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A novel interaction between 12-lipoxygenase and β4 integrin plays a role in cancer progression

Sangeeta Joshi
Wayne State University,

A NOVEL INTERACTION BETWEEN 12-LIPOXYGENASE AND β4 INTEGRIN PLAYS A ROLE IN CANCER PROGRESSION

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SANGEETA JOSHI

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DEDICATION

I would like to dedicate my dissertation work to my family, my dear parents and my younger brother for their love, support, encouragement, and understanding throughout my life in both personal and professional aspects.

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LIST OF ABBREVIATIONS

AA   Arachidonic acid

aa   Amino acid residue

ADB   Antibody dilution buffer

A431   Human epidermoid Carcinoma

β4   ITGβ4

BHPP   Benzyl-N-Hydroxy-5-PhenylPentanamide), P-12-LOX inhibitor

BMD122   Biomide Compound 122 (formerly know as BHPP-benzyl-N-

            hydroxy-

            5-phenylpentanamide) P-12-LOX inhibitor

cc $(Cm)^3$

ECL   Enhanced chemiluminescence

FNIII   Fibronectin type 3 domain

GFP   Green Fluorescent Protein

HD   Hemidesmosomes

LCMS   Liquid chromatography–mass spectrometry,

LOX   Lipoxygenase

COX   Cyclo-oxgenase

HETE   Hydroxyeicosatetraenoic acid

LN   Laminin

ECM   Extracellular Matrix

EIA   Enzyme Immunoassay

EGF   Epidermal growth factor

EGFP   Enhanced Green Fluorescent Protein

IACUC   Institutional Animal Care and Use Committee

HPETE   12(S) - hydroperoxyeicosaterarnoic acid

12-LOX   12-Lipoxygense

5-LOX   5-Lipoxygenase

MAPK   Mitogen activated protein kinase

mAb   Monoclonal antibody

ODS   Octadecyl silane -silica cartridges

pAb   Polyclonal antibody

PBS   Phosphate buffered saline

PG   Prostaglandin

PI3K   PI3 kinase, a phosphoinositide 3-OH kinase

PKB/Akt   Protein Kinase B

RP-HPLC   Reverse phase high performance liquid chromatography

SEM   Standard error measurement or mean

SFK   Srk family kinases

SDS-PAGE   Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBST   Tris buffered saline with tween-20

TGF   Transforming growt

CHAPTER 1 : Introduction

1.1 General Overview

The arachidonate or eicosanoid cascade produces oxygenated metabolites, which are derived from arachidonic and related fatty acids through a series of complex interrelated biosynthetic pathways. Arachidonic acid (AA) is a polyunsaturated fatty acid found in the membrane phospholipids (especially phosphatidylethanolamine, phosphatidylinositides, and phosphatidylcholine) of the body's cells, and is abundant in the brain, muscles, and liver. The first step in the biosynthesis is the production of free arachidonic acid in tissues from membrane phospholipids upon stimulation of the enzyme phospholipase A_2 by various physiological and pathological factors, including hormones and cytokines as well as eicosanoids (Murakami, Nakatani et al. 1997; Fitzpatrick and Soberman 2001). Eicosanoids are lipid mediators that are involved in many homeostatic biological functions and inflammation (Funk 2001). The three main pathways for eicosanoid metabolism, involve cyclooxygenases (COXs), lipoxygenases (LOXs) and epoxygenases or the cytochrome P-450 (CYP-450) (Needleman, Turk et al. 1986; Spector, Gordon et al. 1988; Serhan 1994). The cyclooxygenase pathway (COX-1 and COX-2, two major isoforms) generates PGG2 and PGH_2, which get converted further to other prostaglandins, prostacyclin and thromboxanes (TXs) (Samuelsson, Goldyne et al. 1978; Smith, Marnett et al. 1991). Lipoxygenases are named depending upon the insertion of molecular oxygen at different predominant positions on the arachidonic acid,

mainly 5, 12 and 15, but also 8-lipoxygenase is relevant in cancer for production of various hydroperoxyeicosatetraenoic acids (HPETEs), which are converted by peroxidases into hydroxyeicosatetraenoic acids (HETEs) and other products (Hammarstrom 1983; Samuelsson 1983; Samuelsson, Dahlen et al. 1987; Brash 1999). For example, 5-HETE yields, leukotriene LTA_4, which is in turn a precursor for LTB4, cysteinyl-leukotrienes (CysLTs) and lipoxins (LXs) (Samuelsson, Dahlen et al. 1987). The cytochrome P-450 epoxygenase pathway produces epoxy eicosatetraenoic acids (EpETrE). Many of the essential enzymes, precursors and products are cell type specific (Fitzpatrick and Murphy 1988; Fisslthaler, Popp et al. 1999)
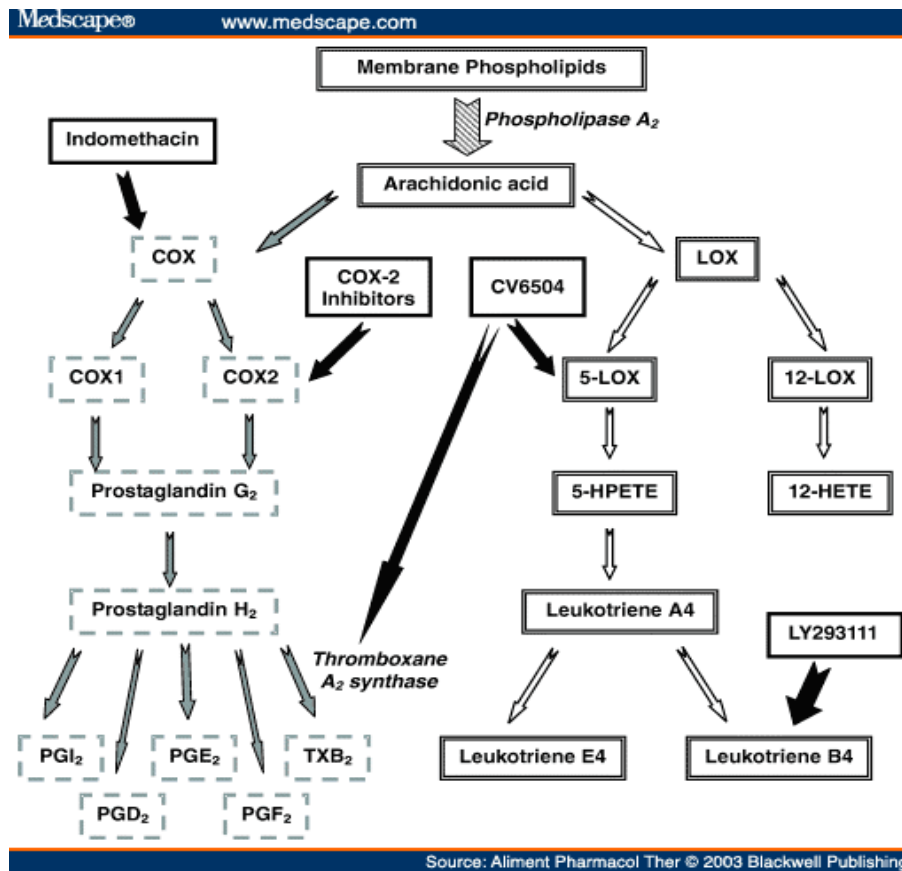
Figure 1.1: Arachidonic Acid Pathway
Source: Aliment Pharmacol Ther 18(11):1049-1069, 2003

Lipoxygenases comprise a group of non-heme iron dioxygenases that catalyze oxidation of polyunsaturated fatty acids in animals and plants (Yamamoto 1992; Brash 1999; Kuhn and Thiele 1999). Lipoxygenases insert molecular oxygen with regional specificity into arachidonic acid at C-5, C-12, and C-15 of arachidonic acid, respectively generating various hyperperoxides (Brash 1999). So far three major lipoxygenases are identified in mammalian tissue i.e. 5-, 12-, 15-lipoxygenases depending on the predominant position of insertion of molecular oxygen in arachidonic acid moiety (Brash 1999). Arachidonic acid is metabolized by 5-LOX to hydroperoxyeicosatetraenoic acid (5-HPETE) which subsequently gets converted to 5-HETE and leukotrienes.

12-lipoxygenase metabolizes AA to 12-HPETE, which is eventually transformed to 12(S)-hydroeicosatetranoic acid, or 12(S)-HETE. Similarly 15-LOX-1 and 15-LOX-2 isoforms generate 15-HPETE, which is ultimately converted to 15-HETE and lipoxins (Figure 1.1). 8(R)-lipoxygenase and 8(S)-lipoxygenase have been cloned from mouse skin and coral plexaura homomalla respectively (Brash, Boeglin et al. 1996). The metabolites of all the lipoxygenases have been reported to potentially form signaling lipid mediators that exert their effects through G protein–coupled receptors (GPCR) (Brash 1999; Serhan, Gotlinger et al. 2004; Guo, Zhang et al. 2011). These Lipoxygenases are expressed predominantly in specific tissues. For example, 5-LOX is expressed in higher amounts in polymorphonuclear leukocytes (Funk, Hoshiko et al. 1989; Funk, Furci et al. 1991), and 15-LOX is expressed in reticulocytes during anemia (Rapoport, Schewe et al. 1979; Fleming, Thiele et al.

1989), whereas 12-LOX is expressed constitutively by platelets (Chen and Funk 1993). However, the distinction between 12- and 15-LOXs becomes blurred with respect to some isoenzymes (e.g. leukocyte-type 12-LOX) due to the multiple sites of oxidation on arachidonic acid (Yamamoto, Suzuki et al. 1997).

1.2   12-Lipoxygenase

To date, three isoforms of 12-LOX have been identified in humans accordingly to their origin or cell type: epidermis, leukocyte, and platelet (Yoshimoto and Takahashi 2002). These are distinct enzymes by sequence catalytic properties, and function (Yamamoto, Suzuki et al. 1997; Limor, Weisinger et al. 2001; Siebert, Krieg et al. 2001). Platelet type 12-LOX is absent in normal tissues or is expressed at a lower level in normal tissues compared to cancer cells. The human platelet-type 12-LOX has been cloned from human erythroleukemia cells and is found primarily in human platelets, but also in epithelial cells, megakaryocytes, keratinocytes, umbilical vein endothelial cells and several tumor cells. Platelet type 12-LOX metabolizes C-20 fatty acids, such as AA to form exclusive product 12(S)-HETE (Kitt, Park et al. 1988; Funk, Funk et al. 1992; Krieg, Kinzig et al. 1995). Another LOX, a porcine leukocyte type 12-LOX that has been isolated and cloned from porcine leukocytes, porcine pituitary cells, and bovine tracheal cells, metabolizes both AA to give 12(S)-HETE and small amounts of 15(S)-HETE and linoleic acid to15(S)-HETE (Limor, Weisinger et al. 2001). There is evidence for the presence of a leukocyte-type 12-LOX in human adrenal glomerulosa cells. A third type termed epithelial-type 12-LOX has been

cloned from bovine tracheal epithelial cells and shares more homology with leukocyte 12-LOX and 15-LOX than with platelet type 12-LOX and catalyzes the formation of both 12(S)-HETE and 15(S)-HETE. Additionally, a structural class of mammalian lipoxygenases has been identified as epidermis-derived lipoxygenase-3 (ALOXE3), and an epidermal 12(R)-LOX was cloned from human skin and shares 54% homology to each other. No enzymatic activity has been detected by using linoleic or arachidonic acids as substrates for 12(R)-LOX (Krieg, Kinzig et al. 1995). (Figure 1.2, Phylogenetic tree of mammalian lipoxygenases;
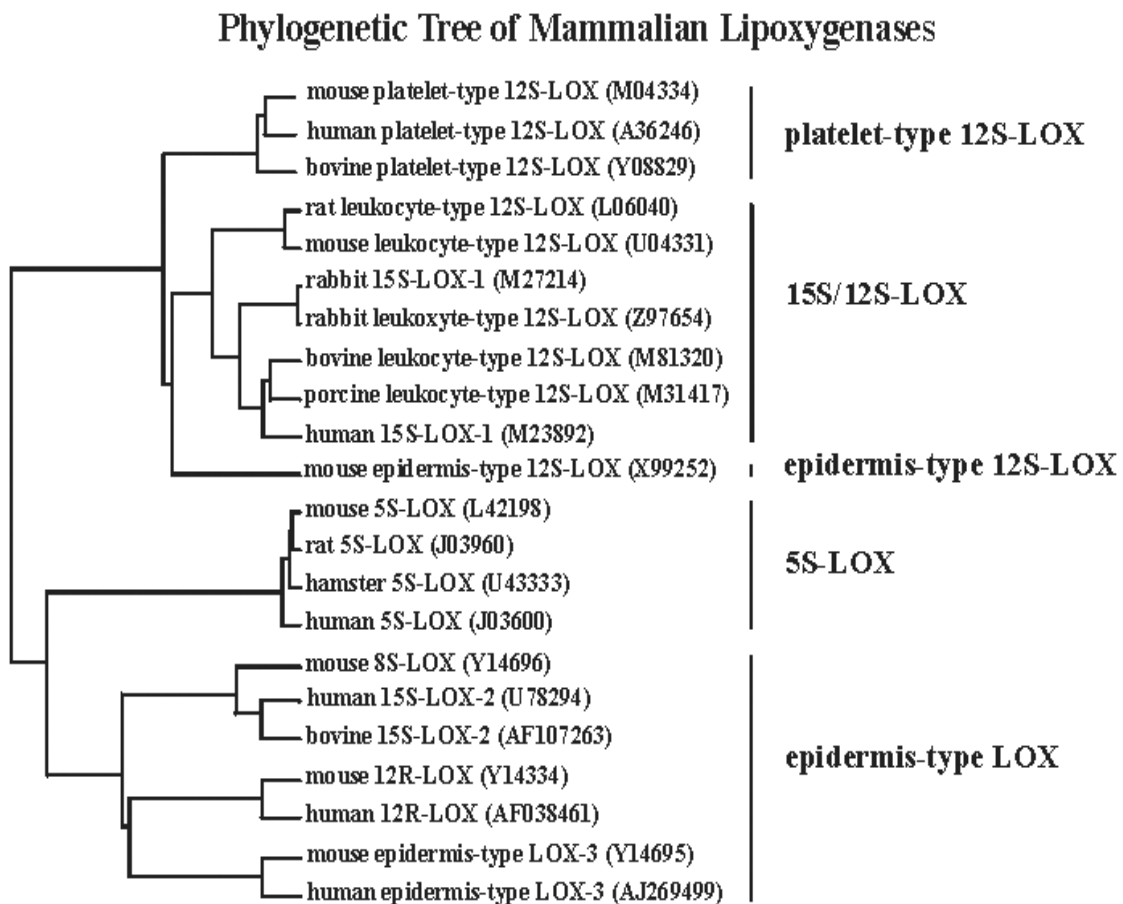
Figure 1.2: Phylogenetic tree of mammalian lipoxygenases.
Source: http://www.glycosciences.de/spec/lox-db/lipoxygenase-info)

Lipoxygenase protein is a single polypeptide chain. The molecular mass of the 12-LOX protein is 75-81 kDa (≈662-711 amino acids) in mammals and 94-103 kDa (≈838-923 amino acids) in plants (Shibata and Axelrod 1995; Brash 1999). LOX are members of a multi-gene family exhibiting an overall sequence identity of ≈ 25-40%, while close functional homologues across species share 70-95% identity (LOX-Dbase). 12-LOX genes from porcine, human and murine sources have been isolated from genomic libraries (Yoshimoto, Yamamoto et al. 1990; Funk, Funk et al. 1992; Yamamoto 1992). They have a highly conserved structure of 14 exons and 13 introns. 12-LOX proteins are comprised of highly conserved domains and sequence motifs that are important for their unique structure and also for binding of catalytic iron (His-X_4-His-X_4-His-X_{17}-His-X_8-His). Plant and mammalian lipoxygenase alignment reveals two domains; the **PLAT** (Polycystin-1, lipoxygenase, Alpha-Toxin) domain at the N-terminus or **LH2** (Lipoxygenase homology) domain, and a catalytic C-terminal part (Ivanov, Di Venere et al. 2011). Iron binds to the 3 conserved histidines, one His/Asn/Ser, and isoleucine at the C-terminus in an octahedral arrangement (LOX-Dbase) (Minor, Steczko et al. 1996). All mammalian lipoxygenases are homologous, which indicates that they have a common ancestral gene origin. All human LOX are localized to the sub-band at p13.1 of chromosome 17 with the exception of 5-LOX (Funk, Funk et al. 1992). Among all the LOX metabolites, 12(S)-HETE, the sole product of platelet type 12-LOX metabolism of AA, promotes the most divergent biological functions such as stimulating endothelial retraction, actin

polymerization, tumor cell adhesion, motility, invasion and metastasis (Timar, Silletti et al. 1993; Honn, Tang et al. 1994; Astudillo, Perez-Chacon et al. 2011; Astudillo, Balgoma et al. 2012).

1.3 Platelet- type 12-Lipoxygenase

12-Lipoxygenase is a cytosolic enzyme. This enzyme is present in higher amounts in platelets, but is also present in smooth muscle cells (Kim, Gu et al. 1995), keratinocytes (Krieg, Kinzig et al. 1995), and endothelial cells. 12-LOX is regulated by alterations in cellular oxidation and reduction conditions that are dependant on relative concentrations of reduced cytosolic glutathione and lipid hydroperoxidase (Shornick and Holtzman 1993; Kuhn and Thiele 1999). The enzymatic activity of 12-LOX is influenced by extracellular growth factors, cellular differentiation state, and sub-cellular compartmentalization (Tang and Honn 1999). Recently our group has demonstrated that there is positive feedback on 12-LOX expression and that 12-LOX activity is regulated by Src phosphorylation (Guo, Zhang et al. 2011) and Dilly A.K et al. to be submitted). 12-LOX localization is triggered by EGF (Chang, Liu et al. 1993; Hagmann, Gao et al. 1996), phorbal ester (Izumi, Hoshiko et al. 1990), Ca^{+2} or thrombin (Baba, Sakuma et al. 1989; Hagmann, Gao et al. 1996; Ozeki, Nagamura et al. 1999) and stimulation of β4 (Tang et al., unpublished observation). 12-LOX is pro-thrombotic when activated. This pro-thrombotic activity is linked to mobilization of calcium (Nyby, Sasaki et al. 1996), regulation of tissue factor activation, and generation of thrombin in the platelet (Thomas, Morgan et al. 2010).

1.4 Expression of 12-Lipoxygenase in Cancer

Different LOX isoforms vary in the individual mechanism and their role in cancer. In general 5-LOX and platelet type 12-LOX play a role in carcinoma and are considered procarcinogenic, the role of 15-LOX-1 is unclear and being debated, while 15-LOX-2 has a unique role and is known to suppress carcinogenesis (van Leyen, Duvoisin et al. 1998; Shureiqi and Lippman 2001). LOX expression and activity in both normal and cancer epithelial tissues from humans and mice were examined. The result revealed that human 15-LOX-1 and -2, or the corresponding mouse orthologs leukocyte 12-LOX and 8-LOX, are usually preferentially expressed in normal tissues and benign lesions, but not in carcinomas of the bladder, breast, colon, lung or prostate. In contrast, 5-LOX and platelet type 12-LOX are generally not expressed in normal epithelia but can be induced by pro-inflammatory stimuli. Several lines of evidence implicate 12-LOX as a regulator of human cancer development. The expression of platelet-type 12-LOX has been confirmed in various tumor cell lines of different tissue origins. It is over expressed in a variety of tumors including melanoma, breast cancer, cervical cancer, ovarian cancer, colorectal cancer, prostate cancer, skin cancer, and pancreatic cancer (Chang, Liu et al. 1993; Kim, Gu et al. 1995; Krieg, Kinzig et al. 1995; Natarajan, Esworthy et al. 1997; Nie, Hillman et al. 1998; Ding, Kuszynski et al. 1999; Timar, Raso et al. 1999; Aran, Bryant et al. 2011). Recently it has been shown that 12-LOX and its metabolite 12(S)-HETE are important regulators of ovarian cancer growth (Guo, Liu et al. 2011). Gao et al. in his study involving over 130 prostate cancer patients, found that the 12-

LOX mRNA expression level is correlated with tumor stage and grade (Gao, Grignon et al. 1995). The 12-LOX RT-PCR sequence products from human epidermoid A431 cancer cells, prostate cancer cultured cell lines as well as prostate tissues have complete homology to the platelet-type 12-LOX (Gao, Grignon et al. 1995; Hagmann, Gao et al. 1996). The product of 12-LOX activity, 12(S)-HETE, was identified in tumor cells and its structure was confirmed by mass spectrometry (Liu, Marnett et al. 1994). Just as with prostate cancer, elevated levels of 12-LOX were found to be of great prognostic value for TNM staging in the case of breast cancer (Jiang, Douglas-Jones et al. 2003; Jiang, Douglas-Jones et al. 2006)

1.5 Role of 12-Lipoxygenase and its product 12(S)-HETE in Tumor Metastasis

Eicosanoids and other bioactive lipids are known to be involved in various aspects of neoplasia including cell transformation, tumor promotion, and tumor cell growth, and metastasis (Honn, Tang et al. 1994). During metastasis tumor cells interact with platelets, endothelial cell, and matrix protein undergoing various cell-host interactions. It has been demonstrated that 12-LOX stimulates prostate carcinoma tumor growth *in vivo*. This effect on tumor growth is closely related to increased angiogenesis (Nie, Hillman et al. 1998). The growth and metastasis of solid tumors is dependent on the ability of tumor cells to induce angiogenesis. 12(S)-HETE, has been shown to protect tumor cells from

apoptosis and induce invasion, motility, and angiogenesis (Honn, Tang et al. 1994; Gao and Honn 1995; Nie, Tang et al. 2000; Nie, Tang et al. 2000), as well as surface expression of $\alpha_v\beta_3$ integrin (Tang, Grossi et al. 1993). Addition of exogenous 12(S)-HETE alters the cancer cell cytoskeleton through phosphorylation of cytoskeletal proteins (Tang and Honn 1997), increases tumor cell adhesion and spreading on *in vitro* matrices (Timar, Chen et al. 1992; Timar, Silletti et al. 1993), secretion of proteinases (Honn, Timar et al. 1994; Liu, Connolly et al. 1996), expression of integrins (Chopra, Timar et al. 1991; Tang, Diglio et al. 1995), and it increases invasion. Pre-treatment of tumors with 12(S)-HETE stimulates cell motility (Silletti, Timar et al. 1994) and increases the surface expression of $\alpha_v\beta$ integrins, specifically $\alpha_v\beta_3$ integrin. (Honn, Tang et al. 1994). The adhesion of B16 murine melanoma cells to micro-vascular endothelial cells was enhanced via up-regulation of $\alpha_v\beta_3$ integrin resulting from treatment with 12(S)-HETE (Tang, Grossi et al. 1993). Furthermore, it has been demonstrated by Honn et al. in 2003 that 12-LOX regulates cell survival and apoptosis by affecting the expression and localization of integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in both PC3 and A431 human cancer cells. It has been reported in certain circumstances that metastasis of tumor cells is enhanced by low dose radiation, where 12(S)-HETE biosynthesis leads to induced onset of elevated 12(S)-HETE (Onoda, Piechocki et al. 1992; Onoda, Kantak et al. 1994). Another positive correlation between biosynthesis of 12(S)-HETE and the metastatic potential of murine B16a cells was observed. The highly metastatic cells demonstrated a five to ten-fold increase in lung colonizing ability, over the low metastatic cell

lines, due to elevated levels of 12(S)-HETE and 12-LOX mRNA (Honn, Tang et al. 1994). 12(S)-HETE is a crucial intracellular signaling molecule involved in activation of various signaling pathways. It activates PKCα *via* PLCγ1 and stimulates PKCζ *via* inositide kinase. It also activates MAPK kinase and mediates biological functions of many growth factors such as bFGF, PDGF, EGF and AMF (Liu, Khan et al. 1995; Wen, Nadler et al. 1996; Hagerman, Fischer et al. 1997; Wen, Scott et al. 1997). Studies have identified the role of 12-LOX in tumor cell metastasis using the selective 12-LOX inhibitor BHPP (now called BMD122). BMD122 was demonstrated to inhibit 12-HETE metabolism while not inhibiting production of 15-HETE or other mono-hydroxy fatty acids (Chen, Duniec et al. 1994). BMD122 treatment led to apoptosis of cancer cells, which was counteracted by over expression of 12-LOX, or by exogenously added 12(S)-HETE. Thus, the role of 12(S)-HETE in these signaling events was confirmed by inactivation of endogenous 12(S)-HETE production with a specific 12-LOX inhibitor

1.6 Regulation of 12-Lipoxygenase by Extracellular and Intracellular Factors

12-LOX activity is regulated by both extracellular and intracellular factors, which influence the expression of 12-LOX at the mRNA and protein level. Extracellular factors like EGF, AMF, PDGF, TPA, TGF-α , IL-3, 4, 8 (Natarajan, Bai et al. 1996; Heydeck, Thomas et al. 1998; Liaw, Liu et al. 1998; Chen, Chen et al. 1999; Tang and Honn 1999) and the intracellular factors like glucose,

angiotensin II, serum, fibronectin and glucocorticoids (Natarajan, Gu et al. 1993; Kuchinke and Funk 1994; sChang, Kao et al. 1995; Hagmann, Gao et al. 1996) are known to regulate 12-LOX activity. It is also regulated by the oxido-reduction changes in the cell that depend on the relative concentration of glutathione and lipid hydroperoxides in the cytosol (Shornick and Holtzman 1993). 12-LOX is regulated by the positive feedback of 12(S)-HETE, and sub-cellular compartmentation (Hagmann and Borgers 1997; Tang and Honn 1999). In HEL cells 12-LOX is suppressed by transcription factors like NF-κB which play an important role in the control of cell proliferation and apoptosis (Arakawa, Nakamura et al. 1995). In turn it has been shown that either over-expression of the platelet-type 12-LOX or exogenously added 12(S)-HETE activates NF-κB (Kandouz, Nie et al. 2003). In a study by Chang et al., 12-LOX protein expression and activity both were induced by EGF in A431 human epidermoid carcinoma cells but the pretreatment of A431 cells with glucocorticoids down regulated both expression and activity through the glucocorticoid receptor (sChang, Kao et al. 1995).

1.7   Regulation of 12-Lipoxygenase by Subcellular Compartmentalization

The distribution of 12-LOX activity varies among different cell lines and does not correspond to the distribution of 12-LOX protein. The 12-LOX activity within a cell varies by compartments and can be found both in the cytosolic and particulate or membranous fraction. In human cancer cells such as A431, HEL

cells and murine B16a melanoma cells, the 12-LOX activity reported by 12(S)-HETE production, is mostly localized to the membranous fraction, though the 12-LOX protein is mainly localized in the cytosol (Chang, Ning et al. 1992; Hagmann, Kagawa et al. 1993; Hagmann, Gao et al. 1996). In Lewis lung carcinoma cells (3LL), 12-LOX activity and protein are both localized in the cytosol. Though 12-LOX gets translocated from the cytosol to the membrane in a Ca^{+2} dependent manner, the enzymatic activity is lost at the membrane (Marnett, Leithauser et al. 1991; Hagmann, Gao et al. 1995). In A431 cells, EGF stimulation can promote 12-LOX translocation and activation (Chang, Ning et al. 1992; Hagmann, Gao et al. 1996), while in HEL cells translocation can be stimulated by TPA treatment (Hagmann, Kagawa et al. 1993). It has been observed in HEL, 3LL cells and platelets that there is a dependence of 12-LOX on transient elevation of intracellular free calcium for its translocation to the membrane (Baba, Sakuma et al. 1989; Hagmann, Kagawa et al. 1993). Thus 12-LOX activity is modulated by subcellular distribution and translocation from the cytosol to membrane.

1.8   12-Lipoxygenase Interacting Partners

In order to identify the potential regulators of platelet type 12-lipoxygenase Tang et al., performed a yeast two-hybrid screen using full-length human platelet type 12-LOX as bait (Tang, Finley et al. 2000). Using this yeast two-hybrid screen approach they screened a human epidermoid carcinoma A431 cDNA library for 12-LOX interacting proteins. Following the screening of $3X10^6$ cDNA clones, four

cellular proteins were identified that specifically interact with platelet type 12-LOX (Tang, Finley et al. 2000). Sequence analysis of the 12-LOX interacting proteins revealed that one of these encoded the cytoplasmic domain of the human β4 integrin subunit. The other three proteins are type-II keratin K5, nuclear envelope protein lamin A and C8FW phosphoprotien. These proteins provide first candidate regulators of 12-LOX. β4 integrin is the only integrin protein with a unique long cytoplasmic tail (1004 amino acid) and is a major component of hemidesmosomes. The following chapters discuss why Integrin β4 is so important to the interaction between platelet type 12-LOX and integrin β4 subunit.

1.9 Integrins

Integrins are heterodimeric transmembrane receptors that mediate either attachment of cells to extracellular matrix, or direct cell to cell interaction. They have a large extracellular domain, a transmembrane region and a short cytoplasmic tail. In mammals, 18 α subunits combine with 8 β subunits to produce a total of 24 integrin molecules with distinct, but often overlapping ligand binding abilities. Integrins link the intracellular and extracellular environment by transducing signals across the membrane in both directions. Binding of the ligand to the extracellular domains of integrin triggers a variety of signaling pathways due to the conformational changes that are transmitted through the plasma membrane to their cytoplasmic tails (Clark and Brugge 1995; Schwartz, Schaller et al. 1995; Hynes 2002). At focal adhesions integrins are clustered only after they are activated and bound to ligand (Cluzel, Saltel et al. 2005). In

addition to binding ligand and organizing the cytoskeleton, integrins trigger the intracellular signaling pathways that control cell survival and proliferation (Hynes 1992; Giancotti 1997). Among the super family of integrins, $\alpha6\beta4$ is unique and characterized by the presence of a long cytoplasmic tail about 1004 amino acid long, which has distinctive cytoskeleton and signaling functions (Giancotti and Tarone 2003).

1.10 Integrin $\alpha6\beta4$

The $\alpha6\beta4$ integrin is an essential component of hemidesmosomes; junctional complexes in the basal layer of stratified epithelia that connect the intermediate filament system to the extracellular matrix (ECM) (Lee, Lotz et al. 1992; Spinardi, Ren et al. 1993; Niessen, Hogervorst et al. 1994; Borradori and Sonnenberg 1996; Dowling, Yu et al. 1996). The distinguishing structural feature of $\alpha6\beta4$ is the atypical cytoplasmic domain of the $\beta4$ subunit. The large $\beta4$ cytoplasmic tail is encoded by ~1000aa, compared to ~50aa for all other known β integrin subunits (Hogervorst, Kuikman et al. 1990; Suzuki and Naitoh 1990). Sequence analysis of the integrin β subunit shows the presence of four regions that are homologous to fibronectin type III (FNIII) domains. The FNIII regions are arranged as two pairs of tandems that are separated by a connecting segment (Suzuki and Naitoh 1990). Most of the known functions of the cytoplasmic domain reside in the first pair of FNIII domains and the **c**onnecting **s**egment (FN1:FN2:CS). In epithelial cells this region (FN1:FN2:CS) is essential for mediating incorporation of $\beta4$ into the hemidesmosomes (Spinardi, Ren et al.

1993). The second FNIII-like domain of the $\beta4$ cytotail and a small region of the adjacent CS are known to interact with plectin (Rezniczek, de Pereda et al. 1998). Distal regions of the $\beta4$ tail have also been implicated in interactions with plectin as well as with two other major components of hemidesmosomes, namely BPAG1 and BPAG2 (Wilhelmsen, Litjens et al. 2006). Yeast two-hybrid studies have shown that the same region also interacts with p27$^{BBP/eIF6}$, an elongation initiation factor that localizes to hemidesmosomes in epithelial cells expressing $\alpha6\beta4$ (Biffo, Sanvito et al. 1997; Sanvito, Piatti et al. 1999). (Figure 1.3 structural biology of hemidesmosome;
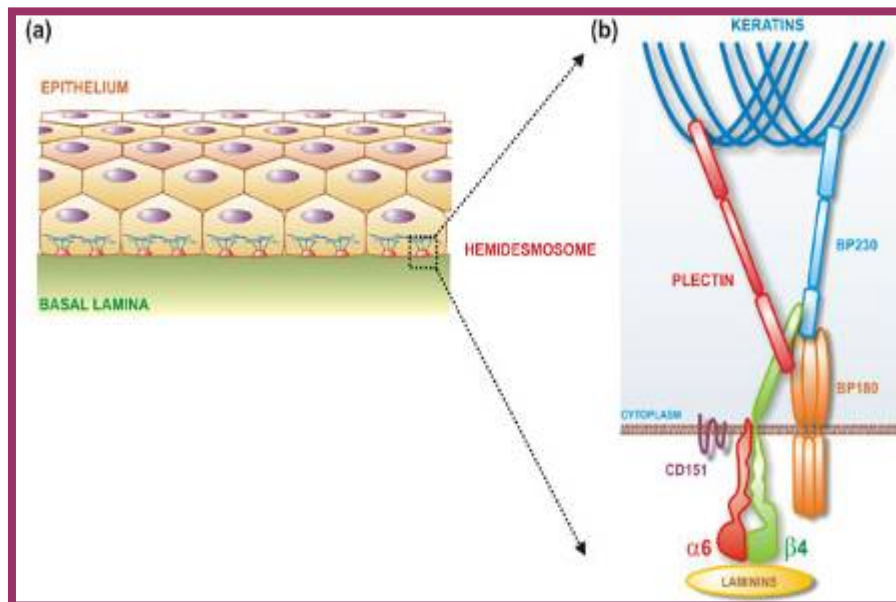
Figure 1.3: Structural biology of hemidesmosome

Source: xtal.cicancer.org/.../hemidesmosome_scheme01.jpg)

The primary function of $\beta4$ is to maintain epithelial integrity especially in the epidermis (Dowling, Yu et al. 1996; van der Neut, Krimpenfort et al. 1996).

Binding of laminin to α6β4 integrin leads to activation of Ras-ERK and Rac-JNK mitogen activated protein kinase (MAPK) pathways in keratinocytes (Mainiero, Pepe et al. 1995; Mainiero, Murgia et al. 1997). Additionally laminin also activates phosphatidylinositol-3 kinase (PI-3K) (Shaw, Rabinovitz et al. 1997). Protein-protein interaction of β4 is governed by its phosphorylation state. Dephosphorylation of this β4 cytoplasmic tail leads to association with keratin cytoskeleton and assembly of hemidesmosomes (Spinardi, Ren et al. 1993; Murgia, Blaikie et al. 1998; Dans, Gagnoux-Palacios et al. 2001). α6β4 cross-talks with multiple receptor tyrosine kinases (RTK's). Activation of EGF receptor (EGF-R) and Met leads to increased phosphorylation of β4 and Shc signaling causes disassembly of hemidesmosomes, and leads to increased cell motility and proliferation (Mariotti, Kedeshian et al. 2001; Trusolino, Bertotti et al. 2001). This joint regulation of α6β4 and tyrosine kinase signaling promotes epithelial cell survival, proliferation and migration (Mainiero, Murgia et al. 1997; Mariotti, Kedeshian et al. 2001; Jonkman, Pas et al. 2002; Weaver, Lelievre et al. 2002; Iacovacci, Cicuzza et al. 2003). Deregulation of this system plays an important role in carcinoma invasion and growth (Shaw, Rabinovitz et al. 1997; Wen, Scott et al. 1997). In the absence of α6β4 skin retains normal morphology, but the epidermis detaches in response to mechanical stress resulting in death shortly after birth (DiPersio, van der Neut et al. 2000). Compound heterozygote for premature termination-codon mutations (C738X/4791delCA), and missense mutation involving a cysteine residue (C61Y) in α6β4, both causes a lethal type of epidermolysis bullosa, a disorder associated with severe skin blistering (Uitto,

Pulkkinen et al. 1997; Jonkman, Pas et al. 2002; Iacovacci, Cicuzza et al. 2003).

In contrast to its function in regulating stable adhesion through hemidesmosome

formation in epithelial cells, studies have revealed that $\alpha6\beta4$ integrin promotes

motility and invasion in carcinoma cells (Borradori and Sonnenberg 1996). It is

also interesting to note that invasion in breast cancer induces $\beta4$ integrin

expression in stromal fibroblasts to protect them from anoikis during tissues

remodeling (Kim, Gao et al. 2012).

1.11 $\beta4$ Integrin and Cancer

The $\beta4$ integrin was initially identified as a **t**umor **s**pecific **p**rotein (TSP180)

unregulated in metastatic variants of mouse lung carcinoma and melanoma cell

lines (Kennel, Foote et al. 1981; Falcioni, Kennel et al. 1986). Many studies have

provided evidence that the expression of $\beta4$ increases significantly during

malignant progression in squamous carcinoma of multiple tissues, including head

and neck, skin, cervix and lung (Van Waes, Kozarsky et al. 1991; Carico, French

et al. 1993; Savoia, Trusolino et al. 1993; Boelens, van den Berg et al. 2007).

High levels of $\beta4$ integrin have been linked to poor prognosis in breast cancer

(Tagliabue, Ghirelli et al. 1998) and bladder cancer (Grossman, Lee et al. 2000),

and also in tumor cells invading gastric cancer (Tani, Karttunen et al. 1996). It is

also interesting to note that invasion in breast cancer induces $\beta4$ integrin

expression in stromal fibroblasts to protect them from anoikis during tissue

remodeling (Kim, Gao et al. 2012). High levels of $\beta4$ integrin have been detected

in colorectal, pancreatic and epitheloid pleural malignant mesothelioma (Giancotti

2007). In addition, α6β4 is also expressed in tumor blood vessels thereby promoting the invasive phase of tumorogenesis (Falcioni, Kennel et al. 1986). Thus, the expression of β4 integrin confers evolutionary advantage during tumorigenesis. Interestingly β4 perform two contrasting roles, stable adhesion, and pro-invasive signaling. It promotes assembly of hemidesmosomes to mediate stable adhesion, and restrains cell migration by connecting the keratin cytoskeleton to the basement membrane. The disassembly of hemidesmosomes takes place when β4 integrin is relocated from hemidesmosomes to the leading edge in migrating keratinocytes. In carcinoma cells it is enriched at lamellipodia causing migration. This shows that there is a dynamic regulation that allows both normal cell migration and carcinoma invasion (Mariotti, Kedeshian et al. 2001; Mercurio and Rabinovitz 2001; Litjens, de Pereda et al. 2006). It has been reported earlier that β4 contains multiple tyrosine phosphorylation sites in the cytoplasmic domain namely, Y1422, Y1440, Y1494, Y1526, and Y1642. It combines with SRC family kinases (SFK) and also mediates recruitment of signaling adapter protein Shc and activation of Ras to stimulate the ERK signaling pathway (Mainiero, Pepe et al. 1995; Mainiero, Murgia et al. 1997; Dans, Gagnoux-Palacios et al. 2001; Gagnoux-Palacios, Dans et al. 2003). In addition it also activates PI-3K kinase. Binding of commercial antibody 3E1 (Chemicon Inc.) or laminin to β4 activates Ras and PI-3K signaling (Mainiero, Pepe et al. 1996; Mainiero, Murgia et al. 1997; Shaw, Rabinovitz et al. 1997). Assembly of hemidesmosomes is prevented by both SFK mediated tyrosine phosphorylation and PKC-mediated serine phosphorylation of the β4 cytoplasmic

tail (Dans, Gagnoux-Palacios et al. 2001; Rabinovitz, Tsomo et al. 2004). β4 integrin ligation is sufficient to promote cell migration but RTK activation is necessary for cell proliferation and sustained migration (Giancotti and Tarone 2003; Giancotti 2007). β4 combines with numerous RTKs including EGF-R, ErbB2, Met and Ron (Mariotti, Kedeshian et al. 2001; Trusolino, Bertotti et al. 2001; Santoro, Gaudino et al. 2003; Guo, Pylayeva et al. 2006). RTKs induce activation of SFKs on stimulation of β4, and leading to phosphorylation of the β4 cytoplasmic tail (Mariotti, Kedeshian et al. 2001; Santoro, Gaudino et al. 2003; Giancotti 2007). Additionally, while ERK and Akt are efficiently activated by mitogeic concentrations of EGF they fail to induce nuclear translocation of ERK, JNK and NF-kB when plated on lamimin-5. Therefore β4 signaling also controls nuclear translocation of many transcription factors (Nikolopoulos, Blaikie et al. 2004; Nikolopoulos, Blaikie et al. 2005). β4 combine with EGF-R, ErbB-2 and Met RTKs, which are often mutated or amplified during tumor progression. This suggests that dysregulated joint β4- RTK signaling contributes to carcinoma invasion and growth (Giancotti 2007). This is supported by the observation that expression of wild type β4 in β4-negative breast carcinoma cells that express the HGF receptor Met, activates signaling from PI-3K to Rac and enhances their invasive properties (Shaw, Rabinovitz et al. 1997) whereas dominant negative inhibition of β4 impairs survival and adhesion of breast carcinoma cells (Weaver, Lelievre et al. 2002) . Further, over expression of β4 promotes ErbB-2 dependent cell invasion and proliferation in cancer cells (Falcioni, Kennel et al. 1986). Tyrosine phosphorylation of β4 also occurs through Fyn, as inhibition of

Fyn suppresses tyrosine phosphorylation of β4 thereby leading to hemidesmosome reassembly and reduced invasion of squamous carcinoma cells expressing elevated levels of EGF-R (Mariotti, Kedeshian et al. 2001; Giancotti 2007). The β4 tail functions as an adapter and amplifier for pro-invasive signals elicited by Met in cells that undergo Met-mediated oncogenesis (Trusolino, Bertotti et al. 2001). Likewise, binding of Shp2 to β4 integrin enhances activation of the Src signaling pathway, which is required for further induction of anchorage independent growth, mediated by β4 in breast carcinoma cells (Bertotti, Comoglio et al. 2006). Collectively these studies suggest that unliganded β4 amplifies signaling by oncogenic RTKs (Giancotti 2007), while on other hand, liganded β4 is required for Ras-mediated transformation in keratinocytes, thus promoting cell survival and proliferation (Dajee, Lazarov et al. 2003). β4-mediated assembly of hemidesmosome leads to cell growth and polarity in normal cells however β4 signaling promotes cell proliferation and invasion in cancer cells. Thus β4 integrin plays a very important role as a signaling hub in cell proliferation, apoptosis, differentiation, adhesion, invasion and metastasis.

1.12 Interaction between P-12-LOX and β4 Integrin Subunit

Initial studies by Tang have shown by yeast two-hybrid screening the binding partners of P-12-LOX using full length human P-12-LOX as bait to screen a human epidermoid carcinoma A431 cell line cDNA library (Tang, Finley et al. 2000). Following the screening of $3X10^6$ cDNA clones, four cellular proteins

were found to interact specifically with 12-LOX. Sequence analysis identified one of them as cytoplasmic domain of human β4 integrin subunit (Tang, Finley et al. 2000). The uniqueness of β4 integrin and its important role in cell proliferation, apoptosis, differentiation, adhesion, invasion and metastasis have been already discussed. Therefore, the main focus of this project was on the interaction of P-12-LOX and the β4 subunit both physically and for functional relevance (Tang et al., unpublished observation). The study confirms that P-12-LOX physically interacts with the cytoplasmic domain of β4 by yeast two-hybrid screening but also by co-immunoprecipitation from the mammalian cells and co-localization by confocal microscopy. Tang reported that stimulation of A431 cells with the anti-β4 mAb 3E1 increased P-12-LOX association with β4, and found that the extracellular domain of the β4 subunit appears to serve as a receptor for extracellular stimuli which triggers a signaling mechanism that plays a role in the interaction of the cytoplasmic tail with 12-LOX (Tang et al., unpublished observation). Confocal microscopic analyses of the two proteins revealed that 12-LOX is primarily localized in the cytosol and upon stimulation of β4 integrin becomes co-localized at the membrane. The data suggested that β4 subunit mediates the translocation of 12-LOX from the cytoplasm to the membrane and provided the first regulatory protein that impacts 12-LOX localization. The co-immunoprecipitation data and confocal microscopy data provide significant evidence for a physical association between 12-LOX and the cytoplasmic domain of β4. This association between 12-LOX and the cytoplasmic domain of β4 after integrin ligation promotes cell migration in response to EGF as a chemo-

attractant. Additionally, the functional relevance of this association for enzymatic activity was demonstrated by the RP-HPLC and EIA data where 12(S)-HETE biosynthesis was increased following cell stimulation with β4 mAb 3E1. The physical interaction of P-12-LOX with β4 integrin subunit up-regulates its enzymatic activity which is measured by 12(S)-HETE production. It also protects cells from apoptosis induced by the 12-LOX inhibitor, BMD122, suggesting a relationship between β4 ligation, enhanced 12-LOX activity, and protection from apoptosis. Also the migration data suggest that 12-LOX activity is important for EGF-stimulated, α6β4-dependent, cell migration upon treatment with 3E1 mAb antibody or laminin. Tang et al. demonstrated that 12-LOX interacts with the integrin β4 both physically and functionally, thus providing insight for both integrin and eicosanoid biology (Tang, Finley et al. 2000). The known correlation between 12-LOX and tumor progression and metastasis, and the insight from this study reveals another facet of the complicated role of the integrin α6β4 in cancer and other human diseases and may provide a new target for therapy and treatment. The focus of this study was to establish which residues or motifs in the cytoplasmic domain of β4 are critical for 12-LOX binding and to associate these with phenotypes that are functionally relevant to cancer progression. Future studies will determine what reciprocal sites of the 12-LOX protein are responsible for the interaction with β4.

1.13 INTEGRIN β4 REGULATION OF 12-LIPOXYGENASE

Keqin Tang[*], Daotai Nie[*ψ], Yinlong Cai[§], William T. Repaskey[†], Sangeeta Joshi[§], Stephanie C. Tucker[§], Krishna Rao Maddipati[§], John D. Crissman[§], and Kenneth V. Honn[§]

Department of Radiation Oncology[*] and Pathology[§], Wayne State University School of Medicine, Karmanos Cancer Institute, Detroit, MI 48202; Department of Internal Medicine[†], University of Michigan, Ann Arbor, MI 48109; Department of Medical Microbiology, Immunology and Cell Biology[ψ], Southern Illinois University School of Medicine, Simmons Cooper Cancer Institute, Springfield, IL 62794-9626

NOTE: This section is part of introduction and indicates my work done for this paper.

1.13.1   Results and Discussion

1.13.1.1   12-LOX-β4 interaction is selectively specific and β4 stimulating antibody 3E1 has the same effect as its natural ligand laminin

We have previously identified the potential regulators of 12-LOX by the yeast two-hybrid screen of an A431 library and found that 12-LOX interacts with the C-terminal cytoplasmic domain of the integrin β4 subunit (Tang, Finley et al. 2000). In this study we confirmed endogenous interaction of 12-LOX with β4 using the A431 cell line by performing a co-immunoprecipitation assay. We performed immunoprecitation on 3E1 or laminin-stimulated A431 cells with anti 12-LOX and probed with β4 and found 12-LOX co-immunoprecipitated with β4. Regardless of stimulus, the association of 12-LOX with β4 was time-dependent and began at 5 minutes post stimulation, peaked at 60 minutes, and declined at 90 minutes (Figure 1.4).   This was suggestive of a specific association between 12-LOX and β4. These data suggests that the interaction between 12-LOX and β4 is specific and that a β4 stimulating antibody elicits the same response as laminin with respect to 12(S) - HETE production and associated phenotypes.

1.13.1.2   The β4 and 12-LOX interaction increases 12-LOX enzymatic activity

The enzymatic activity of 12-LOX, followed by β4 integrin stimulation was determined by LC-MS analysis, for its sole AA metabolite 12(S)-HETE.   Lipid extraction was performed as described in Materials and Methods.   CHO stable transfectant cells were stimulated either with β4 stimulating antibody (3E1), or its natural ligand (laminin) followed by incubation with 10 μM AA for 6 hours.   The results revealed that 12-LOX activity was increased when 12-LOX interacts with β4 integrin and was further enhanced on stimulation (Figure 1.5).   Taken together, we conclude that the interaction of 12-LOX with the β4 subunit up-regulates 12-LOX enzymatic activity.

1.13.1.3   12-LOX plays a role in EGF-stimulated cell migration

It has been previously shown that EGF enhances α6β4-dependent cell migration of A431 cells on laminin (Mainiero, Pepe et al. 1996; Rabinovitz, Toker et al. 1999).   As 12-LOX interacts physically and functionally with β4 in A431 cells, we tested whether 12-LOX modulates α6β4 -dependent migration upon stimulation with laminin or β4 antibody 3E1.   We demonstrated that EGF induced A431 cell migration by 2-2.5 fold.   Interestingly pretreatment of cells with 12-LOX specific inhibitor BMD122 inhibits 12-LOX and reduces A431 cell migration to the level observed in the absence of EGF stimulation (Figure 1.6), which is in agreement with previous reports (Rabinovitz, Toker et al. 1999).   Laminin and 3E1 antibody

induced migration through matrigel equally well. However, migration was inhibited to a greater degree in laminin treated cells. Our data suggest that 12-lipoxygenase activity is important for EGF-stimulated, $\alpha6\beta4$-dependent, cell migration upon stimulation with laminin or 3E1.

1.13.1.4   Intracellular Co-localization of 12-LOX and β4 Integrin

The physical association between 12-LOX and the β4 subunit was confirmed by assessing intracellular co-localization by double labeling and examination by confocal microscopy. Sub-confluent A431 cells were serum starved overnight and treated with 5 μg/ml laminin for two hours and β4 antibody 3E1, or non-specific mouse IgG for one hour. Cells were labeled simultaneously with P-12 LOX antibody and β4 antibody followed by the labeling with the respective secondary antibodies $Alexa_{488}$ (green) and $Alexa_{594}$ (red) (Figure 1.7). Overlapping areas of staining appear yellow in confocal images in the cells stimulated with laminin and 3E1, indicating 12-LOX-β4 colocalization (Fig. 1.7 A and B). The unstimulated cells or the cells treated with mouse IgG, showed no co-localization (Fig. 1.7 C and D). Controls were stained with secondary antibody alone and showed no cross reactivity with the background (Fig. 1.7 E). These data show 12-LOX-β4 co-localization which supports the biochemical evidence that 12-LOX physically interacts with β4.

1.13.2   Summary

In summary, we have demonstrated that 12-LOX interacts with the cytoplasmic domain of the β4 subunit in mammalian cells. 12-LOX colocalizes with β4 at the edge of the cell membrane and some peri-nuclear staining was also observed. 12-LOX-β4 interaction results in an increase in 12-LOX enzymatic activity that is further enhanced upon stimulation with 3E1 or laminin, and culminates in increased cell migration. These data represents the novel interaction between 12-LOX and integrin β4, both physically and functionally, thus providing a new paradigm for both integrin and eicosanoid biology. Given the demonstrated correlation between 12-LOX expression and tumor progression and metastasis, results from this study reveal a complicated role of the integrin α6β4 in cancer and other human diseases and may provide new potential therapeutic targets. Studies presented in this dissertation established which residues or motifs in the cytoplasmic domain of β4 are critical for 12-LOX binding

Laminin

0 min 5 min 15 min 30 min 60 min 90 min

β4 204kDa

β4 204kDa

0 min 5 min 15 min 30 min 60 min 90 min

3E1Ab
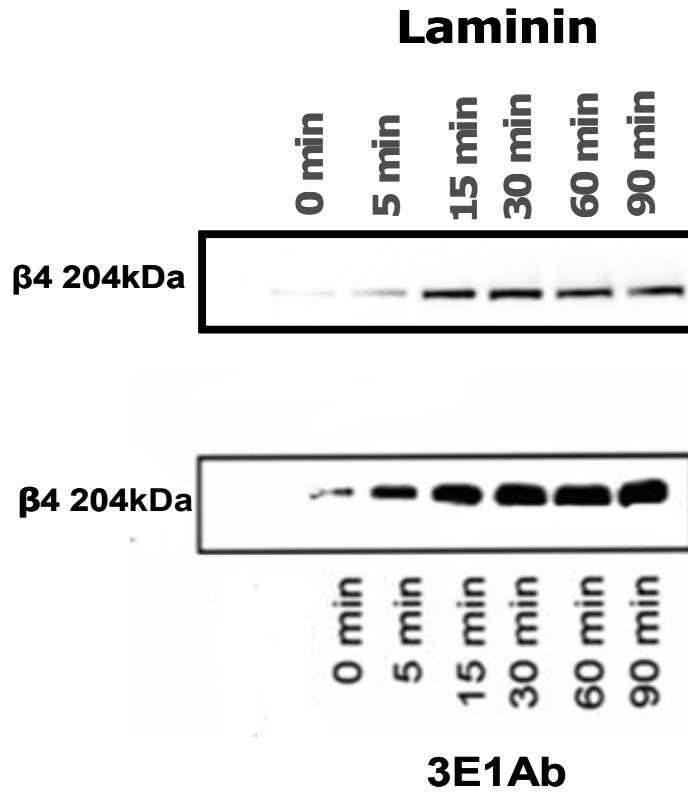
Figure 1.4: P-12-LOX interacts with integrin β4 subunit *in vitro*. A431 cells were stimulated with mAb β4 3E1 or laminin and harvested at timed intervals from 5-90 minutes. 12-LOX and the β4 subunit were co-immunoprecipitated from untransfected A431 cells (A431). β4 subunit was detected with mAb 450-11A.
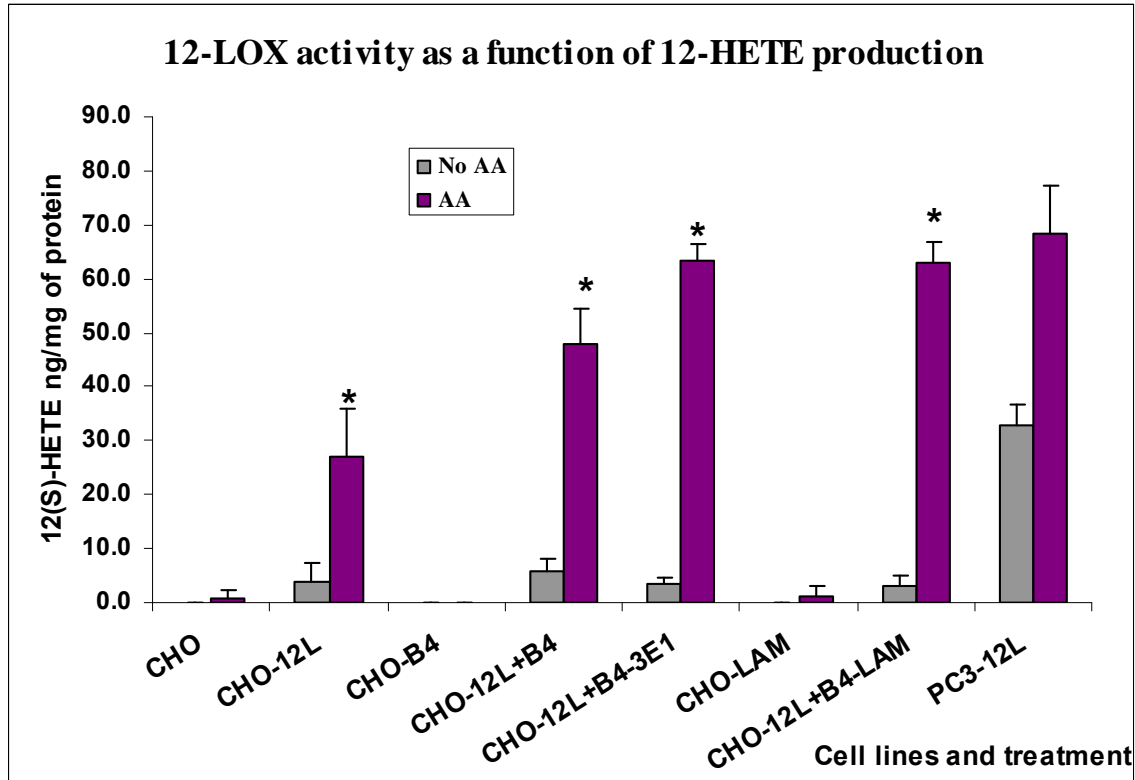
Figure 1.5: LC-MS analysis of 12-LOX products to determine 12-LOX activity. CHO transfectant cells were stimulated with mAb β4 or laminin for 30 minutes followed by treatment with 10 μM AA for 6 hour. Cell lipids were extracted and analyzed as described in Materials and Methods. The data show increased 12(S)-HETE production in cells co-transfected with 12-LOX and β4 that is enhanced when stimulated with laminin or 3E1. The data shown are the mean value (±SEM) from three experiments as represented in (A), error bars indicate SEM. Data is significant since $p < 0.05$ from student t test.
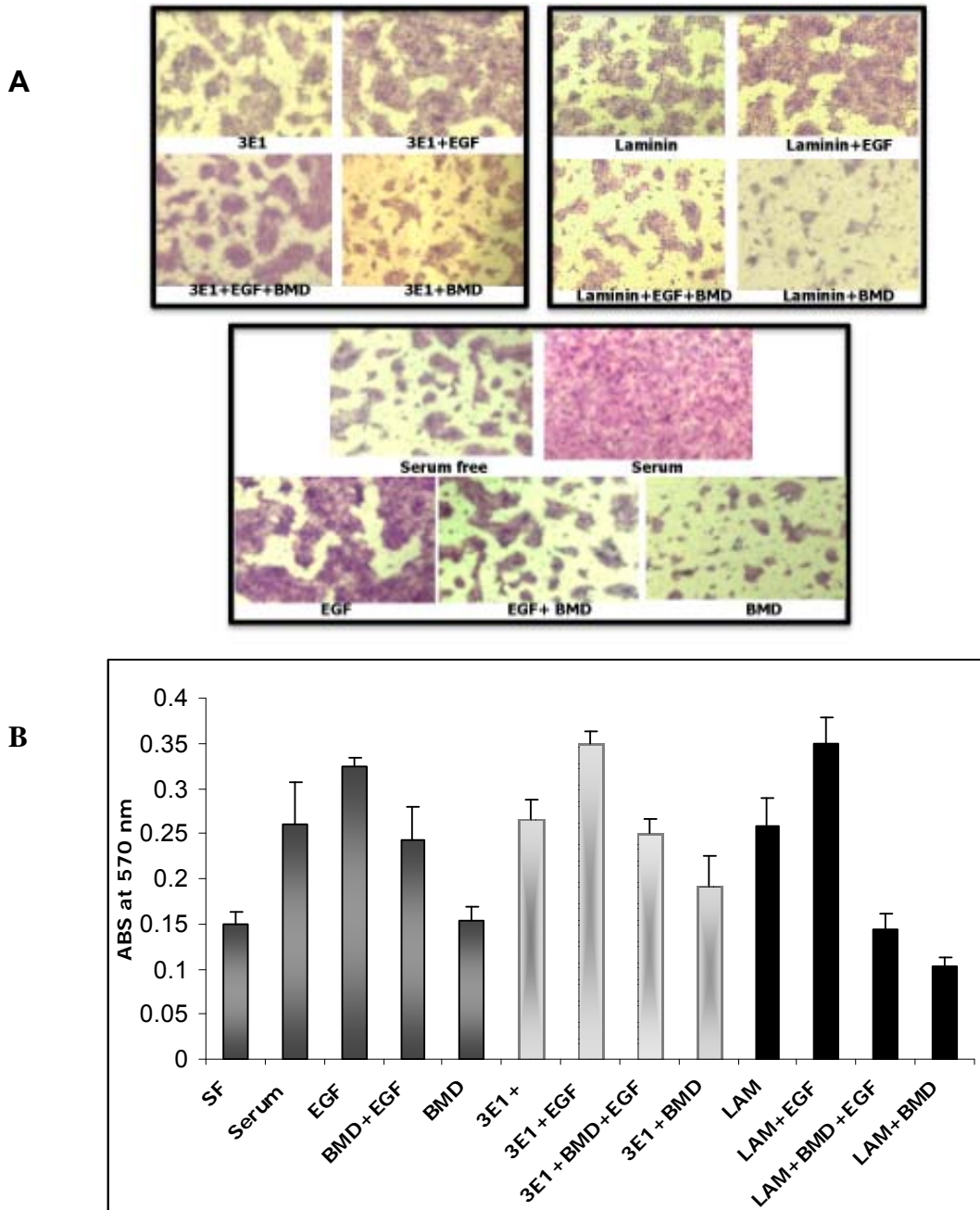
Figure 1.6: The α6β4 integrin and 12-LOX activity function in EGF-induced cell migration. (A) Comparison of cell migration of A431 cells in the presence of EGF chemoattractant when cells stimulated with either mAb 3E1 or laminin in the presence or absence of the 12-LOX specific inhibitor BMD122. (10X magnification).

(B) A431 cells were pretreated with 3E1 antibody for 20 minutes, or laminin with or without EGF (1 ng/ml) for 3 hours in the presence or absence of pharmacological inhibitors as described in Materials and Methods. Data represent the mean number of transmigrated cells/microscopic field ($\pm$ SE).

Figure 1.7

C

C'

D'

D
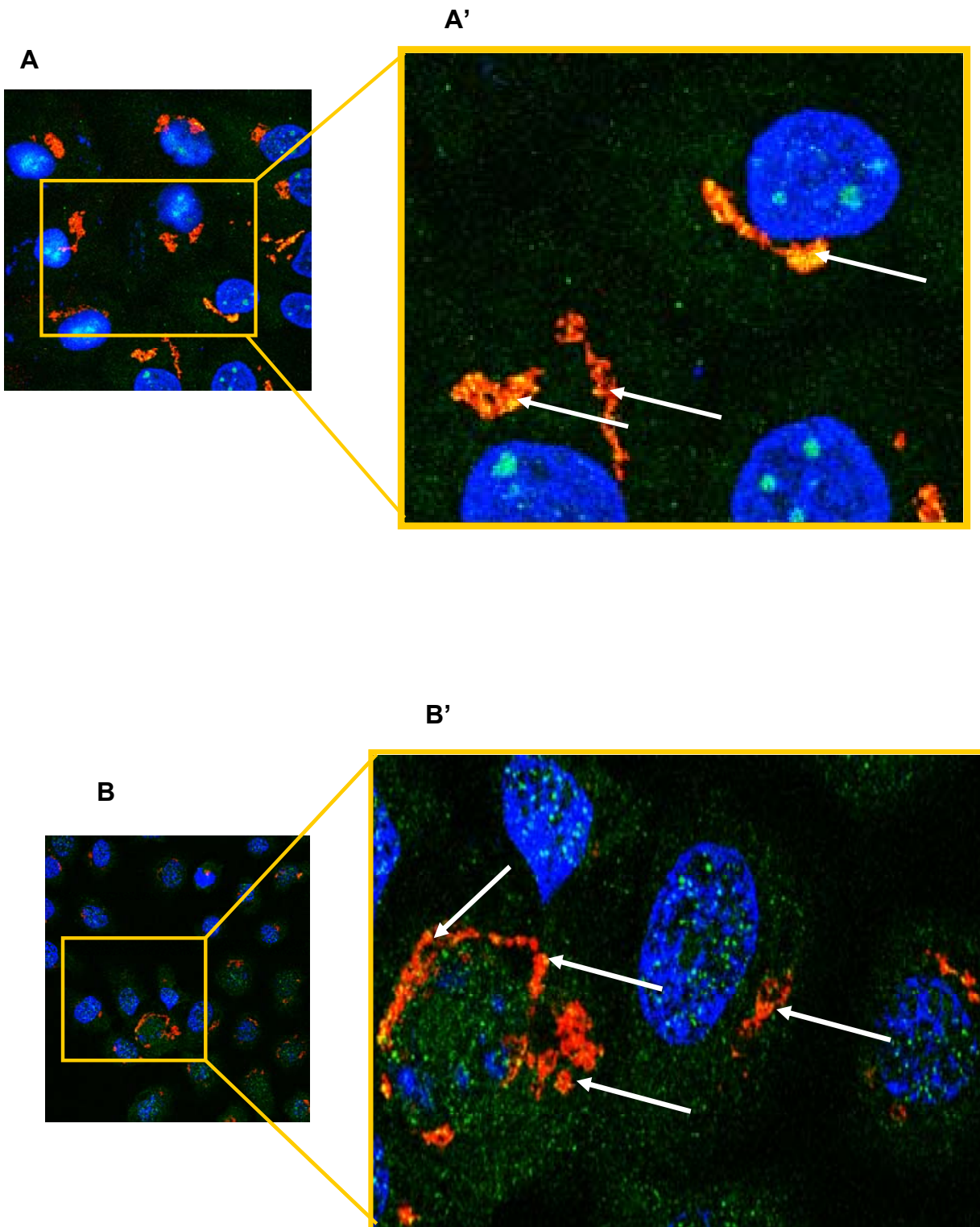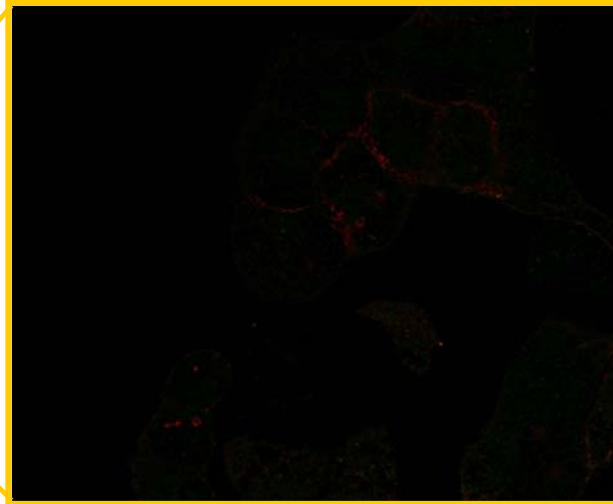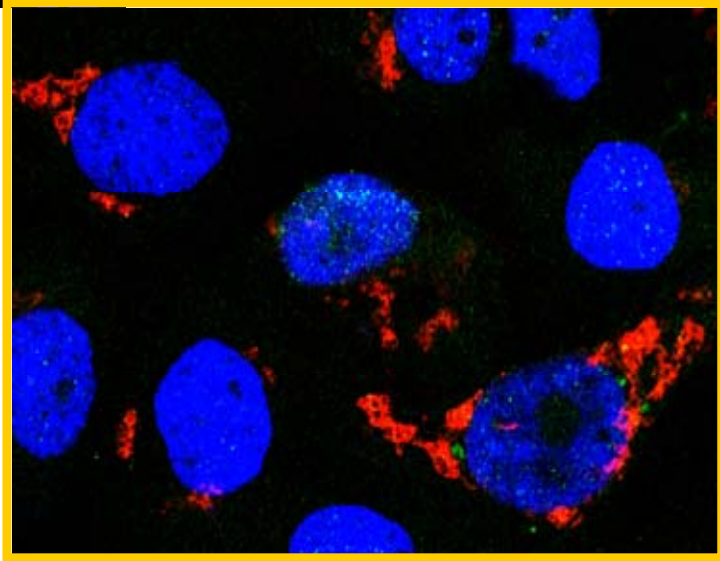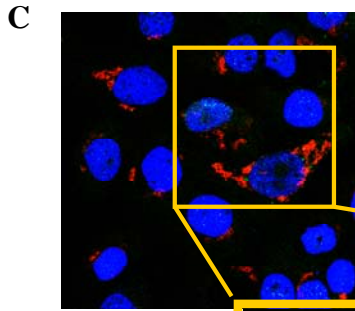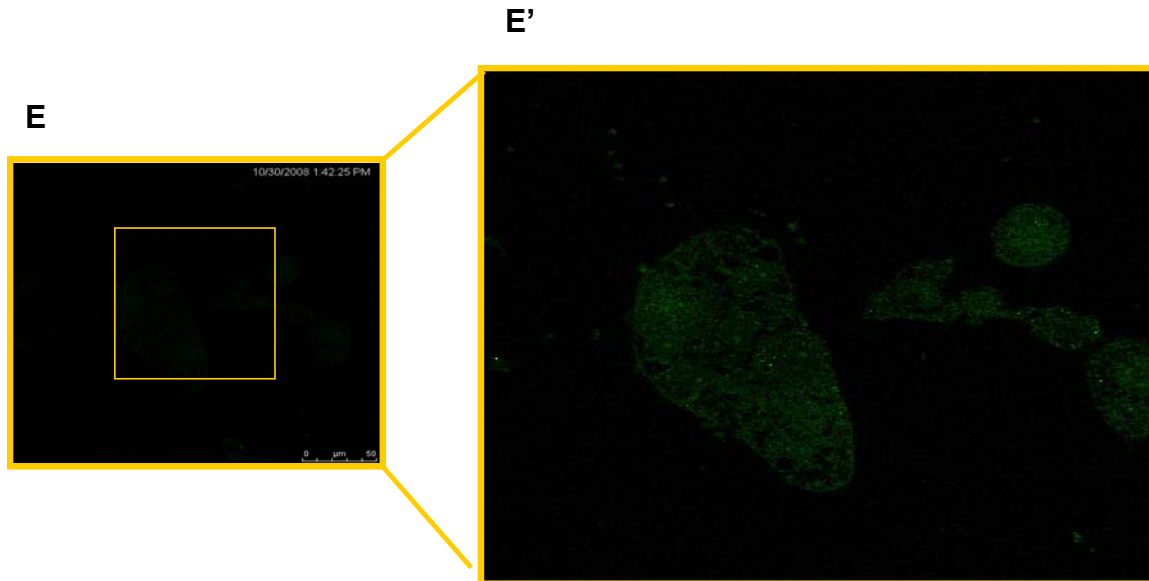
Figure 1.7: 12-LOX co-localizes with β4 by laser confocal immunofluorescence microscopy. A431 cells were treated, fixed, permeabilized, and sequentially incubated with primary antibodies 12-LOX (green) Oxford pAb and/or mouse anti-β4 (red) mAb 3E1 antibodies. Conjugated secondary antibodies Alexa$_{488}$ (green) or Alexa$_{594}$ (red) were used for detection: Yellow color indicates colocalization of 12-LOX (green) and integrin subunit β4 (red); (A) Laminin stimulated cells (B) 3E1 stimulated cells, (C) Unstimulated cells (D) & (E) Both secondary antibodies controls. A', B', C', D', and E' are magnified regions of interest.

1.14 Statement and Hypothesis

A yeast two-hybrid screen approach was used to identify the potential, direct regulators of 12-LOX. The study revealed four interacting partners including the cytoplasmic domain of the human β4 integrin subunit (Tang, Finley et al. 2000). This putative interaction was confirmed by P-12-LOX co-immunoprecipitation with the cytoplasmic domain of β4, and co-localization with β4 integrin as observed by confocal laser microscopy in mammalian cells (Tang et al., unpublished observation). β4 integrin upon stimulation with its natural ligand laminin, or β4-activating 3E1 (mAb), induced 12-LOX translocation from the cytosol to membrane. This provided the first candidate protein that may directly mediate 12-LOX translocation. Additionally, the association of 12-LOX with the β4 subunit up-regulates 12-LOX activity as demonstrated in the RP-HPLC and EIA data where 12(S)-HETE biosynthesis was increased following cell stimulation with β4 mAb 3E1. The increase in enzymatic activity was due to increased interaction of 12-LOX with the cytoplasmic domain of β4. The results demonstrated that the ligation of β4 increased resistance of A431 cells to apoptosis induced by BMD122, suggesting a relationship between β4 ligation, enhanced 12-LOX activity, and protection from apoptosis.

This association also promotes migration in the presence of EGF as a chemo-attractant, which is decreased in the presence of 12-LOX inhibitor. Thus it was demonstrated for the first time that 12-LOX interacts with the integrin β4 both physically and functionally, thereby providing a new insight for both integrin and

eicosanoid biology (Tang et al., unpublished observation). However, it remained to be established which residues or motifs in the cytoplasmic domain of β4 were critical for 12-LOX binding, and conversely, which domains or motifs of the 12-LOX protein are responsible for interaction with β4. In this study we describe for the first time the interaction domain of β4 integrin for 12-LOX. Determination of the structural basis for protein-protein interaction may provide a target for pharmaceutical intervention. This physical interaction has functional implications with regard to cell survival, cell migration and proliferation, and tumor growth. Blocking the 12-LOX β4 interaction will directly interfere with 12-LOX mediated signaling originating from β4 integrin or other proteins that function through this axis.

1.15.1 Aims

- Determine the structural basis for the interaction between β4 integrin subunit and 12-LOX.
- Determine how β4 regulates 12-LOX cellular localization and activity.
- Determine the functional implications with regard to cell survival, cell migration, proliferation, and tumor growth.

1.15.2 Hypothesis

Does interference with the point of interaction between the β4 integrin subunit and 12-LOX have functional consequences by reducing tumor cell survival, invasion and metastasis.
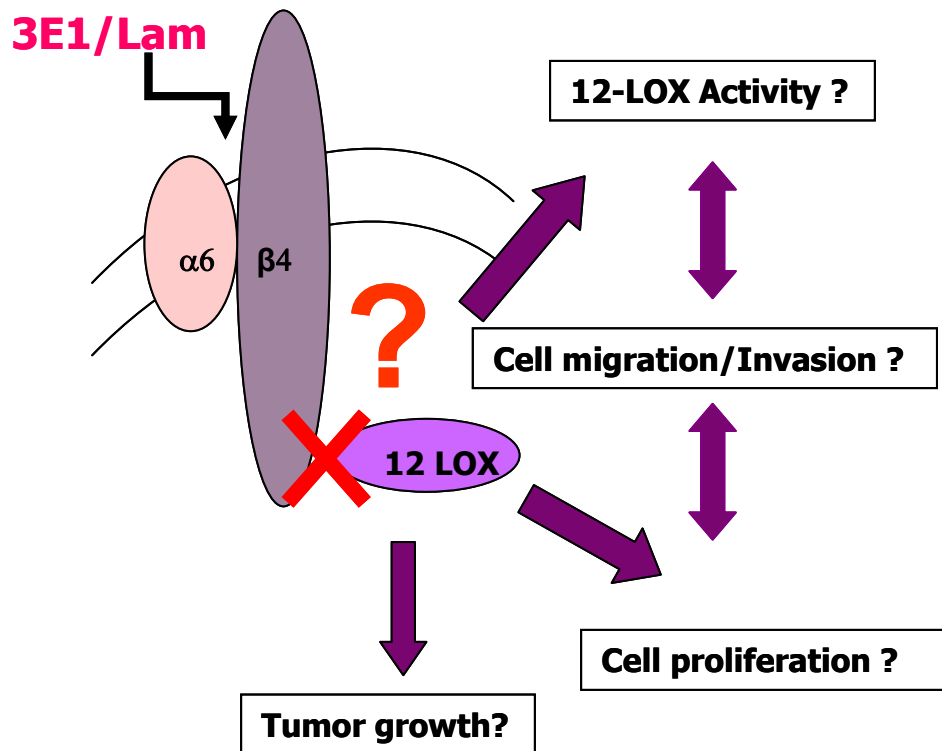
Figure 1.8:   Do 12-LOX and β4 integrin collaborate to regulate cell survival/ apoptosis, migration, proliferation, and tumor progression in carcinoma?

CHAPTER 2 : Materials and Methods

2.1   Antibodies and Reagents:

Polyclonal antibody 12-LOX that specifically recognize platelet type was obtained from Oxford Biomedical Research, (Oxford MI).   Anti-12-LOX 7225, clone 25.20 and 7261, clone 61.39 mAbs were obtained from American Diagnostica, Inc., (Stanford CT). A mAb specific for the extracellular domain of human β4 integrin subunit, clone 3E1, and an anti-human β1 pAb 1952 specific to the C-terminal cytoplasmic domain were obtained from Chemicon International, (Billerica, MA). The anti- human β4 integrin mAb (clone 450-11A), specific to the cytoplasmic domain, was obtained from BD Pharmingen, (San Diego, CA).   The actin antibody was from Affinity BioReagents, (Golden, CO).   Myc mAb was purchased from Millipore, (Billerica, MA).   Horseradish peroxidase-conjugated secondary anti-IgG (mouse and rabbit) were purchased from GE Healthcare, (Piscataway, NJ).   Internal standard 12(S)-HETE d8 and arachidonic acid were from Cayman Chemical Research Products, (Ann Arbor, MI).   Human laminin, EGF and proteosome inhibitor MG132 were purchased from Sigma Aldrich, (St. Louis, MO). BHPP (Biomide compound BMD122), was a generous gift from Biomide Corp, (Grosse Pointe Farms, MI), and Baicalein was purchased from Calbiochem, (San Diego, CA).

2.2 Primers:

A

FN1 deletion (ΔFN1)

FN2 deletion (ΔFN2)

B
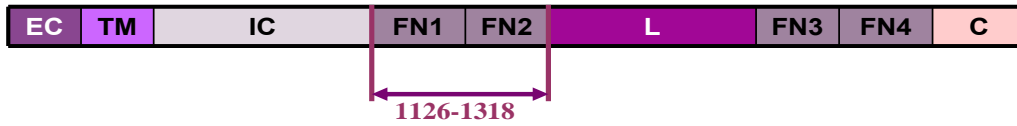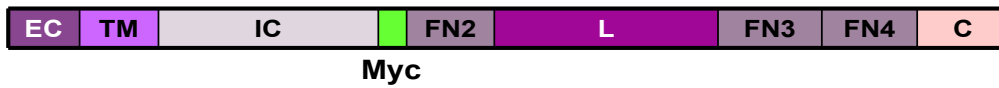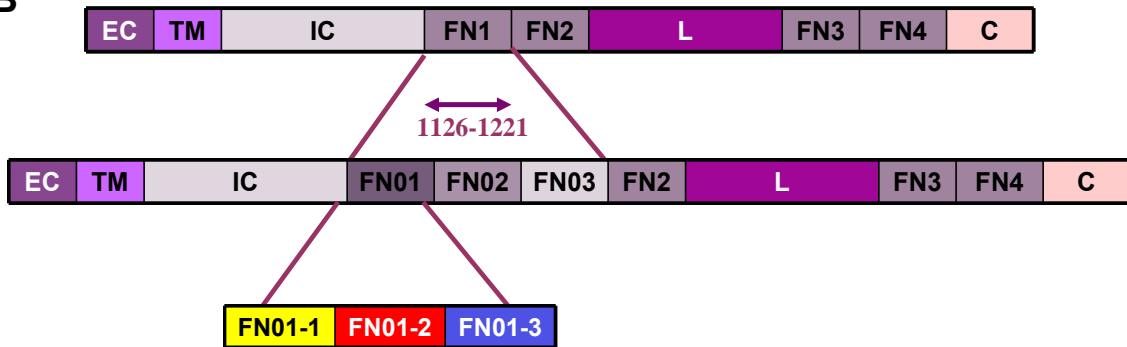
β4 FN1 and FN2 sequences (1126-1318)

FN1

S	Q	P	P	P	H	G	D	L	G	A	P	Q	N	P	N	A	K	A	A
G	S	R	K	I	H	F	N	W	L	P	P	S	G	K	P	M	G	Y	R
V	K	Y	W	I	Q	G	D	S	E	S	E	A	H	L	L	D	S	K	V
P	S	V	E	L	T	N	L	Y	P	Y	C	D	Y	E	M	K	V	C	A
Y	G	A	Q	G	E	G	P	Y	S	S	L	V	S	C	R	T	H	Q	E

FN2

V	P	S	E	P	G	R	L	A	F	N	V	V	S	S	T	V	T	Q	L
S	W	A	E	P	A	E	T	N	G	E	I	T	A	Y	E	V	C	Y	G
L	V	N	D	D	N	R	P	I	G	P	M	K	K	V	L	V	D	N	P
K	N	R	M	L	L	I	E	N	L	R	E	S	Q	P	Y	R	Y	T	V
K	A	R	N	G	A	G	W	G	P	E	R	E	A	I	I	N	L	A	T

Figure 2.1: Schematic design of areas deleted by primers

Primer Name	Primer
FN1 (+)	5'-CAGCCACCCCCTCACGGCGAACAAAAACTTATCAGTGAAGAA GATCTGCACCAG GAAGTGCCCAGC -3'
FN1 (-)	5'-GCTGGGCACTTCCTGGTGCAGATCTTCTTCACTGATAAGTTTTT GTTCGCCGTGAGGGGGTGGCTG -3'
FN2 (+)	5'- CGCACCCACCAGGAAGTGGAACAAAAACTTATCAGTGAAGA AGATCTGCAGCCCAAGAGGCCCATG -3'
FN2 (-)	5'- CATGGGCCTCTTGGGCTGCAGATCTTCTTCACTGATAAGTTT TTGTTCCACTTCCTGGTGGGTGCG -3'
FN01 (+)	5'-CAGCCACCCCCTCACGGCGAACAAAAACTTATCAGTGAAGAA GATCTGAGGGTAAAGTACTGGATT -3'
FN01 (-)	5'- AATCCAGTACTTTACCCTCAGATCTTCTTCACTGATAAGTTTTT GTTCGCCGTGAGGGGGTGGCTG -3'
FN02 (+)	5'- GGCAAGCCAATGGGGTACGAACAAAAACTTATCAGTGAAGAA GATCTGTGCGACTATGAGATGAAG -3'
FN02 (-)	5'- CTTCATCTCATAGTCGCACAGATCTTCTTCACTGATAAGTTTTT GTTCGTACCCCATTGGCTTGCC -3'
FN03 (+)	5'- ACCAACCTGTACCCGTATGAACAAAAACTTATCAGTGAAGAAG ATCTGGAGCCAGGGCGTCTGGCC -3'
FN03 (-)	5'- GGCCAGACGCCCTGGCTCCAGATCTTCTTCACTGATAAGTTTTT GTTCATACGGGTACAGGTTGGT -3'
FN01-1(+)	5'- CAG CCA CCC CCT CAC GGC GCC GCT GGG TCC AGG AAG -3'
FN01-1(-)	5'- CTT CCT GGA CCC AGC GGC GCC GTG AGG GGG TGG CTG -3'
FN01-2(+)	5'- CAG AAC CCC AAT GCT AAG CTG CCC CCT TCT GGC AAG -3'
FN01-2(-)	5'- CTT GCC AGA AGG GGG CAG CTT AGC ATT GGG GTT CTG -3'
FN01-3(+)	5'- AAG ATC CAT TTC AAC TGG AGG GTA AAG TAC TGG ATT -3'
FN01-3(-)	5'- AAT CCA GTA CTT TAC CCT CCA GTT GAA ATG GAT CTT -3'

Table 2-1 List of primers used to generate β4 deletion mutants of FN1 and FN2, the three sub-domains of FN1 and finally the three micro-domains of FN01, namely FN01-1, FN01-2 and FN01-3.

2.3 Cell Culture:

Cell lines A431 (human epithelial carcinoma) and CHO (Chinese hamster ovary) were obtained from ATCC, (Manassas, VA) and grown in RPMI containing 10% FBS. CHO cells were transfected with 12-LOX in combination with either β4 full-length or β4 deletion mutants using lipid-based Mirus, (Mirus Bio; Madison, WI). A431 cells were transfected with β4 mutants using Geneporter, (AMS Biotechnology Ltd.; Lake Forest, CA) and also lipofectamine LTX (Invitrogen Inc.; Carlsbad, CA). For treatment with β4-stimulating antibody 3E1, A431 cells were grown to 90% confluence in 100 mm petri dishes and serum-starved overnight prior to experimental use. Cells were then washed with PBS (3x) and incubated with β4 stimulating antibody for 30 minutes at a concentration of 5 µg/ml in serum-free RPMI medium. Cells were harvested at defined time intervals for further examination.

2.4 Co-immunoprecipitation:

 Cells were washed with 1X PBS (3x) and placed in cold lysis buffer (1% Triton X-100; 150 mM NaCl; 10 mM Tris, pH 7.4; 1 mM EDTA; 1 mM EGTA, pH8.0; 0.2 mM sodium ortho-vanadate; 0.2 mM PMSF; 0.01% aprotinin; 5 µg/ml leupeptin; 0.5% NP-40). The cell lysate was sonicated and clarified by centrifugation (10,000 x g; 10 minutes). Supernatants were pre-cleared with 40 µl Sepharose 4B conjugated protein G beads at 4°C for 1 hour followed by immunoprecipitation with 4 µl of antibody (1 µg/µl) to human P-12-LOX or the β4 integrin subunit 4°C for 3 hours with rotation. Immunoprecipitate was pulled down with 40 µl

Sepharose 4B conjugated protein G beads at 4°C overnight with rotation. Immune-complexes were washed with PBS buffer (3x), and beads were resuspended in SDS sample buffer (85 mM Tris-HCL, pH6.8 containing 1.4 (w/v) glycerol, 5% (v/v) mercaptoethanol, and a trace of bromophenol blue), boiled for 5 minutes, and subjected to SDS-PAGE on 10% or 15% acrylamide gel. Gels were run at 4°C at 130V. Proteins were electrophoretically transferred to nitrocellulose membranes at 4°C for 1 hour and 15 minutes at constant 0.4A. After transfer, nonspecific sites were blocked with 5% (w/v) nonfat-dry milk in TBST (0.1% Tween-20; 20 mM Tris base; 137 mM NaCl; 3.8 mM HCl, pH 7.6) for 1 hour at 25°C followed by primary antibody overnight at 4°C. After washing the blot in TBST (3X), the membranes were incubated (1 hour; 25°C) with horseradish peroxidase-conjugated secondary anti-IgG (dilution: 1:4500, GE Healthcare, Piscataway, NJ). The blot was washed again in TBS-T, and developed using ECL according to the manufacturer's instruction (GE Healthcare, Piscataway, NJ).

2.5   Western Blotting:

Immune complexes or aliquots of total cell lysates were mixed with 1 volume of SDS sample buffer (85 mM Tris-HCL, pH6.8 containing 1.4 (w/v) glycerol, 5% (v/v) mercaptoethanol, and a trace of bromophenol blue). Whole cell lysates in SDS sample buffer, boiled for 5 minutes, and loaded at 30-60 µg of protein, and separated by SDS-PAGE on a 10% or 15% acrylamide gel. The gels were run at 4°C at 130V and electrophoretically transferred to nitrocellulose membranes at 4°C for 1 hour and 15 minutes with constant 0.4A. After transfer, nonspecific

sites were blocked with 5% (w/v) nonfat-dry milk in TBST (0.1% Tween-20; 20 mM Tris base; 137 mM NaCl; 3.8 mM HCl, pH 7.6) for 1 hour at 25°C followed by primary antibody overnight at 4°C. After washing the blot in TBST (3X), the membranes were incubated (1 hour; 25°C) with horseradish peroxidase-conjugated secondary anti-IgG (dilution: 1:4500, GE Healthcare, Piscataway, NJ). The blot was washed again in TBS-T, and developed using ECL according to the manufacturer's instruction (GE Healthcare, Piscataway, NJ).

2.6   12-LOX Activity Measurement:

LC-MS was used to determine the enzymatic activity of 12-LOX by measuring 12(S)-HETE production in relation to an internal standard.   Transiently transfected CHO cells or A431 stable cell lines expressing the GR16 β4 fragment or empty vector were serum-starved overnight.   Prior to serum starvation 'conditioned' medium was harvested directly as a control. Subsequently, cells were treated with laminin or the β4-stimulating antibody 3E1 for 1 hour followed by a 6 hour treatment with 10 μM AA. When used, inhibitors such as BMD122 were given prior to stimulation with 3E1 antibody.   Treatment with 10 μM BMD122 was performed for 2 hours in serum-free medium. After treatment the media was collected and centrifuged, and the resulting supernatants (cell lipids) were extracted. An internal standard, 10 ng of 12(S)-HETE-d8, was added to the samples and then applied to ODS-silica cartridges. Solid phase extraction for mass spectrometry determination of 12-(S) HETE was performed as previously described by Suzuki et al (Suzuki, Hishinuma et al. 2003). The samples were

passed through the mobilized columns (methanol treated) and eluted from the cartridges with Hexane: ethyl-acetate solution (1:3). Solvent was evaporated under a stream of nitrogen gas and lipids were reconstituted in methanol. Prior to LC-MS analysis, 50 mM of ammonium acetate before was added to each sample. Data shown are significant where $p < 0.05$ by student's t test.

2.7   Expression Constructs and Transfection:

A panel of bacterial expression plasmids that yield recombinant protein in *E.coli* and mammalian expression constructs that express various mutant forms of the β4 cytoplasmic tails were generously provided by Dr. Gerhard Wiche (Rezniczek, de Pereda et al. 1998). Stable cell lines were generated that express the β4 variants as fusion proteins with a GFP reporter. Expression constructs encoding wild-type and truncated human β4 subunits in the pRC-CMV vector were generous gifts from Dr. Filippo Giancotti (Giancotti, Stepp et al. 1992). Briefly, the pRC-CMV vector contains either the full length β4 subunit cDNA, β4 Δ FN1 (aa 1126-1215), or β4 Δ FN2 (aa 1220-1318). Additional constructs with macro deletions, pCMV-β4 Δ FN01 (aa 1126-1157), pCMV-β4 Δ FN02 (aa 1158-1189), and pCMV-β4 ΔFN03 (1190-1221), are reported in Table-2.1. These constructs were Myc tagged so as to differentiate them from full length β4, and detected using anti- Myc antibody. Though the deletion mutants could also be detected using anti- β4 mAb, the Myc tag was opted for making stable A431 transfectants. A431 cells contain endogenous full length β4 and transfecting them with the β4 fragments with Myc tag makes it easier to differentiate them from full length β4.

Thus, Myc tag was used to design all of the β4 deletion mutants. Fine mapping was done by making micro deletions within this FN01 macro domain; pCMV-β4 Δ FN01-1 (aa 1126-1136), pCMV-β4 Δ FN01-2 (aa 1137-1147), and pCMV-β4 ΔFN01-3 (aa 1148-1157). The full length cDNA encoding human P-12-LOX (pCMV-P-12-LOX) was provided by Dr. Colin D. Funk (Queen's University, Kingston Ontario, CA). This cDNA was subsequently subcloned into the EcoRI/ XbaI sites of pcDNA3.1 (Invitrogen). Stable A431 transfectants were generated expressing Myc tagged β4 fragment constructs pRC-CMV-GR16 (200 aa) and pRC-CMV-FN01-02 (10 aa). Ampicillin was used for selection of all bacterial expression vectors (50 µg /ml). Transfectants were selected with 350 µg/ ml G418 (Sigma Aldrich; St. Louis, MO).

2.8 Immunofluorescence:

A431 cells were grown to 90% confluency on glass cover slips and serum-starved overnight followed by stimulation with either 5 µg/ml laminin (Sigma Aldrich) for 2 hour, or 5 µg/ml mAb 3E1 to β4 integrin (Chemicon) for 1 hour. Cover slips were washed with PBS (3x) and fixed with 3.7% formaldehyde in PBS for 5 minutes at $37^{o}C$, followed by ice cold methanol fixation for 3 minutes. Cells were then permeabilized in an antibody dilution buffer (ADB: Tris-buffered saline pH 7.4 containing 0.1% Triton X-100 and 2% BSA) for 10 minutes. Primary (1:100) and secondary antibodies (1:500) were diluted in ABD. Cells were incubated first with primary antibody (rabbit anti-human 12-LOX Ab) for 1 hour at room temperature (RT), followed by (3x) PBS washes. Later they were

incubated for 30 minutes with secondary fluorescent antibody (Alexa$_{488}$-conjugated goat anti-rabbit antibody) and washed with PBS (3x). Further they were incubated with mouse anti-human β4 integrin antibody for 1 hour at RT with subsequent 3x PBS washes, and 30 minutes Alexa$_{594}$-conjugated goat anti-mouse antibody followed by (3x) PBS washes. Nuclei were visualized by incubating for 10 minutes at RT with DAPI (4', 6-diamidino-2-phenylindole) followed by (3X) washes. Cover slips were mounted in Mowiol with 0.5% phenylenediamine (anti-fade reagent). (http://www3.niaid.nih.gov/labs/aboutlabs/rtb/biologicalImaging/protocols/). The slides were observed with a Leica TCS SP5 laser scanning confocal microscope.

2.9   Cell Invasion and Migration Assay:

 Cell migration and invasion assays were carried out in modified Boyden chambers using 24 well cell culture plates fitted with 8-um transwell inserts (BD Falcon). The insert membranes were coated with 100 μl of 250 μg/ml Matrigel, (BD Pharmingen) for 2 hour at 37°C. Stable transfectants expressing β4 mutants or harboring an empty vector were trypsinized and washed using serum free medium, and 5×10^5 cells were added to the inserts. When used, inhibitor BMD122 (25 μM) was added prior to 3E1 stimulation for 1 hour. Cells were treated with 3E1 antibody (3 μg) for 1 hour. Medium containing 0.3% serum was added to the lower chamber. Following 24 hours, cells that migrated to the other side of the membrane were stained using the "Diff Quick" staining kit (IMEB Inc., San Marcos, CA) and photographed. Quantitative analysis was performed by

dissolving the cells and membrane in 10% acetic acid and measuring the absorbance at 570nm. Data are taken as significant where $p< 0.05$ by student t test.

2.10 Cell Proliferation Assay:

Cell proliferation was measured using the MTS-PMS colorimetric assay that produces a colored formazan product in the presence of phenazine methosulphate that absorbs at 470 nm. Wild type and stably transfected A431 cells were trypsinized, neutralized, and counted. Approximately $2x10^3$ cells were seeded in quadruplicate in 8 different 96 well plates to cover from day 0 to day 7. Plates from each daily time point were washed (2x) with HBSS solution. Cells were subsequently incubated with a mixture of 100 μl of phenol red free RPMI and 20 μl of MTS-PMS reagent for 2 hour at 37°C. The absorbance was measured at 470nm. Data taken are significant where $p< 0.05$ by student t test.

2.11 Colony Formation Assay:

$1.5x10^3$ cells were seeded in 60 mm petri-dishes in triplicate. Medium was changed every 3 days. Colony formation was visualized and the assay was terminated before the edges of colonies came into contact. Cells were washed (3x) with 1X PBS, followed by fixation with 3.7% formalin for 15 minutes. Fixed cells were stained with 0.5% crystal violet for 30 minutes, washed with distilled water to remove all extra stain, and air dried. The colonies were visualized under the microscope and counted. Quantitative measurements were obtained

by measuring dye extracted from cells with 10% acetic acid and comparing absorbance measurements at 570 nm.

2.12 Cell Adhesion Assay:

96-well plates were coated with laminin at $37^{o}C$ for 1 hour and were washed twice with washing buffer. Uncoated wells served as negative controls. Plates were blocked with blocking buffer at $37^{o}C$ in 5% CO_2 for 45-60 minutes, and subsequently washed with washing buffer and chilled on ice. $5x10^{4}$/ml cells in 100 µl were added to each well, and Incubated in 5% CO_2 at $37^{o}C$ for 30 minutes. Unbound cells were removed by shaking the plate at 2000 rpm for 10-15 seconds, followed by 2-3 washes with washing buffer. Adherent cells were fixed with 4% paraformaldehyde at RT for 10-15 minutes, washed with washing buffer, stained with Crystal Violet for 10 minutes, and finally washed with water. Plates were turned upside down and air dried completely.   Cells were incubated in 10% Acetic acid at RT for 30 minutes and the extracted dye was quantified at an absorbance of 570 nm. Data taken are significant as $p< 0.05$ by student's t test.

2.13 Soft Agar Colony Formation:

Soft agar colony formation assays were performed in 60 mm plates, coated with a bottom layer of 1% agar in 2X RPMI medium. The upper layer was prepared by mixing 1×10^{3} A431 transfectant cells with 0.8% agarose in 2X RPMI-10% FBS medium. Liquid RPMI-10% FBS medium was added on top of the agar layers

and replaced weekly. The assay was performed in triplicate. Plates were stained with 0.5% crystal violet solution, and colonies were counted and photographed.

2.14 Animal Study:

Athymic nude mice (Athymic nude-Foxn1^{nu} nude, 5–6 weeks old) were obtained from Harlan Laboratories, (Haslett, MI), and housed in micro-isolator cages. IACUC approval was obtained for all experiments. The animals involved were provided care in accordance with Wayne State University's Animal Investigation Committee (AIC) guidelines. The animals were allowed to adapt for 1 week under sterile conditions. Xenografts were established by injecting $3{\times}10^6$ exponentially growing A431-GR16 or A431-EV stable cells in 100 µl HBSS (Hanks balanced salt solution) subcutaneously with no Matrigel. The tumor size was measured by vernier caliper every three days, and the tumor volume was computed and reported in cc from the formula for an ellipsoid body (volume = length × breadth × height × π /6), where for small tumors, height (= thickness) was assumed to correspond to the smaller of the two diameters. At the end of the experiment, mice were sacrificed, and tumors excised, shock-frozen in liquid N_2, and stored at –80°C for further analysis.

2.15 Tissue Analysis- RNA and Protein Isolation:

Fresh tissue was homogenized using MagNA Lyser (Roche Applied Science) according to the protocol consisting of 2 pulses for 29 seconds at 3000 rpm separated by 90 seconds of cooling. Total RNA was extracted from the frozen

tissues using Nucleospin RNAII isolation kit, according to the manufacturer's instructions. One microgram of total RNA was reverse-transcribed to a single strand cDNA in a final volume of 20 μL in two steps according to the manufacturer's instructions (Applied Biosystems). In the first step, a pre-mix with RNA, random primers, dNTPs, and water were denatured at 70°C for 5 minutes. In the second step, SuperScript™ III Reverse Transcriptase (Cat. No. 18080-093, Invitrogen), buffer, DTT, and RNase inhibitor were added and left for 5 minutes at room temperature, followed by heating for 60 minutes at 55°C, 15 minutes at 70°C, and 5 minutes at 95°C. All reagents were obtained from Invitrogen.

Relative mRNA quantification was performed using a fluorescence-based real-time detection method with Applied Biosystems 7500 real time Fast Sequence Detection System. Oligonucleotide primers and probes for VEGF, BAX, Bcl2, HIF-1 and *β*-actin gene were purchased from Applied Biosystems. A 12-μL reaction mixture containing 2.5 μL of cDNA template, 6.25 μL TaqMan Universal PCR master mix (Cat. No. 4326708, Applied Biosystems), and 0.62 μL primer probe mixture was amplified using the following thermal cycler parameters: incubation at 50°C for 2 minutes and denaturation at 95°C for 10 minutes, followed by 40 cycles of the amplification step (denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute). All amplification reactions were performed in triplicates. The *β*-actin gene was used as endogenous control. Data taken are significant; $p < 0.05$ by student's t test.

CHAPTER 3 : Results

3.1 Domain mapping of the cytoplasmic tail of β4 that interacts with 12-LOX in tumor cells

Our initial studies demonstrated by yeast two-hybrid screen that 12-LOX interacts with the C-terminus of β4 integrin (Tang, Finley et al. 2000). Furthermore, we showed that 12-LOX translocates to the cell membrane on β4 stimulation with laminin or 3E1 (β4 stimulating antibody) where its interaction with β4 leads to increased 12-LOX activity and 12(S)-HETE production (Tang et al., unpublished observation). Therefore we sought to determine the structural basis for the functional interaction between the integrin β4 subunit and 12-LOX. The cytoplasmic tail of the β4 integrin subunit (~1,007 aa) is uniquely large among known β4 subunits (~50 aa) (Giancotti and Tarone 2003). It includes two pairs of fibronectin type III (FNIII) domains, separated by a 143-amino acid linker segment (Hogervorst, Kuikman et al. 1990; Suzuki and Naitoh 1990). The first pair of FNIII repeats, FN1 and FN2, spans aa residues 1126 – 1315 of the β4 cytoplasmic tail. Using a panel of bacterial expression plasmids that yield recombinant protein in E.coli and mammalian expression constructs that express various mutant forms of the β4 cytoplasmic tail for the interaction study (Figure 3.1), we were able to delineate the β4 domain necessary for 12-LOX interaction. We chose to start the analysis using pGR38 (L), pGR39 (F3, F4, C), pGR40 (F1, F2, L, F3), pGR45 (F1, F2, L), pGR46(TM, F2), pGR47(TM, C) as these GFP fusion constructs contain overlapping fragments of the cytoplasmic tail of β4

integrin. Utilizing fluorescence activated cell sorting (FACS), a pool of GFP-positive cells were selected.   We confirmed the expression of β4 mutants as GFP-fusion proteins by Western blot and then performed co-immunoprecipitation on whole cell lysates to determine the interaction of 12-LOX with various β4 mutants. As shown in Figure 3.1A, 12-LOX is able to bind to fusion proteins, expressed by pGR40 (Tx40), pGR45 (Tx45), pGR46 (Tx46), and pGR47 (Tx47), but not to the fusion protein with only the linker region, pGR38 (Tx38). Weak 12-LOX binding to the fusion protein encoded by pGR39 (Tx39) was observed. All the above mutants that show strong binding have 22 residues (aa 1126-1315) in common, which suggested the presence of a strong binding site(s) for 12-LOX, located in the region spanning these residues 1126 – 1315 of the β4 cytoplasmic tail.

3.2   Identification of integrin β4 macro domains important for 12-LOX interaction

Integrin α6β4 exists on the cell surface and forms part of a large complex containing numerous growth factor receptors, tetraspanins and other molecules that influence the approach to study this integrin. The minimum functional unit for targeting β4 into hemidesmosomes and controlling the cellular distribution of plectin is contained within the first two type III fibronectin domains (FN1 and FN2) plus the 1st 37 residues of the connecting segment. Therefore this region may have potential binding motifs that contribute either directly or indirectly to binding its target proteins (Litjens, de Pereda et al. 2006). After we identified the region spanning FN1-FN2 (Figure 3.1A) within β4 as containing a putative 12-LOX-

interaction domain, we began assessing sub-domains of this region (Figure 3.2A).   The expression construct pRC-CMV, a generous gift from Dr. Filippo Giancotti (Memorial Sloan-Kettering Cancer Center, NY), contains the full-length β4 subunit cDNA (Giancotti, Stepp et al. 1992) and was used to make deletions in the region we identified, namely aa 1126 – 1315 of the β4 cytoplasmic tail. These also received a myc-tag insertion (Figure 3.2A and 3.2B).   The Myc tag was introduced so as to differentiate between β4 deletion mutants and full length β4 integrin. We have confirmed that there is no interference of this Myc tag with binding to 12-LOX (data not shown).   Using these deletion mutant co-immunoprecipitation studies were performed to identify the structural determinants responsible for the physical interaction between 12-LOX and β4 integrin. CHO cells were co-transfected with β4 deletion mutants in combination with 12-LOX and the expression of both proteins was confirmed by Western blot using Myc and 12-LOX antibody (Figure 3.2D).   The co-immunoprecipitation results revealed that 12-LOX interacts with the FN1 domain in β4, as binding was unaffected by the absence of FN2 (ΔFN2-β4) (Figure 3.2C).   However, in the absence of FN1, binding was markedly attenuated (ΔFN1-β4) (Figure 3.2C and 3.3D). Also the treatment with β4 stimulating antibody (3E1) showed no effect on the interaction. The mass spectrometry results show an increase in 12(S)-HETE production in cells expressing the ΔFN2-β4 that retains FN1 domain while 12(S)-HETE production was attenuated in the absence of the FN1 domain (ΔFN1-β4) (Figure 3.2E). Data shown is statistically significant,∗ $p < 0.05$ by student t test when comparing HETE production of 12L+ΔFN1 in presence of AA with

12L+ΔFN2 and 12L+ β4. While it shows no significance comparing 12L+ΔFN2 and 12L+ β4. Thus, from the above results we conclude that β4-FN1 is crucial for β4 integrin interaction with 12-LOX.

3.3 Micro-domain mapping of integrin β4-FN1 region necessary for 12-LOX interaction

After determining that the FN1 domain (aa 1126-1215) of the cytoplasmic tail of β4 encodes a 12-LOX binding element, we generated Myc-tagged constructs with successive micro deletions (32 aa each) within this putative FN1 domain (Figure 3.3A). Primers were designed accordingly and β4 deletion constructs (ΔFN01-β4, ΔFN02-β4 and ΔFN03-β4) were generated (Figure 3.3A). These expression constructs were then co-transfected in CHO cells along with 12-LOX vectors. Protein expression from the constructs was confirmed by detection with Myc mAb (Figure 3.3C) as well as 12-LOX. Subsequently co-immunoprecipitation results showed that when the FN01 domain is deleted, binding with 12-LOX is hindered (Figure 3.3B). Over-expression of the proteins led to immunoprecitation that was independent of the 3E1 stimulus but specific to the constructs. Thus we confirmed that this 32 amino acid region between residues 1126-1157, is critical for β4 interaction with 12-LOX.

3.4 Fine-mapping of integrin β4-FN01 domain

Fine mapping was performed within the FN01 (1126-1157, 32 aa residue) domain to identify the residues relevant for 12-LOX -β4 protein interaction. The

FN01 region was divided further into three parts and deletion mutants were generated. The β4 deletions [ΔFN01-**1**, (aa 1126—1136); ΔFN01-**2**, (aa 1137—1147); and ΔFN01-**3**, (aa 1148—1157)] constructs were co-transfected with 12-LOX in CHO cells and their respective expression was confirmed by Western blot. Expression of β4 micro-domain deletion mutants and also full length β4 were detected with β4-450-11A antibody specific for the cytoplasmic domain of integrin β4, and were reprobed with 12-LOX pAb and actin mAb for actin loading control (Figure 3.4B). Co-immunoprecitation studies were performed using the whole cell lysates of these transfectants (Figure 3.4C). 12-LOX antibody was used to pull down 12-LOX and the immunoblots were detected with β4 450-11A mAb. The result revealed weak interaction of integrin β4 with 12-LOX in absence of the FN01-2 micro-domain (Figure 3.4C). Stimulation of β4 with 3E1 did not affect the interaction. Thus, we have identified the 10 amino acid region (aa 1137-1147) within integrin β4 that is required for interaction and activation of the 12-LOX enzyme.

3.5 Mutations of important phosphorylation sites within the FN01 sub domain affect the interaction of full length β4 with 12-LOX

A predictive algorithm was used to locate the putative phosphorylation sites S1140, S1151, and Y1157 within the FN01 sub domain of full length β4 (Figure 3.5A). Of these three, S1140 is within the region of interest that is important for 12-LOX interaction. Single point mutations were made in these phosphorylation

sites by changing serine amino acids to alanine, and tyrosine to phenylalanine. In addition, we also mutated Y1494 as it has been shown to be a major regulatory signaling site and also plays an important role in promoting cancer progression (Dutta and Shaw 2008). The mutations were confirmed by sequencing and these β4 phosphorylation mutant constructs were co-transfected with 12-LOX into CHO cells. Western blots whole cell lysates were probed with β4 mAb 450-11A that is specific for the cytoplasmic domain of integrin β4. Blots were successfully reprobed with 12-LOX and actin (Figure 3.5B). Immunoprecitation of 12-LOX was performed on cell lysates from these transfectants and co-immunoprecipitated β4 was again detected with 450-11A mAb. The results show that the S1140A mutation in β4 weakens the interaction with 12-LOX, while Y1157F and Y1494F markedly reduce the interaction compared to wild-type β4 binding (Figure 3.5C, lane 6). From the above results we incur that S1140, Y1157 and Y1494 are important phosphorylation sites that impact the interaction of full length β4 with 12-LOX.

3.6   12-LOX phosphorylation is important for activity but not interaction

In previous studies we have shown that 12-LOX and β4 association is functionally relevant and results in an increase in 12-LOX enzymatic activity as measured by HPLC where 12(S)-HETE biosynthesis was increased following cell stimulation with β4 mAb 3E1 (Tang et al., unpublished observation). We have also shown that the cytoplasmic domain of β4 integrin can interact specifically

with phosphorylated Src (pSrc) after β4 integrin ligation in A431 cells. The ligation of β4 integrin induces Src activation, which resulted in tyrosine phosphorylation of 12-LOX (Dilly et al., to be published). Here we show that treatment with Src inhibitor PP2 (5 μM) leads to decreased 12-LOX activity as measured by 12(S)-HETE production. CHO transfectant cells were stimulated with 3E1 stimulating antibody followed by treatment with PP2 inhibitor concentration of (5 μM) for 1 hour. Arachidonic acid (10 μM) substrate is added and incubated for 6 hours. Medium was analyzed for 12(S)-HETE by using the LC/MS (Figure 3.6). Thus, the interaction between 12-LOX and β4 is independent of 3E1 stimulus in CHO cells but activity is dependent on 3E1 stimulus, which leads to phosphorylation of 12-LOX via Src. Data shown above is significant, as,*, $p < 0.05$ by student t test when comparing 12L+β4-PP2 with 12L+β4 and also the 3E1 treated set of 12L+β4 with 12L+β4-PP2.

3.7 Ectopic expression of GR16 (FN1 and FN2 region) blocks full length β4 and 12-LOX interaction

The A431 human epidermoid carcinoma cell line endogenously expresses 12-LOX and β4. Therefore, we used A431 to perform a competition assay using the plasmid construct pGR16 that encodes the Myc-tagged FN1 and FN2 portion (residues 1126-1315) of the full length β4 integrin (Figure 3.1). In addition to 12-LOX and integrin β4, the expression of GR16 (FN1 and FN2), in A431 transfectants was confirmed by Western blot (Figure 3.7A). Myc antibody was used to detect the GR16 fragment of β4 integrin (23 kDa) and 12-LOX pAb for

12-LOX (78 kDa) and β4 -450-11A for full length β4 integrin (β4 ~200 kDa). Actin mAb was used for actin loading control. A431 (pGR16) cell lysates were immunoprecipitated with a polyclonal antibody against 12-LOX and immunoblotted for, the Myc tag of GR16 and also full length integrin β4. As shown in Figure 3.7A, when ectopically expressed in A431 cells, the GR16 region (FN1and FN2) interacts with 12-LOX competitively inhibiting the 12-LOX-β4 interaction (Figure 3.7B, lane2). The lack of detectable interaction between 12-LOX and full-length β4 integrin is not due to any change in β4 expression (Figure 3.7A), but is due to the competition by the GR16 region (FN1 and FN2) (Figure 3.7B). Endogenous levels of Myc (115 kDa, faint band) are not detectable in Western blots since the signal for β4- Myc construct is very strong. Thus, the results suggest that the β4 FN1-FN2 region, encoded by pGR16, is able to block the interaction between 12-LOX and full-length β4 integrin in a dominant negative manner.

3.8   Ectopic expression of GRFN01-2 (1137-1147) competes with GR16 (1126-1315) or full length β4 and blocks the interaction with 12-LOX to ~ 50%

From the previous section it is clear that GR16 (FN1 and FN2) fragment competes with full length β4 and blocks the interaction with 12-LOX. Since the fine mapping shows that the FN01-2, 11 aa fragment (residues 1137-1147) of β4 is important for interaction, we used this fragment to compete with either GR16 or full length β4. We transfected, CHO cells with triple constructs expressing

GRFN01-2, GR16 and 12-LOX or GRFN01-2, β4 and 12-LOX individually and then made recombinant lysates. The individual lysates from cells transfected with GRFN01-2 and 12-LOX were combined with lysates from cells expressing GR16 or full length β4. Expression of all transfected constructs, GR16 (Myc tagged, 23 kDa), 12-LOX (78 kDa), and full length β4 integrin ($\sim$ 200 kDa) is shown in Figure 3.8A by Western blot. GR-FN01-2, Myc-tagged construct (1 kDa) expression is not shown on the blot due to the size constraint.   Co-immunoprecipitation of these cell lysates with 12-LOX and blot detection with Myc antibody shows that GRFN01-2 reduced the interaction with GR16 (Figure 3.8B).   Similarly immunoblot with β4 antibody showed reduction in 12-LOX interaction with full length β4 in the presence of GR-FN01-2 (Figure 3.8B). The β4 integrin FN01-2 domain encoded by pGRFN01-2 binds to 12-LOX competes with GR16 (aa 1126-1315), and full length β4, and reduces the interaction between 12-LOX and GR16 or full length β4 integrin. Endogenous level of Myc (115 kDa, faint band) is not detectable due to strong signal of the GR16-Myc construct.

3.9   Ectopic expression of GR16 decreases cell migration

After confirming that GR16 could block the biochemical interaction of 12-LOX with the full length β4 integrin subunit, we focused on the functional aspect of this association. 12-LOX and β4 integrin have both been shown to play a role in the migration of tumor cells. The sole metabolic product of 12-LOX metabolism of arachidonic acid, 12(S)-HETE, can modulate several parameters related to the metastatic potential of tumor cells like cell motility and cell invasion (Timar, Silletti et al. 1993; Honn, Tang et al. 1994). The α6β4 integrin has a significant role in

signaling pathways of epithelial and carcinoma cells leading to enhanced migration and invasion (Mercurio and Rabinovitz 2001). We have reported previously that β4 stimulation leads to 12-LOX membrane translocation in A431 cells. Additionally, we also showed that 12-LOX activity may be important for $\alpha6\beta4$-dependent cell migration on laminin following EGF stimulation (Tang et al., unpublished observation). Therefore we examined the effect of ectopic expression of GR16 on A431 cell migration through a transwell insert in the presence and absence of β4 stimulation. The A431 stable transfectant expressing the β4 fragment GR16 shows 40% less cell migration compared to parental A431 and A431-EV (Figure 3.9). Addition of 12(S)-HETE to the A431-GR16 stable transfectant rescued cell migration. This shows that 12-LOX activity resulting from β4 interaction is important for cell migration. Also the data shown in figure 3.9 is significant, *, $p < 0.05$, comparing cell invasion of A431-GR16 with A431 wild-type and A431-EV cells. Migration of A431-GR16 with 12(S)-HETE add back was similar to A431-EV.

3.10 Ectopic expression of GR16 decreases 12-LOX activity

The arachidonate metabolite 12(S)-HETE, the sole product of 12-LOX activity, induces a plethora of responses in tumor cells and is linked to tumor progression and metastasis (Honn, Tang et al. 1994; Gao and Honn 1995; Nie, Tang et al. 2000; Nie, Tang et al. 2000). We have reported previously that 12-LOX and β4 association is functionally relevant and shows an increase in 12-LOX enzymatic activity by HPLC where 12(S)-HETE biosynthesis was increased following cell stimulation with β4 mAb 3E1 (Tang et al., unpublished observation). Therefore,

in this study we measured the 12-LOX enzymatic activity after blocking the interaction of 12-LOX with full length β4 by ectopic expression of β4 fragment GR16. Arachidonic acid was administered for 6 hours to parental A431 cells and A431 transfectants. Following incubation, conditioned medium from cell cultures was processed as described and 12(S)-HETE production was measured. The results from the mass spectrometry analysis reveal a significant decrease in 12(S)-HETE production when the interaction between 12-LOX and full-length β4 is blocked by GR16 (A431-GR16) compared to the A431-EV stable transfectant (Figure 3.10). Thus ectopic expression of β4 fragment GR16 can effectively reduce 12-LOX activity. Data shown is significant since, *, $p < 0.05$ by student t test there is significant decrease in 12-LOX activity in A431-GR16 stable transfectant when compared to A431-EV or A431 wild-type.

3.11 Ectopic expression of GR16 decreases cell proliferation

Competitive inhibition of the association between 12-LOX and β4 with the GR16 fragment resulted in reduced cell migration and 12-LOX activity compared to parental A431 cells or A431-EV stable transfectants. Consequently the reduction in 12(S)-HETE production should result in reduced cell proliferation, as we and others have shown that 12(S)-HETE is mitogenic. Moreover, the α6β4 integrin triggers signaling pathways involved in cell differentiation, adhesion, invasion and metastasis (Giancotti and Ruoslahti 1999; Gleason, Adley et al. 2005). Likewise, 12(S)-HETE affects cell proliferation, motility, invasion, angiogenesis, and inhibits apoptosis (Nie, Hillman et al. 1998). One possible integrator of these 12(S)-

HETE signaling mechanisms in tumor cells is the pleiotropic transcription factor NF-κB, which controls cell proliferation and apoptosis and was shown in PC-3 cells, to be activated by over expression of the platelet-type 12-LOX or exogenously added 12(S)-HETE (Kandouz, Nie et al. 2003) . Therefore, we examined if cell proliferation was affected when 12-LOX is blocked by GR16 fragment of β4 integrin. We performed an MT-PMS assay to study cell proliferation. We seeded 2 x 10^3 A431 stable transfectants expressing GR16 or EV into a 96 well plate. The cells were seeded in triplicate in 8 plates starting from day-0 to day-7. There is significant decrease in cell proliferation in A431-GR16 stable transfectant when compared to A431-EV everyday over the period of 6 days. The seventh day results show that here was 30% reduction in cell proliferation in the A431-GR16 stable transfectant compared to EV (Figure 3.11). Data shown is significant, *, $p < 0.05$, by student t test.

3.12 Ectopic expression of GR16 reduces colony formation

The functional relevance of using GR16 to block the interaction of 12-LOX with full length β4 integrin is significant, as it reduced 12-LOX enzymatic activity, cell migration and cell proliferation. Therefore we decided to perform an *in vitro* cell survival assay using colony formation assay. We have shown previously 12-LOX activity increases by interaction with β4 integrin (Tang et al., unpublished observation). The 12-LOX metabolite 12(S)-HETE acts as a survival factor in many tumor cells involving a complex signaling mechanism leading to tumorgenesis (Samuelsson 1983; Honn, Tang et al. 1994). We seeded A431-GR16 and A431-EV stable transfectant cells in 60 mm plates. The assay was

terminated after 7 days before the edges of colonies came into contact. The colonies were counted under microscope using10 fields/ plate. The average colony count for A431-EV was 35-45 colonies per field (20-30 cells/colony), while it was only 20-25 colonies per field for A431-GR16 (15-20 cells/colony) (Figure 3.12A). There is significant decrease in cell colony formation in A431-GR16 stable transfectant when compared to A431-EV. Further quantitative analysis showed 40% less colony formation with A431-GR16 stable transfectant cells (Figure 3.12B). Data shown is significant, *, $p < 0.05$, by student t test.

3.13 Ectopic expression of GR16 and its interaction with 12-LOX shows no effect on full length β4 cell adhesion function

Integrins are heterodimeric receptors mediating adhesion of cells to extracellular matrices and other cells (Buck and Horwitz 1987; Ruoslahti and Pierschbacher 1987; Ginsberg, Loftus et al. 1988; Springer 1990; Hynes 1992). α6β4 integrin is a basement membrane receptor for laminin and an important component of hemidesmosomes where it mediates adhesion of epithelial cells to underlying basement membrane (Kajiji, Tamura et al. 1989; Carter, Kaur et al. 1990; De Luca, Tamura et al. 1990; Sonnenberg, Linders et al. 1990; Sonnenberg, Linders et al. 1990; Sonnenberg, Calafat et al. 1991; Lee, Lotz et al. 1992). Above we have shown that, we could successfully block the interaction of full length β4 integrin with 12-LOX by ectopically expressing the GR16 fragment. However, this appeared to have no effect on the cell adhesion function of β4 integrin (Figure 3.13). A431 wild type cells and the A431 transfectants ectopically

expressing GR16 or empty vector cells were plated on the laminin coated 96 well plates and the cell adhesion was quantified by measuring the absorbance of stained cells at 550 nm. The results showed no difference in cell adhesion between A431 wild type and A431 transfectants. Since, *, $p > 0.05$, by student t test.

3.14 Ectopic expression of GR16 suppresses tumor growth in an animal model (*in vivo* study)

In this study we show that the interaction between 12-LOX and β4 mediates the invasive role of α6β4 integrin in the growth of carcinoma. When this interaction is blocked using the β4 fragment GR16 we saw a decrease in the tumor growth *in vivo*. The athymic nude mice were separated into 2 groups. The control group (n=6) was subcutaneously injected with A431-EV and the test group (n=6) with A431-GR16 stable transfectant cells. We evaluated the xenograft tumor growth after the A431 cell inoculation every 3 days for 4 weeks. The tumor growth progression in the test group showed delay (day3 onwards till day15 where it plateaus) compared to the control group and the average tumor volume of the test group was comparatively smaller in size to the control group (Figure 3.14). Thus the data suggest that when the 12-LOX- β4 interaction is blocked by the β4 fragment GR16, tumor growth is reduced. These results support our *in vitro* data that interference of this interaction reduced cell migration, 12-LOX activity, and cell proliferation. A431-GR16 shows significantly reduced tumor weight, since, *, $p < 0.05$, by student t test.

3.15 Tumor tissue analysis for Real time PCR for gene expression (*in vivo study*)

Real time PCR was performed on tumor tissue cDNA using Taqman primers and reagents using actin as the house keeping gene. Bcl2, an anti-apoptotic gene is elevated in most cancers. Results from our laboratory have previously shown that the Bcl family of proteins is involved in apoptosis during 12-LOX inhibition in rat Walker-256 carcinoma cells (Tang, Chen et al. 1996). When 12-LOX specific antisense oligonucleotides were transfected, W526 cells underwent apoptosis; there was a decrease in Bcl2 and therefore a decrease in Bcl2: Bax ratio. Over expression of Bcl2 protein partially overcame apoptosis (Tang, Chen et al. 1996) Also the Pidgeon et al. study showed a similar decrease in Bcl2 levels and the reduction was coupled to an increase in Bax levels when cells were treated with 12-LOX inhibitor (Pidgeon, Kandouz et al. 2002). Inhibition of 12-LOX-β4 interaction with GR16 translated to a similar shift in the Bcl2: Bax ratio. The relative gene expression of Bcl2 decreased while that of Bax increased in A431-GR16 transfectants tumor tissue samples compared to the A431-EV tumor tissue samples (Figure 3.15A and 3.15B).

VEGF is a potent tumor angiogenesis factor. Agents that target VEGF and its signaling pathways inhibit tumor growth and propagation in various experimental models (Ferrara 2002). 12-LOX and 12-HETE both have been known to play a role in angiogenesis (Gao and Honn 1995; Nie, Hillman et al. 1998; Nie, Tang et al. 2000; Nie, Tang et al. 2000) as well as increase the surface expression of $\alpha_v\beta_3$ integrin (Honn, Tang et al. 1994). Residue Y1494 of

β4 integrin regulates VEGF expression and stimulates angiogenesis, thus contributing to tumor progression (Dutta and Shaw 2008). Therefore, we examined what, if any, effect GR16 would have on VEGF production. In tumor tissue samples expressing A431-GR16 transfectants compared to A431-EV, the relative expression of VEGF is significantly decreased in the presence of the GR16 fragment of β4 (Figure 3.15C). This was also confirmed by immunostaining for VEGF. The data shown is significant from Figure 3.15 A, B and C, as,*, $p < 0.05$, by student t-test when comparing the A431-GR16 group to A431-EV which indicates there is increase in apoptosis and a significant decrease in angiogenesis.

The tissue slide from A431-GR16 transfectants showed less staining for VEGF compared to A431-EV control slide (Figure 3.16). VEGF expression is regulated by a variety of transcription factors. The promoter region of the VEGF gene has binding sites for transcription factors like Sp1/Sp3, AP-2, Egr-1, STAT-3, and HIF-1 (Pages and Pouyssegur 2005). Of these factors, HIF-1 is responsible for transcriptional regulation under hypoxic conditions (Forsythe, Jiang et al. 1996; Krishnamoorthy, Jin et al. 2010) and our group has shown that 12-LOX regulates HIF-1α (Krishnamoorthy, Jin et al. 2010).

Therefore we examined the effect of GR16 on HIF-1α expression (Figure 3.15D). While the relative levels of HIF-1α was decreased in tumors from GR16 transfectants, this was not very significant since the, *, $p > 0.05$, from student t test. The increased angiogenicity of A431-EV transfectants was also confirmed by immunostaining and RT-PCR for VEGF. Expression of GR16 protein (23

kDa) was confirmed by Western blot and was present in tumor tissue samples from the A431-GR16 transfectant group (Figure 3.17).

| TM | IC | F1 | F2 | L | F3 | F4 | C |

Figure 3.1: Schematic representation of β4 integrin.

Name	Position (aa)	N-Terminal HIS-tag, T7 promoter	C-terminal myc-tag, CMV promoter	C-terminal EGFP, CMV promoter
B_4-$F_{1,2}$	1126-1315	pGR1	pGR16	-
B_4-$F_{1,2}$L	1126-1457	pGR36	-	pGR45
B_4-$F_{1,2}$L(F_3)	1126-1485	pGR5	-	pGR40
B_4-F_1	1126-1218	pJP1	-	-
B_4-F_2	1219-1314	pJP2	-	-
B_4-L	1316-1457	pGR2	pGR17	pGR38
B_4-F_2L	1219-1457	pGR4	pGR18	-
B_4-F_3	1457-1546	pJP3	-	-
B_4-F_4	1570-1662	pJP4	-	-
B_4-$F_{3,4}$	1457-1662	pJP5	-	-
B_4-$F_{(3),4}$C	1486-1752	pGR3	pGR13	pGR39
B_4-$LF_{3,4}$C	1316-1752	pGR37	-	pGR41
B_4-$F_{3,4}$C	1458-1752	pGR44	-	-
B_4-$F_{1,2}LF_{3,4}$C	1126-1752	pGR6	pGR20	pGR30
B_4-TM-F_2	708-1315	pGR42	-	pGR46
B_4-TM-C	708-1752	pGR43	-	pGR47

F... Fibronectin type3-like repeat
I.... Linker region between fibronectin pairs
C... C-terminal region (C-terminal of second fibronectin pair)
TM... Transmembrane domain

Table 3-1: List of bacterial and mammalian expression constructs of β4 fragments. Bacterial plasmids encode the HIS-tag under control of the T7 promoter. Mammalian expression constructs are either Myc-tagged or EGFP-tagged and are regulated by the CMV promoter. TM, transmembrane; IC, intracellular; F1, F2, F3, and F4 are fibronectin type III repeats.

Courtesy: Dr. Gerhard Wiche

NOTE: Elsewhere F1, F2, F3, F4 are labeled as FN1, FN2, FN3, and, FN4 respectively.
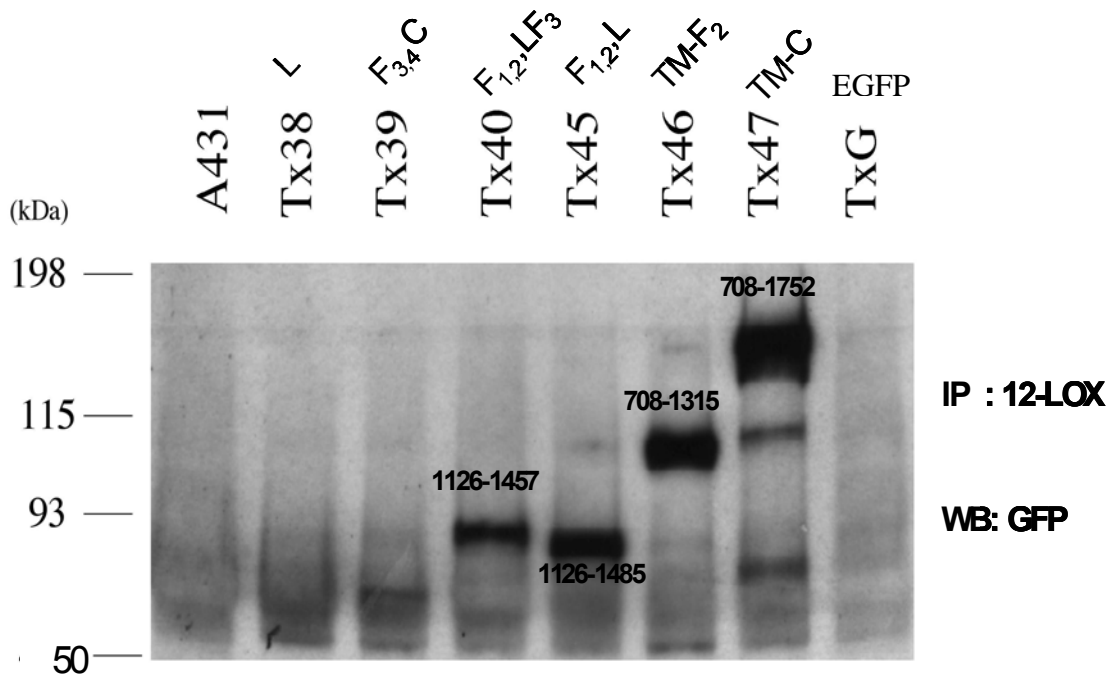
Figure 3.1A: Co-immunoprecipitation shows 12-LOX binds with the fusion proteins expressed by pGR40 (Tx40), pGR45 (Tx45), pGR46 (Tx46), and pGR47 (Tx47), but not with pGR38, pGR39, or pEGFP (TxG). Immunoprecipitation was done using pAb against 12-LOX and antibody mAb GFP was used to detect β4 – GFP fusion fragments. 10% SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C. The binding site localizes to amino acids 1126 – 1315 of the cytoplasmic tail. L: Linker, F: FN, TM: Transmembrane, C: Cytoplasmic

Source: Preliminary data for grant, experiment performed by Dr. Keqin Tang. Reprinted with permission.

A

| EC | TM | IC | FN1 | FN2 | L | FN3 | FN4 | C |

1126-1318

FN1

```
S  Q  P  P  P  H  G  D  L  G  A  P  Q  N  P  N  A  K  A  A
G  S  R  K  I  H  F  N  W  L  P  P  S  G  K  P  M  G  Y  R
V  K  Y  W  I  Q  G  D  S  E  S  E  A  H  L  L  D  S  K  V
P  S  V  E  L  T  N  L  Y  P  Y  C  D  Y  E  M  K  V  C  A
Y  G  A  Q  G  E  G  P  Y  S  S  L  V  S  C  R  T  H  Q  E
```

FN2

```
V  P  S  E  P  G  R  L  A  F  N  V  V  S  S  T  V  T  Q  L
S  W  A  E  P  A  E  T  N  G  E  I  T  A  Y  E  V  C  Y  G
L  V  N  D  D  N  R  P  I  G  P  M  K  K  V  L  V  D  N  P
K  N  R  M  L  L  I  E  N  L  R  E  S  Q  P  Y  R  Y  T  V
K  A  R  N  G  A  G  W  G  P  E  R  E  A  I  I  N  L  A  T
```
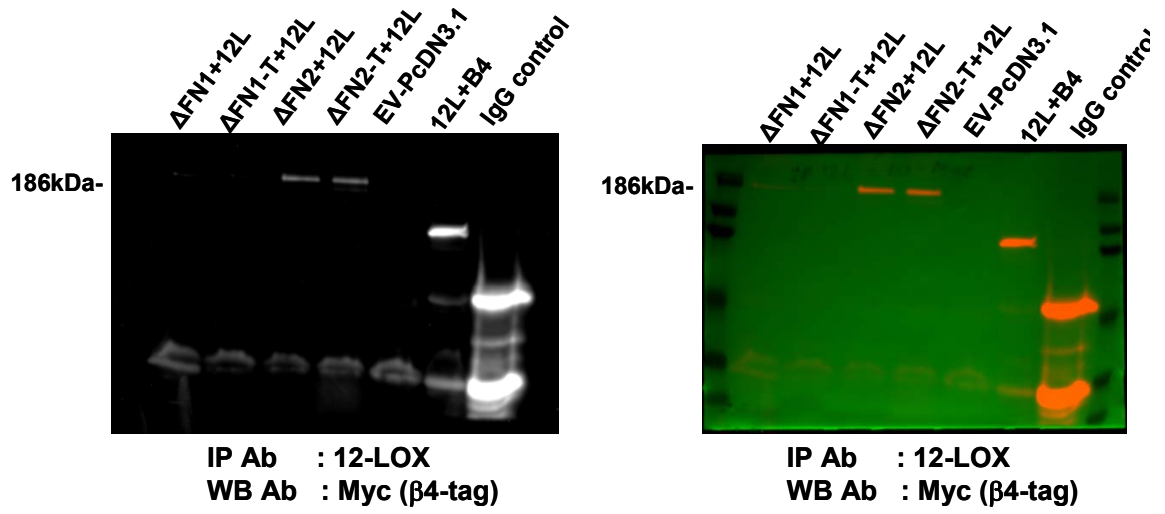
B

FN1 deletion (ΔFN1)

| EC | TM | IC | | FN2 | L | FN3 | FN4 | C |

Myc

FN2 deletion (ΔFN2)

| EC | TM | IC | FN1 | | L | FN3 | FN4 | C |

Myc

	Number of deleted amino acids	MW: 194,997 Da; full-length -β4
ΔFN1-β4	90 aa (1126—1215)	185,056; (+myc186,259)
ΔFN2-β4	99 aa (1220—1318)	183,994; (+myc185,197)

Figure 3.2 A&B

C

IP Ab   : 12-LOX
WB Ab : Myc (β4-tag)

Strip and reprobe with 12-LOX Antibody:

Figure 3.2 C

D

WB Ab : Myc (β4-tag)

186kDa-

WB Ab : Myc (β4-tag)

186kDa-

WB Ab : 12-LOX

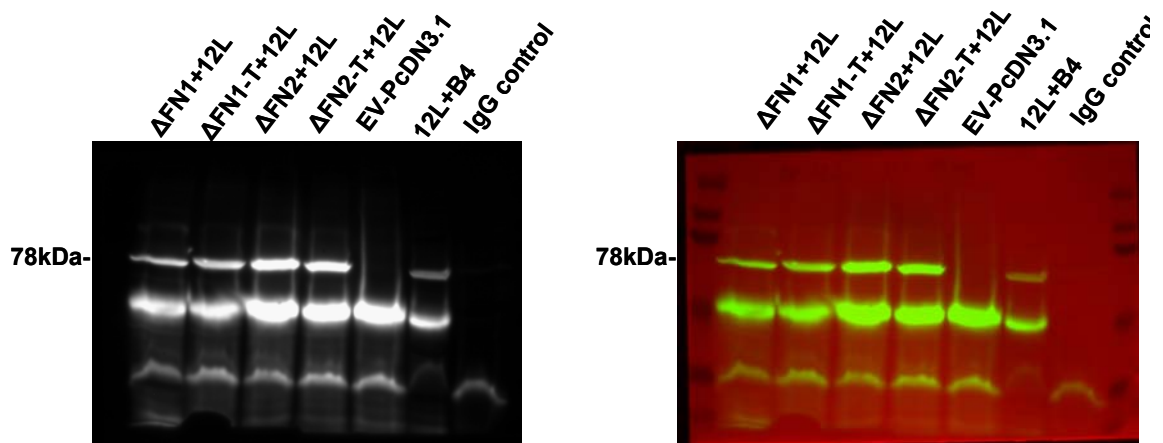78kDa-

WB Ab : 12-LOX
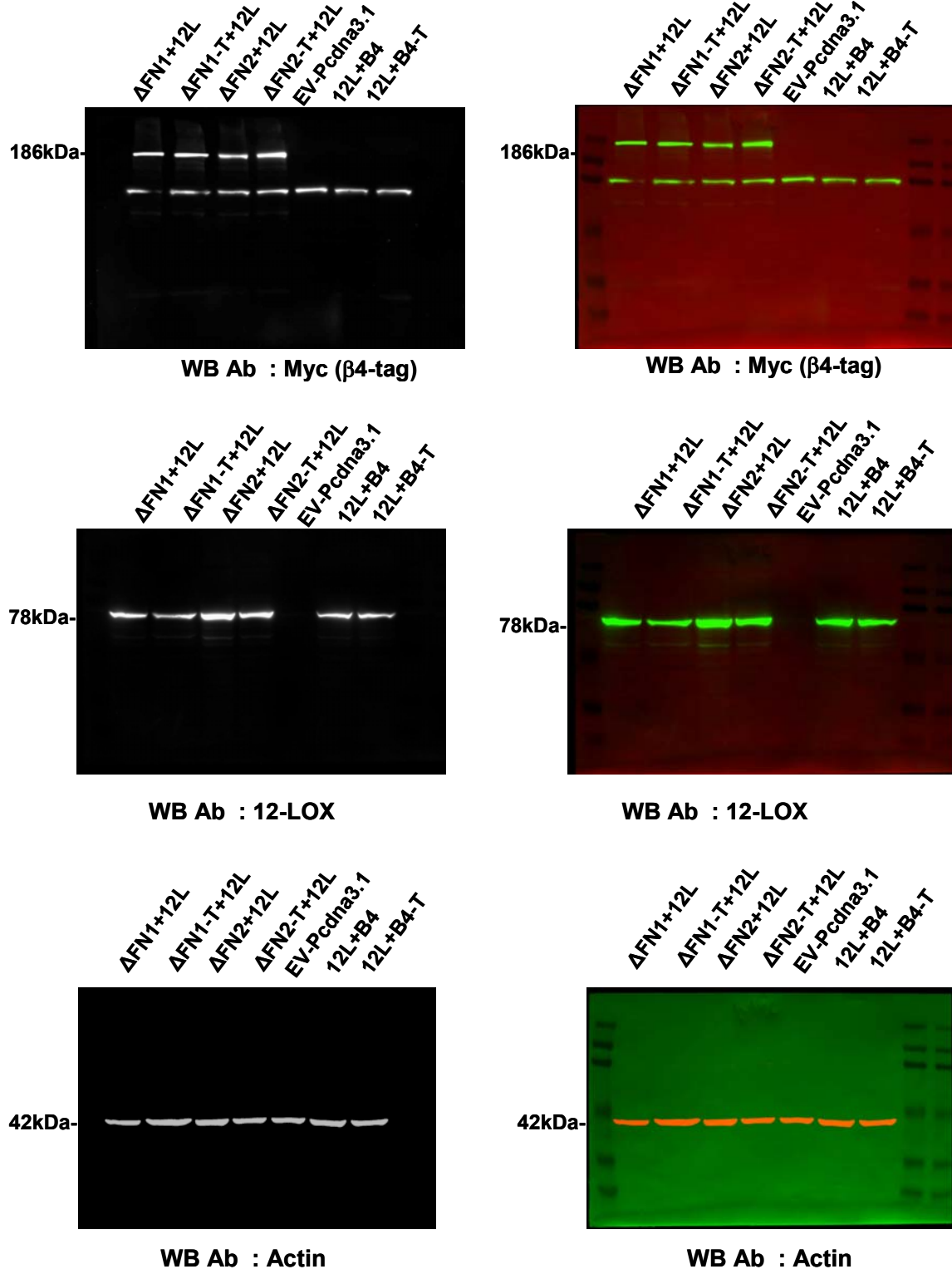
78kDa-

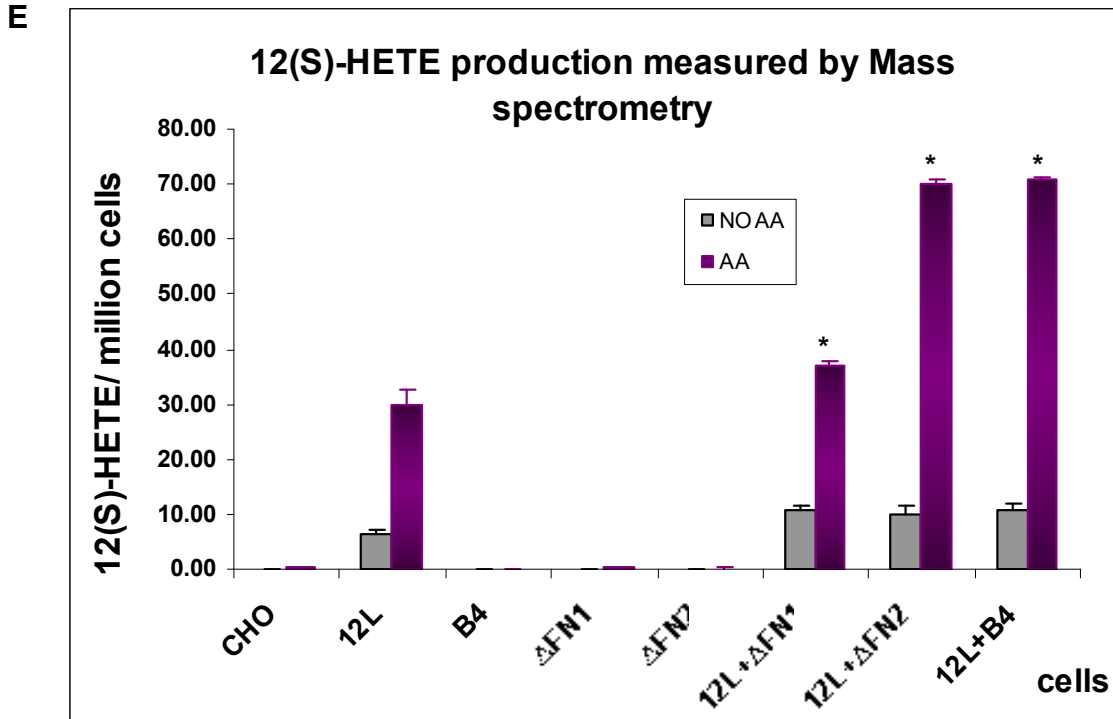

WB Ab : Actin

42kDa-

WB Ab : Actin

42kDa-

E

Figure 3.2: Mapping of integrin β4 macro domains necessary for 12-LOX interaction.

Whole cell lysates were immunoprecipitated with 12-LOX antibody and probed for β4 mutants with Myc antibody. Blots were stripped and reprobed for 12-LOX. (A) Schematic of integrin β4 sub-domains. (B) Description of deleted sub-domains in the cytoplasmic tail of β4 integrin. (C) *Co-immunoprecipitation* experiments from CHO cells transiently transfected with ΔFN1-β4 or ΔFN2-β4 constructs. 12-LOX was pulled down with pAb 12-LOX antibody and the immunoblots were probed with mAb for Myc. 10% SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C (D) *Western blot* of *whole cell lysates* showing expression of ΔFN1-β4, ΔFN2-

β4 (upper) and 12-LOX constructs (middle) in transiently transfected CHO cells along with actin control (lower). pAb 12-LOX, mAb Myc and actin antibodies were used to detect 12-LOX and β4 deletion constructs. (E) 12-LOX activity measured as a function of 12(S)-HETE production by mass spectrometry with β4 mutant constructs ΔFN1 and ΔFN2. Data are statistically significant, $* p < 0.05$ by student t test when comparing 12(S)-HETE production of 12L+ΔFN1 in presence of AA with12L+ΔFN2 and 12L+ β4, while it shows no significant difference when comparing 12L+ΔFN2 and 12L+ β4.

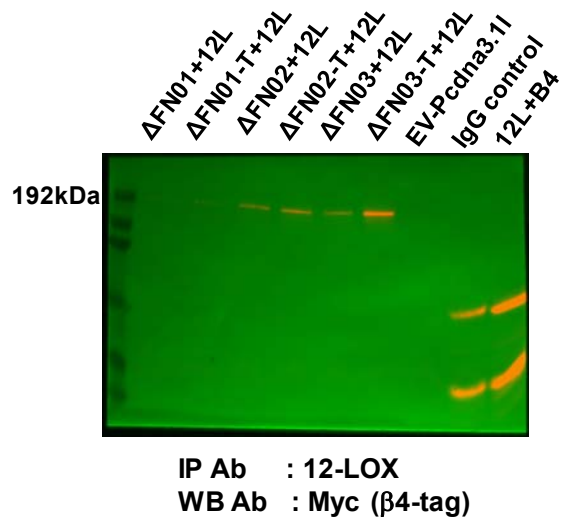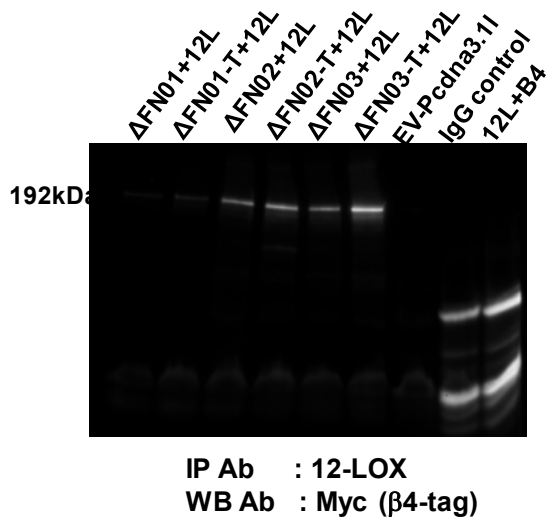**Note: In (B) and (C) images on the right are overlays to show molecular weight marker.**

A
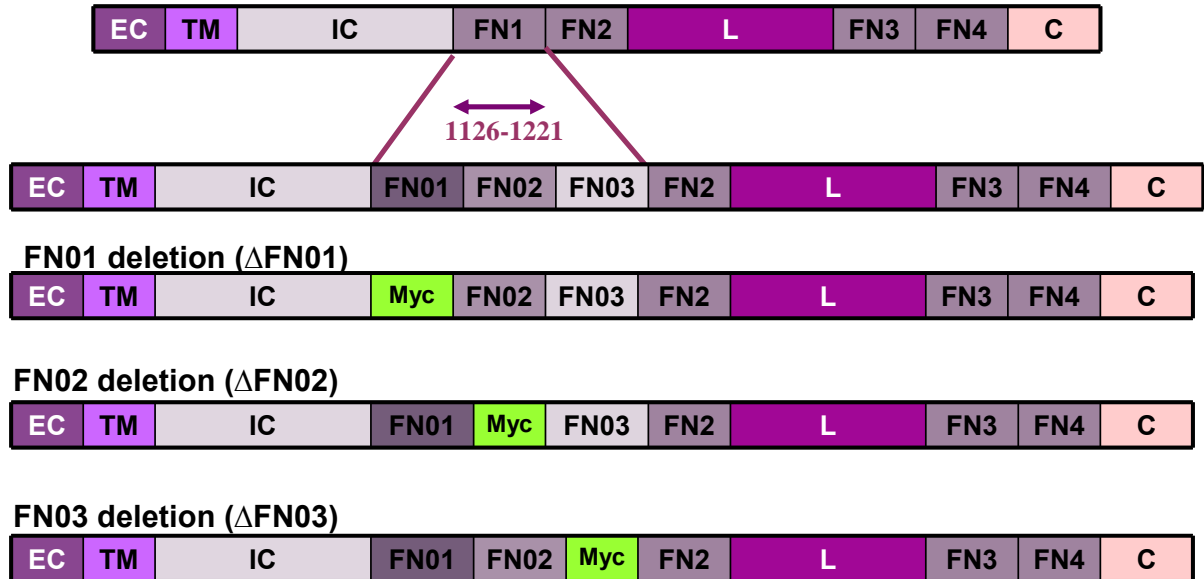
	Number of deleted amino acids	MW: 194,997 Da ; full length - β4
ΔFN01-β4	32 aa (1126—1157)	191,576; (+myc192,779)
ΔFN02-β4	32 aa (1158—1189)	191,259; (+myc192,462)
ΔFN03-β4	32 aa (1190—1221)	191,501; (+myc192,704)

FN1 Sequence: FN01 -32 aa, FN02- 32 aa, FN03- 32 aa

FN01-S Q P P P H G D L G A P Q N P N A K A A G S R K I H F N W L P P
FN02-S G K P M G Y R V K Y W I Q G D S E S E A H L L D S K V P S V E
FN03-L T N L Y P Y C D Y E M K V C A Y G A Q G E G P Y S S L V S C R T

B

IP Ab : 12-LOX
WB Ab : Myc (β4-tag)

IP Ab : 12-LOX
WB Ab : Myc (β4-tag)

Strip and reprobe with 12-LOX Antibody:

WB Ab : 12-LOX

WB Ab : 12-LOX

C

WB Ab : Myc (β4-tag)

WB Ab : Myc (β4-tag)

192kDa

192kDa

WB Ab : 12-LOX

WB Ab : 12-LOX

78kDa

78kDa

WB Ab : Actin

WB Ab : Actin

42kDa

42kDa
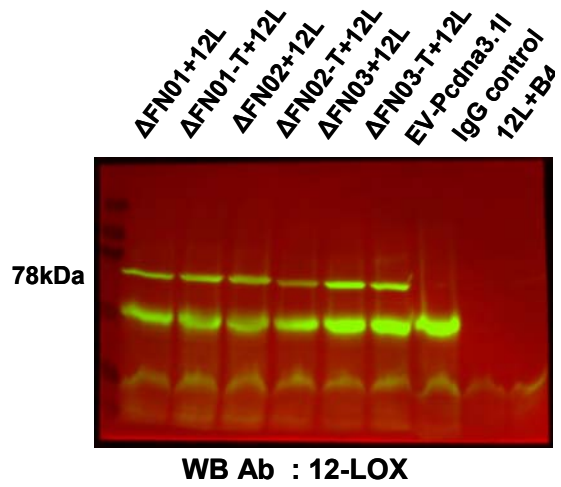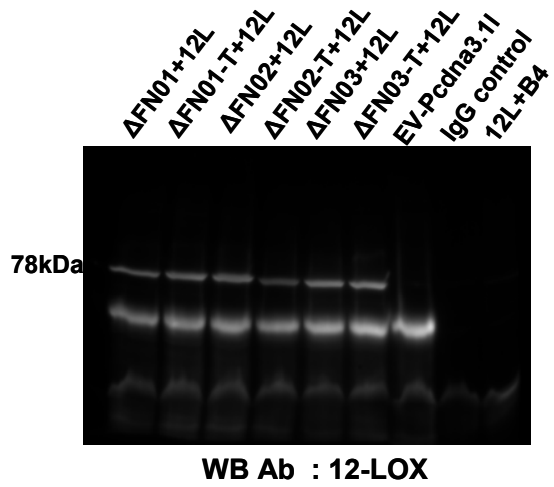
Figure 3.3: Mapping of micro-domains within integrin β4-FN1 region necessary for 12-LOX interaction. 12-LOX interacts with the FN01 sub-domain within the FN1 macro-domain, and maps to residues 1126-1157. (A) Schematic of integrin β4 macro-domains (B) *Co-immunoprecipitation* of 12-LOX in CHO cells transiently transfected with β4 deletion mutants, ΔFN01, ΔFN02 and ΔFN03 shows that there is no binding of 12-LOX with ΔFN01, while its binding was unaffected by ΔFN02 and ΔFN03. 12-LOX antibody was used to pull down 12-LOX and the immunoblots were detected with mAb Myc. 10% SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C (C) Western blot of *whole cell lysates* showing expression of β4 deletion mutants, ΔFN01, ΔFN02, ΔFN03, and 12-LOX constructs in transiently transfected CHO cells along with the actin control. Constructs were detected with Myc antibody for β4 deletion mutants, and 12-LOX pAb for 12-LOX, and actin mAb was used for the actin loading control.

Note: In (B) and (C) images on the right are overlays to show molecular weight marker.

A

1126 - `D L G A P Q N P N A K` `A A G S R K I H F N W` `L P P S`
`G K P M G Y` - 1157

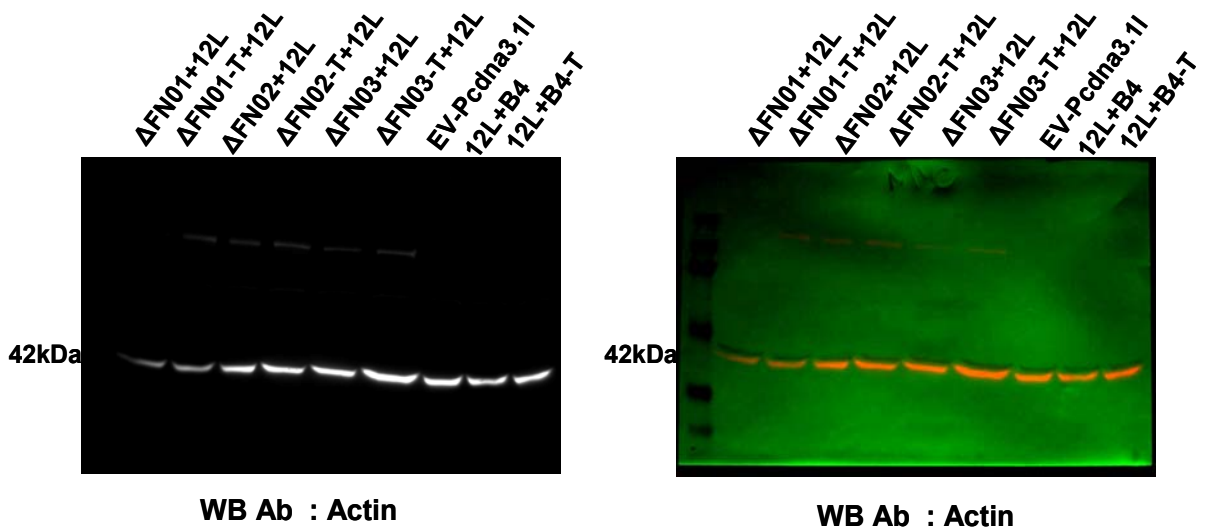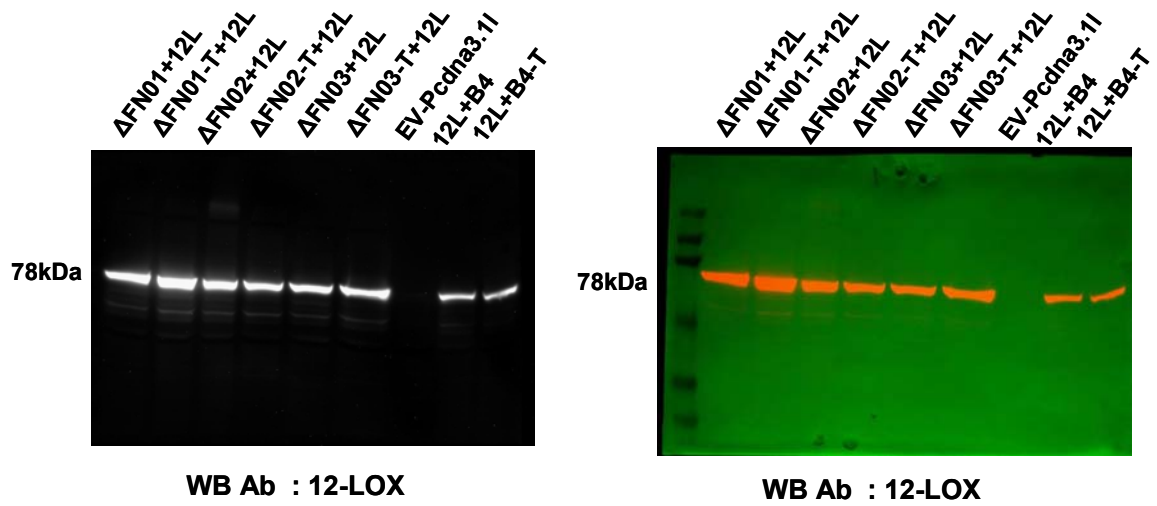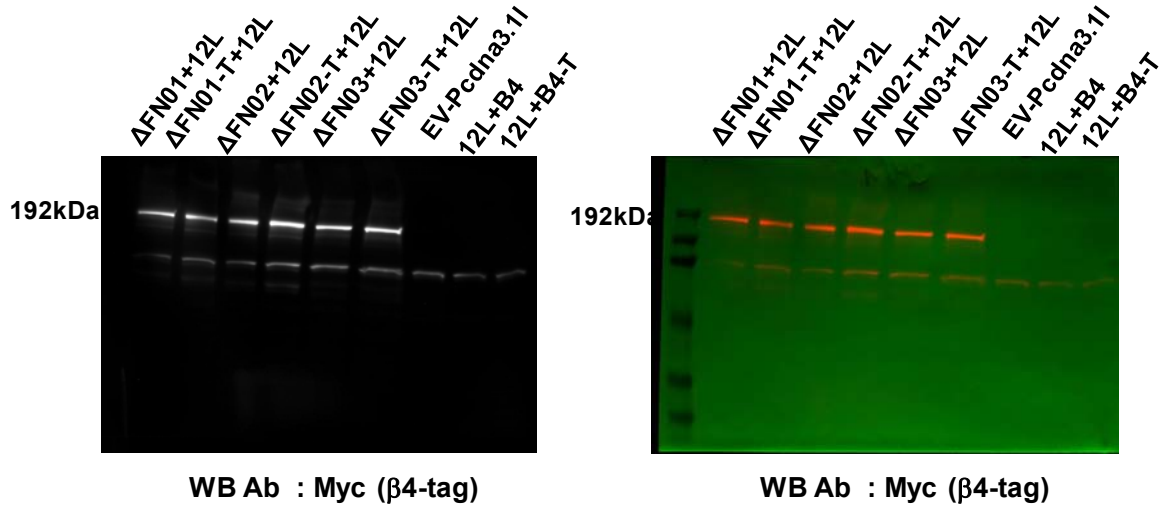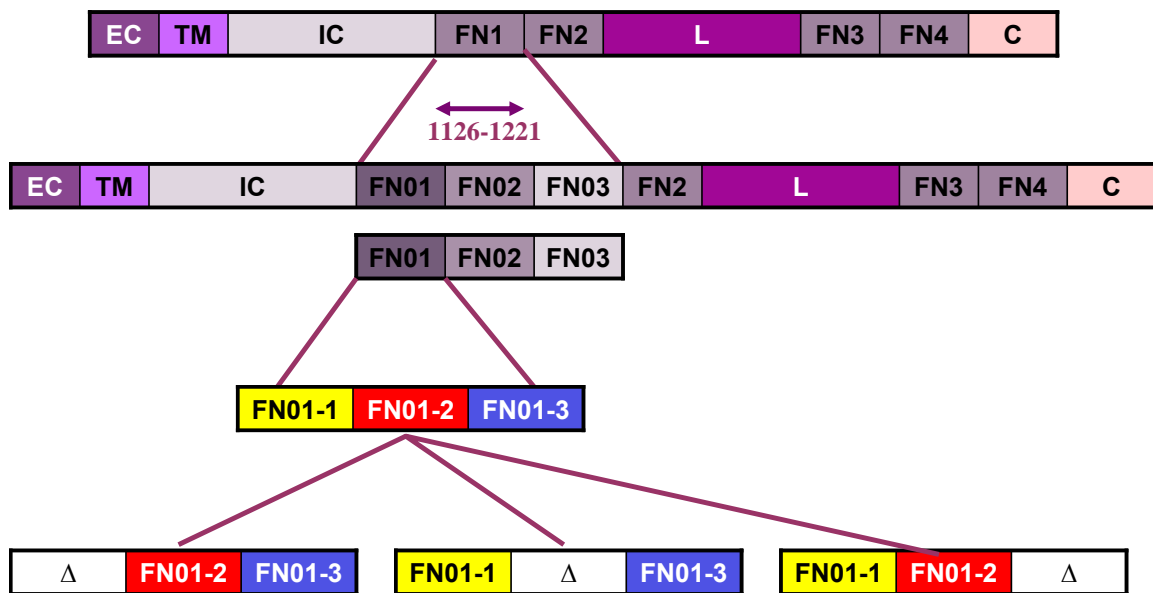|	Number of deleted amino acids	MW: 194,997 Da
ΔFN01-1-β4	11 aa (1126—1136)	191,576; (+myc192,779)
ΔFN01-2-β4	11 aa (1137—1147)	191,259; (+myc192,462)
ΔFN01-3-β4	10 aa (1148—1157)	191,501; (+myc192,704)

B

WB Ab : 450-11A-β4

WB Ab : 450-11A-β4

WB Ab : 12-LOX

WB Ab : 12-LOX

WB Ab : Actin

WB Ab : Actin

C

IP Ab : 12-LOX
WB Ab : 450-11A-β4

IP Ab : 12-LOX
WB Ab : 450-11A-β4

Strip and reprobe with 12-LOX Antibody:

WB Ab : 12-LOX

WB Ab : 12-LOX
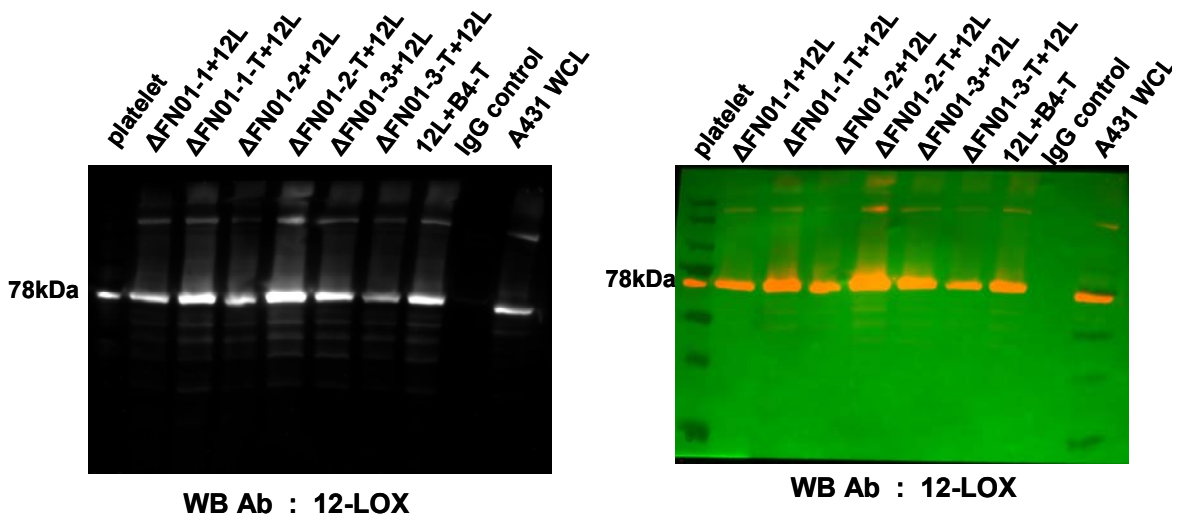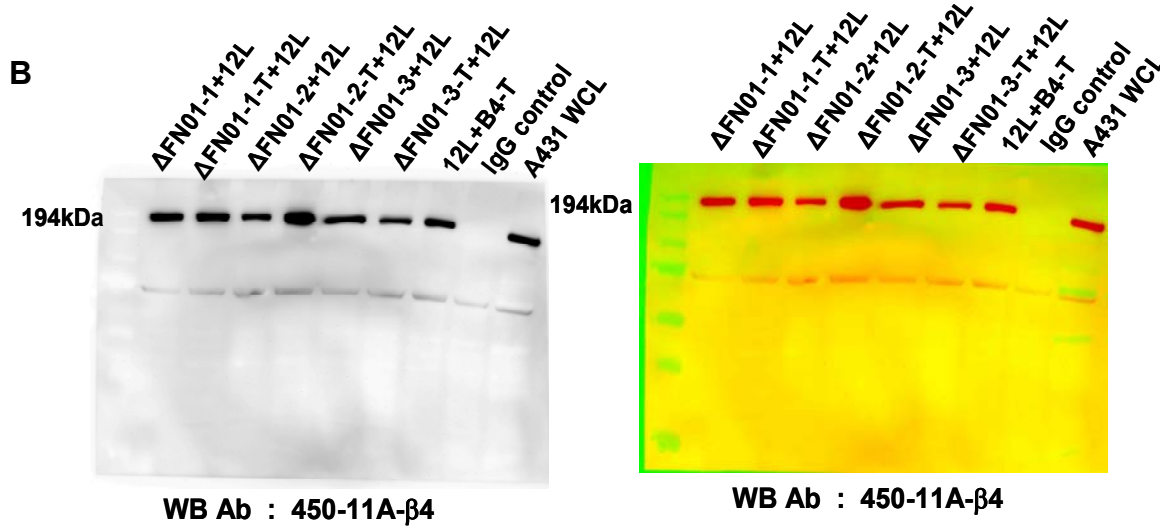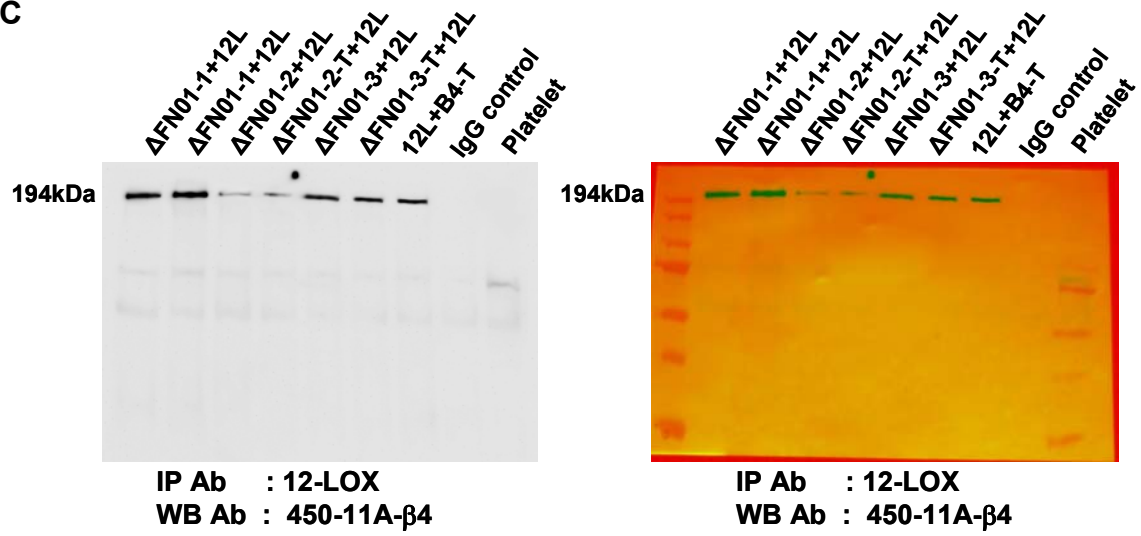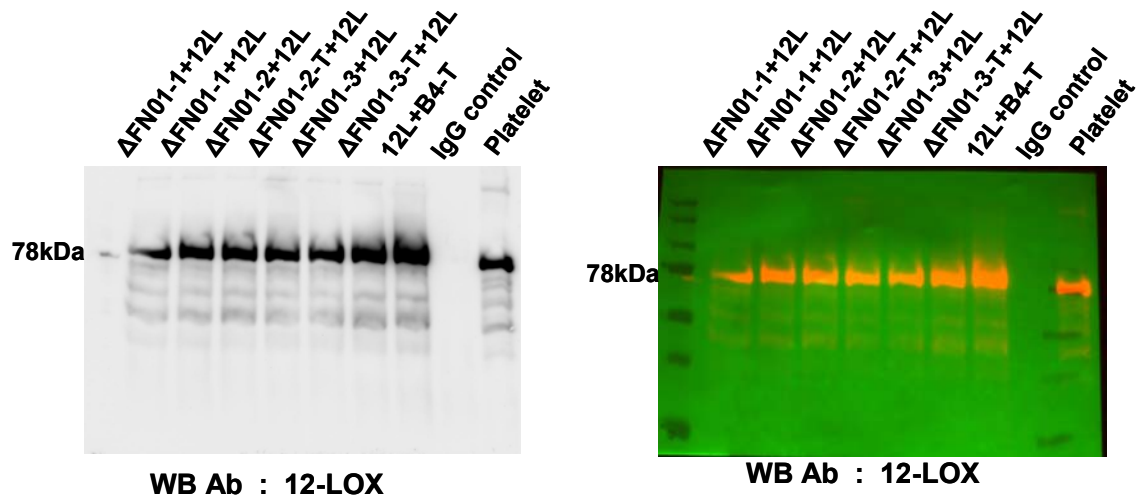
Figure 3.4: Fine-mapping of integrin β4 domains. P-12-LOX interacts with the FN01-2 micro-domain and maps to residues 1137-1147. (A) Schematic of β4 integrin micro sub-domains. (B) *Western blot* of *whole cell lysates* showing expression of β4 micro-domain deletion mutants ΔFN01-1, ΔFN01-2, ΔFN01-3 and 12-LOX constructs in transiently transfected CHO cells along with the actin control. Integrin β4 constructs, and full length β4 were detected with β4-450-11A antibody (against the cytoplasmic domain of integrin β4), while 12-LOX pAb was used to detect 12-LOX, and actin mAb was used for the actin loading control. (C) *Co-immunoprecipitation* with β4 micro-domain deletion mutants ΔFN01-1, ΔFN01-2, and ΔFN01-3 constructs in transiently transfected CHO cells, shows no binding of 12-LOX with β4 mutant ΔFN01-2, while it shows binding with ΔFN01-1 and ΔFN01-3. 12-LOX antibody was used to pull down 12-LOX and the immunoblots were detected with β4 450-11A mAb. 10% SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C.
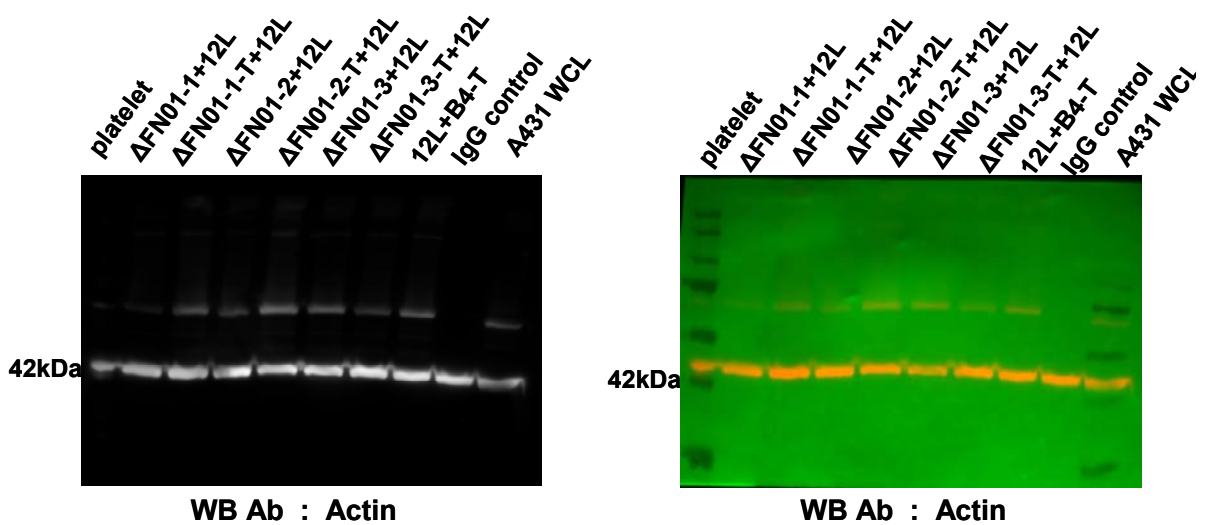
Note: In (B) and (C) images on the right are overlays to show molecular weight marker.

A

EC | TM | IC | FN01 | FN02 | FN03 | FN2 | L | FN3 | FN4 | C
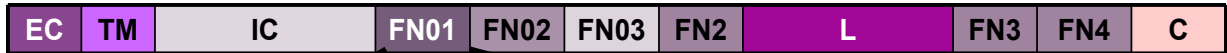
1126 -D L G A P Q N P N A K A A G **S** R K I H F N W L
P P **S** G K P M G **Y** - **1157**

FNO1-S1140
FNO1-S1151
FNO1-Y1157
Y1494 Important phosphorylation site (PMCID: PMC2586898)

FNO1-S1140A
FNO1-S1151A
FNO1-Y1157F
Y1494F

B

204kDa

WB Ab : 450-11A-β4

204kDa

WB Ab : 450-11A-β4

WB : 12-LOX

WB : 12-LOX

WB : Actin

WB : Actin

C

IP Ab   : 12-LOX
WB Ab : 450-11A-β4

IP Ab   : 12-LOX
WB Ab : 450-11A-β4

Strip and reprobe with 12-LOX Antibody:

WB : 12-LOX

WB : 12-LOX
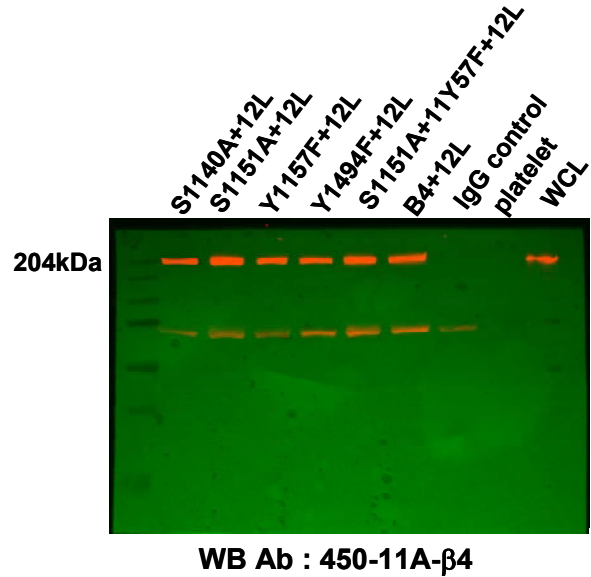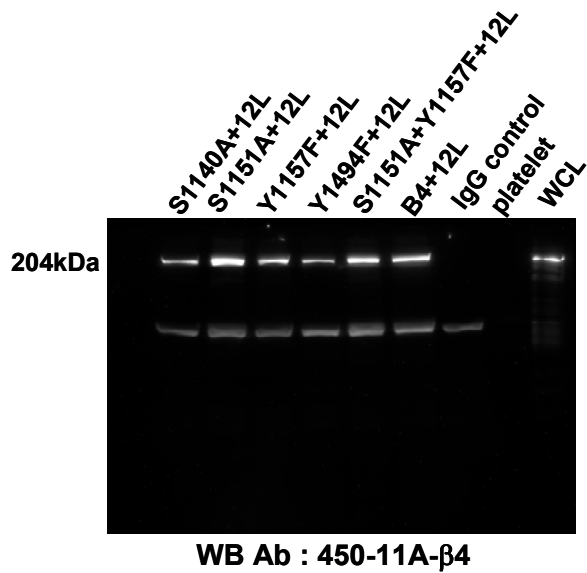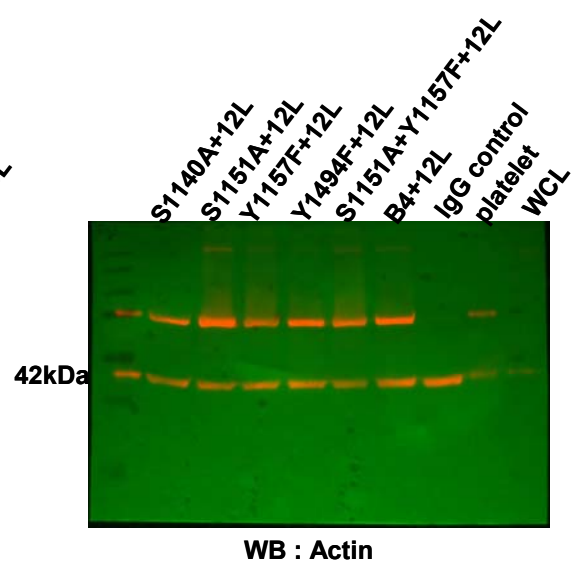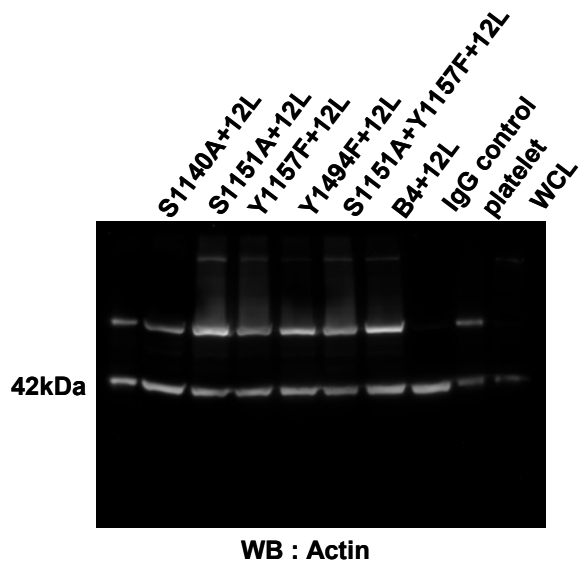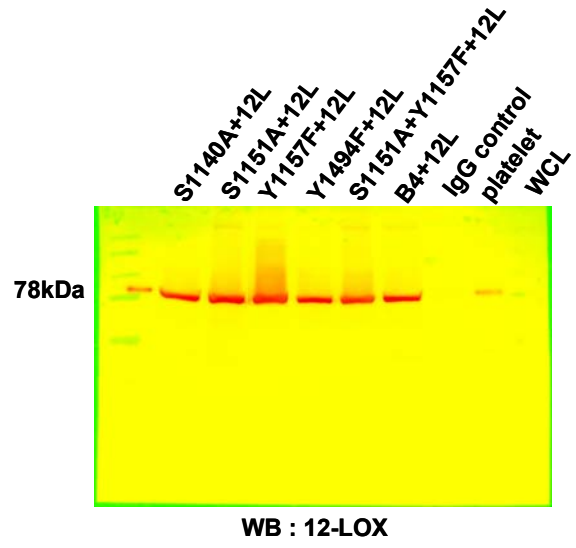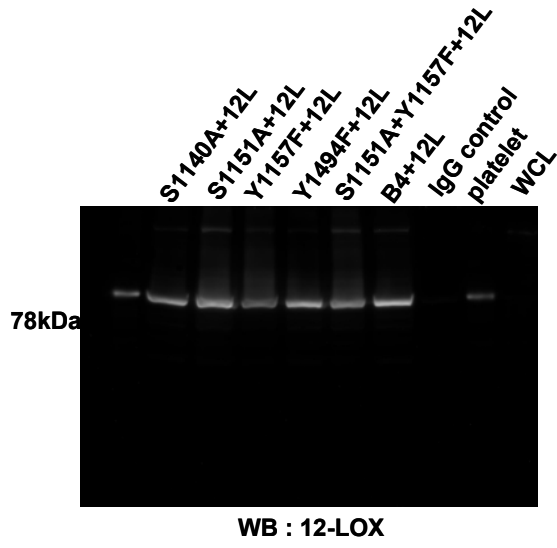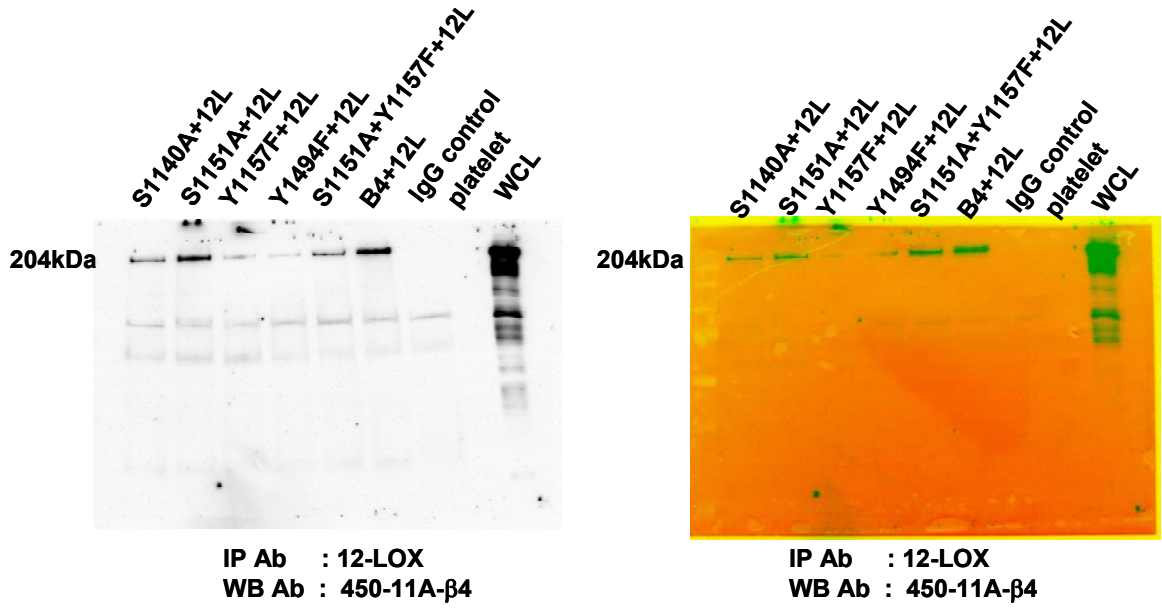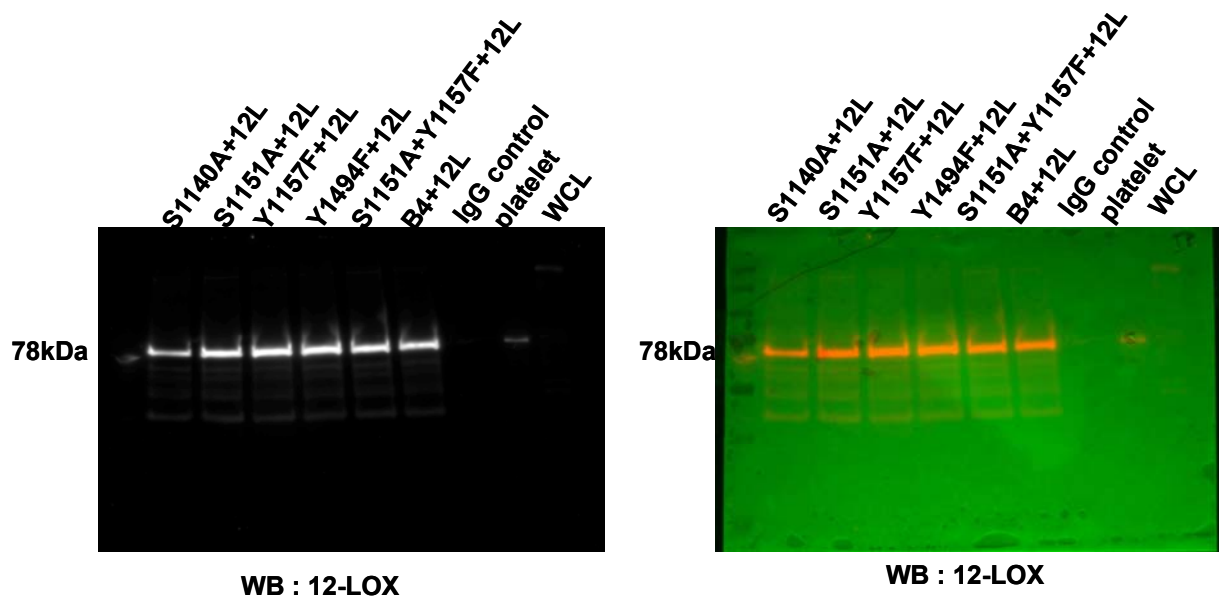
Figure 3.5: Phosphorylation sites in the FN01 domain of integrin β4 necessary for 12-LOX interaction. 12-LOX interacts with the FN01 sub-domain in FN1, within in a region spanning residues 1126-1157. (A) Schematic of predicted important phosphorylation sites to be mutated. (B) *Western blot* of *whole cell lysates* showing expression of β4 mutants, S1140A, S1151A, Y1157F, Y1494F, and 12-LOX constructs in transiently transfected CHO cells along with actin control. β4-450-11A mAb (against the cytoplasmic domain of integrin β4) was used to detect β4 phosphorylation mutants while 12-LOX was detected with 12-LOX pAb and actin mAb for the actin loading control. (C) *Co-immunoprecipitation* of 12-LOX from CHO cells transiently transfected with β4 phosphorylation mutants. The β4 mutants S1140A, Y1157F and Y1494F show weak or no binding with 12-LOX, while S1151A shows no effect on interaction. 12-LOX antibody was used to pull down 12-LOX and the immunoblots were probed with β4 450-11A mAb. 10% SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C.

Note: In (B) and (C) images on the right are overlays to show molecular weight marker.
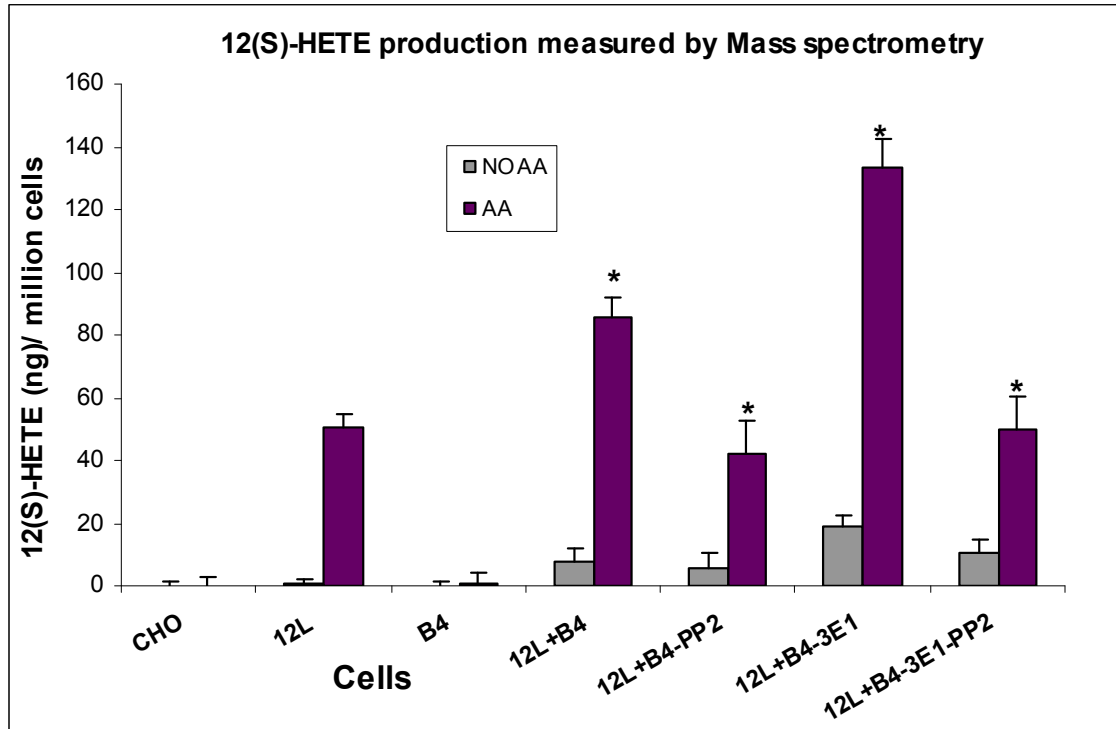
Figure 3.6: 12-LOX phosphorylation is important for activity. Mass spectrometry measurements of 12-LOX activity from CHO transfectant cells. 12(S)-HETE production is elevated in CHO cells co-transfected with 12-LOX and β4 integrin and is enhanced when transfectants are stimulated with 3E1. Treatment with Src-family inhibitor PP2 (5 μM) for 2 hours attenuates activity. Data shown above are significant, *, $p < 0.05$ when comparing 12L+β4 −PP2 with 12L+β4, and also the 3E1 treated set of 12L+β4 with 12L+β4-PP2.

A

WB: β4 -450-11A

WB: β4 -450-11A

WB: Myc

WB: Myc

WB: 12-LOX

WB: 12-LOX

WB: Actin

WB: Actin

B

IP Ab   : 12-LOX
WB Ab : 450-11A-β4

IP Ab   : 12-LOX
WB Ab : 450-11A-β4

IP Ab   : 12-LOX
WB Ab : 450-11A-β4

IP Ab   : 12-LOX
WB Ab : 450-11A-β4

IP Ab   : 12-LOX
WB Ab : 12-LOX

IP Ab   : 12-LOX
WB Ab : 12-LOX
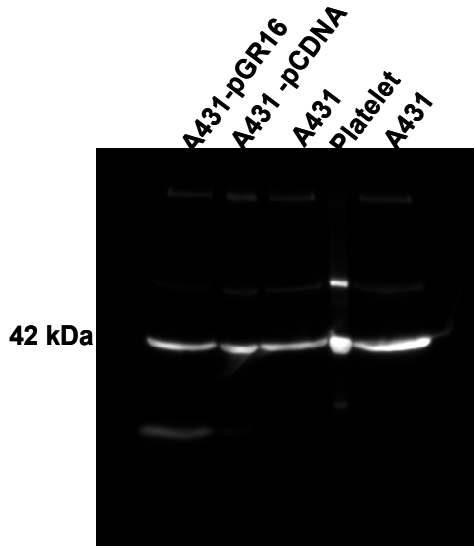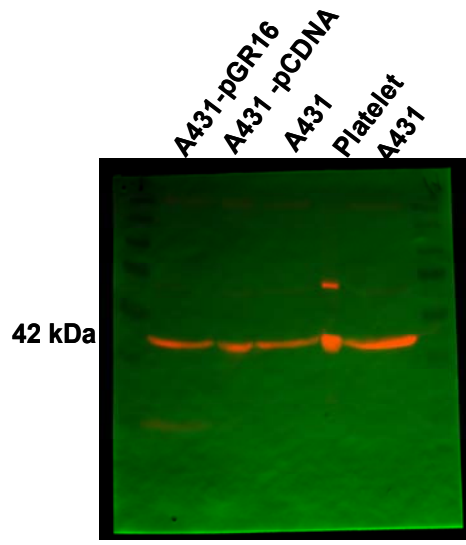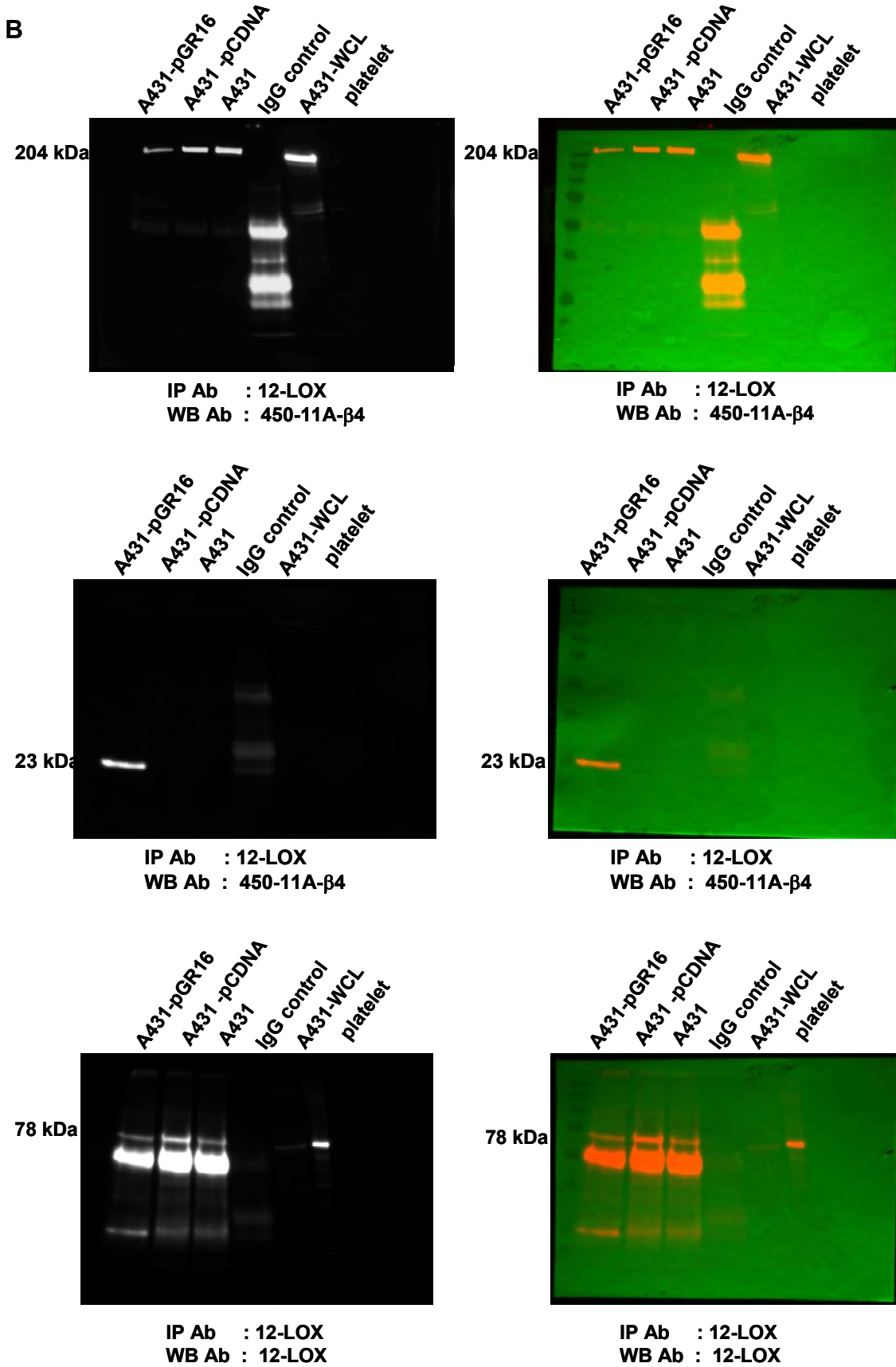
Figure 3.7:   The β4 integrin region GR16, encoding FN1 and FN2 domains (aa 1126-1315 in pGR16) binds to 12-LOX and blocks the interaction between 12-LOX and full length β4 integrin. (A) A431 cells were transiently transfected with the expression construct pGR16. *Western blot* of *whole cell lysates* shows the expression of GR16 (Myc-tagged), 12-LOX and β4 integrin. Myc antibody was used to detect the GR16 fragment of β4 integrin (23 kDa) and 12-LOX pAb for 12-LOX (78 kDa) detection and β4 -450-11A for full length β4 integrin (~200 kDa). Actin mAb was used for the actin loading control. (B) Cell lysates were *immunoprecipitated* with a polyclonal antibody against 12-LOX and immunoblotted for the Myc tag of GR16 (target band ~ 23 kDa), or full-length β4 integrin (target band, ~205 kDa) and also 12-LOX (78 kDa). 10% SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C. Endogenous Myc (115 kDa) band is below the level of detection unless figure contrast is increased.

Note: In (A) and (B) images on the right are overlays to show molecular weight marker.

WB : Myc (GR16- tag)

WB : Myc (GR16- tag)

WB : 12-LOX

WB : 12-LOX
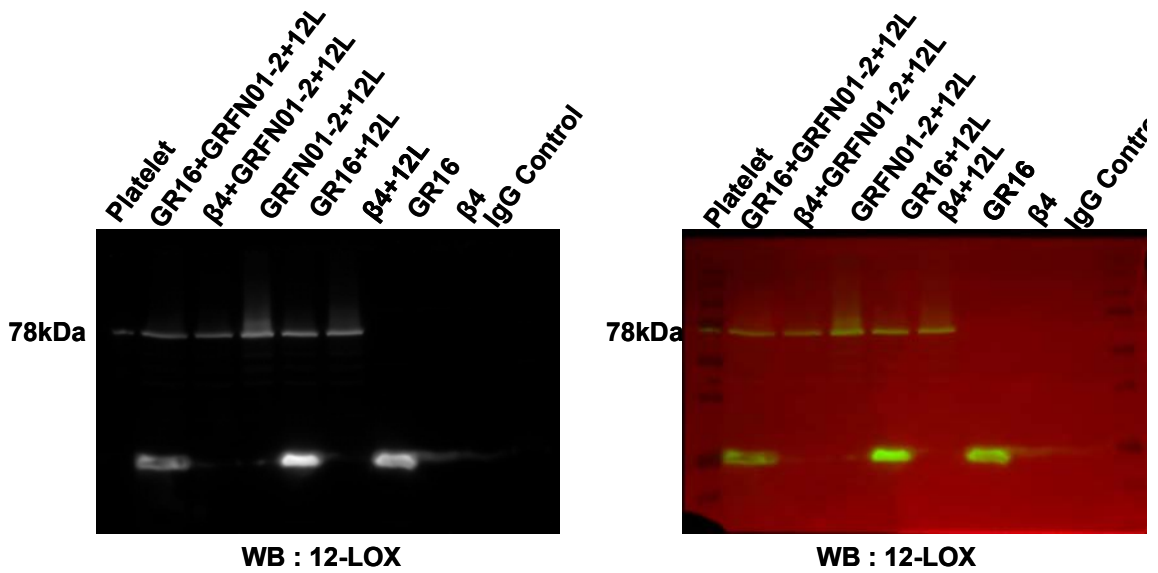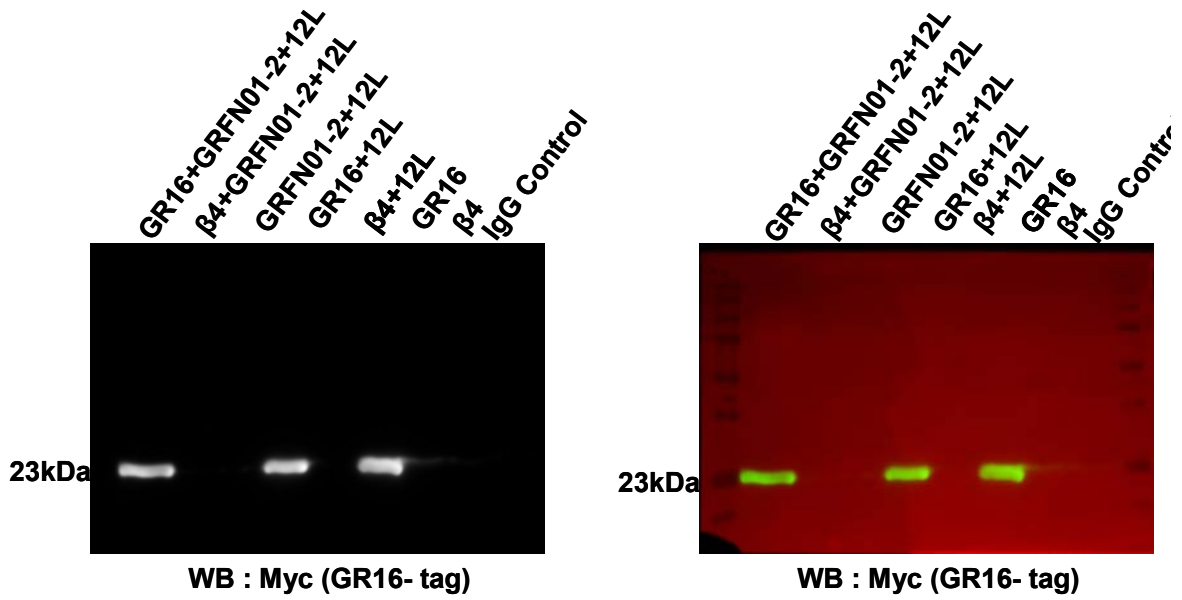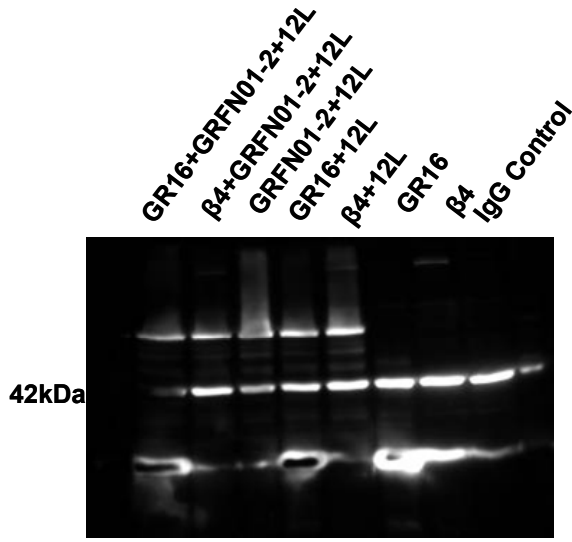
WB : Actin

WB : Actin

WB : 450-11A β4

WB : 450-11A β4

B

23kDa

IP Ab   : 12-LOX
WB Ab : Myc (GR16-tag)

23kDa

IP Ab   : 12-LOX
WB Ab : Myc (GR16-tag)

Reprobe with β4 Antibody 450-11A:

203kDa

WB Ab : 450-11A β4

204kDa

WB Ab : 450-11A β4
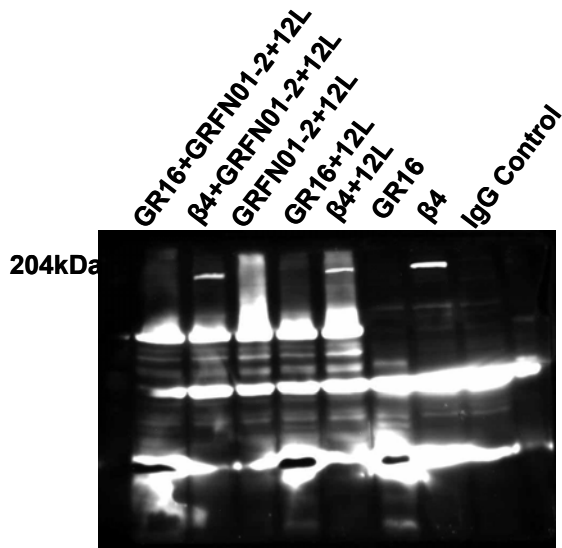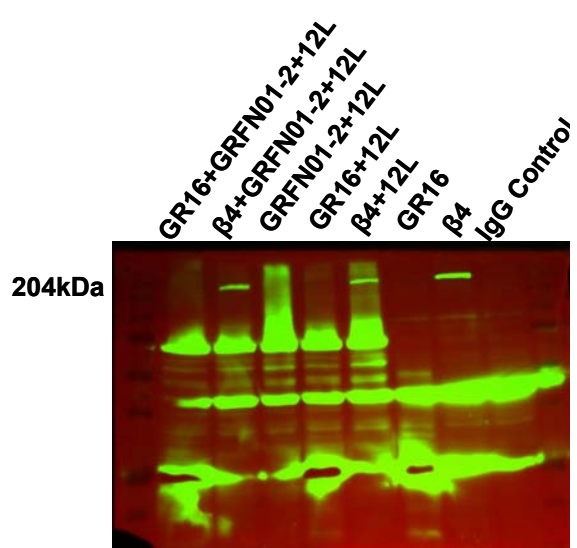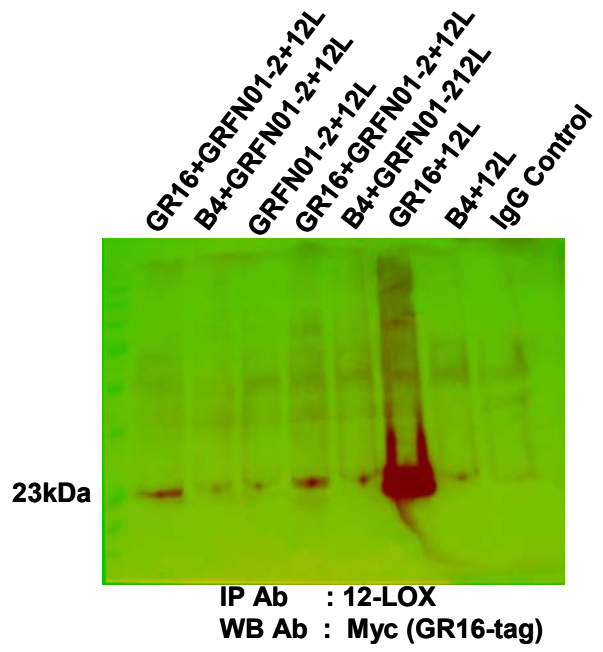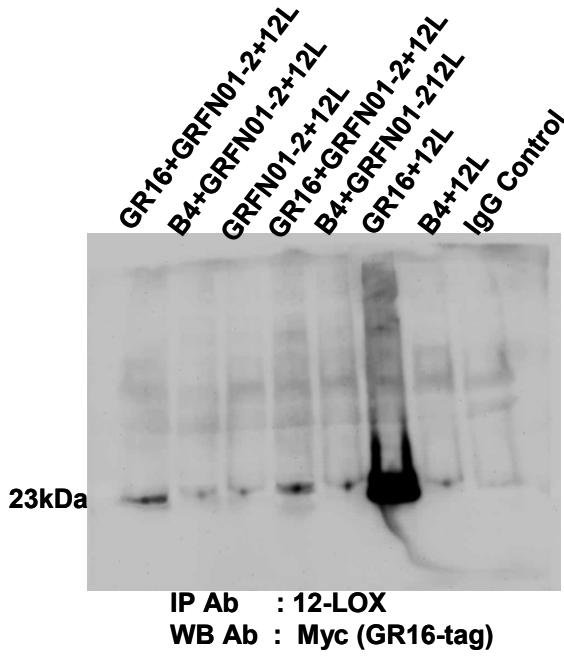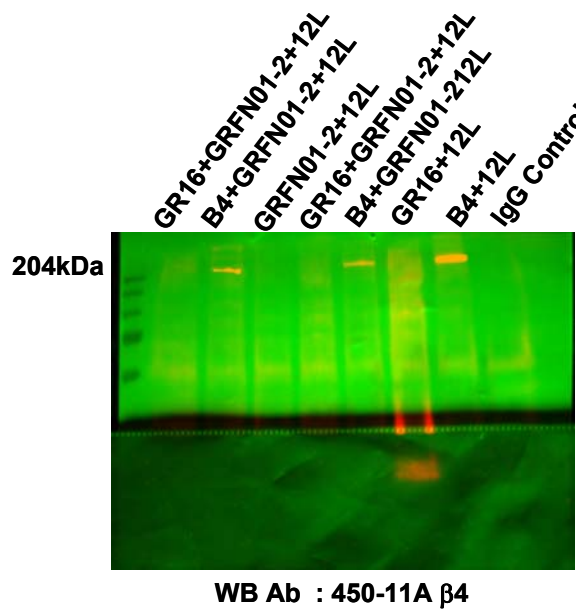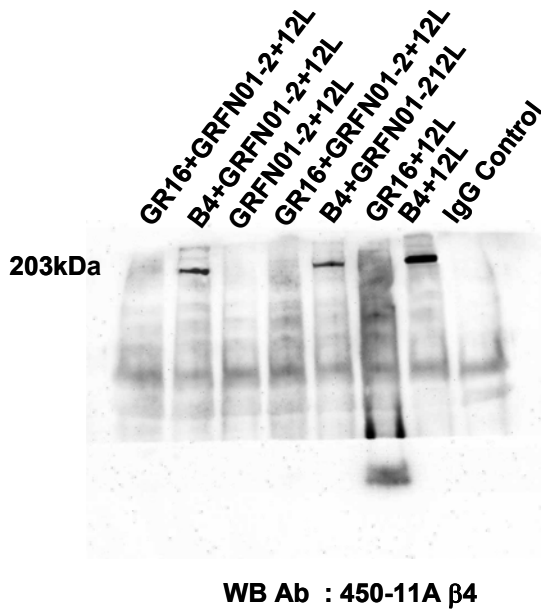
Reprobe with 12-LOX antibody 7225:

WB Ab : 12-LOX   **WB Ab : 12-LOX**
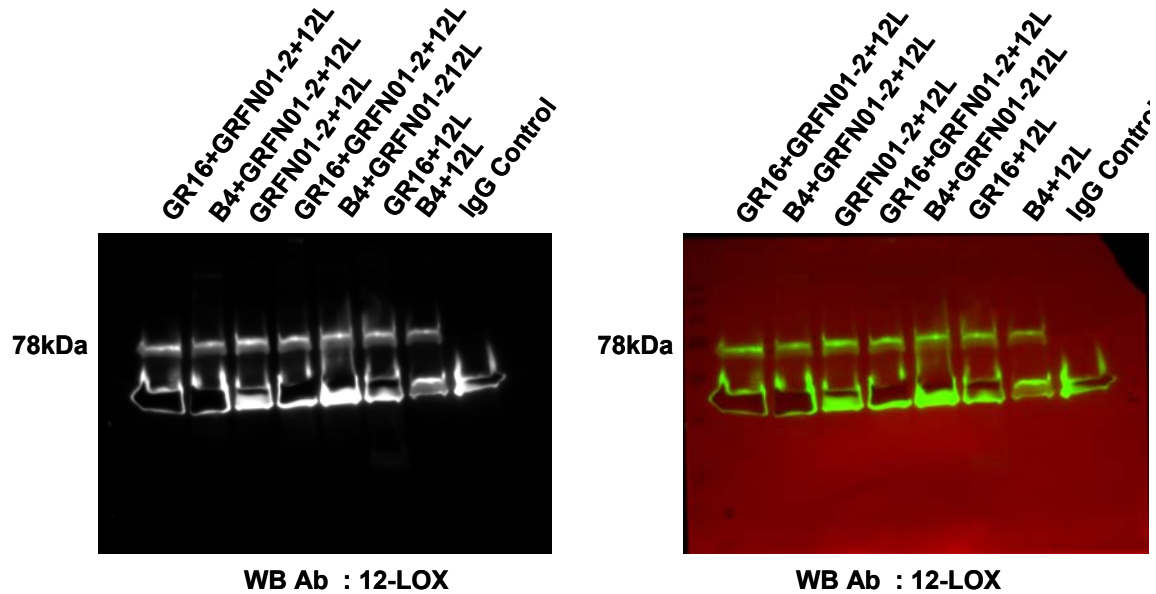
Figure 3.8: The β4 integrin FN01-2 domain (residues 1137-1147), encoded by pGRFN01-2, binds to 12-LOX, competes with GR16 (1126-1315) and full length β4, and reduces the interaction between 12-LOX and GR16 or full length β4 integrin. CHO cells were transiently transfected with the expression construct pGRFN01-2, pGR16, β4, and, 12-LOX in different combinations. (A) Western blot of *whole cell lysates* shows the expression of all transfected constructs, GR16 (Myc tagged, 23kDa), 12-LOX (78 kDa), full length β4 integrin (~ 200 kDa) and actin loading control (42 kDa). Note: Expression of GR-FN01-2 is not shown here as 1 kda band is not visible. (B) Cell lysates were *immunoprecipitated* with pAb against 12-LOX and immunoblotted for Myc (target band ~ 23 kDa), or full-length β4 integrin (target band, ~205 kDa). 12-LOX was also confirmed.   10%

SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C.

Note: In (A) and (B) images on the right are overlays to show molecular weight marker.
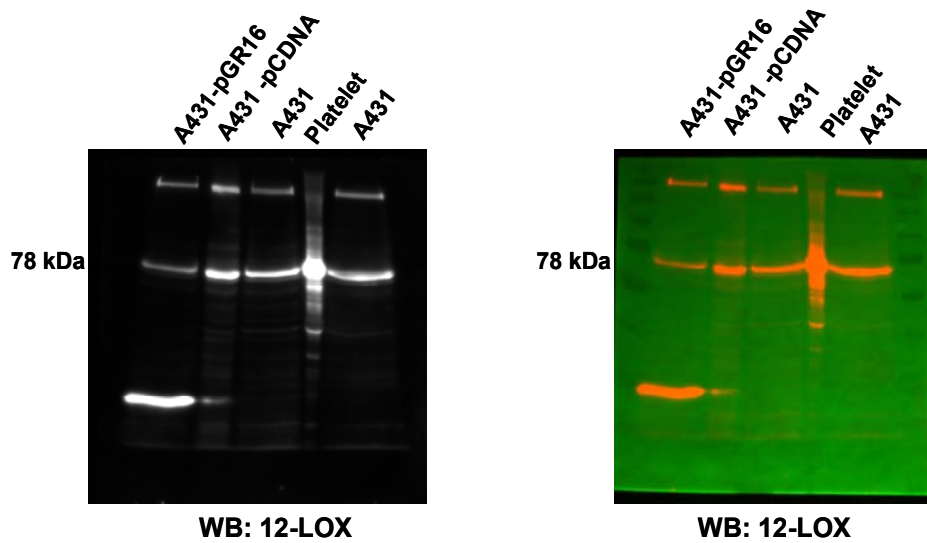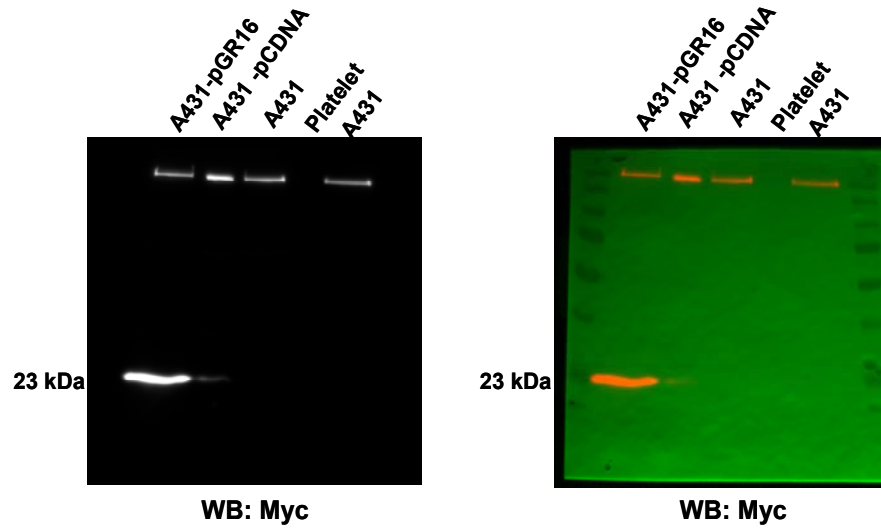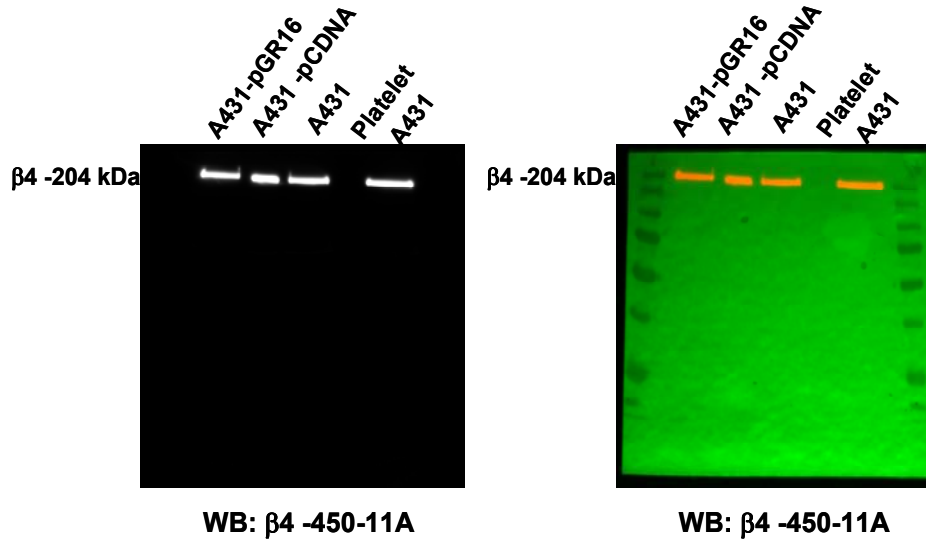
Figure (B) , Due to quenching of β4 probe, covered myc signal so digital camera picks β4.

A

B

| A431 | A431-GR16 | A431-PcDNA (EV) | A431-GR16-12-HETE | Serum Free |
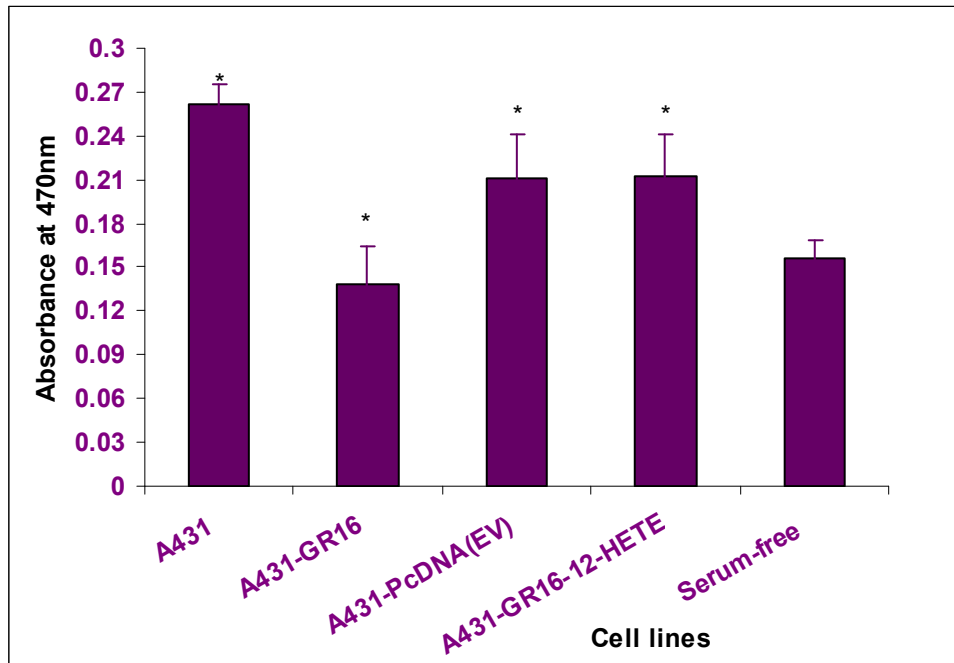
Figure 3.9: Cell migration and invasion assay using parental A431 cells and the A431-GR16 stable cell line. A431-GR16 shows 40% less migration compared to A431-EV and parental A431. Add back of 12(S)-HETE rescued A431-GR16 cell migration. (A) Quantitative colorimetric analysis (OD_{570}) of the migrated cells, (B) Bright field images of transwell migrated cells stained with Diff-Quick at 100X magnification. Data shown are significant, *, $p < 0.05$, comparing cell invasion of A431-GR16 with A431 wild-type and A431-EV cells.
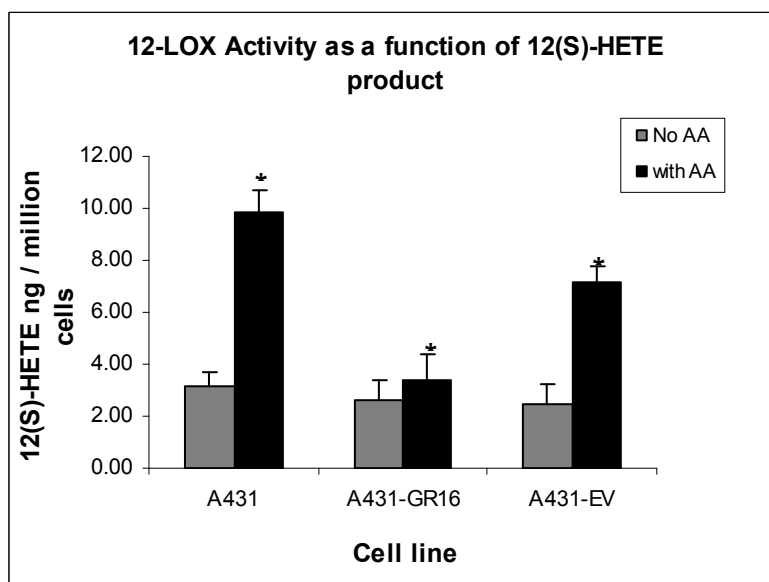
Figure 3.10: LC-MS measurements of 12-LOX activity from an A431 stable cell line expressing the GR16 construct or pCDNA3.1 -empty vector (EV).

The cells were treated with 10 μM AA for 6 hours and the medium was collected.

The samples were processed as described in Methods and subjected to mass spectrometry. A431-GR16 significantly attenuates 12(S)-HETE production compared to A431-EV. Data shown are significant, *, $p < 0.05$.
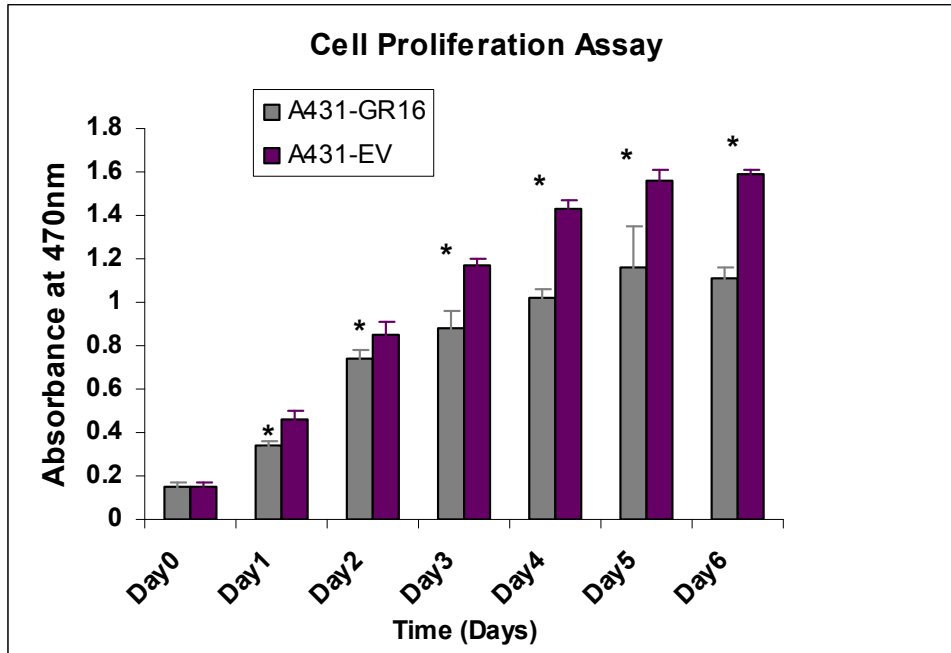
Figure 3.11:   Cell proliferation assay (MT-PMS assay). A431-GR16 or A431-EV

cells were seeded in a 96 well plate and assayed every day for 6 days. The

reading was measured at absorbance 470 nm. There is a 30% reduction in cell

proliferation when 12-LOX is blocked by the GR16 fragment of β4 integrin (Gray)

compared to EV (purple). Data shown are significant, *, $p < 0.05$, by student t

test; there is a significant decrease in cell proliferation in the A431-GR16 stable

transfectant when compared to A431-EV everyday over the period of 6 days.
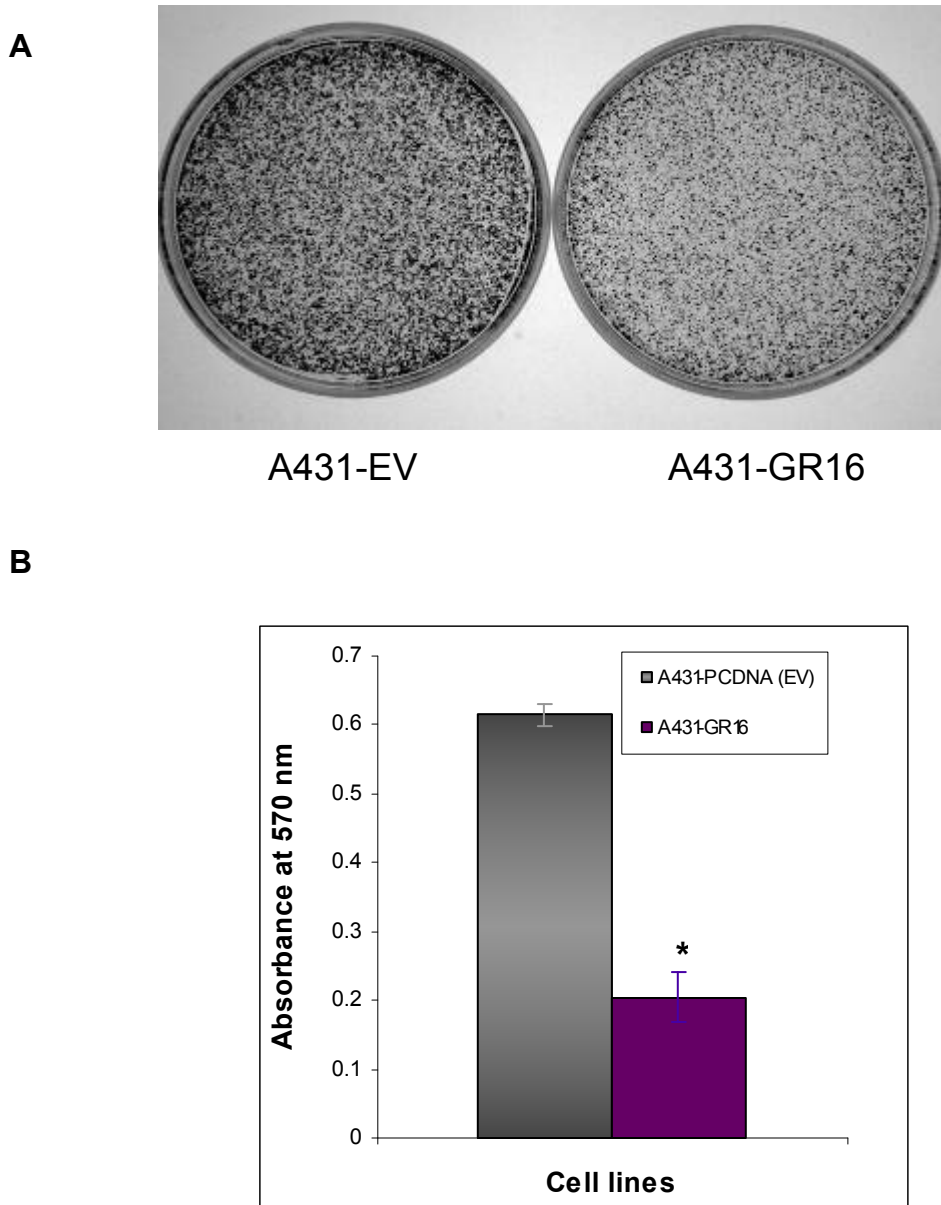
Figure 3.12: Colony formation assay. 1.5×10^3 cells either A431-GR16 or A431-EV cells were plated in triplicate in 60 mm plates and grown for 7 days. (A) The colonies were stained with crystal violet and photographed. Ten fields per plate were counted with bright field microscopy. (B) Quantification of cell-associated crystal violet was done by destaining the colonies and reading the absorbance at 570 nm. There is a 30% reduction in colony formation when 12-LOX is blocked

by the GR16 fragment of $\beta 4$ integrin compared to EV. Data shown are significant, *, $p < 0.05$, by student t test. There is significant decrease in cell colony formation in the A431-GR16 stable transfectant when compared to A431-EV.
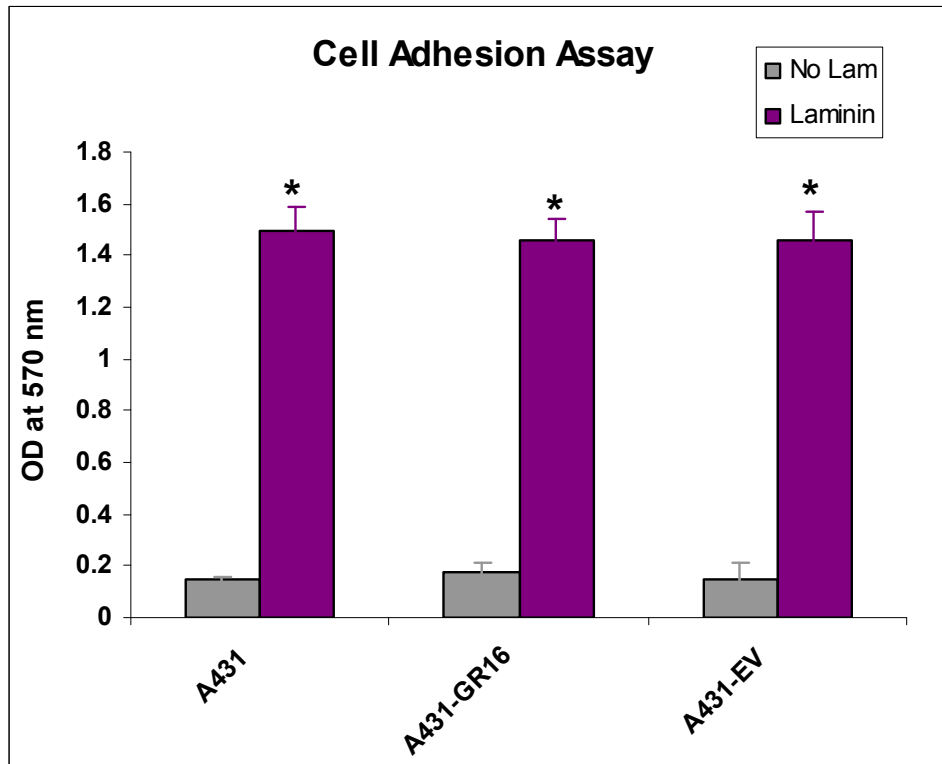
Figure 3.13: Cell Adhesion Assay. Parental A431, or transfectant A431- GR16 or A431-EV cells were plated in triplicate 5 x10^3 cells on laminin-coated 96 well plates and incubated for 1 hour. Adherent cells were fixed and stained with crystal violet. Absorbance was measured at 570 nm by destaining the cells with 10% acetic acid. The results show no difference in cell adhesion function when 12-LOX is blocked by the GR16 fragment of β4 integrin compared to EV and parental A431. There is no significant difference between samples since, *, $p >$ 0.05, by student t test. There is no significant change in cell adhesion in the A431-GR16 stable transfectant when compared to A431-EV and A431 wild-type.

A
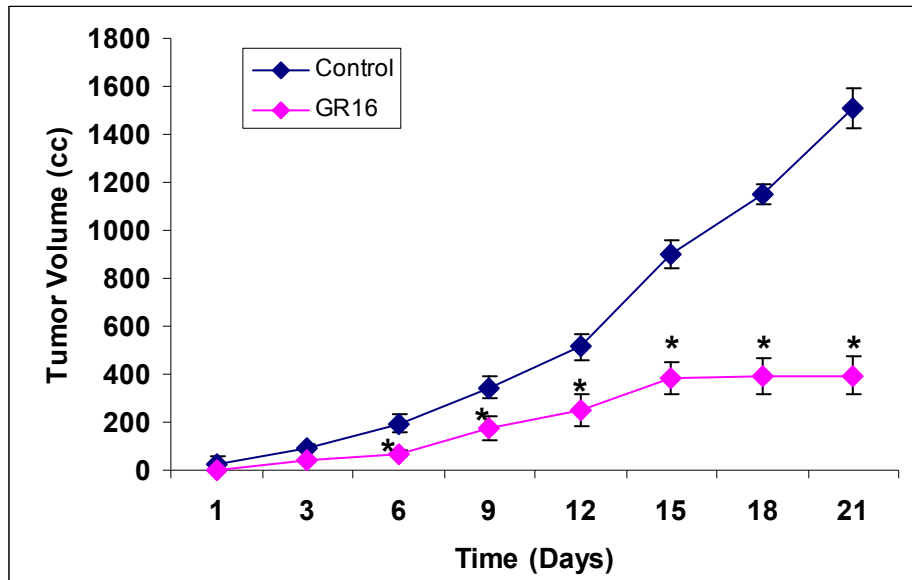
B

Control-A431-EV

Test-A431-GR16

C

Control-Empty Vector
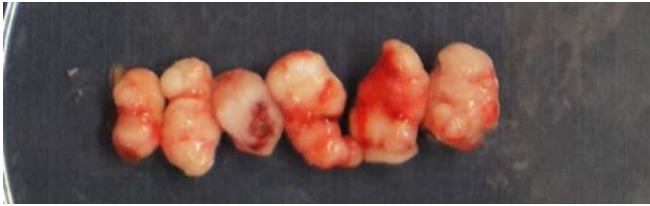
Test-GR16
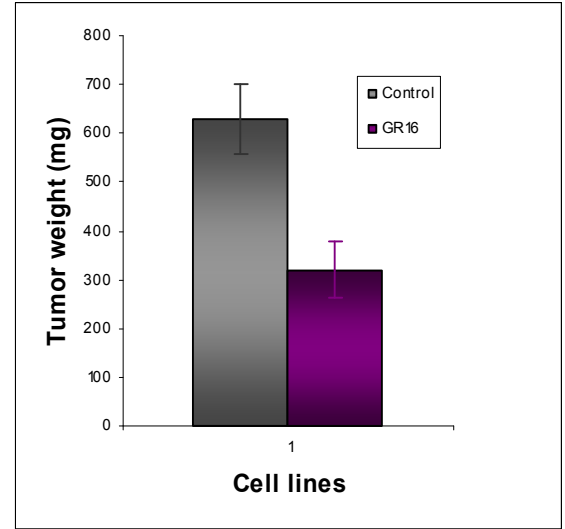
Figure 3.14:   Decreased tumor growth in GR16 transfected A431 cells.   (A) Growth kinetics of tumors derived from mice with A431 transfectants over a period of 3 weeks shows decreased growth in mice with the GR16 fragment compared to EV. Data shown are statistically significant, *, $p < 0.05$, by student t test.   A431-GR16 shows significantly reduced tumor growth compared to the A431-EV group over the period of 4 weeks. (B) Tumors are attenuated ~ 2 fold in mice receiving A431-GR16, encoding the competitive binding fragment of β4 integrin, (C) Tumors excised from mice with A431-GR16 are half the size and weight compared to A431-EV.   Data shown are statistically significant, *, $p <$ 0.05, by student t test. A431-GR16 shows significantly reduced tumor weight.

A
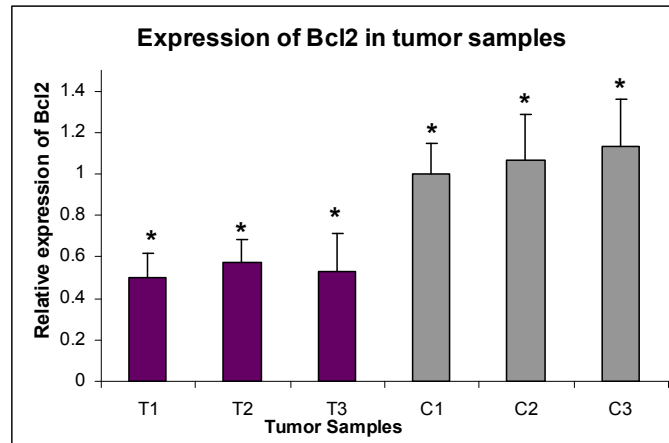
B
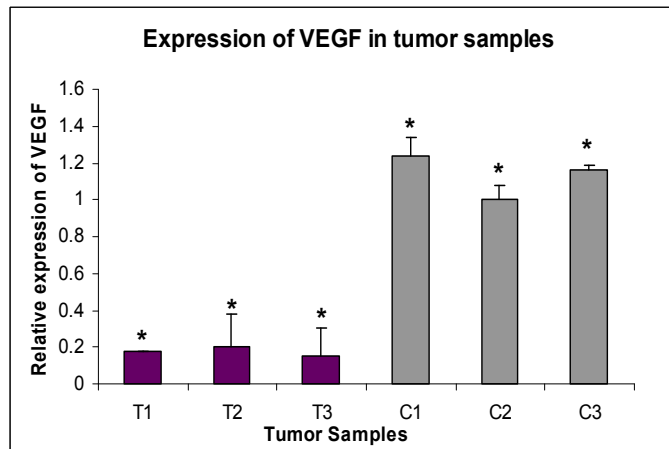
C
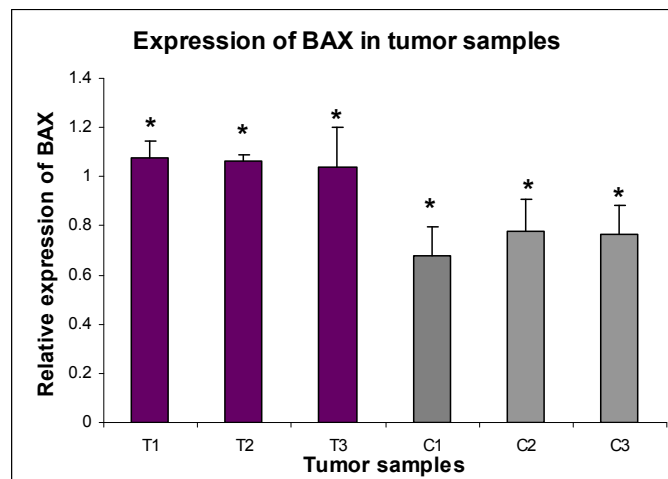
D
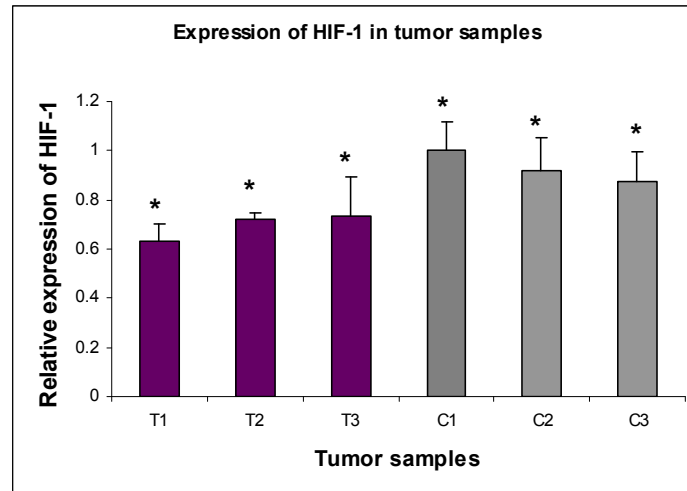
Figure 3.15: Real time PCR results from cDNA samples of tumor tissue, 3 tumor tissue samples per group were analyzed. (A) Relative expression of anti-apoptotic gene Bcl2 is higher in A431-EV compared to A431-GR16. (B) Relative expression of angiogenesis marker gene VEGF is higher in A431-EV compared to A431-GR16. (C) Relative expression of pro apoptotic gene BAX is higher in A431-GR16 compared to A431-EV. (D) The difference in the relative expression of the HIF1 gene in A431-GR16 and A431-EV is not very significant since the, *, $p > 0.05$, from student t test. The data shown are significant in A, B and C, as,*, $p < 0.05$, by student t-test when comparing the A431-GR16 group to A431-EV, which indicates there is an increase in apoptosis and a significant decrease in angiogenesis.

A   **B**
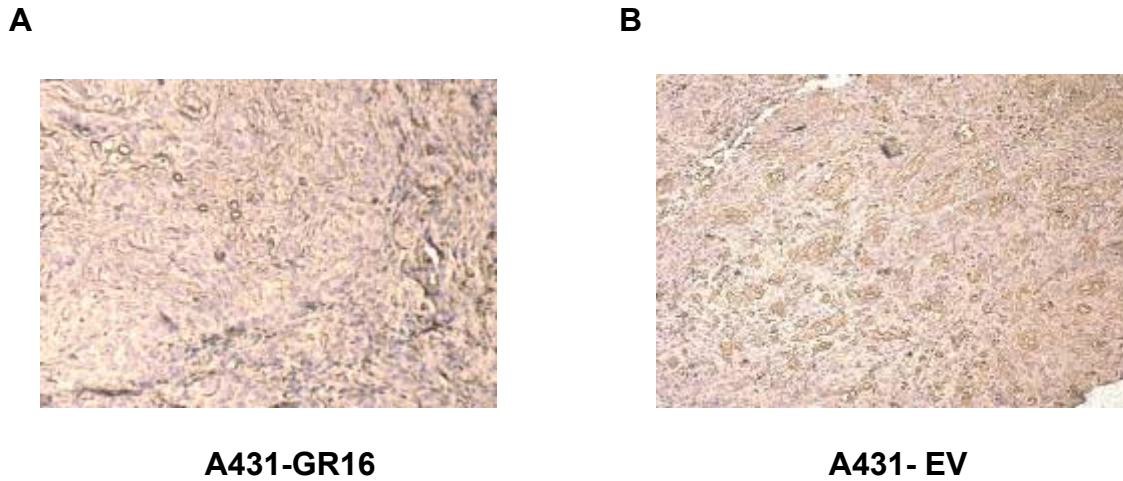
A431-GR16   **A431- EV**

Figure 3.16: Immunohistochemical staining with VEGF antibody showing brown Immunostaining for VEGF of A431 transfectant tumor tissue samples (magnification 40X). A431- GR16 (A) shows less VEGF staining compared to A431-EV (B), indicative of more angiogenesis in A431-EV, which is blocked by the presence of the GR16 fragment. Data are based on the evaluation of 3 sections in each group and a similar trend was observed in all samples.

A   **Test-GR16**

1 2 3 4 5 6 7 8

Myc-23kDa

WB : Myc (GR16-tag)

1 2 3 4 5 6 7 8

23kDa

WB : Myc (GR16-tag)

Test-GR16 reprobed for Actin

1 2 3 4 5 6 7 8

42kDa

WB : Actin

1 2 3 4 5 6 7 8

42kDa
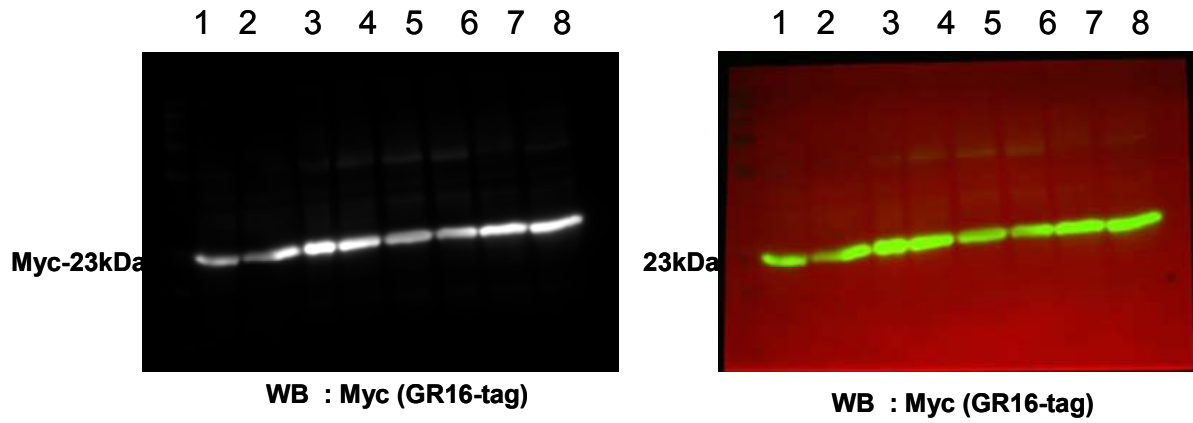
WB : Actin

B   Control-Empty Vector

1 2 3 4 5 6 7 8

WB : Myc

1 2 3 4 5 6 7 8

WB : Myc
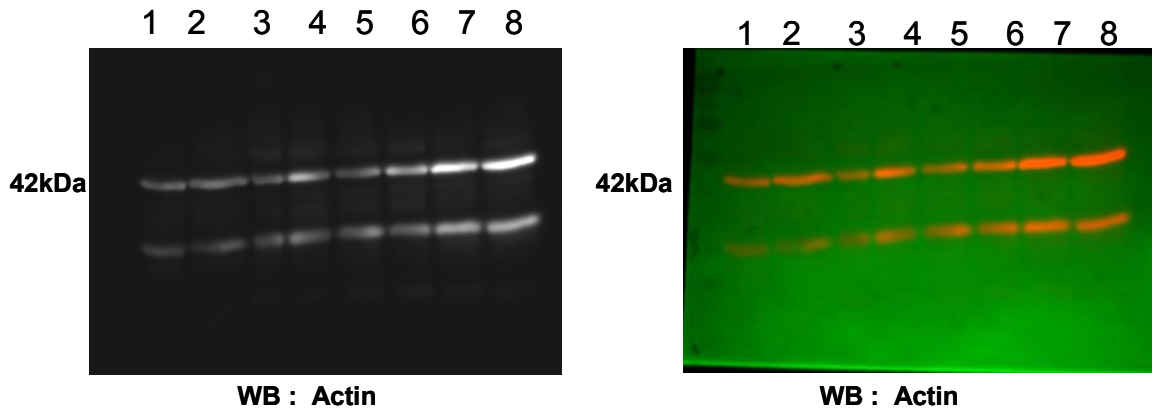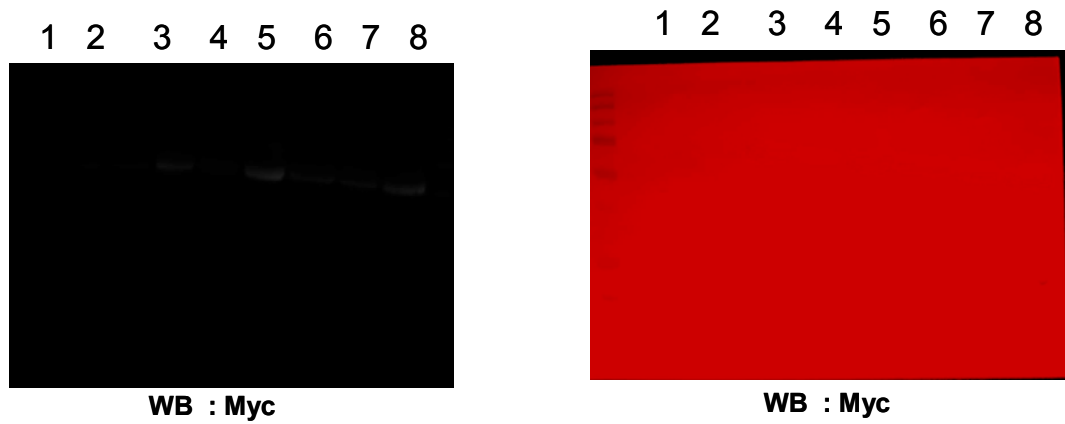
Control-Empty vector reprobed with Actin
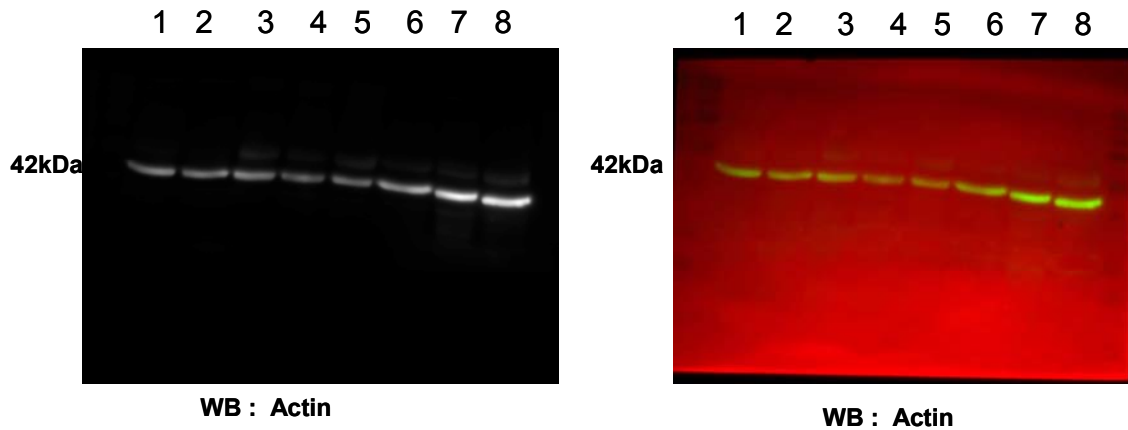
Figure 3.17: Western blot results of protein derived from the homogenized tumor tissue from 8 mice showing the expression of Myc-tagged GR16 (23 kDa) in A431-GR16 (A), and its absence in control mice injected with A431-EV transfectants (B). 10% SDS gel was run at 130V at 4°C and transferred to nitrocellulose membrane at 0.4A for 1.5 hours at 4°C. Myc mAb was used to detect the GR16 fragment of β4 integrin. Endogenous Myc (115 kDa) is present in all blots as a faint band 115 kDa.

Note: In (A) and (B) images are overlays to show molecular weight marker.

CHAPTER 4 : Discussion

4.1 Discussion

Evidence from previous studies has shown that enzymes from the AA pathway of eicosanoid biosynthesis, such as cycloxgenase and lipoxygenase, play important roles in cancer progression and metastasis (Honn, Tang et al. 1994; Yamamoto, Suzuki et al. 1997). Lipoxygenases are involved in various cancers including prostate, breast, pancreatic, and lung cancer. They have been recently identified in ovarian cancer (Chang, Liu et al. 1993; Kim, Gu et al. 1995; Krieg, Kinzig et al. 1995; Natarajan, Esworthy et al. 1997; Nie, Hillman et al. 1998; Ding, Kuszynski et al. 1999; Timar, Raso et al. 1999; Aran, Bryant et al. 2011; Guo, Liu et al. 2011). The AA metabolite, 12(S)-HETE, was shown to be involved in various cellular functions important in the metastatic cascade of tumors such as survival, adhesion, invasion and motility as well as angiogenesis, due to fact that 12-LOX is involved in variety of signaling pathways (Honn, Tang et al. 1994; Tang, Chen et al. 1996; Trikha, Timar et al. 1997; Tang, Nie et al. 1999; Nie, Tang et al. 2000; Krishnamoorthy, Jin et al. 2010; Krishnamoorthy and Honn 2011). Therefore, given the wide distribution of P-12-LOX in various human cancers (Honn, Tang et al. 1994) and its correlation with pathologic stage and grade (Gao, Grignon et al. 1995), and the effects elicited by its metabolite, understanding the regulation of this enzyme may have important therapeutic regulations. The physical interaction between 12-LOX and $\beta4$ was first identified through yeast two-hybrid screening, confirmed by co-immunoprecipitation in A431 cells, and further recapitulated by ectopically expressed 12-LOX and the

cytoplasmic tail of β4 integrin in CHO or PC-3 cells (Tang, Finley et al. 2000). Another yeast two hybrid study identified CLP, DTRAP-1, and, the ΔK12H4.8 as potential interacting partners and regulators of 5-LOX (Provost, Samuelsson et al. 1999). The direct interaction between 5-LOX and CLP has been demonstrated to inhibit actin polymerization and to interfere with the binding of CLP to F-actin (Provost, Doucet et al. 2001). Similarly, our group has shown direct interaction of 12-LOX with β4 integrin and also has demonstrated that extracellular stimulation of β4 by 3E1 in A431 cells subsequently triggers a signaling mechanism that recruits 12-LOX to the cytoplasmic domain of β4 and also increases 12-LOX activity (Tang et al., unpublished observation). The uniquely long cytoplasmic domain of the β4 integrin subunit protein has been implicated in connecting the α6β4 integrin complex with the cytoskeletal elements *via* cytoplasmic components of hemidesmosomes (Schwarz, Owaribe et al. 1990; Spinardi, Ren et al. 1993; Borradori and Sonnenberg 1996). The distal regions of the β4 tail have been implicated in interactions with plectin and the other major hemidesmosomal components BPAG1 and BPAG2 (Wilhelmsen, Litjens et al. 2006). In this study we have defined for the first time the region in the cytoplasmic tail of β4 critical for interacting with 12-LOX. CHO cells were used to carry out these interaction studies as they express α6 but not β4 and 12-LOX. We performed domain mapping by making deletion mutants of β4 integrin and co-transfecting them with 12-LOX in CHO cells and looking for the loss of interaction. Co-immunoprecitation studies revealed that the FN01-2 (1136 -1147 aa) domain in the cytoplasmic tail of β4 integrin is critical for interacting with 12-

LOX. Residues 1137-1147 span a portion of the FN1 region of β4 integrin that is within the first FNIII repeat. Until now not much has been known about this FN1 region and no important interactions have been characterized. The first tandem pair of FNIII domain has been reported to provide a high level of insight that should facilitate structure-function studies (de Pereda, Wiche et al. 1999). The other fibronectin pairs have been reported to be involved in protein binding. FN2 domain has been known to interact with the hemidesmosomal components such as plectin, BPAG1, and BPAG2. In a similar study, Biffo et al, showed that p27^{BBP} interacts with β4 integrin *in vivo* thus linking β4 integrin to intermediate filament cytoskeleton. A yeast two-hybrid screen using β4 cytodomain confirmed the interaction. Both by yeast two-hybrid and *in vitro* assay they showed that p27^{BBP} interacts with the two NH$_2$ -terminal FNIII region that is important for hemidesmosome formation and also signaling (Biffo, Sanvito et al. 1997). Many phosphorylation sites have been located in the connecting segment (CS) and FNIII pair 3 and 4, which when phosphorylated by Src family kinase (SFK) leads to recruitment of Shc and Shp2 mediating downstream signaling (Dans, Gagnoux-Palacios et al. 2001; Shaw 2001; Bertotti, Comoglio et al. 2006). Recent studies have identified Y1494 as an important phosphorylation site on β4 integrin for signaling and promoting tumor development and progression (Dutta and Shaw 2008). We made point mutations in the most important phosphorylation sites as predicted by internet tool, Netphos; S1140, S1151, Y1157, and double mutation S1151+Y1175 within the FN01 region and also Y1494 as mentioned in literature (Dutta and Shaw 2008). S1140 and Y1157

when mutated show weak binding with 12-LOX when compared to S1151 mutation and full length β4 and the double β4 mutants, S1151+Y1157 (changed to A1151+F1157) which show strong interaction with 12-LOX. Interestingly Y1494 mutation, shown previously to play an important role in tumor progression, resulted in loss of interaction between 12-LOX and β4. Thus, we infer from these results that tyrosine mutations play an important role. S1140, Y1157 and Y1494 are potentially important phosphorylation sites that hinder the interaction of 12-LOX with integrin β4. We have shown that FN01-2 (11aa, 1137-1147 residues) is important for interaction with 12-LOX; the S1140 phosphorylation site is within the region of interest while both the tyrosine sites which hinder the interaction are located outside this region. Even these sites, located at a distal end are able to have an impact on the interaction which of 12-LOX and β4 which demonstrated their potential role in cancer progression. Shaw et al have already shown Y1494 as the master phosphorylation site in β4 and its role in tumorgenesis. Interestingly when the region (11aa, 1147-1157) containing this phosphorylation site Y1157 is deleted we still see interaction of integrin β4 with 12-LOX. This may be because of structural stability of the integrin β4 on deletion of this region. The other possibility may be it leads to exposure of the FN01-2 region (11aa, 1137-1147), which makes it more accessible for 12-LOX. Proteins have complex 3D structure that makes it difficult to answer this question. Designing experiments to study the role of this phosphorylation site Y1157 would help us to better understand this inconsistency.

The interaction of 12-LOX with β4 or the β4 deletion mutants when over expressed in CHO cells was found to be independent of 3E1 stimulus. Furthermore, mass spectrometry data for 12-LOX activity revealed that these interactions were able to induce increased 12(S)-HETE production in the presence of extracellular stimuli. Our lab has also shown that β4 integrin ligation regulates 12-LOX activity through c-Src kinase (Dilly et al., Unpublished data). Treatment with Src- family kinase inhibitor PP2, showed decrease12-LOX activity on integrin ligation indicating Src phosphorylation is important for 12-LOX activity.

Furthermore, we have shown that GR16 (FN1 and FN2), fragment of β4 integrin is sufficient to block the interaction of 12-LOX with full length β4 in A431 cells. The A431 cell line is well characterized with respect to its expression of α6β4 integrin and 12-lipoxygenase protein. There are numerous papers in the literature documenting the role of α6β4 integrin in A431 cell motility and invasion. Previously we have shown that the cytoplasmic tail of β4 was able to block the interaction of 12-LOX with full length β4 (Tang K., et al unpublished observation). Here we have shown that the β4 fragment called β4-GR16 about 23 kD size (~200 aa) consisting of FN1 and FN2 region when stably transfected in A431 cells could block the interaction of 12-LOX and full length β4. This β4-GR16 fragment interacts with the 12-LOX may be in the cytoplasm even before its translocation to the membrane. Also we showed that GR-FN01-2 (1137-1147, 11 aa fragment of β4) could compete with GR16 and full length β4 to interact with 12-LOX, thus confirming that the 11 aa acid region GR-FN01-2 is important for interaction with 12-LOX.

It has been previously demonstrated that $\beta4$ integrin plays an important role in tumorogenesis and tumor progression (Lipscomb and Mercurio 2005). Our studies have previously shown that 12-(S)HETE protects tumor cells from apoptosis and stimulates invasion, motility and angiogenesis (Honn, Tang et al. 1994; Gao and Honn 1995; Tang, Renaud et al. 1995; Nie, Tang et al. 2000; Nie, Tang et al. 2000) as well as increases integrin expression (Tang, Grossi et al. 1993) . Studies have reported that suppression of integrins affects downstream eicosanoids, indicating integrins indirectly regulate eicosanoids. For example, Mitchel K. et al have shown that suppression of integrin $\alpha3\beta1$ in breast cancer cells reduces COX-2 gene expression and inhibits tumorigenesis. They showed COX-2 as the downstrean effector of $\alpha3\beta1$ in tumor cells (Mitchell, Svenson et al. 2010). Recent studies by Chuang JY. et al have shown connective tissue growth factor (CTGF), binds to integrins and inhibits COX-2 expression and cell motility in oral cancer through signal transduction pathways that involve FAK, PI3K, Akt and c-Jun (Chuang, Yang et al. 2011). We have for the first time shown that an integrin can directly interact and regulate an eicosanoid enzyme, namely 12-LOX (Tang et al , unpublished observation). The impact of blocking this interaction was seen in 12-LOX activity, cell migration, and cell proliferation and tumor growth. In this study we have not only mapped the domain of $\beta4$ cytoplasmic tails that inteacts with 12-LOX but we have also shown that, 12-LOX activity, and cell migration is regulated by integrin interaction with 12-LOX.

As mentioned above, 12(S)-HETE induces a plethra of cellular responses in tumor cells. It is shown to protect tumor cells from apoptosis and induce

invasion, motility, and angiogenesis (Honn, Tang et al. 1994; Gao and Honn 1995; Nie, Tang et al. 2000; Nie, Tang et al. 2000; Nie, Tang et al. 2000; Guo, Liu et al. 2011) as well as surface expression of $\alpha_v\beta_3$ integrin (Tang, Grossi et al. 1993). We previously showed that $\beta4$ ligation mediates translocation of 12-LOX from the cytosol to the membrane and interacts with $\beta4$ integrin. This interaction causes the increase in 12-LOX activity which results in increased 12-(S)HETE production (Tang et al , unpublished observation). In this study we have shown here that when interaction of the full length $\beta4$ integrin and 12-LOX is blocked by the ectopically $\beta4$ fragment GR16, there is 40% reduction in 12-LOX activity and exogenous addition of 12-HETE rescues the activity.

Several studies have shown chemotactic factors such as EGF stimulate rapid dissembly of hemidesmosmes in cells with rapid coincident with formation of lamilipodia and membrane ruffles (Rabinovitz, Toker et al. 1999). Phosphorylation of $\beta4$ cytoplasmic domains increases in response to stimuli such as EGF and PMA, both of which dissemble hemidesmosomes . It is reported that activation of PKC $-\alpha$ plays an important role in phosphorylation of the cytoplasmic tail but also in dissembly of hemisdesmosomes (Rabinovitz, Toker et al. 1999; Dans, Gagnoux-Palacios et al. 2001). Our group has also shown that ligation of $\beta4$ integrin likely contributes to cell migration via the phosphorylation of 12-LOX and subsequent production of 12(S)-HETE (Dilly A. et al., to be submitted) and also reported that 12-LOX also plays a role in cell migration (Tang et al., unpublished observation). Migration of A431 cells stimulated with laminin or commercially available $\beta4$ stimulating antibody (3E1) following

treatment with EGF was reduced in the presence of BMD 122, a select P-12-LOX enzymatic inhibitor. Additionally exogenously added 12(S)-HETE has been shown to stimulate tumor cell motility, invasion and migration, endothelial cell retraction, release of proteases, and processes dependant on PKC activation (Liu, Marnett et al. 1994; Liu, Khan et al. 1995)

in the present study, we showed that when 12-LOX interaction with β4 integrin is blocked by the β4 fragment GR16, there was a 40% reduction in cell migration and cell proliferation. Addition of exogenous 12(S)-HETE rescued the cells and increased migration of the A431 –GR16 cells similar to A431-EV stable transfectant cells. Also we have shown decreased cell proliferation and colony formation.

We believe that the β4 fragment GR16 functions as a dominant negative by interacting with 12-LOX in the cytosol and preventing it's binding to native, full-length, membrane-associated β4 and thereby prevents 12-LOX activation. In support of this observation, when A431 stable transfectant cells ectopically expressing the β4 fragment GR16 were injected subcutaneously into athymic nu/nu mice, there was a significant decrease in tumor growth when compared to A431 stable transfectant cells expressing the empty vector.

12(S)-HETE has been shown to protect tumor cells from apoptosis and induce angiogenesis (Honn, Tang et al. 1994; Gao and Honn 1995; Nie, Tang et al. 2000; Nie, Tang et al. 2000) as well as surface expression of $\alpha_v\beta_3$ integrin (Tang, Grossi et al. 1993). In W256 cells, apoptosis induced by antisense oligonucleotides, and 12-LOX inhibitors was followed by a rapid down regulation

of Bcl-2 protein, and thus a dramatic decrease in the Bcl-2/Bax ratio that could be suppressed by Bcl-2 over-expression (Tang, Chen et al. 1996). A study by Pidgeon et al. showed a similar decrease in Bcl-2 levels response to Baicalein or BHPP and the reduction was coupled to increase in Bax levels (Pidgeon, Kandouz et al. 2002). The results suggest that the P-12-LOX pathway plays an important physiological role in regulating apoptosis (Tang, Chen et al. 1996). Our RT-PCR data show decreased expression of Bcl-2 in murine tumors of A431-GR16 transfectants where the interaction of 12-LOX with full length $\beta 4$ is blocked. Immunostaining with CD31 antibody, which detects the presence of endothelial cells, showed that the vascular networks in tumors derived from A431-EV transfectant cells were sinusoidal in pattern and well- developed in structure. In contrast, in tumors derived from A431-GR16 cells, endothelial cells were present but were randomly distributed and did not form an organized vascular network. The increased angiogenicity of A431-EV transfectants was also confirmed by RT-PCR for VEGF gene expression and brown immunostaining of tumor tissues for VEGF. 12(S)-HETE is known to increase the surface expression of $\alpha v \beta 3$ integrin, an integrin predominantly associated with angiogenic blood vessels in tumors and human wound tissue in both rat aorta endothelial cells and murine pulmonary micro-vascular endothelial cells (Honn, Tang et al. 1994), (Brooks, Clark et al. 1994). 12-HETE also acts as a mitogen for micro-vascular endothelial cells at low concentration of serum (Tang, Diglio et al. 1995). In a study by Nie et al., they showed that 12-LOX transfected PC3 cells have enhanced tumor angiogenicity in a mouse model; and a similar

study on breast cancer by Conolly and Rose found that 12-LOX over-expression enhanced tumor angiogenesis in a fat pad animal model (Connolly and Rose 1998; Nie, Hillman et al. 1998). Thus, 12(S)-HETE and 12-LOX appear to act as potent angiogenesis enhancers in cancer cells.   VEGF and bFGF (basic fibroblast growth factor) are known to elevate expression and activation of various integrins that are involved in angiogenesis (Klein, Bikfalvi et al. 1996; Byzova, Goldman et al. 2000). Shaw et al. have shown that phosphorylation state of residue Y1494 of β4 integrin is important for VEGF expression and stimulates angiogenesis thereby contributing to tumor progression (Dutta and Shaw 2008). Thus, β4 signaling domain is also known to drive VEGF- mediated retinal neo-vascularization (Nikolopoulos, Blaikie et al. 2004).   Pharmaceuticals that disrupt β4 signaling have been sought as useful therapy agents, for VEGF driven retinal neo-vascularization and angiogenesis (Giancotti 2007). Our RT-PCR data show decreased VEGF expression when the interaction of 12-LOX with full length integrin is blocked in A431-GR16 transfectant tumor cells.

This additional data supports our hypothesis that 12-LOX association with membrane-bound native β4 alters 12-LOX activity resulting in increased cellular migration, and proliferation while the fragment of β4 that contains the 12-LOX binding motif, can be used to prevent this interaction.

Integrins are known to predominantly signal through recruitment and activation of Src- family kinases. Dilly et al have recently shown that Src kinase activity is required for β4 integrin mediated regulation of 12-LOX, which may in turn regulate metastatic potential of cancer cells (Dilly et al, unpublished data).

Most integrins recruit FAK through their β subunit. As well as activating signaling through PI3K to AKT / protein kinase B (PKB) through phosphotidylinositol-3, 4, 5-triphosphate (PtdIns (3, 4, 5) P3), FAK functions as a recruiter of Src to focal adhesions. Src phosphorylates CAS which recruits Crk-Doc-180 complex thereby activating Rac which further activates PAK, JNK, JUN and NFkB. FAK also activates extracellular signal regulated kinase (ERK) and mitogen activated protein kinase (MAPK). Certain integrins $\alpha_5\beta_1$, $\alpha_1\beta_1$, $\alpha v\beta_3$ are coupled to SFKs such as Fyn and Yes through α subunits (Miranti and Brugge 2002; Schwartz and Ginsberg 2002; Giancotti and Tarone 2003; Guo and Giancotti 2004) Signaling of β4 function has been studied and has been shown that it contains multiple tyrosine phosphorylation sites in its cytoplasmic tails thus it behaves as binary RTK (Mainiero, Pepe et al. 1995; Mainiero, Murgia et al. 1997; Gagnoux-Palacios, Dans et al. 2003). $\alpha 6\beta 4$ can directly interact with Src, phosphorylating five major tyrosine phosphorylation sites thereby activating Ras- ERK/MAPK and PI3K signaling (Mainiero, Pepe et al. 1996; Shaw, Rabinovitz et al. 1997; Dans, Gagnoux-Palacios et al. 2001). These pathways activated through SFK are sufficient to induce cell migration, cell survival and proliferation. Both SFK mediated tyrosine phosphorylation and PKC mediated serine phosphorylation of β4 cytotail prevents assembly of hemidesmosome, thus opposing its cytoskeleton bonding(Dans, Gagnoux-Palacios et al. 2001; Rabinovitz, Tsomo et al. 2004). Interestingly β4 also combines with several RTKs, including EGF-R, ErbB2, Met and Ron (Mariotti, Kedeshian et al. 2001; Santoro, Gaudino et al. 2003; Guo, Pylayeva et al. 2006). Apart from its classical signalling pathway it

also has been shown that integrin signalling is mediated by 12-LOX by various researchers (as mentioned below).

Pidgeon et al demonstrated that over-expression of platelet-type 12-LOX in prostate cancer PC3 cells or epithelial cancer A431 cells significantly extended their survival and delayed apoptosis when cultured under serum-free conditions by affecting the expression and localization of the vitronectin receptors, $\alpha v\beta 3$ and $\alpha v\beta 5$, in PC3 and A431 cancer cell lines (Pidgeon, Kandouz et al. 2002).

The study by Yeung et al showed that inhibition of 12-LOX or PKC resulted in inhibition of dense granule secretion and attenuation of both, $\alpha IIb\beta 3$ aggregation and activation. However, activation of PKC downstream of 12-LOX inhibition rescued agonist-induced aggregation and integrin $\alpha IIb\beta 3$ activation. Further, inhibition of 12-LOX had no effect on PKC-mediated aggregation indicating that 12-LOX is upstream of PKC (Yeung, Apopa et al. 2012). Also it has been observed that the expression of platelet type 12-lipoxygenase upregulated Integrin αv mRNA and showed increased angiogenesis in esophageal squamous cell carcinoma (ESCC) (http://www.rescancer.com/stomach-cancer/32887.html, FEB 2012).

One recent study have shown that suppression of $\alpha_6\beta_4$ in breast cancer cells reduced cycloxgenase -2 gene expression and also inhibits tumorogenesis, and invasion . Thus they showed a novel role for COX-2 as a downstream effecter of $\alpha 3\beta 1$ in tumor cells, thereby identifying potential therapeutic target to inhibit breast cancer (Mitchell, Svenson et al. 2010). Dilly et al have recently shown 12-LOX is regulated through $\beta 4$ integrin via Src activation, which may in

turn regulate metastatic potential of cancer cells (Dilly et al, unpublished data). Similarly by expressing GR16 or using a commercial 11 aa small peptides, crossbinding to the 12-LOX binding domain and we can suppress full length β4 integrin   interaction with 12-LOX as a potential therapeutic target for cancer therapy.

Collectively our previous studies in combination with the novel findings here show that 12- LOX and β4 interaction enhances cell motility, invasion, and increases activity leading to   tumor progression (Tang et al., unpublished observation). However when this interaction is blocked by using this small 11 aa fragment, we were able to reduce tumor progression by reducing cell motility, invasion and 12-LOX activity. Validation of these observations using the *in vivo* model confirms the functional relevance of blocking 12-LOX and β4 interaction. Further studies are required to understand the potential role of this interaction in assembly or disassembly of hemidesmosomes in cancer progression. The work presented in this study has established a new conceptual framework with significant implications for carcinomas and other pathologies that involve β4 integrin signaling through 12-LOX. This study has provided a novel target for therapeutic intervention and has revealed a new dimension in integrin and eicosanoid biology.
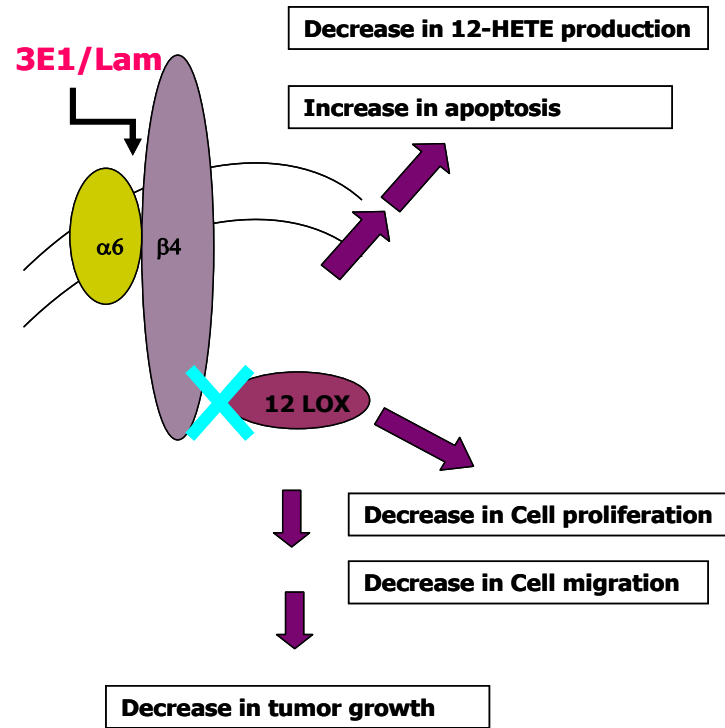
Figure 4.1: Schematic overview of the study. We have demonstrated that the interaction of 12-LOX with full length $\beta 4$ can be blocked with $\beta 4$ fragment that decreases 12-LOX activity, cell invasion, cell proliferation and also attenuates tumor growth. This represents a novel therapeutic target.

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ABSTRACT

A DEFINED DOMAIN OF INTEGRIN SUBUNIT β4 DETERMINES INTERACTION WITH 12-LOX AND PLAYS A ROLE IN TUMOR PROGRESSION.

by

SANGEETA JOSHI

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Advisor: Dr. Kenneth V. Honn

Major: Pathology

Degree: Doctor of Philosophy

A yeast two-hybrid screen and subsequent studies identified the cytoplasmic tail of β4 integrin as a 12-Lipoxygenase (12-LOX)-interacting protein. The physical interaction of β4 with 12-LOX regulates 12-LOX activity and leads to cell migration, which suggests that 12-LOX interacts both physically and functionally with β4 to promote cancer progression._ Here we describe for the first time the critical interaction domains that unite β4 integrin and 12-LOX. The amino acid residues spanning 1126-1157, correspond to a domain in the cytoplasmic tail of β4 that is important for 12-LOX binding and activation.

Expressed fragments of the β4 (FN1 and FN2) domains are sufficient to block the interaction of 12-LOX with full length β4 leading to decreased cell survival, cell migration, and proliferation and tumor growth. These domains represent novel therapeutic targets.

AUTOBIOGRAPHICAL STATEMENT

SANGEETA JOSHI

Education

- **Ph.D. in Pathology**   Wayne State University, Detroit, MI; 2012

- **M.S. in Molecular Biotechnology** Wayne State University, Detroit, MI; 2005

- **M.S. in Medical Lab Technology** Mumbai University, Mumbai, India; 1999

- **B. Sc in Microbiology** Mumbai University, Mumbai, India; 1997

Honors/ Awards
- 3rd place poster presentation, Wayne State University 13th Annual Graduate Student Research Day (2008)
- 2nd place poster presentation, BAM, 10th Microbiology Research Colloquium (1997),University of Mumbai

Publications

- Yande Guo, Wenliang Zhang, Craig Giroux, Yinlong Cai, Prasanna Ekambaram, Ashok-kumar Dilly, Andrew Hsu, Senlin Zhou, Krishnarao Maddipati, Jingjing Liu, **Sangeeta Joshi**, Stephanie C. Tucker, Menq-Jer Lee, Kenneth V. Honn. *Identification of the Receptor for the Human Lipoxygenase Product 12(S)-HETE that Regulates Prostate Tumors and their Progression* (J Biol Chem. 2011 Sep 30;286(39):33832-40. Epub 2011 Jun 28)

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- **Sangeeta Joshi,** Keqin Tang, Yande Guo, Yinlong Cai, , Ashok Kumar Dilly, Stephanie C. Tucker and Kenneth V. Honn. *A Novel Interaction Between 12-Lipoxygenase and Integrin subunit β4 plays a role in cancer progression*. (In progress will be submitting soon to Cancer Research)