 20886-20893
13. Lee, S. J., and Nathans, D. (1988) *J Biol Chem* **263**, 3521-3527
14. Faust, P. L., Chirgwin, J. M., and Kornfeld, S. (1987) *J Cell Biol* **105**, 1947-1955
15. Ikushima, H., Munakata, Y., Ishii, T., Iwata, S., Terashima, M., Tanaka, H., Schlossman, S. F., and Morimoto, C. (2000) *Proc Natl Acad Sci U S A* **97**, 8439-8444
16. Godar, S., Horejsi, V., Weidle, U. H., Binder, B. R., Hansmann, C., and Stockinger, H. (1999) *Eur J Immunol* **29**, 1004-1013
17. Dennis, P. A., and Rifkin, D. B. (1991) *Proc Natl Acad Sci U S A* **88**, 580-584
18. Yang, L., Tredget, E. E., and Ghahary, A. (2000) *Wound Repair Regen* **8**, 538-546
19. Metcalf, D. (2003) *Stem Cells* **21**, 5-14
20. Gonzalez-Perez, O., Jauregui-Huerta, F., and Galvez-Contreras, A. Y. (2010) *Curr Immunol Rev* **6**, 167-173
21. Sims, N. A., and Walsh, N. C. (2010) *BMB Rep* **43**, 513-523
22. Ruan, M., Pederson, L., Bradley, E. W., Bamberger, A. M., and Oursler, M. J. (2010) *Endocrinology* **151**, 1713-1722
23. Slaets, H., Hendriks, J. J., Stinissen, P., Kilpatrick, T. J., and Hellings, N. (2010) *Trends Mol Med* **16**, 493-500
24. Shin, J. E., Park, S. H., and Jang, Y. K. (2011) *Mol Cells* **31**, 181-189
25. Maruta, S., Takiguchi, S., Ueyama, M., Kataoka, Y., Oda, Y., Tsuneyoshi, M., and Iguchi, H. (2009) *Clin Exp Metastasis* **26**, 133-141

26. Kapoor, M., Martel-Pelletier, J., Lajeunesse, D., Pelletier, J. P., and Fahmi, H. (2011) *Nat Rev Rheumatol* **7**, 33-42
27. Abramoff, M. D., Magelhaes, P.J., Ram, S.J. (2004) *Biophotonics International* **11**, 36-42
28. Godard, A., Heymann, D., Raher, S., Anegon, I., Peyrat, M. A., Le Mauff, B., Mouray, E., Gregoire, M., Virdee, K., Soulillou, J. P., and et al. (1992) *J Biol Chem* **267**, 3214-3222
29. Sleat, D. E., Zheng, H., Qian, M., and Lobel, P. (2006) *Mol Cell Proteomics* **5**, 686-701
30. Lim, J. M., Aoki, K., Angel, P., Garrison, D., King, D., Tiemeyer, M., Bergmann, C., and Wells, L. (2009) *J Proteome Res* **8**, 673-680
31. Kraushaar, D. C., Yamaguchi, Y., and Wang, L. (2010) *J Biol Chem* **285**, 5907-5916
32. Steet, R., and Kornfeld, S. (2006) *Mol Biol Cell* **17**, 2312-2321
33. Steet, R., Lee, W. S., and Kornfeld, S. (2005) *J Biol Chem* **280**, 33318-33323
34. Hering, K. W., Karaveg, K., Moremen, K. W., and Pearson, W. H. (2005) *J Org Chem* **70**, 9892-9904
35. Lazzarino, D. A., and Gabel, C. A. (1989) *J Biol Chem* **264**, 5015-5023
36. Niwa, H., Ogawa, K., Shimosato, D., and Adachi, K. (2009) *Nature* **460**, 118-122
37. Katayama, H., Yamamoto, A., Mizushima, N., Yoshimori, T., and Miyawaki, A. (2008) *Cell Struct Funct* **33**, 1-12
38. Sleat, D. E., Wang, Y., Sohar, I., Lackland, H., Li, Y., Li, H., Zheng, H., and Lobel, P. (2006) *Mol Cell Proteomics* **5**, 1942-1956
39. Sleat, D. E., Della Valle, M. C., Zheng, H., Moore, D. F., and Lobel, P. (2008) *J Proteome Res* **7**, 3010-3021
40. Mi, Y., Shapiro, S. D., and Baenziger, J. U. (2002) *J Clin Invest* **109**, 269-276
41. Steirer, L. M., Park, E. I., Townsend, R. R., and Baenziger, J. U. (2009) *J Biol Chem* **284**, 3777-3783
42. Park, E. I., Mi, Y., Unverzagt, C., Gabius, H. J., and Baenziger, J. U. (2005) *Proc Natl Acad Sci U S A* **102**, 17125-17129
43. Ohtsubo, K., Takamatsu, S., Minowa, M. T., Yoshida, A., Takeuchi, M., and Marth, J. D. (2005) *Cell* **123**, 1307-1321
44. Dustin, M. L., Baranski, T. J., Sampath, D., and Kornfeld, S. (1995) *J Biol Chem* **270**, 170-179
45. Cuozzo, J. W., Tao, K., Cygler, M., Mort, J. S., and Sahagian, G. G. (1998) *J Biol Chem* **273**, 21067-21076
46. Warner, J. B., Thalhauser, C., Tao, K., and Sahagian, G. G. (2002) *J Biol Chem* **277**, 41897-41905
47. Baranski, T. J., Cantor, A. B., and Kornfeld, S. (1992) *J Biol Chem* **267**, 23342-23348
48. Cantor, A. B., Baranski, T. J., and Kornfeld, S. (1992) *J Biol Chem* **267**, 23349-23356
49. Nishikawa, A., Gregory, W., Frenz, J., Cacia, J., and Kornfeld, S. (1997) *J Biol Chem* **272**, 19408-19412
50. Nishikawa, A., Nanda, A., Gregory, W., Frenz, J., and Kornfeld, S. (1999) *J Biol Chem* **274**, 19309-19315
51. Knight, D. A., Lydell, C. P., Zhou, D., Weir, T. D., Robert Schellenberg, R., and Bai, T. R. (1999) *Am J Respir Cell Mol Biol* **20**, 834-841
52. Elias, J. A., Zheng, T., Whiting, N. L., Marcovici, A., and Trow, T. K. (1994) *Am J Physiol* **266**, L426-435

53. Wang, J., Chen, Q., Corne, J., Zhu, Z., Lee, C. G., Bhandari, V., Homer, R. J., and Elias, J. A. (2003) *J Biol Chem* **278**, 31226-31232
54. Grimaud, E., Blanchard, F., Charrier, C., Gouin, F., Redini, F., and Heymann, D. (2002) *Cytokine* **20**, 224-230
55. Lotz, M., Moats, T., and Villiger, P. M. (1992) *J Clin Invest* **90**, 888-896
56. Villiger, P. M., Geng, Y., and Lotz, M. (1993) *J Clin Invest* **91**, 1575-1581
57. Upadhyay, A., Sharma, G., Kivivuori, S., Raye, W. S., Zabihi, E., Carroll, G. J., and Jazayeri, J. A. (2009) *Cytokine* **46**, 332-338
58. Richards, C. D., Langdon, C., Deschamps, P., Pennica, D., and Shaughnessy, S. G. (2000) *Cytokine* **12**, 613-621
59. Pohl, S., Tiede, S., Marschner, K., Encarnacao, M., Castrichini, M., Kollmann, K., Muschol, N., Ullrich, K., Muller-Loennies, S., and Braulke, T. (2010) *J Biol Chem* **285**, 23936-23944
60. Altmann, S. W., and Prystowsky, M. B. (1992) *Arch Biochem Biophys* **293**, 349-355
61. Okamoto, M., Nakai, M., Nakayama, C., Yanagi, H., Matsui, H., Noguchi, H., Namiki, M., Sakai, J., Kadota, K., Fukui, M., and et al. (1991) *Arch Biochem Biophys* **286**, 562-568

Figures

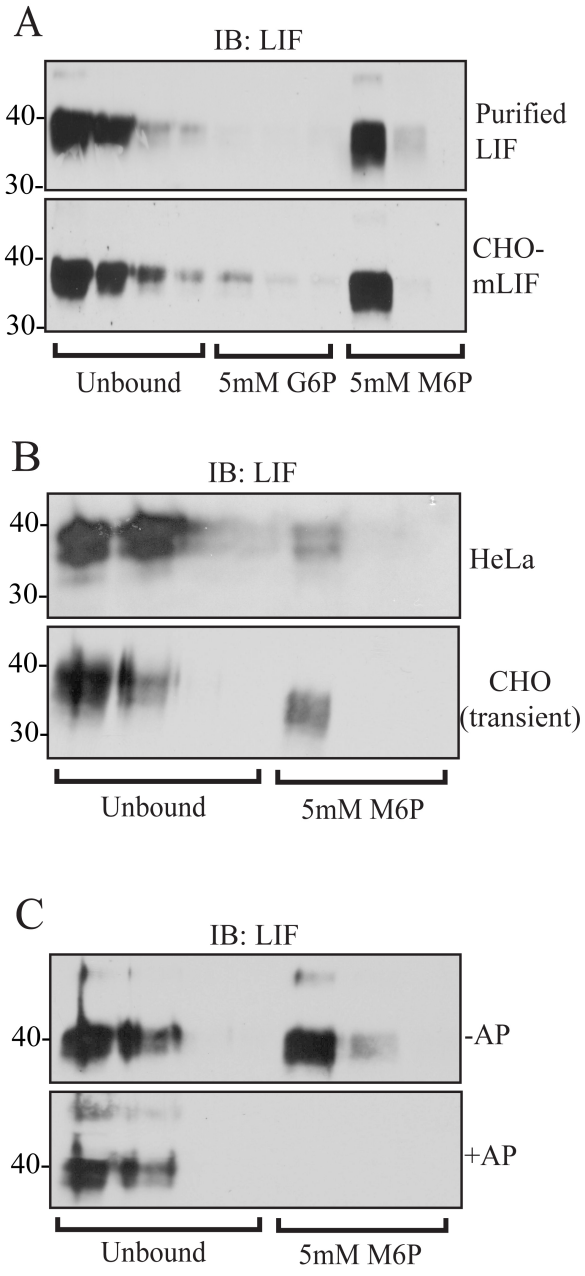


Figure 2.1. Secreted mouse and human LIF are modified with Man-6-P residues. (A) Immunoblot analysis of purified human LIF (hLIF; *top panel*) and secreted mLIF from stably overexpressing CHO cells (CHO-mLIF; *bottom panel*) fractionated on a CI-MPR affinity column. **(B)** Immunoblot analysis of precipitated column fractions following transient expression of hLIF in HeLa and CHO. **(C)** Treatment of CHO-LIF media with alkaline phosphatase (AP) attenuates binding of LIF to the CI-MPR column, demonstrating that the interaction is specifically mediated by Man-6-P-modified glycans.

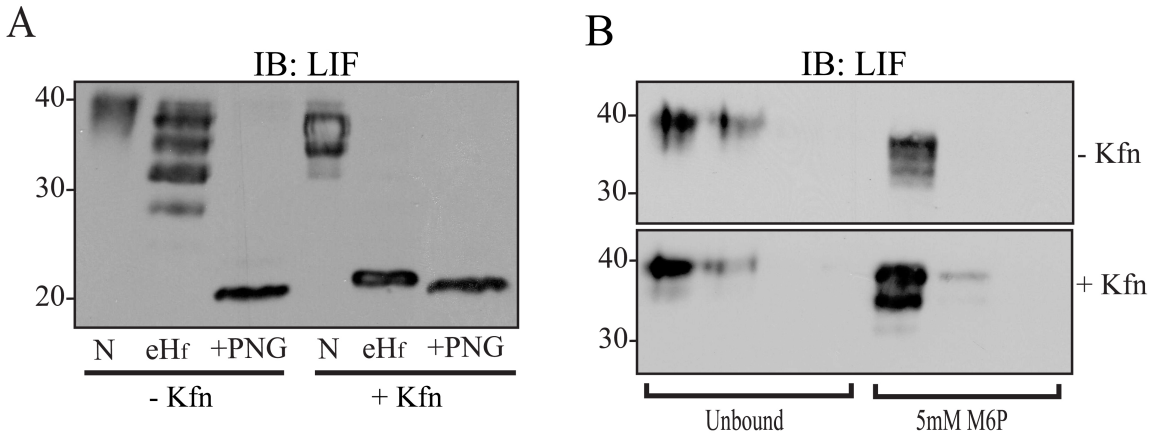


Figure 2.2. Increasing the high mannose glycoforms on LIF does not potentiate further mannose phosphorylation. (A) Immunoblots of secreted hLIF following Endo Hf (eH_f) and PNGase F (PNG) treatment of transfected CHO cells. **(B)** Immunoblot analysis of hLIF from transfected CHO cells following kifunensine (Kfn) treatment (2 μg/mL) and CI-MPR column chromatography. Note that although Kfn treatment completely inhibits glycan maturation, mannose phosphorylation of LIF does not increase.

SPLPITPV**NAT**CAIRHPCHNNLMNQIRSQLAQL**NGS**ANALFILYYTAQG
EPFPNNLDKLCGP**NVT**DFPPFHA**NGT**EKAKLVELYRIVVYLGTSLG**NIT**
RDQKILNPSALSLHSKL**NAT**ADILRGLLSNVLCRLCSKYHVGHVDVTY
GPDTSGKDVFQKKKLGCQLLGKYKQIIAVLAQAF

Figure 2.3. The amino acid sequence, tryptic peptides and N-glycan sites of human LIF. hLIF is a 181 amino acid protein containing six potential N-glycan sites (shown in bold). These N-linked sites are conserved in the mouse sequence. Digestion of hLIF with trypsin yields five glycopeptides (underlined) with one peptide bearing two glycans. The LIF sequence is shown without the signal peptide.

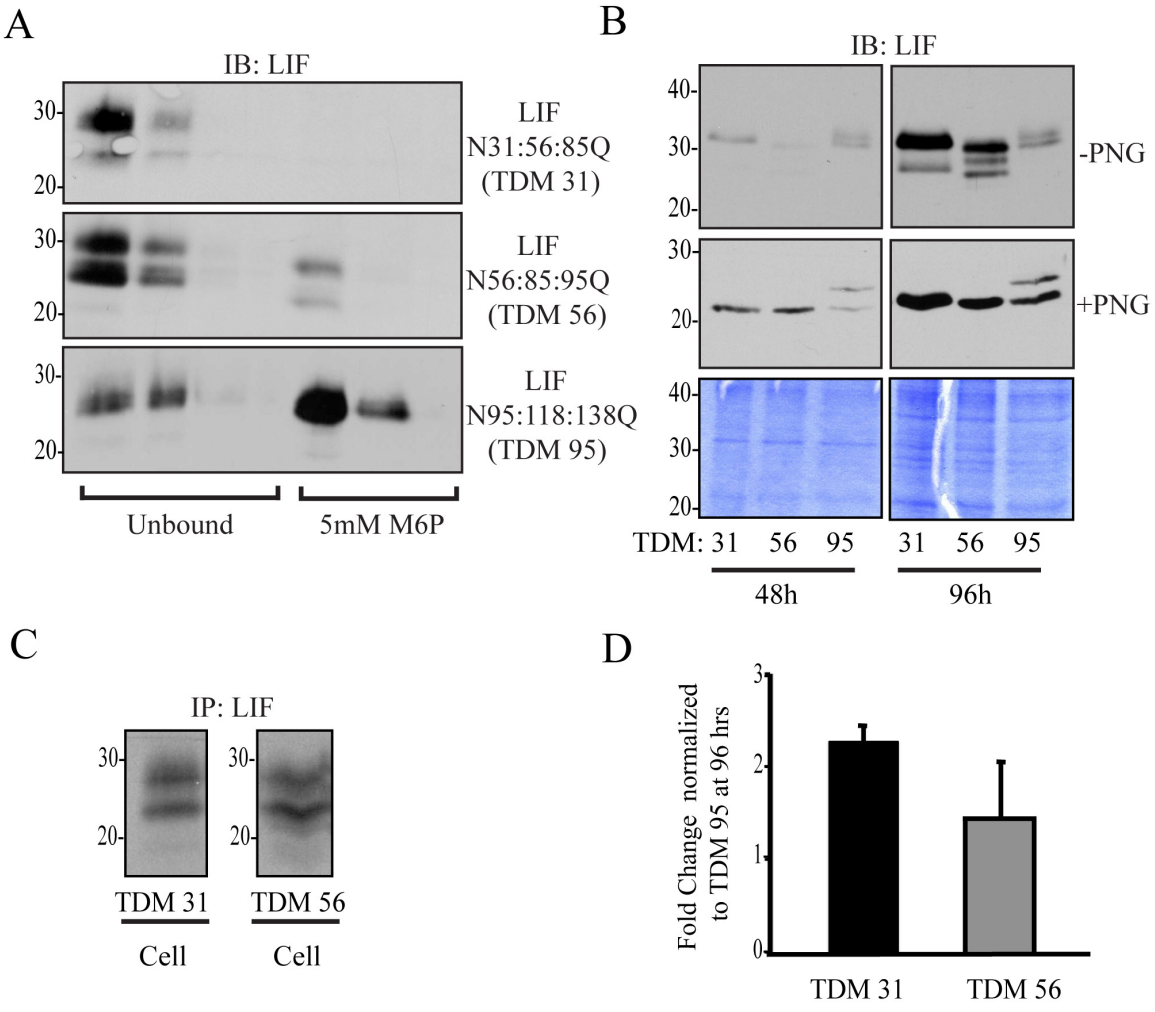


Figure 2.4. Loss of mannose phosphorylation results in the extracellular accumulation of LIF. (A) Representative immunoblots of triple glycan-deleted LIF mutants (TDMs) fractionated on the CI-MPR affinity column: TDM 31, N31:56:85Q; TDM 56, N56:85:95Q and TDM 95, N95:118:138Q. (**B**) CHO cells were transfected with TDM 31, 56 or 95 constructs and secreted LIF levels were assayed by Western blot (n=3) over the various culture times, with and without PNGase F (PNG). As a loading control, secreted protein was normalized using micro BCA protein assay in addition to Coomassie blue stains (**B**, bottom panel). This analysis revealed that similar amounts of protein were synthesized for the various glycan-deleted mutants, suggesting that removal of N-glycan sites does not generally affect the synthesis or glycosylation of these mutants. (**C**) Autoradiograph of immunoprecipitated TDM 31 and TDM 56 LIF mutants from CHO cells pulse-labeled for 2 h with ^{35}S -methionine. (**D**) Quantification of densitometry analysis comparing the fold change of TDM 31 and 56 to TDM 95 at 96 h post transfection.

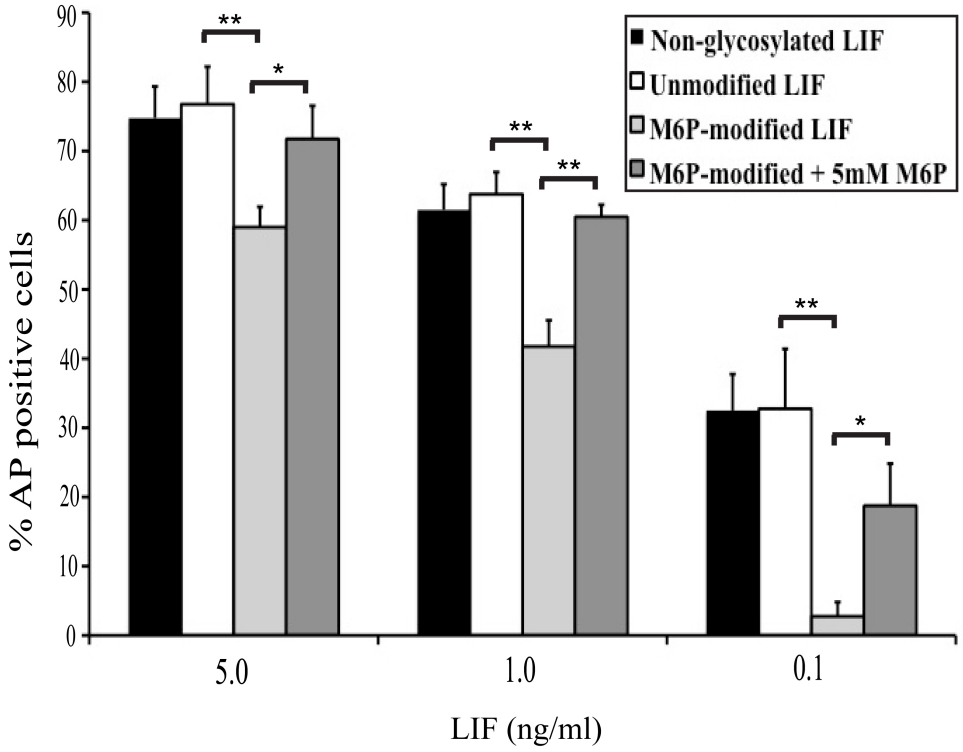


Figure 2.5. The activity of Man-6-P-modified LIF in ESC self-renewal is reduced due to internalization by cell surface Man-6-P receptor. Non-glycosylated ESGRO® LIF (*black*), Unmodified LIF (*white*), Man-6-P modified LIF (*grey*) and Man-6-P modified LIF + 5mM Man-6-P (*dark grey*) were added to separate ESC cultures plated at clonal density. After five days, alkaline phosphatase (AP) staining was performed and the number of AP positive colonies (undifferentiated) compared to the number of unstained colonies (differentiated) was scored based on overall AP stain intensity and cell morphology. Assays were performed as triplicates of the same experiment. Error bars represent the SEM of independent triplicate experiments. P-values were given based on *t-test* determined from the independent triplicate experiments ($P < 0.05$, *; $P < 0.001$, **).

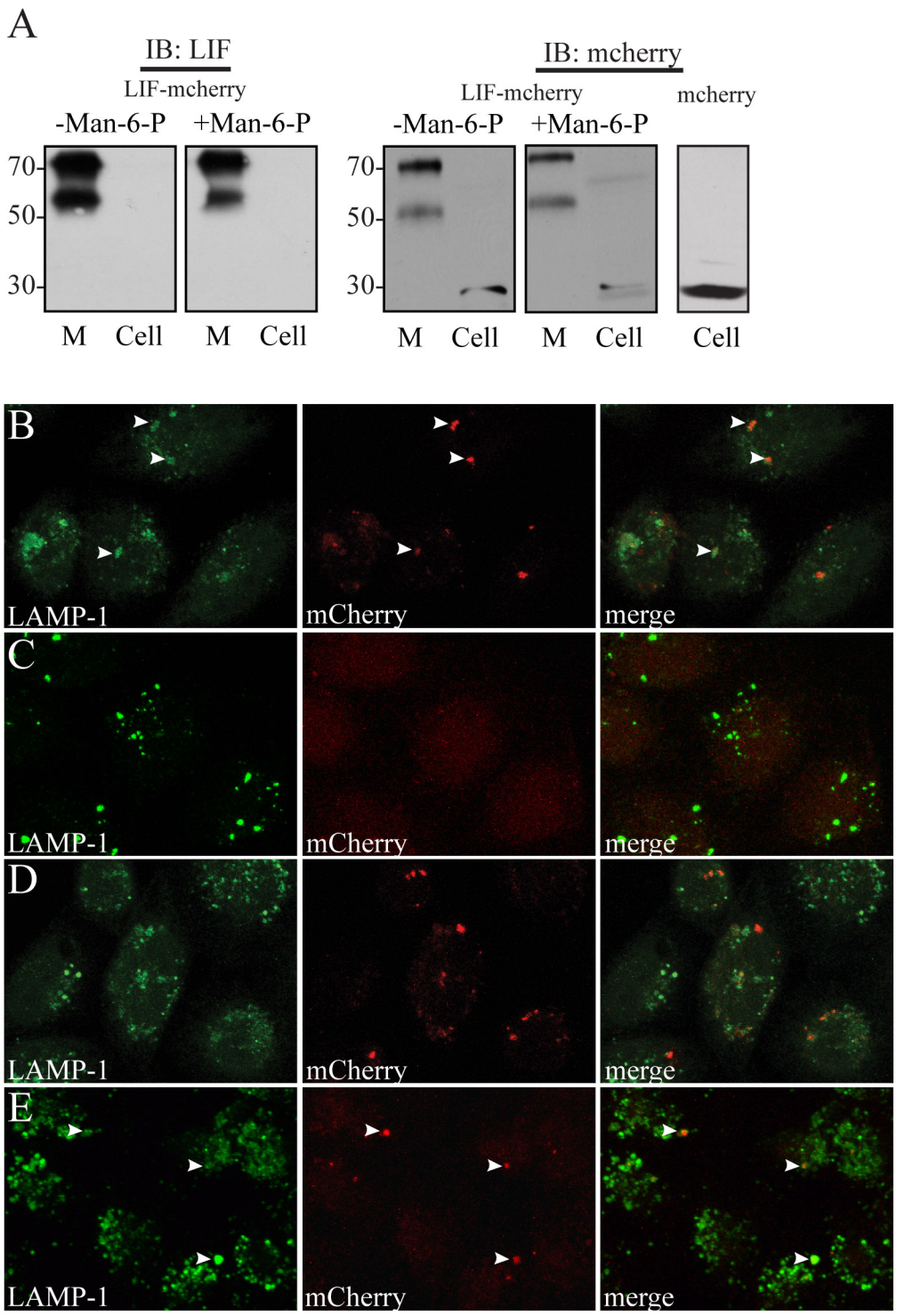


Figure 2.6. Man-6-P-modified LIF is subject to intracellular lysosomal targeting and degradation. **(A)** HeLa cells were transfected with various LIF-mCherry or mCherry (with

signal peptide) constructs and grown in the presence or absence of 5 mM Man-6-P. Cell and media (M) pools were then analyzed by Western blot using either anti-LIF or anti-mCherry antisera. (**B-E**) Confocal analysis of HeLa cells transfected with LIF-mCherry or mCherry constructs (red) and co-stained with the lysosomal marker LAMP-1 (green). **(B)** LIF-mCherry. Co-localization indicates lysosomal targeting of LIF (*see arrowheads*). **(C)** Signal peptide-containing mCherry. **(D)** LIF-mCherry transfected cells grown in the presence of 5 mM Man-6-P. **(E)** TDM31 LIF-mCherry.

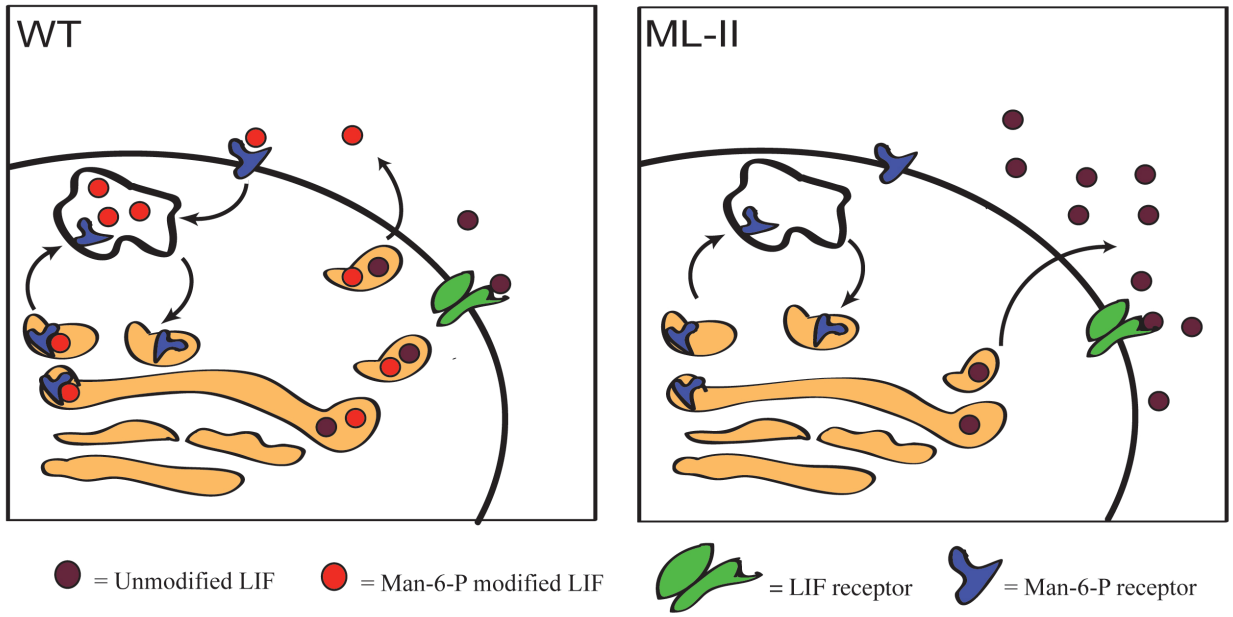
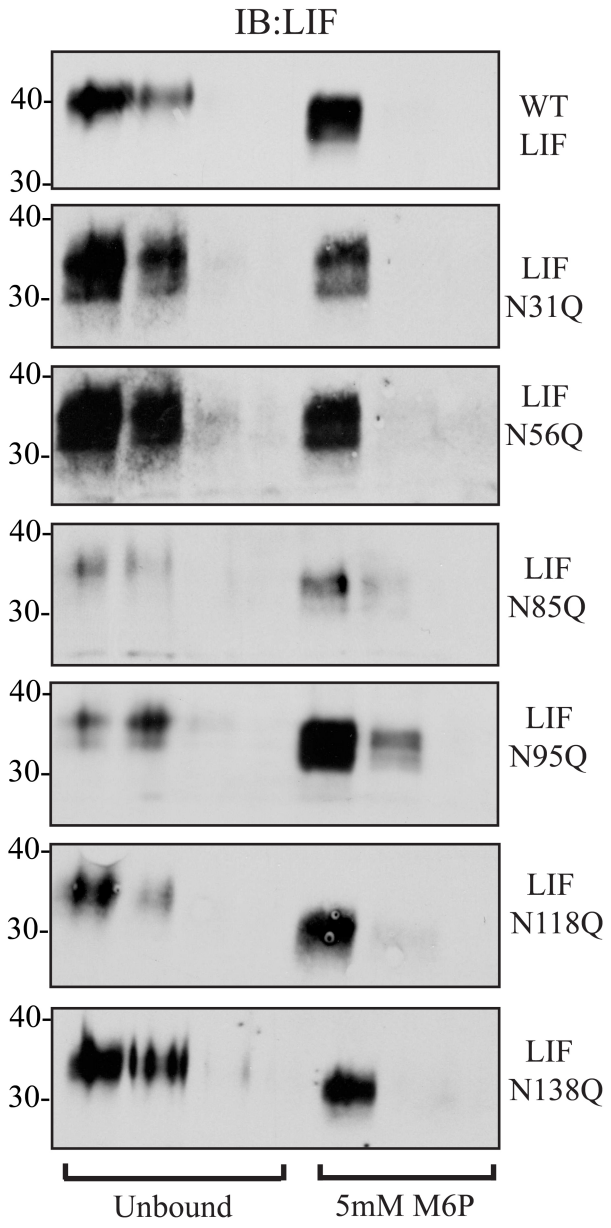
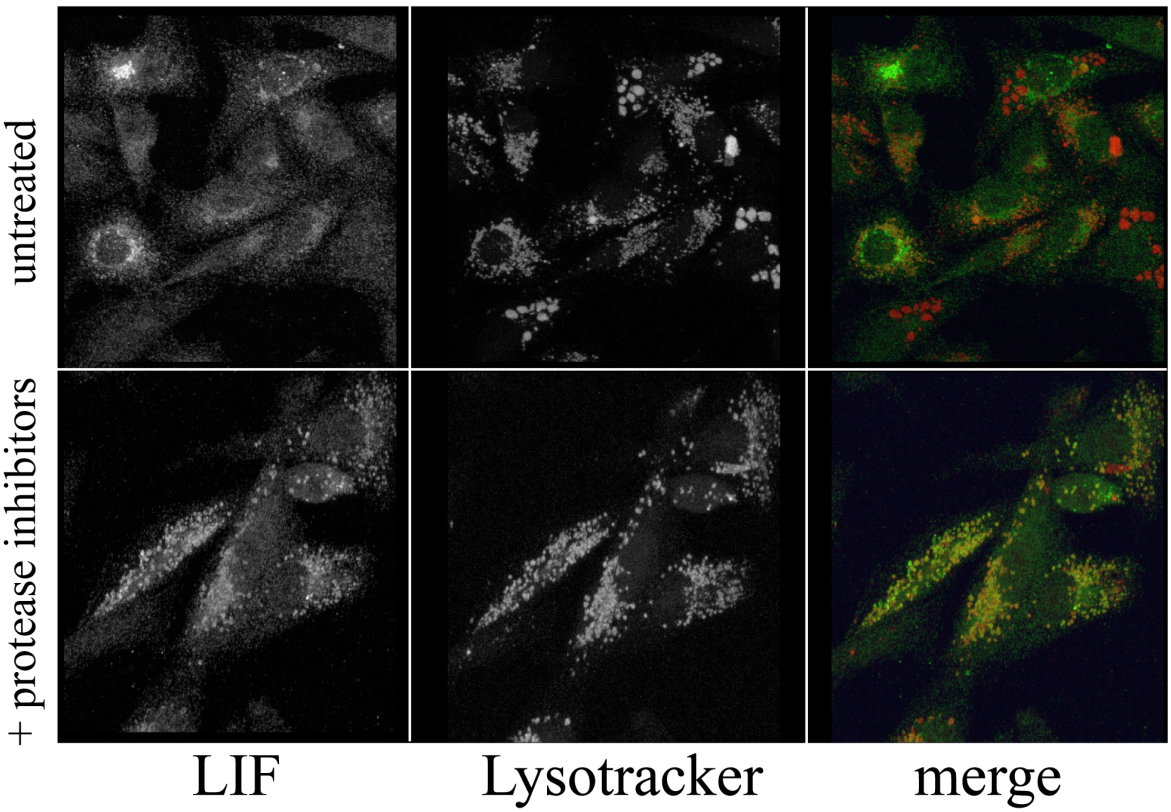


Figure 2.7. Mannose phosphorylation controls LIF extracellular levels by multiple mechanisms. In WT cells, mannose phosphorylation and subsequent lysosomal trafficking of newly synthesized LIF can limit its secretion while uptake of secreted LIF via cell surface CI-MPR can also help to reduce its extracellular levels. In ML-II cells, loss of GlcNAc-1-phosphotransferase activity and impaired mannose phosphorylation of proteins will "unhinge" these control mechanisms leading to accumulation of extracellular LIF and increased LIF signaling and activity.



S. Figure 2.1. Single glycan-deleted LIF mutants bind to the CI-MPR column to a similar degree as the wild type protein. Site-directed mutagenesis was performed to mutate glycan-bearing asparagines to glutamine residues. All single glycan-deletions bound to the CI-MPR column similar to wild type LIF.



S. Figure 2.2. Mouse LIF is targeted to the lysosome and readily degraded. High-expressing CHO-mLIF cells were immunostained with an anti-LIF antibody and Lysotracker and visualized by confocal microscopy. In the absence of protease inhibitors, LIF does not co-localize with Lysostracker-positive late endosomes and lysosomes. Slowing lysosomal degradation of LIF by treatment with a lysosomal protease inhibitor cocktail resulted in accumulation of LIF and robust co-localization with Lysotracker-positive compartments.

TABLE 2.1

Tandem Mass Spectrometry Identification of Modified Peptides Following CI-MPR Chromatography Enrichment

LIF Glycosylated Peptides	Charge State (Z)	Data Assignment	
		Xcorr	Mass Accuracy (ppm)
(-)SPLPITPV**N***ATCAIR(H)	2	4.37	-2.4
	3	4.12	-1.7
(R)SQLAQL**N***GSANALFILYYTAQGEPFPNNLDK(L)	3	5.64	-4.6
(K)LCGP**N***VTDFPPFHA**N***GTEK(A)	2	3.93	0.3
	3	4.27	-3.2
(R)IVVYLGTSLG**N***ITR(D)	2	4.34	-2.9
(K)L**N***ATADILR(G)	2	3.49	-2.2

N* represents a +3 dalton modification (conversion of glycosylated Asn to Asp by PNGaseF in ^{18}O-H_2O)

CHAPTER 3: THE LATENCY-ASSOCIATED PEPTIDE OF TRANSFORMING GROWTH FACTOR-β1 IS NOT SUBJECT TO PHYSIOLOGICAL MANNOSE PHOSPHORYLATION[2]

[2] Jarrod Barnes, Debra Warejcka, Jennifer Simpliciano, Sally Twining, and Richard Steet. Manuscript submitted to *The Journal of Biological Chemistry*

Abstract

Latent TGF-β1 was one of the first non-lysosomal glycoproteins reported to bear mannose 6-phosphate (Man-6-P) residues on its N-glycans. Prior studies have suggested that this sugar modification regulates the activation of latent TGF-β1 by allowing it to bind cell surface-localized Man-6-P receptors. Man-6-P has also been proposed as an anti-scarring therapy based on its ability to directly block the activation of latent TGF-β1. A complete understanding of the physiological relevance of latent TGF-β1 mannose phosphorylation, however, is still lacking. Here we investigate the degree of mannose phosphorylation on secreted latent TGF-β1 and examine its Man-6-P dependent activation in primary human corneal stromal fibroblasts. Contrary to earlier reports, minimal to no Man-6-P modification was found on secreted and cell-associated latent TGF-β1 produced from multiple primary and transformed cell types. Results showed that the inability to detect Man-6-P residues was not due to masking by the latent TGF-β1 binding protein (LTBP). Moreover, the efficient processing of glycans on latent TGF-β1 to complex type structures was consistent with the lack of mannose phosphorylation during biosynthesis. We further demonstrated that the conversion of corneal stromal fibroblast to myofibroblasts, a well-known TGF-β1 dependent process, was not altered by Man-6-P addition when latent forms of this growth factor were present. Collectively, these findings indicate that Man-6-P dependent effects on latent TGF-β1 activation are not mediated by direct modification of its latency-associated peptide.

Background

The delivery of soluble acid hydrolases to the lysosome is primarily mediated by the mannose 6-phosphate (Man-6-P) targeting system in vertebrates. The biosynthesis of Man-6-P residues on newly synthesized hydrolases is initiated by the heterohexameric enzyme UDP-N-acetylglucosamine:lysosomal enzyme N-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase) in the early Golgi. This enzyme selectively transfers GlcNAc-1-phosphate to mannose residues on lysosomal enzymes to form phosphodiester intermediates. The subsequent removal of the GlcNAc residues by the enzyme GlcNAc-1-phosphodiester α-N-acetylglucosaminidase, or "uncovering enzyme" (UCE), in the trans-Golgi network produces Man-6-P monoesters (1,2). These monoesters serve as recognition markers for the sorting of newly synthesized lysosomal proteins by the cation-dependent and the cation-independent mannose-6-phosphate receptors (CD-MPR and CI-MPR, respectively) (3,4).

In addition to acid hydrolases, several non-lysosomal proteins - including latent transforming growth factor-β1 (TGF-β1) (5), leukemia inhibitory factor (LIF) (6,7), proliferin (8), renin precursor (9), and T-cell activation antigen CD26 (10) – have been identified as Man-6-P-modified glycoproteins. An understanding of the mechanisms by which mannose phosphorylation governs the properties of these proteins, however, remains limited. In light of the fact that many Man-6-P modified non-lysosomal proteins are secreted glycoproteins, mannose phosphorylation is thought to facilitate their uptake from the extracellular space by cell surface Man-6-P receptors. Recent work has also demonstrated that addition of phosphomannosyl residues on LIF can limit its extracellular levels by another mechanism - direct sorting and disposal within lysosomes (11).

The functional relevance of mannose phosphorylation on non-lysosomal proteins, however, is not restricted to the regulation of extracellular half-life, as past evidence suggests that this modification may regulate activation of latent TGF-β1 (12-17). Following secretion, latent TGF-β1 must be activated and released from latency-associated peptide (LAP) before it can bind to its receptor and exert its biological activity (18-21). Activation can occur by various mechanisms *in vivo*, including those governed by integrins and thrombospondin (22-27). Mannose phosphorylation has also been implicated in this activation process (12-14,28). Stockinger and colleagues have proposed a model in which cell surface-localized CI-MPR is not only responsible for the binding of Man-6-P-modified latent TGF-β1 but also serves as a scaffold for the proteolytic cleavage of plasminogen to plasmin (29). Along these lines, Dennis and Rifkin demonstrated that the presence of free Man-6-P blocked the plasmin-dependent activation of TGF-β1 in co-cultures of bovine endothelial and smooth muscle cells supporting a role for the CI-MPR in this process (14). Similar results were obtained by Ghahary and co-workers who showed that addition of Man-6-P to the culture media or use of CI-MPR deficient MS-9 cells results in loss of activation of latent TGF-β1 in keratinocyte/fibroblast co-culture assays (12).

Supporting these studies, earlier reports provided biochemical evidence for the presence of Man-6-P on the N-glycans of overexpressed, latent TGF-β1 (5,14,29,30). These experiments did not yield any information, however, regarding the percentage of TGF-β1 molecules that were modified. Many of the subsequent studies addressing the Man-6-P dependent activation of TGF-β1 were based on indirect effects mediated by the addition of free Man-6-P to culture media. Therefore, the possibility that other Man-6-P sensitive glycoproteins contributed to the altered activation of latent TGF-β1 could not be ruled out. In light of these considerations, we felt that a quantitative determination of the extent of mannose phosphorylation on latent TGF-β1 and

further investigation into its Man-6-P dependent activation was needed in order to elucidate the glycosylation-dependent mechanisms that govern TGF-β1 activation.

In this report, we undertook a biochemical evaluation of latent TGF-β1 mannose phosphorylation and explored its Man-6-P dependent activation in human corneal stromal fibroblasts. Contrary to earlier studies, we observed no mannose phosphorylation on latent TGF-β1 secreted from several lines, with the exception of CHO- and HeLa-expressed protein. Our results further showed that the inability to detect high levels of mannose phosphorylation was not due to masking by the latent TGF-ß1 binding protein (LTBP). Consistent with a lack of mannose phosphorylation, we were unable to detect Man-6-P dependent effects on activation in either primary cell line, suggesting that effects on exogenous Man-6-P addition in previously reported co-culture systems may arise due to indirect effects not associated with the mannose phosphorylation of latent TGF-β1 itself.

Materials and Methods

Cell lines, plasmids and reagents – CHO, HeLa, and human erythroleukemia (HEL and K562) cells were obtained from ATCC. CHO, HEL, and K526 cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS) supplemented with 100 U/mL penicillin and streptomycin in a humidified 5% CO_2 atmosphere, while HeLa cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin. Primary HUVEC cells, maintained in EBM-2 Basal medium (Lonza, Walkersville, MD, USA) containing 2% FCS, were used no later than passage 7. The cation-independent Man-6-P receptor (CI-MPR) lectin affinity column was a generous gift from Dr.

Peter Lobel (Robert Wood Johnson Medical School, NJ). LAP goat polyclonal and LTBP mouse monoclonal antibodies, purified LAP (from sf21 cells) and latent TGF-β1 (derived from CHO cells) were obtained from R&D Systems, USA. The anti-cathepsin D rabbit polyclonal antibody was a generous gift from Dr. Stuart Kornfeld (Washington University in Saint Louis). Mannose 6-phosphate and glucose 6-phosphate (both as disodium salt hydrate) as well as phorbol 12-myristate 13-acetate (PMA) and plasmin from human plasma were purchased from Sigma (St. Louis, MO). Endoglycosidase H_f (Endo H_f) and N-glycosidase F (PNGase F) were obtained from New England Biolabs (Ipswich, MA). The transfection reagents Lipofectamine PLUS and Opti-MEM were obtained from Invitrogen (Carlsbad, CA, USA). Human donor corneas were obtained from the Wisconsin Lion's Eye Bank (Madison, WI) under the approval of the Medical College of Wisconsin IRB.

PMA treatment of human erythroleukemia and chronic myelogenous leukemia cells − HEL and K562 cells were cultured in a roller bottle system and maintained at a cell density under 1.0 x 10^6 cells/mL. PMA treatment was performed as previously described (21,31). Upon differentiation, cells were cultured in serum free media for 48 hrs and collected for analysis.

CI-MPR lectin chromatography and Western blot analysis − HeLa and CHO cells were transfected as previously described (11). Cell lysates were prepared in detergent-containing buffer and total protein concentration determined using a BCA protein assay kit (Pierce, Rockford, IL). Media from transfected CHO and HeLa cells and PMA-treated erythroleukemia cells was collected, concentrated using Centricon 10 spin columns (Amicon), and fractionated using an immobilized CI-MPR affinity column as previously described (11). Column fractions

were precipitated with ethanol and resolved by SDS-PAGE. Immunoblot detection was performed with either a mouse monoclonal antibody to LTBP or a goat polyclonal antibody to LAP. In some cases, aliquots of concentrated media treated with either 50 U of Endo H_f or 15 U of PNGase F for 2 h prior to SDS-PAGE and Western blot analysis.

Plasmin treatment of media aliquots from human erythroleukemia cells − Media aliquots from HEL cells were concentrated (from 1 mL to 200 µl) and treated with and without 1 U of plasmin buffered to pH 8.0 and supplemented with 3 mM $MgCl_2$ and $CaCl_2$ for 2 hrs at 37 $^{\circ}$C. Media aliquots were then boiled and brought to a volume of 1 mL followed by application to the CI-MPR chromatography column.

Latent TGF-β1 plasmid construction and transfections − The human latent TGF-β1 plasmid used in this study was synthesized by GeneArt (Invitrogen, Carlsbad, CA, USA) and ligated into a pcDNA3.1 expression vector using flanking BamHI and XbaI restriction sites. Cell transfections were performed as described (11).

Purification of latent TGF-β1 deficient of mannose phosphorylation glycoforms − Purified latent TGF-β1 (R&D systems, USA) was fractionated in unbound and bound pools using the CI-MPR column equilibrated in sodium phosphate, pH 6.9, as previously described (11). Total protein concentration was determined in the unbound pool (containing only unmodified TGF-β1 glycoforms) and this fraction was reserved for the corneal stromal fibroblast assays.

Assessment of myofibroblast differentiation in human corneal stromal fibroblasts – Human corneal stromal cells were obtained by digesting corneas with collagenase. Stromal fibroblasts were maintained in DMEM hi glucose, MITO+ and 5 % FBS. To differentiate to myofibroblasts, stromal cells were grown on collagen coated plates in serum free medium (DMEM hi glucose containing 1% RPMI vitamin mix, 100 μM non-essential amino acids, 1mM pyruvate, and 100 μg/mL ascorbic acid) containing 10 ng/ml TGF-β1 for seven days. Myofibroblast transformation was assessed morphologically by immunofluorescence using a monoclonal antibody to alpha-smooth muscle actin (α-SMA). Fixed cells were imaged using a Nikon Diaphot 300 equipped with a DVC camera and C-View software.

Results

Latent TGF-β1 secreted from differentiated human erythroleukemia cells is not mannose phosphorylated. The identification of phosphomannosyl residues on latent TGF-β1 was initially done via the analysis of secreted protein overexpressed in transfected or transduced cultured cells (5). To estimate the extent of mannose phosphorylation on endogenously expressed latent TGF-β1, we took advantage of the fact that human erythroleukemia cell lines (HEL and K562) express and secrete latent TGF-β1 in a dose-dependent manner following stimulation with phorbol 12-myristate 13-acetate (PMA), a potent inducer of megakaryocyte differentiation in these cells (21,31). Since treatment of both HEL and K562 with 16 nM PMA was found to result in the highest production of latent TGF-β1 (data not shown), this concentration was used for subsequent experiments. Following PMA stimulation, media samples were resolved by non-reducing and reducing SDS-PAGE and Western blot analysis performed using antisera against

the LAP portion of TGF-β1. As shown, latent TGF-β1 was secreted in multiple forms in PMA-stimulated HEL and K562 cells, representing the different complexes of this growth factor (Figure 3.1A-B). Under non-reducing conditions, latent TGF-β1 was primarily secreted as a 270 kDa protein, corresponding to the large latent complex (LLC), containing the small latent complex (SLC) disulfide linked to latent TGF-β1 binding protein (LTBP). In addition, a 100 kDa band corresponding to the dimerized SLC was observed in the non-reducing samples. Upon reduction with DTT (and liberation of the mature TGF-β1 ligand), monomeric LAP migrated at a molecular weight of 40 kDa in both cell lines.

To determine whether latent TGF-β1 was mannose phosphorylated, media samples from PMA-stimulated erythroleukemia cells were subjected to CI-MPR affinity chromatography and Western blot analysis as described in the Methods section. The results of this analysis clearly showed that none of the secreted forms of latent TGF-β1 from HEL (Figure 3.1C-E) or K562 (data not shown) cultures bound to the column. As a control, PMA-stimulated HEL cell lysates were subjected to CI-MPR affinity chromatography as before, followed by Western blot analysis using antisera against cathepsin D, a highly Man-6-P modified lysosomal protease containing a comparable amount of N-glycans as latent TGF-β1 (Figure 3.1F). The high level of cathepsin D binding to the affinity column indicated that PMA stimulation did not alter global mannose phosphorylation in these cells. This analysis was extended to two other cultured cells, NCI-H23 adenocarcinoma and hFOB 1.19 osteoblasts, both of which produce large quantities of latent TGF-β1, but no detectable mannose phosphorylation on this protein was observed in either cell line.

The failure of latent TGF-β1 to bind immobilized CI-MPR is not due to masking of N-glycans by LTBP. The presence of Man-6-P on LAP could be masked by its interaction with LTBP, and thus prevent its binding to the CI-MPR. Indeed, previous reports have suggested that the proteolytic liberation of LLC from the ECM releases a hinge region within LTBP that can mask the glycans present on LAP (15). To explore this possibility, the interaction of LTBP with LAP was disrupted by reduction of disulfide bonds with DTT in HEL media aliquots prior to Man-6-P binding analysis using the immobilized CI-MPR. The results of this experiment demonstrated that separation of LTBP from latent TGF-β1 does not increase binding the Man-6-P receptor affinity column (Figure 3.2A). To determine whether serine protease cleavage of LTBP might lead to a conformational change in LLC and increased accessibility of the N-glycans on latent TGF-β1, HEL media samples were incubated in the presence or absence of 1 U of plasmin prior to analysis. Again, no increase in column binding was detected (Figure 3.2B, *left panel*). Proteolysis of LTBP was demonstrated in Figure 3.2B (*right panel*) as a control for efficiency of plasmin digestion. These data suggest that LTBP masking of the N-glycan residues on latent TGF-β1 was not responsible for lack of detectable binding to Man-6-P receptor affinity column.

Prior experiments using co-cultured systems of endothelial and smooth muscle cell lines have indicated that latent TGF-β1 can be activated through its interaction with Man-6-P receptors and specifically blocked by the addition of free Man-6-P (14). A human endothelial cell line, HUVEC, was used to assess TGF-β1 mannose phosphorylation levels in these cells and the possibility of glycan masking by LTBP. Western blot analysis confirmed that latent TGF-β1 is not mannose phosphorylated in fractionated cell lysates or media samples (Figure 3.2C and D, *top panel*). In addition, latent TGF-β1 reduced from LTBP does not bind to the CI-MPR column

Figure 3.2C and D, *bottom panel*). Collectively, these data show that the latent TGF-β1 is poorly modified in human endothelial cells and LTBP does not mask CI-MPR binding.

Latent TGF-β1 expressed in CHO and HeLa cells contains low levels of mannose phosphorylation. In light of the fact that phosphomannosyl residues were first identified on latent TGF-β1 overexpressed in CHO cells, we chose to analyze mannose phosphorylation levels in transfected CHO and HeLa cultures to gauge whether the extent of this modification is a cell type-specific phenomenon. Media from cultures transfected with a latent TGF-β1 expression vector was collected and analyzed by Western blot under reducing conditions to determine the molecular forms of the molecule secreted by these cell lines. Equivalent media samples were also subjected to mannose phosphorylation analysis as described above. The majority of latent TGF-β1 in transfected HeLa cells (cell-associated and secreted) was detected as the SLC or LAP monomer in HeLa cells (Figure 3.3A), indicating that the majority of secreted TGF-β1 was processed from the precursor (49 kDa) to the latent form (~38 kDa) by the TGN localized furin convertase (32). As shown in Figure 3.3B (upper panel), precursor TGF-β1 (~100 kDa and 90 kDa doublet (31)) and latent TGF-β1 (82 kDa SLC) from HeLa cell lysates does not bind the Man-6-P receptor column, but a small amount of bound processed latent TGF-β1 from HeLa (<1%, Figure 3.3B, *lower panel*) as well as precursor and processed latent TGF-β1 from CHO (~3%, Figure 3.3C) media samples was detected. These data demonstrate that mannose phosphorylation on latent TGF-β1 overexpressed in CHO cells is present, but only at low levels.

Secreted latent TGF-β1 predominantly bears complex type N-glycans. The lack of phosphomannosyl residues on latent TGF-β1 suggests that no mannose phosphorylation occurred

during biosynthesis (an outcome that would result in processing of the N-glycans to complex type). It is equally possible, however, that these residues are effectively removed by phosphatases upon secretion into the culture media (an outcome that would result in the preservation of the N-glycans as high mannose type). To address these two possibilities, media samples were treated with either endoglycosidase H (Endo H_f), which removes high mannose N-glycans or N-glycanase, which removes all N-glycans, followed by immunoblot analysis. In HEL media, the results show that fully processed latent TGF-β1 glycoforms are highly resistant to Endo H_f, consistent with the processing of N-glycans to complex-type (Figure 3.4A). A minor shift in the molecular weight of precursor and latent TGF-β1 from HeLa and CHO cell media (Figure 3.4B) was observed following Endo H_f, however, these glycoforms were mostly complex type. The marginal shift upon endo H_f treatment is again consistent with a low level of mannose phosphorlyation observed in Figure 3.3B, *lower panel* and 3.3C.

Latent TGF-β1 is not able to stimulate myofibroblast conversion of corneal stromal fibroblasts in a Man-6-P dependent fashion. Human corneal stromal fibroblasts are thought to exhibit Man-6-P-dependent effects on TGF-β1 activation. These effects are based on observations that addition of Man-6-P to cultured keratocytes suppresses TGF-β1 myofibroblast transformation (33). Moreover, the presence of Man-6-P was shown to reduce corneal haze following refractive surgery in rabbits (34). While these outcomes are believed to arise due to direct effects on the binding of latent TGF-β1 to cell surface CI-MPR, this mechanism is inconsistent with the present observations that latent TGF-β1 is only marginally modified with Man-6-P residues. To address whether latent forms of TGF-β1 were capable of stimulating myofibroblast differentiation and whether these molecules could be activated in a Man-6-P-

dependent manner, cultured human corneal fibroblasts were incubated with various forms of active and latent TGF-β1 in the presence or absence of free Man-6-P. Expression of the myofibroblast-specific protein, α-smooth muscle actin (α-SMA), was used to gauge the extent of myofibroblast transformation. As shown in Figure 3.5, the active TGF-β1 ligand was the only form capable of robustly increasing α-SMA levels and stimulating assembly of α-SMA fibers characteristic of myofibroblast transformation in corneal fibroblasts. Although a minor amount of transformation was observed in the corneal fibroblast cultures upon addition of latent TGF-β1 purified from CHO cells (but not LAP expressed in sf21 insect cells), this stimulation was not sensitive to Man-6-P addition. We confirmed that these sources of latent TGF-β1 and LAP were poorly modified with Man-6-P residues (Figure 3.5B, *see also Figure 3C*). The lack of myofibroblast stimulation with LAP expressed in sf21 insect cells was not surprising since this form does not contain mature ligand.

Discussion

Latent TGF-β1 was one of the first secreted glycoproteins reported to contain Man-6-P residues (5,30). Subsequent studies have been aimed at understanding how mannose phosphorylation of latent TGF-β1 may regulate its physiological function. Much of this past work has focused on the role of latent TGF-β1 mannose phosphorylation in its activation at the cell surface, where the binding of modified latent TGF-β1 to the CI-MPR is thought to mediate its proteolytic activation (12,14-17). In these studies, activation of latent TGF-β1 (gauged using reporter cell-based or growth inhibition assays that respond to liberated TGF-β1 ligand) was reduced by the addition of free Man-6-P or antisera against the CI-MPR to the culture media. In

nearly all cases, however, the extent of mannose phosphorylation on the latent TGF-β1 molecules employed in these experiments was never determined, leaving open the possibility that Man-6-P dependent effects on activation were not mediated by direct perturbation of latent TGF-β1.

We demonstrated here that latent TGF-β1 was poorly modified with Man-6-P residues; ranging from undetectable in differentiated erythroleukemia cells to 1-3% in transfected CHO and HeLa cells. The highest level of Man-6-P modified latent TGF-β1 could be detected in transfected CHO cells, a noteworthy observation since these cells were also employed in the initial studies (5). Utilizing metabolic labeling experiments with ^{32}P-orthophosphate and subsequent carbohydrate analysis, these investigators accurately identified Man-6-P on the N-glycans of latent TGF-β1 but also estimated in another study that only 5% of the ^{125}I labeled TGF-β1 precursor bound to CI-MPR-coated plates (30). This minimal binding is consistent with our findings that less than 3% of CHO-derived protein binds to the affinity column. Due to the sensitivity of metabolic labeling, very low levels of Man-6-P might be detected on liberated glycans. Thus, one might reason that the original determination of Man-6-P modified glycans on latent TGF-β1 was simply based on high sensitivity detection of a low abundance modification. This likelihood is supported by the fact that many serum glycoproteins can be identified as Man-6-P modified using mass spectrometric-based enrichment and detection strategies (35). We believe these examples serve to reinforce the necessity of quantifying the extent of mannose phosphorylation on non-lysosomal glycoproteins prior to the analyses of its functional relevance.

The very low level of mannose phosphorylation on latent TGF-β1 suggests that this modification is a cell type specific artifact or a function of its overexpression. It is possible, however, that the Man-6-P modified fraction of the molecule is relevant to its activation or

regulation in specific physiological contexts. By virtue of its ability to associate with the plasma membrane following secretion, Man-6-P modified latent TGF-β1 may be more readily activated in cell types where cell surface activation mechanisms, such as interaction with integrins, are present.

Over the past two decades, multiple reports have described the Man-6-P dependent activation of latent TGF-β1. Ghahary and co-workers showed, using a keratinocyte/fibroblast coculture system, that ECM expression was mediated by Man-6-P dependent activation of latent TGF-β1 (12). Along these lines, Rifkin and colleagues demonstrated similar effects on TGF-β1 activation in a bovine aortic endothelial and smooth muscle cell coculture system when free Man-6-P was present (14). However, these studies left an open question as to what other co-factors might be necessary for the activation mechanism, since only the combined co-culture media stimulated an effect. For instance, Godar et al. later proposed an *in vitro* model for Man-6-P dependent activation of TGF-β1 that involved the organization of a proteolytic cascade including plasminogen and urokinase-type plasminogen activator receptor (uPAR) at the cell surface by the CI-MPR (29). Indeed, work by MacDonald and colleagues have determined that uPAR itself is mannose phosphorylated (36). Therefore, it is possible that addition of free Man-6-P abrogates the interaction of uPAR with cell surface CI-MPR. Failure to recruit the uPAR/uPA/plasminogen complex would potentially decrease TGF-β1 activation. Similar results have been determined through inhibition of plasmin suggesting that the conversion of plasminogen to plasmin is an important part of the activation mechanism (28,37).

More recently, effects of free Man-6-P and its analogues on both TGF-β1 activation and downstream indicators of this activation have been documented in models of flexor tendon injury and renal fibrosis (38-40). How can the prior work demonstrating Man-6-P dependent effects on

TGF-β1 activation be justified in light of the findings presented here? Because proteases are capable of latent TGF-β1 activation, one possibility is that the addition of free Man-6-P is displacing Man-6-P modified enzymes from cell surface-localized CI-MPR or interfering with their uptake in some manner that is indirectly influencing the activation. Moreover, free Man-6-P has been reported to decrease TGF-β1 receptor expression at the cell surface (40,41), providing another mechanism to reduce TGF-β1 signaling. Our results in human corneal stromal fibroblasts clearly demonstrated that latent forms of TGF-β1 were not capable of stimulating myofibroblast transformation.

Mannose 6-phosphate and its analogues have been proposed as candidate molecules to prevent haze following refractive surgery on the cornea (33,34). The proposed mechanism of action is thought to occur via decreased activation of latent TGF-β1, which would in turn limit the production of ECM and reduce pathology. Although the level of mannose phosphorylation on endogenous latent TGF-β1 has not been determined from these tissues, careful interpretation of such results and investigation into the possibility that other Man-6-P modified proteins mediate the effects of activation is warranted.

Acknowledgements

We would like to acknowledge Dr. Peter Lobel for his gift of the CI-MPR affinity matrix and Dr. Eduard Condac for providing HUVEC cells. We are grateful to Dr. Nancy Dahms for critical reading of the manuscript. This work was funded by grants from NIGMS (1R01GM086524-01 to RS), NEI (1 R01 EY012731-07 to SST) and a graduate fellowship from the Cousins Foundation (to JB).

References

1. Do, H., Lee, W. S., Ghosh, P., Hollowell, T., Canfield, W., and Kornfeld, S. (2002) *J Biol Chem* **277**, 29737-29744
2. Rohrer, J., and Kornfeld, R. (2001) *Mol Biol Cell* **12**, 1623-1631
3. Dahms, N. M., Olson, L. J., and Kim, J. J. (2008) *Glycobiology* **18**, 664-678
4. Kornfeld, S. (1987) *Faseb J* **1**, 462-468
5. Purchio, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L. E., Kovacina, K. S., Roth, R. A., and Marquardt, H. (1988) *J Biol Chem* **263**, 14211-14215
6. Blanchard, F., Duplomb, L., Raher, S., Vusio, P., Hoflack, B., Jacques, Y., and Godard, A. (1999) *J Biol Chem* **274**, 24685-24693
7. Blanchard, F., Raher, S., Duplomb, L., Vusio, P., Pitard, V., Taupin, J. L., Moreau, J. F., Hoflack, B., Minvielle, S., Jacques, Y., and Godard, A. (1998) *J Biol Chem* **273**, 20886-20893
8. Lee, S. J., and Nathans, D. (1988) *J Biol Chem* **263**, 3521-3527
9. Faust, P. L., Chirgwin, J. M., and Kornfeld, S. (1987) *J Cell Biol* **105**, 1947-1955
10. Ikushima, H., Munakata, Y., Ishii, T., Iwata, S., Terashima, M., Tanaka, H., Schlossman, S. F., and Morimoto, C. (2000) *Proc Natl Acad Sci U S A* **97**, 8439-8444
11. Barnes, J., Lim, J. M., Godard, A., Blanchard, F., Wells, L., and Steet, R. (2011) *J Biol Chem* **286**, 24855-24864
12. Ghahary, A., Tredget, E. E., and Shen, Q. (1999) *J Cell Physiol* **180**, 61-70
13. Yang, L., Tredget, E. E., and Ghahary, A. (2000) *Wound Repair Regen* **8**, 538-546
14. Dennis, P. A., and Rifkin, D. B. (1991) *Proc Natl Acad Sci U S A* **88**, 580-584
15. Gleizes, P. E., Munger, J. S., Nunes, I., Harpel, J. G., Mazzieri, R., Noguera, I., and Rifkin, D. B. (1997) *Stem Cells* **15**, 190-197
16. Ghahary, A., Tredget, E. E., Shen, Q., Kilani, R. T., Scott, P. G., and Houle, Y. (2000) *Growth Factors* **17**, 167-176
17. Ghahary, A., Tredget, E. E., Mi, L., and Yang, L. (1999) *Exp Cell Res* **251**, 111-120
18. Annes, J. P., Munger, J. S., and Rifkin, D. B. (2003) *J Cell Sci* **116**, 217-224
19. Annes, J. P., Rifkin, D. B., and Munger, J. S. (2002) *FEBS Lett* **511**, 65-68
20. Flaumenhaft, R., Kojima, S., Abe, M., and Rifkin, D. B. (1993) *Adv Pharmacol* **24**, 51-76
21. Miyazono, K., Olofsson, A., Colosetti, P., and Heldin, C. H. (1991) *EMBO J* **10**, 1091-1101
22. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T. A. (2011) *Nature* **474**, 343-349
23. Murphy-Ullrich, J. E., and Poczatek, M. (2000) *Cytokine Growth Factor Rev* **11**, 59-69
24. Ribeiro, S. M., Poczatek, M., Schultz-Cherry, S., Villain, M., and Murphy-Ullrich, J. E. (1999) *J Biol Chem* **274**, 13586-13593
25. Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P., and Bouck, N. (1998) *Cell* **93**, 1159-1170
26. Wipff, P. J., and Hinz, B. (2008) *Eur J Cell Biol* **87**, 601-615
27. Aluwihare, P., Mu, Z., Zhao, Z., Yu, D., Weinreb, P. H., Horan, G. S., Violette, S. M., and Munger, J. S. (2009) *J Cell Sci* **122**, 227-232
28. Odekon, L. E., Blasi, F., and Rifkin, D. B. (1994) *J Cell Physiol* **158**, 398-407

29. Godar, S., Horejsi, V., Weidle, U. H., Binder, B. R., Hansmann, C., and Stockinger, H. (1999) *Eur J Immunol* **29**, 1004-1013
30. Kovacina, K. S., Steele-Perkins, G., Purchio, A. F., Lioubin, M., Miyazono, K., Heldin, C. H., and Roth, R. A. (1989) *Biochem Biophys Res Commun* **160**, 393-403
31. Miyazono, K., Thyberg, J., and Heldin, C. H. (1992) *J Biol Chem* **267**, 5668-5675
32. Dubois, C. M., Laprise, M. H., Blanchette, F., Gentry, L. E., and Leduc, R. (1995) *J Biol Chem* **270**, 10618-10624
33. Angunawela, R. I., and Marshall, J. (2010) *J Cataract Refract Surg* **36**, 121-126
34. Sutton, G., Patmore A.L., Joussen, A.M., Marshall, J. (1996) *Lasers and Light in Ophthalmology*, 117-119
35. Sleat, D. E., Wang, Y., Sohar, I., Lackland, H., Li, Y., Li, H., Zheng, H., and Lobel, P. (2006) *Mol Cell Proteomics* **5**, 1942-1956
36. Kreiling, J. L., Byrd, J. C., Deisz, R. J., Mizukami, I. F., Todd, R. F., 3rd, and MacDonald, R. G. (2003) *J Biol Chem* **278**, 20628-20637
37. Flaumenhaft, R., Abe, M., Mignatti, P., and Rifkin, D. B. (1992) *J Cell Biol* **118**, 901-909
38. Bates, S. J., Morrow, E., Zhang, A. Y., Pham, H., Longaker, M. T., and Chang, J. (2006) *J Bone Joint Surg Am* **88**, 2465-2472
39. Xia, C. S., Yang, X. Y., Yingzhen, W., Sun, K., and Tian, S. (2010) *Orthopedics* **33**, 727
40. Xia, C., Yang, X. Y., Wang, Y., and Tian, S. (2011) *Orthopedics* **34**, 21
41. Yang, R., Xia, C., Wang, X., Sun, K., Yang, X., Tian, S., and Hong, G. (2010) *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* **24**, 64-68

Figures

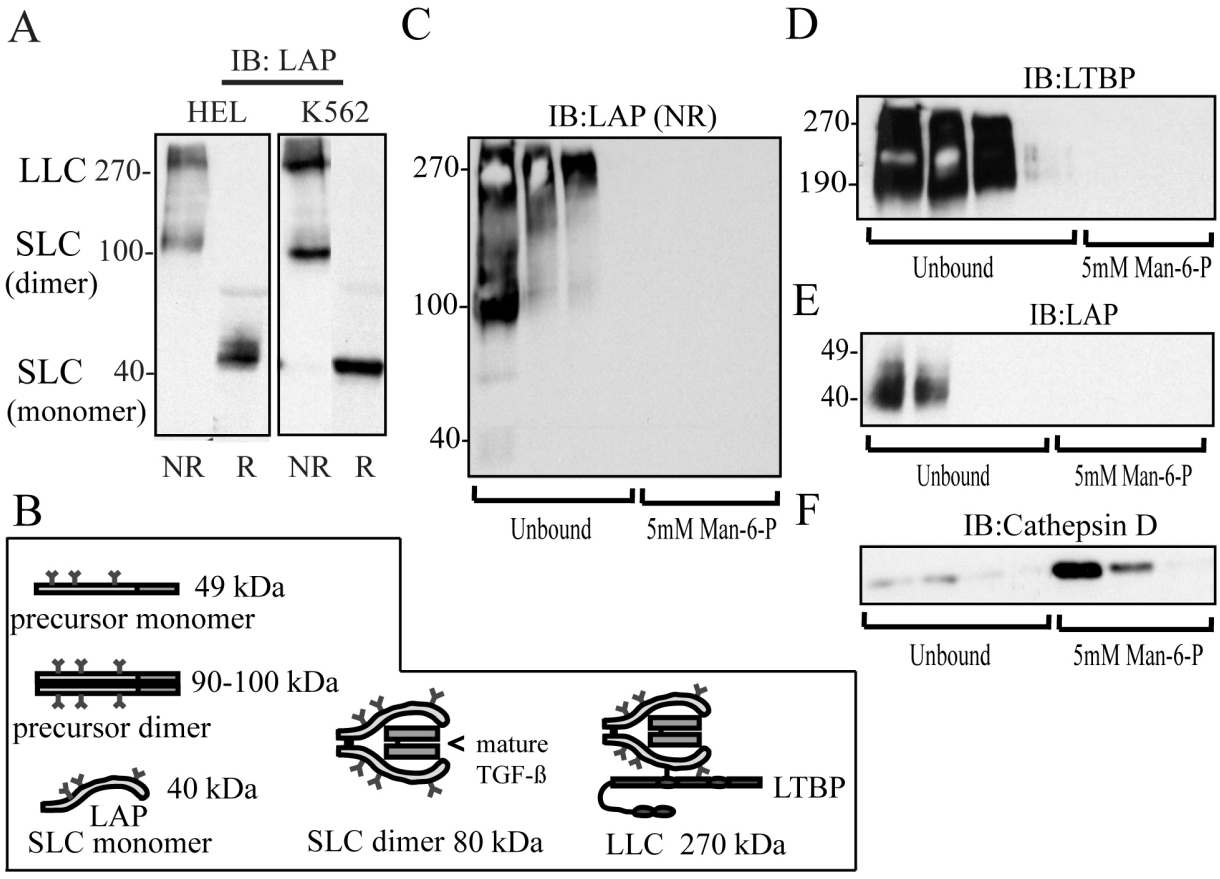


Figure 3.1. The mannose phosphorylation of secreted latent TGF-β1 from PMA-stimulated human erythroleukemia cultures is undetectable. (**A**) Immunblots of PMA-treated human erythroleukemia (HEL and K562) media samples using an anti-LAP antibody. NR, non-reducing; R, reducing. (**B**) Illustration of the different forms of TGF-β1. (**C-E**) Immunoblots of precipitated fractions from a CI-MPR affinity column using antibodies to either LAP or LTBP run under non-reducing (**C** and **D**) or reducing conditions (**E**). Note the lack of latent TGF-β1 binding to the immobilized CI-MPR column in any form. **(F)** Immunoblot of cathepsin D from HEL cell lysates following CI-MPR affinity chromatography. Unbound denotes the protein eluted with column buffer and 5mM Man-6-P is the pool of Man-6-P bearing glycoproteins.

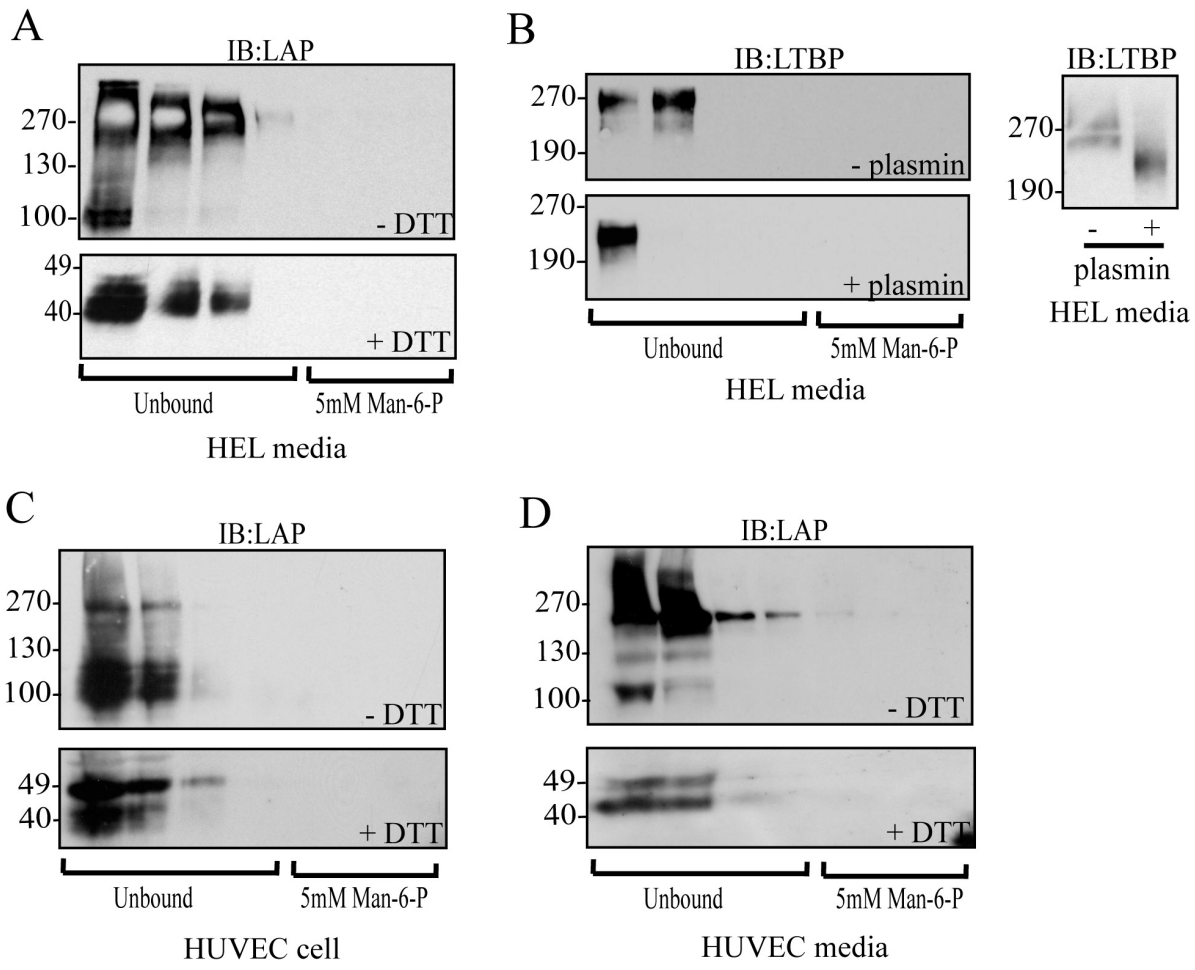


Figure 3.2. Unmasking of the N-glycans on latent TGFβ1 from PMA-stimulated HEL cultures does not increase CI-MPR column binding. **(A)** Immunoblots of CI-MPR column fractions under reducing and non-reducing conditions using the anti-LAP antibody. Media samples were to the CI-MPR column under non-reducing (*top panel*) or reducing (40 mM DTT) column application (*bottom panel*). **(B)** Non-reducing Immunoblots of the large latent complex (LLC) using the anti-LTBP antibody. Media was concentrated and treated without (*left top panel*) and with (*left bottom panel*) the serine protease plasmin (1 U) at 37˚C for 2h prior to column application. Plasmin digestion was performed on HEL media aliquots as a control (*right panel*). HUVEC cell lysates (**C**) and media aliquots (**D**) were applied to the CI-MPR column with and without 40 mM DTT and analyzed by Immunoblot using antisera against LAP.

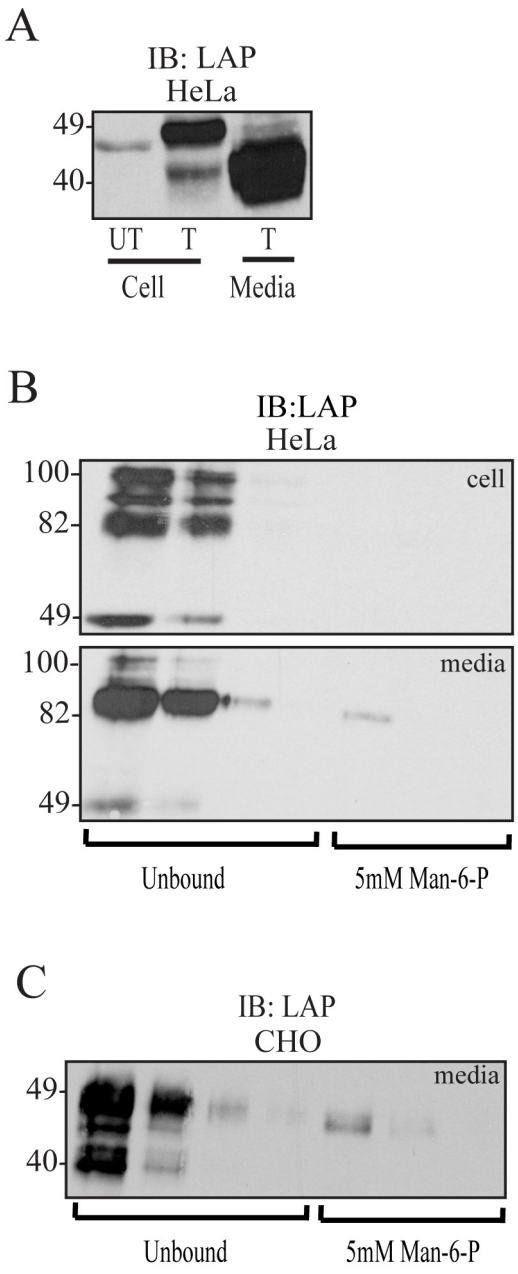


Figure 3.3. Latent TGF-β1 overexpressed in HeLa and CHO cells minimally binds the immobilized CI-MPR. (**A**) Reducing Immunoblot of LAP from latent TGF-β1 in untransfected (UT) and transfected (T) HeLa cell and media. (**B**) CI-MPR column fractionation of cell lysates (*top panel*) and media samples (*bottom panel*) from HeLa cells (non-reducing) overexpressed with latent TGF-β1. (**C**) Immunoblot analysis of transiently transfected latent TGF-β (reducing) from CHO cell culture media after CI-MPR column fractionation.

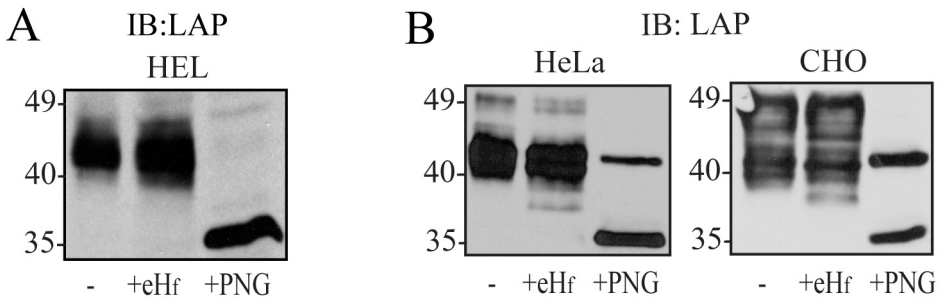


Figure 3.4. The majority of N-glycans on media-derived latent TGF-β1 from PMA-stimulated HEL and transfected CHO and HeLa cells are processed to complex-type. (A) Immunoblot analysis of latent TGF-β1 from PMA-stimulated HEL media treated with endoH_f, PNGase F or no enzyme (control). (**B**) EndoH_f and PNGase F N-glycan digestion profiles of overexpressed latent TGF-β1 from HeLa (*left panel*) and CHO (*right panel*) media samples.

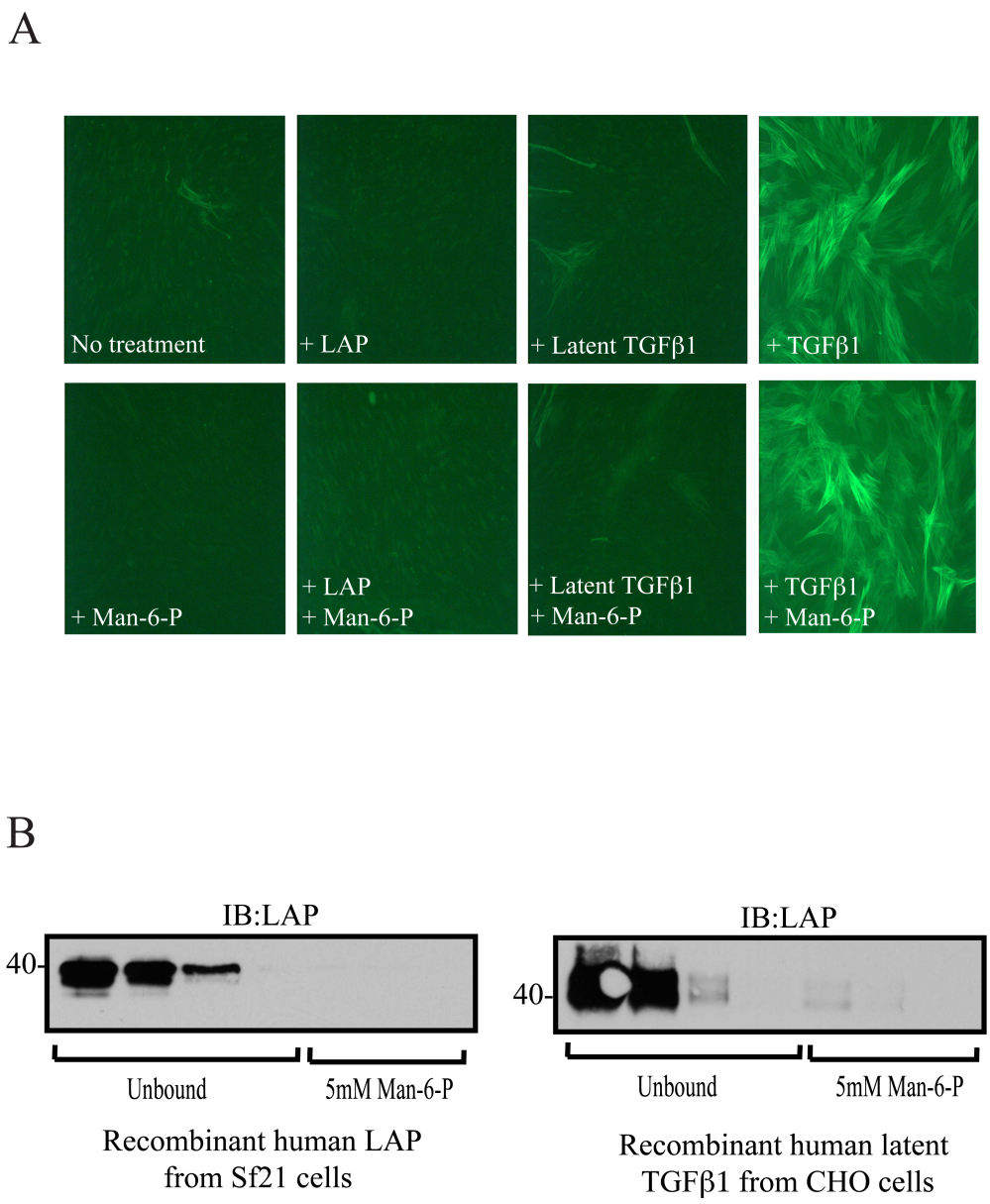


Figure 3.5. Addition of active TGF-β1 ligand, but not latent TGF-β1 or LAP, stimulates differentiation of corneal fibroblasts to myofibroblasts in a Man-6-P independent manner. **(A)** Immunofluorescence staining of α-smooth muscle actin represents differentiation of primary corneal fibroblasts to myofibroblasts**.** **(B)** Western blot analysis of recombinant human LAP from sf21 insect cells and recombinant latent TGF-β1derived from CHO cells following fractionation on the CI-MPR receptor column. Minimal binding of both molecules was detected.

CHAPTER4: UPREGULATION OF TRANSGLUTAMINASE ALTERS TRANSFORMING GROWTH FACTOR-β1 IN MUCOLIPIDOSIS TYPE II CELLS[3]

[3] Jarrod Barnes, Mark Haskins, Richard Steet. Manuscript to be submitted to *Journal of Cell Biology*.

Abstract

Dysregulation of transforming growth factor-β1 (TGF-β1) signaling underlies many skeletal and connective tissue disorders. Recent *in vivo* evidence suggests that this pathway might also be involved in the pathogenesis of certain lysosomal storage disorders including mucolipidosis II (ML-II) and mucopolysaccharidosis I (MPS-I). To address this possibility, we analyzed TGF-β1 biosynthesis and regulation in cultured human fibroblasts from ML-II and MPS-I patients. Examination of Smad 2/3 phosphorylation determined that TGF-β1 signaling was reduced in both cell lines. This reduction was also associated with decreased wound closure in ML-II fibroblasts. Metabolic labeling and analysis of steady-state levels determined that secretion of latent TGF-β1 was altered in ML-II cultures. Loss of soluble secreted forms of latent TGF-β1 in ML-II was associated with the increased deposition of this latent growth factor within detergent-insoluble fractions. Immunofluorescent staining of fibrillar network proteins confirmed that the increased insolubility of latent TGF-β1 was associated with its deposition into the extracellular matrix (ECM). These observations were demonstrated in feline primary fibroblasts-like synoviocytes, indicating increased ECM deposition and altered TGF-β1 bioavailability is a general feature of ML-II disease. In light of the fact that latent TGF-β1 is believed to deposit into the ECM via cross-linking, we examined whether levels of the transamidase cross-linking enzyme, transglutaminase 2 (TG2), were altered in ML-II cells. Results indicated that the steady-state level of this protein was significantly increased in both human and feline ML-II fibroblasts. Furthermore, TG2 was found in the insoluble ECM of ML-II but not WT fibroblasts. Upregulation of TG2 levels in WT fibroblasts following retinoic acid treatment resulted in

greater ECM levels and transamidation activity of this enzyme as well as increased insolubility of latent TGF-β1. Collectively, our data suggests that TG2 may be responsible for the abnormal deposition and altered TGF-β1 bioavailability in ML-II. A discussion for the implications of TG2 function as well as the altered TGF-β1 signaling in ML-II pathogenesis will be presented.

Background

TGF-β1 mediates a broad spectrum of biological processes including wound repair, angiogenesis and immunity and plays specific roles in the development of cartilage, connective tissue and bone (1-4). TGF-β1 and its related isoforms are initially synthesized as pre-proproteins consisting of three peptide domains: the signal peptide, the latency-associated peptide (LAP), and the mature TGF-β1 peptide (see Figure 1.7). Prior to secretion, the TGF-β1 precursor undergoes extensive proteolytic and post-translational modification in the endoplasmic reticulum and Golgi. During these processing steps, the LAP portion is cleaved from the TGF-β1 ligand but re-associates with it in a non-covalent manner to form the small latent complex (SLC). The SLC can become covalently attached to latent TGF-β1 binding proteins (LTBPs) to generate the large latent complex (LLC) (5,6). The LTBPs facilitate more rapid secretion of latent TGF-β1 and are responsible for extracellular targeting and storage of the LLC to the ECM via interactions with several matrix components, including fibrillins, fibronectin and heparan sulfate (7-9). Extracellular latent TGF-β1 must be activated and released from LAP before it can bind to its receptor and exert its biological activity (10-13). One critical step that precedes the activation of TGF-β1 is the transamidation event carried out by the multi-functional enzyme

transglutaminase 2 (TG2; for review see (14,15) and Chapter 1) that has been shown to cross-link this growth factor to the ECM (16). Once cross-linked, *in vivo* activation can occur by various mechanisms, including those governed by integrins and thrombospondin-1 (17-26). In some cases, liberation of the LLC from the ECM is required for activation. Within bone and cartilage, both activation steps are mediated by proteases including plasmin, cathepsins and MMPs (27-30).

Numerous skeletal and connective tissue diseases have been described in which the regulation and activation of latent TGF-β1 is altered (see Table 1.4). In the case of Camurati-Engelmann disease, enhanced activation of latent TGF-β1 arises due to mutations in the LAP portion of the precursor molecule (17,31). In other disorders, however, TGF-β1 dysregulation occurs due to defects in extracellular matrix proteins (i.e., fibrillin-1) that are involved in the deposition and sequestration of this growth factor at the cell surface (32-35). Indeed, there is growing evidence that differences in ECM architecture can modulate TGF-β1 activities during many physiological and pathological processes (36).

The lysosomal diseases mucolipidosis type II (ML-II) and mucopolysaccharidosis type I (MPS-I) are characterized by pronounced skeletal manifestations and cartilage defects. The molecular and cellular mechanisms that drive pathology in the various affected tissues of ML-II and MPS-I patients are incompletely understood. Elucidating such mechanisms will provide a starting point for the development of much needed therapies for these disorders. Both diseases are characterized by upregulation of proteases (37-39). In addition, ML-II cells hypersecrete proteolytic enzymes due to impaired Man-6-P dependent lysosomal targeting. Aberrant activation of TGF-β1 has been suggested in these diseases (40-42) and may account for the skeletal phenotypes, however the mechanisms whereby this growth factor is impacted are not

known. In the case of ML-II, mannose phosphorylation has been proposed to mediate the proteolytic activation of TGF-β1 (43,44). Hence, loss of this modification may directly alter its cell surface activation. Our current studies, however, have challenged the idea that mannose phosphorylation of latent TGF-β1 plays a direct role in its activation (see Chapter 3). Since altered biosynthesis of ECM proteins has also been noted in ML-II, indirect effects on the deposition and bioavailability might also influence TGF-β1 activation in specific tissues. Regardless of whether direct or indirect mechanisms are evoked, aberrant activation of latent TGF-β1 would be expected to have profound consequences on ECM deposition, ECM homeostasis, and tissue development.

We analyzed TGF-β1 biosynthesis and regulation in cultured fibroblasts from ML-II and MPS-I patients in an effort to investigate the possible role of TGF-β1 in these diseases. Our results show increased deposition of latent TGF-β1 within insoluble ECM pools of ML-II fibroblasts. The abnormal deposition of latent TGF-β1 was associated with impaired wound closure, decreased Smad2/3 phosphorylation, reduced isoforms of secreted latent TGF-β1, and more rapid deposition of the ECM protein fibrilin-1 and this growth factor into the fibrillar network. Further investigation of this phenomenon using a TGF-β1 luciferase reporter system revealed that there was a three-fold excess of latent TGF-β1 (capable of activation by heat treatment) in the insoluble ECM of ML-II fibroblasts. The specificity of these findings was clearly demonstrated by parallel observations in a primary feline ML-II fibroblast-like synoviocyte line. One potential mechanism that may account for the shift in solubility of latent TGF-β1 is the upregulation and translocation of TG2 into the insoluble ECM of these cultures. In support of a role for TG2, we were able to shift the solubility of latent TGF-β1 in WT fibroblast by increasing the expression of TG2 using retinoic acid treatment. Together, our

results provide the first known example whereby the solubility of latent TGF-β1 is decreased in a pathological context and suggest that upregulation of TG2 in this disease drives this effect. Implications for the impaired bioavailability of latent TGF-β1 and TG2 upregulation in ML-II disease are discussed.

Materials and Methods

Cell lines, plasmids and reagents – Human WT, ML-II and MPS-I skin fibroblasts were obtained from Coriell (Camden, NJ) and cultured in Dulbecco's modified Eagle's medium containing 18% fetal bovine serum (FBS) supplemented with 100 μg/mL penicillin in addition to streptomycin and maintained in a humidified 5% CO_2 atmosphere. Primary feline fibroblast-like synoviocytes and skin fibroblasts were isolated from feline synovium and skin, respectively, from unaffected and ML-II littermates reared and cared for by Dr. Mark Haskins as described (45). All feline primary cell lines were grown in RPMI media supplemented with 10% FBS with the addition of 100 μg/mL streptomycin and penicillin and maintained in humidified 5% CO_2 atmosphere. Human skin fibroblast cultures were metabolically labeled with TRAN35S-label (MP Biomedicals, Solon, OH) in methionine- and cysteine-free DMEM (Invitrogen, USA). Goat antisera against human LAP was purchased from R & D systems (USA), while the monoclonal antibodies γ-tubulin and fibrillin-1 were obtained from Sigma (St. Louis, MO, USA) and Millipore (Billerica, MA), respectively. Rabbit antisera against human phospho-Smad 2/3 and monoclonal anti-human Smad 2/3 were purchased from Millipore, where as rabbit anti-human transglutaminase 2 was obtained from Abcam (Cambridge, MA, USA). The transglutaminase 2 inhibitors cystamine HCl and monodansyl cadaverine (MDC) were purchased from TCI Fine

Chemicals (USA) and Sigma (St. Louis, MO, USA), respectively. Retinoic acid and the protease inhibitor cocktail were obtained from TCI Fine Chemicals and Sigma, respectively as well. TGF-β1 mink lung epithelial cells (T-MLECs) stably transfected with a luciferase gene driven by a TGFβ-responsive plasminogen activator inhibitor-1 promoter sequence was a kind gift from D. Rifkin (New York University, New York, NY).

Isolation of primary feline fibroblasts-like synoviocytes, skin fibroblasts and chondrocytes − Fibroblast-like synoviocytes and feline skin fibroblasts were isloated from normal and ML-II synovial membrane and skin, respectively, through collagen 1A digestion. Chrondrocytes were extracted from articular cartilage by collagenase B digestion. All primary cells were cultured as monolayers in RPMI containing 10% FBS as stated above until confluent and scraped in PBS.

qPCR − WT, ML-II and MPS-I human skin fibroblasts were cultured for 3 days followed by RNA isolation and qRT-PCR data analysis as previously described (46).

Preparation of SDS whole cell lysates, DOC-soluble, DOC-insoluble fractions and media collection − In preparation for western blot analysis, human and feline fibroblasts were plated at equal density (2.5 x 10^5 cells/mL), cultured for times indicated and collected. Feline chondrocytes were isolated and cultured upon removal of collagenase and collected after 4 days. Cells were scraped with a rubber policeman followed by centrifugation. For SDS whole cell lysate preparations, cell pellets were treated with 1% SDS in PBS 7.4, plus protease inhibitors followed by a brief sonication and centrifugation. These cell preparations result in full solubility

of cellular components (including ECM). DOC-soluble and insoluble fractions were prepared as described (47) with a few modifications. Briefly, cells were lysed in 1% DOC containing 2 mM EDTA in 20 mM Tris pH 8.3 generating two pools: (1) a DOC-soluble (cell-associated) and (2) DOC-insoluble (matrix) fraction. The insoluble matrix fraction was solubilized in SDS containing buffer (3 % SDS, 15% glycerol, and 75 mM Tris pH 6.5). Both SDS whole cell and DOC preparations were normalized to total protein using micro BCA protein assay (Pierce/Thermo Scientific, Rockford, IL) and prepared for SDS-PAGE and Western blot analysis as described (48). As a loading control for SDS whole cell preps, nitrocellulose membranes were lightly stripped and re-probed for γ-tubulin. For the additional collection of conditioned media, cells were washed twice with PBS and incubated in 1 mL of serum free DMEM overnight, 24 hours before collection. Media was then concentrated from 1 mL to 100 μL, in the presence of protease inhibitors, using centricon 10 tubes (Milllipore) and assayed for equivalent loading concentration using microBCA protein assay kit (Pierce). Cells were prepared as described above using sodium deoxycholate and analyzed by Western blotting.

Metabolic ^{35}S labeling − Pulse-chase analysis of latent TGF-β1 was performed as described (48) with modifications. WT, MLII and MLIII fibroblasts were cultured until 80% confluent in DMEM supplemented with 20% FBS. The media was removed and the cells were methionine deprived for 1 hr in met/cys free media and subsequently labeled with ^{35}S methionine (0.75 mCi/mL) in met/cys free media with 10% dialyzed FBS for 2 hrs. Media was change to DMEM supplemented with 20% FBS to initiate the chase for times indicated in each experiment. Samples were immunoprecipitated overnight with anti-LAP antibody, incubated with protein A sepharose beads at 4 °C for 1hr and washed extensively, followed by boiling for 10 minutes at

100 °C to elute protein. Samples were then separated by SDS-PAGE and detected by autoradiography.

TGF-β1 mink lung luciferase activity assay − WT, ML-II and MPSI human skin fibroblasts were cultured for 3-4 days in a 60 mm dish and scraped with a rubber policeman in PBS. Cells were then pelleted by centrifugation and subsequently lysed with 1% DOC and prepared as described above. For the TGF-β1 reporter experiments, DOC-insoluble pellets were washed and broken apart using a pipette in 200 μL of DMEM (serum free). Homogenized samples were split into two 100 μL aliquots. One set was added directly to the mink lungs, while the other was subjected to 80 °C heat activation for 10 minutes, followed by cooling and addition directly to previously plated mink lung reporter cells (1.6×10^5 cells/mL). The experiments were performed by a pre-determined protocol from the lab of Dr. Dan Rifkin (49).

Wound healing assays − Wild type and ML-II cells were plated and grown to confluency followed by washing with PBS and culturing cells overnight in 0.1 % FBS supplement DMEM. After serum starvation, cells were scratched with a pipette tip and washed extensively to remove cell debri. Fresh culture media containing 2% FBS was added to cultures and incubated for 24 h in the presence or absence of TGF-β1 ligand (10 ng/mL). Images were prepared at 0 and 24 h post scarring using a 10x Plan C objective attached to an Olympus CX41 compound light microscope. While pictures of wound closure fields were taken with a Retiga 2000R Fast 1394 camera Qcapture software v2.8.1. Measurement of wound closure was determined within several fields by counting cells that migrated into the wound at the times indicated.

Transglutaminase 2 inhibition assays − WT and ML-II skin fibroblast were plated (2.5 x 10^5 cells/mL) in the absence or presence of the TG2 inhibitor, cystamine (100 μM) or monodansylcadaverine (MDC; 100 μM) and incubated for 4 days. Cells were collected and DOC fractionated followed by western blot analysis.

Retinoic acid treatment and transglutaminase 2 activity assays – WT human fibroblast were plated in the presence or absence of 5 μM retinoic acid (RA) and cultured for 72 hours. After the incubation period, 1 mL of fresh media was applied to the cells with and without 1 mM biotinylated pentylamines and further incubated for 1 hr at 37 °C as described (50). Fibroblasts were then collected and lysed with 1% SDS and prepared for Western blot analysis as described above.

Immunofluorescence and Confocal Microscopy - For immunofluorescence experiments, cells were seeded on coverslips and cultured for 2 or 7 days then analyzed with mouse anti-human fibrillin-1 and goat anti-human LAP. Cells were washed with phosphate-buffered saline, fixed in 3.7% formaldehyde in PBS and stained as described previously (51). The standard dilution buffer for both primary and secondary antibody incubations contained PBS, pH 7.4, 0.1% Triton X-100 and 1 mg/mL bovine serum albumin. Confocal images were acquired using an Olympus FV1000 laser scanning microscope equipped a 60x oil immersion (numerical aperture 1.4) objective. Stacks of 0.25 μm (based on calculated optimums) optical sections were collected in the z-dimension, and subsequently collapsed into a single image (maximum intensity or z-projection) unless otherwise noted.

Results

TGF-β1 signaling is reduced in ML-II fibroblasts. Prior studies in zebrafish models of both ML-II and MPS suggested that the TGF-β1 signaling was altered (41,42). While such studies provide a correlative link between abnormal lysosomal function and dysregulation of TGF-β1 signaling, the mechanisms and cell type specificity of these effects have yet to be investigated. To explore whether this pathway is also sensitive in cells isolated from ML-II and MPS-I patients, the level of phosphorylated Smad 2/3, a downstream readout of TGF-β1 signaling, was first analyzed in wild type, ML-II and MPS-I dermal fibroblasts. The levels of phosphorylated Smad2/3 (p-Smad 2/3) were significantly decreased in ML-II and MPS-I fibroblasts (2.09 ± 0.31-fold reduction in ML-II), indicating a reduction in TGF-β1 mediated signaling (Figure 1A,B). Because wound closure and healing are well established to be TGF-β1 dependent processes, wound closure assays were performed to assess whether decreased TGF-β1 signaling impacted the behavior of ML-II fibroblasts (Figure 1C and D). For these experiments, confluent cultures of WT and ML-II fibroblasts were wounded and incubated in the presence or absence of TGF-β1 ligand. The extent of wound closure was qualitatively and quantitatively determined after 24 h in culture. Wound closure in ML-II fibroblasts was reduced by 63.1 $\pm$ 6.8% compared to WT fibroblasts in the absence of TGF-β1 ligand. The presence of TGF-β1 ligand (10 ng/mL) did not alter wound closure in WT cells but did result in partial rescue in ML-II cells (39.3 $\pm$ 13.5 %, $p < 0.01$). Surprisingly, no decrease in wound closure was observed in MPS fibroblasts despite the reduction in pSmad 2/3 levels. These data indicate that the delayed wound healing in ML-II is TGF-β1 dependent and correlative with the decreased TGF-β1 signaling observed in ML-II human skin fibroblast.

Steady-state level of latent TGF-β1 is increased in ML-II fibroblasts. Several mechanisms can account for the decreased TGF-β1 signaling observed in ML-II fibroblasts, including downregulation of TGF-β1 biosynthesis. To test this, qPCR analysis was performed to address any differences in the expression of the TGF-β1 precursor. Alterations in transcript abundance did not appear to account for the lower pSmad2/3 levels, as the level of TGF-β1 transcripts was comparable in the three cell lines (Figure 2A). Strikingly, however, Western blot analysis demonstrated a clear increase in total latent TGF-β1 protein in ML-II fibroblasts, as detected by presence of the 40 kDa latency associated peptide (LAP) (Figure 2B). Collectively, these findings suggest that the decrease in TGF-β1 signaling in ML-II is associated with increased levels of the latent form of this cytokine.

TGF-β1 is processed and secreted normally in ML-II cells. Since increased transcription of TGF-β1 precursor could not account for the increased level of protein detected in ML-II fibroblasts, metabolic labeling experiments were performed next to monitor the processing and trafficking of newly synthesized latent TGF-β1 in ML-II fibroblasts. As shown in Figure 3, comparable amounts of 35S-labeled latent TGF-β1 were detected at 0 h chase in WT and ML-II fibroblasts. The protein appeared to be secreted at the same rate at 5 and 24 h, suggesting that its early processing and trafficking is not altered (Figure 3). Analysis of media fractions, however, revealed a lower abundance of the large latent complex (LLC) as well as the absence of the small latent complex (SLC) and latency associated peptide (LAP) in ML-II cells. The appearance of a reactive band at 70 kDa was noted in the ML-II media cultures comparable to WT. In several pulse-chase experiments, the 70 kDa band, along with LTBP, were the only bands detected in ML-II media (Figure 3B and data not shown). The 70 kDa band may correspond to dimerized

LAP (the presence of SDS dissociates the mature ligand). Several attempts were made to reduce this form, but our efforts were unsuccessful (data not shown). These data indicate that while the processing and trafficking of latent TGF-β1 is normal, deposition into the media is drastically altered. This altered deposition may account for the impaired signaling and wound closure (as shown in Figure 1).

The increased insolubility is associated with decreased latent TGF-β1 secretion into the culture media of ML-II fibroblasts. Thus far, we have determined that there is impaired TGF-β1 signaling that can be attributed to a lower abundance of readily activated forms of latent TGF-β1 in the media of ML-II cultures (Figure 3B). However, this still does not address the increase in total latent TGF-β1 levels as determined in the Western blots in Figure 1D. To assess the partitioning of latent TGF-β1 in ML-II cells, we used a standard sodium deoxycholate (DOC) solubility separation method (*see Materials and Methods*) utilized by Mosher and McKeown-Longo(52) and Keski-Oja (47). Briefly, this method generates two pools: (1) a detergent-soluble *cell-associated* pool and (2) an detergent-insoluble *ECM* pool. Using this method, Western blot analysis was performed on DOC soluble and insoluble cellular lysates and media from WT, ML-II and MPS I cultures to determine the partitioning of latent TGF-β1 in these fractions. As shown in Figure 4A, the majority of latent TGF-β1 (both LLC and SLC) was recovered in the DOC-soluble fractions of WT and MPS-I 3-4 day fibroblast cultures. In contrast, these complexes were highly enriched within the DOC-insoluble ECM fraction of ML-II cells as well as the cell-associated (DOC-soluble pool), indicating increased levels and enhanced insolubility of latent TGF-β1. Similar results were determined from 7 day cultures of WT, ML-II and MPS-I (Figure 4B). To determine the partitioning of latent TGF-β1 in the cell soluble, insoluble ECM,

and cultured media, 6 day cultures of WT and ML-II fibroblasts were incubated in serum free media for another 24 hours in these conditions. Cells were DOC-fractionated and media was collected and concentrated followed by Western blot analysis. Cultured WT fibroblasts showed a striking difference in partitioning of latent TGF-β1 deposition compared to ML-II cells (Figure 4C). Levels of latent TGF-β1 in the media of ML-II were absent compared to WT cultures, consistent with the pulse-chase analysis shown in Figure 3. Conversely, ML-II insoluble pools contained more latent TGF-β1 than WT insoluble fractions. To address whether the latent forms of TGF-β1 in insoluble ECM pools from ML-II fibroblasts could be activated, insoluble pellets from WT and ML-II 3-4 day cultures were heat-treated and tested for TGF-β1 activity using a luciferase reporter assay. A three-fold increase in TGF-β1 activity was detected in the insoluble fraction of ML-II cells following heat treatment, indicating that the deposited TGF-β1 was in fact a latent protein capable of activation (Figure 4D). Collectively, these findings suggest that the increased insolubility of latent TGF-β1 is associated with its decreased secreted levels, altered activation and reduced bioavailability.

Insolubility of latent TGF-β1 is associated with increased fibrillar network incorporation in ML-II cultures. Previous studies have determined that in normal cell cultures, latent TGF-β1 is incorporated with fibrillin-1 during ECM microfibril assembly, a pericellular interaction that governs the storage and extracellular deposition of this growth factor (9). Since our data suggests that latent TGF-β1 has increased insoluble ECM forms in ML-II, we sought to determine whether this insolubility corresponded with greater ECM deposition of both TGF-β1 and ECM components. Immunofluorescence staining of latent TGF-β1 and fibrillin-1 was performed on human WT, ML-II and MPS-I cultures (Figure 4.5). Within two days of culture

(Figure 4.5A), fibrillin-1 was shown to deposit into the ECM in greater amounts compared to WT and MPS-I fibroblasts. Similar to this, latent TGF-β1 was incorporated into more tightly co-localized regions of fibrillin-1 microfibrils at the early 2 day time point. Strikingly, fibrillin-1 microfibril assembly at the 7 day culture time points (Figure 4.5B) was comparable between cell types. However, the increased co-localization of latent TGF-β1 within the ECM of ML-II fibroblasts was vastly different at 7 day compared to WT, MPS-I as well as the 2 day ML-II cultures. These results are consistent with our biochemical results shown in Figure 4.4 and clearly demonstrate a more rapid incorporation and increased deposition of latent TGF-β1 within the fibrillar network in ML-II fibroblasts.

Tissue transglutaminase is upregulated in ML-II fibroblast. Prior studies by several groups have suggested that latent TGF-β1 is cross-linked to the ECM by the action of TG2 (16,44,53). TG2 has been demonstrated to contain both transamidation and protein disulfide isomerase activity, but can also increase the association of matrix proteins in an activity-independent manner (14). Since the decreased solubility of latent TGF-β1 in ML-II fibroblasts might be due to the TG2 cross-linking activity, the level of this protein in whole SDS lysates from WT and patient fibroblasts was analyzed by Western blot. As shown in Figure 6A, the level of TG2 was upregulated compared to WT and MPS-I. Similar findings were determined in DOC-soluble and insoluble fractions, with a high abundance of TG2 found in the insoluble pool (Figure 6B). These findings indicate that the upregulation of TG2 is a feature of ML-II fibroblasts and may be the basis for the insolubility of latent TGF-β1 in human ML-II fibroblasts.

Upregulation of TG2 and abnormal deposition of latent TGF-β1 into the ECM are reproducible in feline fibroblast, but not in chondrocytes. To address the specificity of the upregulation of TG2 and altered ECM deposition of latent TGF-β1 in feline tissues, Western blots on whole SDS cell lysates, DOC-soluble and –insoluble as well as media fractions were performed in feline fibroblasts-like synoviocytes (FLS), while chondrocytes isolated from unaffected and affected ML-II litters. FLS were used at very early passage number (n=3-10) to avoid adaptations in culture and to minimize effects of senescence. Chondrocytes were cultured 4 days upon removal from collagenase and collected. Whole SDS cell Western blot analysis revealed that TG2 protein levels were similar between WT and ML-II FLS (Figure 7A). However, DOC fractionation determined that higher TG2 levels are found in the DOC-insoluble pools (Figure 7B). Furthermore, DOC preparations revealed a similar abnormal increase of latent TGF-β1 into the ECM (DOC-insoluble) of 3-4 day cultured ML-II feline skin fibroblasts (data not shown) and ML-II feline FLS compared to wild type, (Figure 7C). Note a similar 70 kDa band that was observed in Figure 3 is present in the ML-II insoluble ECM fractions shown in Figure 7C. Strikingly, latent TGF-β1 is not found in the media of 7 day cultured ML-II FLS, consistent with the finding in human ML-II fibroblast (Figure 4C) and substantially different from WT media cultures as shown in Figure 7D. Similar to the observations noted in human fibroblasts, phosphorylation of Smad 2/3 is decreased (2.78 $\pm$ 1.22 fold) in ML-II FLS compared to WT (Figure 7E). These results mirror the findings in human fibroblast and demonstrate a general ML-II disease phenomenon.

To address the systemic nature of these findings, TG2 upregulation in feline chondrocytes was also assessed. As shown in Figure 8A, protein levels of TG2 in ML-II are comparable to WT in SDS cell lysates and DOC prepared lysates. In addition, latent TGF-β1

solubility and deposition is not altered in ML-II feline chondrocytes, however, there is a slight increase in soluble latent TGF-β1 in ML-II chondrocytes (Figure 8B, SDS and DOC preps). These results suggest that the upregulation of TG2 and altered latent TGF-β1 solubility may be a feature of some, but not all, cultured ML-II cells.

Inhibitors of transglutaminase activity do not alter solubility of latent TGF-β1. Cross-linking of latent TGF-β1 to the insoluble ECM can be inhibited using the compounds cystamine HCl or monodansyl cadaverine (MDC) (ref). In order to determine whether inhibition of transglutaminase activity can alter latent TGF-beta solubility, cells were cultured for 4 days in the presence or absence of either 150 μM cystamine-HCl (Figure 4.9) or 100 μM MDC (data not shown). As shown, cystamine-HCl does not appear to alter the deposition of latent TGF-β1 into the insoluble pool.

Induction of transglutaminase expression in human WT fibroblasts by retinoic acid treatment leads to increased insolubility of latent TGF-β1. One potential reason why inhibitors to TG2 cross-linking of latent TGF-β1 did not seem to work is the fact that cumulative cross-linking of latent TGF-β1 over time could mask any effects of the inhibitors in these cells. Normal cells that have sustained expression of TG2 have been shown to exhibit increased cell surface cross-linking as a result of translocation of TG2 from the cytoplasm to the ECM (54). Retinoic acid (RA), the vitamin A metabolite, has been demonstrated to upregulate the expression of TG2 due to the fact the TG2 gene contains a retinoic acid response element (RARE) (55,56). To directly test whether RA treatment of WT fibroblasts could increase TG2 levels and shift the solubility of latent TGF-β1, WT human fibroblasts were seeded overnight

followed by the addition of 5 μM of RA for 72 h and subjected to DOC fractionation and Western blot analysis. As shown in Figure 4.10A, TG2 cell soluble levels were equivalent after RA treatment, however the insoluble ECM fraction showed a striking difference in TG2 protein levels, suggesting this material has translocated to the ECM after RA stimulation. The shift in TG2 solubility is very similar to the trend shown in human and feline ML-II fibroblast (Figure 4.6B and 4.7A and B). To determine if the expression and shift in TG2 solubility results in increased activity, we incubated cultured cells with and without (control for non-specific biotin) 1 mM biotinylated pentylamines (BP). These peptides have been previously used to measure cross-linking activity of TG2 (50,57). Western blot analysis, using antisera against biotin, determined that BP incorporation into cellular protein substrates of TG2 was increased with the addition of RA (Figure 4.10C). Consistent with these findings, Western blot analysis of LTBP (Figure 4.10C, *top panel*; non-reducing to visualize LLC) and latent TGF-β1 (Figure 4.10C, *lower panel*) after RA treatment resulted in a shift in solubility of LLC and latent TGF-β1. These data suggest that increased expression of TG2 results in its partial translocation to the ECM. Moreover, increased transamidation activity of this enzyme corresponds to the increased deposition of latent TGF-β1 into the insoluble ECM. Increased cross-linking of latent TGF-β1 may also explain why this insolubility form of the molecule was not altered upon treatment of the pellets with reducing agents.

Discussion

The mechanisms that underlie tissue-specific pathology of lysosomal diseases such as ML-II remain poorly understood. In addition, there is limited information regarding the role of

specific Man-6-P modified proteins in the disease process. Here, we provide novel evidence that transglutaminase 2 (TG2) is upregulated in ML-II cells, correlating with the increased insolubility of latent TGF-β1. Latent TGF-β1 insolubility was demonstrated in human as well as feline fibroblasts, indicating that the altered deposition is a general disease phenomenon. In addition, the increased deposition of latent TGF-β1 was consistent with a more rapid fibrillar network assembly in ML-II, suggesting that the regulation of other ECM components may be influenced by TG2.

Our data suggest that the increased incorporation of latent TGF-β1 is directly associated with the increase in TG2 expression and transamidation activity. However, our preliminary attempts to determine the role of TG2 in ML-II were not successful. In particular, we tried to inhibit the deposition of latent TGF-β1 into the insoluble ECM by use of the TG2 inhibitors cystamine and MDC, both of which were not capable of inhibiting the TG2 enzyme. We can only speculate at this point why these inhibitors did not seem to work. Due to the previous accumulation of protease insensitive cross-linked latent TGF-β1 in ML-II, it is possible that one would never observe the inhibition under steady-state conditions. Optimization of metabolic labeling conditions is necessary to determine a difference in deposition of this growth factor. Moreover, there is no data available that addresses the efficacy or how well these compounds penetrate cell membranes. Because of this, it is plausible that concentrations we used were not effective. However, increases in the concentration of these inhibitors, in our hands, were cytotoxic (data not shown). Current work is ongoing to determine and manipulate the overall activity of TG2 in these cells.

Even though the TG2 inhibitors did not block the deposition of latent TGF-β1 in ML-II cells, TG2 is likely responsible for the altered solubility for several reasons. First, previous

literature has indicated that this protein cross-links latent TGF-β1 to the ECM via the N-terminal region of LTBP (16,53,54). Secondly, our data suggests that upregulation of TG2 activity by RA treatment can shift the solubility of latent TGF-β1 in normal cells (see Figure 4.10). Lastly, the presence of TG2 in the insoluble ECM is consistent with earlier work demonstrating the translocation of TG2 from the cytoplasm to the ECM and an increase in its extracellular cross-linking activity (54,58). In fact, it has been demonstrated that extracellular TG2 has activity independent functions associated with cell adhesion and migration (14), two characteristics that are impaired in ML-II (Figure 4.1). Thus, it is possible that the role of TG2 in ML-II extends beyond its cross-linking activity.

It is has been documented that TG2 has calcium-dependent and independent functions. The majority of TG2 (over 80%) is localized to the cytoplasm where it performs its calcium-independent functions, such as GTPase activity and signal transduction mediation (through G-protein coupled receptors) (59). In addition, this multi-functional enzyme has been shown to act as a protein kinase (60) and disulfide isomerase (61). The most notable function of TG2 is its calcium dependent transamidation activity. Cytoplasmic TG2 does not have calcium-dependent transamidation activity due to the low concentration of cytoplasmic calcium and the high levels of cytoplasmic GTP, an inhibitor of transamidase activity (62,63). Indeed, TG2 may be abnormally activated in diseases where calcium homeostasis is altered (64). Recent literature has hypothesized that calcium imbalance may be important in the pathology of lysosomal storage diseases (65,66) and ML-II has been shown to exhibit autophagic dysfunction associated with abnormal mitochondrial fragmentation, resulting in calcium flux (67,68). Therefore, secondary changes in calcium homeostasis in ML-II might lead to aberrant activation of TG2 and increased transamidation activity in the cytoplasm or the pericellular microenvironment.

Stress inducers such as oxidative stress and abnormal matrix remodeling can stimulate translocation of TG2 to the ECM (69,70) or the possibly to the ER where calcium is present. It is conceivable that abnormal ECM fragmentation could stimulate TG2 translocation and activity, since fragments can induce stress responses (71-75). In support of this mechanism, we have shown that fibronectin is abnormally fragmented in ML-II (Appendix Figure 1A and B). This fragmentation profile is not found in MPS-I or the milder form of ML-II disease, ML-III (Appendix Figure 1B). In addition, we have shown that this abnormal fragmentation can be generated by specific cathepsin proteases (Appendix Figure 2). Recent observations have also shown that fibrillin-1 is susceptible to cathepsin proteases K and V (76), another ECM component that may be fragmented in ML-II. As shown in Appendix Figure 2, treatment with recombinant cathepsin D results in a similar fragmentation profile as that seen in ML-II fibroblasts (Appendix Figure 1). This generated fragmentation profile by cathepsin D is distinct from the other cathepsin proteases tested. It is interesting to speculate that cathepsin generated ECM fragments may elicit a stress response that upregulates and translocates TG2 to the ECM increasing its cross-linking activity (model; Figure 4.11).

As mentioned above, TG2 may be translocated to the ER under stress conditions. A recent report proposed a novel finding, in which TG2 co-localized within ER associated granules in Parkinson's disease brain (77). Placement of TG2 within the ER would result in abnormal transamidase activity due to the high levels of calcium present in this organelle. Abnormal TG2 localization within the ER of ML-II cells could lead to increased cross-linking of proteins as well as secretion of this protein out of the cell to the ECM or cell surface where it would be active. Experiments are currently underway to investigate the possible localization of TG2 in the ER.

In addition to the increased deposition of latent TGF-β1, our data also determined that TGF-β1 signaling is decreased in human and feline ML-II fibroblasts as assessed by phosphorylation of Smad 2/3 levels and scratch-wound assays (Figure 4.1 and 4.7). However, it is not known if the increased deposition and storage of latent TGF-β1 is responsible for the decreased signaling. Previous literature suggests that increased cross-linking of latent TGF-β1 by TG2 leads to activation of this growth factor, indicating that the cross-linking step is a requirement for activation (78-80). This is not consistent with our findings, which suggest that increased deposition leads to impaired signaling of TGF-β1. Since ML-II disease has multiple complications (i.e., lysosomal dysfunction, upregulation of matrix-degrading enzymes), control of growth factor activation may be important for cell survival or regulation of abnormal ECM remodeling. So, it is plausible that TG2 cross-linking of growth factors to the ECM is important, since sequestration may reduce activation.

Another possible reason for the decreased TGF-β1 signaling may be linked to the hypersecretion of cathepsin proteases. Dieter Bromme and colleagues have suggested that secreted cathepsin K can regulate TGF-β1 signaling by means of degradation (81). Hypersecretion of cathepsin proteases could potentially degrade the activated ligand in ML-II cells. Our model (Figure 4.11), proposes that increased cathepsin protease secretion diminishes TGF-β1 signaling. These cathepsin proteases may generate the ECM fragments (as discussed above), which could upregulate TG2 activity resulting in abnormal cross-linking of ECM components as well as latent TGF-β1. There are possible reasons why it would be necessary for upregulation in TG2 cross-linking activity. First, the transamidation of ECM components would serve as a defense mechanism to block ECM destruction by cathepsin proteases. Indeed, TG2 isopeptide cross-links have been shown to be resistant to lysosomal enzymes (82). Last,

increased cross-linking of latent TGF-β1 may be coordinated to compensate for the impaired signaling of this growth factor in ML-II.

The potential role that TG2 in ML-II disease is novel and could shed light on several pathologic characteristics associated with the disease. Patients with ML-II and the related disorder ML-III typically suffer from stiffness in their joints, a phenotype that has been attributed to lysosomal storage within connective tissue. In addition, these children typically present with hyperplastic tissues (i.e., gingiva) and thick and waxy skin, however, no evidence for classic fibrosis has been observed in this disease. It is possible that the joint immobility, skin thickness, and tissue hyperplasia may arise from increased TG2 activity. In fact, a recent report suggested that altered ECM protein deposition (i.e., fibrillin-1) leads to aberrant TGF-β1 activity and the disease stiff skin syndrome (35). Others have shown that altered TGF-β1 signaling causes alterations in ECM deposition resulting in connective tissue disorders (see Chapter 1 and Table 1.4). Our data presented here as well as the discussion of TG2 function in ML-II and other connective tissue disorders presents a basis for further investigation.

Acknowledgements

We would like to acknowledge Dr. Daniel Rifkin for his generous gift of the TGF-β1 mink lung reporter cells. We are grateful to Dr. Heather Flanagan-Steet for her assistance with the confocal imaging. This work was funded by grants from NIGMS (1R01GM086524-01 to RS), and a graduate fellowship from the Cousins Foundation (to JB).

References

1. Massague, J., Cheifetz, S., Boyd, F. T., and Andres, J. L. (1990) *Ann N Y Acad Sci* **593**, 59-72
2. Massague, J. (2000) *Nat Rev Mol Cell Biol* **1**, 169-178
3. Janssens, K., ten Dijke, P., Janssens, S., and Van Hul, W. (2005) *Endocr Rev* **26**, 743-774
4. Cohen, M. M., Jr. (2006) *Am J Med Genet A* **140**, 2646-2706
5. Gentry, L. E., Webb, N. R., Lim, G. J., Brunner, A. M., Ranchalis, J. E., Twardzik, D. R., Lioubin, M. N., Marquardt, H., and Purchio, A. F. (1987) *Mol Cell Biol* **7**, 3418-3427
6. Gentry, L. E., Lioubin, M. N., Purchio, A. F., and Marquardt, H. (1988) *Mol Cell Biol* **8**, 4162-4168
7. Dabovic, B., Levasseur, R., Zambuto, L., Chen, Y., Karsenty, G., and Rifkin, D. B. (2005) *Bone* **37**, 25-31
8. Chen, Q., Sivakumar, P., Barley, C., Peters, D. M., Gomes, R. R., Farach-Carson, M. C., and Dallas, S. L. (2007) *J Biol Chem* **282**, 26418-26430
9. Massam-Wu, T., Chiu, M., Choudhury, R., Chaudhry, S. S., Baldwin, A. K., McGovern, A., Baldock, C., Shuttleworth, C. A., and Kielty, C. M. (2010) *J Cell Sci* **123**, 3006-3018
10. Annes, J. P., Munger, J. S., and Rifkin, D. B. (2003) *J Cell Sci* **116**, 217-224
11. Annes, J. P., Rifkin, D. B., and Munger, J. S. (2002) *FEBS Lett* **511**, 65-68
12. Flaumenhaft, R., Kojima, S., Abe, M., and Rifkin, D. B. (1993) *Adv Pharmacol* **24**, 51-76
13. Miyazono, K., Olofsson, A., Colosetti, P., and Heldin, C. H. (1991) *EMBO J* **10**, 1091-1101
14. Belkin, A. M. (2011) *FEBS J*
15. Kiraly, R., Demeny, M., and Fesus, L. (2011) *FEBS J*
16. Nunes, I., Gleizes, P. E., Metz, C. N., and Rifkin, D. B. (1997) *J Cell Biol* **136**, 1151-1163
17. Shi, M., Zhu, J., Wang, R., Chen, X., Mi, L., Walz, T., and Springer, T. A. (2011) *Nature* **474**, 343-349
18. Aluwihare, P., Mu, Z., Zhao, Z., Yu, D., Weinreb, P. H., Horan, G. S., Violette, S. M., and Munger, J. S. (2009) *J Cell Sci* **122**, 227-232
19. Aluwihare, P., and Munger, J. S. (2008) *Am J Respir Cell Mol Biol* **39**, 499-502
20. Annes, J. P., Chen, Y., Munger, J. S., and Rifkin, D. B. (2004) *J Cell Biol* **165**, 723-734
21. Cambier, S., Gline, S., Mu, D., Collins, R., Araya, J., Dolganov, G., Einheber, S., Boudreau, N., and Nishimura, S. L. (2005) *Am J Pathol* **166**, 1883-1894
22. Crawford, S. E., Stellmach, V., Murphy-Ullrich, J. E., Ribeiro, S. M., Lawler, J., Hynes, R. O., Boivin, G. P., and Bouck, N. (1998) *Cell* **93**, 1159-1170
23. Murphy-Ullrich, J. E., and Poczatek, M. (2000) *Cytokine Growth Factor Rev* **11**, 59-69
24. Murphy-Ullrich, J. E., Schultz-Cherry, S., and Hook, M. (1992) *Mol Biol Cell* **3**, 181-188
25. Ribeiro, S. M., Poczatek, M., Schultz-Cherry, S., Villain, M., and Murphy-Ullrich, J. E. (1999) *J Biol Chem* **274**, 13586-13593
26. Schultz-Cherry, S., Ribeiro, S., Gentry, L., and Murphy-Ullrich, J. E. (1994) *J Biol Chem* **269**, 26775-26782
27. Jenkins, G. (2008) *Int J Biochem Cell Biol* **40**, 1068-1078
28. Hornebeck, W., Emonard, H., Maquart, F. X., and Bellon, G. (2003) *J Soc Biol* **197**, 25-30

29. Maeda, S., Dean, D. D., Gay, I., Schwartz, Z., and Boyan, B. D. (2001) *J Bone Miner Res* **16**, 1281-1290
30. Pedrozo, H. A., Schwartz, Z., Gomez, R., Ornoy, A., Xin-Sheng, W., Dallas, S. L., Bonewald, L. F., Dean, D. D., and Boyan, B. D. (1998) *J Cell Physiol* **177**, 343-354
31. Saito, T., Kinoshita, A., Yoshiura, K., Makita, Y., Wakui, K., Honke, K., Niikawa, N., and Taniguchi, N. (2001) *J Biol Chem* **276**, 11469-11472
32. Le Goff, C., Mahaut, C., Wang, L. W., Allali, S., Abhyankar, A., Jensen, S., Zylberberg, L., Collod-Beroud, G., Bonnet, D., Alanay, Y., Brady, A. F., Cordier, M. P., Devriendt, K., Genevieve, D., Kiper, P. O., Kitoh, H., Krakow, D., Lynch, S. A., Le Merrer, M., Megarbane, A., Mortier, G., Odent, S., Polak, M., Rohrbach, M., Sillence, D., Stolte-Dijkstra, I., Superti-Furga, A., Rimoin, D. L., Topouchian, V., Unger, S., Zabel, B., Bole-Feysot, C., Nitschke, P., Handford, P., Casanova, J. L., Boileau, C., Apte, S. S., Munnich, A., and Cormier-Daire, V. (2011) *Am J Hum Genet* **89**, 7-14
33. Ramirez, F., and Dietz, H. C. (2007) *Curr Opin Genet Dev* **17**, 252-258
34. Olivieri, J., Smaldone, S., and Ramirez, F. (2010) *Fibrogenesis Tissue Repair* **3**, 24
35. Loeys, B. L., Gerber, E. E., Riegert-Johnson, D., Iqbal, S., Whiteman, P., McConnell, V., Chillakuri, C. R., Macaya, D., Coucke, P. J., De Paepe, A., Judge, D. P., Wigley, F., Davis, E. C., Mardon, H. J., Handford, P., Keene, D. R., Sakai, L. Y., and Dietz, H. C. (2010) *Sci Transl Med* **2**, 23ra20
36. Balemans, W., and Van Hul, W. (2002) *Dev Biol* **250**, 231-250
37. Metcalf, J. A., Linders, B., Wu, S., Bigg, P., O'Donnell, P., Sleeper, M. M., Whyte, M. P., Haskins, M., and Ponder, K. P. (2010) *Mol Genet Metab* **99**, 396-407
38. Ma, X., Tittiger, M., Knutsen, R. H., Kovacs, A., Schaller, L., Mecham, R. P., and Ponder, K. P. (2008) *Mol Genet Metab* **94**, 298-304
39. Petrey, A. C., Flanagan-Steet, F., Johnson,S., Fan, X., De la Rosa, M., Haskins, M., Nairn, A.V., Moremen, K.W., and Steet, R. (2011) *Disease Models and Mechanisms*
40. Lyons, J. A., Dickson, P. I., Wall, J. S., Passage, M. B., Ellinwood, N. M., Kakkis, E. D., and McEntee, M. F. (2011) *Lab Invest* **91**, 665-674
41. Flanagan-Steet, H., Sias, C., and Steet, R. (2009) *Am J Pathol* **175**, 2063-2075
42. Moro, E., Tomanin, R., Friso, A., Modena, N., Tiso, N., Scarpa, M., and Argenton, F. (2010) *Matrix Biol* **29**, 43-50
43. Dennis, P. A., and Rifkin, D. B. (1991) *Proc Natl Acad Sci U S A* **88**, 580-584
44. Yang, L., Tredget, E. E., and Ghahary, A. (2000) *Wound Repair Regen* **8**, 538-546
45. Mazrier, H., Van Hoeven, M., Wang, P., Knox, V. W., Aguirre, G. D., Holt, E., Wiemelt, S. P., Sleeper, M. M., Hubler, M., Haskins, M. E., and Giger, U. (2003) *J Hered* **94**, 363-373
46. Nairn, A. V., dela Rosa, M., and Moremen, K. W. (2010) *Methods Enzymol* **479**, 73-91
47. Taipale, J., Miyazono, K., Heldin, C. H., and Keski-Oja, J. (1994) *J Cell Biol* **124**, 171-181
48. Barnes, J., Lim, J. M., Godard, A., Blanchard, F., Wells, L., and Steet, R. (2011) *J Biol Chem* **286**, 24855-24864
49. Abe, M., Harpel, J. G., Metz, C. N., Nunes, I., Loskutoff, D. J., and Rifkin, D. B. (1994) *Anal Biochem* **216**, 276-284
50. Shin, D. M., Jeon, J. H., Kim, C. W., Cho, S. Y., Kwon, J. C., Lee, H. J., Choi, K. H., Park, S. C., and Kim, I. G. (2004) *J Biol Chem* **279**, 15032-15039
51. Steet, R., and Kornfeld, S. (2006) *Mol Biol Cell* **17**, 2312-2321

52. McKeown-Longo, P. J., and Mosher, D. F. (1983) *J Cell Biol* **97**, 466-472
53. Le, M., Gohr, C. M., and Rosenthal, A. K. (2001) *Connect Tissue Res* **42**, 245-253
54. Verderio, E., Gaudry, C., Gross, S., Smith, C., Downes, S., and Griffin, M. (1999) *J Histochem Cytochem* **47**, 1417-1432
55. Shimada, J., Suzuki, Y., Kim, S. J., Wang, P. C., Matsumura, M., and Kojima, S. (2001) *Mol Endocrinol* **15**, 1677-1692
56. Antonyak, M. A., Boehm, J. E., and Cerione, R. A. (2002) *J Biol Chem* **277**, 14712-14716
57. Jeon, J. H., Kim, C. W., Shin, D. M., Kim, K., Cho, S. Y., Kwon, J. C., Choi, K. H., Kang, H. S., and Kim, I. G. (2003) *FEBS Lett* **534**, 180-184
58. Hodrea, J., Demeny, M. A., Majai, G., Sarang, Z., Korponay-Szabo, I. R., and Fesus, L. (2010) *Immunol Lett* **130**, 74-81
59. Fesus, L., and Piacentini, M. (2002) *Trends Biochem Sci* **27**, 534-539
60. Mishra, S., and Murphy, L. J. (2004) *J Biol Chem* **279**, 23863-23868
61. Hasegawa, G., Suwa, M., Ichikawa, Y., Ohtsuka, T., Kumagai, S., Kikuchi, M., Sato, Y., and Saito, Y. (2003) *Biochem J* **373**, 793-803
62. Achyuthan, K. E., and Greenberg, C. S. (1987) *J Biol Chem* **262**, 1901-1906
63. Singh, U. S., Erickson, J. W., and Cerione, R. A. (1995) *Biochemistry* **34**, 15863-15871
64. Gibson, G. E., Starkov, A., Blass, J. P., Ratan, R. R., and Beal, M. F. (2010) *Biochim Biophys Acta* **1802**, 122-134
65. Vitner, E. B., Platt, F. M., and Futerman, A. H. (2010) *J Biol Chem* **285**, 20423-20427
66. Lloyd-Evans, E., and Platt, F. M. (2011) *Cell Calcium* **50**, 200-205
67. Otomo, T., Higaki, K., Nanba, E., Ozono, K., and Sakai, N. (2009) *Mol Genet Metab* **98**, 393-399
68. Otomo, T., Higaki, K., Nanba, E., Ozono, K., and Sakai, N. (2011) *J Biol Chem* **286**, 35283-35290
69. Shin, D. M., Jeon, J. H., Kim, C. W., Cho, S. Y., Lee, H. J., Jang, G. Y., Jeong, E. M., Lee, D. S., Kang, J. H., Melino, G., Park, S. C., and Kim, I. G. (2008) *FASEB J* **22**, 2498-2507
70. Gross, S. R., Balklava, Z., and Griffin, M. (2003) *J Invest Dermatol* **121**, 412-423
71. Homandberg, G. A., Hui, F., Wen, C., Purple, C., Bewsey, K., Koepp, H., Huch, K., and Harris, A. (1997) *Biochem J* **321 (Pt 3)**, 751-757
72. Chowdhury, T. T., Schulz, R. M., Rai, S. S., Thuemmler, C. B., Wuestneck, N., Bader, A., and Homandberg, G. A. (2010) *Arthritis Res Ther* **12**, R82
73. Ding, L., Guo, D., and Homandberg, G. A. (2009) *Osteoarthritis Cartilage* **17**, 1385-1392
74. Homandberg, G. A., Hui, F., and Wen, C. (1996) *Biochim Biophys Acta* **1317**, 143-148
75. Homandberg, G. A., Hui, F., and Wen, C. (1996) *Biochim Biophys Acta* **1317**, 134-142
76. Kirschner, R., Hubmacher, D., Iyengar, G., Kaur, J., Fagotto-Kaufmann, C., Bromme, D., Bartels, R., and Reinhardt, D. P. (2011) *J Biol Chem* **286**, 32810-32823
77. Wilhelmus, M. M., Verhaar, R., Andringa, G., Bol, J. G., Cras, P., Shan, L., Hoozemans, J. J., and Drukarch, B. (2011) *Brain Pathol* **21**, 130-139
78. Shweke, N., Boulos, N., Jouanneau, C., Vandermeersch, S., Melino, G., Dussaule, J. C., Chatziantoniou, C., Ronco, P., and Boffa, J. J. (2008) *Am J Pathol* **173**, 631-642
79. Huang, L., Haylor, J. L., Fisher, M., Hau, Z., El Nahas, A. M., Griffin, M., and Johnson, T. S. (2010) *Nephrol Dial Transplant* **25**, 3897-3910

80. Telci, D., Collighan, R. J., Basaga, H., and Griffin, M. (2009) *J Biol Chem* **284**, 29547-29558
81. Zhang, D., Leung, N., Weber, E., Saftig, P., and Bromme, D. (2011) *Respir Res* **12**, 72
82. Fesus, L., Tarcsa, E., Kedei, N., Autuori, F., and Piacentini, M. (1991) *FEBS Lett* **284**, 109-112

Figures

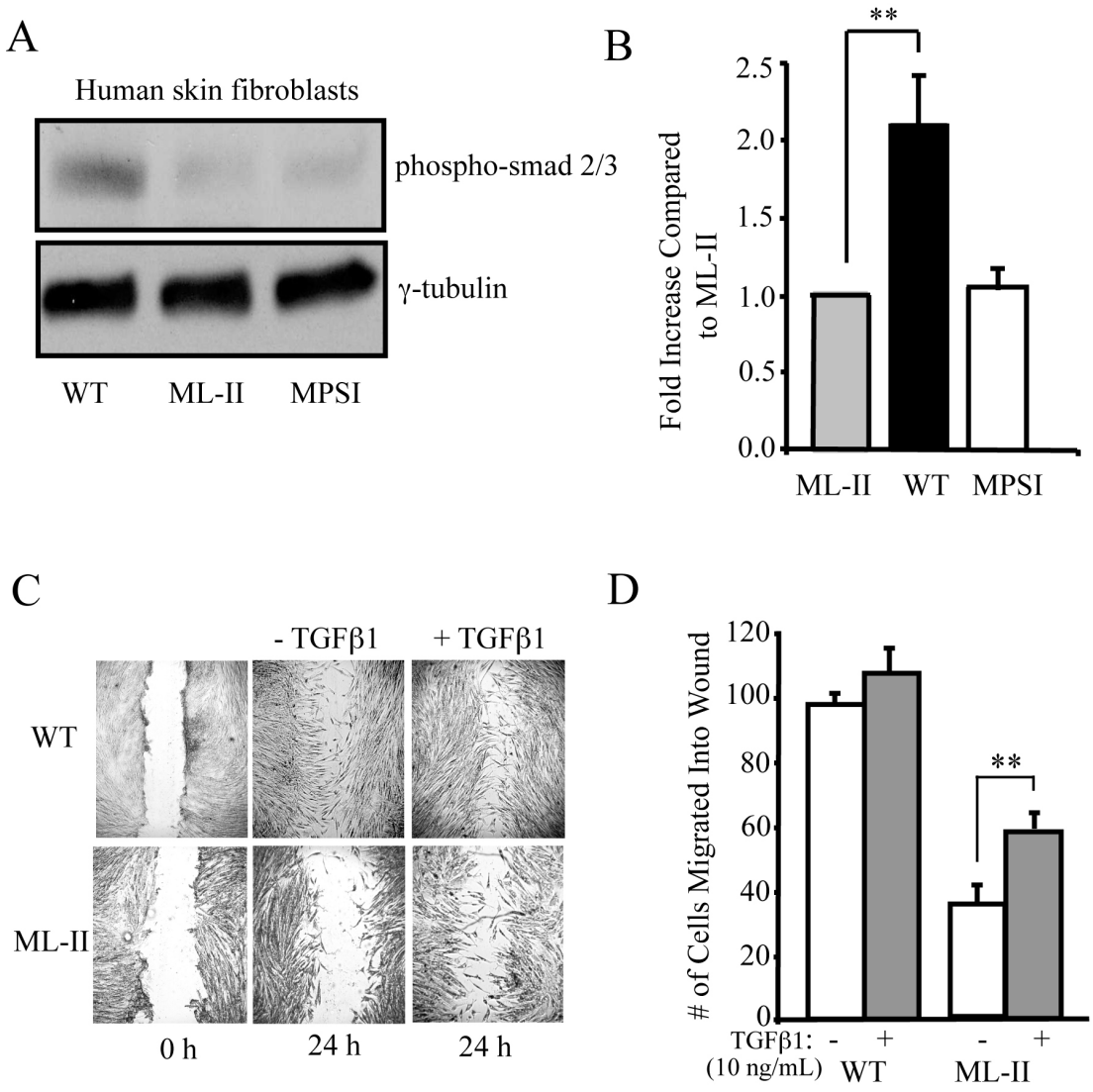


Figure 4.1. TGF-β1 signaling is altered in human ML-II fibroblasts. A representative Western blot of Smad 2/3 phosphorylation (n = 3) in 3-4 day WT, ML-II and MPS-I fibroblasts is shown (**A**). Quantitative densitometry analysis, using Image J (224), of Smad 2/3 phosphorylation normalized to the γ-tubulin loading control in WT and MPS-I is compared to ML-II (**B**). Wound closure was assessed in WT and ML-II human fibroblasts at 0 and 24h with and without 10 ng/mL TGF-β1 ligand (**C**). Wound closure was defined as the number of cells per field that migrate into the wound area. Closure experiments were performed in triplicate and a total of five fields were counted per assay and quantitated (**D**). Error bars represent the SEM of the independent triplicate experiments. P-values were given based on *t-test* determined from the independent triplicate experiments ($p < 0.05$, *; $p < 0.001$, **).

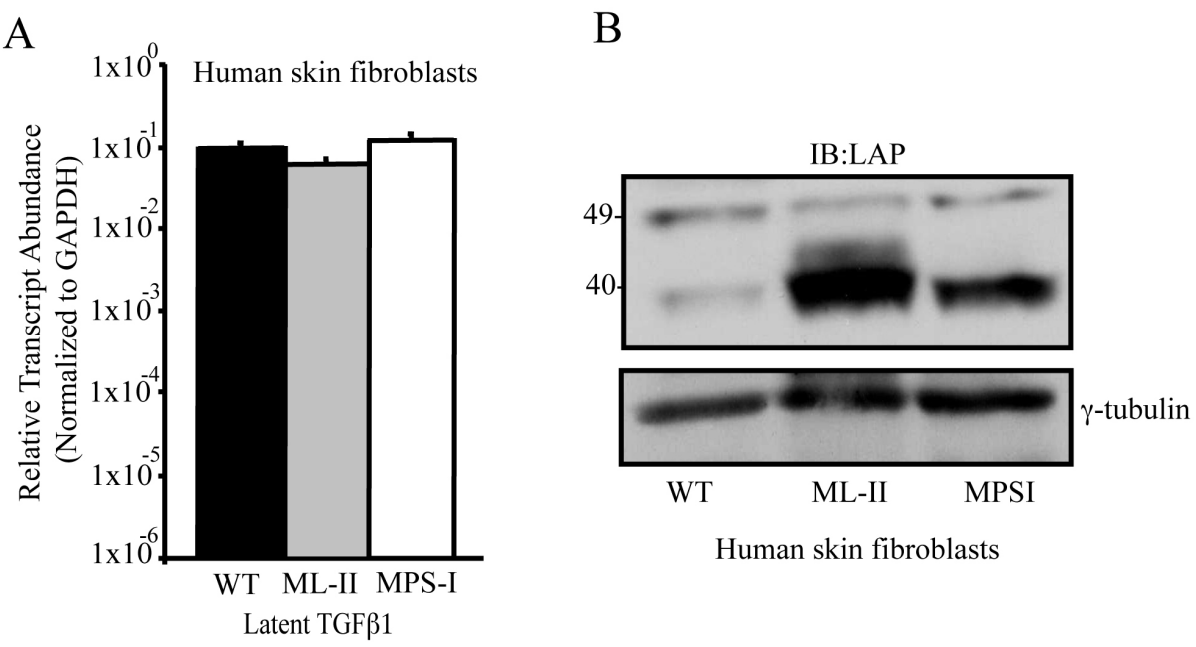


Figure 4.2. A decrease in transcription or steady-state levels of TGF-β1 does not account for the altered TGF-β1 signaling. (**A**) qPCR transcript abundance of latent TGF-β1 was determined in 3-4 day WT, ML-II and MPS-I human fibroblast. (**B**) Whole SDS cell lysates were subjected to Western blot analysis using antisera against LAP. Cellular protein was normalized to total protein using a microBCA protein assay kit. To ensure efficiency in loading, anti-γ-tubulin was used as a control.

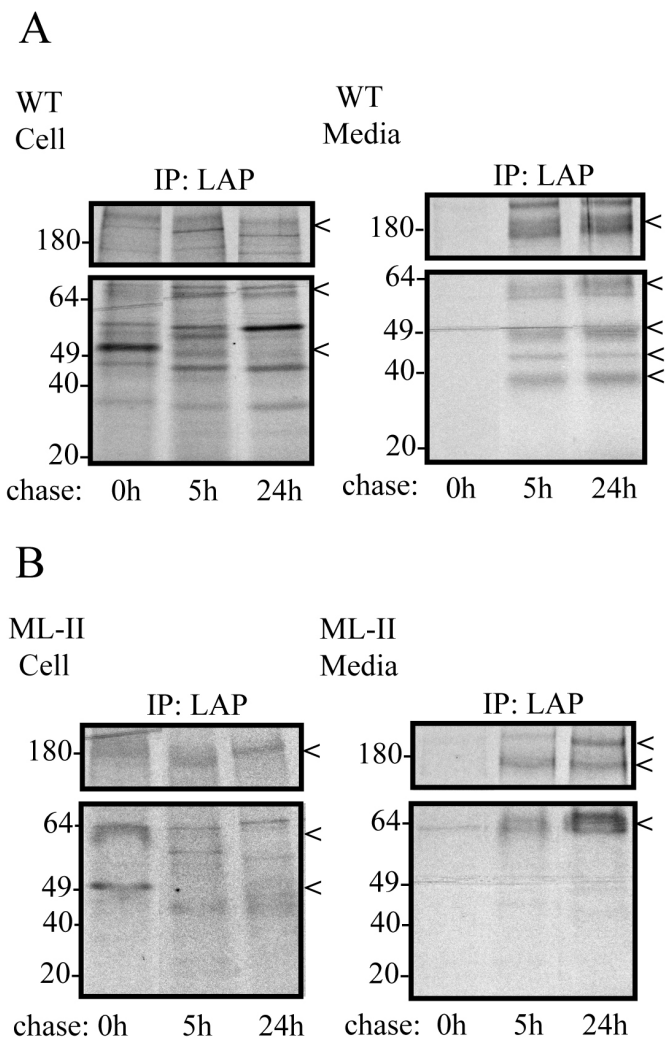


Figure 4.3. Media isoforms of latent TGF-β1 are reduced in human ML-II fibroblasts as determined by ^{35}S metabolic labeling. Human skin fibroblasts were pulse labeled with S^{35} methionine for 2 hours and chased for 0, 5 and 24 hours. Reduced cell and media samples are shown from (**A**) WT and (**B**) ML-II skin fibroblast. Note the detection of the lower molecular weight form of latent TGF-β1 in WT that is not found in ML-II media. Arrowheads denote forms of latent TGF-β1.

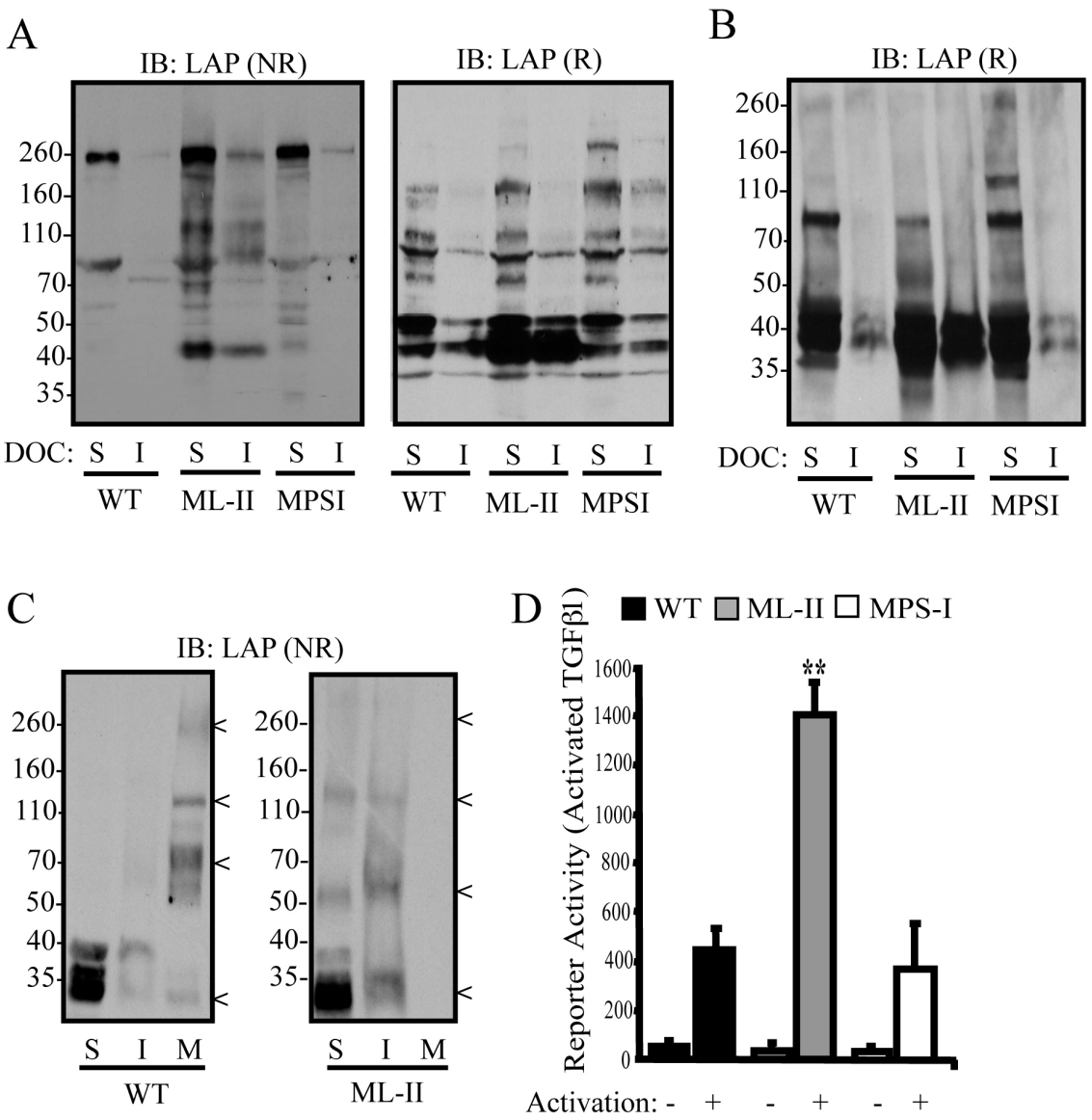


Figure 4.4. Latent TGF-β1 is abnormally deposited into the DOC-insoluble (ECM) fractions as a storage reservoir in ML-II fibroblasts. DOC-soluble and –insoluble fractions from WT, ML-II and MPS-I human fibroblasts were subjected to SDS-PAGE and Western blot analysis (**A** and **B**). 3-4 day cultures were probed with antisera against LAP under non-reducing (NR) and reducing (R) conditions (**A**), while 7 day cultures were determined under reducing conditions (**B**). (**C**) Western blot (non-reducing) of DOC-soluble, insoluble and media (serum free) from WT and ML-II 7 day fibroblasts cultures. (**D**) A representative TGF-β1 mink lung luciferase assay performed on DOC-insoluble (ECM) pools with and without heat activation (80°C for 10 min). Luciferase assays were performed in triplicate. Error bars represent the SEM of the independent triplicate experiments. P-values were given based on *t-test* determined from the independent triplicate experiments ($p < 0.001$, **). S = DOC-soluble (cell-associated), I = DOC-insoluble (ECM), and M = media. Arrowheads denote forms of TGF-β1.

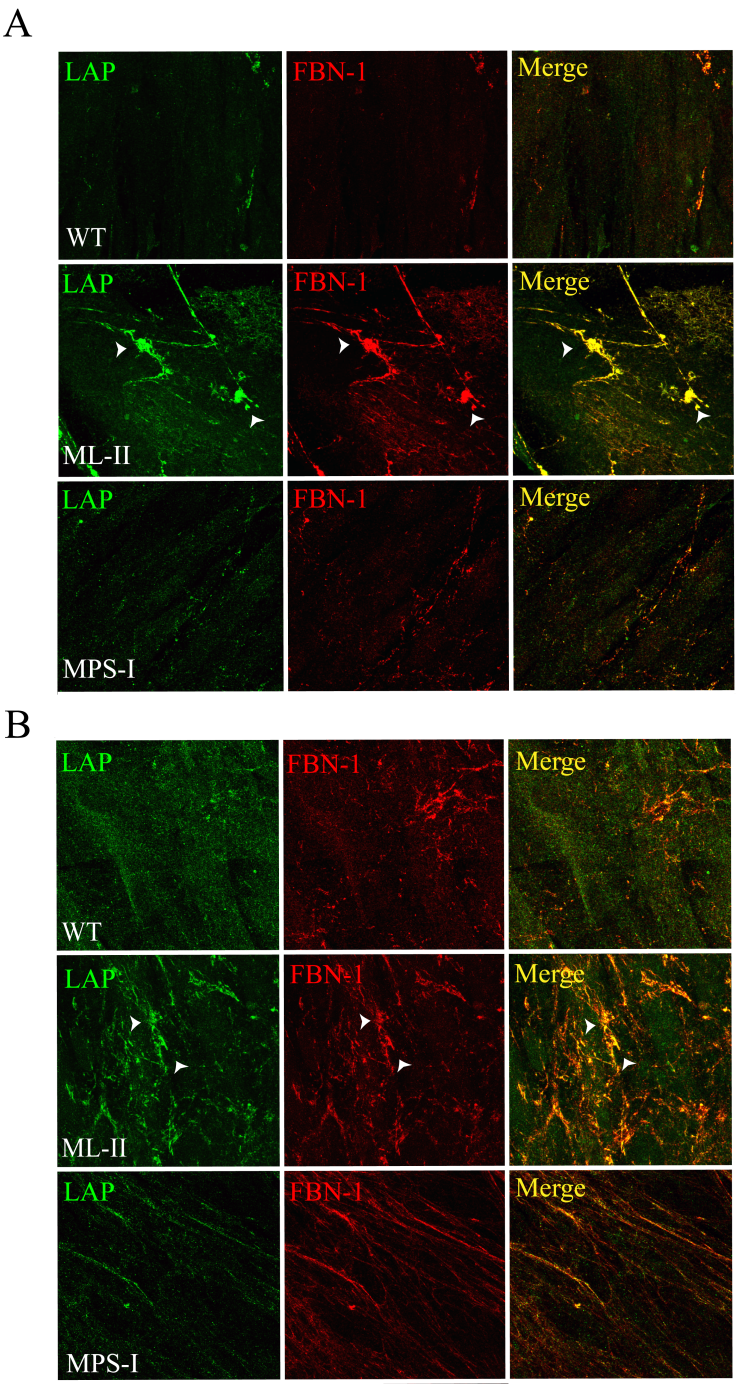


Figure 4.5. Latent TGF-β1 and fibrillin-1 are more rapidly deposited into the fibrillar network of human ML-II fibroblasts. WT, ML-II and MPS-I human fibroblasts were cultured for 2 days (**A**) or 7 days (**B**) and immunostained for latent TGFB1 (LAP; green) or fibrillin-1 (red). White arrows denote regions within the fibrillar network that contain tight co-localization.

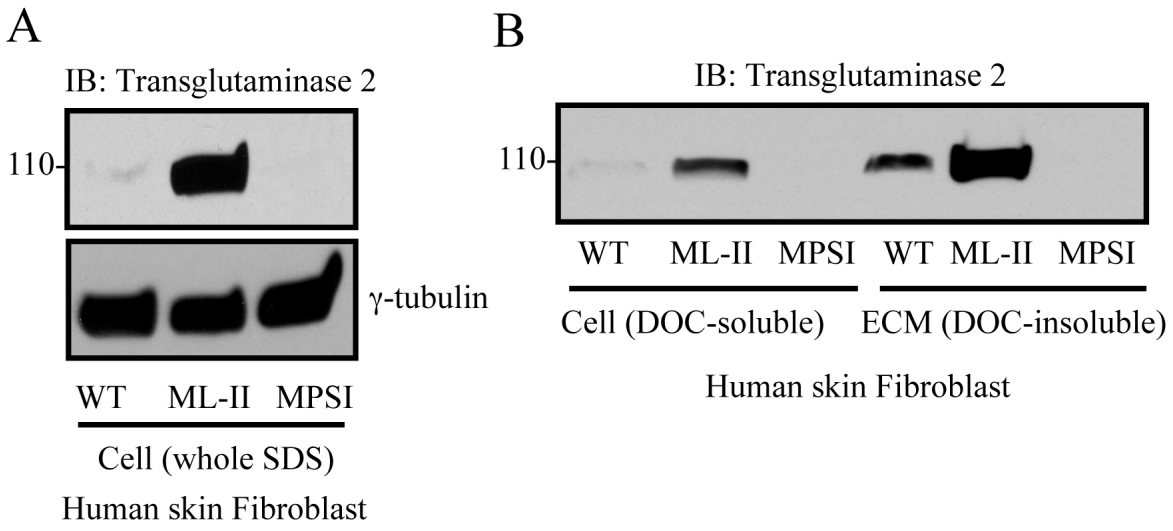


Figure 4.6. The transamidase enzyme, transglutaminase 2, is upregulated and observed in high quantities in the insoluble ECM of human ML-II fibroblasts. (**A**) Western blot of TG2 from whole SDS cell lysates from WT, ML-II and MPS-I human skin fibroblasts. (**B**) DOC-soluble and –insoluble fractions from human skin fibroblasts. S = DOC-soluble (cell-associated), I = DOC-insoluble (ECM).

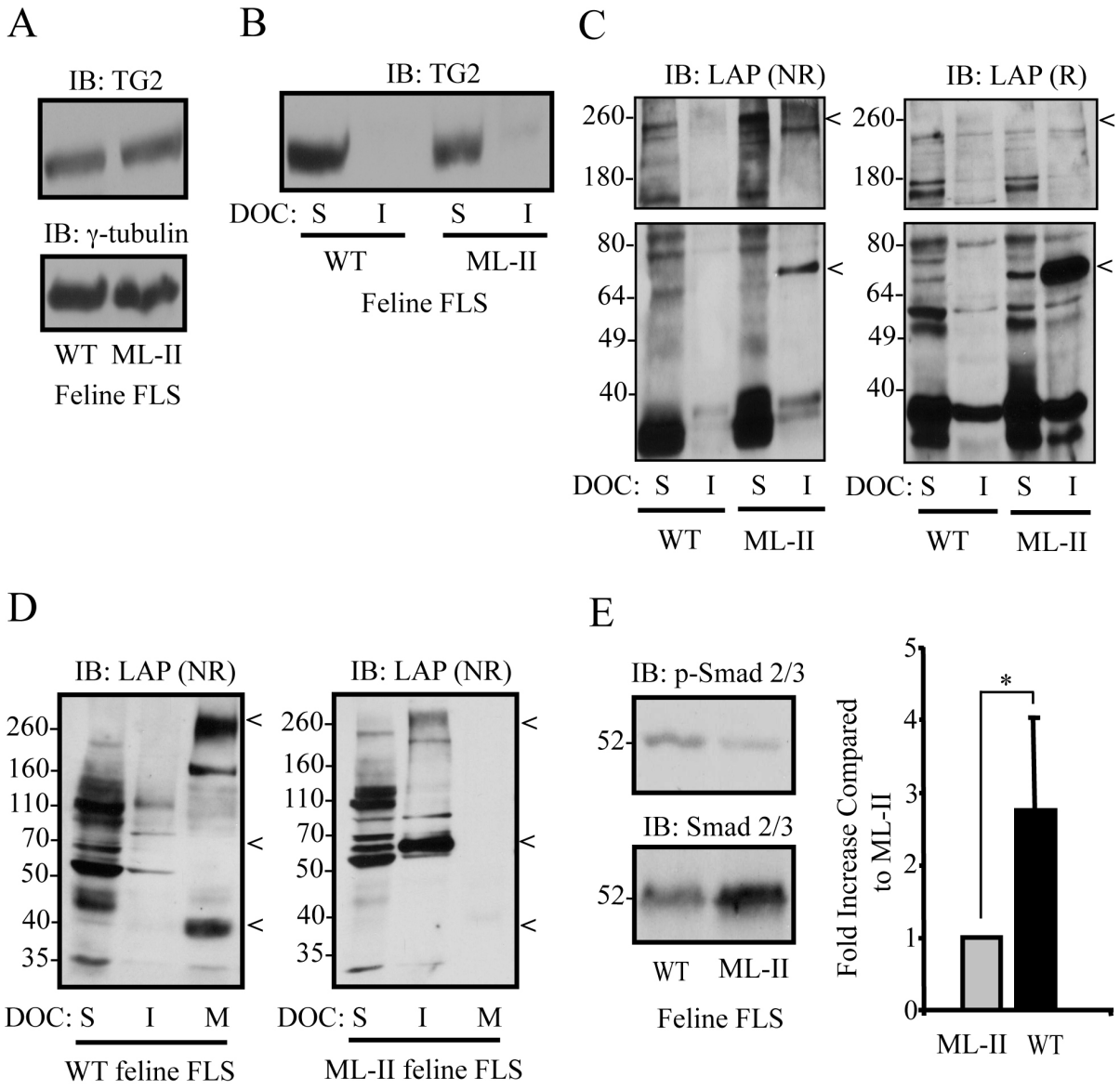


Figure 4.7. The increased insoluble ECM forms of latent TGF-β1 and TG2 are reproducible in feline ML-II synovial fibroblasts. (**A**) SDS whole cell lysate Western blots of TG2 in 3-4 day WT and ML-II feline FLS. (**B**) DOC-soluble and insoluble fractions from 3-4 day WT and ML-II feline FLS. (**C**) Western blot of LAP from 3-4 day feline FLS cultures after DOC solubility fractionation. (**D**) Western blot of LAP (non-reducing) after DOC solubility fractionation and serum free media collection from 7 day feline FLS cultures. (**E**) A representative Western blot of phosphorylated Smad 2/3 from 3-4 day feline FLS and densitometry quantitation of p-Smad relative to Smad 2/3 in WT and ML-II. Fold difference is relative to ML-II. Error bars represent the SEM of the independent triplicate experiments. P-values were given based on *t-test* determined from the independent triplicate experiments ($p < 0.05$, *). S = DOC-soluble (cell-associated), I = DOC-insoluble (ECM), and M = media. Arrowheads denote forms of latent TGF-β1.

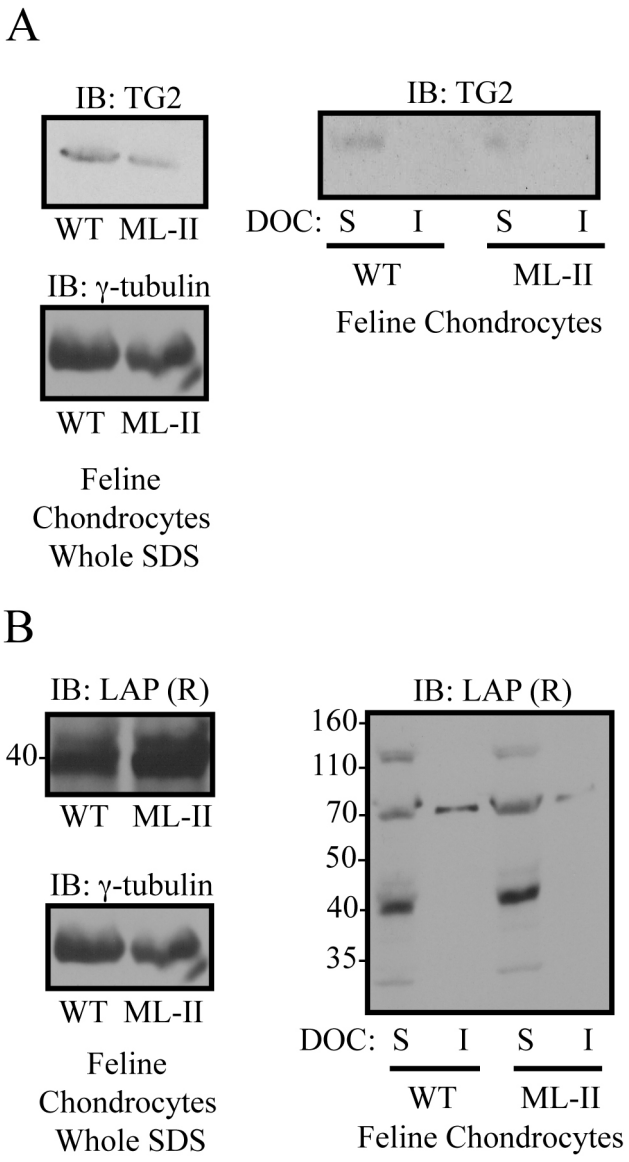


Figure 4.8. Latent TGF-β1 solubility is not altered in 4 day chondrocytes and corresponds to normal levels of TG2. Western blot analysis of TG2 (**A**) and LAP (**B**) from 4 day feline primary chondrocytes. S = DOC-soluble (cell-associated), I = DOC-insoluble (ECM).

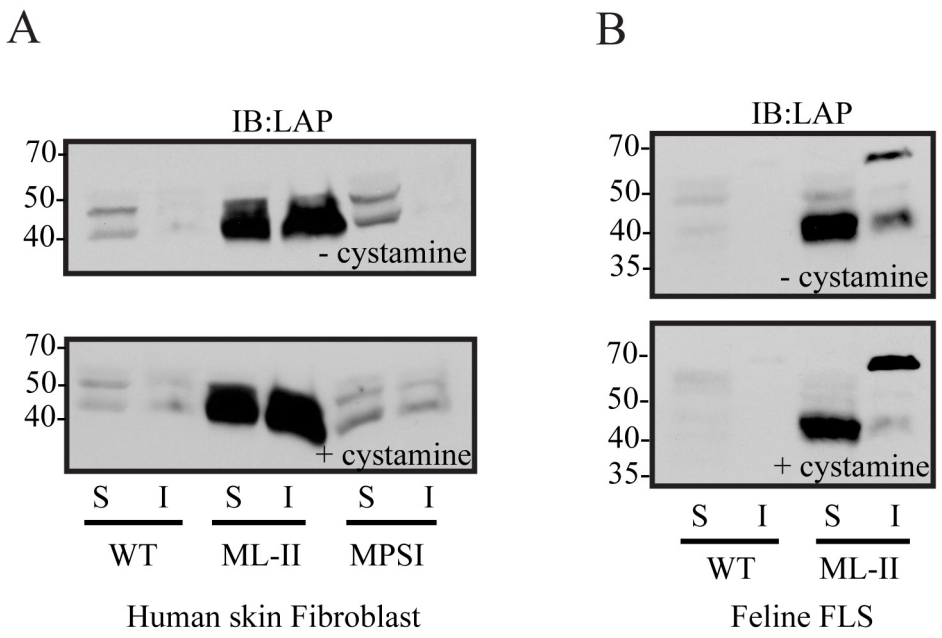


Figure 4.9. Cystamine and MDC (not shown) do not shift the solubility of latent TGF-β1 in human or feline ML-II fibroblasts. Western blot analysis of LAP deposition in DOC pools after the addition of cystamine (150 μM) in human skin fibroblasts (**A**) or feline FLS (**B**) cultures. S = DOC-soluble (cell-associated) and I = DOC-insoluble (ECM).

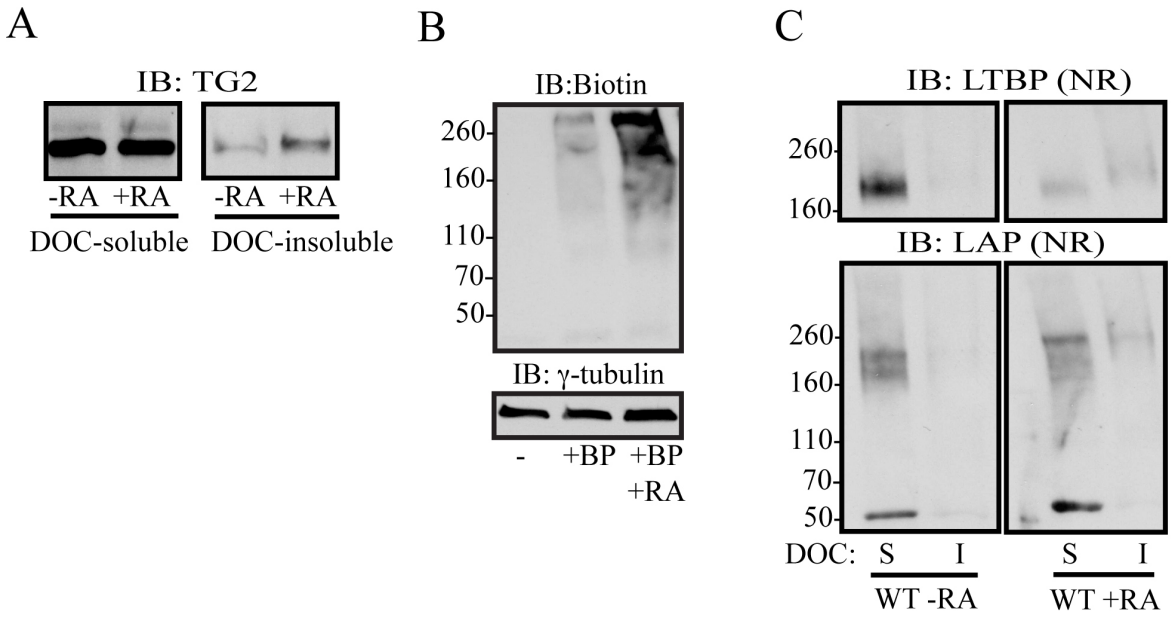


Figure 4.10. Addition of RA increases the insoluble ECM quantities and transamidation activity of TG2, resulting in greater amounts of latent TGF-β1 insolubility in human WT fibroblasts. (**A**) DOC soluble fractionation and Western blot analysis of TG2 after 5 μM retinoic acid (RA) treatment. (**B**) Whole SDS Western blot analysis of TG2 activity assessed by incorporation of biotinylated pentylamines (BP) into cellular protein in the presence or absence of RA. DOC solubility fractionation and Western blot analysis of (**C**, *top panel*) LTBP (non-reducing) and (**C**, *bottom panel*) LAP (non-reducing) from WT human fibroblasts in the presence and absence of RA. S = DOC-soluble (cell-associated), I = DOC-insoluble ECM.

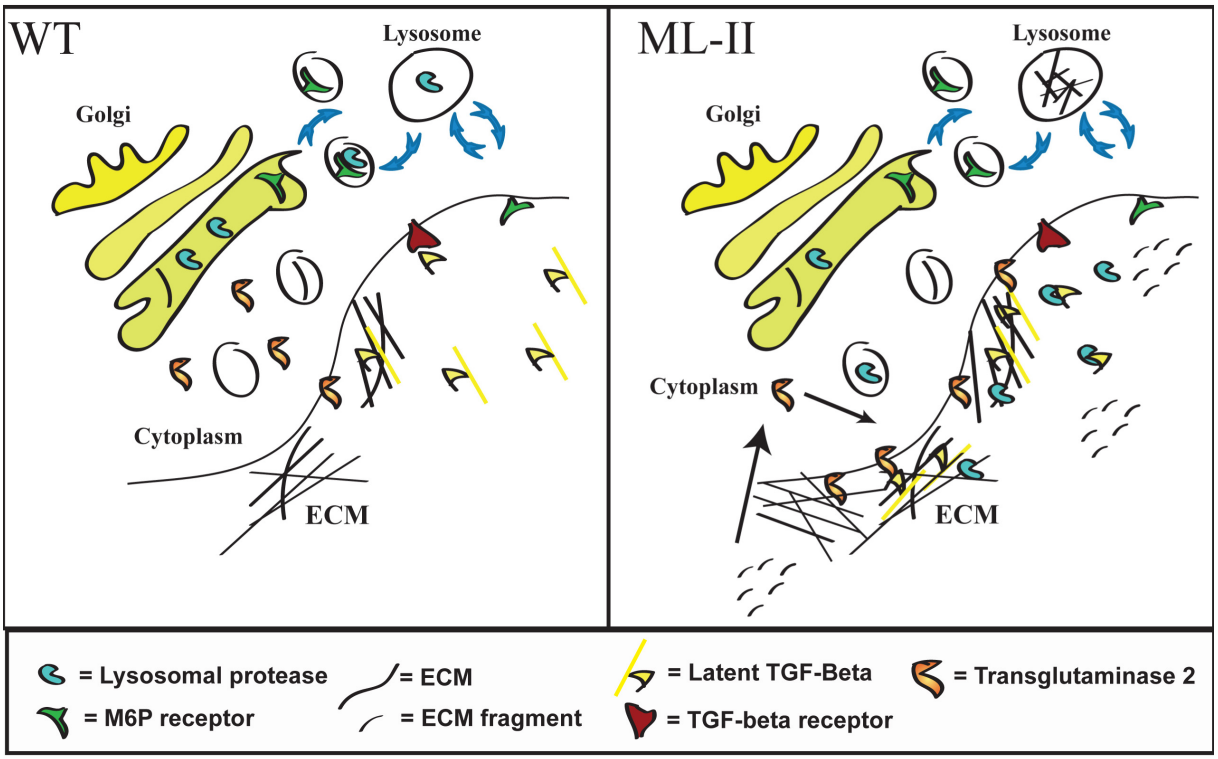


Figure 4.11. **Model of potential mechanisms that may account for altered ECM deposition of latent TGF-β1 and impaired bioavailability.** In normal cells, latent TGF-β1 is processed and secreted outside of the cell. Some molecules of TGF-β1 are cross-linked by transglutaminase 2 to the ECM and others are readily activated and utilized. In ML-II cells, stress inducers like impaired lysosomal function and abnormal ECM fragmentation generated by hypersecreted cathepsin proteases may lead to upregulation and translocation of transglutaminase 2 to the cell surface. Because of this, the latent TGF-β1 and ECM components that are made by ML-II cells are rapidly cross-linked into an insoluble ECM via transglutaminase 2 to combat abnormal ECM destruction. In normal cells, increased transglutaminase 2 cross-linking typically results in increased activation of latent TGFB1. In ML-II, however, any release of activated TGF-β1 molecules from the ECM may be proteolyzed by active extracellular cathepsin proteases resulting in decreased TGF-β1 signaling.

CHAPTER 5: DISCUSSION AND FUTURE PERSPECTIVES

The Role of Non-Lysosomal Man-6-P-Modified Proteins in the Pathogenesis of ML-II

LIF and latent TGF-β1 as well as several other secretory proteins have been shown to bear Man-6P (discussed in Chapter 1). The extent of mannose phosphorylation and the functional role of the Man-6-P modification on these molecules have not been fully elucidated. Chapters 2 and 3 of this thesis demonstrate that the importance of determining the extent of mannose phosphorylation as it is clear that not all the Man-6-P modified secretory proteins initially identified represent bona fide substrates for the GlcNAc-1-phosphotransferase enzyme. In the case of LIF, 30-45% of this protein was shown to bind to the immobilized CI-MPR affinity column, where as latent TGF-β1 was shown to bind only marginal (0-3%).

LIF Is A Bona Fide Mannose Phosphorylated Protein

In addition to the assessment of LIF mannose phosphorylation with the CI-MPR affinity column, mass spectrometric analysis of LIF glycopeptides validated this molecule as a bona fide Man-6-P modified protein containing mannose phosphorylation at multiple N-glycan sites. It was shown, using an embryonic stem cell internalization assay, that the Man-6-P modification has a specific function in controlling the extracellular levels of LIF through rapid internalization and degradation of this cytokine in the lysosome. Furthermore, intracellular localization studies using a LIF and mCherry fusion demonstrated that LIF is trafficked to the lysosome via Man-6-P targeting and rapidly degraded, suggesting another regulation mechanism to control LIF

extracellular levels. Loss of the Man-6-P addition by mutation of specific N-glycan sites on LIF resulted in excess accumulation of LIF in cultured media as detected by secretion assays. This result demonstrates a potential consequence whereby impaired Man-6-P biosynthesis would affect the extracellular levels of LIF.

Future Perspectives – Man-6-P modified LIF and Its Role in ML-II Pathogenesis

Although the present work established the mechanisms whereby mannose phosphorylation controls the extracellular levels of LIF, there are several questions that should be addressed in future studies, both in regard to the structural basis for LIF mannose phosphorylation and the potential impact on LIF regulation in an ML-II context. How does the binding affinity of LIF compare to other known lysosomal proteases? Does LIF contain the specific protein determinant, found in other hydrolases, which is required for GlcNAc-1-phosphotransferase recognition? In an ongoing collaboration, we have now determined that LIF is a good substrate for GlcNAc-1-phosphotransferase (Appendix Figure 3A). The results of this binding assay demonstrated that LIF has a comparable binding affinity to immobilized GlcNAc-1-phosphotransferase to the lysosomal glycosidase, alpha-iduronidase. This assay was also performed on purified LIF that was subjected to acetylation prior to analysis to neutralize all the lysine residues on the protein. Since lysines are important determinants for efficient mannose phosphorylation, loss of these residues was predicted to reduce GlcNAc-1-phosphotransferase binding. Surprisingly, we found that binding of acetylated LIF to GlcNAc-1-phosphotransferase was only modestly inhibited, suggesting that lysines are not the solely required for GlcNAc-1-phosphotransferase interaction (Appendix Figure 3B). Using a complementary approach, we have been performing site-directed mutagenesis experiments on specific lysine residues to

determine the specificity as well as spatial positioning of these residues compared to N-glycan sites within LIF (Appendix Figure 3C). Preliminary results suggest that certain lysines are critical for efficient mannose phosphorylation of LIF as their mutation resulted in a lack of CI-MPR binding on secreted protein. Our plan going forward will be to combine specific lysine mutants that reduce GlcNAc-1-phosphotransferase recognition, such as K99Q and K136Q (Appendix Figure 3C) and perform secretion assays similar to our published findings using N-glycan deletion mutants (Figure 2.4). We believe that using lysine mutants for the secretion assay will be more quantitative and allow us to address any questions concerning improper folding as a consequence of the removal of N-glycans.

The ultimate goal of the LIF project is to determine how impaired Man-6-P biosynthesis on LIF can impact the disease process in ML-II. Using ML-II animal models, ongoing and future studies will continue to explore *in vivo* the potential consequences of excess LIF. Tissues that would be impacted by excess LIF are bone and connective tissues. We are in the process of determining if LIF levels are elevated in feline ML-II bone sections by immunostaining. In addition, ongoing collaborations using microCT technology have been initiated to investigate bone mass and density, osteoblast/clast cell number and osteoblast differentiation - all of which may be affected by excess LIF signaling.

With the use of cell-culture based *in vivo* models, proposed studies include the knockdown GlcNAc-1-phoshotransferase in bone mesenchymal stem cells and the stimulation of LIF expression and secretion using the inducers, LPS or parathyroid hormone (PTH). Alternatively, calvarial osteoblasts from WT and GNPTAB$^{-/-}$ mice can be obtained and cultured. Measurement of secreted levels of LIF by Western blot or ELISA will allow us to determine whether loss of mannose phosphorylation on LIF results in rapid and linear secretion in ML-II

cells. The possible finding that LIF accumulates extracellularly in a model for ML-II disease would provide valuable insight into the potential contribution of this cytokine to the bone and cartilage pathogenesis associated with ML-II.

LIF exists in multiple splice variants (1,2). Two of these forms have been shown to be secreted, with one being highly soluble and the another localized within the ECM (1,3). The present data only addressed the mannose phosphorylation on the secreted soluble form (see Chapter 2). It would be interesting to determine the levels of mannose phosphorylation of the extracellular matrix bound LIF. If it is modified, what is the function? Does this ECM localize splice variant of LIF get readily synthesized and secreted in ML-II? Does it play a role in the bone phenotypes associated with ML-II disease? Perhaps LIF is abnormally deposited into the insoluble ECM of ML-II cultures similar to latent TGF-β1 (see Chapter 4). All of these questions will be productive areas of future research.

TGF-β1 is Not Subject to Physiological Mannose Phosphorylation

The findings in this thesis demonstrate that latent TGF-β1 has low binding affinity for the immobilized CI-MPR and is poorly mannose phosphorylated during its biosynthesis. In particular, latent TGF-β1 from several cell lines (including HEL, human fetal osteoblast, and HUVEC) was not Man-6-P modified at all. Overexpression in CHO and HeLa cells resulted in only a slight increase of in binding of secreted latent TGF-β1, suggesting that a small portion of this molecule may be Man-6-P modified at very low levels. Furthermore, it was determined that LTBP does not shield the N-glycans on latent TGF-β1 from binding to the CI-MPR column, as DTT reduction and coordinated plasmin proteolysis of LTBP to "unmask" TGF-β1's N-glycan sites did not increase the binding interaction. We further demonstrated that direct activation of

TGF-β1 through binding to M6PRs in cultured corneal fibroblast is not mediated via Man-6-P on its latency-associated peptide.

Future Perspectives – Latent TGF-β1

Previous literature suggested that latent TGF-β1 contains Man-6-P residues and, more importantly, can be activated through its interaction with the M6PRs at the cell surface. How can we reconcile our findings compared to the previous literature? Our data suggests that there is negligible binding to the CI-MPR column. We cannot rule out the possibility that the small percentage of secreted man-6-P modified TGF-β1 observed in our results may be activated directly by the cell surface CI-MPR. Our data suggests that this is probably not the case, since free Man-6-P had no effect on the activation of latent TGF-β1 and subsequent stimulation of corneal fibroblast differentiation to myofibroblast (Figure 3.5). One valid argument to consider is that our experiments were performed under monoculture conditions, yet previous reports determined Man-6-P dependent effects in co-culture systems. Future studies need to be aimed at deciphering the activation mechanism in the endothelial/smooth muscle co-culture system. The mannose phosphorylation of latent TGF-β1 from an endothelial cell line was addressed directly in our studies. Our analysis of the primary endothelial cell line, HUVEC, suggested that latent TGF-β1 is not modified. Previous literature, along with our findings, does not rule out the possibility that a co-factor (i.e., plasmin, UPAR) binds to and complexes with the cell surface CI-MPR, facilitating the recruitment and subsequent activation latent TGF-β1 (4). Identifying this secondary target of free Man-6-P will be important since it may lead to additional therapies to prevent scarring and fibrosis of several tissues sensitive to overactive TGF-β1 signaling.

The Impact of Other Non-Lysosomal Man-6-P-Modified Proteins in ML-II Disease

Many other non-lysosomal proteins have been shown to bear Man-6-P. Since the methodology to investigate the mannose phosphorylation of these proteins has been established, similar experiments to those applied to LIF and latent TGF-β1 can be undertaken for additional secretory glycoproteins. It will be of interest to investigate other growth factors, like proliferin and CSF1, which have been determined to be Man-6-P modified, since dysregulation of these molecules may also be relevant to ML-II phenotypes. If these secreted proteins contain high levels of mannose phosphorylation, we plan to explore the role of these proteins and their potential impact in ML-II.

TGF-β1 – Reduced Signaling, Increased Insolubility and Possible Mechanisms

In Chapter 4, we showed that TGF-β1 signaling was altered in human ML-II fibroblasts as determined by detection of the phosphorylated Smad 2/3 levels and assessment of wound closure. Decreased signaling was not attributed to transcript or steady-state protein levels as both were either unchanged or upregulated, respectively. Metabolic labeling of newly synthesized protein suggested that processing and intracellular trafficking of latent TGF-β1 from ML-II fibroblasts was normal, however the secretion of this growth factor was altered, lacking multiple secreted forms. Deoxycholate (DOC) solubility assays determined that latent TGF-β1 was largely deposited in a DOC-insoluble ECM pool compared to human fibroblast cultures from WT and MPS-I. This characteristic was also noted in feline fibroblast-like synoviocytes and skin fibroblasts, indicating that this shift in solubility is a general phenomenon of ML-II

disease. In addition, we determined that latent TGF-β1 more tightly co-localizes with fibrillin-1 in fibrillar network assemblies at a higher intensity than WT and MPS-I. Consistent with this, latent TGF-B1 and fibrillin-1 were more rapidly deposited into the ECM, suggesting that multiple components of the ECM may be altered. In support of these findings, we determined that the deposition of latent TGF-β1 into the ECM results in storage of this growth factor as an "activatible" reservoir. The increased deposition of latent TGF-β1 in ML-II fibroblasts is also consistent with a shift in solubility and an increase in tissue transglutaminase 2 (TG2) levels, a multi-functional protein that is known to cross-link latent TGF-β1 as well as other matrix components together in the ECM (5-7). Our attempts to inhibit latent TGF-β1 deposition into the insoluble ECM, however, were not successful possibly due to the large quantities of previously deposited cross-linked latent TGF-β1. Our experiments inducing the expression of TG2 in WT fibroblasts (with retinoic acid) led to an increase in latent TGF-β1 insolubility, suggesting that TG2 may be regulating the solubility shift by cross-linking this growth factor to the ECM in ML-II. The factors responsible for upregulation of TG2, altered solubility of latent TGF-β1 and decrease TGF-β1 bioavailability are discussed.

Increased ECM Cross-linking of Latent TGF-β1 And Decreased TGF-β1 Signaling – An Affect of Hypersecreted Cathepsins?

In addition to our findings describing the altered bioavailability of latent TGF-β1, Western blot analysis of fibronectin from ML-II patient fibroblast determined that this protein was heavily fragmented compared to WT and MPS-I (Appendix Figure 1A and B). In support of these findings, this fragmentation profile was seen in feline ML-II FLS chondrocytes and synovial fluid (Appendix Figure 1C and D), indicating that these Fn-fs are specific to ML-II

disease and found in a fluid surrounding tissues. Using an *in vitro* model, we digested purified plasma fibronectin with several recombinant cathepsins (Appendix Figure 2). Surprisingly, cathepsin D generated an identical fragmentation profile to that observed in ML-II fibroblasts. We hypothesize that ECM fragmentation is caused by hypersecretion of cathepsins. Recently, fibrillin-1 was shown to be susceptible to several proteases including cathepsin K and V (8). Fibrillin-1 and other ECM components may be abnormally fragmented, similar to fibronectin in ML-II. Previous literature has suggested that ECM fragments can facilitate stress responses (9-13). Furthermore, other reports have shown that TG2 upregulation and activity is associated with stress activators (7,14-17). It is interesting to speculate that these ECM fragments in ML-II may elicit stress responses that result in increased TG2 activity. Upregulation of TG2 transamidation activity in ML-II may result in protease resistant cross-links of ECM components, a defense mechanism to combat abnormal cathepsin proteolysis outside the cell (*see model* Figure 4.11). Stress-related increases of TG2 levels in ML-II may be a consequence of autophagosome and lysosomal dysfunction, both of which have been shown to be associated with patient fibroblast (18-20) and similarly linked to altered TG2 levels (21,22).

Several reports have shown that increased TG2 activity and cross-linking of latent TGF-β1 to the ECM results in greater TGF-β1 activation (23-26). However, our results suggest that increased sequestration of latent TGF-β1 in the ECM by TG2 cross-linking may account for the decreased activation and signaling. Work is currently underway to determine and understand the underpinnings of this phenomenon. It was recently reported that cathepsin K deficient mice have increase TGF-β1 signaling in lung fibroblasts (27). In line with this, inhibitors to cathepsin K activity increased TGF-β1 signaling resulting in improved migration and proliferation rates in these cultures. These results suggest that TGFβ1 signaling can also be regulated by cathepsin

proteases. Since hypersecretion of acid hydrolases is one of the hallmarks of ML-II disease, it will also be necessary to consider how altered localization or activity of these proteases might also account for the deficits in TGFβ1 signaling in ML-II fibroblasts.

Future Directions – Determining the Role of TG2 in Latent TGF-β1 Solubility and ML-II Disease

Ongoing experiments are aimed at determining whether a shift in solubility is observed for other ECM proteins in addition to latent TGF-β1 and whether suppression of TG2 levels in ML-II fibroblasts is sufficient to alter latent TGF-β1 solubility. We will assess knockdown of TG2 levels by WB analysis and maintain a TG2 deficient cultures to perform experiments such as the DOC-solubility assays to measure latent TGF-β1 deposition as well as other ECM components. More extensive studies will be carried out to assess the activity levels of TG2 in ML-II fibroblasts using biotinylated pentylamines. Proteins that are cross-linked by TG2 in the presence of biotin-pentylamine can also be immunoprecipitated and analyzed by MS analysis, providing insight into the range of extracellular protein substrates for this cross-linking enzyme.

Other Mechanisms To Account for Increased Levels of Insoluble ECM

Several reports have suggested that other proteins such as Factor XIIIa and lysyl oxidase can cross-link protein within the ECM. Factor XIIIa transglutaminase is known for its role in cross-linking fibrin to form insoluble clots during thrombosis. Lysyl oxidase is important in cross-linking collagen and elastin fibers. Since these proteins have no previously described role in cross-linking latent TGF-β1 to the ECM, we do not believe they are relevant to this

phenomenon. However, these enzymes may be involved in abnormal cross-linking of other ECM components (collagens and elastins) and thus deserve further investigation.

The Association of Stress Inducers and Upregulation of TG2 in ML-II

As mentioned in Chapter 4, ECM fragments have been shown to function as stress inducers. It is conceivable that TG2 expression may be exacerbated by the abnormal fragmentation. Since cathepsin proteases may be actively degrading the ECM, it is possible that TG2 expression is increased in ML-II cells in response to this insult to counteract the effects of ECM fragmentation by generating protease resistant cross-links between ECM components. To address this mechanism, the extracellular activity of several cathepsins (D, K, and L) can first be determined using cathepsin activity assays. Since cathepsin proteases can be blocked with active-site specific inhibitors, we propose to gauge the fragmentation of ECM by Western blot analysis. In particular, we can determine the expression levels of TG2 and solubility of latent TGF-β1 and fragmentation of fibronectin after protease inhibition by Western blot analysis.

Recently, autophagic and mitochondrial impairment as well as several other cellular phenotypes associated with ML-II were reported to be corrected with the supplementation of acid hydrolases to these cells (28). Using normal cultured medium containing secreted acid hydrolases after treatment with NH_4Cl, we aim to correct the autophagic and lysosomal impairment associated stress. With this in mind, our expectations are that TG2 activity levels will be restored and abnormal cross-linking will be reduced.

Numerous reports have documented the various locations of TG2 within and outside the cell (see (29) for review). Our proposed model from Chapter 4 suggests that TG2 may be translocated to the ECM where its transamidase activity is stimulated by the presence of calcium

(see Figure 4.11). However, it is conceivable that TG2 could be brought into the ER under stress conditions. A recent report proposed a novel finding, in which TG2 co-localized within ER associated granules in a Parkinson's disease brain (17), which is characterized by accumulation of α-synuclein aggregates and subsequent degeneration. Abnormal ER localization of TG2 would position this enzyme to cross-link the α-synuclein monomers and oligomers. In essence, high calcium levels would result in TG2 transamidase activity within the ER. Abnormal TG2 localization within the ER of ML-II cells could lead to increased cross-linking of proteins as well as secretion of this protein out of the cell to the ECM or cell surface resulting in an active enzyme. Experiments are currently underway to investigate the localization of TG2 in the ER in ML-II diseased fibroblasts.

A Potential Role for Cathepsins and Decreased TGF-β1 Signaling in ML-II

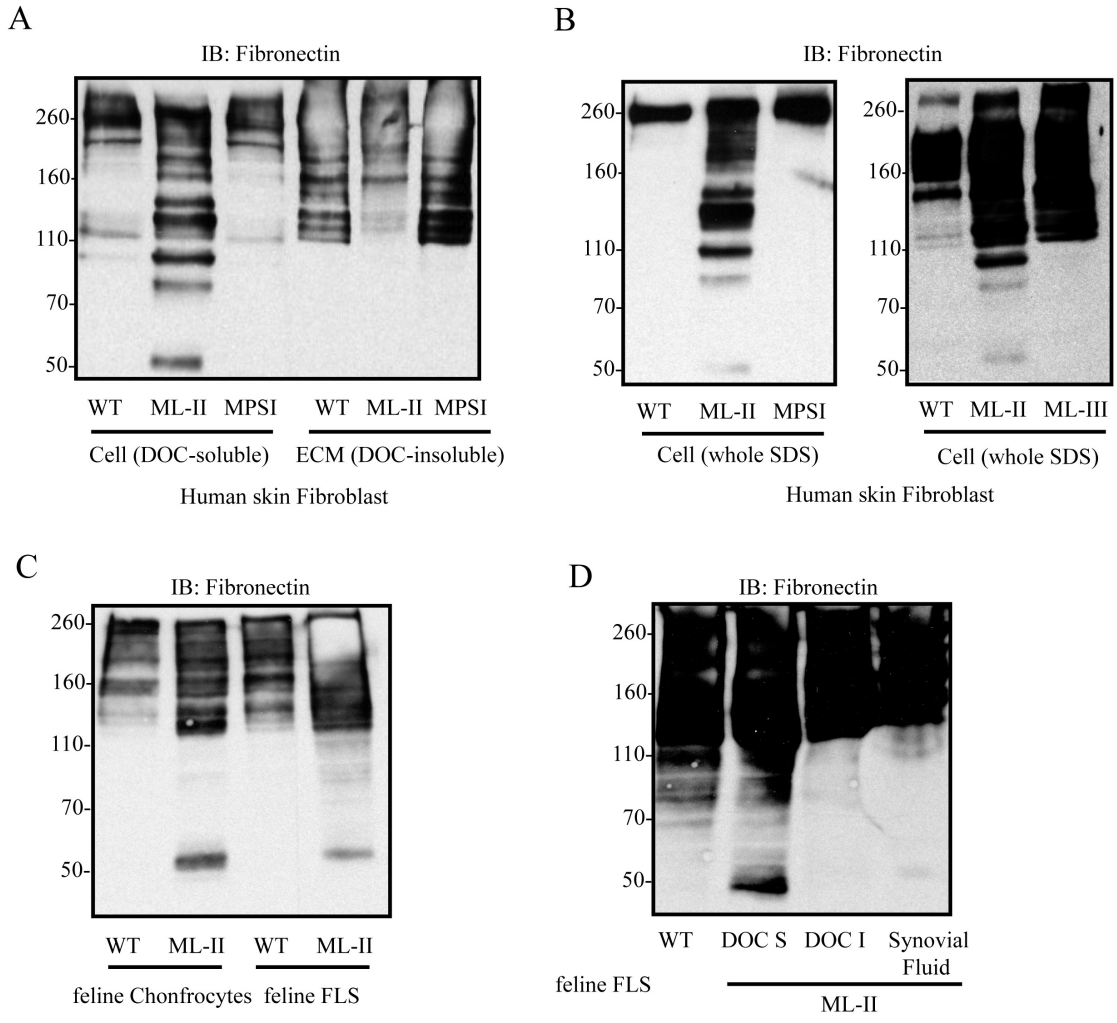
Cathepsin proteases may be responsible for the decrease in TGF-β1 signaling. Our data from human and feline ML-II fibroblast media cultures suggest that latent TGF-β1 is deficient compared to WT (Figure 4.4C and 4.5D), which may be due to rapid proteolysis by cathepsin during culture. It would be of interest to perform Western blots on cultured media (serum free) with and without protease inhibitors and reproduce these experiments, with the possibility of blocking latent TGF-β1 degradation in ML-II cultures. In addition, TGF-β1 signaling and wound closure assays can be performed on cultured ML-II fibroblasts in the presence and absence of protease inhibitors (i.e., cathepsin K, L, and D) to determine whether there is a link between elevated extracellular protease activity and altered TGF-β1 signaling.

References

1. Rathjen, P. D., Toth, S., Willis, A., Heath, J. K., and Smith, A. G. (1990) *Cell* **62**, 1105-1114
2. Haines, B. P., Voyle, R. B., and Rathjen, P. D. (2000) *Mol Biol Cell* **11**, 1369-1383
3. Mereau, A., Grey, L., Piquet-Pellorce, C., and Heath, J. K. (1993) *J Cell Biol* **122**, 713-719
4. Gleizes, P. E., Munger, J. S., Nunes, I., Harpel, J. G., Mazzieri, R., Noguera, I., and Rifkin, D. B. (1997) *Stem Cells* **15**, 190-197
5. Belkin, A. M. (2011) *FEBS J*
6. Fesus, L., and Piacentini, M. (2002) *Trends Biochem Sci* **27**, 534-539
7. Kiraly, R., Demeny, M., and Fesus, L. (2011) *FEBS J*
8. Kirschner, R., Hubmacher, D., Iyengar, G., Kaur, J., Fagotto-Kaufmann, C., Bromme, D., Bartels, R., and Reinhardt, D. P. (2011) *J Biol Chem* **286**, 32810-32823
9. Homandberg, G. A., Hui, F., Wen, C., Purple, C., Bewsey, K., Koepp, H., Huch, K., and Harris, A. (1997) *Biochem J* **321 (Pt 3)**, 751-757
10. Chowdhury, T. T., Schulz, R. M., Rai, S. S., Thuemmler, C. B., Wuestneck, N., Bader, A., and Homandberg, G. A. (2010) *Arthritis Res Ther* **12**, R82
11. Ding, L., Guo, D., and Homandberg, G. A. (2009) *Osteoarthritis Cartilage* **17**, 1385-1392
12. Homandberg, G. A., Hui, F., and Wen, C. (1996) *Biochim Biophys Acta* **1317**, 143-148
13. Homandberg, G. A., Hui, F., and Wen, C. (1996) *Biochim Biophys Acta* **1317**, 134-142
14. Gibson, G. E., Starkov, A., Blass, J. P., Ratan, R. R., and Beal, M. F. (2010) *Biochim Biophys Acta* **1802**, 122-134
15. Shin, D. M., Jeon, J. H., Kim, C. W., Cho, S. Y., Kwon, J. C., Lee, H. J., Choi, K. H., Park, S. C., and Kim, I. G. (2004) *J Biol Chem* **279**, 15032-15039
16. Shin, D. M., Jeon, J. H., Kim, C. W., Cho, S. Y., Lee, H. J., Jang, G. Y., Jeong, E. M., Lee, D. S., Kang, J. H., Melino, G., Park, S. C., and Kim, I. G. (2008) *FASEB J* **22**, 2498-2507
17. Wilhelmus, M. M., Verhaar, R., Andringa, G., Bol, J. G., Cras, P., Shan, L., Hoozemans, J. J., and Drukarch, B. (2011) *Brain Pathol* **21**, 130-139
18. Kobayashi, H., Takahashi-Fujigasaki, J., Fukuda, T., Sakurai, K., Shimada, Y., Nomura, K., Ariga, M., Ohashi, T., Eto, Y., Otomo, T., Sakai, N., and Ida, H. (2011) *Mol Genet Metab* **102**, 170-175
19. Otomo, T., Higaki, K., Nanba, E., Ozono, K., and Sakai, N. (2009) *Mol Genet Metab* **98**, 393-399
20. Boonen, M., van Meel, E., Oorschot, V., Klumperman, J., and Kornfeld, S. (2011) *Mol Biol Cell* **22**, 1135-1147
21. D'Eletto, M., Farrace, M. G., Falasca, L., Reali, V., Oliverio, S., Melino, G., Griffin, M., Fimia, G. M., and Piacentini, M. (2009) *Autophagy* **5**, 1145-1154
22. Rossin, F., D'Eletto, M., Macdonald, D., Farrace, M. G., and Piacentini, M. (2011) *Amino Acids*
23. Huang, L., Haylor, J. L., Fisher, M., Hau, Z., El Nahas, A. M., Griffin, M., and Johnson, T. S. (2010) *Nephrol Dial Transplant* **25**, 3897-3910

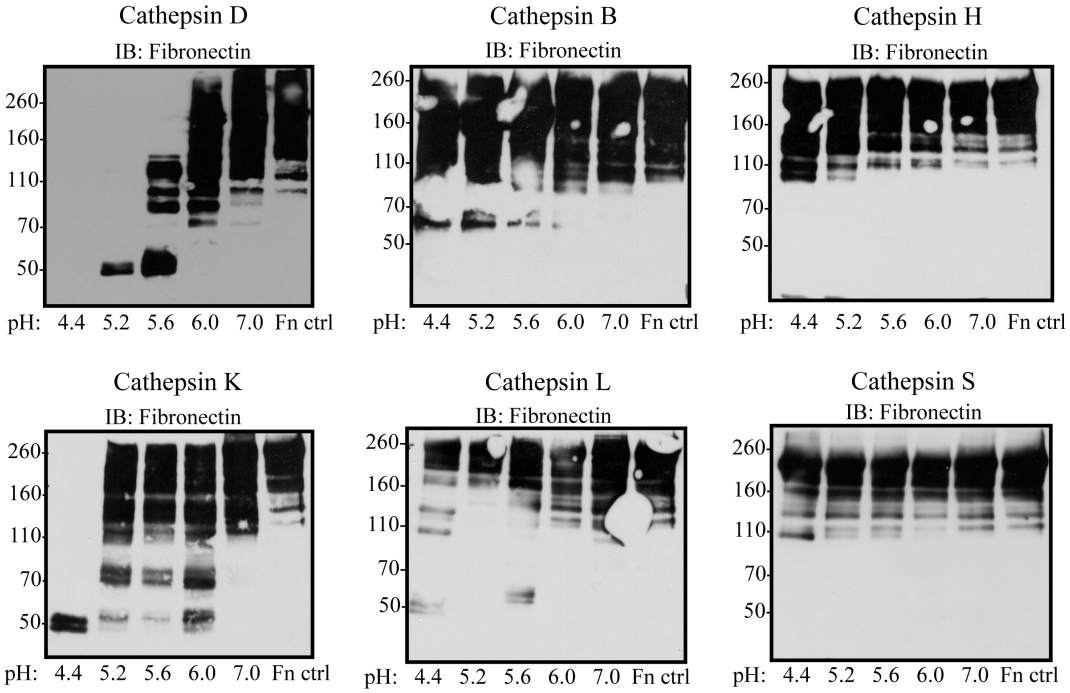
24. Kojima, S., Nara, K., and Rifkin, D. B. (1993) *J Cell Biol* **121**, 439-448
25. Le, M., Gohr, C. M., and Rosenthal, A. K. (2001) *Connect Tissue Res* **42**, 245-253
26. Telci, D., Collighan, R. J., Basaga, H., and Griffin, M. (2009) *J Biol Chem* **284**, 29547-29558
27. Zhang, D., Leung, N., Weber, E., Saftig, P., and Bromme, D. (2011) *Respir Res* **12**, 72
28. Otomo, T., Higaki, K., Nanba, E., Ozono, K., and Sakai, N. (2011) *J Biol Chem* **286**, 35283-35290
29. Gundemir, S., Colak, G., Tucholski, J., and Johnson, G. V. (2011) *Biochim Biophys Acta*

APPENDIX A: SUPPLEMENTAL FIGURES

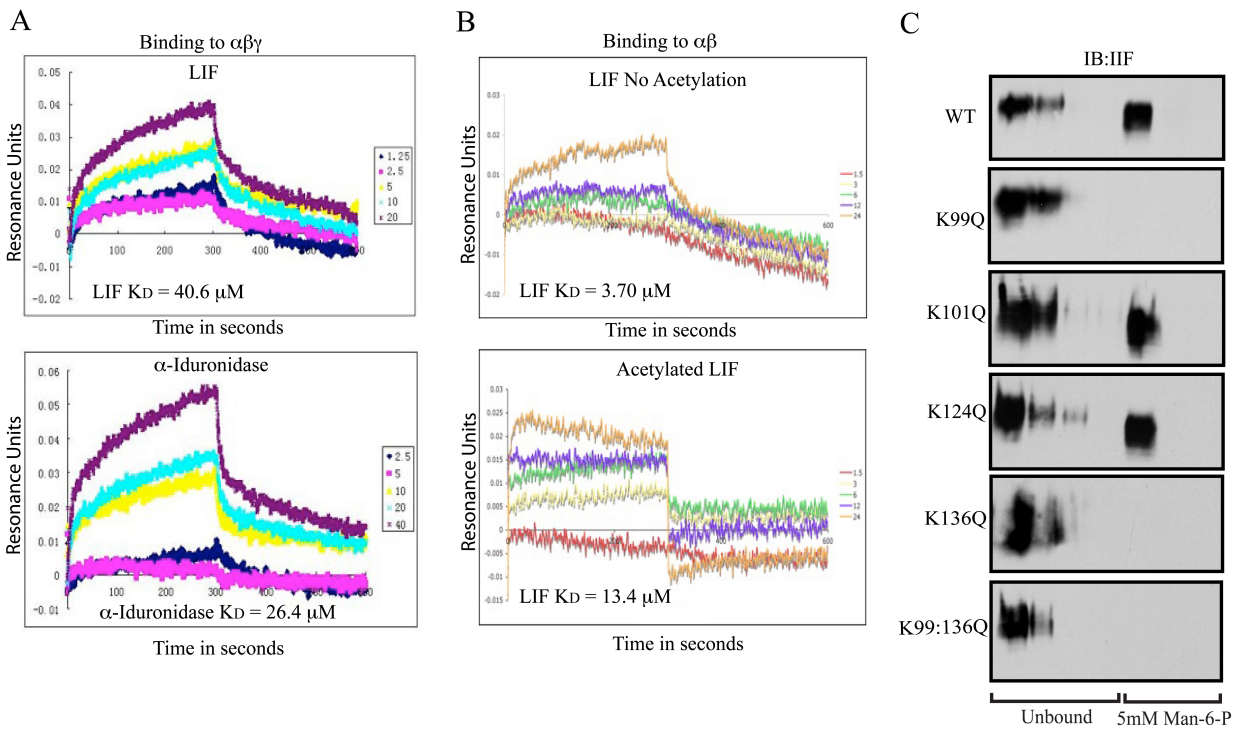


Appendix Figure 1. Fibronectin is abnormally fragmented in human and feline ML-II cells. **(A)** WT, ML-II and MPS-I human skin fibroblasts were grown to confluency in DMEM supplemented with 15% FBS at 37 °C. Cell lysates were prepared with sodium Deoxycholate (DOC) detergent and protease inhibitors. The lysis of the cells consisted of two pools: (1) a detergent soluble (cell associated) and (2) detergent insoluble (ECM) pool. Both pools were collected and prepared for SDS-PAGE electrophoresis and WB analysis. Immunoblot analysis determined that fibronectin was substantially fragmented in the MLII patients in comparison to the WT and MPSI fibroblasts. (**B**) Whole SDS cell lysates of WT, ML-II and MPS-I (left panel) as well as ML-III (right panel) were subjected to Western blot analysis. ML-II human skin fibroblast exhibit the same fragmentation profile as that seen using DOC fractionation. These fragments are not seen in MPS-I or ML-III. (**C**) Feline chrondrocytes were extracted from articular cartilage by collagenase B digestion while synovial fibroblasts were extracted from the synovial membrane by collagen 1A. The cells were subjected to whole SDS cell lysis and analyzed by Western blot using antisera against fibronectin for fibronectin fragments. The feline

ML-II chondrocytes as well as ML-II synovial fibroblasts show a fragmentation pattern as that seen in human ML-II skin fibroblasts with a prominent 49 kDa band. (**D**) WT synovial fibroblasts were prepared as SDS whole cell lysates as described above, however, the ML-II synovial fibroblasts were DOC fractionated as described. Feline synovial fluid was extracted from a ML-II feline joint and analyzed for fibronectin fragments as well. Western blot analysis confirmed the presence of Fn-fs in the MLII DOC soluble samples as well as synovial fluid.



Appendix Figure 2. Fibronectin fragments generated by various recombinant cathepsin proteases and analyzed by Western blot. Recombinant cathepsin proteases were incubated in the presence of purified plasma fibronectin at 37 °C for 16 hours over a pH range. Samples were subjected to Western blot analysis and probed with antisera against fibronectin. The above figure shows the fragmentation profile of fibronectin after treatment with the various proteases. Note the fragmentation profile generated by cathepsin D compared to that observed in ML-II cells. Units of recombinant protease used: Cathepsin D = 5 U, Cathepsin H = 1.3 mU, Cathepsin K = .75 mU, Cathepsin L = .31 mU, and Cathepsin S = 183 mU. All samples used 1 mg/mL purified plasma fibronectin.



Appendix Figure 3. LIF is a bona fide substrate for GlcNAc-1-phosphotransferase. (**A**) The binding profile of purified LIF and α-Idurondiase (lysosomal enzyme control) to the αβγ subunits of GlcNAc-1-Phosphotransferase in resonance units. LIF binds to the GlcNAc-1-Phosphotransferase comparable to α-Iduronidase (40.6 μM and 26.4 μM, respectively). (**B**) Purified LIF was subjected to lysine acetylation, followed by binding experiments to the αβ subunit of the enzyme. Acetylated LIF does not bind as well to the GlcNAc-1-phosphotransferase as the non-acetylated control. This suggests that the lysines on LIF are necessary, but not a requirement for interaction with GlcNAc-1-Phosphotransferase. (**C**) Site-directed mutagenesis and Western blot analysis of transiently transfected LIF K to Q mutants from CHO media cultures. Notice that a single lysine mutation (K99Q and K136Q) results in reduced levels of mannose phosphorylation as assessed by CI-MPR affinity chromatography.