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Mechanistic paradigm of Leptin receptor activation revealed by complexes with wild type and antagonist leptins

by

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Dissertation presented to the faculty of Sciences in partial fulfillment of the requirements for the degree of Doctor of Science: Biochemistry
Academic year 2013-2014

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Front Cover illustration:

The figure presents artistic impression of the proposed octameric signaling complex formed by the leptin induced dimerization of two pre-dimerized receptors. Each of the pre-dimerized receptor has been colored as green-light pink or yellow-grey while Leptin has been colored as deep pink. While predimerized receptor structure is based on the SAXS solution structure of monomeric receptor, the structure shown for 4:4 complex was obtained from the SAXS based solution structure of the quaternary complex.

K. Moharana – Mechanistic Paradigm of Leptin Receptor Activation Revealed by its Complexes with Wild type and Antagonist Leptins.

Ph.D. Thesis, Faculty of Sciences, Ghent University, Ghent, Belgium. Publicly defended in Ghent, 10 January 2014.

ACKNOWLEDGEMENT

First and foremost, I would like to thank my promoter Prof. Dr. Savvas N. Savvides. It has been my privilege to conduct my doctoral dissertation under his supervision. Even though my research work has gone through many ups and downs, his persistent support has been the center of the success of this research project. I would be grateful to him for all his contributions in the form of time, effort and funding because of which now I can materialize my childhood dream of becoming a research scientist. I would also like to thank my co-promoter Prof. Dr. Jan Tavernier. His continual support and contributions are so crucial without which this project would not have completed.

My special thanks to Dr. Lennart Zabeau, Prof. Dr. Frank Peelman, Dr. Kenneth Verstraete and Dr. Bjorn Vergauwen for their relentless brain-storming discussions and crucial suggestions which have enlighten me time to time and helped me establishing many decisive steps during my doctoral research work. I am gratefully thankful to Bert Remmerie and Kenneth Verstraete for teaching me the mammalian cell culture techniques. Special thanks to Kenneth Verstraete and Jonathan Elegheert for their support in learning the experimental and computational aspects of small angle X-ray scattering.

I would like to thank all my present and former colleagues of the Unit for Structural biology and biophysics at Laboratory of Protein Biochemistry and biomolecular engineering: Jan Felix, Wouter Van Putte, Jonathan Elegeert, Geraldine Buysschaert, Nathalie Bracke , Ruben Van der Meeren, Bjorn Vergauwen, Bert Remmerie, Ann Dansercoer, Yehudi Bloch, Aleksandra Fulara, Erwin Panneckouke and Sammy detry. All of them have always extended their helpful hand whenever I was in need. I would also like to thank all the members of Mass spectrometry group including Isabel Vandenberghe and Dr. Gonzalez Van Driessche for all their support during my doctoral study. All the efforts contributed by our LProBE secretaries Mrs. Ingrid Anseau and Mrs. Rita Becu have helped in smooth functioning of the lab and thus I am thankful to them too. I would like to thank all the members of the doctoral examination committee for their time and effort in testing the rationality as well as intellectual capability of this research work. Many thanks to our collaborators Dr. Philippe Ringler and Prof. Dr. Henning Stahlberg (C-CINA, University Basel) for their important contributions in this study.

I would like to thank my family back in India, who have provided me much-needed emotional support during my stay in Belgium. Finally, I conclude by saying that I really had a great time in the city of Gent and the credit goes to my good old friends who have never made me feel as if I am away from my family, away from my home. So thanks to all my friends including Barsha, Sukumar, Anil and Ark.

Sincerely,

Kedar Moharana

OUTLINE

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ABBREVIATIONS

ΔG	Change in Gibbs free energy
ΔH	Change in enthalpy
ΔS	Change in entropy
µM	Micromolar
2D	Two dimensional
3D	Three dimensional
ACTH	Adreno-corticotrophic hormone
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
AgRP	Agouti-related protein
AH	Autoimmune hepatitis
AMPK	5' AMP-activated protein *kinase*
APB	Acceptor photobleaching
APC	Antigen processing cell
BCL-2	B-cell lymphoma-2
BCL-XL	B-cell lymphoma-extra large
β-ME	β-mercaptoethanol
BRET	Bioluminescence resonance energy transfer
CD	Clusters of differentiation
cDNA	Complementary Deoxyribo-Nucleic Acid
CLC	Cardiotrophin-like cytokine
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
CRH	Cytokine receptor homology
CRH1	First cytokine receptor homology
CRH2	Second cytokine receptor homology
CT-1	Cardiotrophin-1
db/db	Leptin receptor deficient strain
Dmax	Maximum dimension
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribo-Nucleic Acid
DTH	Delayed type hypersensitivity
DTT	Dithiothreitol
E. coli	*Escherichia coli*

EBI3	Epstein-Barr virus induced gene 3
EC_{50}	Half maximal Effective concentration
EM	Electron microscopy
eNOS	Endothelial Nitric oxide synthase
EPO	Erythropoietin
EpoR	Erythropoietin receptor
ERK	Extracellular signal-regulated kinase
ESRF	European synchrotron radiation facility
Fab	Antibody
FFF	Field flow fractionation
FLIM	Fluorescence Lifetime *Imaging*
FNIII	Fibronectin type III
FRET	Fluorescence resonance energy transfer
FSH	Follicular stimulating hormone
G-CSF	Granulocyte-colony stimulating hormone
GH	Growth Hormone
GlcNAc	N-Acetyl Glucosamine
GM-CSF	Granulocyte Macrophage-colony stimulating hormone
GMRalpha	GM-CSF Receptor alpha
GnRH	Gonadotropin-releasing hormone
$GnTI^{-/-}$	N-acetylglucosaminyltransferase I
gp130	Glycoprotein 130
GRB2	Growth factor receptor-bound protein 2
HCA	Hydrophobic cluster analysis
HEK	Human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hGH	Human Growth hormone
HIF-1α	Hypoxia inducible factor -1α
HLA-DR	Human leukocyte antigen D Receptor
HMW	High molecular weight
I(0)	Forward intensity
IC50	Half maximal inhibitory concentration
IFN-γ	Interferon γ
IGD	Immunoglobulin like domain
IgG	Immunoglobulin G

IMAC	Immobilized metal affinity chromatography
ITC	Isothermal titration calorimetry
JAK	Janus Kinase
JNK	c-Jun N-terminal kinase
K_D	Dissociation constant
kDa	Kilo Dalton
kV	Kilo volt
LBD	Leptin binding domain
$Leptin_{a1}$	Antagonist Leptin S120A/T121A
$Leptin_{a2}$	Antagonist Leptin L39A/D40A/F41A/I42A
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LMW	Low molecular weight
LPS	Lipo-polysaccharide
LR	Leptin receptor
LR_{ecto}	Leptin receptor ectodomain
LR_{IC}	Leptin receptor intracellular part
LR_{Ig}	Leptin receptor immunoglobulin like domain
LR_{TM}	Leptin receptor trans-membrane part
Luc	Luciferase
MALDI-TOF	Matrix-assisted laser desorption/ionization – Time of flight
MALLS	Multi-angle laser light scattering
Man	Mannose
MAPK	Mitogen-activated protein kinases
mAU	mili absorbance Unit
MIP-α	Macrophage Inflammatory Protein α
MLCK	Myosin light-chain kinase
mM	miliMolar
mRNA	Messanger Ribonucleic Acid
MS	Mass spectrometry
N	Stoichiometry
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer
nM	nanoMolar
NPY	Neuropeptide Y

NPY-Y1R	Neuropeptide Y receptor Y1
NPY-Y2R	Neuropeptide Y receptor Y2
NSD	Normalized spatial discrepancy
NTD	N-terminal domain
ob/ob	Obese mutant
ObR	Obesity receptor
OSMR	Oncostatin M receptor
P(r)	Distance distribution function
PBS	Phosphate Buffer Saline
PDB	Protein Data bank
PEI	Polyethyleneimine
PHA	Polyhydroxyalkanoates
PI3K	Phosphoinositide 3-Kinase
PKC	Protein kinase C
PLC-γ1	Phospholipase C γ1
pM	picoMolar
PMN	Polymorphonuclear Leukocyte
PMOC	Proopiomelanocortin
PTP1B	Protein-tyrosine phosphatase 1B
PYY_{3-36}	Peptide tyrosine tyrosine
RA	Rheumatoid arthritis
Rg	Radius gyration
RMSD	Root mean square deviation
rPAP1	rat pancreatitis-associated protein 1
RPM	Rotation per minute
SAXS	Small angle X-ray scattering
SCF	Stem cell factor
SD200	Superdex 200
SDS PAGE	Sodium dodecyl sulfate Poly acrylamide Gel electrophoresis
SEAP	Secreted embryonic alkaline phosphatase
SEC	Size exclusion chromatography
SH2	Src homology region 2
SHP2	Src homology region 2 domain-containing phosphatase-2
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of Transcription

TCCR	T-cell cytokine receptor
TH1	T helper cell type-1
TM	Transmembrane
TNF-α	Tumor necrosis Factor α
TSLP	Thymic stromal lymphopoietin
UV	Ultra violate
VEGF	Vascular endothelial growth factor
Vex	Exclusion volume
Vp	Porod volume
Wt-Leptin	Wild type leptin
γ_c	Common γ chain
YFP	Yellow fluorescent protein

1

Preface

This doctoral thesis entitled "*Mechanistic paradigm of Leptin receptor activation revealed by its complexes with wild type and antagonist leptins*" encompass research work done during four years of doctoral study. The primary objective has been the structural and mechanistic characterization of leptin mediated receptor activation and to unravel the mechanism of antagonism. The results and conclusions have been described here in following chapters.

Chapter 2, entitled "*Introduction to leptin/leptin receptor system*", gives detailed account of the available information pertaining to physiological and pathophysiological roles of leptin/leptin receptor system, the structural aspects of leptin and its cognate receptor and the proposed mechanisms of leptin mediated leptin receptor activation. In the end of this chapter, objectives of this doctoral research project have been briefly described.

In chapter 3, entitled "*Structural and mechanistic paradigm of leptin receptor activation revealed by complexes with wild type and antagonist leptins*", results obtained from the research work

with full length extracellular part of receptor with wild type and antagonist leptins have been presented. The results encompass first-of-it-kind biochemical, biophysical and structural analysis of the leptin induced receptor extracellular part oligomerization while pointing towards an unconventional mechanistic model for leptin receptor signaling complex. This chapter has been adopted from the manuscript currently undergoing peer review for publication.

Chapter 4, entitled "*Redundant role of FNIII domains in leptin mediated leptin receptor oligomerization*" comprises the biochemical and structural characterization of a truncated variant of leptin receptor extracellular part lacking the membrane proximal fibronectin type III (FNIII) domains. This chapter attributes to the experimental work leading to the discovery of the redundant role of the membrane proximal FNIII domains in agonist induced receptor oligomerization. The biochemical, biophysical and structural aspects of the resultant complexes have been presented. This chapter has been adopted from the manuscript prepared for publication.

In chapter 5, entitled "*Crystallization of leptin receptor and its complexes*" experimental procedures adopted for optimized expression of receptor and crystallization trial of the various receptor constructs alone and in complex with wild type and antagonist leptins have been described.

Finally, in chapter 6, this thesis has been concluded by providing a summary of the results obtained during this research work. The impact and future prospects of these results have been cited.

2

Introduction to Leptin/Leptin Receptor system

2.1 BACKGROUND

Leptin, a term derived from the Greek word "*leptos*" meaning "thin", was originally discovered as a protein hormone regulating food intake and energy homeostasis. Later, highly divergent pleiotropic activities of leptin have been unraveled, raising the necessity to understand its multi-dimensional aspects in human physiology and pathology. Leptin involves in diverse physiological processes ranging from immune system to reproduction to bone metabolism to energy homeostasis. Recent developments in leptin research have unmasked the darker side of leptin (patho)physiology, revealing its role in breast cancer and several autoimmune diseases and demanding investigation for a deeper understanding of leptin physiology. This four helical bundle cytokine transduces its signal via the membrane bound leptin receptor. Ironically, a consensus is still lacking in our understanding of the mechanistic paradigm of leptin dependent receptor activation, even though the scientific community has spent more than two decades studying this system. This chapter summarizes the available information and our current understanding of the structural, functional and mechanistic aspects of leptin and its cognate receptor.

2.2 FUNCTIONS OF LEPTIN

The primary function of leptin is to regulate the food intake by satiating appetite and thereby controlling the energy metabolism. Unlike ghrelin which is a fast acting hormone, leptin is a long term regulator of energy homeostasis. The originally-conceived “anti-obesity” role of leptin has rapidly changed with the discoveries of its broader pleiotropic functions in diverse physiological processes. These functions of leptin have been summarized as follows.

2.2.1 Anti-obesity Hormone

Leptin is primarily produced by stomach adipose tissue. It controls the appetite and increases the energy expenditure by signaling via its cognate receptors expressed in hypothalamic nuclei in brain.

A) Regulation of Leptin production: Sympathetic nervous system, that maintains the homeostasis in mammals, regulates the production of leptin (Rayner, 2001). The circulating leptin concentrations in blood has been found to be proportional to the adipose tissue content (Ahren et al., 1997; Maffei et al., 1995). Beside the adipose tissue mass, food intake also controls the leptin production. *In vitro* studies have shown that insulin and glucocorticoids synergistically act on the adipose tissue to upregulate the leptin mRNA levels and frequency of leptin secretion in a long term fashion (Fried et al., 2000). Increased leptin levels in obesity has been partly attributed to the chronic hormonal condition of hyperinsulinemia and high cortisol turnover. On the contrary, fasting leads reduction in insulin and enhancement in catecholamine levels in blood, thus causing downregulation of leptin production by adipose tissue.

B) Mechanism of appetite control by Leptin: Appetite is a more complicated phenomenon compared to what was conceived at the time of the discovery of leptin. It involves inputs from hypothalamus (parasympathetic nervous system), thyroid (hypothalamic-pituitary-adrenal axis), opoid receptor system along with involvement of hormones like neuropeptide Y (NPY), agouti-related peptide and ghrelin (Figure 2.1). Leptin still plays a critical role in both immediate and long term regulation of appetite. The immediate effects are manifested by the interaction of circulating leptin with the cognate receptor ObRb in brain hypothalamus initiating a cascade that triggers the synthesis of anorexigenic pro-hormone proopiomelanocortin (PMOC)

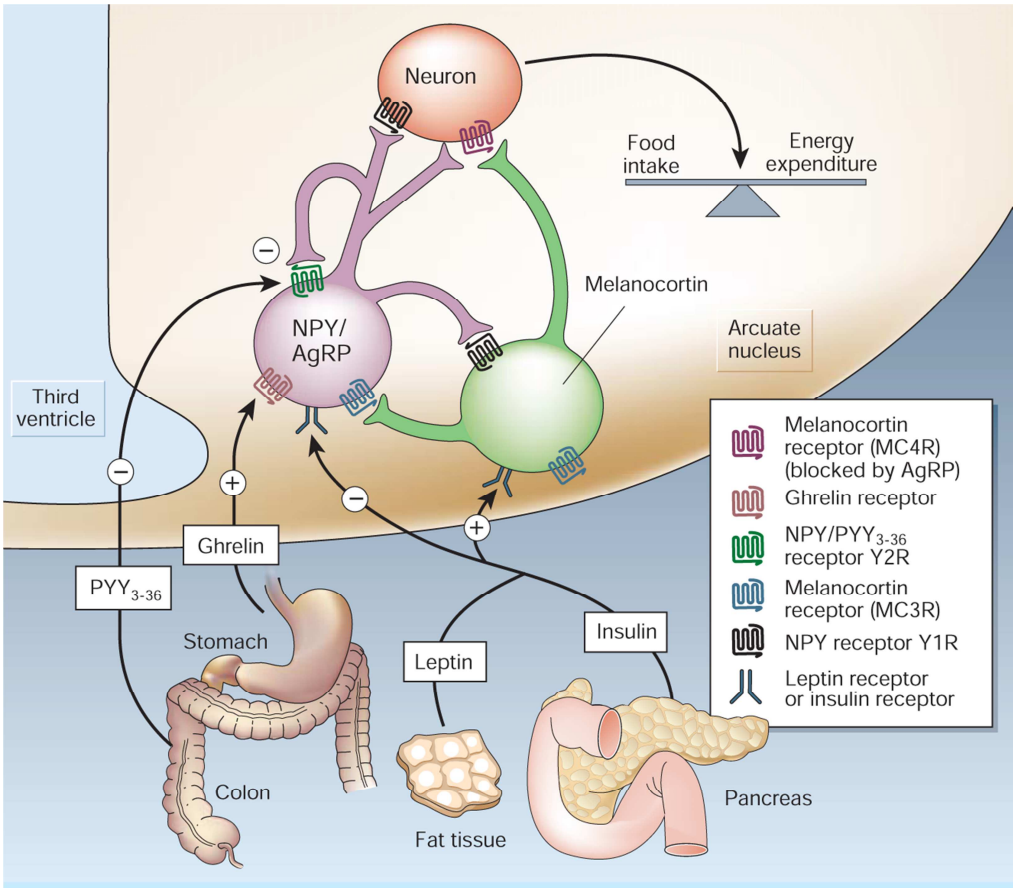

Figure 2.1. Hormones that control eating: Leptin and insulin (lower part of the figure) circulate in the blood at concentrations proportionate to body-fat mass. They decrease appetite by inhibiting neurons (centre) that produce the molecules NPY and AgRP, while stimulating melanocortin-producing neurons in the arcuate-nucleus region of the hypothalamus, near the third ventricle of the brain. NPY and AgRP stimulate eating, and melanocortins inhibit eating, via other neurons (top). Activation of NPY/AgRP-expressing neurons inhibits melanocortin-producing neurons. The gastric hormone ghrelin stimulates appetite by activating the NPY/AgRP-expressing neurons. Batterham *et al.*1 have now shown that PYY_{3-36}, released from the colon, inhibits these neurons and thereby decreases appetite for up to 12 hours. PYY_{3-36} works in part through the autoinhibitory NPY receptor Y2R. (Adopted from Schwartz and Morton, Nature, 2002)

causing decline of appetite and increased energy expenditure (Butler and Cone, 2002; Butler et al., 2000; Chen et al., 2000; Huszar et al., 1997; Marsh et al., 1999; Ste Marie et al., 2000). Leptin action on hypothalamus also causes reduction in production of orexigenic NPY and agouti-related protein (AgRP), thus resulting in loss of appetite and a feeling of satiety.

2.2.2 Role in Immune system: Innate immunity

The major roles of leptin in regulation of innate immunity includes activation of neutrophils, cytokine production by monocytes and macrophages, activation of antigen presenting cells (APCs) and cytotoxicity (Figure 2.2). A more detailed account of these roles are as follow.

A) Role in inflammation: The regulation of innate immunity by leptin is somewhat in twilight zone as not much direct evidence exist in literature. The circulating leptin level has been observed to be high during the inflammation by LPS, turpentine and cytokines (Faggioni et al., 2001; Matarese, 2000). Although there are evidences that the inflammation-induced and tumor-induced anorexia are not mediated by leptin (Lopez-Soriano et al., 1999), the linkage between leptin and inflammation have been demonstrated to be mediated by leptin induced IL-1β production (Luheshi et al., 1999).

B) Activation of monocytes and Macrophages: Leptin has been found to stimulate proliferation and activation of circulating monocytes *in vitro*, upregulating surface markers like HLA-DR, CD11b, CD11c and activation markers like CD69, CD38 and CD71 in human (Santos-Alvarez et al., 1999). Leptin has also found to stimulate proliferation of monocytes and dose-dependent secretion of proinflammatory cytokines like TNF-α, IL-6 in human *in vitro* (Kiguchi et al., 2009). *In vitro*, murine leptin stimulates the expression of CC chemokine ligand via the JAK2-STAT3 pathway. Dendritic cells have been shown to express leptin receptor ObRb on cell surface and in response to leptin, increased production of IL-8, IL-2, IL-6 and TNF-α and decreased production of MIP-1-α have been observed (Mattioli et al., 2005). Based on the observation that mast cells express both leptin and leptin receptor simultaneously, a paracrine mode of leptin stimulation in these cell lines have been proposed (Taildeman et al., 2009).

C) Activation of neutrophils: Neutrophils have been shown to express short forms of leptin receptor. Leptin signaling in these cells increases the CD11b expression, preventing apoptosis (Zarkesh-Esfahani et al., 2004). Similar to G-CSF, leptin has been shown as survival cytokine for the human polymorphonuclear neutrophils (PMN) (Bruno et al., 2005). Several studies have suggested the effect of leptin on the chemotaxis of neutrophils (Caldefie-Chezet et al., 2003; Faggioni et al., 2001; Ottonello et al., 2004). Leptin induces release of inflammatory cytokines by eosinophils and stimulate chemokinesis (Wong et al., 2007).

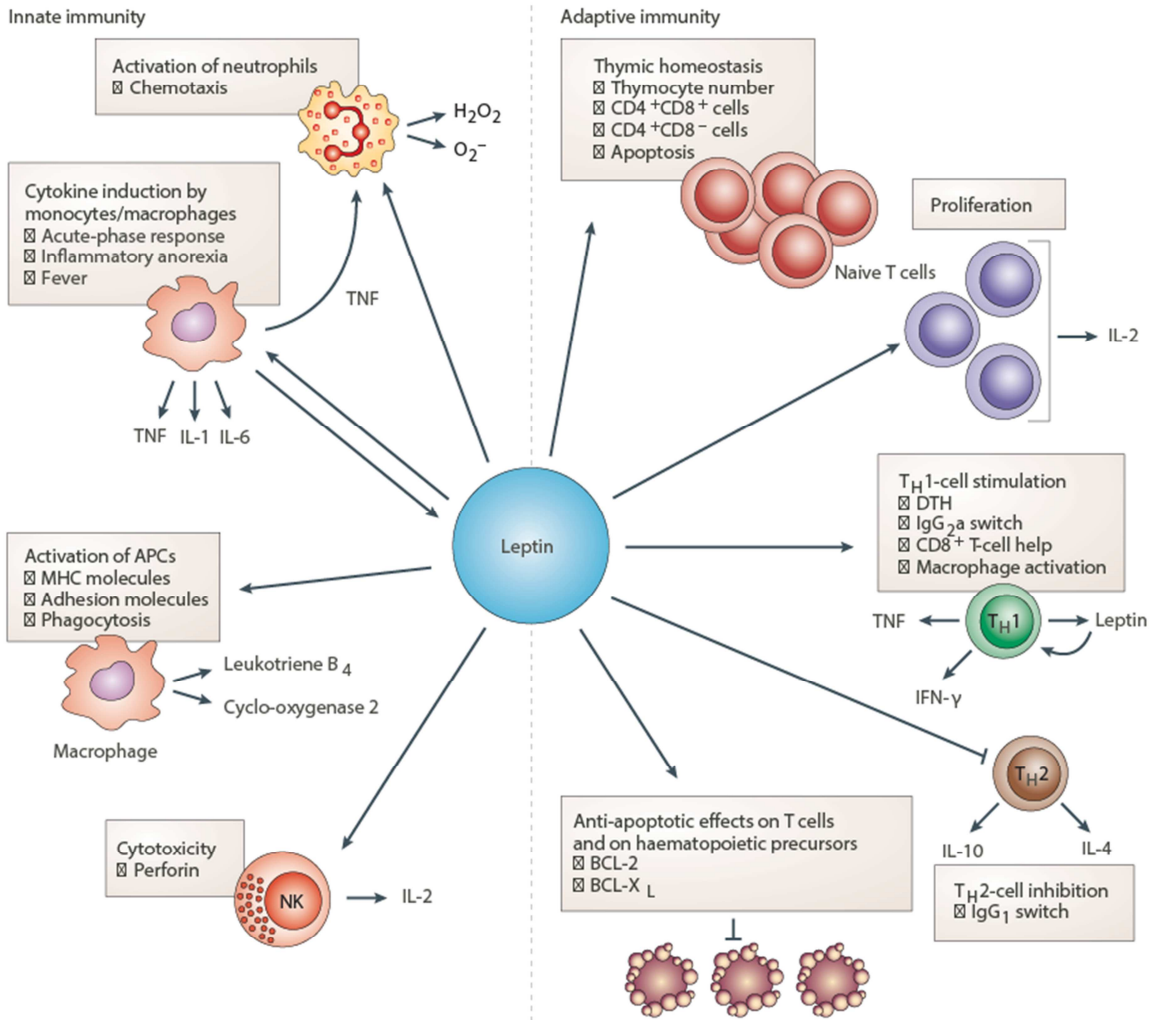

Figure 2.2. Effects of leptin on innate and adaptive immune responses: Leptin affects both innate and adaptive immunity. In innate immunity, it modulates the activity and function of neutrophils by increasing chemotaxis and the secretion of oxygen radicals through direct and indirect mechanisms. In mice, leptin seems to activate neutrophils directly. In humans, the action of leptin seems to be mediated by tumour necrosis factor (TNF) secreted by monocytes. Leptin increases phagocytosis by monocytes/macrophages and enhances the secretion of pro-inflammatory mediators of the acute-phase response and the expression of adhesion molecules. On natural killer (NK) cells, leptin increases cytotoxic ability and the secretion of perforin and IL-2. In adaptive immunity, leptin affects the generation, maturation and survival of thymic T cells by reducing their rate of apoptosis. On naive T-cell responses, leptin increases proliferation and IL-2 secretion through the activation of MAPK and PI3K pathways. On memory T cells, leptin promotes the switch towards TH1-cell immune responses by increasing IFN-γ and TNF secretion, the production of IgG2a by B cells and delayed-type hypersensitivity (DTH) responses8. This process is then sustained by an autocrine loop of leptin secretion by TH1 cells. Finally, leptin has anti-apoptotic effects on mature T cells and on haematopoietic precursors. APC, antigen-presenting cell. (Adopted from La Cava & Matarese, 2004)

D) Activation of Natural Killer (NK) cells: These cells have been shown to expressing long and short LR forms constitutively. Leptin has been shown to upregulate IL-2 and perforin expression in NK cells, an observation that can be correlated with the the impaired NK cell function in mouse models with homozygous inactivating mutation in leptin receptor gene (db/db) (Tian et al., 2002; Zhao et al., 2003). Leptin potentiates the NK cell maturation, differentiation, activation and cytotoxicity; thus directly affecting the innate immunity (Matarese et al., 2005).

2.2.3. Role in Immune system: Adaptive immunity

The various roles of leptin in adaptive immunity includes thymic homeostasis, proliferation of T cells and regulation of T cell response (Figure 2.2). Leptin deficient ob/ob mice show atrophy of lymphoid organs, have lower circulating T cells, higher monocytes and present decreased sensitivity of T-cells, thus suggesting a direct involvement of leptin in the development and regulation of adaptive immunity (Fantuzzi and Faggioni, 2000; Howard et al., 1999; Lord et al., 1998). Leptin induced expression of thymic growth factor IL-7 by thymic epithelial cells suggests a crucial role of leptin in thymic homeostasis (Gruver et al., 2009). Acute hypoleptinemia leads to apoptosis of thymic cells, atrophy of spleen (Montez et al., 2005) in mice. T-lymphocytes express long form leptin receptor (LR) by which it receives the survival signal during thymic maturation (Howard et al., 1999). A direct effect of leptin on T-lymphocytes in the absence of monocytes whereby costimulation of these cells by leptin and PHA or concavaline A drives activation, proliferation and enhanced cytokine (IL2, IFN-γ) production (Martin-Romero et al., 2000) via the long form LR (Sanchez-Margalet et al., 2002). The hyporesponsiveness of T cells in obese subjects with monogenic obesity links the role of leptin for the modulation of T cell function.

2.2.4 Effect of Leptin on Hematopoiesis

The evidence for the involvement of leptin in hematopoiesis comes from the observation that the long form LR has been found to be expressed in yolk sac, fetal liver, bone marrow, fetal marrow stromal cells and megakaryocytes. (Bennett et al., 1996; Cioffi et al., 1996; Gainsford et al., 1996; Konopleva et al., 1999). Leptin has been shown to promote formation of granulocyte-macrophage colonies from murine bone marrow cell and stimulates activity of stem cell factor (SCF) and erythropoietin (Umemoto et al., 1997). Also proliferation of BaF3 and multilineage

progenitor cells have been observed to be induced by leptin (Bennett et al., 1996). Though there are evidences against a direct role of leptin in hematopoiesis (Gainsford et al., 1996), a reduced

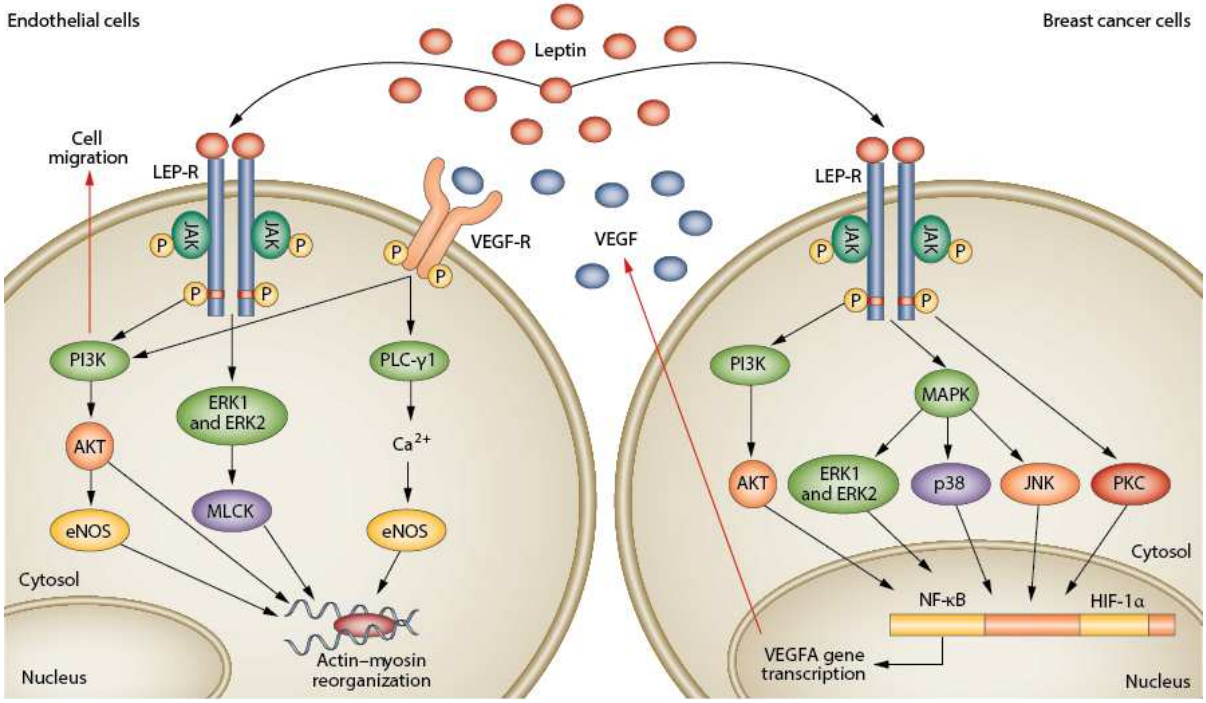

Figure 2.3. Leptin-induced endothelial cell migration and angiogenesis: Binding of leptin to LR on endothelial cells leads to tyrosine (shown by red bands) phosphorylation by JAK and induces their migration through activation of the PI3K–AKT–eNOS and ERK1–ERK2 signaling pathways. Activation of AKT induces reorganization of the actin–myosin cytoskeleton and subsequent cell movement. ERK1 and ERK2 modulate cell motility through downstream phosphorylation and activation of MLCK. Leptin is also able, in breast cancer cells, to increase *VEGFA* gene transcription through effects on the HIF-1α and NF-κB binding sites in the *VEGFA* promoter, via activation of canonical (PI3K, ERK) and noncanonical (p38 MAPK, JNK, and PKC) signaling pathways. VEGF, in turn, induces endothelial cell migration via PI3Kgnaling pathways. Activation of AKT induces reolls. In addition, VEGF activates PLC-γ1 in endothelial cells, increasing levels of intracellular calcium ions, which are also responsible for eNOS activation and nitric oxide production. Abbreviations: AKT, protein kinase B; eNOS, endothelial nitric oxide synthase; ERK, extracellular regulated kinase; HIF-1α, hypoxia inducible factor 1α; JAK, Janus kinase; JNK, mitogen-activated protein kinase; LEP-R, leptin receptor; MAPK, mitogen-activated protein kinase; MLCK, myosin light chain kinase; NF-κB, nuclear factor κB; PI3K, phosphatidylinositol 3 kinase; PKC, protein kinase C; PLC-γ1, phospholipaseC-γ1; VEGF, vascular endothelial growth factor; VEGF-R, VEGF receptor. (Adopted from Andò & Catalano, Nature Reviews Endocrinology, 2012)

level of lymphopoetic progenitor cells, defective erythrocyte production in spleen in db/db mouse (Bennett et al., 1996), decreased number of circulating lymphocytes and elevated levels of monocytes in ob/ob mice (Faggioni et al., 2000) clearly postulates some essential role of leptin particularly in lymphocytic lineage.

2.2.5 Role in Angiogenesis

Expression of leptin receptor on endothelial cells and leptin induced STAT-3 and Erk 1 activation set the premise to investigate the role of leptin in angiogenesis (Figure 2.3) (Bouloumie et al., 1998; Sierra-Honigmann et al., 1998). Later observations of *in vivo* neovascularization and formation of capillary like structures in human umbilical venous endothelial cell culture *in vitro* have provided sufficient evidence to associate leptin with angiogenesis (Bouloumie et al., 1998). Leptin via tyrosine kinase-dependent signaling cascade generates growth signals necessary for formation of new blood vessels. However, knockout mice for leptin or its receptor, do not manifest any defect in angiogenesis due to compensatory mechanisms involving other angiogenic factors. Recent studies have shown the role of leptin as a pro-angiogenic signal in breast cancer inducing vascularization via upregulation of vascular endothelial growth factor (VEGF) (Ando and Catalano, 2012; Gonzalez et al., 2006; Newman and Gonzalez-Perez, 2013; Zhou et al., 2011).

2.2.6 Role in reproduction

Due to localization of leptin receptor in hypothalamus, which is also known to regulate reproduction, leptin was initially hypothesized to be involved in onset of maturity and regulation of reproduction. *In vitro* studies suggested the leptin induced dose-dependent acceleration of gonadotropin releasing hormone (GnRH) pulsatility in the arcuate nucleus of the hypothalamus (Lebrethon et al., 2000). About 90 % of the gonadotropes in the pars tuberalis and 30% of the gonadotropes in pars distalis in the anterior pitutitary have shown to express LR, suggesting gonadotropin (LH and FSH) secretion might be regulated by leptin signaling. The observation of minute to minute oscillation of serum adreno-corticotrophic hormone (ACTH) and cortisol levels in men (Licinio et al., 1997), serum luteinizing hormone (LH) and estradiol levels in women (Licinio et al., 1998), being significantly related to the leptin pulsatile levels in serum, adds an extra dimension to the role of leptin in reproduction. Administration of leptin to non-human

primates and prepubertal mice has shown it to accelerate puberty (Rogol, 1998). Human subjects with an inactivating mutation in leptin receptor gene, remained prepubertal and had hypogonadotrophic hypogonadism (Clement et al., 1998). This suggests an important role played by leptin in onset of puberty. Expression of functional LR in gonads suggest the peripheral role of leptin (Caprio et al., 1999; Karlsson et al., 1997) in reproduction.

2.2.7 Role in embryo implantation and development

Leptin serum level has been shown to be correlated with fetal body weight gain (Harigaya et al., 1999). This cytokine has direct effect on the oocyte maturation and preimplantation embryo development, *in vitro* (Craig et al., 2005). Indirect assessment based on the expression of leptin and leptin receptor at the implantation sites has led to the proposed role of leptin in embryo implantation (Cervero et al., 2005). Leptin's role in various fetal physiological processes including erythroid and myeloid development (Mikhail et al., 1997), bone metabolism (Ogueh et al., 2000) are well established.

2.2.8 Role in bone metabolism

Leptin has been shown to induce growth of chondrocytes of skeletal growth centers (Maor G. et al., 2002). LR has been shown to be expressed in primary adult osteoblasts and chondrocytes while leptin plays essential role in skeletal growth and development (Steppan et al., 2000). Recent work has suggested central leptin injection stimulates bone formation increasing bone mineral density in leptin deficient *ob/ob* mice (Bartell et al., 2011).

2.2.9 Role in wound healing

Leptin whose angiogenic role has already been described, has also been shown to enhance wound re-epithelialization and skin repair when applied exogenously (Frank et al., 2000; Goren et al., 2006; Murad et al., 2003; Ring et al., 2000). Leptin expression is induced in wound tissue and this endogenous leptin plays potential role in wound healing (Marikovsky et al., 2002).

2.3 STRUCTURE OF LEPTIN

The mature leptin is a non-glycosylated 16 kDa protein of 146 amino acid length (Figure 2.4A). As the purified human leptin aggregates extensively, crystal (PDB ID: 1AX8) structure of a point mutant carrying tryptophan to glutamate (W100E) mutation at 100th position (Figure 2.4B), has been successfully solved at 2.4 Å resolution (Zhang et al., 1997a).

2.3.1 Structure of Leptin-E100

Except for a disordered region from T27 to G38 which the crystal structure lacks, Leptin-E100 X-ray crystal structure shows it to be a four helical bundle cytokine with four anti-parallel helices (A, B, C and D) in up-up-down-down fashion, two long crossover links AB and CD, the short BC loop and a kinked 3_{10} helix formed by five residues of Helix D (Q139 to S143). The four helical bundle structure is maintained by four conserved regions : D9-T16, M54-A59, D79-H88, Y119-Q130, contributed by four helices to form a hydrophobic core. The CD loop has a distorted helix E formed by residues L107 to E115 and positioned near-perpendicular to the four helices. Leptin has only two cysteine residues: C96 (on CD-loop) and C146 (C-terminal residue) (Figure 2.4A), which form a solvent exposed disulfide bridge essential for the tethering the C-terminal end of D-helix to the CD loop via a kinked extension formed by residues N139-S143. This disulfide induced kinked extension is essential for the structural stability and/or activity of this cytokine, as the mutation of these conserved cysteines render the cytokine inactive. The short E-helix with 2 turns separated by a kink in the CD loop has many highly conserved hydrophobic residues (L104, L110, L114, V113) that interact closely with hydrophobic residues at the C-end of helix B and N-end of Helix D, thus forming a cap enclosing a lipophilic nucleus that holds the B and D helices in position. Two glycines located in the kink region of helix-E are partly conserved. The W100E mutation is localized on the CD loop between the C helix and the distorted helix E. This tryptophan is not conserved across species and thus may not have any structural or functional role in leptin.

Despite low sequence similarity, when compared with the atomic structures of other related cytokines like G-CSF, LIF, CNTF and hGH, the inter-helical angles and the characteristics of the long crossover loops in Leptin-E100 was found to be similar. The root mean square deviations

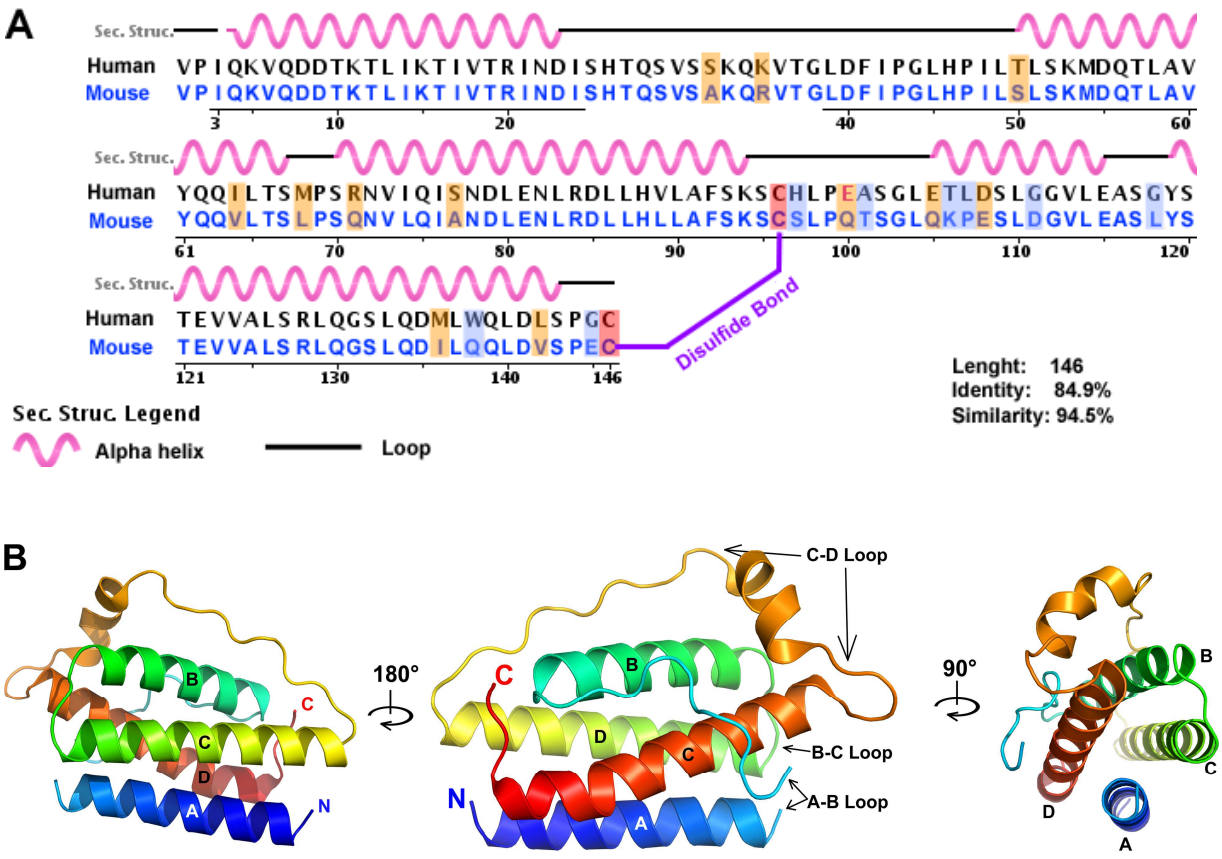

Figure 2.4. Structure of Leptin-E100: A) Pairwise sequence alignment of human and murine leptin shows high sequence conservation between these two species with sequence identity of 84.9%. The unaligned residues are colored blue while similar residues in yellow. Two cysteines have been colored as deep salmon. The corresponding scondary structure of human Leptin as derived from its crystal structure has been depicted as alpha helix (pink) and loop regions (black). The W100E mutation has been shown as red in human leptin sequence. **B)** *center:* X-ray crystallographic structure of human Leptin-E100 (PDB ID:1AX8), shown as cartoon, with rainbow color scheme. The N- and C-termini have been labeled. Four helices have been named as A, B, C and D. All three loops viz. A-B, B-C and C-D loops have been labeled. Left & Right: Two other orientations of human leptin-E100 colored in rainbow scheme.

(RMSDs) for the crystal structures of its homologues are as follows: LIF:2.50Å (85 Cα atoms); G-CSF: 2.37 Å (101 Cα atoms); human GH: 1.95 Å (85 Cα atoms). The low electron density for the T27-G38 region suggests high flexibility which might get stabilized upon interaction with receptor; a phenomenon already seen in IL6 system, where the corresponding region undergoes coil to helix transformation upon receptor interaction. The homology among these cytokines are further pronounced by the existence of the similar exon-intron boundaries in their respective genes (Sprang and Fernando Bazan, 1993).

Unlike Leptin-E100, the helical kinks utilized to maximize the surface contact with the receptor binding epitopes are quite pronounced and localized on A, B and D helix of G-CSF, LIF and human GH, respectively. In Leptin-E100, this kink is as small as involving L139, E140 on helix D. Another difference between leptin-E100 and other classical type 1 cytokines like G-CSF, LIF and human GH is the B helix, which is about two turns shorter in leptin-E100. While an extra helix in the AB-loop is characteristic of GCSF, LIF and hGH, leptin-E100 lacks such helix; rather a small distorted helix-E is found in the CD loop. The highly conserved aromatic residue (F or W) in helix D, found in other type 1 cytokines is missing in leptin; rather Y61 on helix B neighboring the hydrophobic E helix cap is highly conserved and may serve similar purpose. All these distinctive features provokes to classify leptin into a sub class separate from these classical type-1 cytokines.

2.3.2 Computational analyses and mutagenesis:

A) Structural homology based mutagenesis:

Homology modeling of murine leptin followed by superposition onto the structures of well characterized close homologues like IL-6 and GCSF led to discovery of the putative binding sites on leptin. IL-6 whose structure has been solved in complex with its cognate receptor IL-6 receptor α (IL-6Rα) and shared co-receptor gp130, has three binding sites: site I is used to interact with IL-6Rα; sites II and III are used to interact with two gp130 receptors; thus forming a hexameric assembly of two IL-6:two IL-6Rα:two gp130 (Boulanger et al., 2003). GCSF on the other hand uses only site II and III to interact with two cognate receptors; thus forming a tetrameric assembly (Layton and Hall, 2006). Based on the superposition of the homology

modeled murine leptin onto the solved structures of type 1 cytokines like human leptin (1ax8), human CNTF (1cnt), human IL-6

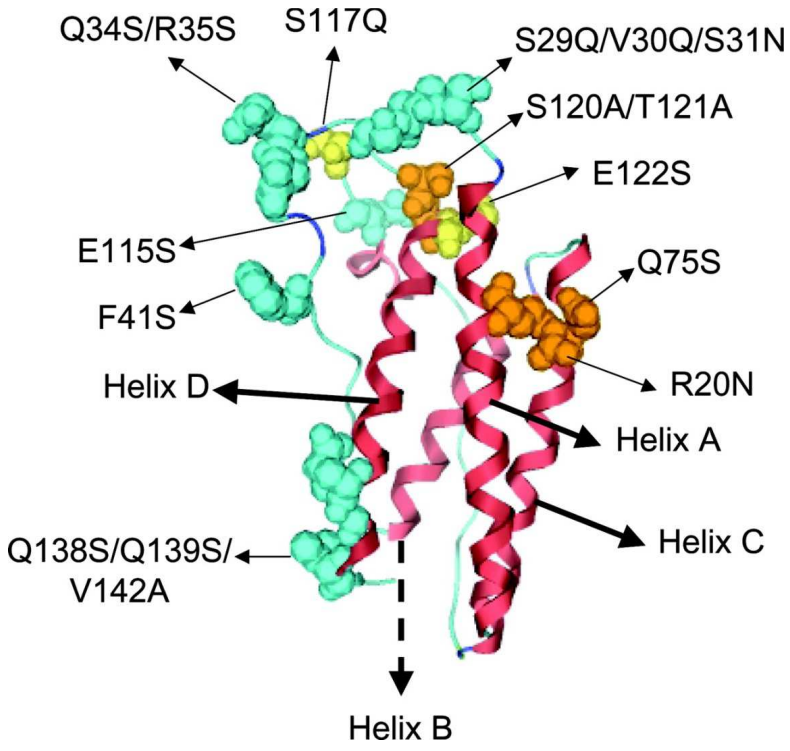

Figure 2.5. Position of mutations that affect LR activation in the mouse leptin model: The secondary structure of mouse leptin is presented by *ribbons*. Residues that are involved in mutations that affect luciferase activity are presented as *space-filling spheres*. The *spheres* are colored according to the residual maximal luciferase activity of the mutant: *yellow*, 0–30% of wild type; *cyan*, 30–60% of wild type. Ser-120, Thr-121, Arg-20, and Gln-75 are presented as *orange space filling spheres*. (Adopted from Peelman et al., 2004)

(1alu), bovine GCSF (1bgc), vIL-6 (1ilr), ovine placental lactogen (1f6f), murine LIF (1lki) and human oncostatin M (1evs) followed by prediction of binding sites on murine model based on solvent accessibility, 31 mutants have been designed by Peelman et al. (2004). Studying the effects of these mutations on the leptin binding to LR and leptin induced LR signaling, has provided immense insight into functional roles of these binding sites on leptin surface. The location of these mutations on the surface of leptin has been represented in figure 2.5 and 2.6. The L13N mutant did not express while among the rest of 30 mutants, those with drastically

reduced activation efficiency can be categorized depending on the site of location into following categories:

***Site III mutants*:** S29Q/V30Q/S31N, Q34S/R35S, E115S, S117Q, E122S, S120A/T121A

Site II mutants: D9S/T12Q, K15S, T16N, R20N, Q75S, N82S/D85S, L86S, L86N, L86Q,

Site I mutants: F41S, Q138S/Q139S/V142A

(i) Binding Site I: Site I in homologue cytokines bind to a CRH domain on the receptor. IL-6 site I interacts with the CRH of the shared receptor gp130; while a similar site on GCSF is absent in GCSF/GCSFR complex. Based on the mutational analysis performed by Peelman et al. (2006), the only site I mutant Q138S/D135S/V142A studied has marked effect on receptor activation, while point mutations Q134S and D135S showed only minimal effect on receptor activation. Although residues in this region are not conserved and the receptor activation potential is only affected by a triple mutation (Q138S/D135S/V142A), possible existence of binding site I on leptin has led to the modeling of a hexameric signaling assembly (described in section 2.6.2).

(ii) Binding site II: This site mediates the high affinity but non-signaling binding to the CRH domain of cognate receptor in type 1 cytokines; so perturbation of this region leads to an inactive ligand incapable of binding to its receptor (Figure 2.6 and 2.11). The IL-6 site I interacts with the CRH domain of IL-6Rα in the hexameric form of its complex (Boulanger et al., 2003). In GCSF/GCSFR complex, site II has been shown to be major binding interaction (Layton et al., 1999). Structural superposition suggested site II to be present on the surface of antiparallel helices A and C. Mutation analysis in the predicted site II on Leptin showed R20N and Q75S to affect both binding and receptor activation. Another mutant R20Q located in site II has also been shown to be incapable of binding leptin receptor (Verploegen et al., 1997). Mutation of polar residues (K15S, D9S/T12Q, N82S/D85S) has shown to hamper the affinity of leptin to CRH2 domain. A hydrophobic cleft formed by L13, L86, between helices A and C may also play significant role for leptin binding (Iserentant et al., 2005). L13 mutants (L13A, L13N and L13S) did not express possibly because L13 is buried and so have important structural role. L86 mutants (L86S, L86Q, L86N) showed drastically reduced affinity for CRH2.

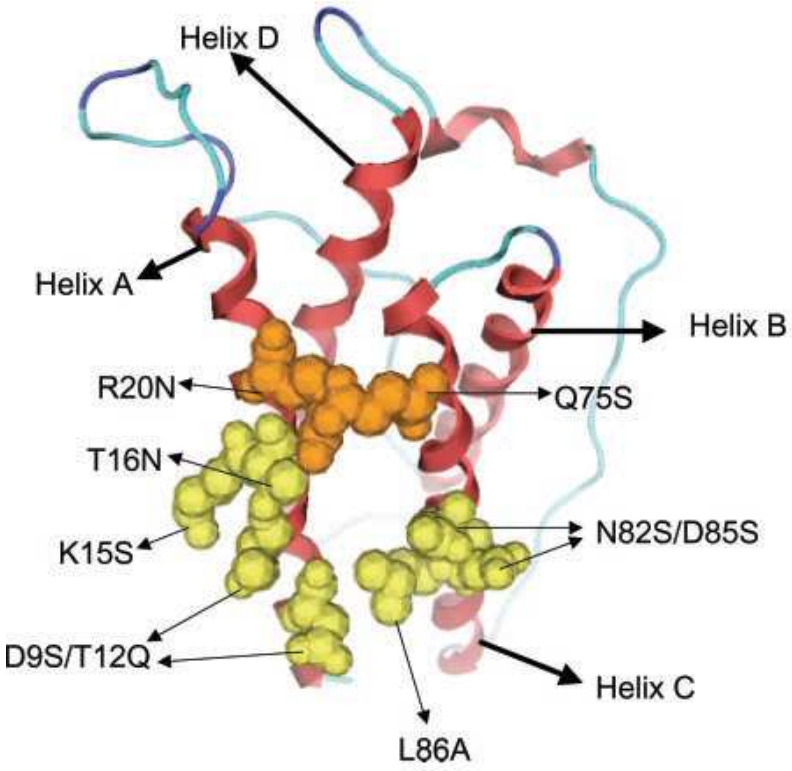

Figure 2.6. Mutations that inhibit the binding to CRH2 in the mouse leptin model: The secondary structure of mouse leptin is presented by *ribbons*. Residues that are involved in mutations that affect binding to CRH2 are presented as *yellow spacefilling spheres*. The Arg-20 and Gln-75 residues are presented as *orange space-filling spheres*. (Adopted from Peelman et al., 2004)

(iii) Binding Site III: The ligand site III has been found to be essential for interaction with the Ig domain of a shared receptor as in IL-6 system (Boulanger et al., 2003) or that of a cognate receptor as in GCSF/GCSFR system (Tamada et al., 2006), enabling the ligand to induce necessary conformational change in the receptor which is inevitable for the signal transduction. So site III mutations will not affect the binding of the ligand, but activation of the receptor after binding (Figure 2.5). Based on Peelman et al.'s mutation analysis, site III was found to be located in leptin on the N-terminus of D helix, C-D loop and A-B loop. Among all site III mutants studied, S29Q/V30Q/S31N, Q34S/R35S, E115S, S117Q, S120A/T121A and E122S affect the receptor activation without altering their binding potential. The double mutant S120A/T121A behaves as an antagonist leptin.

B) Hydrophobic cluster analysis based mutagenesis:

Hydrophobic cluster analysis (HCA), depicts a binary pattern filtering the hydrophobic clusters from the polar background in a protein secondary structure and thus enabling the easier visualization and extraction of hydrophobic motifs stabilizing the structural topology and functional requirement of the proteins. Thus, this method has proven successful in multitude of objectives ranging from distant-homologue search to essential amino acid recognition in proteins by comparison with homologues. HCA of leptin in light of well-characterized vIL-6 (PDB Id: 1I1R) binding epitopes has led to identification of a hydrophobic strand (V^{36}TGLDFI^{42}) in the A-B loop of leptin corresponding to the hydrophobic cluster "IFHLKL motif" in the A-B loop of vIL-6 (Niv-Spector et al., 2005) (Figure 2.7). The leptin-E100 crystal structure (PDB ID: 1AX8) lacks electron density corresponding to the A-B loop and so this region is supposedly unstructured in the unbound leptin. Based on the structure based sequence comparison of leptin A-B loop with the corresponding loop of vIL-6 structure (PDB ID: 1ILR) and IL-6 (PDB:1P9M), no significant information could be obtained due to low sequence similarity. But using HCA strategy, Niv-Spector L. et al. identified a hydrophobic cluster comprising V^{36}TGLDFI^{42} at the center of the A-B loop. The corresponding hydrophobic cluster in vIL-6 forms a typical β-strand "IFHLKL" in vIL-6 at the center of A-B loop as observed in the crystal structure of vIL-6/gp130 complex (PDB: 1ILR). This vIL-6 β-strand engages in interaction with the β-sheet of the Ig-domain of gp130, drives the receptor activation and thus essential for the function of vIL-6. A similar role was hypothesized for the hydrophobic cluster in leptin A-B loop. Further mutagenic analysis corroborated the role of this β-strand to be essential for the Leptin-Ig domain interaction necessary of LR activation.

Mutations in the A-B loop of human and ovine leptins (L39A/D40A, F41A/I42A and L39A/D40A/F41A/I42A) did not affect the binding to LR; but they lost the potential to elicit the STAT signaling. As these residues have been proposed to interact with the Ig-domain of LR in a fashion similar as observed in vIL-6/gp130 crystal structure, these residues should be included in the site III of leptin.

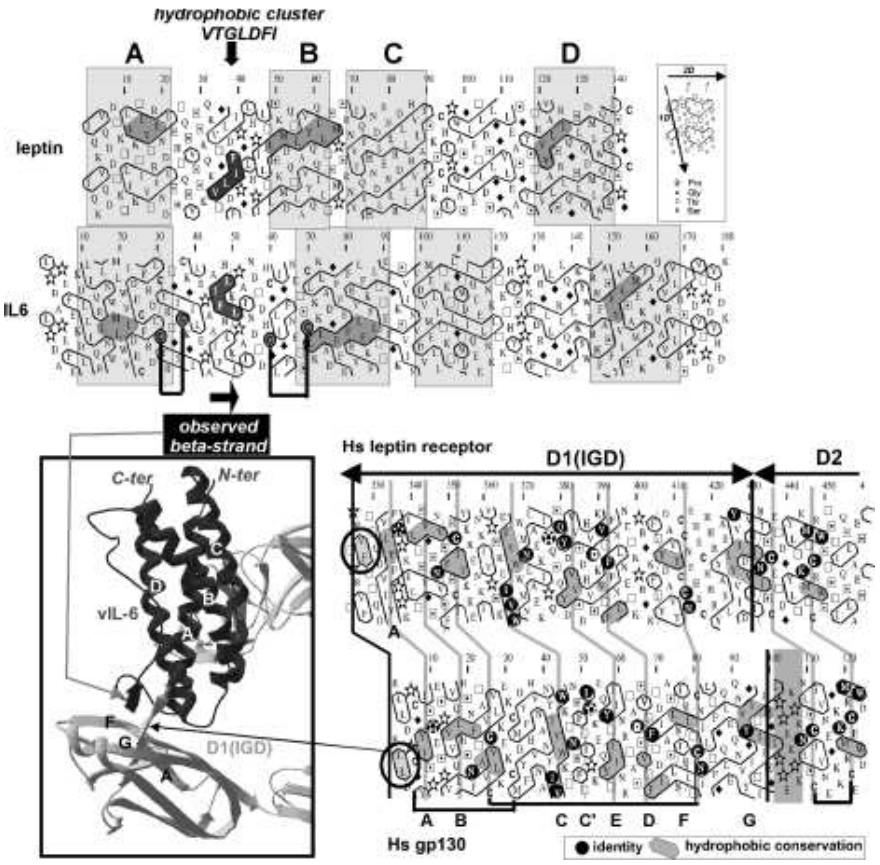

Figure 2.7. Comparison of the HCA plots of human leptin and vIL-6: The IL-6's site III interacts with the gp130's IGD. The region including the short *β*-strand of the A–B loop of vIL-6 interacts with a short extended region preceding the IGD strand *β*A. The corresponding expressions of these short *β*-strands on the two-dimensional representations of the sequences (HCA plots) are shown with grey (vIL-6) and black (gp130) arrows. In the HCA plots, the sequence is shown on a duplicated *α*-helical net, in which the hydrophobic amino acids (V, I, L, F, M, Y and W) are circled. These form two-dimensional hydrophobic clusters, the positions of which have been shown to mainly match those of regular secondary structures. Despite low levels of sequence identity, comparison of the HCA plots indicates the presence of conserved hydrophobicity (shaded in grey) within clusters, indicative of similar secondary structures. Although the experimental structures of leptin are known (PDB ID:1AX8), a poor density was observed for the first part of the leptin A–B loop (no residues are visible from Ser25 to Gly38), indicating a high flexibility of the loop in the uncomplexed form of the cytokine. The cluster associated with the *β*-strand of the vIL-6 A–B loop, which is involved in the interaction with gp130, is shown in black. The two disulfide bonds located at the beginning and at the end of this loop are indicated by black lines. The only hydrophobic cluster of the leptin A–B loop, which is highly typical of an extended structure, is also shown in black. (Adopted from Niv-Spector et al., 2005)

2.3.3 Leptin Antagonists: Perspective tool for delineation of mechanistic principles

Site directed mutagenesis of human leptin has led to discovery of site III mutants with comparable binding potential but no/low activation potential, (Verploegen et al., 1997; Peelman et al., 2004; Niv-Spector et al., 2005). Such antagonist leptin can bind LR without eliciting a signaling and hence they can potentially be used for therapeutic purpose as in auto-immune diseases (La Cava and Matarese, 2004) or as a tool to study the mechanism of signal transduction (Feldman et al., 2005; Shim et al., 2002). Two antagonist leptins S120A/T121A and 39AAAA42 mutants have been described in detail for their relevance in the current doctoral research.

A) Leptin S120A/T121A mutant: This mutation drastically reduced the ability of leptin to activate the cognate receptor while binding with the leptin receptor remained unaffected (Peelman et al., 2004). The maximal luciferase activity that is directly related to the leptin induced signaling intensity was found to have reduced by nearly 14 times for this mutant compared to that for wild type leptin. The half maximal effective concentration (EC50) was found to have increased approximately 10,000 times and thus a higher concentration of mutant is necessary to achieve maximal signal strength. In the competitive binding assay, the half maximal inhibitory concentration (IC_{50}) of S120A/T121A mutant for wt-Leptin/Leptin binding domain interaction was found to be 1.5 nM, emerging as the strongest antagonist of all 31 mutants analyzed by Peelman et al (2004). The site of mutation was found to be located corresponding to the site III of other homologues on the superposed model (Figure 2.8). In vivo studies in mouse showed S120A/T121A mutant to stimulate feeding, reduce energy expenditure and thereby increasing the total body mass, possibly competitively inhibiting the binding of native wt-leptin to leptin receptor. Beyond the fact that S120 and T121 is part of site III and participate either directly or indirectly in leptin-site III/LR-Ig-domain interaction, the exact *in vivo* mechanism of antagonism is largely unknown.

B) Leptin L39A/D40A/F41A/I42A mutant: This stretch of amino acids L39-I42 proceeds the missing electron density region of T25-G38 residues in the crystal structure solved for leptin-E100 and forms the part of the A-B loop (Figure 2.8). This and other related mutants have been characterized to have similar binding affinities as wild type leptin for the ligand binding domain of cognate receptor LR.

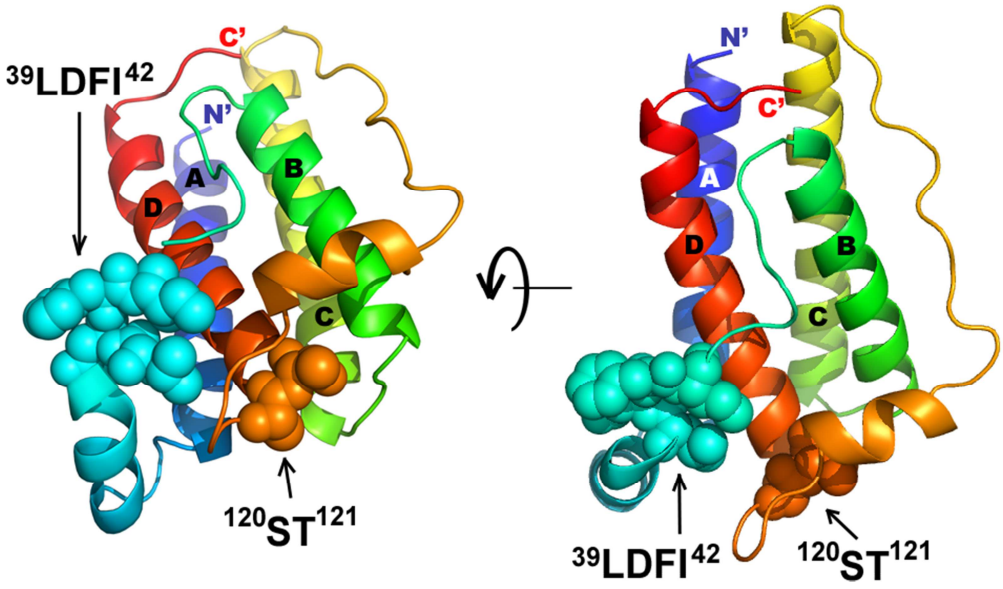

Figure 2.8. Sites of mutation in antagonist leptins: The ^{120}ST^{121} present in binding site III, upon mutation to alanine behaves as an antagonist. The ^{39}LDFI^{42} stretch discovered by hydrophobic cluster analysis upon substitution mutation to alanine also confers antagonism.

Though both of these mutants act as antagonist preventing receptor activation following binding to CRH2 domain of LR, there exists possibility whereby these two may differ in the mechanism of their antagonism. Nonetheless, the events that lead to an active signaling complex following the site II/CRH2 binding would be missing in the antagonist leptin system. So these mutants can be used as molecular tools to study the behavior of resulting complexes by disintegrating the ligand binding from receptor activation. Subsequent blockade in the formation of a signaling complex on membrane can provide important insights into the mechanism of leptin mediated LR activation.

2.4 LEPTIN RECEPTOR

The leptin receptor was originally identified by scanning for tissue types capable of binding alkaline phosphatase tagged leptin and radioactive ^{125}I labeled leptin (Tartaglia et al., 1995). The expression of the receptor in the choroid plexus mouse brain led to understanding of the mechanism of central nervous system regulating energy homeostasis and energy intake via the leptin/leptin receptor system. Expression cloning followed by nucleotide sequencing suggested it to be a single membrane-spanning receptor containing motifs similar to gp130. In spite of a heavy volume of data available today mostly based on indirect methods like mutagenesis and fluorescence spectroscopy, a mechanism for the leptin dependent activation of LR compatible with all available data is still to need be deciphered, largely because of the higher degree of complexity that exists in this system.

2.4.1 Structure of Leptin receptor ectodomain

The full length leptin receptor is 1162 residue long with following three regions: extracellular part (LR_{ecto}), single pass helix trans-membrane domain (LR_{TM}) and an intracellular part (LR_{IC}) (Figure 2.9A). Leptin receptor belongs to the type 1 receptor family characterized by having a four helical bundle ligand, disulfide cluster and WSXWS motif.

LR_{ecto} (Figure 2.9A) has a membrane-distal N-terminal secretion signal sequence which undergoes cleavage during insertion of the receptor into the membrane. This signal sequence is followed by the n-terminal domain (NTD), approximately of 100 residues but no sequence similarity to any known proteins. The rest part can be divided into 4 functional regions: first cytokine receptor homology (CRH1) domain, immunoglobuline (Ig) like domain, a second cytokine receptor homology (CRH2) domain, followed by two membrane proximal fibronectin type III domains. This modular structure shows striking similarity with many receptors belonging to gp130 family of shared receptors in having an Ig-like domain (gp130, G-CSFR, IL-6Rα) and having multiple CRH domains (as in LIFR and OSMR).

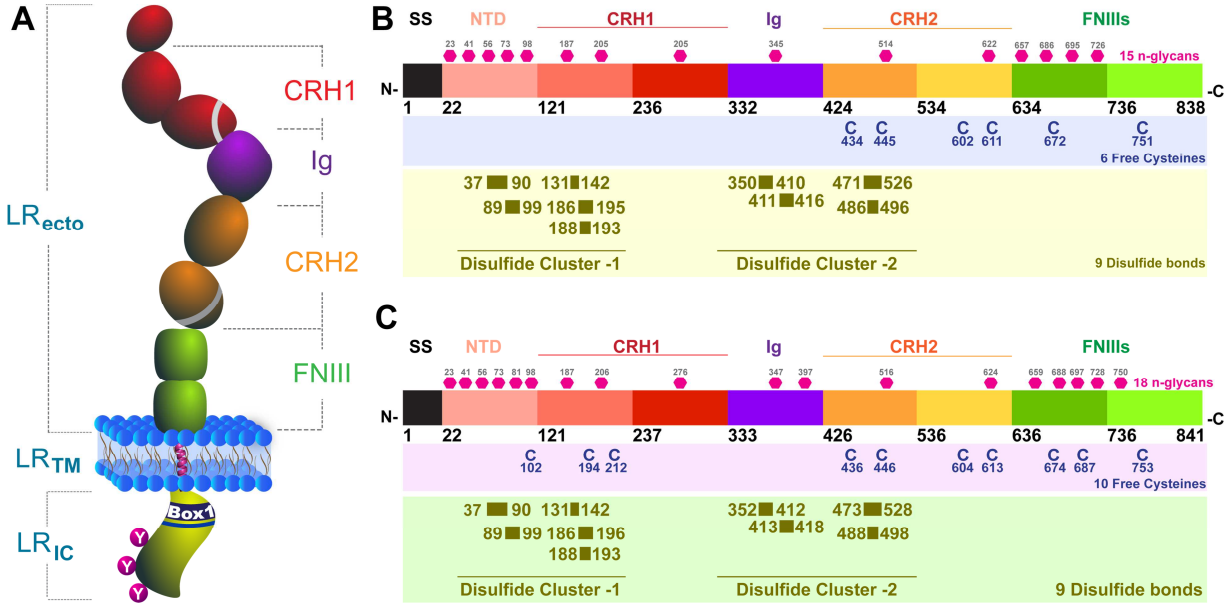

Figure 2.9. Schematic structure of leptin receptor: ***(A)*** Leptin receptor can be divided into three parts: extracellular (LR_{ecto}), transmembrane (LR_{TM}) and intracellular (LR_{IC}). The extracellular part LR_{ecto} has a n-terminal domain (NTD) (*red*) which do not show any homology to any other known protein folds. The rest part can further be divided into four functional regions: distal most 1st cytokine receptor homology, CRH1 (*red*), immunoglobulin like, Ig domain (*violet*), 2nd cytokine receptor homology, CRH2 (*orange*) and two membrane proximal fibronectin type III domains, FNIII (*green*). The transmembrane region (LR_{TM}) is formed by a single pass helix. The intracellular part (LR_{IC}) has a box1 motif that harbors janus kinase (JAK) binding site. Following receptor activation, JAK gets activated by cross-phosphorylation. Activated JAK phosphorylates three highly conserved tyrosine residues, which then acts as the platform for the binding of Signal Transducer and Activator of Transcription (STAT3) proteins. Such bound STAT molecules undergo JAK mediated phosphorylation. This follows with dissociation of STAT 3 and regulation of gene expression in nucleus. ***(B-C)*** Primary sequence of murine (top, B) and human (bottom, C) leptin receptor annotated to show different domains, predicted domain borders, N-linked glycosylation sites, position of free cysteins and disulfide clusters. The color scheme for different domains are as follows: secretion signal, SS (*black*); n-terminal domain, NTD (*salmon*); 1st cytokine receptor homology domain, CRH1 (*red*); Ig domain (*purple*); 2nd cytokine receptor homology domain (*orange*) and fibronectin type III domains, FNIIIs (*green*).

Each of the CRH domains in LR_{ecto} has a WSXWS motif and conserved disulfide cluster, characteristic of the type 1 cytokine receptor family. In erythropoietin receptor, WSXWS motif

has been shown to be crucial for efficient folding of CRH domain (Hilton et al., 1996; Yoshimura et al., 1992). Recently, this motif has been shown to act as a molecular switch stabilizing the dimeric prolactin receptor upon prolactin binding and thus playing essential role in formation of a sustainable signaling complex (Dagil et al., 2012). In IL21 system, another type 1 receptor system, WSXWS motif has been shown to be essential for the A-shaped architecture of the receptor-ligand complex by the formation of hydrogen bond between two C-mannosylated tryptophans (1st W of WSXWS) of this motif coming from two CRH domains in single IL21R (Hamming et al., 2012). Though there is not much information of such role of WSXWS motif in leptin receptor, the WSXWS motif of CRH2 domain has asparagine (N^{624}) at X position which has been reported to be glycosylated (Haniu et al., 1998).

The extracellular part of the human LR_{ecto} has at least 20 putative N-linked glycosylation sites as determined by the signature motif NXS/T motif (Figure 2.9B-C). Mass spectrometric analysis showed hLR_{ecto} has unusually high amount N-glycosylation, accounting for a total of 18 sites (i.e. 1 in every 46.61 residues in average) and equivalent to 36% of the total molecular mass of the protein (Haniu et al., 1998). Only two putative N-glycosylation sites which were not found to be glycosylated are N433 (in CRH2) and N670 (in distal FNIII). Distribution of these experimentally determined N-glycosylation sites across different domains are as follows: 9 sites in CRH1, 2 in Ig domain, 2 in CRH2, 4 in distal FNIII and 1 in proximal FNIII domain. The glyosylation of the asparagine residue which is part of the conserved WSXWS motif in CRH2 may assist in folding or the stabilization of the LR structure (Haniu et al., 1998).

i) 1st Cytokine Receptor Homology domain (CRH1): Like CRH domains of other type 1 receptors, this region is formed of two structural domains. It has no known function as it does not interact with leptin nor required for LR activation in vitro. Although recent evidences show its involvement in obesity among certain ethnic groups (Quinton et al., 2001; Duarte et al., 2006) and increased susceptibility towards protozoan infection in children (Duggal et al., 2011) due to a naturally occurring single nucleotide polymorphism Q223R in this region. This suggests CRH1 might have some physiological role to play which is yet to be discovered. Deletion of CRH1 domain results in reduced signaling intensity (Zabeau et al., 2004).

ii) Ig-like domain (Ig): Ig domain is essential for the activation of LR mediated by leptin site III interaction followed by ligand binding to CRH2 (Zabeau et al., 2004). There are two N-glycosylation sites on N347 and N397. Two disulfide bridges (C352-C412 and C413-C418) have been shown to stabilize the Ig domain in human LR (Haniu et al., 1998).

iii) 2nd Cytokine Receptor Homology domain (CRH2): Based on domain truncation study, Fong TM et al. (1998) has shown the deletion of the CRH2 domain led to loss of LR's binding ability to leptin; thus suggesting CRH2 is the high affinity leptin binding domain on LR. Structurally, CRH2 region has two sub-domains. CRH2 domain expressed in isolation in *Escherichia coli* has shown to bind leptin with a K_D of 15.4nM and a 1:1 stoichiometry (Sandowski et al., 2002). Structural superposition with homologues and mutagenesis suggests site II of leptin interacts with this domain (Peelman et al., 2004). Human CRH2 domain has 2 glycosylation sites, 4 free cysteines and 2 disulfide bridges as shown by Haniu M. et al. (1998). Though there is no crystal structure available for leptin receptor or its subdomains alone or in complex with its cognate ligand, recently solved crystal structure of CRH2 domain expressed in prokaryotic expression system in complex with a monoclonal antibody 9F8 Fab (PDB ID: 3V6O), provides the first structural details of this domain at atomic resolution (Carpenter et al., 2012) (Figure 2.10). Presence of two copies of CRH2/9F8 Fab complex in the asymmetric unit with the two CRH2 in proximity to each other in a cross-shaped erythropoietin receptor (EPO) like conformation and sharing an interface with buried surface area of 700Å may not suggest ruling out the possibility of low affinity interaction via this interface on cell surface. Though authors have stressed on the crystal packing interaction to be more likely for this kind of dimerization interface. Interestingly, the IFLLS cluster shown to involve in high affinity binding with leptin (Iserentant et al., 2005) is located in the CRH2-CRH2 interface in the 3V6O crystal structure.

This crystal structure shows CRH2 to be composed of two subdomains, each adopting a fibronectin type III fold containing beta sheet sandwich (Figure 2.10D and 2.9E). Missing electron density includes loop regions in B-C loop, J-K loop, L-M loop and accounting for 39

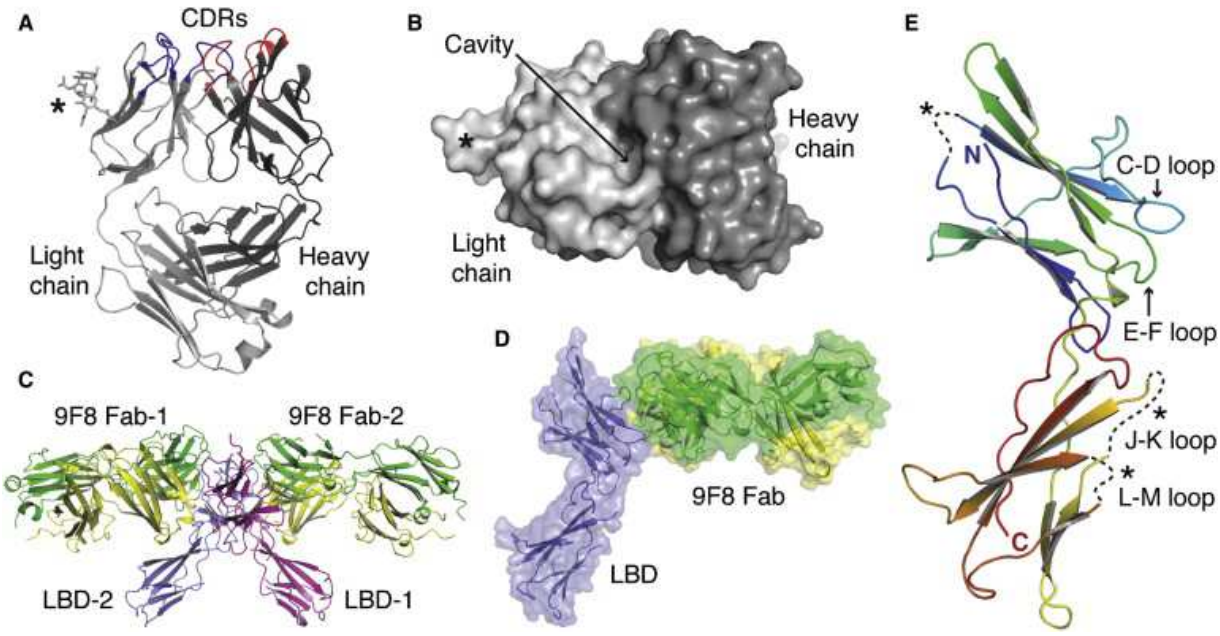

Figure 2.10. Crystal structure of a CRH2 domain binding monoclonal antibody 958 Fab in isolation and in complex with human Leptin expressed in *Escherichia coli*:*(A)* Ribbon representation of the 9F8 Fab. The CDRs from the light chain (light gray) and heavy chain (dark gray) are colored blue and red, respectively. The glycosylation of Asn-22 of the light chain is shown as sticks and marked by an asterisk. ***(B)*** Surface representation of the CDR regions of 9F8 Fab. The deep cavity at the interface between the light and heavy chains is clearly visible; the glycosylation of Asn-22 of the light chain is marked by an asterisk. ***(C)*** Ribbon representation of the two copies of the LBD-9F8 Fab complex in the asymmetric unit. The two LBD molecules are colored blue and magenta, the 9F8 Fab molecules are colored yellow (heavy chain) and green (light chain). ***(D)*** A single copy of the LBD-9F8 Fab complex showing that 9F8 Fab binds to the N-terminal subdomain of LBD. ***(E)*** Secondary structural elements of LBD colored by rainbow: N terminus, blue; C terminus, red. Key loops, which are discussed in the text, are labeled; unmodeled loops are indicated by dashed lines and marked with an asterisk. (Adopted from Carpenter et al., 2012)

residues. The cysteinylation of the exposed cysteine C604 is a byproduct of refolding of CRH2 in the presence of excess cysteine and have been shown to act as an important crystal lattice contact. The WSXWS motif, characteristic of type 1 receptor family, forms a π-cation stack with R573, W583, R612 and K614.

Disulfide map of CRH2 domain: Mass spectrometric analysis of CRH2 region from human LR has suggested existence of two disulfide bridges (C473-C526 and C486-C498) (Haniu et al., 1998) (Figure 2.9B-C). Structural superposition and sequence comparison of murine Leptin with other long chain cytokine receptor CRH domains has led the assumption that C434 (C436 in human) and C445 (C447 in human) would be disulfide linked (Iserentant et al., 2005). Recently, the solved structure of human CRH2 domain in complex with a neutralizing Fab fragment (PDB ID:3V6O) has proven existence of three disulfide bridges in human CRH2: C436-C447, C473-C528, C488-C498 (Carpenter et al., 2012). The 7^{th} and 8^{th} cysteines in murine CRH2, C602 (C604 in human) and C611 (C613 in human), does not form any disulfide bridge. Crystal structure of human CRH2 shows C613 to be completely buried in the core while C604 is solvent exposed. Earlier studies by Zabeau L. et al. (2005) suggesting involvement of free cysteine in CRH2 domain in homotypic interaction responsible for leptin-independent covalent-dimerization of LR have been contradicted by Carpenter B. et al. (2012), based on evidences of existence of intra-receptor disulfide bridge between C604 of CRH2 and C-674 of FNIII based on CRH2 crystal structure alignment with gp130 ectodomain crystal structure (PDB ID: 3L5H). Out of total 4 disulfide bonds in CRH2 domain, 3 are present in the distal domain and only 1 in proximal connecting CRH2 with FNIII, suggesting proximal domain might have a greater conformational plasticity to accommodate any leptin induced structural reorganization.

iv) Fibronectin type III domains (FNIII): The membrane proximal tandem FNIII domains do not take part in ligand binding, but are essential for LR activation (Fong et al., 1998; Zabeau et al., 2005). Mass spectrometric analysis has revealed FNIII of human LR has 3 free cysteines (Haniu et al., 1998): C674, C687 and C753 (Figure 2.9B-C). The FNIII domain of murine LR has only 2 free cysteines: C672 and C751. Substitution mutation C672S in murine FNIII drastically reduces the STAT3-dependent signaling; while C751S has very limited effect (Zabeau et al., 2005). The double mutant C672S/C751S is devoid of signaling capacity, suggesting a critical role played by FNIII cysteines in LR activation. Presence of these surface exposed cysteines has resulted in a very high propensity of inter-FNIII covalent clustering in vitro when this domain was expressed in isolation. On the premise of the ligand induced receptor-receptor disulfide bridge formation in IL-3 receptor system and the critical role of FNIII cysteines in LR, a ligand-induced inter-receptor FNIII-FNIII disulfide bridge formation model have been put forth (Zabeau et al., 2005). A truncated receptor with only FNIII on extracellular part is constitutively active (Zabeau et al., 2005). Comparing the results of Zabeau et al. (2005) showing that LR without CRH1 domain is

active and work of Fong TM et al. (1998) showing inactivity of LR without CRH1 and FNIII domains, precisely suggest that FNIII domains may play a major role in orienting the intracellular domains from a non-signaling conformation into a signaling conformation upon binding of ligand. On the other hand, constitutively active FNIII receptor suggests, CRH1, Ig and CRH2 domains orient the intracellular domains in an inactive configuration; so either ligand binding or removal of CRH1, Ig and CRH2 domains makes the receptor active. Mass spectroscopic analysis has shown the two FNIII domains to have 5 N-glycosylation sites in human (Haniu et al., 1998) (Figure 2.9B-C).

2.4.2 Mapping of Interface between Leptin and CRH2

Alanine scanning mutation of interface residues based on homology model of CRH2/Leptin has provided important insights into the binding interface (Iserentant et al., 2005). Six out of sixteen mutants studied: I501A, F502A, L503A, L504A, S505A and D615A showed lesser sensitivity to leptin, distinct reduction in LR signaling. I501A, F502A, L503A, L504A and S505A mutants reduced the LR signaling drastically and increased the EC_{50} to 33.5-55.0nM vs. 6.44nm of wild type LR. This motif IFLLS is present on the E-F loop close to the hinge region on the distal CRH2 domain (Figure 2.11A-C). The D615A mutant is rather less sever with EC_{50} value of 24.1nM. This residue is located on the loop connecting the last β-strand "N" to the distal FNIII domain on the proximal CRH2 domain close to the inter-domain hinge at a distance of 16.6 Å and 16.8 Å from the L503 and L504 respectively. D615 may interact with the K5 in leptin via polar salt bridge. The effect on the Leptin binding have been assessed to be most severe for the I501A mutant with the *in vivo* measured K_D increased by 66 times. This appreciable reduction in the binding affinity for these mutants clearly suggests the crucial role played by these residues in leptin binding.

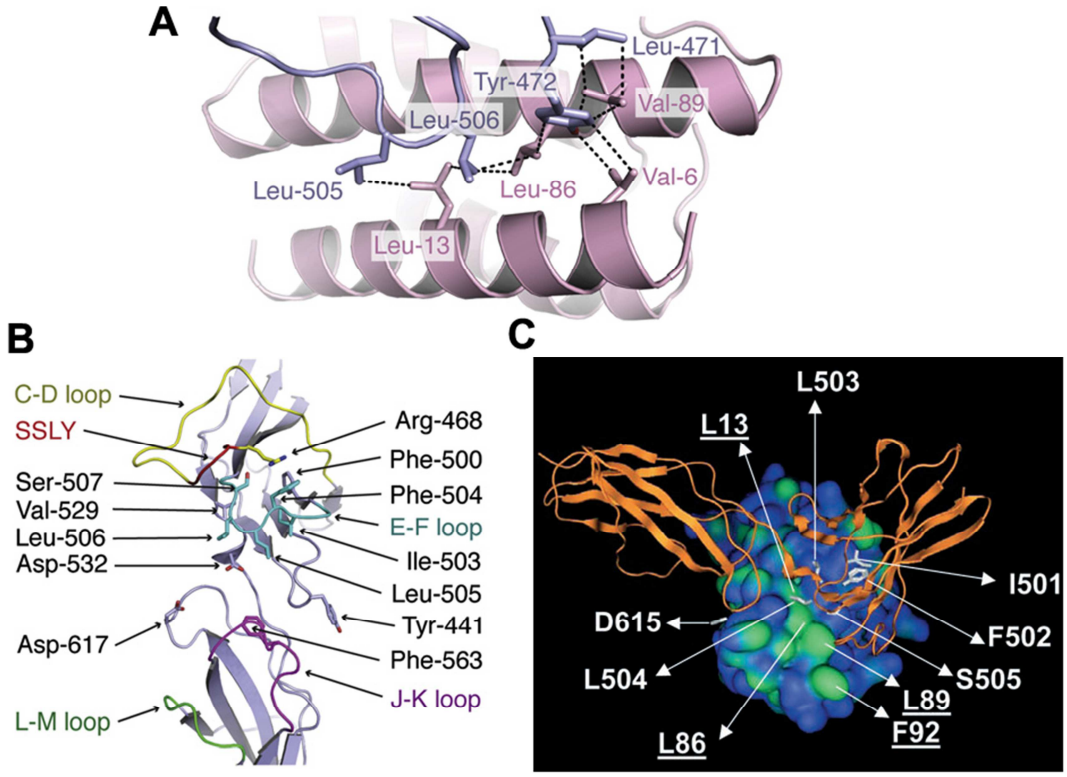

Figure 2.11. Molecular models of binding site II in leptin: ***(A)*** Hydrophobic and aromatic residues from the C-D loop (471–472) and E-F loop (505–506) of LBD (*light blue*) aligned with the hydrophobic cavity between helices 1 and 3 of leptin (*pink*). All contacts shown are less than 4.2 Å . ***(B)*** Leptin binding domain residues implicated in leptin binding by mutagenesis studies are shown as sticks. Important loops are C-D loop (*yellow*), E-F loop (*cyan*), J-K loop (*magenta*), L-M loop (*green*). The SSLY motif (*red*) is located within the C-D loop. (Adopted from Carpenter et al., 2012) **(B)** Residues in binding site II that affect binding to CRH2 are colored yellow. Residues in binding site II that affect both binding to CRH2 and LR activation are colored orange. (Adopted from Carpenter et al., 2012) **(C)** Model of the mouse leptin/CRH2 complex. The molecular surface of leptin is colored according to the surface hydrophobicity (*blue*, hydrophilic; *green*, hydrophobic). The CRH2 model is presented as ribbons, the Cα atom and heavy side chain atoms of residues I501, F502, L503, L504S and D615 are displayed as white sticks. L504 of CRH2 fits into the hydrophobic cleft of leptin and interacts with L13 and L86 of leptin (Adopted from Iserentant et al., 2005)

The role of the hydrophobic patch "IFLLS" have further been assessed with hydrophobicity-reversal mutations by mutating IFLL residues to serine (Iserentant et al., 2005). F502S and L503S did not differ much from their alanine counterparts in signaling. I501S and

L503S showed sever effect on signaling; while L504S lost its signaling capacity. Interestingly, L504S not only lost the signaling capacity, but also the leptin binding affinity was reduced by more than 10 times. Thus L504 may provide the most of the buried surface area (210Å) for the leptin/LR interaction. Homology based modeling suggests this hydrophobic patch would be interacting the hydrophobic cleft (L13, L86, L89, F92) formed in A-C helix in leptin. Computational modeling has suggested the involvement of E565, R615 beside D617 (E563, R613 and D615 in mouse) of CRH2 with Leptin via polar interactions.

While Y441A mutation has only limited effect, double mutant of chicken CRH2 with Y441A (Y442 in human) and F501A (F500 in human) showed complete loss of binding to ovine leptin (Niv-Spector et al., 2005). The authors have suggested important role of F501 in binding. Based on the location of the F500 (corresponding ovine F501) at the junction of the β-strand D and DE-loop in the crystal structure of human CRH2 (PDB ID:3V6O) (Figure 2.11A-B), F501A mutation in ovine CRH2 domain can have a profound effect on the position and/or secondary structure of the DE-loop harboring the conserved motif $I^{503}FLLS^{507}$, shown crucial for leptin binding (Isenterant et al., 2005). The importance of IFLLS motif has been shown in chicken by corresponding 504-VFLL-507 substitution mutation resulting loss of binding capacity. Mutagenesis of A533D/D534V in chicken leptin abolished its binding potential (Niv-Spector et al., 2005); although sequence alignment with human CRH2, does not show any conservation of these residues.

2.4.3 Physiological role of N-linked glycans in LR

N-linked glycosylation is a posttranslational modification of proteins characteristic of eukaryotes, archaea, but very rarely found in bacteria. Evidences suggest N-linked glycans play important role both in structure and function of the protein. LR has been shown to be unusually heavily glycosylated with 18 glycosylation sites in human LR_{ecto} (Haniu et al., 1998) (Figure 2.9). Analysis of importance of n-linked glycans in class I cytokine receptor neighbors has demonstrated crucial roles played by these glycans in some receptor systems if not in all. In GM-CSF receptor β, substitution of N-glycosylation site within CRH domain and 2 in close proximity interferes in ligand binding (Niu et al., 2000). In the shared receptor gp130, N-linked glycosylation has been shown to be highly crucial for the structural stability; though it has no role to play in signaling (Waetzig et al., 2010). In case of LR, treatment of short form LR with N-

glycosidase F has shown to reduce the leptin binding on cell surface by nearly 80% (Kamikubo et al., 2008). When LR was expressed in megakaryoblastic cell line MEG-01, similar results were obtained; treatment of receptor with N-glycosidase F led to decrease in leptin binding. On the contrary, removal of N-linked glycans from isolated CRH2 domain did not abolish the leptin binding potential (Kamikubo Y. et al., 2008). Other groups have also showed unglycosylated CRH2 domain expressed in bacterial host binds leptin with a nanomolar affinity (Carpenter et al., 2012; Sandowski et al., 2002). It has been suggested that n-linked glycosylation in LR has no direct role in leptin binding; but these glycans may stabilize the active conformation of the full length receptor on cell surface (Kamikubo Y. et al., 2008).

2.4.4 Leptin Receptor Isoforms

The leptin receptor, also called obesity receptor (ObR) receives an extra dimension of complexity by having at least 6 isotypes which differ from each other both at structural and functional levels yet to be understood completely (Figure 2.12). The 6 isotypes named ObRa, ObRb and so on upto ObRf, differ from each other in their intracellular part. All these isotypes except ObRe have identical extracellular parts and a single pass transmembrane helix. But they differ from each other in the length and the C-terminal sequence of the intracellular part. All these isotypes are membrane bound except ObRe, composed of the extracellular part of LR. All these isotypes are formed by alternate splicing; while ObRe has been reported to be formed by membrane shedding and proteolytic cleavage of membrane bound LR isotypes in human (Ge et al., 2002; Maamra et al., 2001). The longest receptor isotype is the ObRb, which is also the only isotype with well-studied signaling capability. All other isotypes are signaling-incapable; though they may play crucial roles in modulating leptin mediated signaling in vivo (Yang et al., 2004; Zhang and Scarpace, 2009).

ObRb is the only isotype functionally capable to signal upon leptin binding and thus controlling appetite and energy homeostasis. It is also known as long form LR owing it to be longest with 1162 residues in human. The intracellular part has a functional JAK binding box1 motif and a putative box2 motif.

ObRa, ObRc and ObRd, all are short form Leptin receptor; but they vary in their C-termini. ObRa has 894 residues with the unique C-terminal sequence RTDTL. ObRc has 892 residues ending with VTV as the unique C-terminus. ObRd is the longest of all short form LR isotypes

with 901 residues and the c-terminal sequence specific to this isotype is DISHFHEVFIFR-901. The expression of ObRa has been found to be ubiquitous in murine heart, brain, spleen, lung, liver, pancreas, adipocyte, skeletal muscle, kidney and testes; while ObRc and ObRd expression was extremely low (Fei et al., 1997) in these tissue types. But high level of expression have been reported for ObRa and ObRc in choroid plexus and brain microvessels, possibly playing role in blood-brain barrier transport of leptin (Tartaglia et al., 1995). All these three isotypes lack box1 motif and thus signaling incompetent.

Initially discovered as a defective mutant (Lee et al., 1996), ObRe is unique from all other isotypes in that it is not membrane-bound and is secreted directly into blood plasma in rodents. In human this isoform is formed by ectodomain shedding of other membrane bound isoforms (Ge et al., 2002). ObRe isotype represent the major binder of leptin in circulating in human blood (Lammert et al., 2001). After binding leptin, ObRe regulates the bioactivity of leptin and modulates its bioavailability. The generation of ObRe by the proteolytic cleavage of membrane bound isoforms in human is not a random phenomenon; rather regulated by a disintegrin and metalloproteinase 10 (ADAM10), with increased levels of shedding during induced lipotoxicity and apoptosis and reduced levels during high leptin concentration and ER stress(Schaab et al., 2012). The upregulation of ObRe antagonizes the effect of leptin by reducing levels of unbound leptin. The downregulation of ObRe levels in blood results from the decreased membrane expression of LR.

Ob-Rf was first discovered in rat brain cDNA library (Wang et al., 1996). It has 895 residues in total with the first 889 residues aligning well with human and mouse leptin receptor ObRb with 77 and 92% identity, suggesting homology in this region. The six C-terminal residues (IMPGRN) from 890 to 895 are unique without any known homologue in human or mouse at the time of its discovery, produced by splicing of discrete 3’ exons. There is no literature describing the specific role of this isotype. This isotype does not have box2 motif and so can not signal in a leptin dependent way.

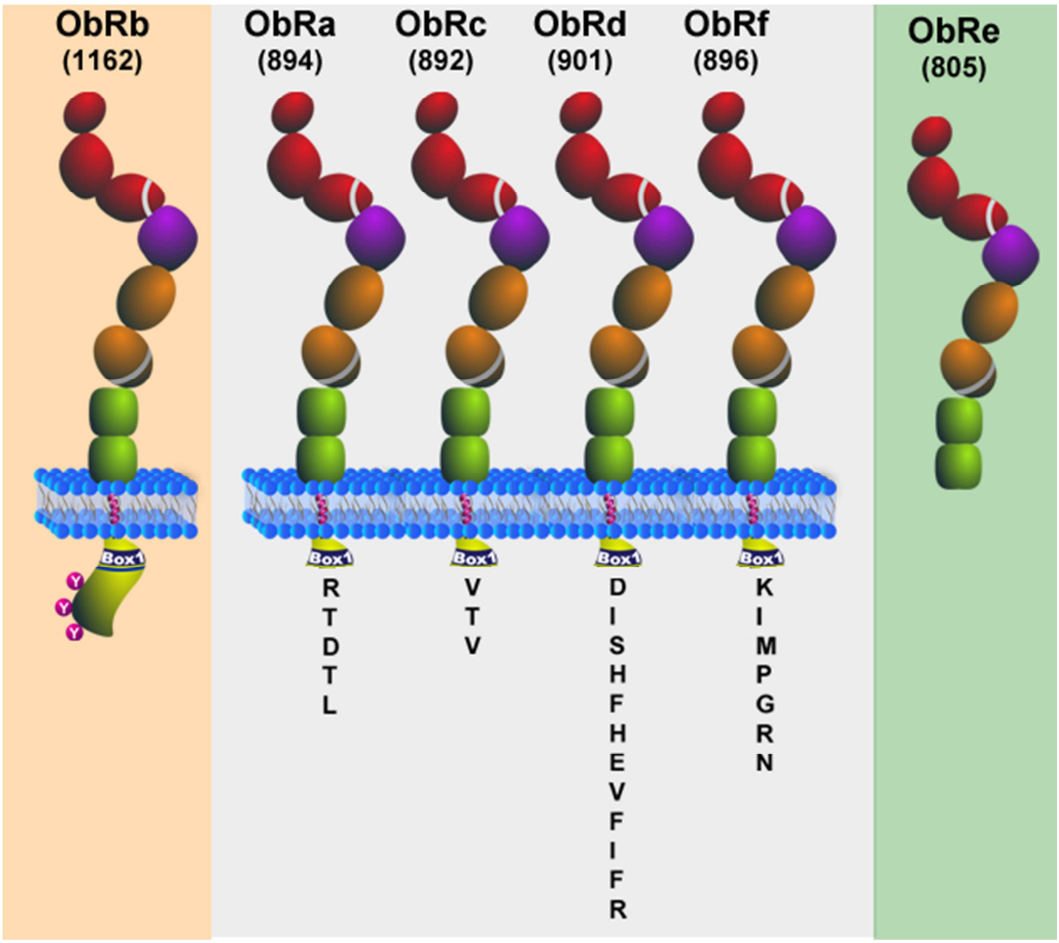

Figure 2.12. Leptin receptor isoforms: At least six different LR isoforms are found in human named ObRa to ObRf. ObRb is the longest one with fully functional intracellular parts and thus is the only functional isotype. All other isotypes, except ObRe, have intracellular parts with JAK binding box1 motif, but lacking STAT binding region. Each of these four (ObRa, ObRc, ObRd and ObRf) have unique C-terminal stretch of amino acids. The only soluble isotype is ObRe which lacks trans-membrane and intracellular parts. All these isoforms are formed by alternative splicing while ObRe is formed by ectodomain shedding in human.

2.4.5 Leptin Receptor Signaling pathways

The leptin receptor lacks intrinsic kinase activity and thus depends on the extrinsic kinases for phosphorylation of its intracellular part. The receptor juxtamembrane region has the box1 motif (6^{th} to 17^{th} amino acid of LR_{IC}) characterized by two highly conserved proline residues, providing a binding site for Janus Kinase 2 (JAK2) (Kloek et al., 2002) (Figure 2.9 and 2.13). JAK2 is constitutively bound to inactive receptor and only activated upon ligand induced receptor oligomerization and conformational change. There is a second conserved motif box2 (49^{th} to 60^{th} amino acid on LR_{IC}), a putative JAK2 interacting region which may play role in attaining maximal signaling activity (Bahrenberg et al., 2002; Kloek et al., 2002).

While JAK2-binding box1 motif is present in all LR isoforms, three highly conserved tyrosine residues (Y_{985}, Y_{1077}, Y_{1138} in murine LR) (Eyckerman et al., 2000; Myers, 2004) present only on the intracellular part of long form LR (ObRb) play role in recruitment of Signal Transducer and Activator of Transcription type 3 (STAT3) molecules. Activated JAK2 phosphorylates these three tyrosines and phosphorylated tyrosines form the binding sites for STAT3 molecules. Activated JAK2 phosphorylates bound STAT3 and activates it. Such phosphorylated STAT3 proteins dimerizes and translocates to nucleus for further modulation of gene regulation.

Although JAK2/STAT3 mediated signal transduction is the well-studied pathway of signal transduction in LR system, several other pathways have been shown to be associated with it (Figure 2.13). Non-canonical JAK1 activation has been demonstrated (Carpenter et al., 1998). STAT1 and STAT5B have been shown to linked with Leptin induced LR activation (Baumann et al., 1996). The first tyrosine Y_{985} has been shown to be involved in the recruitment of SH2-containing-Phosphatase 2 (Carpenter et al., 1998; Li and Friedman, 1999). Numerous evidences exist for the involvement of suppressor of cytokine signaling (SOCS) in Leptin signaling (Bjorbaek et al., 2000; Waelput et al., 2000). Leptin and insulin signal crosstalk via the phosphatidylinositol 3-kinase (PI3K) pathway (Margetic et al., 2002; Szanto and Kahn, 2000).

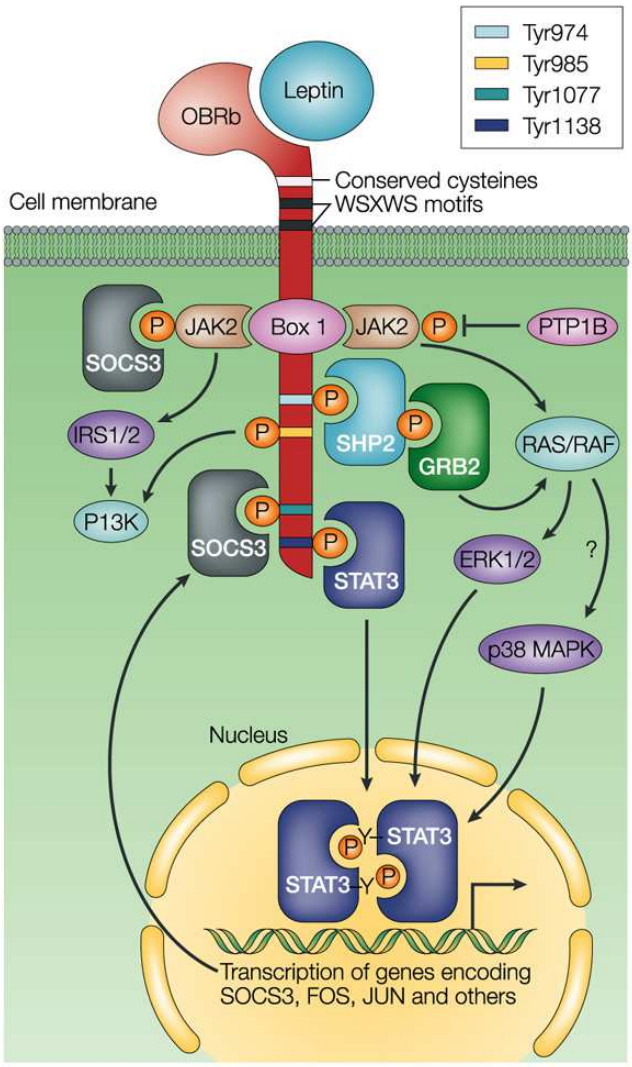

Figure 2.13. Signaling pathways activated by the leptin receptor: Only the long form of the leptin receptor (OBRb) can signal intracellularly, whereas the short forms of the leptin receptor do not. The total length of OBRb is 1162 amino acids; the extracellular domain consists of 816 amino acids and is a class I cytokine receptor that contains two cytokine-like binding motifs, Trp-Ser-Xaa-Trp-Ser (WSXWS), and a fibronectin type III domain. After binding leptin, OBRb-associated Janus-family tyrosine kinase 2 (JAK2) becomes activated by auto- or cross-phosphorylation and tyrosine phosphorylates the cytoplasmic domain of the receptor. Four of the phosphorylated tyrosine residues function as docking sites for cytoplasmic adaptors such as signal transducer and activator of transcription (STAT) factors, particularly STAT3 (in some cases, also STAT1 and STAT5). The membrane distal tyrosine (position 1138) functions as a docking site for STAT3, which is a substrate of JAK2. After subsequent dimerization, STAT3 translocates to the nucleus and induces the expression of suppressor of cytokine signaling 3 (*SOCS3*) and other genes. SOCS3 takes part in a feedback loop that inhibits leptin signaling by binding to phosphorylated tyrosines. SRC homology 2 (SH2) domain-containing phosphatase 2 (SHP2) is recruited to Tyr985 and Tyr974, and activates extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) pathways through the adaptor protein growth factor receptor-bound protein 2 (GRB2), ultimately inducing the expression of FOS and JUN. After leptin binding, JAK2 can induce phosphorylation of the insulin receptor substrate 1/2 (IRS1/2) proteins that are responsible for the activation of phosphatidylinositol 3-kinase (PI3K). Phosphotyrosine phosphatase 1B (PTP1B), which is localized on the surface of the endoplasmic reticulum, is involved in negative regulation of OBRb signaling through the dephosphorylation of JAK2 after internalization of the OBRb complex. (Adopted from La cava and Matarese, 2004)

2.5 TYPE 1 RECEPTOR ACTIVATION MECHANISMS

Although type 1 receptors show similarity in various aspects like sharing similar structural modules comprised of domains with Ig-like fold and FNIII folds, lack of intrinsic kinase activity and presence of the WSXWX motif etc., their mechanism of activation on cell membrane differs significantly (Figure 2.14). Based on the usage of a shared receptor, they can be classified into following two groups:

A) ***Shared cytokine receptors:*** This group shares common receptors among each other and so the signaling complex is always a heteromeric complex.

B) ***Non-shared cytokine receptors:*** These receptors do not share their receptors with each other. Thus ligand binding leads to homo-oligomerization.

As the mechanistic aspects and oligomeric nature of the leptin receptor signaling complex is not known yet, detailed analysis of the mechanistic principles followed by other members of type 1 receptors will provide important insights into the commonalities shared with other members, while simultaneously contrasting the distinctiveness of LR system from others in the class. So far, leptin receptor has shown not to be using any shared coreceptor for signal transduction (Nakashima et al., 1997); thus it can be categorized in the non-shared cytokine receptor group. On the other hand, the modular structure of LR_{ecto} comprising CRH, Ig-like and FNIII domains, shows striking similairity with members of shared cytokine receptors utilizing gp130 as co-receptor (Figure 2.14).

2.5.1 Shared Cytokine Receptors

This group of receptors utilize both a cognate receptor as well as a shared receptor to form a heteromeric signaling complex. Depending on the usage of shared receptors, these cytokines and their receptors can be divided into following four families:

A) γ-chain family
B) β-chain family
C) gp130 family
D) Interleukin 12 (IL-12) family

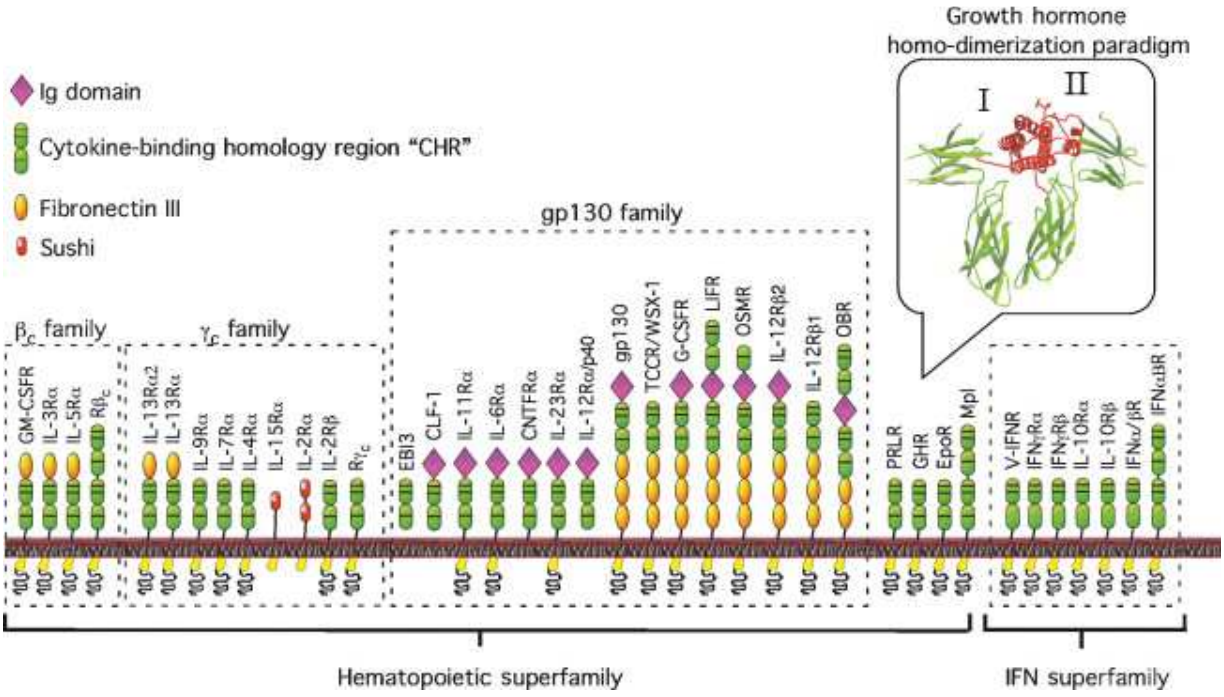

Figure 2.14. Modular structure and classification of type 1 cytokine receptors: Grouping of hematopoietic cytokine receptors by shared receptor usage. The majority of the hematopoietic cytokine receptors incorporate one of three shared signaling receptors, either the common beta (β_c) chain, the common gamma (γ_c) chain or gp130. The crystal structure of the growth hormone (*red*) complex (inset) was the first structure of a four helix bundle cytokine in complex with its receptor (*green*) (de Vos et al., 1992) and established the paradigm of cytokine/receptor complex formation. (Adopted from Boulanger and Garcia, 2004)

A) γ-chain Family

Members of this family are monomeric cytokines having a heterodimeric cognate receptor and sharing a common gamma chain among them. So they signal via a quaternary signaling complex on cell surface (Figure 2.15). This family includes following cytokine systems: IL-2, IL-4, IL-7, IL-9, IL-15, IL-21 and Thymic stromal lymphopoietin (TSLP).

(i) Interleukin 2/Interleukin 15 Cytokine System

IL-2 imparts its effect via its cognate receptor IL-2R composed of 2 subunits IL-2Rα, IL-2Rβ and a shared receptor IL2Rγ_c. This γ_c receptor is shared by other ILs like IL-4, IL-7, IL-9 and IL-15 (He and Malek, 1998). IL-2 rather than binding a preformed heterotrimeric receptor (IL-2R$\alpha\beta\gamma_c$), follows a more efficient stepwise assembly mechanism whereby it interacts with the highly expressing IL-2Rα cognate receptor via a long range, relatively weak and nanomolar ionic interaction with high K_{on} and K_{off} rates (Forsten and Lauffenburger, 1994). This induces a minor conformational change on the IL-2 enabling IL-2/IL-2Rα complex to interact with relatively low expressing IL-2Rβ solely via IL-2:IL-2Rβ polar interactions resulting in the heterotrimeric complex (IL-2/IL-2Rα/IL-2Rβ). Eventually, this heterotrimeric complex interacts with the common γ_c receptor, with major contributions from the IL-2Rβ:IL-2Rγ_c degenerate binding sites and a minor contribution from IL-2:IL-2Rγ_c interactions, thus forming the active quaternary signaling complex with picomolar affinity. The formation of this signaling complex is followed by the phosphorylation of 3 tyrosine residues on cytoplasmic part of IL-2Rβ mediated by JAK1 and JAK3 associated with β and γ_c receptors (Nelson and Willerford, 1998).

In case of IL-15, the mechanism of activation is very much similar to IL-2 system along with participation of the IL-2Rβ/γ_c complex in the formation of the active quaternary signaling complex. The major difference is the trans-presentation of IL-15 leading to a juxtacrine signaling, whereby IL-15 bound to IL-15Rα is presented to the IL-2Rβ/γ_c binary complex from another cell (Dubois et al., 2002; Sandau et al., 2004). The binding of IL-15 to IL-15Rα is a high affinity interaction with K_D value of 30-100 pM (Anderson et al., 1995; Bernard et al., 2004; Giri et al., 1995; Mortier et al., 2006). However, cis-presentation has also been found in IL-15 system, where by both the cognate and the shared receptors exist on the same cell surface (Olsen et al., 2007).

(ii) Interleukin 4/ Interleukin 13 Cytokine System

IL-4 binds to its cognate receptor IL-4Rα with sub-nanomolar affinity (LaPorte et al., 2008). The IL-4/IL-4Rα binary complex, depending on the cell type, can form two different types of signaling complexes: in hematopoietic cells, this complex interacts with the γ_c shared receptor with a dissociation constant (K_D) of 559 nM while in non-hematopoietic cells, this binary complex interacts with IL-13Rα1 receptor with binding constant K_D of 487nM (LaPorte et al., 2008). An experimental set up causing forced homodimerization of IL-4Rα chimeric receptor has resulted

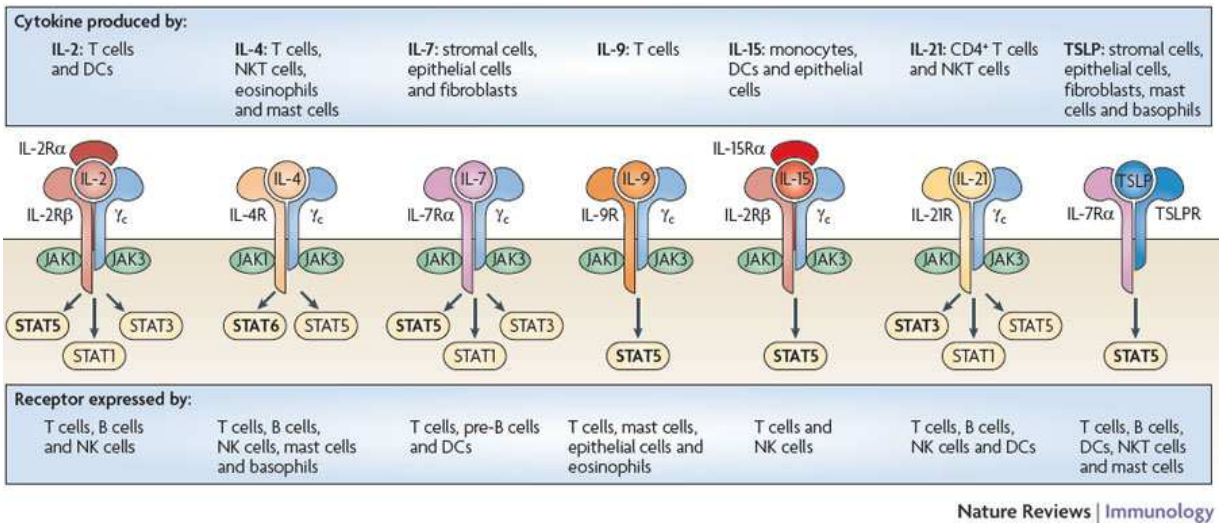

Figure 2.15. Receptors for γ_c family cytokines and TSLP: Shown are the receptors for interleukin-2 (IL-2), IL-4, IL-7, IL-9, IL-15, IL-21 and thymic stromal lymphopoietin (TSLP). IL-2 and IL-15 are the only two of these cytokines to have three receptor chains. The receptors for these two cytokines share the common cytokine receptor γ-chain (γ_c; also known as IL-2Rγ) and IL-2Rβ, and the receptors for IL-7 and TSLP share IL-7Rα. Of the cytokines shown, only TSLP does not signal through a receptor containing γ_c. There are three classes of IL-2 receptor that bind IL-2 with low affinity (IL-2Rα alone), intermediate affinity (IL-2Rβ and γ_c) and high affinity (IL-2Rα, IL-2Rβ and γ_c); only the high-affinity IL-2 receptor is shown. The receptor for each γ_c family cytokine activates Janus kinase 1 (JAK1) and JAK3, whereas the receptor for TSLP has been reported to not activate any JAK. The main signal transducer and activator of transcription (STAT) proteins that are activated by these cytokine receptors are shown in bold. STAT5 refers to both STAT5A and STAT5B. DC, dendritic cell; NK cell, natural killer cell; NKT cell, natural killer T cell. (Adopted from Rochman et al., 2009).

in the IL-4 induced receptor activation in the absence of the co-receptors. But *in vivo*, IL-4Rα always works via the ternary IL-4/IL-4Rα/γ_c or IL-4/IL-4α/ IL-13α1 complex, suggesting inevitable requirement of the co-receptors in signaling. Interestingly, IL13 receptor has another isoform called IL-13Rα2 (Caput et al., 1996; Zhang et al., 1997b). It has been shown to be a non-signaling decoy receptor, with a short, non-functional cytosolic part (Donaldson et al., 1998). Its major function is the regulation of IL-13 and IL-4 signaling (Andrews et al., 2006).

(iii) Interleukin 7/ Thymic stromal lymphopoietin (TSLP) Cytokine System

The IL-7 cognate receptor IL-7Rα, in the absence of IL-7 has been shown to pre-exist as both inactive homodimer and as inactive heterodimer in complex with the common gamma chain γ_c (Rose et al., 2009; Rose et al., 2010). In vitro studies show little self-association of IL-7Rα to form homodimer and high affinity interaction between IL-7Rα EC and γ_c EC with a K_D value of 140µM (McElroy et al., 2012). IL-7 signals via the ternary complex of IL-7/IL-7Rα/γ_c. Thymic stromal lymphopoietin (TSLP) (Ray et al., 1996), which shows striking similarity to IL-7 in its signaling mechanism and functionality, signals via a ternary complex formed of its cognate receptor TSLPR and shared co-receptor IL-7Rα. The exact mechanism of association of the receptors during complex formation are yet to be revealed.

(iv) Interleukin 9 Cytokine System

IL-9 signals via its cognate receptor IL-9R (Renauld et al., 1992) and common gamma chain γ_c (Kimura et al., 1995) for signal transduction. IL-9 signaling complex is a ternary complex of IL-9/IL-9Rα/γ_c shared receptor (Bauer, 1998). The details of the interaction of the extracellular domains have not been revealed till now.

(v) Interleukin 21 Cytokine system

The shared coreceptor γ_c has been shown to be indispensable for IL-21 signaling (Asao et al., 2001; Habib et al., 2002). IL-21 interaction with its cognate receptor IL-21R has been characterized to be high affinity with K_D of 70pM (Zhang et al., 2003). Beside, this independent binding of IL-21 to IL-21R (Mehta et al., 2004), IL-21 can directly interact with γ_c, but with relatively low affinity (K_D=160µM). The mechanism of IL-21 has been proposed to be via a ternary complex of IL-21/IL-21R/γ_c common receptor.

B) β_c-chain Family

The common β-chain (β_c) is shared between at least 3 cytokines: Granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-5. Human β_c is a 881 residue long, glycoprotein receptor with an extracellular part of 427 residues comprising two pairs of FNIII domains and an intracellular part of 437 residues. It is the major signal transducing unit harboring the docking sites for STAT, MAPK, PI3K etc.

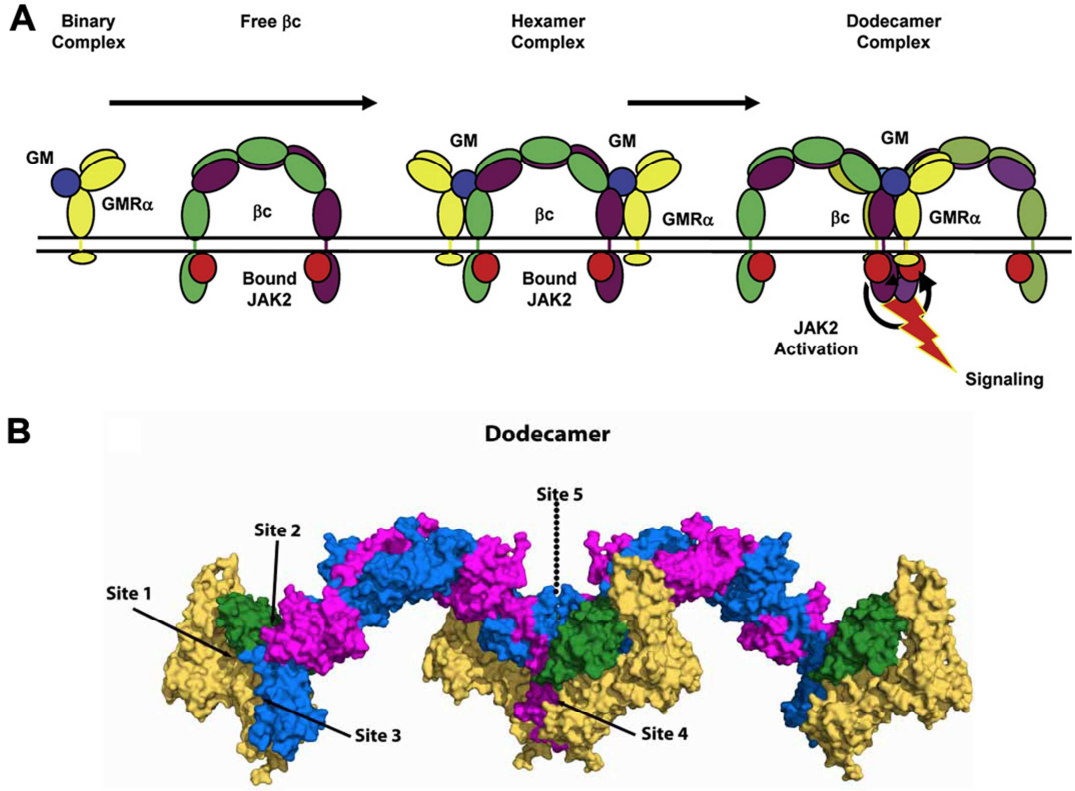

Figure 2.16. Model of Signal Transduction for GM-CSF: ***(A)*** The low-affinity complex consists of GM-CSF bound to GMRα. Interaction with free βc forms the high-affinity hexamer complex. Dodecamer (or higher-order) complexes form by lateral aggregation of hexamer complexes to form a fully competent signaling complex. JAK2 associated with βc (*red* spheres) is able to dimerize and transphosphorylate in the dodecamer complex but not in the hexamer complex. (Adopted from Hansen et al., 2008); ***(B)*** Surface representation of the GM-CSF receptor dodecamer complex, showing two interacting hexamer complexes and the five sites of interaction. (Adopted from Hercus et al., 2012)

(i) Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) Cytokine System

GM-CSF is one of the most well studied systems in this group. It is a four helical bundle cytokine like most other belonging to type-1 class (Rozwarski et al., 1996; Wong et al., 1985). GM-CSF interacts with its cognate receptor GMRα to form the binary complex with interactions contributed by Ig and FNIII domains of GMRα (Hercus et al., 1994; Mirza et al., 2010). The binding has been shown to be relatively high affinity (K_D=100-200 pM) in the presence of the shared co-receptor β_c compared to GMRα alone (K_D=1-10 nM) (Gearing et al., 1989; Hayashida

et al., 1990). GMRα has a N-terminal Ig-domain, two membrane proximal FNIII domains and an intracellular part of 54 residues without any role in activation. This GM-CSF/GMRα binary complex interacts with the homodimeric βc shared receptor, thus forming the hetero-hexameric complex with two GM-CSF/two GMRα/homodimer βc (Hansen et al., 2008). Such two heterohexamers interact via sites 4, 5 and 6 (Figure 2.16B) to form a dodecameric signaling complex (Figure 2.16A).

(ii) Interleukin 3 Cytokine System

While only a solution structure of IL-3 is available since 1996 (Feng et al., 1996) , structure of IL-3 receptor has not been solved yet. Based on mutation analysis, domain truncation and homology modeling, a putative dodecameric model of activation has been proposed by independent investigators (Broughton et al., 2012; Dey et al., 2009). IL3 binds the ligand-specific IL-3Rα receptor; this binary complex recruits a dimeric shared receptor β_c forming a 2IL3:2IL-3α:2βc hexameric assembly. The extracellular part of IL-3Rα has a N-terminal Ig like domain followed by two membrane proximal tandem repeats of fibronectine like type III domains. The receptor β_c is shared with other members GM-CSF and IL5 of this family. While IL-3 interaction with IL-3Rα is relatively low affinity (120nM), interaction of IL-3 with IL-3Rα and β_c is high affinity (140pM). Unlike human single β_c, mouse β receptor is formed of two subunits a shared β_c and IL-3 specific βIL-3. The interactions stabilizing this hexamer can be divided into three sites: (i) IL-3 interacts with IL-3Rα via the site-1 (ii) dimerization interface between two β_c dimer formed by domain-4 of one β_c and domain 1 of another β_c, referred as site 2 (iii) membrane proximal domain 3 of IL-3Rα and domain 4 of β_c forms the site 3. Two such hexamer units dimerize via 2 (Broughton et al., 2012) or 3 (Dey et al., 2009) interaction sites to form the active signaling dodecameric signaling complex. Membrane proximal domains of IL-3Rα and β_c between hexamer units form the fourth interaction site : site 4. Mutation in this region prevents dodecamerization and receptor activation, without affecting the IL-3 mediated hexamerization. The interaction of neighboring IL-3 in the interface of two hexamers have been predicted to be a leucine zipper interaction and it forms the site 5. This interaction has been hypothesized to be the driving force for the hexamer-hexamer dodecamerization. Based on model proposed by Dey et al. (2009), a site 6 may exist where N-terminal domains of adjacent IL-3Rα would be interacting with each other; while signaling complex model proposed by Broughton et al. (2012) contradicts the existence of such site 6.

(iii) Interleukin 5 Cytokine System

IL-5 is a homodimeric cytokine (Milburn et al., 1993) with two units of four helical bundle cytokines linked to each other via ionic, hydrophobic and disulfide interaction, in contrast to the monomeric IL-3 and GM-CSF (Wells et al., 1994). Although IL-5 is a dimer and it has two binding sites each accessible to interact with one cognate receptor, binding of one receptor sterically hinders the second binding site. Thus one IL-5 dimer binds to IL-5Rα with 1:1 stoichiometry (Johanson et al., 1995; Morton et al., 1994; Patino et al., 2011). The crystal structures of only binary complex of IL-5/IL-5Rα extracellular part are available (Kusano et al., 2012; Patino et al., 2011) suggesting a wrench-like architecture. The binary IL-5 signaling interactions follow a similar trend as IL-3, recruiting the shared receptor β_c to form the active signaling complex. Computational modeling based on the ternary complex of GM-CSF/GMRα/β_c crystal structure (Hansen et al., 2008), shows a octameric complex with two IL-5 dimers: two IL-5Rα:one βc dimer. Analogous to IL-3 and GMCSF dodecameric signaling complexes, a hexadecameric signaling complex of IL-5 has been proposed (Broughton et al., 2012; Patino et al., 2011).

C) Glycoprotein 130 (gp130) Family

There are numerous cytokine receptor systems sharing gp130 framework for signal transduction some of which are: IL-6, IL-11, IL-27, IL-30, IL-31, Oncostatin M (OSM), Leukocyte inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and cardiotrophic factor 1 (CTF1). These receptors follow mechanistic paradigms which utilize gp130 as the shared co-receptor and involve either a hetero-trimeric or a hetero-hexameric signaling complex (Figure 2.17). IL-6 and IL-11 signals via dimeric gp130 and a signaling deficient cytokine specific receptor to form a hexameric signaling complex. On the other hand, members like OSM, LIF, CNTF and CTF1 bind gp130 and then recruit their cognate receptors to form the heterotrimeric signaling complex.

(i) Interleukin 6 Cytokine System

This represent the most well-studied system utilizing gp-130 as its shared co-receptor. IL-6 is a four helical bundle cytokine (Somers et al., 1997) with both proinflammatory and anti-inflammatory functionalities. The first step of signaling complex formation nucleates with IL-6 interacting with its cognate receptor IL-6Rα. IL-6Rα lacks the intracellular JAK-STAT binding

regions and thus IL-6/IL-6Rα binary complex is signaling incompetent. The major interactions in this binary complex comes from the A and D helices of IL-6, forming the binding site I on IL-6. The D3 domain of CRH region of IL-6Rα alone, contributes 70% of binding surface (Boulanger et al., 2003). The binding constants for this interaction has been determined to be 9 nM with 1:1 stoichiometry. This inactive binary complex then recruits the bivalent constitutively dimeric gp130 (Tenhumberg et al., 2006) to form a hexameric assembly with two copies of IL-6, IL-6Rα and gp130 (Boulanger et al., 2003). This interaction with gp130 is mediated by two sites: site II is a composite site formed by both IL-6 and IL-6α, while site III formed completely of epitopes from IL6.

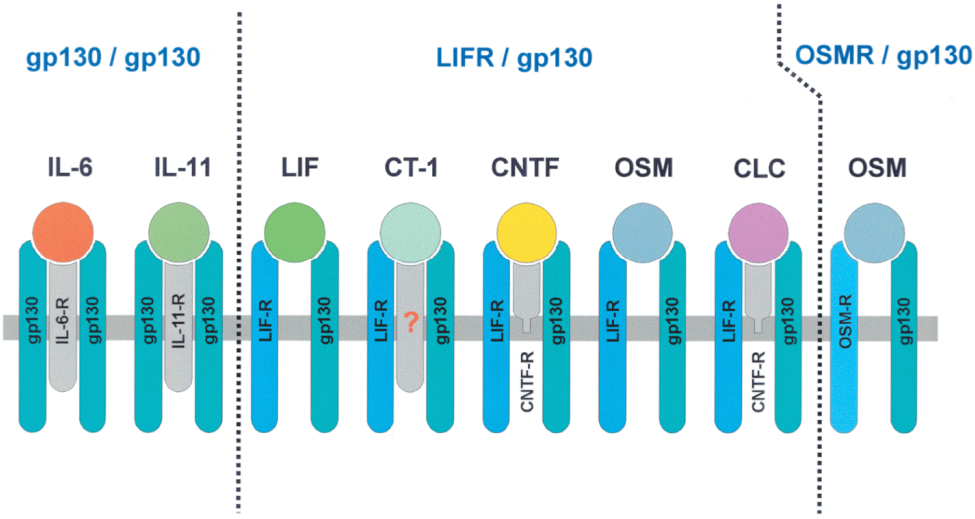

Figure 2.17. Receptor complexes of IL-6-type cytokines: IL-6-type cytokine receptor complexes signal through different combinations of the signaling receptor subunits gp130, LIFR and OSMR, with gp130 being used by all the family members. (Adopted from Heinrich et al., 2003)

(ii) Interleukin 11 Cytokine System

The atomic structure of IL-11 is not known yet. But it has been proposed to be a long four helical bundle cytokine (Yanaka et al., 2011) . Its cognate receptor, IL-11Rα (Yin et al., 1992) has at least two isoforms IL-11Rα1 and IL-11Rα2, formed by alternate splicing (Cherel et al., 1995). IL-11Rα1 has a cytoplasmic part while IL-11Rα2 lacks one. IL-11Rα extracellular part

has N-terminal Ig-domain followed by two FNIII domains forming the cytokine receptor homology (CRH) domain. The membrane proximal FNIII sub-domain of CRH2 can bind IL-11 (Schleinkofer et al., 2001). IL-11 interacts with the CRH domain of its cognate receptor via the site I (Barton et al., 1999) with a stoichiometry of 1:1 (Barton et al., 2000) and dissociation constant K_D=10-50nM (Curtis et al., 1997). This binary complex can then interact with gp130 to form the signaling complex. The final signaling complex has been suggested to be hexameric by independent investigators, formed by two IL-11/two IL-11α1/two gp130 (Barton et al., 2000; Neddermann et al., 1996).

(iii) Oncostatin M (OSM)

Signalling by human OSM differs from other type 1 cytokine receptors in a way that it can form a heterotrimeric signaling complex using common receptor gp130 and either leukemia inhibitory factor receptor (LIFR) or the cognate receptor oncostatin M receptor (OSMR) (Gearing and Bruce, 1992; Mosley et al., 1996). The sequential binding proceeds via OSM binding to gp130 and then recruiting either LIFR or OSMR in human (Modrell et al., 1994). On the contrary, in mouse OSM can only signal via the OSMR receptor and gp130 (Lindberg et al., 1998). Though these receptors have been shown to mediate the signaling, the exact oligomeric nature of the signaling complex is still lacking in literature and require more structural and functional studies to obtain mechanistic insights.

(iv) Leukemia inhibitory factor (LIF)

Leukemia inhibitory factor receptor (LIFR) is the cognate receptor of LIF; however it is shared by many other cytokines like OSM, ciliary neurotrophic factor (CNTF), neurotrophin-1/cardiotrophin-like cytokine and cardiotrophin-1. LIF signals via sequential formation of LIF/LIFR binary complex, followed by recruitment of common gp130 (Gearing and Bruce, 1992; Hilton and Nicola, 1992; Stewart et al., 1992; Zhang et al., 1998). Among the IL-6 family of receptors signaling via a trimeric signaling complex, LIF system has been studied most extensively, along with successful crystallographic structure determination (Huyton et al., 2007). LIF signaling complex presents an unusual cytokine-receptor interaction revealing an unique paradigm for the receptors sharing gp130 and having a hetero-trimeric signaling complex. The major binding site is formed by the non-canonical interaction of site III from ligand with Ig-domain and CRH loops of the receptor, thereby presents a sharp contrast to hexameric

signaling complexes of IL-6 family where the “ligand site I/II-receptor CRH” is the major binding interactions.

D) Interleukin 12 (IL-12) Family

The very unique feature of this family is the heterodimeric nature of its cytokines. Some of the well-studied IL-12 family cytokines include IL-12 itself, IL-23, IL-27 and IL-35 (Figure 2.18). This family resembles the IL-6 family by having gp-130 as the shared co-receptor and recruitment of this co-receptor by the cytokine-cognate receptor binary complex is inevitable for the activation.

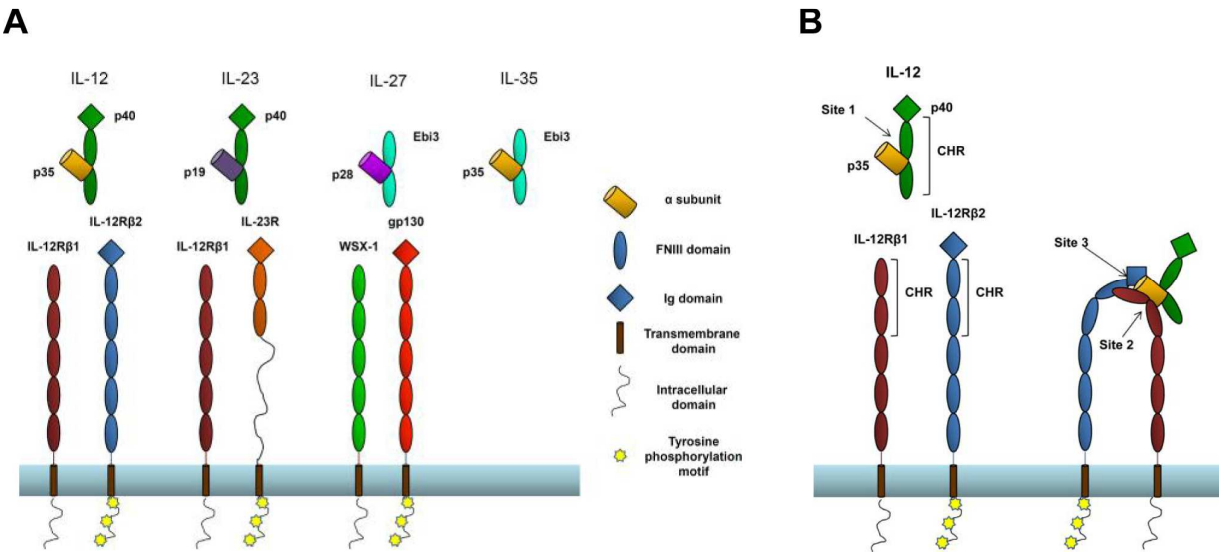

Figure 2.18. IL-12 Family of cytokines receptors and predicted binding sites: (A) Four different IL-12 family of cytokines presenting the heterodimeric ligands and corresponding modular receptors. **(B)** Predicted binding site 1-3 in IL-12 system (Adopted from Jones and Vignali, 2011).

(i) Interleukin 12 Cytokine System

IL-12 (also called IL-12p70) is a disulfide linked heterodimer composed of p35 and p40 subunits. Its subunit p35 is homologue of other type 1 cytokines like IL-6 and GCSF (Merberg et al., 1992). On the other hand, p40 shows similarity with the IL-6Rα and the CNTF (Gearing and

Cosman, 1991). Beside IL-12, it also exists as a subunit in IL-23 cytokine. It is composed of a N-terminal Ig-domain followed by two C-terminal CRH domains. The receptor for IL-12 is a heterodimer formed of the cognate receptor IL-12Rβ2 and a shared co-receptor IL-12Rβ1 (Presky et al., 1996). Both IL-12 and IL-23 utilizes IL-12Rβ1 as the shared co-receptor. Only IL-12Rβ2 has three cytoplasmic tyrosine residues participating in IL-12 signaling. IL12 can interact with IL-12Rβ1 with nanomolar affinity to form a non-signaling complex. But with IL-12Rβ1/ β2 heterodimer, it interacts with 5-20pM affinity to form the hetero-tetrameric signaling complex.

(ii) Interleukin 27 Cytokine System

IL-27 is a heterodimeric cytokine formed of two subunits: IL-27B (also called Epstein-Barr Virus-induced gene 3 or EBI3) and IL-27-p28(also called IL-30) (Pflanz et al., 2002). IL-27B is a 34 kDa glycoprotein without any membrane anchoring motif (Devergne et al., 1996) and considered as the soluble cytokine receptor for IL-30. The structural analysis of IL-27B shows it to be composed of a CRH domain comprising two FNIII subdomains, conserved disulfide bridge and a C-terminal WSXWS motif (Rousseau et al., 2010). IL-30 is a four helical bundle cytokine of 28kDa. The cognate receptor for IL-27 heterodimer has been shown to be a type-1 receptor called IL-27Rα (also known as WSX-1 or TCCR) (Pflanz et al., 2004); though this cognate receptor can bind IL-27 independently, the IL-27/IL-27Rα is signal-inactive. The shared coreceptor for IL-6 family, gp130, has been shown to mediate the signaling. This system has been proposed to signal via the quaternary complex of IL-27/IL-27Rα/gp130. (Pflanz et al., 2002).

2.5.2 Non-shared Cytokine Receptors

These type 1 cytokine receptors do not share their receptors, thus forms homomeric signaling complex via their respective cognate receptors only. Well-studied systems include: growth hormone/growth hormone receptor, granulocyte colony-stimulating factor/granulocyte colony-stimulating factor and erythropoietin/erythropoietin receptor. Although leptin receptor does not use any shared receptors, unlike these receptors, the exact oligomeric nature of the signaling complex of leptin/LR is not known yet.

(i) Growth Hormone

Growth hormone (GH) drives the dimerization of its cognate receptor GHR by binding to one receptor with high affinity followed by recruitment of a second receptor, thus forming a trimeric signaling complex (Cunningham et al., 1991; de Vos et al., 1992) (Figure 2.19A). The ligand-receptor interaction has been quantified to be high affinity with K_D value of 2 nM (Uchida et al., 1999). However, ligand-independent, pre-dimerized inactive GHRs have been observed (Frank, 2002; Gent et al., 2002). Structural study of the crystallographic structure of GH/GHR complex vs. unbound GHR suggests that ligand induced activation is achieved by relative rotation of subunits within the constitutive receptor dimer without significant conformational changes (Brown et al., 2005). Similar to erythropoietin receptor, in GHR the transmembrane region has been shown to playing role in ligand independent oligomerization (Yang et al., 2007).

(ii) Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor (G-CSF) can drive the dimerization of its cognate receptor G-CSFR to form a stable 2:2 complex (Figure 2.19B) with cross-over receptors (Tamada et al., 2006). Formation of quaternary complex involves interaction of ligand site II with the CRH domain from one receptor followed by second interaction between site III of G-CSF and Ig-like domain of other GCSFR, thus forming a crossover structure. Although the 1:1 and 1:2 stoichiometry of G-CSF/G-CSFR is well established (Hiraoka et al., 1995a; Hiraoka et al., 1995b) and the crystal structures of both 1:1 (Aritomi et al., 1999) and 2:2 complex (Tamada et al., 2006) have been solved, the oligomeric nature of the signaling complex on cell surface is ambiguous with three proposed possibilities: 1:1, 2:2 (Figure 2.19B) and 4:4 complexes (Hiraoka et al., 1995a; Hiraoka et al., 1994; Hiraoka et al., 1995b; Horan et al., 1996; Horan et al., 1998).

(iii) Erythropoietin

In contrast to the G-CSFR which is present as monomer on membrane and activated by ligand induced dimerization, Erythropoietin receptor (EpoR) is present as inactive constitutive dimers on membrane (Livnah, 1999) and activated by Epo-induced conformational change . Physical interactions driving this ligand independent dimerization has been attributed and localized to both Epo binding site on extracellular part of receptor (Livnah, 1999) as well as the transmembrane region (Ebie and Fleming, 2007). The actvation mechanism is quite similar to that of growth hormone system, as single Epo interacts with pre-dimerized EpoR to bring

conformational changes required for receptor activation and thus signals via a trimeric signaling complex (Figure 2.19C).

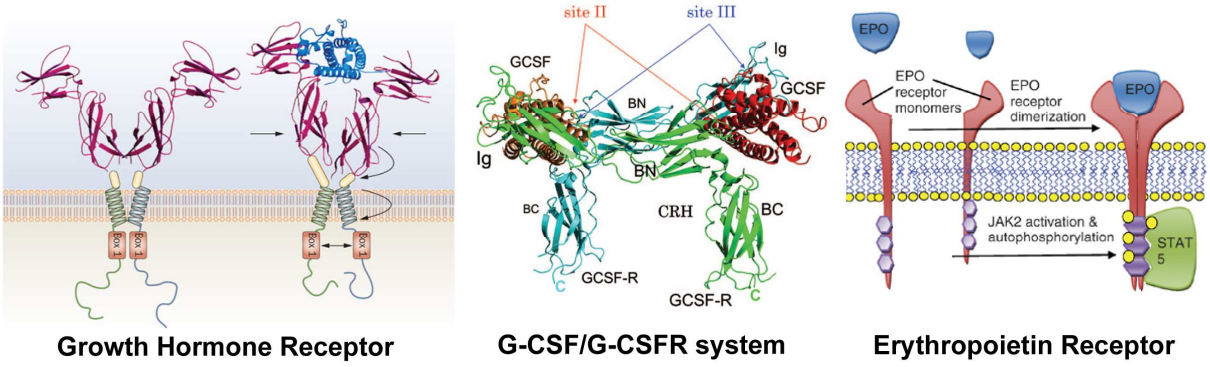

Figure 2.19. Three non-shared cytokine receptor systems: These receptor systems function via ligand induced formation of homo-oligomeric signaling complex. The systems include growth hormone (GH), granulocyte colony stimulating factor (G-CSF) and erythropoietin (Epo) and their respective receptors. GH receptor (GHR) exists as constitutive dimer on membrane and activated by the ligand induced conformation change in 1 ligand:2 receptor stoichiometry. G-CSF induce dimerization of its receptor G-CSFR, thus forming a homodimeric crossover complex of 2:2 stoichiometry. Epo receptor has been shown to exist as constitutive dimer and activated by Epo induced conformation rearrangement. (Adopted from Brooks and Waters, 2010 for GH/GHR system; from Tamada et al., 2005 for G-CSF/G-CSFR system; from Patnaik and Tefferi, 2009 for Epo/EpoR system)

2.6 MECHANISM OF LEPTIN RECEPTOR ACTIVAION

2.6.1 Leptin independent receptor oligomerization

Numerous receptors have been shown to exist as inactive, pre-oligomerized receptor complexes before the interaction with the ligand. Dimeric erythropoetin receptor (Constantinescu et al., 2001; Livnah, 1999; Remy et al., 1999), dimeric growth hormone receptor (Brown et al., 2005; Gent et al., 2002), interferon-γ receptor (Krause et al., 2002), interleukin-6 receptor (Schuster et al., 2003) and shared β-receptor of IL-3 family of receptors are examples of ligand-independent oligomeric assembly of receptors. Though not much structural evidence exist for pre-assembled leptin receptor oligomers on membrane, indirect analysis has proven their presence *in vivo*.

i) Evidences supporting ligand-independent LR oligomerization

Based on the gel filtration analysis, Devos R. et al. (1997) has concluded that extracellular part of LR expresses as preformed homodimer when expressed in baculovirus-infected insect cells. Coimmunoprecipitation of two different epitope-tagged receptor from COS7 cells provides evidences in support of leptin independent LR oligomerization on cell surface (Nakashima et al., 1997).

Ligand independent oligomerization has also been shown both in LR_{long} and LR_{short} using immunoprecipitation and immunoblotting (White and Tartaglia, 1999). The bioluminescence resonance energy transfer (BRET) study of pre-ligand LR on cell surface (Couturier and Jockers, 2003) provides a better assessment of the ligand independent oligomerization by analyzing at a maximum possible physiological state and minimizing the effects of external factors as in (co)immunoprecipitation etc. Direct measurement of BRET signal from receptors fused with firefly luciferase (Luc) and yellow fluorescent protein (YFP) from living cells, showed a very high basal signal, suggesting the two fluorophores and so the receptors are at less than 100 Å apart from each other. Similar observations were obtained in intact cells, isolated plasma membrane and light membrane preparation, suggesting LR oligomerization not only exists on plasma membrane, but also in other compartments inside cell.

Based on the western blot analysis of a series of murine LR deletion variants lacking one or more than one extracellular domains, LR has been proposed to exist as preformed disulfide linked dimer and oligomer on the cell surface (Zabeau et al., 2005).

ii) Sites and interactions necessary for ligand independent LR oligomerization

Use of truncated receptors to study the leptin-independent LR oligomerization shows the putative sites of interaction stabilizing these oligomers. The short form LR (LR_{short}) differs from the long form LR (LR_{long}) in their cytoplasmic side by lacking the box2 and box3 motif, essential for STAT signaling. The box1 motif is still present and so JAK2 kinase can still bind LR_{short} to the PNP motif in box1. Basal BRET signal for both form LR_{long} and LR_{short} has been shown to be high suggesting both forms exist as oligomers in cells and in extracted membranes (Couturier and Jockers, 2003). This nullifies any role of the major part of LR_{IC} that is missing in LR_{short} to be contributing towards ligand independent oligomerization. This leaves the possibility that LR_{ecto}, transmembrane helix and/or the short LR_{IC} containing box1 motif would be responsible for the leptin independent oligomerization.

Similar evidences for the existence of LR homo-oligomers of LR_{long} and LR_{short} and absence of LR hetero-oligomers have been demonstrated using quantitative FRET (Biener et al., 2005).

Zabeau et al. (2005) has also demonstrated that FNIII domains when expressed in isolation, formed disulfide linked dimer and oligomer, suggesting the existence of homotypic interactions contributed by FNIII domains to stabilize pre-formed oligomer. As a double mutant receptor LR:ΔCRH1-ΔIg-C72S/C751S with substituted FNIII cysteines exist as dimers under non-reducing condition, Zabeau et al. has concluded that the CRH2 domain may also involve in the ligand independent clustering of LRs on membrane. This result has been supported by the sequence analysis showing cross-species conservation of cysteines in LR. Similar ligand independent dimerization has been seen in erythropoetin receptor (EpoR), where the ligand interacting domain plays major role for receptor dimerization in the absence of its ligand erythropoietin (Livnah, 1999).

Recent evidence from the electron microscopy of LR_{ecto} has suggested it to be monomeric in solution; but the very low concentration of LR_{ecto} used in this kind of experiment would be a limiting factor especially to rule out existence of any concentration dependent oligomerization.

So based on all these observations, LR seems to exist as a pre-assembled dimer on the membrane, the binding epitopes involved being contributed by the transmembrane helix, short intracellular part having box1 motif and possibly the LR_{ecto} parts.

2.6.2 Leptin/Leptin Receptor complex

Evidence for an oligomeric LR signaling complex first came from the study of GCSFR/LR and LR/GCSFR chimera (White et al., 1997). GCSFR/LR chimera has extracellular part of GCSFR and trans-membrane and intracellular part of human LR (aa 829-1165). White et al. has shown that ligand dependent oligomerization of intracellular part of LR is the pre-requisite for signaling by showing that GCSF can drive the dimerization of the extracellular part of GCSFR/LR chimera and elicit the LR_{IC} mediated STAT signaling. However this experiment could not prove the dimerization capability of leptin. GCSFR, being the closest homologue with a sequence identity of 24% with human LR, a 2:2 homology model has been proposed for leptin/LR complex (Hiroike et al., 2000). Nonetheless, GCSFR receptor shows dominant negative repression, a distinct behavior LR has been found to be resistant to. While co-transfection of increasing quantity of truncated non-functional GCSFR represses the signaling capability of functional GCSFR, due to increasing dimers having at least one partner as nonfunctional receptor, LR was found to be relatively resistant to such repression. Such a different behavior shown by LR has been attributed to the possibility of an oligomeric signaling complex higher than quaternary 2:2 complex (White et al., 1997)

The second evidence for oligomeric leptin/leptin receptor came from chemical crosslinking of ^{125}I-labelled leptin with LR expressed on cell surface followed by SDS PAGE (Devos et al., 1997). But the exact oligomeric state of the complex could not be determined because of the presence of the higher order oligomers beside major monomer band and a second major dimer bands on the SDS PAGE analysis. The formation of a quaternary complex (2 Leptin: 2 receptor) between leptin and extracellular part of leptin receptor have been demonstrated by same group. According to Devos R. et al., as the receptor exists as a preformed dimer on membrane and the final signaling complex upon leptin exposure is a homodimeric quaternary complex, leptin signals via conformational change upon leptin binding to predimerized LRs; unlike G-CSFR, no leptin induced oligomerization was postulated.

Bioluminescence resonance energy transfer (BRET) study has provided important insights into LR signaling complex (Couturier and Jockers, 2003). These experiments suggested that leptin induced conformational change of preassembled LR leads to activation of LR. BRET signal increased upon leptin exposure to LR_{short} but not in case of LR_{long} possibly due to larger spatial movements expected for BRET donor and acceptor present at the juxta-membrane

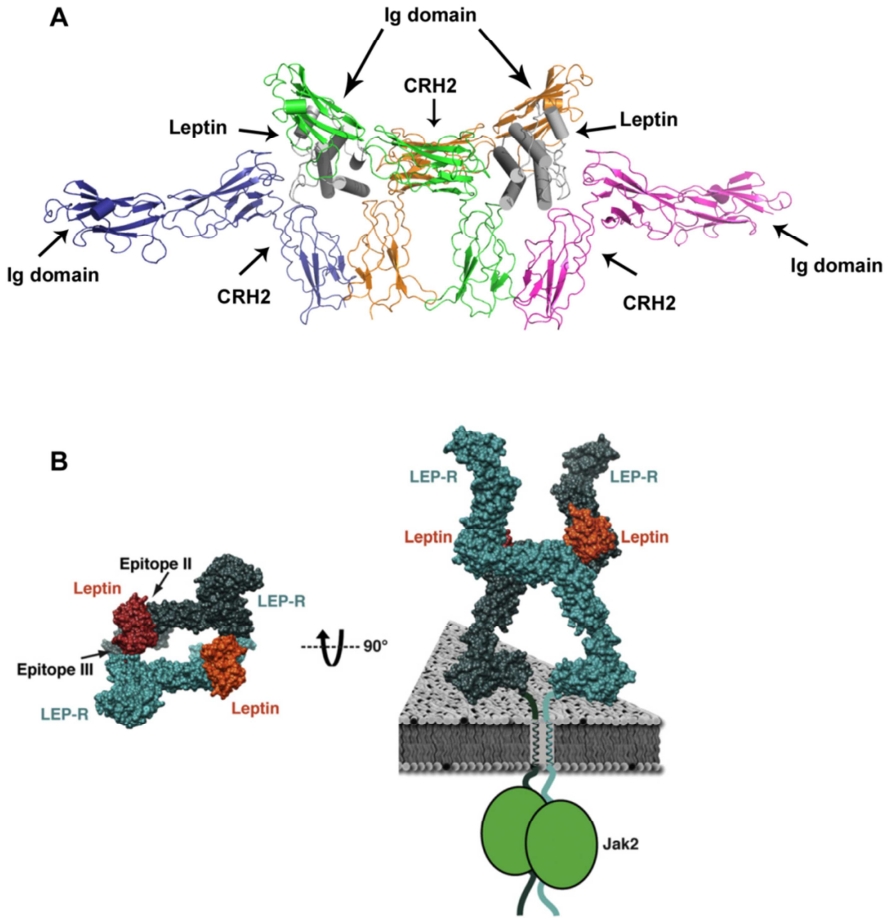

Figure 2.20. Proposed models of signaling complex of Leptin/Leptin Receptor complex: ***(A)*** Homology model for a hexameric leptin/LR complex. LR1 (*green*) and LR2 (*orange*) forms core of the complex by forming a homo-dimeric crossover via site-2/CRH2 and site-3/Ig interaction. Two additional receptors LR3 (*blue*) and LR4 (*pink*) are recruited via site 1 of two leptins and bound to the CRH2 domains of two receptors. (Adopted from Peelman et al., 2006); ***(B)*** Electron microscopy based 2 Leptin :2 LR model: Two leptin dimerize two Leptin receptor via site-2/CRH2 and site-3/Ig domain interaction, forming a homo-dimeric cross-over structure (Adopted from Mancour et al., 2012)

regions of LR_{short} than for LR_{long} in which the they are relatively far away. BRET donor saturation assay suggested a 32±3% that forms significant population of LR_{short} to be monomeric in equilibrium with rest of ~68% dimeric LR_{short}. Also the proportion of monomeric to dimeric LR_{short} does not change upon ligand binding; so the increase in BRET signal is due to conformational change induced by leptin interaction. This may complement earlier observation by David D.W. &

Tartaglia L.A. (1999), that leptin treatment does not change the LR homo-oligomer formation. Couturier and Jockers have concluded based on this BRET study that leptin induces only conformational change upon binding to the predimerized receptor o form the active signaling complex.

Formation of leptin/LR signaling complex and its kinetics has been studied in single cell technique involving quantitative fluorescent resonance energy transfer (FRET) (Biener et al., 2005; Ramanujan et al., 2005). Based on this FRET study, leptin exposure causes both conformational reorganization of preformed LR_{long} homo-oligomers and formation of de-novo LR_{long} homo-oligomers. This conclusion contradicts previous results of Couturier and Jockers proposing that leptin binding only induces conformational change and no leptin-induced *de novo* LR oligomerization.

The weak negative dominant repression by LR_{short} for LR_{long} signaling, a phenomenon unique to leptin receptor system has been explained based on earlier cellular work (White and Tartaglia, 1999). This work involving immunoprecipitation and immunoblotting of two differently C-terminally tagged LR_{long} and LR_{short} has shown that LR sequence 868-965 containing box1 motif is essential for LR to repress the constitutively active LR-fa mutant receptor, possibly by ligand dependent oligomerization with LR-fa mutant. On the premise that this part of LR is essential for JAK binding and that LR_{short} shows inability to efficiently recruit JAK kinase (Bjorbaek et al., 1998; Ghilardi and Skoda, 1997), the weak negative dominant repression shown by LR_{short} can be described as a result of its inefficient JAK binding. The key role of JAK binding to stabilize the leptin/LR complex comes from the work of Couturier and Jokers (2003), where they have shown decrease in BRET signal upon digitonin mediated solubilization of leptin/LR complex happens only in case of LR_{short} while that of leptin/LR_{long} complex remains unaffected.

Based on the domain deletion experiments of Fong et al. (1998) and site directed mutagenesis by Zabeau et al. (2004), FNIII has been shown to play crucial role in formation of the ligand induced signaling complex. As LR with only FNIII domain as extracellular part was found to be constitutively active, FNIII-FNIII homotypic interaction between receptor partners have been suggested as crucial for ligand dependent LR activation (Zabeau et al., 2005). Biochemical and cellular work (Zabeau et al., 2004) has shown that the signaling complex of leptin/LR is formed of more than two receptors per signaling complex. In this experimental set up, two types of LR were engineered: "*LR-F3*" and "*LR-FFY Δbox1*". Three conserved tyrosines (Y985, Y1077, Y1138) in the cytosolic part which are crucial for STAT3 signaling were substituted with phenylalanines in "*LR-F3*". In case of "*LR-FFY Δbox1*", box1 in LR_{IC} part, the

site for the JAK2 binding, was truncated. Also Y985 and Y1077 were substituted to phenylalanine leaving Y1138 intact which upon phosphorylation provides site for STAT3 interaction. Hence, "*LR-F3*" even if can phosphorylate and activate JAK upon ligand binding, can not recruit STAT for signaling; while "*LR-FFY Δbox1*" lacking a JAK binding motif, can not phosphorylate the Y1138 and thus incapable for STAT mediated signaling. When either of these two types of LR expressing in HEK-293T cells were treated with leptin separately, neither of the two LR types could be activated. But co-expression of these two receptor variants resulted in successful complementation and LR activation. This complementation can only be explained via a mechanism that involves a signaling complex formed of > 2 receptors. Use of chimeric receptors with extracellular part corresponding to erythropoietin receptor (EpoR) while transmembrane and intracellular parts corresponding to "*LR-F3*" and "*LR-FFY Δbox1*", did not produced any leptin induced signal when co-expressed. EpoR is known to signal via the conformational change of pre-dimerized receptors. By applying similar constraint to chimeric EpoR/LR system and forcing the formation of a leptin induced 2:2 quaternary complex could not generate STAT3 signaling. This suggests a signaling complex model involving more than 2 LRs per signaling complex.

Based on the above work (Zabeau et al., 2004), mutagenesis of FNIII cysteines to LR:ΔCRH1-ΔIg-C672S/C751S (Zabeau et al., 2005) and epitope mapping on leptin (Peelman et al., 2004), a hexameric model of LR signaling complex (4 LR/2 leptin), based on the hexameric IL6/IL6Rα/gp130 has been proposed (Peelman et al., 2006) (Figure 2.20A). Each leptin, in the signaling complex interacts with CRH2 domains from 2 LRs via putative binding sites I and II. Two such subunits (2 LR:1 Leptin), interacts with each other via a cross-over involving leptin site III/LR_{Ig} domain of the other subunit.

Recently, single particle electron microscopy has been employed to generate 2D images and 3D reconstructions of Leptin/LR quaternary complex (Mancour et al., 2012). Leptin has been shown to induce dimerization of LR_{ecto}, culminating into a quaternary 2:2 complex, which have been proposed as the signaling complex for leptin/LR system (Figure 2.20B). The EM structure shows high degree of flexibility between the subdomains of CRH2 domain, needed for ligand interaction in an induced-fit fashion. An interesting inference is that the ligand interaction rigidifies of the membrane proximal CRH2 subdomain thus stabilizing the FNIII domains into a "leg like" single conformation, essential for signaling. Based on 3D reconstruction and modeling, site III of leptin has been shown to interact the Ig domain of the 2nd receptor chain. CRH1 has been shown to not interacting leptin as expected based on Fong T.M. et al (1998) domain truncation study. The mode of complex formation has been hypothesized to be cooperative, with

formation of 1:1 “leptin-site II”/”LR-CRH2” complex followed by “leptin-site III”/”LR-Ig” domain interaction. Essentially, this study shows the low resolution architecture of the leptin induced quaternary complex structure of leptin/LR_{ecto}. Such a 2:2 quaternary signaling complex contradicts previous biochemical (Peelman et al., 2006; Zabeau et al., 2004) and cellular (White et al., 1997) studies pointing towards a signaling complex requiring more than two receptors/signaling complex.

2.6.3 Hetero-oligomerization of Leptin/leptin receptor complex

Hetero-oligomerization refers to the formation of a signaling complex involving more than one leptin receptor isotype. Hetero-oligomerization in leptin receptor system has been in discussion, but without much of a consensus, so far. Hetero-dimerization has been shown in GCSFR system, by showing how a signaling incompetent G-CSFR inhibits the signaling potential of wild type G-CSFR upon heterodimerization; also proving that G-CSFR signals by dimerization of its receptors. Absence of such heterodimerization in the LR system has been shown by crosslinking on cell surface (Devos R. et al., 1997). Based on this observation, Devos R. et al. has explained the marginal dominant negative repression by the LR_{short} on LR signaling observed earlier by White D.W. et al. (1997).

White and Tartaglia (1999) on the basis of immunoprecipitation and immunoblotting of two differently tagged LR_{long} and LR_{short} showed that both receptor subtypes exist as pre-assembled homo-oligomers only; but leptin interaction leads to receptor hetero-oligomer formation; thus contradicting earlier observations of Devos R. et al. (1997).

Use of chimeric receptors with extracellular part made up of IL-5Rα and IL-5Rβ, transmembrane and intracellular parts from LRb and LRa, has enabled specific, targeted hetero-oligomeric clustering and analysis of STAT-3 signaling capability of such receptors (Bahrenberg et al., 2002). Such an experimental set up suggested the signaling incompetence of IL5 induced heterodimeric complex with LRa and LRb transmembrane and intracellular parts. This signaling incapability has been shown to be due to the incomplete JAK binding motif 1 in LRa.

Fluorescence resonance energy transfer (FRET) experiments investigating the hetero-oligomerization of LR *in vivo*, suggested the absence of leptin-induced hetero-oligomerization on cell surface (Biener et al., 2005). Recent work utilizing BRET and optimized coimmunoprecipitation has suggested otherwise the formation of such heterodimeric signaling complexes (Biener et al., 2010).

2.7 SUMMARY

Leptin being a pleiotropic cytokine regulates a broad range of physiological processes which include appetite, energy metabolism, body weight, both innate and acquired immunity, reproduction, bone metabolism, hematopoiesis and angiogenesis. This system has been associated with many pathological conditions including obesity, multiple sclerosis, rheumatoid arthritis, autoimmune hepatitis and more recently in breast cancer. Due to such important physiological and pathophysiological roles played by leptin, understanding of leptin-mediated signaling can provide important insights into this system assisting in drug development for related disorders. Further understanding of the structural and mechanistic principles driving the LR activation via leptin binding will enrich our current knowledge of cytokine/cytokine receptor systems. Even though the scientific community has spent more than a decade solving this puzzle, little is known about the structure and higher order architecture of this receptor and its leptin-bound assembly on cell surface.

Unraveling the comprehensive structural insights into leptin/LR assemblies has been the central objective of this doctoral research project. To achieve this goal, I have employed a hybrid approach based on multiple structural biology method such as small angle X-ray scattering and electron microscopy.

Feature comparison of LR system with shared and non-shared cytokine receptors strongly suggests it to be a member of the later class. The LR system exhibits many distinct organizational features; such as it exists as an ensemble of ligand independent homo-oligomers as well as monomers on cell surface, it has multiple isoforms with potential to form heterodimers, an extra membrane-distal CRH1 domain not participating in ligand binding or receptor activation but having discrete effect on the signaling intensity. Such peculiar features suggests that the LR system may employ an unconventional assembly and mechanistic principle. Therefore the second objective is to dissect the mechanistic paradigm of leptin induced LR activation using antagonist leptins as tools and to converge available information to an universal leptin/LR signaling model.

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3

*Structural and mechanistic paradigm of Leptin receptor activation revealed by complexes with wild type and antagonist leptins**

*This chapter has been adapted from the manuscript submitted for publication.

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Contribution: LZ is credited for the production of wild type and antagonist leptin, preparation of receptor constructs , the cell surface biotinylation experiment and cysteine mutation in the leptin receptor. For electron microscopy of leptin receptor and its complex ,credit goes to PR. All other results has been contributed by KM.

3.1 ABSTRACT

Leptin, an adipocyte-derived cytokine, signals via its cognate receptor (LR) to serve as a metabolic switch, thereby regulating body weight and processes with a high metabolic cost such as reproduction and immune responses. The downside of such benevolent pleiotropy has been the possible role of leptin-mediated signaling in autoimmune diseases and breast cancer, which has raised interest in the targeted antagonism of leptin signaling. We here present comparative biochemical and structural studies of the ectodomain of LR (LR_{ecto}) in complex with wild type and antagonist leptin variants and reveal that the basic assembly principle leading to leptin signaling is a 2:2 stoichiometric complex. The cornerstone of the quaternary complex is high-affinity binding of leptin to the second cytokine receptor homology domain (CRH2) of LR_{ecto}, which structurally primes additional interactions with the Ig domain of a second leptin-bound LR_{ecto}. This leads to dimerization of LR_{ecto}, which is otherwise devoid of inherent dimerization propensity. In contrast, antagonist leptin variants carrying mutations at the epitope interacting with LR_{Ig} are only able to establish high affinity 1:1 complexes with LR_{ecto}. Acetylation of free cysteines in LR_{ecto} also abrogates quaternary complexes with leptin suggesting a role for intra-receptor disulfides upon receptor activation. Mechanistic integration of our studies in light of diverse prior findings supports an assembly mechanism whereby leptin conformationally activates pre-dimerized LR molecules at the cell-surface to seed a higher-order complex with 4:4 stoichiometry. This mechanistic proposal establishes a new conceptual framework for future studies of leptin-mediated signaling.

Keywords: Leptin | Leptin Receptor | Signaling | Receptor activation

3.2 INTRODUCTION

Leptin receptor (LR), a type I cytokine receptor, is activated by its cognate ligand leptin secreted by the adipose tissue, to regulate a host of essential body functions, such as the homeostasis of body weight and energy (Friedman and Halaas, 1998; Halaas *et al.*, 1995; Varela and Horvath, 2012), the immune system and hematopoiesis (Carbone *et al.*, 2012; Procaccini *et al.*, 2012) reproduction and fetal development (Hausman *et al*., 2012), angiogenesis (Cao *et al.*, 2001; Park *et al.*, 2001), and bone formation (Motyl and Rosen, 2012). Leptin-mediated activation of

LR signals through the JAK/STAT pathways and also modulates the activity of phosphoinositide 3-kinase (PI3K), mitogen activated protein kinase (MAPK), extracellular-signal related kinase (ERK) and AMP-activated protein kinase (AMPK) (Wauman *et al.*, 2008). The pleiotropic signaling via the Leptin-LR axis and its potential in treating a number of pathologies, have been a cynosure for more than a decade. Although leptin monotherapy has been ineffective to curb obesity, it has found applications in type-I and type-II diabetes (Wang *et al.*, 2010; Cummings *et al.*, 2011), in rare cases of congenital leptin deficiency (Ramachandrappa and Farooqi, 2011), as a potential anti-depressant (Lu *et al.*, 2006) and as a biomarker in breast cancer progression (Artac and Altundag, 2012). The possible involvement of leptin in autoimmune diseases like multiple sclerosis (MS) (De Rosa *et al.*, 2007), rheumatoid arthritis (RA) (Otvos *et al.*, 2011) and autoimmune hepatitis (AH) (Sennello *et al.*, 2005) and in cancers (Otvos *et al.*, 2011) has raised the interest in leptin and LR antagonists.

The LR ectodomain (LR_{ecto}) comprises two cytokine receptor homology domains (CRH1 and CRH2) flanking an Immunoglobulin-like motif (Ig), followed by two membrane-proximal fibronectin type III (FNIII) domains (Figure 1A). A single helix transmembrane domain (TM) connects LR_{ecto} to the intracellular segments, which carry the conserved juxtamembrane motifs box1 and box2 for binding of JAKs (Ghilardi and Skoda, 1997). The CRH2 domain is responsible for leptin binding (Fong *et al.*, 1998; Iserentant *et al.*, 2005; Peelman *et al.*, 2004) while the Ig-like domain is required for receptor activation (Zabeau *et al.*, 2004). Whereas LR has been classified as a type 1 cytokine receptor due the presence of conserved WSXWS sequence motifs in such receptors, it exhibits a number of distinct organizational features suggesting it may employ unique assembly and mechanistic principles. For instance, LR does not recruit a co-receptor in the signaling complex; it can undergo ligand-independent oligomerization at the cell-surface; it has multiple isoforms that may assemble as hetero-oligomers (Bacart *et al.*, 2010), the N-terminal CRH1 segment has a hitherto unidentified role in receptor activation; LR_{ecto} harbors an unprecedented combination of CRH, Ig-like, and FNIII domains; These architectural eccentricities are manifested at the mechanistic level as well, which is not well understood but was shown to involve pre-oligomeric receptors at the cell-surface (Biener *et al.*, 2005; Couturier and Jockers, 2003) that upon leptin binding may oligomerize further to form a signaling-competent complex of higher order (Zabeau *et al.*, 2004).

Homology-based structural analysis of leptin, a monomeric cytokine that adopts a four-helix bundle fold (Zhang et al., 1997), complemented by mutagenesis studies led to proposals for three distinct binding sites on leptin (Iserentant *et al.*, 2005; Peelman *et al.*, 2004; Niv-

Spector *et al.*, 2005; Peelman *et al.*, 2006). The canonical site II interacts with CRH2 domain on LR and is essential for high affinity binding, while site III interacts with the Ig domain on LR and likely invokes conformational changes necessary for signal transduction. On the other hand the role of putative binding site I has been rather controversial due to the lack of robust mutagenesis and functional data to fully substantiate its importance. The relevance of site III of leptin has inspired the design of leptin variants with potent antagonistic properties (Peelman *et al.*, 2004; Gertler *et al.*, 2006), whereby the leptin variants retain their ability to interact with LR but are unable to support signaling. Besides their therapeutic potential, such leptin variants could serve as excellent tools to probe structural and mechanistic aspects of leptin-LR signaling.

Despite great progress in our understanding of the structural basis of several extracellular signaling assemblies mediated by type I cytokine receptors such as the Interleukin-6 receptor (IL-6R) (Boulanger *et al.*, 2003; Skiniotis *et al.*, 2005), IL-2R and IL-15R (Wang *et al.*, 2005; Ring *et al.*, 2012), and the GM-CSF receptor (Hansen *et al.*, 2008), our view of leptin-LR complexes and the mechanism of LR activation has not yet come to full circle despite recent developments (Carpenter et al., 2012; Mancour et al., 2012). We here employ structural, biophysical, biochemical and cellular studies to study the assembly and mechanistic principles of mouse LR in complex with wild-type leptin and leptin variants with antagonistic properties. In light of a wealth of prior studies, we have arrived to a mechanistic proposal for the assembly of leptin-LR signaling complexes, which can now serve as a new working paradigm for future mechanistic interrogation of leptin-mediated signaling.

3.3 RESULTS

3.3.1. The LR ectodomain adopts a monomeric extended structure

To enable structural and biophysical studies of the LR_{ecto} and its complexes with leptin and leptin antagonists we expressed LR_{ecto} (CRH1, Ig, CRH2, and tandem FNIII domains) (Figure 3.1A) in transiently transfected HEK-293T cells in the presence of the mannosidase inhibitor kifunensine (Chang *et al.*, 2007) as a recombinant protein construct carrying a C-terminal hexahistidine tag. LR_{ecto} purified via immobilized metal-ion affinity chromatography and size-exclusion chromatography (SEC) could be characterized as a monodisperse species using multi-angle laser light scattering (MALS) after sample resolution via Field-Force-Fractionation

(FFF) consistent with monomeric glycosylated LR_{ecto} (Figure 3.1B). In addition, we investigated the possible role of glycosylation in the oligomerization propensity of LR_{ecto} by expressing LR_{ecto} in stably transfected HEK-293S $GnTI^{-/-}$ cells producing LR_{ecto} with homogeneous N-linked $GlcNAc_2Man_5$ glycans and found that it retains its monomeric character.

We subsequently undertook structural studies of LR_{ecto} in solution by small-angle X-ray scattering (SAXS). In the first instance, this allowed us to probe the oligomerization tendency of LR_{ecto} within a broad range of concentrations up to 17µM (3.1 mg/mL) (Table 3.S1, Figure 3.S1a). To facilitate structural modeling of the SAXS data we first constructed models of LR_{ecto} based on structure-based sequence alignments, homology modeling, and structure-prediction approaches. Indeed, modeling of the SAXS data using *ab initio* approaches (Franke and Svergun, 2009) and restrained rigid-body refinement protocols (Petoukhov and Svergun, 2005) coupled to modeling of oligo-mannose glycan trees at 16 N-linked glycosylation sites using Glyprot (Bohne-Lang and von der Lieth, 2005; Haniu *et al.*, 1998; Felix et al., 2013) established that LR_{ecto} adopts a monomeric and extended structure measuring ~80Å x 80Å x 225Å and which is hallmarked by an elbow bend centered at the Ig domain (Figure 3.1C and 3.1D, Figure 3.S1b). The N-terminal CRH1 segment can be described by three structural domains, followed by the Ig segment, a CHRH2 segment with two domains, and ends with two FN-III domains. A parallel structural study of LR_{ecto} via negative-stain electron microscopy (EM), albeit at inherently much lower sample concentrations, led to class-averages bearing features closely resembling the structural models obtained via SAXS (Figure 3.S1c).

Our finding that LR_{ecto} lacks inherent dimerization propensity even at such high concentrations came as a surprise because of previous reports that LR exists in a dimeric form at the cell surface (Biener *et al.*, 2005; De Vos *et al.*, 1997; White and Tartaglia, 1999) and that such predimerized assemblies might be mediated by disulfide bridges between LR molecules (Zabeau *et al.*, 2005). In addition, we had also observed that shorter constructs of the extracellular segment of LR ($LR_{CRH1\text{-}Ig\text{-}CRH2}$ or $LR_{Ig\text{-}CRH2}$) showed the tendency to form covalent oligomers. To cross-validate the possible role of covalent receptor-receptor interactions we selectively captured LR at the cell surface labeled with sulfo-NHS-SS-biotin in the presence of iodoacetamide, followed by detection via Western-blotting. Our analysis showed LR as a single band corresponding to the monomeric molecular weight (Figure 3.1E), thereby establishing the non-covalent character of LR at the cell surface. Thus, in the absence of any inherent oligomerization propensity in LR_{ecto}, dimerization of LR at the cell surface is likely driven by the

transmembrane and intracellular segments of the receptor, and may be enhanced by the dimensionality of the membrane (Wu *et al.*, 2011). In this regard, earlier studies investigating constitutive LR dimers at the cell-surface demonstrated that the short form of LR lacking the intracellular segment was still able to form constitutive dimers (Biener *et al.*, 2005), and that constitutive dimers consisting of full length LR are far more stable than LR lacking the intracellular segment (Couturier and Jockers, 2003).

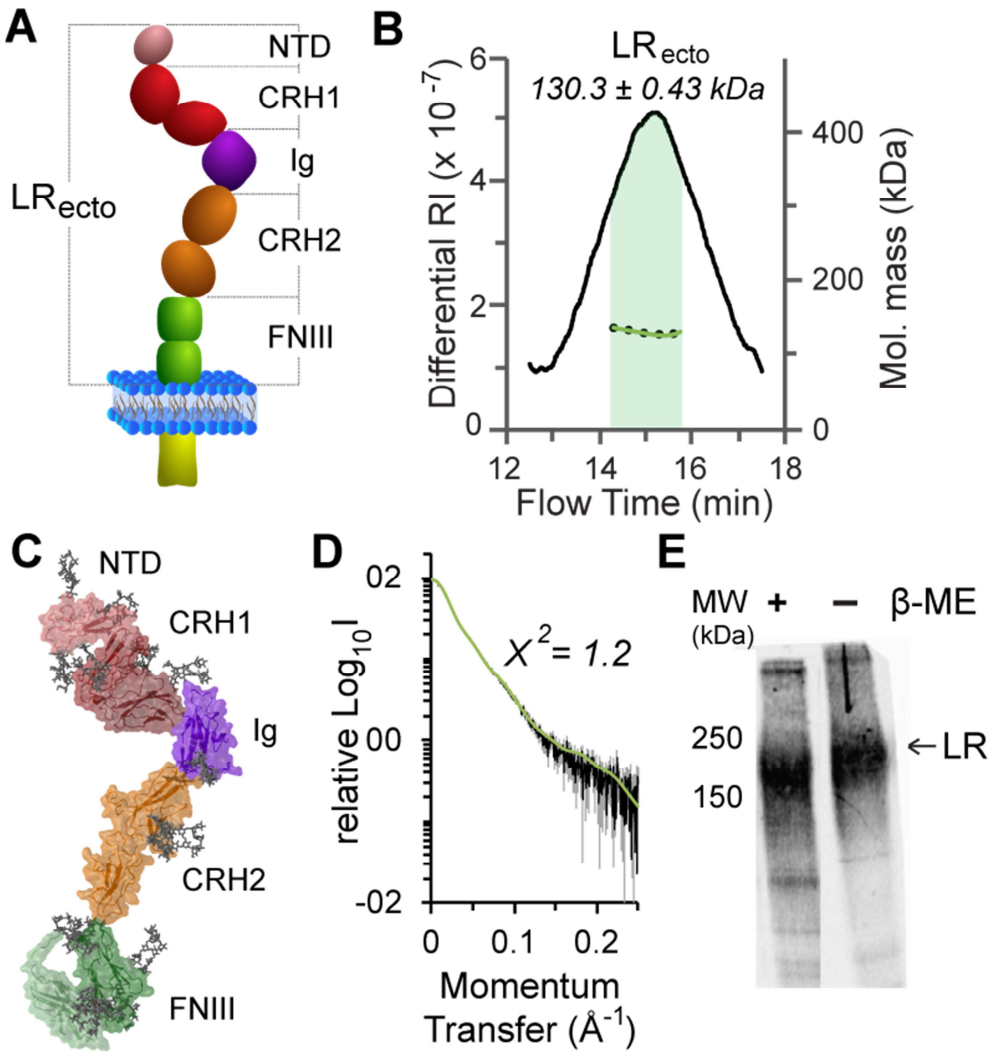

Figure 3.1. Characterization of LR_{ecto} *in vitro* and LR in vivo: (A) Schematic representation of LR: LR can be divided into an extracellular part (LR_{ecto}), a transmembrane part formed of single-pass helix and intracellular part. LR_{ecto} has a modular structure with four functional domains: distal 1st cytokine receptor homology (CRH1) domain (*red*), immunoglobulin like (Ig) domain (*violet*), 2nd cytokine receptor

homology (CRH2) domain (*orange*) followed by two membrane proximal fibronectine type III (FNIII) domains (*green*). **(B)** Western blot analysis lysis of cell surface LR expressed in HEK293 cells in the presence and absence of β-mercaptoethanol (β-ME) showing absence of disulfide linkage in the preassembled receptor dimer. **(C)** Multi-angle laser light scattering (MALLS) analysis of LR_{ecto} suggests it to be monomeric in solution **(D)** The calculated scattering from the rigid body model obtained from the X-ray scattering of LR_{ecto} produces a chi square fit of 1.2 against experimental scattering data. **(E)** SAXS based rigid body model of LR_{ecto} with following functional domains: CRH1 (*red*), Ig-domain (*violet*), CRH2 (*orange*) and membrane proximal FNIII (*green*).

3.3.2 Leptin and LR_{ecto} engage in a non-covalent quaternary complex with 2:2 stoichiometry

We employed several lines of experimentation to characterize binding of leptin to LR_{ecto} and to obtain structural insights into the assembly of extracellular leptin:LR_{ecto} complexes. Incubation of purified recombinant LR_{ecto} with a molar excess of leptin, followed by chromatographic analysis by SEC typically resulted in two incompletely resolved peaks: a minor high molecular weight peak and a major low molecular weight peak. This suggested that the leptin-LR_{ecto} complex might be a dynamic equilibrium of high and a low molecular-weight complexes with dilution favoring the low molecular-weight species. To better characterize these two possible states we subjected assembled leptin-LR_{ecto} complexes to MALLS-FFF analyses and found that the two assemblies are consistent with a binary 1:1 stoichiometry and a quaternary 2:2 complex, respectively (Figure 3.2A). Subjecting complexes with 2:2 stoichiometry to a new round of SEC resulted in partial dissociation of the 2:2 species to the 1:1 stoichiometric assembly indicating the concentration dependence of the assemblies in vitro. Both types of stoichiometric complexes could be further stabilized by crosslinking with formaldehyde (Leitner *et al.*, 2010) and isolated by SEC, resulting in better preparative resolution of the two candidate molecular assemblies.

In light of these findings we sought to characterize the thermodynamic and stoichiometric profile of the leptin-LR interaction by isothermal titration calorimetry (ITC). Our approach centered on carrying out measurements at concentrations that would be representative of those required to maintain the two apparent types of complexes we could identify chromatographically (Figure 3.2B and 3.2C). Our measurements show that both types of complexes are characterized by nearly identical K_D values consistent with high affinity binding, while exhibiting very similar stoichiometric and thermodynamic profiles. Upon subjecting the post-ITC protein solutions to SEC we obtained elution profiles that cross-validated the exclusive presence of binary 1:1

complex in the ITC titration at low concentration (Figure 3.S2a). On the other hand, the post-ITC solution at high concentration resolved as mixture of complexes with 2:2 and 1:1 stoichiometries (Figure 3.S2b), confirming the lability of the quaternary assembly in a concentration dependent manner and the fact that a 2:2 stoichiometry is the maximum attainable for the leptin-LR_{ecto} complex.

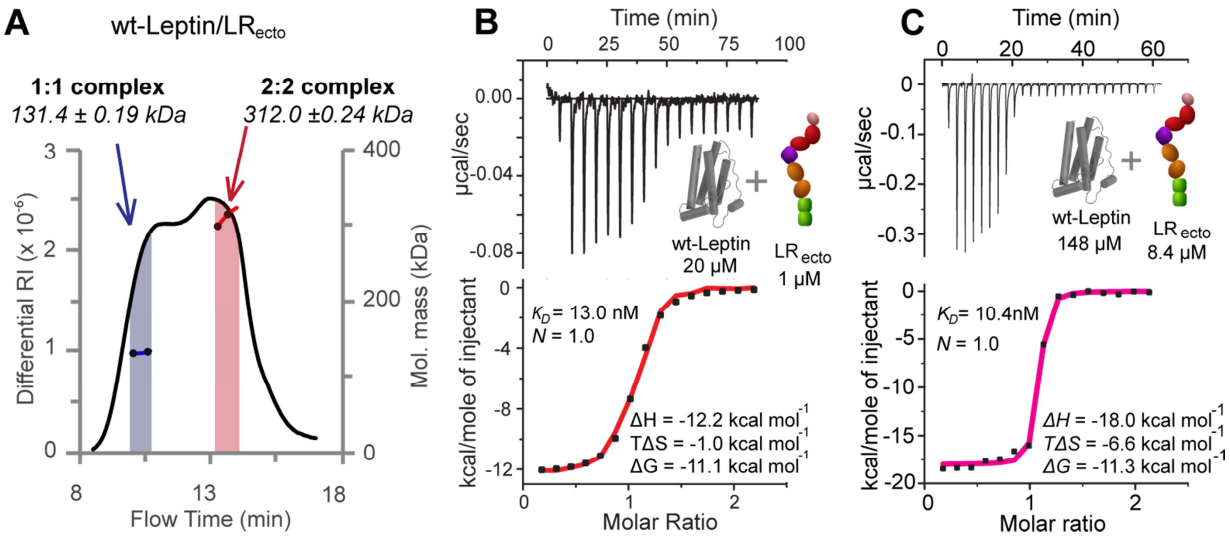

Figure 3.2: Molecular and biophysical characterization of wt-leptin/LR_{ecto} complex

(A) MALS analysis of two incompletely resolved peaks of wt-Leptin/LR_{ecto} complex produced molecular masses corresponding to 1:1 (blue) and 2:2 (red) complexes **(B)** ITC of wt-Leptin into LR_{ecto} relatively lower concentration **(C)** ITC of wt-Leptin into LR_{ecto} at relatively higher concentration that promotes formation of 2:2 complex. Both ITC runs were carried out at a temperature of 37º C.

3.3.3 Structural studies of leptin in complex with LR_{ecto}

To obtain structural insights into the assembly principles of the leptin-LR_{ecto} complex we employed structural studies by small-angle X-ray scattering (SAXS), which in the first instance served as an orthogonal method for corroborating the molecular sizes of the two apparent stoichiometric complexes of leptin-LR_{ecto} with 1:1 and 2:2 stoichiometries (Table 3.S1). Modeling of SAXS data measured from purified recombinant leptin-LR_{ecto} complex with 2:2 stoichiometry after cross-linking using *ab initio* and rigid-body refinement protocols revealed a frontally

symmetric Y-shaped assembly measuring ~225 Å x 300 Å x 160 Å composed of two 1:1 leptin-LR_{ecto} subcomplexes (Figure 3.3A, curve (i) and Figure 3.3B, Figure 3.S3). In a given 1:1 subcomplex a leptin molecule binds to the CRH2 domain of LR_{ecto} consistent to what has been termed a site II binding epitope (Peelman *et al.*, 2004), invoking a drastically different conformational state of LR_{ecto} when compared its unbound state. The apparent consequence of such restructuring in LR_{ecto} is that the Ig domain becomes primed to associate with a leptin molecule in the second 1:1 subcomplex to establish a site-III binding epitope on leptin (Peelman *et al.*, 2006). Furthermore, the two N-terminal CRH1 segments and the two membrane-proximal FNIII domains extend away from the core of the complex. We note that accounting for 16 N-linked $GlcNAc_2Man_9$ glycans greatly improved model agreements with the scattering data as recently shown for other receptors (Felix *et al.*, 2013; Guttman *et al.*, 2013). Upon extrapolating our structural studies to characterize the leptin-LR_{ecto} complex obeying 1:1 stoichiometry (Figure 3.3A curve (ii) and Figure 3.3C) we found that this binary complex adopts a conformation that is quite different from its structure in the quaternary complex and that of unbound LR_{ecto}, as further supported by comparisons of the pair-distance distribution functions (Figure 3.3D).

3.3.4 Antagonist leptin variants abrogate ligand-induced LR oligomerization

Given the capacity of leptin to engage LR_{ecto} into 2:2 stoichiometric complexes we pursued biochemical and structural insights into the possible mode of antagonism by two established antagonist leptin variants (Peelman *et al.*, 2004; Niv-Spector *et al.*, 2005). A parallel reasoning was that by studying LR antagonism we might be able to obtain additional mechanistic insights into the assembly of cognate ligand-receptor complexes. One of the antagonists employed ($Leptin_{a1}$) carries a S120A/T121A double mutation localizing at the beginning of helix D (Peelman et al., 2004) and was designed to interrogate the relevance of site III on leptin, while the second ($Leptin_{a2}$) bears a substitution 39AAAA42 in the loop linking helices A and B and was proposed to impact the leptin-LR_{Ig} interaction (Niv-Spector *et al.*, 2005) (27). We note that the two sets of mutations cluster close to each other on the leptin scaffold, as the AB loop traverses the start of helix D.

Analyses by SEC-MALLS of $Leptin_{a1}$-LR_{ecto} and $Leptin_{a2}$-LR_{ecto} complexes revealed that in contrast to wild type leptin, the two antagonist leptin variants are only able to engage LR_{ecto} in a complex obeying 1:1 stoichiometry (Figure 3.4A). Interestingly, the thermodynamic binding

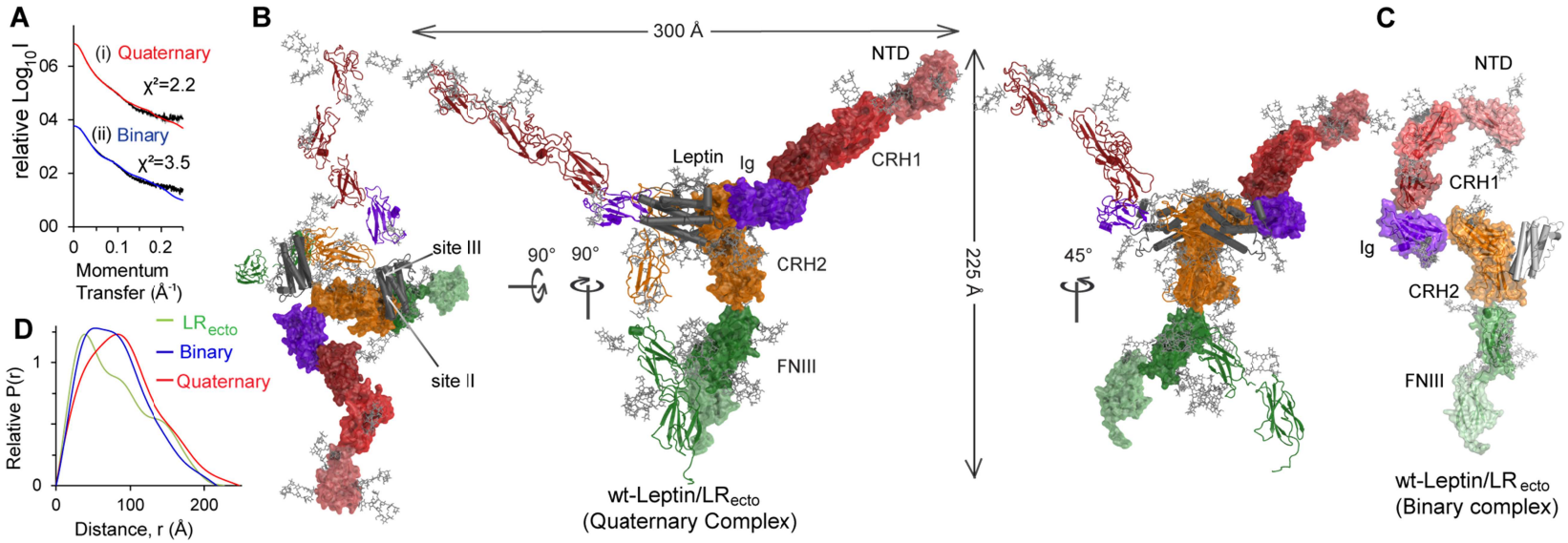

Figure 3.3. Small angle X-ray scattering of wt-Leptin/LR$_{ecto}$ complex: (A) Plot of theoretical scattering curves from rigid body models of wt-Leptin/LR$_{ecto}$ quaternary complex (*red*) and binary complex (*blue*) fitted against experimental scattering curves of quaternary (i) and binary complex (ii), respectively **(B)** SASREF-generated rigid body model of wt-Leptin/LR$_{ecto}$ quaternary complex. Leptin (grey) presented as cylinder and receptor in cartoon and surface views. Color scheme for receptor same as in Fig. 1A **(C)** SASREF-generated rigid body model of wt-Leptin/LR$_{ecto}$ binary complex. Color scheme is as in Fig. 3B. **(D)** Pairwise distance distribution plot of relative probability vs. distance for LR$_{ecto}$ (green), wt-Leptin/LR$_{ecto}$ binary (blue) and quaternary (red) complex.

fingerprints for both antagonists (Figure 3.4B) are very similar to that for wild type leptin (Figure 3.2B), which suggested that the driving force for the assembly of the wild-type leptin complex with LR_{ecto} is the interaction with LR_{CRH2} via site II. Structural modeling of the two binary complexes via *ab initio* and rigid-body modeling of SAXS data (Figure 3.4C-E, Figure 3.S4, Table 3.S2) led to molecular complexes that strongly resemble the binary complex of wild type leptin with LR_{ecto} in 1:1 stoichiometry (Figure 3.3C), as further evidenced by comparisons of the pairwise distance distribution function (Figure 3.4E). Thus, we can now confirm that $Leptin_{a1}$ does target site III of leptin, and that $Leptin_{a2}$ also appears to behave as a site III antagonist, thereby pointing to the mechanistic importance of this interaction in mediating agonist-induced LR dimerization.

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3.3.5 Free cysteines in LR_{ecto} may constitute conformational switches for activation

Delineation of the disulfide-bond network in human LR_{ecto} has shown that LR_{ecto} carries nine free cysteines (Haniu *et al.*, 1998). Six of these are conserved in mouse LR_{ecto} and at least one of them, Cys672 found in the membrane proximal FNIII domain, was shown to be essential for receptor activation (Zabeau *et al.*, 2005) (45). In this study we mutated Cys604 in LR_{CRH2} close to the LR_{CRH2}-LR_{FNIII} domain boundary to serine and found that the mutant receptor can only be activated at very high leptin concentrations (>500 ng/ml) as measured by rPAP1-luciferase reporter activity. Interestingly, the recent crystal structure of LR_{CRH2} in complex with a Fab fragment (Carpenter *et al.*, 2012) provided insights leading to the proposal that a Cys604-Cys672 intra-receptor disulfide might be important for receptor activation. To extend these studies and to obtain insights into the general relevance of unpaired cysteines in the conformational competence of LR_{ecto} towards complex formation, we alkylated the free cysteines in LR_{ecto} using iodoacetamide. We discovered that while the acetylated receptor could readily form a 1:1 complex with leptin, it lost its ability to undergo leptin-dependent dimerization to form a 2:2 quaternary complex (Table 3.S3, Figure 3.S5). Together, these lines of evidence show that Cys-604 and Cys-672 are very likely candidates in engaging in an intra-receptor disulfide in the context of a leptin-LR quaternary complex and receptor activation. Indeed, recent evidence for functionally relevant redox regulation of the common γ_c receptor was reported in the context of IL-2 mediated signaling (Metcalfe *et al.*, 2012).

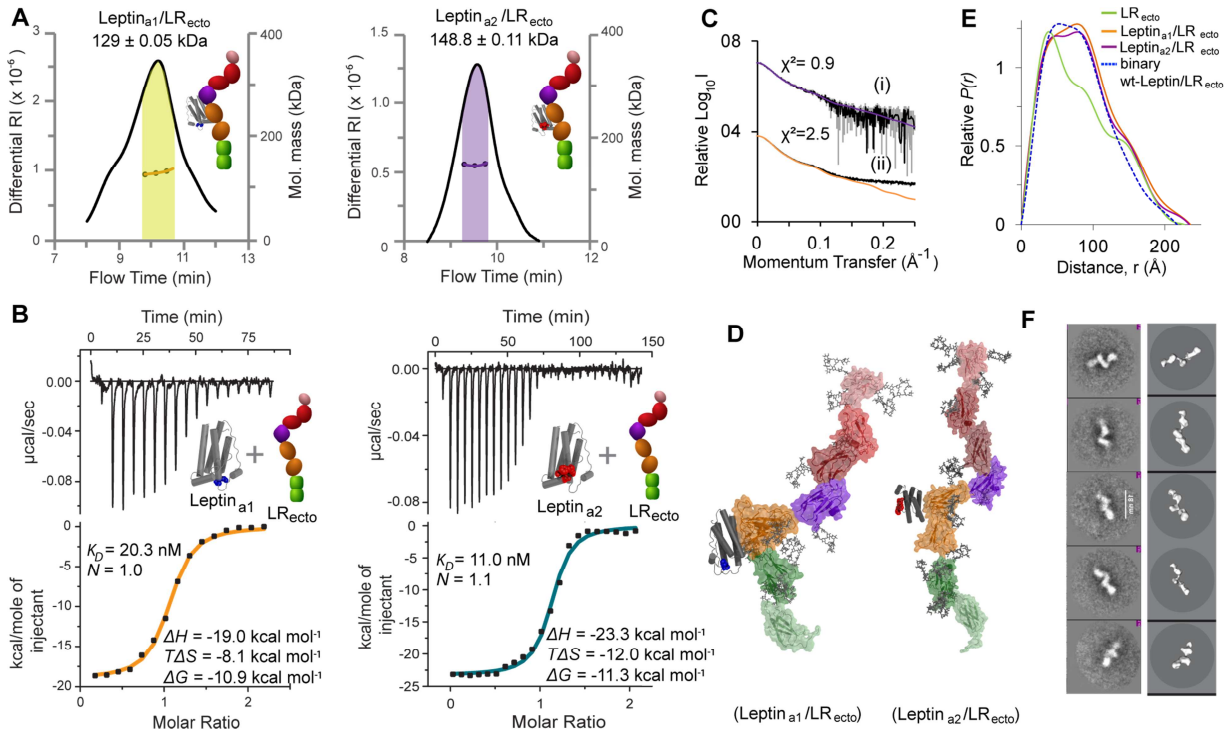

Figure 3.4: Biophysical and structural characterization of Leptin$_{a1}$/LR$_{ecto}$ and Leptin$_{a2}$/LR$_{ecto}$ complexes: (A) MALS of Leptin$_{a1}$/LR$_{ecto}$ and Leptin$_{a2}$/LR$_{ecto}$ produced molecular masses, each corresponding to 1:1 binary complex. **(B)** ITC carried out at 37º C for each type of antagonist Leptin into LR$_{ecto}$ shows an enthalpy-driven interaction with nanomolar affinities and 1:1 stoichiometry. **(C)** Plot of theoretical scattering intensity from rigid body models of Leptin$_{a1}$/LR$_{ecto}$ (orange) and Leptin$_{a2}$/LR$_{ecto}$ (violet) fitted to the experimental scattering (black) from respective complexes. **(D)** Rigid body models of leptin$_{a1}$/LR$_{ecto}$ (from SAXS data at 0.9 mg/ml) and leptin$_{a2}$/LR$_{ecto}$ (from SAXS measurement at 0.7 mg/ml) binary complex. The color scheme is same as used in fig. 3B. **(E)** Pair-wise distance distribution plot of relative probability vs. distance for LR$_{ecto}$ (*green*), leptin$_{a1}$/LR$_{ecto}$ (orange), leptin$_{a2}$/LR$_{ecto}$ (*red*) and binary complex of wt-Leptin/LR$_{ecto}$ (*dotted blue*).**(F)** Five class averages of Leptin$_{a2}$/LR$_{ecto}$ as revealed by negatively stained single particle electron microscopy (*left*). Shown to the right of each class average is the ab initio model from SAXS in comparable orientation.

3.4 DISCUSSION

The principles underlying the assembly of the leptin-mediated extracellular signaling complex of LR had remained unclear despite a large body of literature addressing the physiology and biomedical relevance of signaling along the leptin-LR axis (Guo *et al.*, 2012). Receptor oligomerization culminating with receptor activation has been observed for many type I cytokine receptors but the dependence of receptor oligomerization on ligand binding varies among the members. While the growth hormone receptor (Gent *et al.*, 2002) and the erythropoietin receptor (Livnah *et al.*, 1999) exist in an inactive dimeric form at the cell surface that becomes active upon ligand binding, others like IL-6Rα/gp130 (Boulanger *et al.*, 2003), leukemia inhibitory factor receptor (Huyton *et al.*, 2007), and GMCSF-R (Hansen *et al.*, 2008) proceed via ligand-induced receptor oligomerization coupled to concomitant conformational changes. LR has been reported to exist as a dimer or oligomer at the cell surface (Bacart *et al.*, 2010; Biener *et al.*, 2005; DeVos *et al.*, 1997; White and Tartaglia, 1999), yet the conformational impact of cognate leptin binding in the assembly of signaling complexes has remained unclear.

In this study we employed a diverse set of methods and molecular tools, including two established leptin antagonists, to provide structural and mechanistic insights into this process, an area of research that has recently gained significant momentum (Carpenter *et al.*, 2012; Mancour *et al.*, 2012). By carrying out comparative studies of wild type and antagonist leptins we have now shown that high-affinity binding of leptin to the CRH2 domain of LR_{ecto} leads to structural rearrangements in LR_{ecto} to prime assembly of a complex obeying 2:2 stoichiometry. This is mediated via a new interaction interface, albeit with a small contribution to the stability of the complex and no signs of cooperativity, between leptin participating in a 1:1 complex with LR_{ecto} with the Ig domain of LR_{ecto} in a second 1:1 complex. Indeed, the likely decrease in the number of degrees of freedom due to the dimensionality of the cell membrane may greatly impact the thermodynamics and kinetics of the invoked interaction between site III on leptin and LR_{Ig} (Wu *et al.*, 2011).

In this regard, we have now provided direct evidence for the importance of this interaction by elucidating the structural basis of leptin antagonism by two leptin mutants that stall the leptin-LR interaction to 1:1 stoichiometric complexes. Indeed, the inability of $Leptin_{a2}$ to elicit FRET signal elevation when added to cells expressing LR with intracellular fluorescence tags (Niv-Spector *et al.*, 2005) is consistent with the concomitant loss of oligomerization

capacity when the functionality of site III is abolished. Furthermore, we have shown that LR_{ecto} lacks inherent dimerization propensity, a property that now uncouples the role of LR_{ecto} from the assembly of constitutive, yet inactive, dimeric or oligomeric forms of LR at the cell-surface in the absence of ligand (Bacart *et al.*, 2010; Biener *et al.*, 2005; De Vos *et al.*, 1997; White and Tartaglia, 1999).

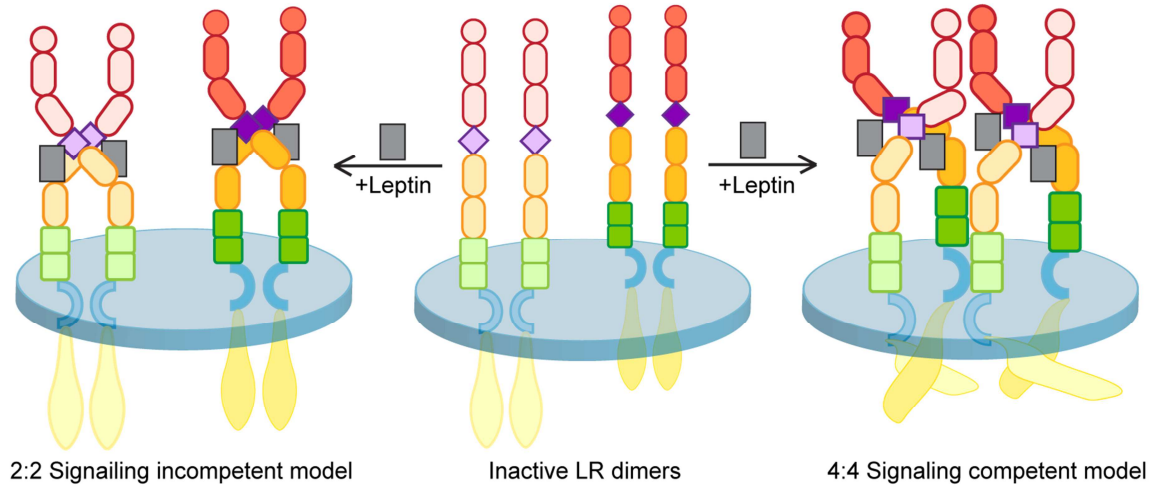

Figure 3.5. Proposed mechanism for LR activation

The main question arising is whether the observed leptin-LR_{ecto} complex represents a signaling-competent assembly. An analogous complex derived from a negative-stain EM analysis also obeys a 2:2 stoichiometry, and has been proposed as a leptin-LR signaling complex (Mancour *et al.*, 2012). The complex is in a drastically different conformational state compared to the one we presented here (Figure 3.S6). Despite the structural differences between the two assemblies, which as such can be attributed to inherent methodological limitations calling for validation via orthogonal methods, it would still be tempting to propose the observed quaternary complexes as signaling-competent states. However, we maintain that careful consideration of previous work in light of our structural data suggests otherwise.

Several studies have established that LR exists predominantly as a constitutive dimer at the cell-surface (Bacart *et al.*, 2010; Biener *et al.*, 2005; Couturier and Jockers, 2003; De Vos *et al.*, 1997; White and Tartaglia, 1999; Bahrenberg *et al.*, 2002). In addition, APB- and FLIM-based quantitative FRET on single cells (Biener *et al.*, 2005) had suggested ligand-induced

conformational changes and *de novo* oligomerization of LR *in situ*. Thus, considering constitutive LR dimers as basal assemblies for the nucleation of signaling complexes and leptin's ability to dimerize LR_{ecto} in vitro, we propose that the model most consistent with a higher-order assembly would be a complex obeying 4:4 stoichiometry (Figure 3.5). In this model, which contrasts previous proposals (Peelman *et al.*, 2006; Mancour *et al.*, 2012), binding of leptin to predimerized but inactive LR at the cell surface induces trans-dimerization of LR-dimers to establish a signaling-competent complex. Importantly, such new assembly paradigm would be consistent with the observed weakly dominant negative repression of LR signaling by the short-form of non-signaling LR (De Vos *et al.*, 1997; White *et al.*, 1997) in the context of heteromeric LR dimers at the cell surface (Bacart *et al.*, 2010), and supports previous studies employing JAK-STAT signaling complementation (Zabeau et al., 2004). Lastly, our proposed mechanistic paradigm further highlights the therapeutic importance of leptin antagonist variants that can abrogate such assemblies while displaying near wild type affinities to LR. We envisage that the herein mechanistic proposal for leptin-mediated activation of LR will set the stage for a more targeted mechanistic interrogation of the system as well as renewed efforts in antagonist development.

3.5 MATERIALS AND METHODS

3.5.1 Expression and purification of LR_{ecto}: pMET7 vector carrying the DNA encoding the extracellular part of murine Leptin Receptor (LR_{ecto}) with the amino-terminal native secretion signal and a carboxyl-terminal His_6-tag was used to transiently transfect 90% confluent HEK-293T cells using Polyethyleneimine (1DNA:1.5 Polyethyleneimine molar ratio) and was further cultured in DMEM medium containing Penicillin (Sigma) and Streptomycine (Sigma) and in the presence of mannosidase inhibitor Kifunensine (Tocris Bioscience) for 4 days at 37 ºC in a 5% CO_2 environment . The medium was harvested by centrifuging at 10,000g and filtered using 0.2 µm bottle-top filter. The secreted receptor was purified by passing it through TALON^{TM} IMAC resin (Clontech), washing with 4mM Imidazole (Sigma Aldrich) to remove non-specifically bound protein to matrix, followed by elution with 300mM Imidazole. The eluted peak was pooled, concentrated to 1 ml and further purified on a 120 ml Superdex 200 HiLoad 16/600 column (GE HealthCare) pre-equilibrated with buffer containing 25mM HEPES pH=7.4 and 100mM Sodium Chloride. The peak fractions were pooled and the purity was assessed using 15% SDS PAGE.

3.5.2 Expression and purification of wild type and antagonist leptins: Heterologous expression of murine Leptin cloned into pET-11a vector in *Escherichia coli*, followed by solubilization of inclusion body in 7 M Urea and refolding produced crude leptin. This preparation was further purified by anion chromatography on an ANX FF Column (GE Healthcare Life Sciences) and later by size exclusion chromatography using a HiLoad Superdex75 column (GE Healthcare). The homogeneity and purity was analyzed by 15% SDS PAGE.

3.5.3 Immuno-precipitation and Western blot of cell surface-biotinylated full length LR: HEK-293T cells were seeded and after one day were transfected with pMET7 vector encoding murine LR with a C-terminal FLAG tag using classical calcium phosphate transfection . After 24 hr of transfection, cells were washed with PBS and cultured further with 10% fetal calf serum (Sigma Aldrich). For cell surface biotinylation, cells were detached with trypsin and washed three times with ice-cold PBS. Cells were resuspended to a final concentration of 25 x 10^6 cells/ml and treated with 0.1% azide to prevent internalization of cell surface receptors. Cells were incubated at room temperature with freshly prepared 0.8 mM Sulfo-NHS-SS for 30 minutes. To remove unreacted biotin, cells were washed with ice-cold PBS containing 0.1% azide. Cells were lysed using modified RIPA buffer containing 50 mM iodoacetamide, to prevent non-physiological clustering during processing and lysate was cleared by centrifuging for 10 minutes at 14000 RPM. In order to enrich the biotinylated receptor using Dymabeads Streptavidin T1 (Invitrogen), beads were washed 3 times with PBS, cleared lysate was added to the beads, incubated at room temperature for 30 min while rotating and washed 3 times with PBS containing 0.1% (v/v) Tween-20 (Sigma). The biotinylated protein was eluted with laemlli buffer. For detection, samples were heated at 65° C for 5 minutes, resolved on a 7.5% SDS PAGE with and without reducing agent 2-mercaptoethanol. The LR was detected via western blotusing anti-FLAG antibody (Sigma).

3.5.4 Purification of complexes of LR_{ecto} with wild type and antagonist leptins: After size exclusion chromatography, the purified LR_{ecto} and wild type or antagonist leptins, each from size exclusion chromatography were concentrated separately to 8 mg/ml and 5mg/ml respectively. A mixture of LR_{ecto} with 6 times molar excess of ligand was incubated at room temperature for 1 hr followed by injection onto a pre-equillibrated Superdex 200 HiLoad 16/600 column. In case of wild type leptin, the resulting 1:1 and 2:2 complex peaks were pooled separately for further studies. In case of the carboxymethylated LR_{ecto}, purified receptor from Superdex 200 was incubated with 5 mM iodoacetamide for 1 hr followed by concentration for complex formation.

3.5.5 Formaldehyde mediated chemical cross-linking of LR_{ecto}/wt-Leptin complex: 30 µl of 36.5-38% aqueous solution of formaldehyde (Sigma) was dissolved in 1M HEPES pH=7.4 to a final volume of 100 µl. 5 µl of this diluted formaldehyde was added to 40 µl of complex of tetrameric LR_{ecto}/wt-Leptin at 0.3-0.8 µM and adjusted to a final volume of 50µl with buffer containing 50 mM HEPES (pH=7.4) and 100

mM sodium chloride followed by 3 hr incubation at room temperature. The cross-linking was stopped by adding 1 M Tris pH=7.4. This sample was further analyzed on non-reducing SDS PAGE followed by silver staining to assess the efficiency of cross-linking.

3.5.6 Isothermal Titration Calorimetry (ITC): The kinetic and thermodynamic parameters of LR_{ecto} interaction with wt-Leptin and antagonist leptin variants were determined by Isothermal Titration Calorimetry. All the protein components were purified in 25 mM HEPES (pH=7.4) containing 100 mM sodium chloride and degased immediately before titration. 20 µM of wt-Leptin was titrated against 1.4 ml of 1 µM LR_{ecto} with 17 discrete injections (3 µl followed by 10 µl each) at 37 ºC and mixing at 307 RPM stirring speed. The heat released was recorded using a MicroCal VP-ITC set up (GE Healthcare) and analyzed using Origin (OriginLab). The best fitting of the experimental data was obtained using built-in "one-set of sites" curve fitting model in Origin and this fit was used to determine the equilibrium constant ($1/K_d$), stoichiometry of interaction (N), enthalpy change (ΔH). The change in Gibbs free energy (ΔG) was calculated using the equation $\Delta G = -RT\ ln\ (1/K_D)$ and the entropy change (ΔS) was back calculated using $\Delta G = \Delta H - T\Delta S$. Similar experiments were performed for $Leptin_{a1}$ and $Leptin_{a2}$ to characterize their interaction with LR_{ecto}. In order to verify the stoichiometry of the high molecular weight complex, similar protocol has been followed using microcal ITC_{200} by titrating wt-Leptin at 2.4 mg/ml (~148 µM) into receptor in cell at 1.2 mg/ml (~8.4 µM).

3.5.7 Binding and Signaling Properties of LR_{C604S}: The cysteine residue on position 604 in the mouse LR, cloned in the pMET7 expression vector, was mutated to a serine using standard mutagenesis techniques. Resulting receptor was transfected together with the pXP2d2-rPAP1 (rat pancreatitis associated protein 1)-luciferase reporter (Eyckerman *et al.*, 2000) with calcium phosphate. Cells were stimulated overnight with a serial dilution of leptin, lysed and light emission was measured in a TopCount chemiluminescence counter. Leptin binding was determined using the leptin-SEAP (secreted alkaline phosphatase) chimera as described earlier (Zabeau *et al.*, 2005) (45). In brief, transfected cells are incubated with fusion-protein for 2 hours. After three washing steps, bound enzymatic activity is measured with the Phospha-Light assay (Life Technologies) in the TopCount counter.

3.5.8 Multi-angle Laser Light Scattering (MALS): The molecular masses and oligomerization state of LR_{ecto} and its complexes with wt-Leptin, $leptin_{a1}$, $leptin_{a2}$ in a concentration range of 18-50 µM were estimated by multi-angle laser light scattering. The purified protein sample was resolved on an asymmetric FFF (Field Flow Fractionation) system (Wyatt Technology) encompassing an ultrafiltration membrane (Microdyn-Nadir GmBH) with 10 kDa cut off and spacer of 350 µm height, operated by HPLC (Shimadzu) and connected with an online UV detector (Shimadzu), recorded by a 18 angle static light scattering detector (DAWN HELEOS) and a refractometer (Optilab T-rEX, Wyatt Technology). In order to enhance the resolution between HMW and LMW peaks of wt-Leptin/LR_{ecto} complex during FFF elution,

the cross flow was optimized. Based on the measured Rayleigh scattering in different angles and the established differential refractive index increment of 0.185 mL/g for proteins in solution with respect to the change in protein concentration (*dn/dc*) (61), weight averaged molar masses (Mw) for each species was calculated using the ASTRA V software.

3.5.9 Small angle X-ray scattering (SAXS):

***Data collection and primary data reduction*:** SAXS data were measured at beamline SWING at SOLEIL Synchrotron (Gif-sur-Yvette, France) with a mounted online HPLC system (David and Perez, 2009) and beamline ID14-3 of the European Synchrotron Radiation Facility (ESRF, Grenoble) equipped with a robotic sample changer. The protein samples of LR_{ecto}, its dimeric complex with $leptin_{a1}$ and $leptin_{a2}$ were measured in bulk mode with at least 3 different concentrations in the range of 0.5-3 mg/ml; while sample of wt-Leptin/LR_{ecto} complex at 10 mg/ml having both dimeric and tetrameric form in dynamic equilibrium, was first resolved through gel filtration column (Shodex KW404-4F) connected online with the X-ray scattering set up. Monochromatic X-ray beam of either 1.003 Å (Soleil) or 1.54 Å (ESRF) scattered through these monodispersing samples at 293 K were recorded covering the momentum transfer range $0.01\ Å^{-1} < s < 0.6\ Å^{-1}$, where $s=4\pi\sin\theta/\lambda$ (2θ=scattering angle, λ=wavelength of incident X-ray). In bulk mode measurement, absence of X-ray induced radiation damage was confirmed by alignment of 10 two dimensional scattering curves obtained from integration of continuous radial scattering data obtained from 10 independent X-ray scattering measurements with 10 second exposure for each sample. For each concentration set, the scattering data was normalized for beam intensity, exposure time and corrected for the detector response. Subtraction of buffer scattering from the sample scattering using PRIMUS (Konarev *et al.*, 2003) eliminated both buffer background as well as parasitic background arising from instrument hardware. For wt-Leptin/LR_{ecto}, after resolution on a size exclusion column, 150 frames with exposure time 1 second and delay of 0.5 second each were collected for buffer and then for protein peak. This 2D data was radially integrated to 1D scattering data and further analyzed with Foxtrot software developed at SWING beamline of Soleil synchrotron. Buffer scattering was subtracted from protein scattering and radius of gyration (Rg) for each scattering was plotted. The regions with stable Rg found to correspond to the peaks corresponding to the 2:2 and 1:1 complex and were used for all further analysis. The forward scattering (I_0) and radius of gyration (Rg) was determined in PRIMUS from infinite dilution extrapolation based on Guinier approximation and compared with corresponding estimates from Gnom (Svergun D., 1992) calculated by indirect fourier transformation of the complete one dimensional scattering data. The distance distribution function P(r) and maximal dimension Dmax for each scatterer was evaluated using Gnom. Molecular masses were calculated based on the forward scattering from a calibrated sample viz. bovine serum albumin (ESRF) or water (Soleil). Molecular mass based on porod volume was estimated using Autoporod (Petoukhov *et al.*, 2012) and compared with corresponding estimates from Guinier approximation. Molecular mass was also determined independent of the

calibration standards using SAXSMoW (Fischer *et al.*, 2010) and compared with corresponding forward scattering and porod volume based masses.

Ab initio modeling: *Ab initio* three dimensional shape reconstructions from one dimensional scattering data was achieved using DAMMIF (Franke and Svergun, 2009). In order to avoid stochastic error while modeling, 20 models were generated by 20 independent runs for each scatterer type. Uniqueness of these *ab initio* shapes were evaluated using DAMAVER (Volkov and Svergun, 2003). When the average normalized spatial discrepancy (NSD) value for the 20 models was found to be less than or equal to 1, the set was considered as single cluster and the model having lowest NSD average value with respect to other 19 models was considered as the representative for the scatterer type. Unlike the receptor and its complex with antagonist Leptins, the wt-Leptin/LR_{ecto} complex, when modeled using DAMMIF followed by DAMAVER analysis, produced average NSD value of ~ 1.6. Visual inspection of these models using the molecular viewer PyMol (D*eLano Scientific)* also showed variability in the models, possibly due to flexibility of this system. These models with multimodal distribution were clustered using DAMCLUST and the cluster centers were considered as the representative conformers of the wt-Leptin/mLR_{ecto} tetrameric complex. Evaluation of these models was carried out by analyzing the chi-square values against the respective experimental scattering curves.

Rigid-body modeling: SASREF (Petoukhov and Svergun, 2005), a heuristic based simulated annealing program in ATSAS package was used to generate low resolution atomic models. The 3D models for 8 individual receptor structural domains (1 for n-terminal domain (NTD), 2 for CRH1, 1 Ig, 2 CRH2 and 2 FNIII) were generated as described in Peelman *et al.*, (2006) except for CRH1 and FNIII, which were predicted using I-TASSER (Zhang Y., 2008; Roy et al., 2010; Roy et al., 2012). These predicted models were used to build ab initio models using SASREF. An inter-domain distance restriction of 3.7 Å was used corresponding Cα- Cα distance between consecutive amino acids from the termini of two tandem domains. Also the CRH2-leptin distance and CRH2-CRH2 distance from the hexameric signaling complex model (Peelman *et al.*, 2004) were used as the input parameters. A P2 symmetry restraint was used for the modeling of 2:2 quaternary complex. In order for the statistical significance 10 parallel runs were carried out with identical parameters and the models generated were evaluated using crysol against the experimental scattering curve. Rigid body models produced in this manner were found to have relatively high chi square values (≥17). In order to improve the fitting, these predicted individual domains were glycosylated *in silico*. Based on the earlier experimental evidence for the sites of glycosylation on LR_{ecto} (Haniu et al., 1998) and predicted N-glycosylation sites, these predicted models of individual domains were *in silico* glycosylated using GlyProt server (Bohne-Lang and von der Lieth, 2005). As the receptor used for SAXS measurement was expressed in the presence of Kifunensine, the glycosylation moieties are expected to be of Glc-NAC_2+$Mannose_{6-9}$ type (Chang *et al.*, 2007) (37). Parallel runs were carried out with predicted domains *in silico* glycosylated with Glc-NAC_2+$Mannose_6$, Glc-NAC_2+$Mannose_7$ and Glc-

NAC_2+$Mannose_9$ respectively. In order for the statistical significance of these parallel run, in each case 10 runs were performed and the resultant models were evaluated using crysol. Best results (Chi square ~5.8) were obtained when "2 Glc-NAC+9 Mannose" trees (GlyProt glycan id: 9146) was used for *in silico* glycosylation. Interestingly, it has been shown using MALDI-TOF experiment that Glc-NAC_2+$Mannose_9$ is the major glycan when expression is performed in the presence of kifunensine (Chang *et al.*, 2007). The initial CRH2-CRH2 distance based on the hexameric signaling complex model (Peelman *et al.*, 2004) was further optimized by using the predicted domains *in silico* glycosylated with Glc-NAC_2+$Mannose_9$. The stretch V^{466}LQRDGFYECVF^{477} from the each of the two partnering CRH2 domains were used as reference and the distance was found to be 11.2 Å in the modeled hexameric complex. So in order to optimize this distance, SASREF runs were carried out in the range of 10.7 Å-11.7 Å with 0.1 Å increment (total 11 different values). The statistical significance was assessed by 10 runs for each of the 11 sampled distances. In subsequent step, the CRH2 (reference Asp^{509})-leptin (reference Ser^{132}) distance was optimized in a similar fashion by sampling 21.5±1.1 Å distance (total 23 distances considering 0.1 Å increment). These two distance optimization steps improved the crysol generated chi square value for the models to 2.9. The final step involved the chi square based optimization of the models by sampling the glycan orientation. This step involved generation of 20 rotamers for the one glycan tree of a domain using UCSF Chimera package and 20 (x5 for statistical significance) parallel runs using these 20 rotamers. Best rotamer evaluated based on crysol fitting was subsequently segregated while sampling the rotamers of the next glycan. Such rotamer sampling method of all the glycans in murine LR_{ecto} could be able improve the fitting of the models to the experimental scattering with a chi square value of 2.2. Hence, a very large conformational space (>1500) has been sampled with a non-random and directed approach in order to obtain a best fitting rigid body model from the experimental SAXS data.

The best fitting rotamers of glycosylated domains from quaternary complex model optimization were subsequently used for the rigid body modeling of LR_{ecto}, wt-Leptin/LR_{ecto} binary complex, $leptin_{a1}$/LR_{ecto} complex and $leptin_{a2}$/LR_{ecto} complex with P1 symmetry restraint. The quality of these models were assessed using Crysol (Svergun *et al.*, 1995) by fitting the models with experimental scattering data.

Normal mode analysis: The conformational transformation of the receptor upon binding with cognate ligand or its antagonist forms were modeled using elNémo server (Suhre and Sanejouand, 2004; Suhre and Sanejouand, 2004b) which models potential low frequency intermediate conformers.

3.5.10 Single-particle negative-stain Electron Microscopy: Samples were diluted in base buffer (25mM HEPES pH=7.4 containing 100 mM sodium chloride) as required and adsorbed for 1 min to glow discharged thin carbon films coating a perforated carbon layer on gold-coated copper grids. These were washed with 4 drops of distillated water and negatively stained with 2% (w/v) uranyl acetate (UAc) and imaged with a CM10 transmission electron microscope (Philips) operating at 80 kV. Electron micrographs where recorded with a 2k charge-coupled device camera (Veleta; Olympus soft imaging solutions GmbH)

at a nominal magnification of × 130 000, yielding a final pixel size corresponding to 0.37 nm on the specimen scale. Particles were manually selected for single-particle analysis and averaged using the EMAN software (Ludtke *et al.*, 1999).

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3.7 SUPPLEMENTARY FIGURES

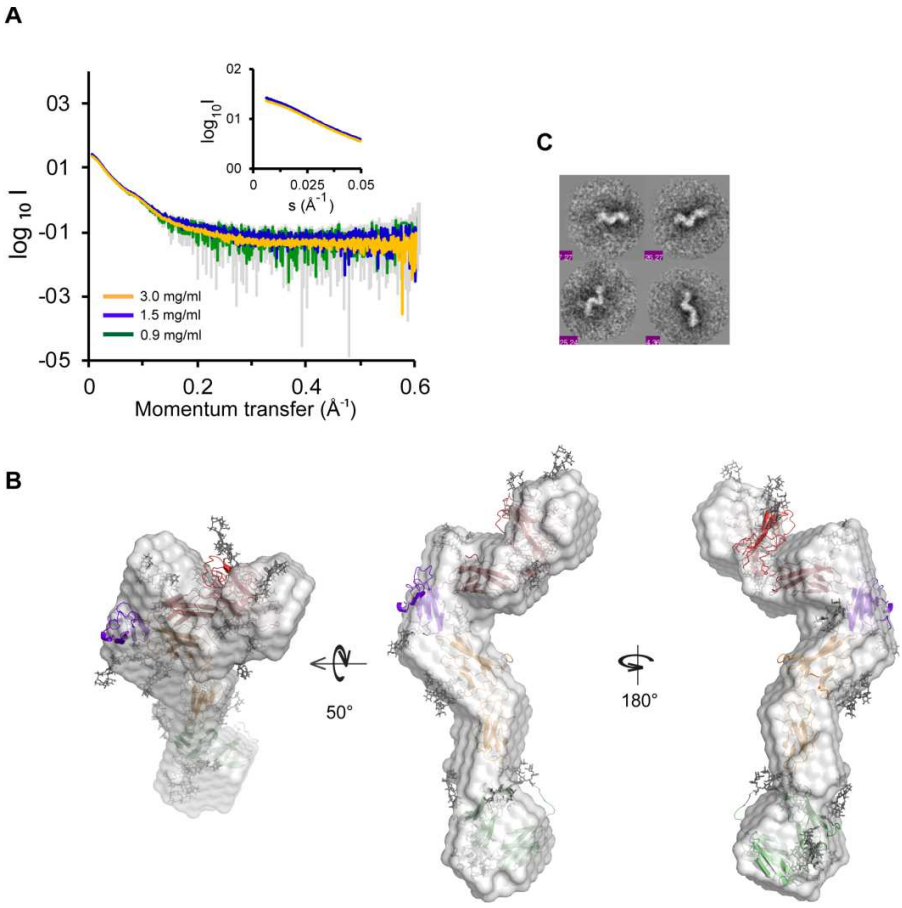

Figure 3.S1. Structural characterization of LR_{ecto} (A) Small angle X-ray Scattering of LR_{ecto}: Scattering intensity was plotted against the momentum transfer measured at three different concentrations of the receptor viz. 3.0 (*yellow*), 1.5 (*blue*) and 0.9 (*green*) mg/ml. *Inset:* Same plot of log I vs. momentum transfer at very low scattering angle showing absence of any concentration dependency for these measurements. **(B)** SAXS based *ab initio* modeling of LR_{ecto}: SAXS data from LR_{ecto} at 0.9mg/ml was used to build 20 *ab initio* models with P1 symmetry constraint using *DAMMIF* from *ATSAS* package. *DAMAVER* was used to average these models and the reference model detected by *DAMAVER* has been presented here in three different orientations in surface view (grey). This dummy bead *ab intio* model has been overlapped with the rigid body model presented as ribbon to show similarity among them in spite of difference in the adopted modeling methods. The color scheme for the rigid body model is same as in figure 3.1: CRH1 in red, Ig domain in violet, CRH2 domain in orange, FNIII domains in green and glycans have been shown as grey sticks. **(C)** Negative stain electron microscopy of LR_{ecto}: Electron microscopy based class averages of LR_{ecto}. These four class averages show striking similarity to the SAXS based *ab initio* models. Also the monomeric nature of LR_{ecto} derived from MALLS and SAXS analysis is supported by this electron microscopy analysis.

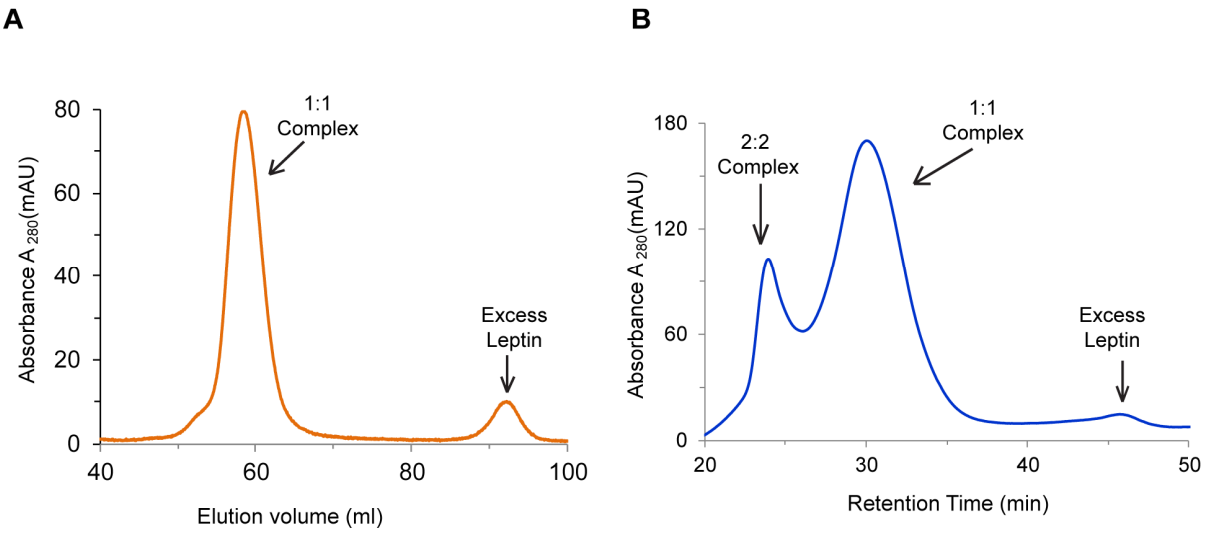

Figure 3.S2. Size exclusion chromatrography of samples recuperated from ITC runs: Size exclusion chromatography of sample recuperated after isothermal titration calorimetry of **(A)** wt-Leptin at 20 µM into LR_{ecto} at 1 µM and **(B)** wt-Leptin at 148 µM into LR_{ecto} at 8.4 µM. Sample from low concentration titration produced a single peak corresponding to the 1:1 binary complex of wt-Leptin/LR_{ecto} while that at high concentration eluted as two peaks: minor 2:2 quaternary complex and major 1:1 binary complex peaks. In each case, excess of wt-Leptin has eluted as minor peak.

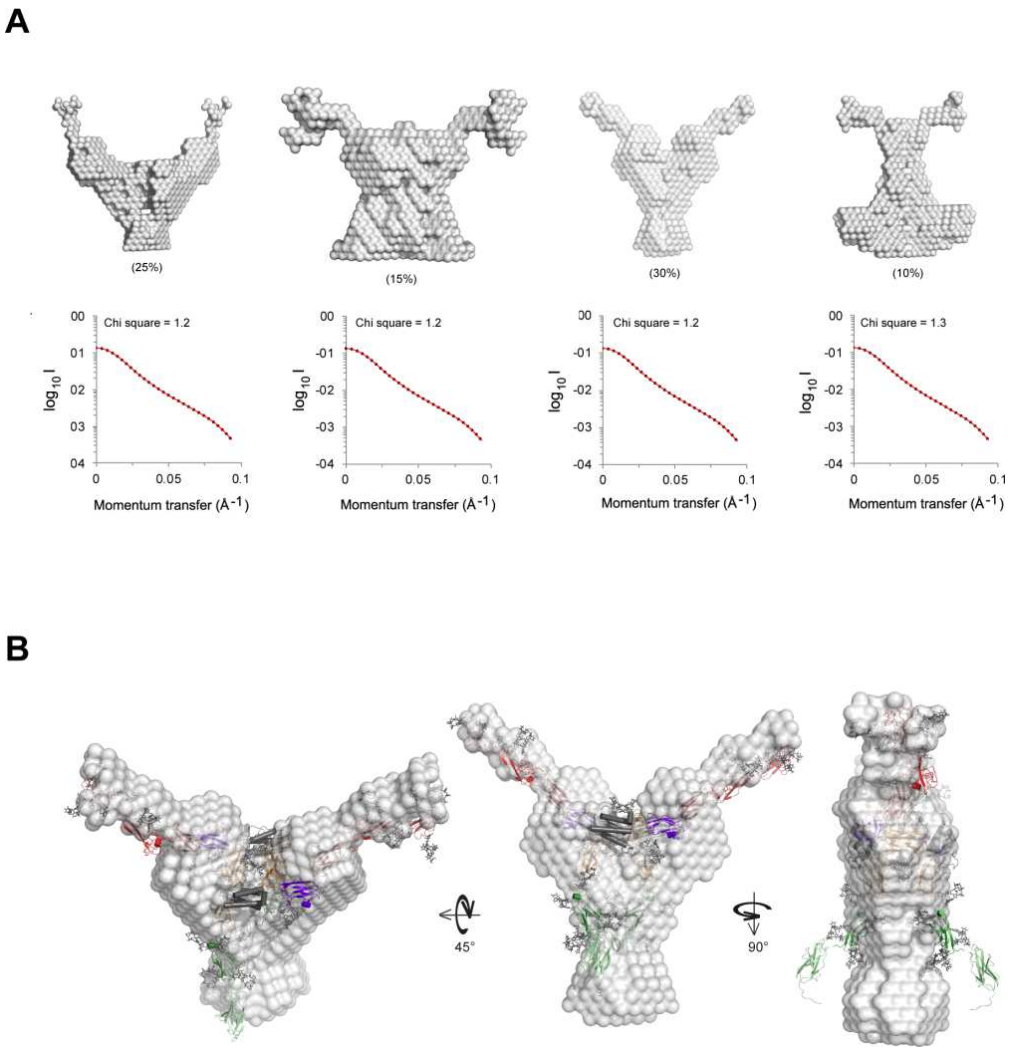

Figure 3.S3. Structural characterization of wt-Leptin/LR_{ecto} quaternary complex by SAXS
(A) Ab initio modeling of wt-Leptin/LR_{ecto} quaternary complex: After integrating the frames with similar radius of gyration (Rg) value corresponding to the quaternary complex peak on the size exclusion chromatography from online SEC-SAXS measurement, scattering data was used for *ab initio* modeling using *DAMMIF* from *ATSAS* package. 20 iterative *DAMMIF* runs followed by clustering of the resulting models using *DAMCLUST*, produced four clusters. Representative models from these four clusters are presented in surface views (*grey*). All of these cluster centers shows quite similarity in having a bulky core and two extended arms. Corresponding fitting of the calculated intensities for each these four cluster centers against the experimental SAXS curves are shown along with the chi square value of the fitting. **(B)** DAMMIF bead model of major cluster center wt-Leptin/LR_{ecto} quaternary complex: The bead model for the major cluster center from the DAMCLUST generated four clusters from 20 *ab initio* models of wt-Leptin/LR_{ecto} quaternary complex has been presented in three different orientations in grey surface view. The rigid body model generated from same scattering curve using SASREF has been presented as cartoon and manually fitted onto the ab initio model. The coloring scheme for different domains of the rigid body model is same as in figure 3.3.

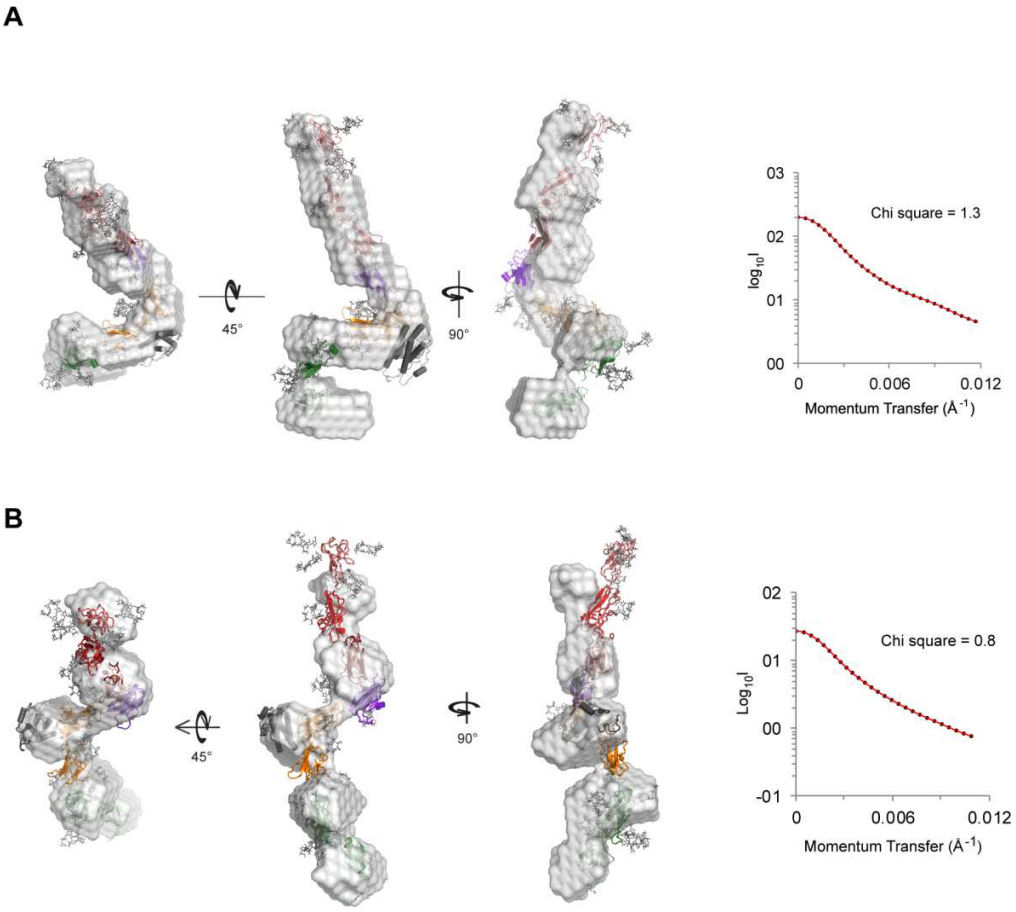

Figure 3.S4. Structural characterization of LR_{ecto} in complex with antagonist Leptins by SAXS (A) Ab initio model of $leptin_{a1}/LR_{ecto}$ binary complex: Small angle X-ray scattering of $leptin_{a1}/LR_{ecto}$ at 0.9 mg/ml was used to generate 20 ab initio models with *DAMMIF* from *ATSAS* package. The reference model from the averaging of these 20 models using *DAMAVER* has been shown here in grey surface view. The rigid body model generated using *SASREF* in cartoon view have been fitted onto the *ab initio* models manually. The color scheme for the rigid body model is same as in figure 4. The fitting of the calculated scattering from the ab intio model onto the experimental scattering and corresponding chi square has been shown. **(B)** Ab initio model of $leptin_{a2}/LR_{ecto}$ binary complex: Small angle X-ray scattering of $leptin_{a2}/LR_{ecto}$ at 0.75 mg/ml was used to generate 20 ab initio models with *DAMMIF* from *ATSAS* package. The reference model from the averaging of these 20 models using *DAMAVER* has been shown here in grey surface view. The rigid body model generated using *SASREF* in cartoon view have been fitted onto the *ab initio* models manually. The color scheme for the rigid body model is same as in figure 4. The fitting of the calculated scattering from the *ab intio* model onto the experimental scattering and corresponding chi square has been shown.

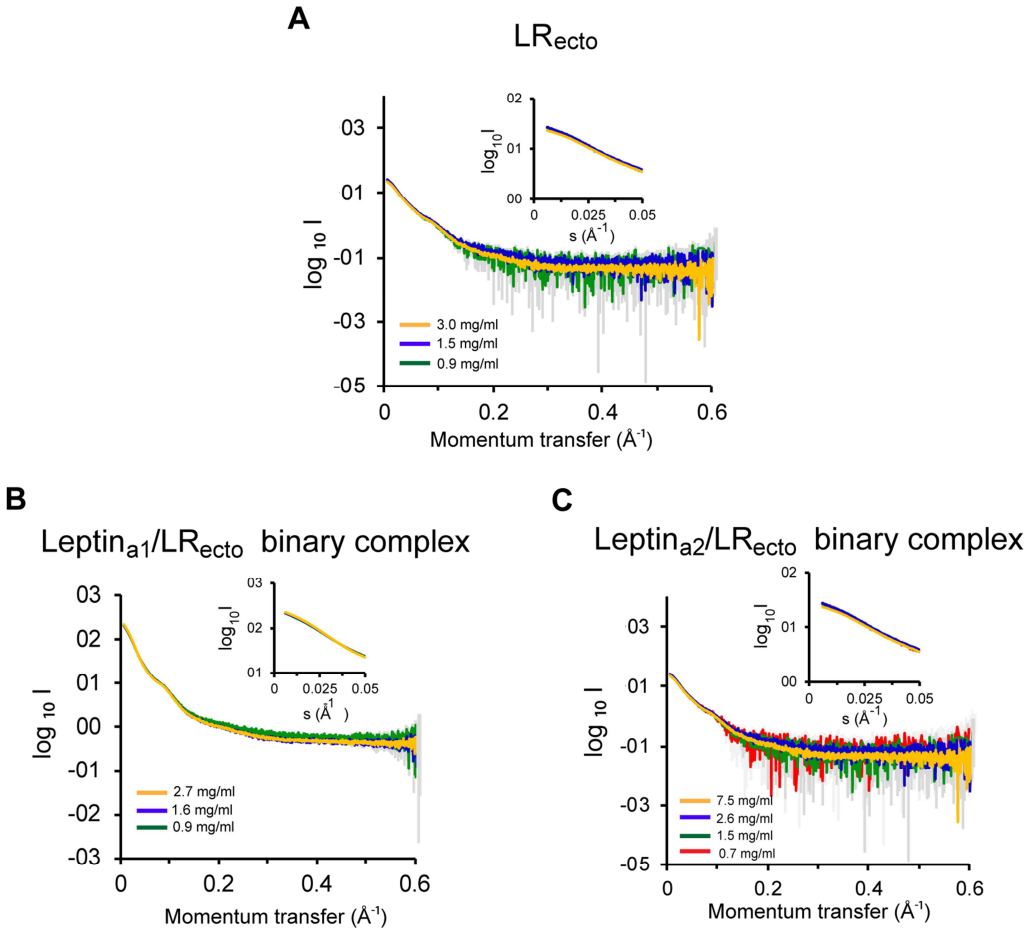

Figure 3.S5. Concentration independent SAXS scattering: Plot of scattered intensity in log scale vs. the momentum transfer, s (in $Å^{-1}$) recorded at different concentrations for ***A.*** LR_{ecto}, ***B.*** $leptin_{a1}/LR_{ecto}$ binary complex and ***C.*** $leptin_{a1}/LR_{ecto}$ binary complex. Different concentration (mg/ml) for each scatterer has been shown as color coded bars. *Inset*: Plot of log I vs. momentum transfer, s ($Å^{-1}$) at very low scattering angle to assess the concentration effect in guinier region.

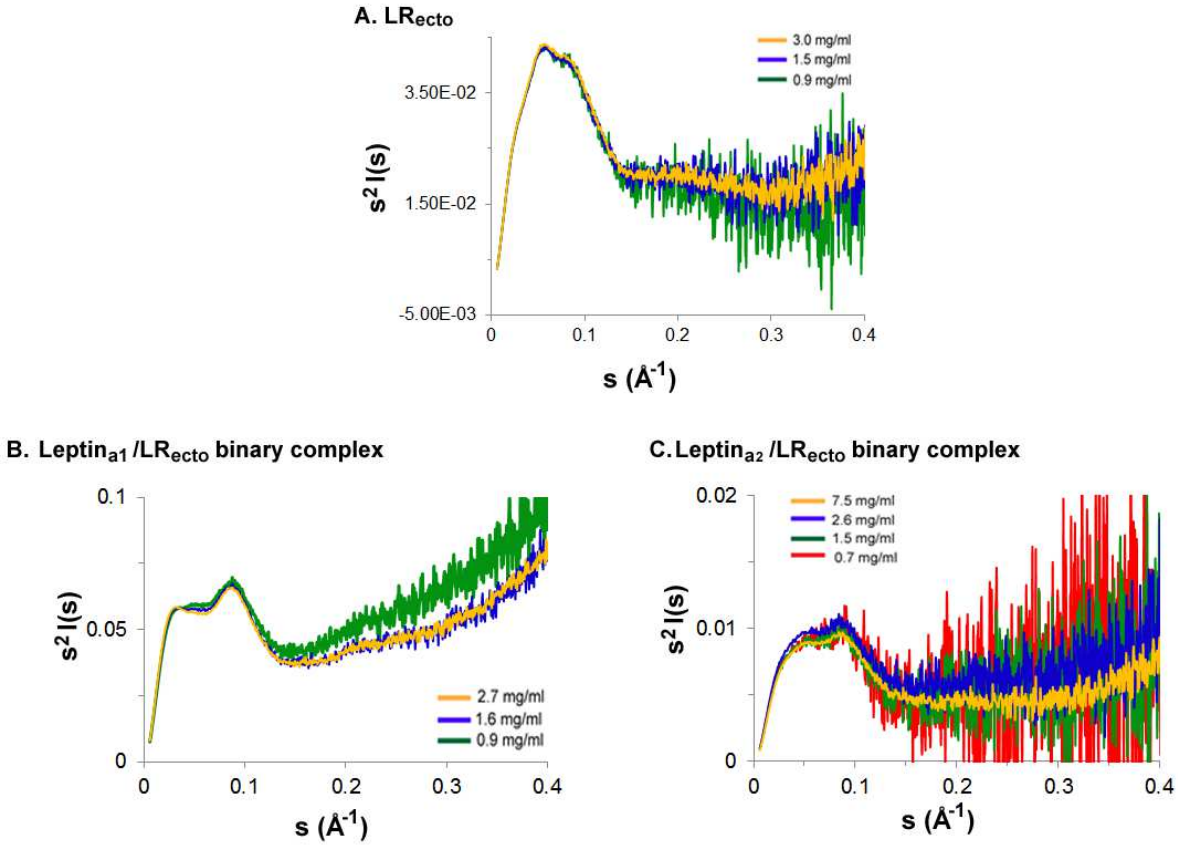

Figure 3.S6. Kratky Plot for A) LR_{ecto}, B) $Leptin_{a1}/LR_{ecto}$ and C) $Leptin_{a1}/LR_{ecto}$ complex

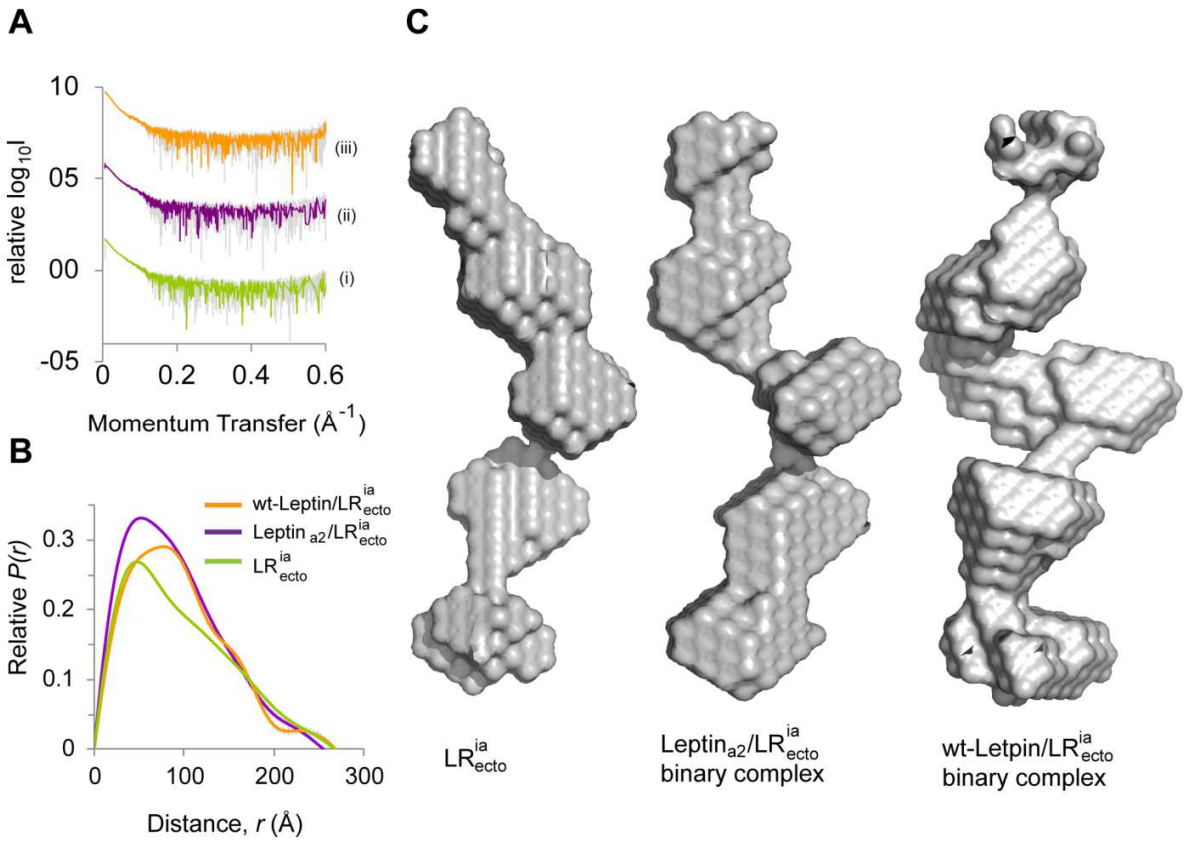

Figure 3.S7. Structural characterization of carboxymethylated LR_{ecto} and its complexes ***(A)*** SAXS scattering of iodoacetamide treated LR_{ecto} and its complexes: SAXS scattering intensity have been plotted for iodoacetamide treated LR_{ecto} (*green*), its complex with $leptin_{a2}$ (*purple*) and with wt-Leptin (*orange*) in log scale against the momentum transfer. The standard deviation for each curve has been shown in grey. ***(B)*** Pairwise distance distribution: The pairwise distance distribution plot for carboxymethylated LR_{ecto} (*green*), its complex with $leptin_{a2}$ (*purple*) and with wt-Leptin (*orange*). ***(C)*** Ab initio modeling: The scattering curves from receptor and its complexes have been used to generate ab initio models using DAMMIF. Forward intensity, I(0) based molecular weight determination suggested a monomeric LR_{ecto} and binary complexes for both types of complex. Thus a P1 symmetry restraint have been used for *ab intio* modeling.

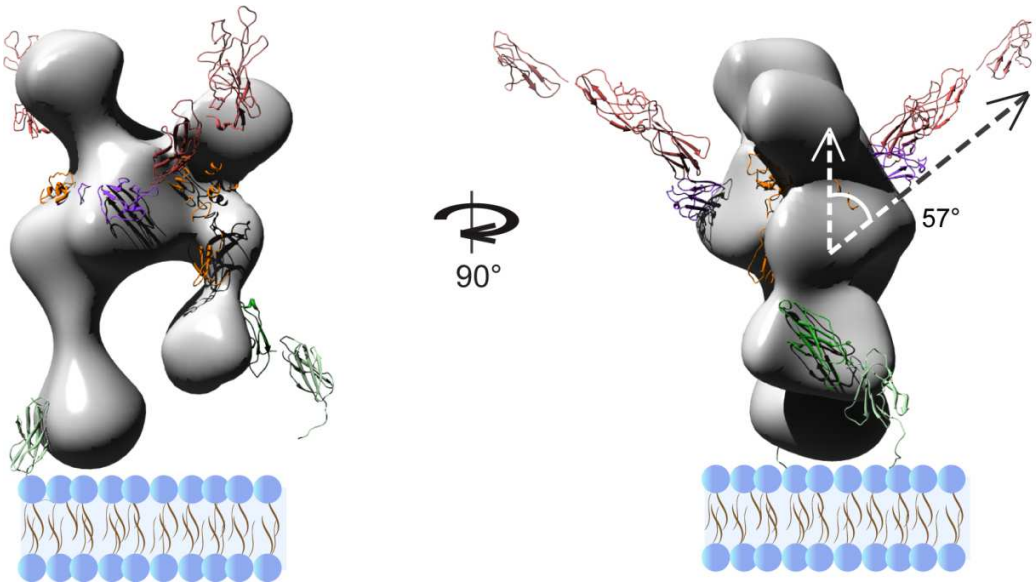

Figure 3.S8. Comparative analysis of SAXS rigid body model and EM model of wt-Leptin/LR$_{ecto}$ quaternary complex: The rigid body model of wt-Leptin/LR$_{ecto}$ quaternary complex obtained from SAXS analysis was manually fitted into the EM map of a leptin-LR quaternary complex (36).

TABLE 3.S1: SAXS analysis of the LR_{ecto} and wt-Leptin/LR_{ecto} quaternary and binary complexes

Data collection parameters	LR_{ecto}	wt-Leptin/LR_{ecto} Quaternary complex	wt-Leptin/LR_{ecto} Binary complex
Beamline	ID 14-3, ESRF	SWING, Soleil	SWING, Soleil
Detector	PILATUS 1M	AVIEX PCCD	AVIEX PCCD
Beam Geometry	0.7 x 0.7 mm^2	0.45 x 0.04 mm^2	0.45 x 0.04 mm^2
Wavelength(Å)	0.931	1.54	1.54
Q range ($Å^{-1}$)	0.15–6.110	0.06–6.125	0.06–6.125
Exposure Time (sec)	100 (10 x 10)	1	1
Concentration range (mg/ml)	0.9 - 3	10 (injected)	10 (injected)
Temperature (K)	293	293	293
Structural Parameters			
I(0) ($Å^{-1}$) from P(r)	151.6 ± 4.3	0.137	0.067
Rg (Å) from P(r)	65.4±0.3	87.0	63.9
I(0) ($Å^{-1}$) from Guinier	151.3±1.4	0.137	0.067±0.0
Rg (Å) (from Guinier)	63.8±1.6	84.9	61.9±0.2
Dmax (Å)	219.2±0.7	297.1	216.7
Porod volume estimate, V_p	244025	1225451	463936
Excluded volume, V_{ex}($Å^3$)	346000	1450000	608000
Molecular Mass (kDa)			
From I(0)*	146.4±4.2	305	149.1
From SaxsMOW	138.5	---	---
From Porod volume (V_p/1.7)	149.6	792.861	289.9
From excl. volume (V_{ex}/1.7)	203.5	852.9	357.6
Peptide (Protparam)	93	218	
Modeling Parameters			
Shape reconstruction	*DAMMIF*	*DAMMIF*	*DAMMIF*
Symmetry	P1	P2	P1
χ^2 of reference model	1.1	1.2 - 1.3	1.3
# of models averaged	20	20	28
DAMAVER NSD (var)	0.808±0.025	---	---
Rigid body modeling	*SASREF*	*SASREF*	*SASREF*
Initial χ^2	1.2	17.4	4.6
Final χ^2	1.2	2.2	3.5
Software/Server			
Data Reduction	*BsxCuBE*	Foxtrot	Foxtrot
Data Processing	PRIMUS	PrimusQt	PrimusQt
Data Evaluation	PRIMUS, Gnom	PrimusQt, Gnom	PrimusQt, Gnom
Structure Modeling	Dammif, Sasref	Dammif, Sasref	Dammif, Sasref
Molecular visualization	PyMOL	PyMOL	PyMOL

*****BSA was used for calibration for data obtained from ESRF, while water was used as molecular weight standard for data collected from SWING, Soleil for all the SAXS measurements discussed in this thesis.

TABLE 3.S2 : SAXS analysis of leptin$_{a1}$/LR$_{ecto}$, leptin$_{a2}$/LR$_{ecto}$ binary complexes

Data collection parameters	Leptin$_{a1}$/LR$_{ecto}$ (1:1)	Leptin$_{a2}$/LR$_{ecto}$ (1:1)
Beamline	ID 14-3, ESRF	ID 14-3, ESRF
Detector	PILATUS 1M	PILATUS 1M
Beam Geometry	0.7 x 0.7 mm^2	0.7 x 0.7 mm^2
Wavelength(Å)	0.931	0.931
Q range (Å^{-1})	0.15–6.110	0.15–6.110
Exposure Time (sec)	100 (10 x 10)	100 (10 x 10)
Concentration range (mg/ml)	0.9 - 2.7	0.7 – 7.5
Temperature (K)	293	293
Structural Parameters		
I(0) (Å^{-1}) from P(r)	145.9±6.2	35.7±2.4
Rg (Å) from P(r)	69.1±0.7	68.6±2.8
I(0) (Å^{-1}) from Guinier	146.1±6.5	51.0±3.6
Rg (Å) (from Guinier)	67.7±0.8	67.0±3.8
Dmax (Å)	236.4±0.3	234.0±1.5
Porod volume estimate, V_p	286541	273948
Excluded volume, V_{ex}(Å^3)	372000	390000
Molecular Mass (kDa)		
From I(0)	140.9±6.0	155.1±10.5
From SaxsMOW	164.9	151.7
From Porod volume (V_p/1.7)	180.4	183.6
From excl. volume (V_{ex}/1.7)	218.8	229.4
Peptide (Protparam)	109	109
Modeling Parameters		
Shape reconstruction	*DAMMIF*	*DAMMIF*
Symmetry	P1	P1
χ^2 of reference model	1.3	0.8
# of models averaged	20	20
DAMAVER NSD (var)	1.018±0.067	0.824±0.049
Rigid body modeling	*SASREF*	*SASREF*
Initial χ^2	3.3	0.9
Final χ^2	2.5	0.9
Software/Server		
Data Reduction	*BsxCuBE*	*BsxCuBE*
Data Processing	PRIMUS	PRIMUS
Data Evaluation	PRIMUS, Gnom	PRIMUS, Gnom
Structure Modeling	Dammif, Sasref	Dammif, Sasref
Molecular visualization	PyMOL	PyMOL

TABLE 3.S3 : SAXS analysis of LR_{ecto}, $leptin_{a1}/LR_{ecto}$, $leptin_{a2}/LR_{ecto}$ in the presence of the sulfhydryl alkylating agent iodoacetamide.

Data collection parameters	LR_{ecto} (+iodoacetamide)	wt-Leptin/LR_{ecto} (+iodoacetamide)	$Leptin_{a2}/LR_{ecto}$ (+iodoacetamide)
Beamline	ID 14-3, ESRF	ID 14-3, ESRF	ID 14-3, ESRF
Detector	PILATUS 1M	PILATUS 1M	PILATUS 1M
Beam Geometry	0.7 x 0.7 mm^2	0.7 x 0.7 mm^2	0.7 x 0.7 mm^2
Wavelength (Å)	0.931	0.931	0.931
Q range ($Å^{-1}$)	0.15–6.110	0.15–6.110	0.15–6.110
Exposure Time (sec)	100 (10 x 10)	100 (10 x 10)	100 (10 x 10)
Concentration range (mg/ml)	0.9 - 3	0.9 - 2.7	0.7 – 7.5
Temperature (K)	293	293	293
Structural Parameters			
I(0) ($Å^{-1}$) from P(r)	20.0±1.0	24.0±0.9	25.6±1.6
Rg (Å) from P(r)	72.5±4.9	71.6±2.8	68.7±2.6
I(0) ($Å^{-1}$) from Guinier	20.0±1.1	24.0±1.1	25.6±1.8
Rg (Å) (from Guinier)	70.6±5.2	71.5±5.0	67.2±3.8
Dmax (Å)	251.2±14.6	245.3±15.8	235.2±13.3
Porod volume estimate, V_p	290529	430354	293806
Excluded volume, V_{ex}($Å^3$)	421000	513000	390000
Molecular Mass (kDa)			
From I(0)	187.5±9.4	211.0±12.0	221.1±14.4
From SaxsMOW	199.9	220.8	193.4
From Porod volume (Vp/1.7)	181.5±21.8	248.7±13.9	183.6±6.5
From excl. volume (Vex/1.7)	247.6	301.7	229.4
Peptide (Protparam)			
Modeling Parameters			
Shape reconstruction	*DAMMIF*	*DAMMIF*	*DAMMIF*
Symmetry	P1	P1	P1
χ^2 of reference model	0.9	0.8	0.8
# of models averaged	20	20	20
DAMAVER NSD (var)	0.779±0.080	1.007±0.070	0.824±0.049
Rigid body modeling	---	---	---
Initial χ^2	---	---	---
Final χ^2	---	---	---
Software/Server			
Data Reduction	*BsxCuBE*	*BsxCuBE*	*BsxCuBE*
Data Processing	PRIMUS	PRIMUS	PRIMUS
Data Evaluation	PRIMUS, Gnom	PRIMUS, Gnom	PRIMUS, Gnom
Structure Modeling	Dammif	Dammif	Dammif
Molecular visualization	PyMOL	PyMOL	PyMOL

4

Redundant role of FNIII domains in Leptin induced Leptin Receptor oligomerization#

Adopted from the second manuscript currently under preparation

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Contribution: For the preparation of leptin and leptin receptor plasmid constructs and production of leptin, credit goes to LZ. All the rest of experimental work is performed by KM.

4.1 Abstract

Despite two decades of studies on the (patho)physiology of leptin-mediated Leptin receptor (LR activation) our current understanding of the leptin-LR signaling assembly is yet to come to full circle with respect to a mechanistic consensus. As we have previously shown, leptin acts via dimerization of its cognate receptor thereby displaying pleiotropic functionality ranging from energy homeostasis to reproduction, from regulation of immune system to angiogenesis and so on. While administration of leptin in diabetes and congenital leptin deficiency established promising therapeutic applications, its pathophysiology in obesity, autoimmune disease and breast cancer raises unequivocal demand for better understanding of structural and mechanistic paradigm of leptin receptor activation. Here we demonstrate that leptin can induce dimerization of LR lacking the membrane proximal fibronectin type III ($LR_{\Delta FNIII}$) domains, thus suggesting a redundant role of these domains in leptin induced LR oligomerization. Earlier studies had shown that the FNIII region is essential for the functionality of LR. The affinity of leptin for this construct was found to be similar to the full length receptor, with nanomolar affinity and stoichiometry of 1:1. Based on light scattering and small-angle x-ray scattering data, we demonstrate that $LR_{\Delta FNIII}$, despite the absence of the membrane proximal domains, can form a quaternary complex with wt-Leptin. In contrast, antagonist leptin bearing S120A/T121A mutation could not dimerize the $LR_{\Delta FNIII}$ receptor. These results evidently suggest a functional dichotomy whereby while FNIII appears to be redundant in the oligomerization of LR it still may be playing a key role in the spatial disposition and orientation of intracellular parts to facilitate a signaling-competent conformation.

Keywords: Leptin | Leptin Receptor | FNIII domain | Receptor oligomerization

4.2 INTRODUCTION

Leptin, a 16kDa four helical bundle (Zhang et al., 1997a), adipocyte hormone, initially discovered as the “obesity causing hormone” has rapidly gained its prominence as a pleiotropic cytokine with functions like regulation of body weight and energy homeostasis (Baile et al., 2000; Bates and Myers, 2003; Halaas et al., 1995; Morton et al., 2005), direct and indirect regulation of immune system (Lord et al., 1998; Matarese, 2000), angiogenesis (Park et al.,

2001), hematopoiesis (Fantuzzi and Faggioni, 2000), bone formation (Cornish et al., 2002), reproduction and fetal development (Hoggard et al., 1997). Structurally, Leptin composed of four helices, running in up-up-down-down fashion and harbors two cysteines bonded by a disulfide bridge. Homology based structure alignment has revealed three distinct putative binding sites on Leptin (Peelman et al., 2004): site II has been shown to be directly involved in binding to its cognate receptor while site III is inevitable for receptor activation. The experimental work described in "Chapter 3" shows that site III is essential for ligand induced receptor oligomerization and thereby it explains the mechanism behind the antagonism manifested by site III mutants. The role of binding site I is still questionable due to limited and weak experimental evidence. Once activated, LR signaling proceeds via JAK2/STAT3 pathway with potential crosstalk with other signaling pathways (Wauman and Tavernier, 2011). The pleiotropic role of Leptin confers it versatility while simultaneously making it susceptible towards mis-regulations and dys-functionalities. Leptin has been shown to be directly linked in the development of autoimmune diseases (La Cava and Matarese, 2004) and cancer (Schaffler et al., 2007), thus demanding in-depth understanding of the molecular mechanism of leptin induced LR activation.

Leptin acts via binding to LR, a type I cytokine receptor without intrinsic kinase activity and with a broad spatial expression ranging from hypothalamic nuclei (Tartaglia et al., 1995), kidney (Serradeil-Le Gal et al., 1997), liver (Cohen et al., 2005), mononuclear cells (Sanchez-Margalet et al., 2003) etc. The extracellular part of LR (LR_{ecto}) is composed of 4 functional regions: distal CRH1 domain, followed by Ig-like domain, leptin binding CRH2 domain and two membrane proximal FNIII domains (Haniu et al., 1998). CRH1 domain which is composed of 3 subdomains, although does not take part in leptin binding or LR oligomerization directly, it has been shown to harboring a single nucleotide polymorphism Q223R, resulting in obesity in certain ethnic group (Duarte et al., 2006; Quinton et al., 2001) and increased susceptibility towards protozoan infection in children (Duggal et al., 2011). CRH2 has been shown to be the major site of leptin binding (Fong et al., 1998). Previously we have shown that Ig-domain interaction with site III on Leptin is inevitable for leptin mediated LR oligomerization and mutation in site III confers antagonism due to loss of oligomerization ability (Chapter 3).

Close homologue gp130 has three membrane proximal FNIII domains, classically known as D4, D5 and D6. Deletion of D4 and D6 has been shown to reduce the affinity of gp130 for IL-6/sIL-6R complexes, while deletion of D5 completely abolishes the LR activation (Kurth et al., 2000).

FNIII domains of GCSFR and gp130 has been shown to orient the intracellular part of respective receptors enabling signaling (Hammacher et al., 2000). This has led to the theory that the FNIII domains enable the ideal relative orientation of receptor proximal regions with respect to each other facilitating the interaction of intracellular domains. FNIII mediated inter-receptor interactions leading to the formation of constitutively active receptor oligomers have been described using chimeric gp130 receptors (Kurth et al., 2000). LR has been shown to be constitutively active when the LR_{ecto} composed of FNIII domains only suggesting a similar behavior of LR_{FNIII} domains. All these evidences point towards a consensus in the functionality of FNIII domains in type 1 receptor family where these domains help orienting the intracellular part.

However, juxtamembrane FNIII domains have never been shown to have any direct role in binding or oligomerization of LR, deletion of this domain makes the LR inactive (Fong et al., 1998). Mass spectrometric analysis of human leptin (Haniu et al., 1998) and alignment of mouse and human leptin receptor suggests, FNIII domains in mouse LR possess two free cysteines: Cys672 and Cys751. Substitution mutation C751S has limited effect on LR activity, while C672S causes substantial reduction in LR activity. Though these cysteines were initially hypothesized to be involved in homotypic interaction in LR dimers (Zabeau et al., 2005), we have shown that LR dimers exist as non-covalently linked on plasmamembrane. But based on homology to gp130, a putative inter-domain but intra-receptor disulfide bond between CRH2 Cys604 and FNIII Cys672 has been modeled from the crystal structure of leptin binding domain solved in complex with an antibody (Carpenter et al., 2012). However, employing antagonist Leptin as a tool, we have shown oligomerization to be essential for activation.

We have used multidisciplinary approach employing various biophysical and biochemical techniques to understand the role of FNIII in the ligand induced LR oligomerization.

4.3 RESULTS

4.3.1. $LR_{\Delta FNIII}$ is expressed largely as monomer: The purified $LR_{\Delta FNIII}$ from Talon purification, when concentrated and loaded onto size exclusion column SD200, the receptor eluted in two

fully-resolved and symmetric peaks: a major low molecular weight (LMW) peak and a minor high molecular weight (HMW) peak.

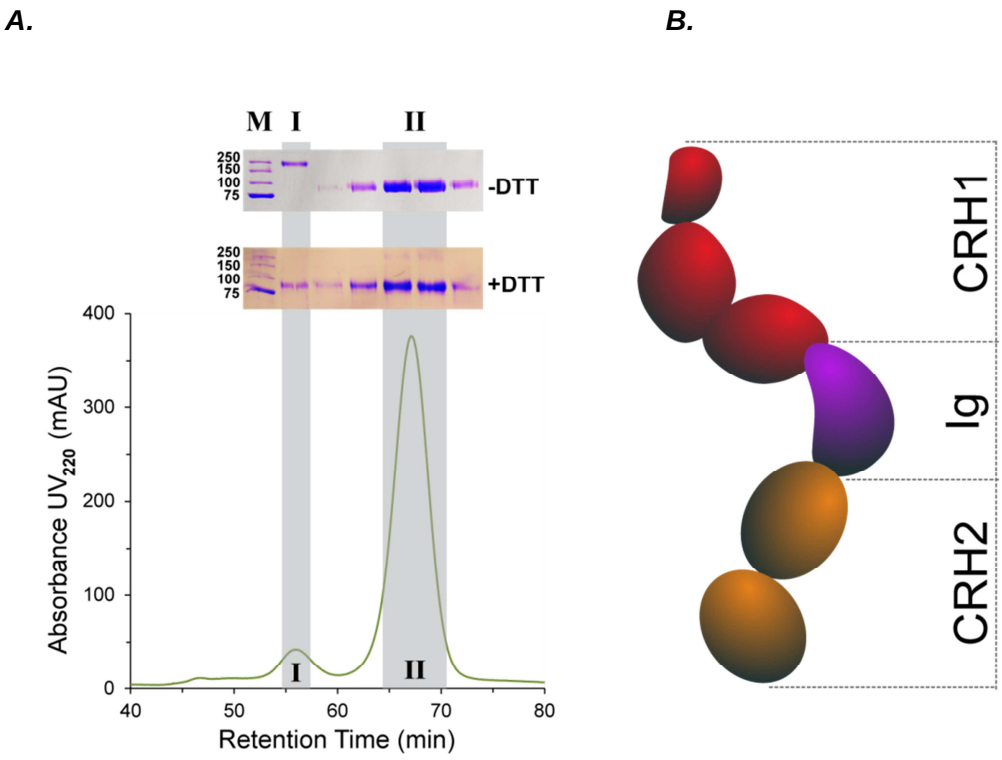

Figure 4.1. Schematic and biochemistry of $LR_{\Delta FNIII}$: ***(A)*** Elution profile of $LR_{\Delta FNIII}$ from size exclusion chromatography results in two peaks: minor Peak 1 composed of disulfide-linked dimeric receptor and major Peak 2 formed by monomeric receptor as observed on reducing and non-reducing SDS PAGE followed by Coomassie Brilliant Blue staining. ***(B)*** Schematic representation of $LR_{\Delta FNIII}$: Amino-terminal 1st cytokine receptor homology domain, CRH1 (*red*), immunoglobulin domain, Ig (*purple*), carboxy-terminal 2nd cytokine receptor homology domain, CRH2 (*orange*).

Further analysis of these peaks on reducing and non-reducing SDS PAGE showed the major LMW peak comprises monomeric receptor running between 100kDa and 150kDa marker while

the minor HMW peak has disulfide-linked dimeric receptor. The monomeric nature of the LMW fraction has also been confirmed by both MALLS and SAXS studies (described elsewhere). We observed disulfide mediated clustering of monomeric $LR_{\Delta FNIII}$ upon storage as analyzed by non-reducing SDS PAGE. Based on this observation, the disulfide linked dimer was considered non-physiological artifact. Monomeric $LR_{\Delta FNIII}$ which represent physiological state of the receptor was used for all further studies.

4.3.2 Elimination of FNIII domains has no effect on wt-Leptin induced receptor dimerization: The purified monomeric $LR_{\Delta FNIII}$ upon incubation with four times molar excess of wt-Leptin and resolution on a SD200 column, produced a broad peak possibly formed by fusion of more than one peak. This elution profile suggested formation of both high molecular weight and low molecular complexes of wt-Leptin/$LR_{\Delta FNIII}$, similar to the full length LR_{ecto} shown earlier. Samples corresponding to left side of this broad peak when analyzed on non-reducing SDS PAGE did not showed presence of any disulfide bonds, thus suggesting a non-covalently linked higher order complex. The stoichiometry, molecular weight information was further characterized.

4.3.3 Antagonist Leptin is unable to dimerize $LR_{\Delta FNIII}$: In contrast to the wt-Leptin, monomeric $LR_{\Delta FNIII}$ when incubated with four times molar excess of antagonist leptin bearing substitution mutation S120A/T121A in site III and resolved on SD200, produced single peak. Further analysis of the fractions corresponding to this peak showed it to be $leptin_{a1}$/$LR_{\Delta FNIII}$ binary complex. Unlike wt-leptin, we did not observed any formation of HMW complex in this case suggesting the role of site III-Ig domain interaction essential for leptin mediated dimerization.

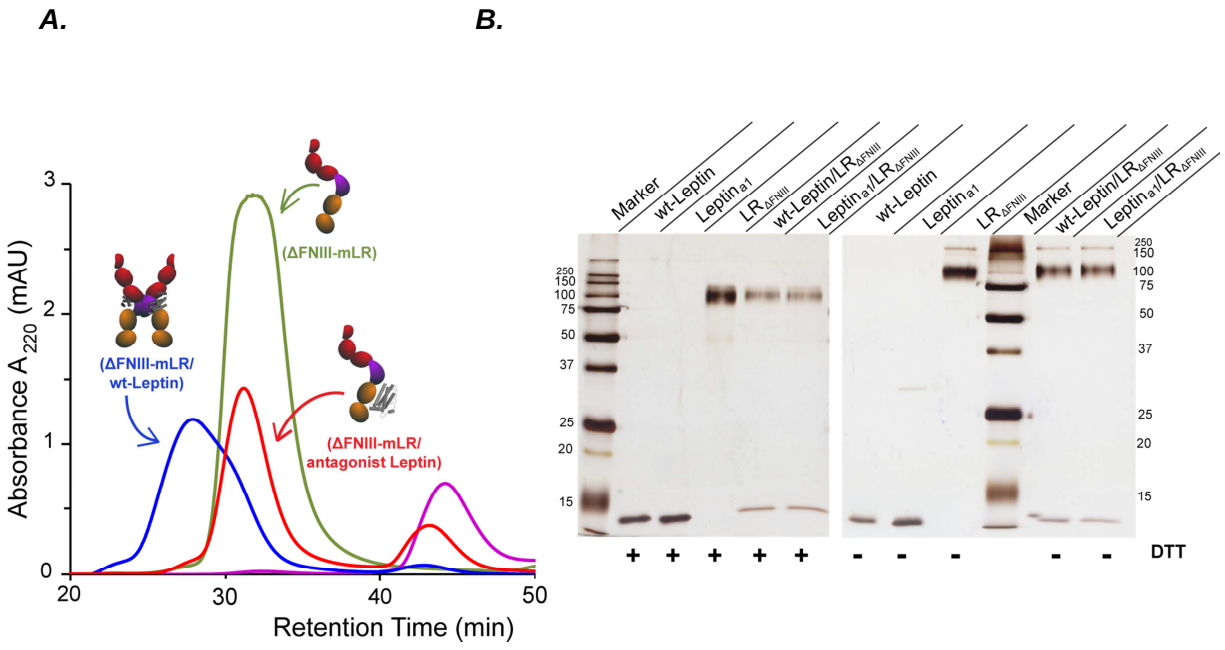

Figure 4.2. Size exclusion chromatography of mLR_{EC}-ΔFNIII and its complexes: ***(A)*** Analytical gel filtration of wt-Leptin (*pink*), $LR_{\Delta FNIII}$ (*green*), $leptin_{a1}/LR_{\Delta FNIII}$ complex (*red*) and wt-Leptin/$LR_{\Delta FNIII}$ Complex (*blue*). The excess Leptin (2^{nd} peak in *red* and *blue*) has eluted corresponding the wt-Leptin (*pink*) peak. For easy interpretation, corresponding schematic representations of $LR_{\Delta FNIII}$ and its binary and quaternary complexes with antagonist $leptin_{a1}$ and wt-Leptin, respectively have been shown. ***(B)*** The eluted peak fractions have been analyzed on reducing and non-reducing SDS PAGE followed by Silver staining.

4.3.4. Isothermal Titration calorimetry: Isothermal titration calorimetry (ITC) was used for the determination of binding constants and stoichiometry of the interaction between $LR_{\Delta FNIII}$ and Leptin variants. $LR_{\Delta FNIII}$ was found to interact with both leptin types with a 1:1 stoichiometry. Based on this result, the broad peak from size exclusion chromatography was assumed to be formed of 1:1 and 2:2 complexes, similar to the complete extracellular part of LR_{ecto} as shown earlier. The dissociation constants for wt-Leptin and $Leptin_{a1}$ was determined to be 3.0 nM and 4.5 nM, respectively.

4.3.5 Small Angle X-ray Scattering: The molecular weight, structural dimension and low resolution architecture of receptor alone and in complex with wt-Leptin and $Leptin_{a1}$ was determined using small angle X-ray scattering (SAXS). $LR_{\Delta FNIII}$ and its complex with antagonist Leptin, did not show any radiation damage or concentration dependency. However, wt-Leptin/ $LR_{\Delta FNIII}$ showed concentration dependency when measured in bulk mode. The size of the receptor and complexes was determined from the Guinier based radius of gyration, while the molecular weight was calculated from the zero intensity extrapolation. These molecular weight data was found to be compatible with the expected molecular weight determined from size exclusion chromatography and chemical crosslinking. SAXS data analysis for $LR_{\Delta FNIII}$ showed it to be monomeric with a molecular weight of 87.8 kDa as extrapolation based molecular weight determination suggested a 230.9 kDa complex. Based on the 1:1 stoichiometry of wt-Leptin and $LR_{\Delta FNIII}$ interaction, a 230.9 kDa suggests a quaternary complex with two receptor and two ligand. The plot of distance distribution function *P(r)* showed a similar size for the receptor and complexes but different form as expected. The open form of the Kratky plot analysis suggested these constructs to be highly flexible. Ab initio shape reconstruction using DAMMIF tool from ATSAS package provided further structural information about the receptor and its complexes. The receptor ab initio dummy bead model built with a P1 symmetry showed an elongated extended multi-domain structure. The ab initio model of 1:1 complex of antagonist Leptin/ $LR_{\Delta FNIII}$ was also reconstructed with a P1 symmetry. This model shows an elongated model similar to the receptor alone. As the wt-Leptin/ $LR_{\Delta FNIII}$ complex is a quaternary complex, it was modeled with a P2 symmetry. Interestingly, limited variability was observed among the models and clustering using *DAMCLUST* produced two different clusters. The cluster centers of the both clusters shows similar feature having two arms extending from a bulky base. The two extended arms in the low resolution structure can easily be interpreted as the CRH1 domain while the bulky base is formed by two CRH2 domains interacting two Leptins. The striking similarity of these models with the distal part of *ab initio* model of the quaternary complex of full length extracellular receptor and Leptin corroborate each other. This robust SAXS study bolsters results obtained by size exclusion chromatography, isothermal titration calorimetry and chemical cross-linking study thus clearly suggesting the redundant role of FNIII domains in Leptin induced LR dimerization.

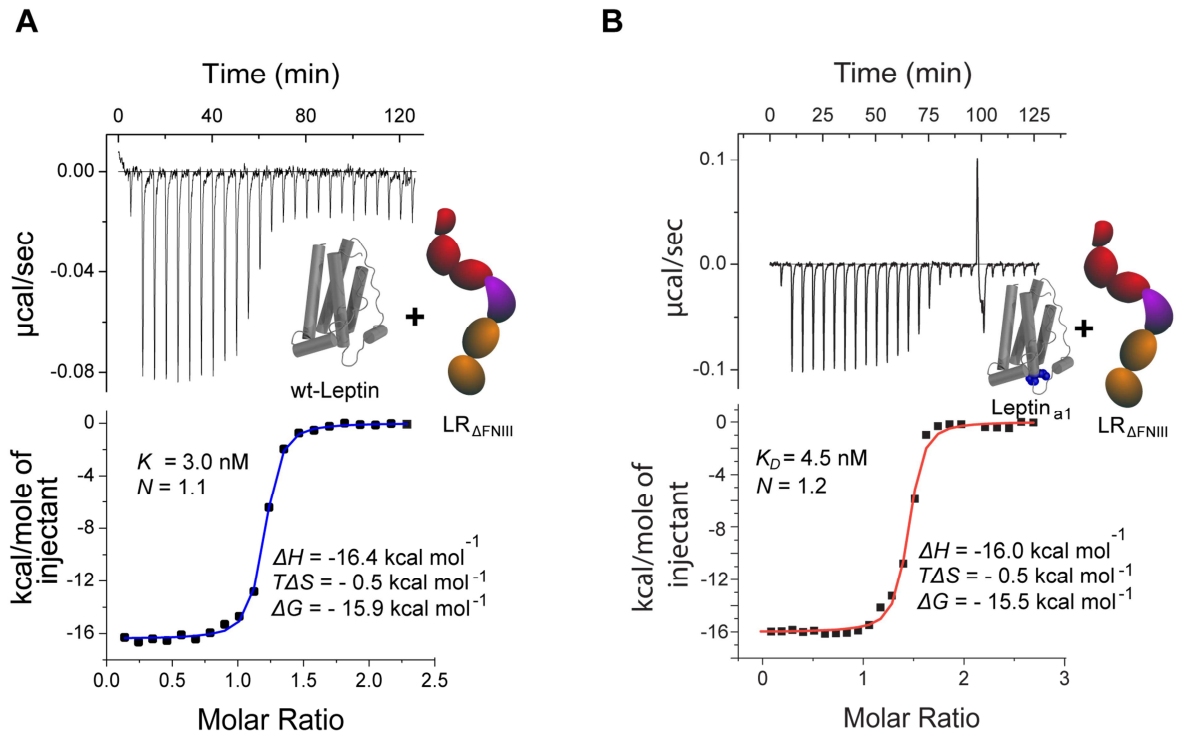

Figure 4.3. Biophysical characterization of $LR_{\Delta FNIII}$ interaction with Leptin: (*A*) Calorimetric isotherm and thermodynamic parameters for interaction of $LR_{\Delta FNIII}$ with wt-Leptin and **(*B*)** Calorimetric isotherm and thermodynamic parameters for interaction of $LR_{\Delta FNIII}$ with antagonist $leptin_{a1}$.

4.4 DISCUSSION

4.4.1. $LR_{\Delta FNIII}$ is monomeric but has higher tendency to form non-physiological covalent clusters: $LR_{\Delta FNIII}$ lacks the membrane proximal two FNIII domains harboring two free cysteine residues (Haniu et al., 1998). These cysteines have been shown to form clusters resulting in covalently linked dimer, trimer and oligomers of FNIII domains when expressed in isolation (Zabeau et al., 2005). In spite of the loss of these two cysteines upon FNIII truncation, this construct showed higher tendency to form non-physiological disulfide clusters unlike the full length LR_{ecto}. Considering earlier observation that free cysteines in CRH2 also take part in non-physiological clustering (Zabeau et al., 2005), such an irony can be explained by our hypothesis that a specific role played by FNIII domains where it prevents the random clustering of the free cysteines in rest of the part of the receptor in solution by hindering their spatial proximity with

steric interference. The majority of monomeric fraction suggests it to be the native form of the receptor, compatible with earlier observation that full length LR_{ecto} expressed as monomeric receptor.

4.4.2. Thermodynamic parameters of agonist and antagonist Leptin interaction with $LR_{\Delta FNIII}$: Although FNIII domains have been shown not to take part in ligand binding (Fong et al., 1998), in order to rule out any indirect effect on the ligand binding by FNIII- mediated modulation of overall orientation and relative organization of leptin binding domains, we have performed quantitative analysis of their interactions. The 1:1 stoichiometry of $LR_{\Delta FNIII}$ with wild type and antagonist Leptins is compatible with earlier result with full length LR_{ecto}. The interactions are enthalpy-driven with nanomolar affinities, similar to our previous observation with full length LR_{ecto} construct.

4.4.3. Absence of FNIII domains has no effect on Leptin-induced quaternary complex formation: In chapter 3 it has been shown that the dimerizing ability of leptin is a prerequisite for the LR activation and loss of dimerizing ability of leptin leads to antagonism. Although this dimerization involves only CRH2-Ig domains, deletion of FNIII or mutation of FNIII cysteines have been shown to affect receptor activation (Zabeau et al., 2005). Our analytical gel filtration experiments show that loss of FNIII has no effect on leptin induced dimerization, suggesting this loss of activation potential is not related to the oligomerization, thereby establishing the dichotomy of receptor function. The CRH2 and Ig is the unit of ligand binding and oligomerization, while FNIII unit plays a redundant role in ligand induced oligomerization. Nonetheless, this domain may act like a lever, controlling the orientation of intracellular parts upon leptin binding. The antagonist Leptin can only form the 1:1 complex unlike the wt-Leptin, corroborating our earlier observation that antagonism is a result of loss of oligomerization by leptin site-III-Ig domain interaction.

4.4.4. Solution structure of the quaternary complex of wt-Leptin/$LR_{\Delta FNIII}$ shows striking similarity to the distal part of wt-Leptin/LR_{ecto} complex: The low resolution solution structure

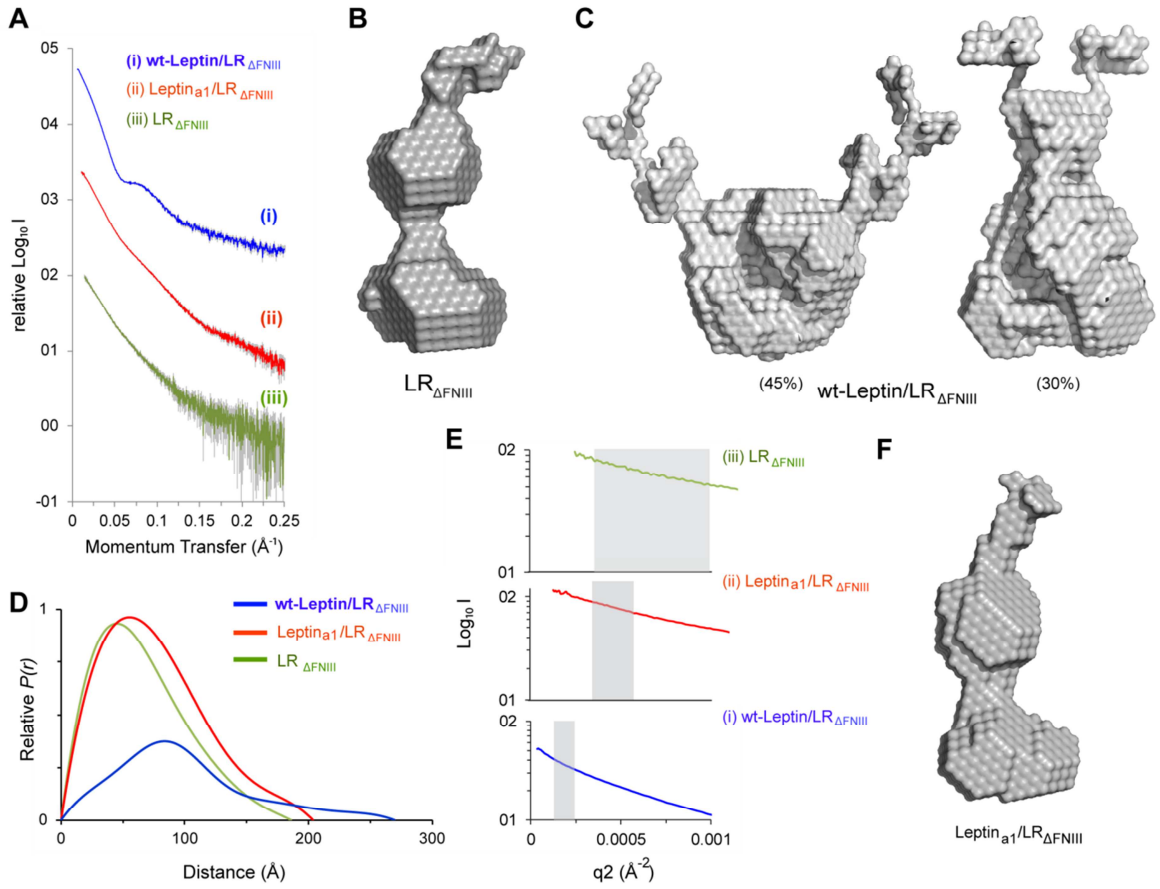

Figure 4.4. Small angle X-ray scattering analysis of $LR_{\Delta FNIII}$ and its complexes: ***(A)*** Relative plot of log of scattering intensity vs. momentum transfer for $LR_{\Delta FNIII}$ (*green*), $Leptin_{a1}/LR_{\Delta FNIII}$ binary complex (*red*) and wt-Leptin/$LR_{\Delta FNIII}$ quaternary complex (*blue*); ***(B)*** Reference *ab initio* model among 20 *DAMMIF* models generated from scattering data of $LR_{\Delta FNIII}$; ***(C)*** Cluster centers from two clusters produced by *DAMCLUST* from 20 *ab initio* models generated using *DAMMIF* from scattering data of wt-Leptin/$LR_{\Delta FNIII}$ complex; ***(D)*** Distance distribution plot *P(r)* for $LR_{\Delta FNIII}$ (*green*), $Leptin_{a1}/LR_{\Delta FNIII}$ binary complex (*red*) and wt-Leptin/$LR_{\Delta FNIII}$ quaternary complex (*blue*); ***(E)*** Guinier region showing linearity at lower angle for $LR_{\Delta FNIII}$ (*green*), $Leptin_{a1}/LR_{\Delta FNIII}$ binary complex (*red*) and wt-Leptin/$LR_{\Delta FNIII}$ quaternary complex (*blue*); ***(F)*** Reference *ab intio* model among 20 *DAMMIF* models generated from scattering data of $Leptin_{a1}/LR_{\Delta FNIII}$ binary complex.

of the full length extracellular part of LR in complex with both wild type and antagonist leptins have described in chapter 3. Here similar SAXS analysis for the $LR_{\Delta FNIII}$ and its complexes hasbeen carried out. The molecular weight determined from zero intensity extrapolation of SAXS curve supports a quaternary complex, thus proving the redundant role of FNIII in leptininduced LR oligomerization. SAXS based molecular weight estimation of $LR_{\Delta FNIII}$/antagonist leptin suggests a binary complex and supports our earlier observation of antagonist Leptin forming only binary complex with full length LR_{ecto}. The striking similarity of the *ab inito* models of wt-Leptin/$LR_{\Delta FNIII}$ quaternary complex with the distal part of wt-Leptin/$LR_{\Delta FNIII}$ complex validates the structural paradigm of Leptin induced LR architecture. The existence of two different clusters in the *ab initio* models of wt-Leptin/$LR_{\Delta FNIII}$ suggests some degree of flexibility exist in the solution structure. But both cluster centers have similar features: a compact broad base formed by the CRH2-Leptin-Ig domains and two CRH1 arms extending from it. *Ab initio* models of both $LR_{\Delta FNIII}$ receptor alone and its binary complex with antagonist Leptin are compatible with the corresponding models with full length LR_{ecto} generated earlier.

In summary, we showed that FNIII domains, though essential for leptin mediated receptor activation, has no role to play in Leptin induced receptor dimerization, thereby establishing a distinct and non-overlapping division of roles played by individual LR_{ecto} domains. We hypothesize that FNIII domain although plays such redundant role in agonist-induced dimerization, its inevitability would be arising from its potential role in channelizing the conformational change upon leptin binding to the intracellular part of the receptor.

4.5 MATERIALS AND METHODS

Production of $LR_{\Delta FNIII}$: In order for expression of $LR_{\Delta FNIII}$ variant containing CRH1-Ig-CRH2 motif of extracellular part of murine Leptin receptor (LR_{ecto}), the pMET7 vector carrying the corresponding ORF from residue 1 to 634 of LR_{ecto} and a C-terminal hexa-His. HEK-293T cells, grown in Roller bottles till 90% confluency, was transiently transfected with 250µg vector per roller bottle (850cm^2) using Polyethyleneimine (1DNA:1.5 Polyethyleneimine molar ratio) and was further cultured in DMEM medium containing Penicillin and Streptomycin in the presence of Kifunensine at 37° C in a 5% CO_2 environment for 4 days. The medium was harvested, centrifuged at 10,000g for 30 minutes to pellet down the cell debris and the supernatant was loaded onto a TALON^{TM} IMAC resin (Clontech) column at 5ml/min. The non-specifically bound proteins to matrix was removed by washing with 4mM Imidazole. Protein of

interest was eluted with 300mM imidazole, concentrated and further resolved on a size exclusion column of Superdex 200 HiLoad 16/600 column (GE HealthCare) prequillibrated with 25mM HEPES (pH 7.4) containing 100mM Sodium Chloride. $LR_{\Delta FNIII}$ eluted as a single peak; the peak fractions were pooled and the purity was assessed by SDS PAGE.

Production of wild type and antagonist Leptins: Similar method was followed as mentioned in chapter 3 "*Materials and methods*" section.

Isolation of complexes of $LR_{\Delta FNIII}$ with Leptin variants: Purified $LR_{\Delta FNIII}$ at 5-7 mg/ml was incubated with 4 times molar excess of wild type or antagonist leptin for 30-60 minutes and then resolved on a size exclusion column SD200 HiLoad 16/600 (GE HealthCare), pre-equilibrated with 25mM HEPES (pH=7.4) containing 100mM Sodium Chloride. The major peak containing monomeric receptor was pooled, concentrated and stored at -80ºC after flash freezing in liquid nitrogen.

Isothermal titration calorimetry: The kinetic and thermodynamic parameters of $LR_{\Delta FNIII}$ interaction with wt-Leptin and $Leptin_{a1}$ were determined by Isothermal Titration Calorimetry. All the protein components were purified in 25mM HEPES (pH=7.4) containing 100mM Sodium Chloride and degased immediately before titration. 20µM of wt-Leptin was titrated against 1.4ml of 1µM $LR_{\Delta FNIII}$ with first discrete injection of 3µl followed by 10µl each at 37 ºC while continuously mixing at 307 RPM stirring speed. The heat released was recorded using a MicroCal VP-ITC set up (GE Healthcare) and analyzed using Origin (version). The best fitting of the experimental data was obtained using built-in "one-set of sites" curve fitting model in Origin and this fit was used to determine the equilibrium constant ($1/K_d$), stoichiometry of interaction (N), enthalpy change (ΔH). The change in Gibbs free energy (ΔG) was calculated using the equation $\Delta G = -RT\ ln\ (1/K_d)$ and the entropy change (ΔS) was back calculated using $\Delta G = \Delta H - T\Delta S$. Similar experiments were performed for $Leptin_{a1}$ to characterize their interaction with $LR_{\Delta FNIII}$.

Small angle X-ray scattering: Following steps were followed in order for SAXS analysis.

a) *Data collection and primary data reduction*: SAXS data was obtained at the beamline BioSAXS station (ID14-3) of ESRF (Grenoble) with a robotic sample changer. The protein samples of $LR_{\Delta FNIII}$, its binary complex with $Leptin_{a1}$ and quaternary complex with wt-leptin were measured in bulk mode with at least 3 different concentrations in the range of 1.0-7.3 mg/ml. Monochromatic X-ray beam of 1.54 Å was scattered through these monodispersing samples at 293 K were recorded covering the momentum transfer range 0.01 $Å^{-1} < s < 0.6\ Å^{-1}$, where $s=4\pi\sin\theta/\lambda$ (2θ=scattering angle, λ=wavelength of incident X-ray). In this bulk mode measurement, absence of X-ray induced radiation damage was confirmed by alignment of 10 two dimensional scattering curves obtained from integration of continuous radial three dimensional scattering data obtained from 10 independent X-ray scattering measurements with 10 second exposure for each sample. For each concentration set, the scattering data was normalized for beam intensity, exposure time and corrected for the detector response. Subtraction of buffer scattering from the sample scattering using PRIMUS (Konarev et al.

2003) eliminated both buffer background as well as parasitic background arising from instrument hardware. A concentration dependency was reproducibly observed for wt-Leptin/$LR_{\Delta FNIII}$ complex scattering, possibly due to covalent clustering that has been observed for this scatterer at higher concentration; thus two concentrations of 0.8 and 1 mg/ml was finally used for further analysis for this complex. The forward scattering (I_0) and radius of gyration (Rg) was determined in PRIMUS from infinite dilution extrapolation based on guinier approximation and compared with corresponding estimates from Gnom (Svergun ,1992) calculated by indirect fourier transformation of the complete one dimensional scattering data. The distance distribution function P(r) and maximal dimension Dmax for each scatterer was evaluated using Gnom. Molecular masses were calculated based on the forward scattering from a calibrated sample viz. bovine serum albumin (ESRF) or water (Soleil). Molecular mass based on porod volume was estimated using Autoporod (Petoukhov MV et al., 2012) and compared with corresponding estimates from Guinier approximation.

b) Ab initio modeling: Ab initio three dimensional shape reconstruction from one dimensional scattering data was achieved using *DAMMIF* (Franke, 2009). In order to avoid stochastic error while modeling, 20 models were generated by 20 independent runs for each scatterer type. Uniqueness of these ab initio shapes were evaluated using *DAMAVER* (Volkov et al., 2003). When the average normalized spatial discrepancy (NSD) value for the 20 models was found to be less than or equal to 1, the set was considered as single cluster and the model having lowest NSD average value with respect to other 19 models was considered as the representative for the scatterer type. Unlike the receptor and its complex with antagonist Leptin, the *DAMMIF* models of wt-Leptin/$LR_{\Delta FNIII}$ complex, upon visual inspection using molecular viewer PyMol (De*Lano Scientific)* showed variability in the models, possibly due to flexibility in this system. So, these models with multimodal distribution were clustered using *DAMCLUST* and the cluster centers were considered as the representative conformers of the wt-Leptin/$LR_{\Delta FNIII}$ quaternary complex. Qualitative evaluation of these models were done by analyzing the chi square values against the respective experimental scattering curves.

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4.7 SUPPLEMENTARY FIGURE

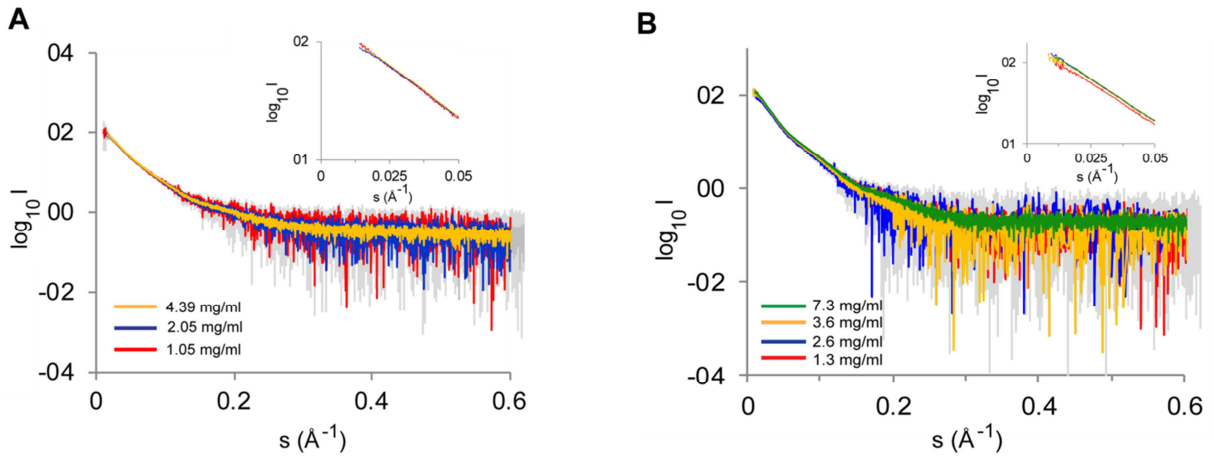

Figure 4.S1. Small angle X-ray scattering intensity vs. scattering angle: ***(A)*** Plot of log of intensity of buffer subtracted scattering intensity from $LR_{\Delta FNIII}$ against momentum transfer (*s*) at three different concentrations. *inset:* Similar log of intensity vs. momentum transfer (*s*) at very low angle showing scattering intensity is independent of concentration of receptor at both lower and higher angles; (***B)*** Similar plot for $Leptin_{a1}/LR_{\Delta FNIII}$ binary complex at four different concentrations. *inset:* Similar log of intensity vs. momentum transfer (*s*) at very low angle showing absence of concentration dependency.

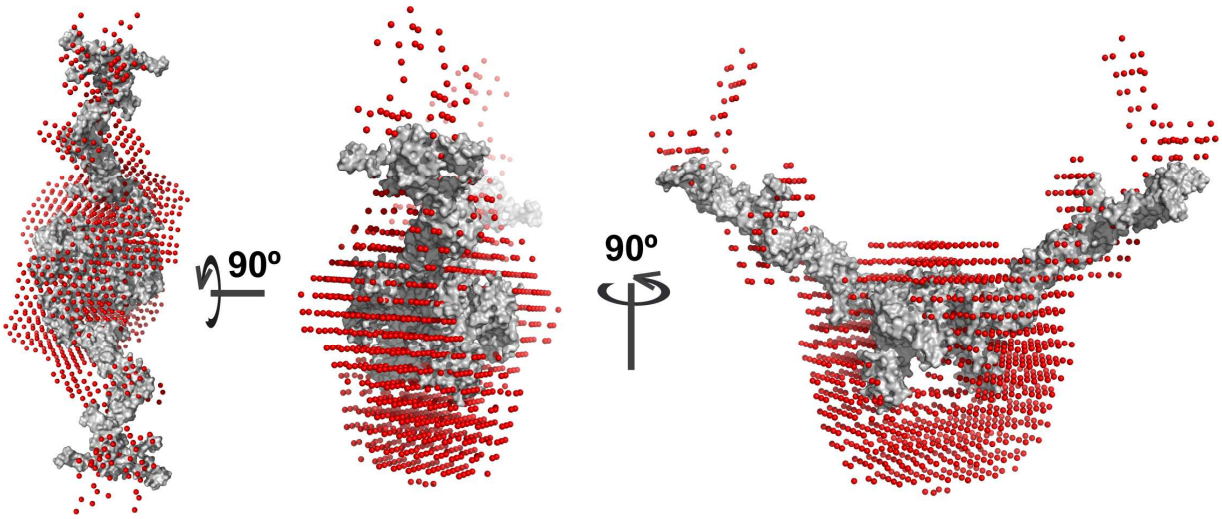

Figure 4.S2. *Ab initio* model of $LR_{\Delta FNIII}$ compared to the rigid body model from LR_{ecto}: After the removal of FNIII domains, the best rigid body model (chi 2.2) obtained from the SAXS analysis of wt-Leptin/LR_{ecto} quaternary complex (*surface view, grey*) was compared with the *ab initio* model of wt-Leptin/$LR_{\Delta FNIII}$ quaternary complex (*sphere view, red*).

Table 4.S1: SAXS analysis of the $LR_{\Delta FNIII}$, $Leptin_{a1}/LR_{\Delta FNIII}$ binary and wt-Leptin/$LR_{\Delta FNIII}$ quaternary complex

Data collection parameters	$LR_{\Delta FNIII}$	$Leptin_{a1}/LR_{\Delta FNIII}$	wt-Leptin/$LR_{\Delta FNIII}$
Beamline	ID 14-3, ESRF	ID 14-3, ESRF	ID 14-3, ESRF
Detector	PILATUS 1M	PILATUS 1M	PILATUS 1M
Beam Geometry	0.7 x 0.7 mm^2	0.7 x 0.7 mm^2	0.7 x 0.7 mm2
Wavelenght (Å)	0.931	0.931	0.931
Q range ($Å^{-1}$)	0.15–6.110	0.15–6.110	0.15–6.110
Exposure Time (sec)	100 (10 x 10)	100 (10 x 10)	100 (10 x 10)
Concentration range	0.9 - 3	0.9 - 2.7	0.9 - 2.7
Temperature (K)	293	293	293
Structural			
I(0) ($Å^{-1}$) from P(r)	103.8 ± 3.1	115.8±9.5	52.2±0.1
Rg (Å) from P(r)	50.5±2.0	58.0±1.5	83.9±0.2
I(0) ($Å^{-1}$) from Guinier	108.9±8.3	120.6±8.9	55.9±0.3
Rg (Å) (from Guinier)	51.8±4.5	58.2±1.4	84.2±2.8
Dmax (Å)	165.3±3.9	189.5±12.9	---
Porod volume	185087	300847	1442936
Excluded volume,	270000	546000	1380000
Molecular Mass (kDa)			
From I(0)	87.8	97.9	230.9
From SaxsMOW	88.2	120.5	191.9
From Porod volume	125.2	208.8	901.8
From excl. volume	127.6	321.1	811.7
Peptide (Protparam)	69.8	85.8	171.6
Modeling			
Shape reconstruction	*DAMMIF*	*DAMMIF*	*DAMMIF*
Symmetry	P1	P1	P2
χ^2 of reference model	0.7	1.5	1.3
# of models	20	20	20
DAMAVER NSD (var)	0.5±0.02	0.6±0.09	1.4±0.1
Rigid body modelling	*SASREF*	*SASREF*	*SASREF*
Initial χ^2	**---**	**---**	**---**
Final χ^2	**---**	**---**	**---**
Software/Server			
Data Reduction	*BsxCuBE*	*BsxCuBE*	Foxtrot
Data Processing	PRIMUS	PRIMUS	PrimusQt
Data Evaluation	PRIMUS, Gnom	PRIMUS, Gnom	PrimusQt, Gnom
Structure Modeling	Dammif	Dammif	Dammif, Damclust
Molecular	PyMOL	PyMOL	PyMOL

5

Crystallization of murine Leptin Receptor and its complexes with wild type and antagonist Leptin

5.1 INTRODUCTION

Protein X-ray crystallography is one of the paramount methods for the structural characterization of proteins at atomic resolution. While electron microscopy and small angle x-ray scattering are limited by their low resolution, crystallographic structures can provide immense information at atomic scale enabling understanding of molecular mechanisms and facilitating intelligent designing of novel agonistic and antagonistic drug molecules.

In Leptin receptor biology, only the crystal structure of the Leptin binding domain has been solved in complex with a competitive Fab fragment (Carpenter et al., 2012). As the structure of receptor or its subdomains have not been characterized structurally in isolation or with its cognate ligand, we decided to set up crystallization trials to obtain diffraction-grade crystals. Crystallization trials of full length LR_{ecto} and $LR_{\Delta FNIII}$ and corresponding results have already been described in Section 3 and 4, respectively. As these constructs did not yield any crystal hits, we have included smaller constructs to increase the probability of getting crystallization hit. The strategies which has been followed to increase the yield and quality of proteins have been described along with results of crystallization trials.

5.2 STABLE CELL LINE PREPARATION

5.2.1 INTRODUCTION

We have used transient transfection of HEK293T cell line for most of the protein production work described earlier. HEK-293 cell line has been a popular protein production platform due to easy handling requirement and efficient transfectability. HEK293T, a variant of original HEK-293, stably expresses hexameric Simian Vacuolating virus 40 (SV40) large T-antigen which can bind SV40 enhancers and increase the protein expression levels. In spite of such optimized system for heterologous expression, HEK-293T system presents specific difficulties for production of glycosylated proteins for the purpose of crystallization. Such glycoproteins due to their inherent complexity pertaining to flexible glycans, pose great challenge in protein crystallization. Glycosylation is imperative for the correct folding and thus Asn residues cannot be substituted without affecting the structural integrity of these proteins. Leptin receptor is one of the highly glycosylated receptor with 18 n-linked glycosylation sites as shown by mass spectrometry of human LR_{ecto} (Haniu et al., 1998). Differential occupancy of different glycosylation sites as well as different chain lengths of the glycans results in very high degree of inhomogeneity in the protein preparation. In this regard, use of glycosylation inhibitors or expression in HEK293S GnTI$^{-/-}$ cell line has been shown to improve the protein homogeneity remarkably (Chang et al., 2007). We have used Kifunensine, the alpha-mannosidase I inhibitor, that results in N-glycans formed of oligomannose moieties only, $Man_{5-9}GlcNAc_2$ (Fig. 5.1.1). Even though complex type glycans were prevented, differential occupancy has produced heterogeneity confirmed by closely running multiple bands of the receptor on SDS PAGE analysis (Fig. 5.1.2). Random mutagenesis based approach using ethyl methanesulfonate as mutagenic agent, a ricin resistant HEK-293 cell line has been created (Reeves et al., 2002). This variant called HEK-293S cell line lacks N-acetylglucosaminyltransferase I (so GnTI$^{-/-}$) activity and is unable to glycosylate proteins using complex N-glycans. MALDI-TOF based mass spectrometric analysis has shown a very high degree of homogeneity in glycosylation when HEK 293S GnTI$^{-/-}$ cell line was used (Chang et al., 2007) (Fig. 5.2.1). So we decided to try this system to get protein preparation with highest homogeneity for the purpose of crystallization trial.

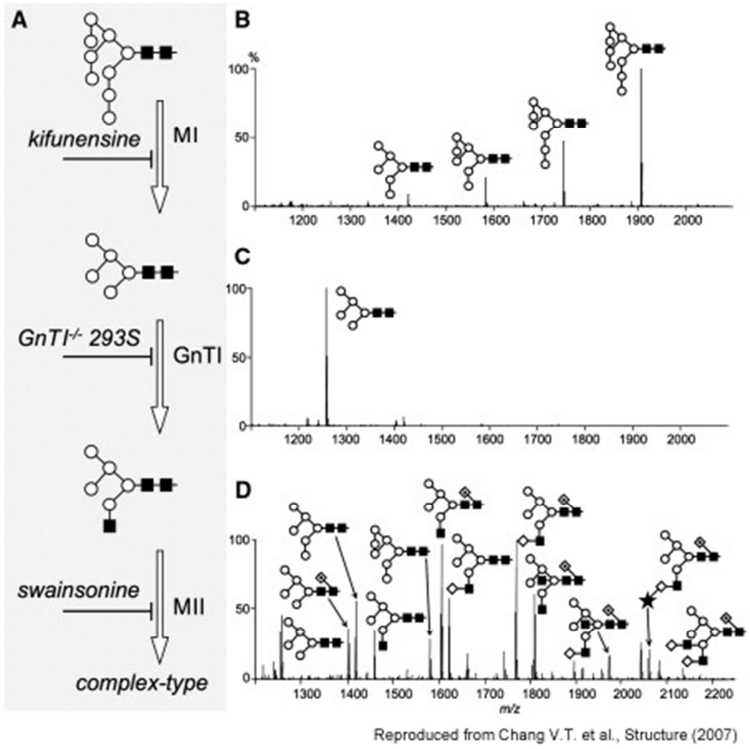

Figure 5.2.1. Manipulation of the Mammalian *N*-Linked Glycosylation Pathway in HEK293 Cells: ***(A)*** A section of the glycosylation pathway is illustrated.***(B–D)*** MALDI-TOF analysis of the glycans of the target glycoprotein (s19A) expressed in (B) HEK293T cells in the presence of the α-mannosidase I (MI) inhibitor, kifunensine, at 5 μM, resulting in the addition of oligomannose-type *N*-glycans, $Man_{5–9}GlcNAc_2$; (C) ricin-resistant HEK293S cells devoid of GnTI activity, resulting in predominantly $Man_5GlcNAc_2$*N*-glycans; and (D) HEK293T cells in the presence of the α-mannosidase II (MII) inhibitor, swainsonine, at 20 μM, resulting in the addition of hybrid-type glycans. For MALDI-TOF MS, glycans were released directly from SDS-PAGE gel bands by overnight PNGase F digestion. Monosaccharide constituents are represented as follows: open diamonds, Gal; closed diamonds, GaINAc; open squares, Glc; closed squares, GlcNAc; open circles, Man; stars, sialic acid; dotted diamonds, Fuc. (Adopted from Chang V.T. et al., Structure, 2007)

Protein production using transient expression in mammalian cell culture systems involves the transfection of cells with vector DNA that remains in the cytoplasm and doesn't get incorporated in genome. Thus the vector does not propagate during cell division and lost within finite period of few days due to cell division or other causes. So this method of protein production by transient transfection requires repeated transfection utilizing large scale DNA preparation; thus

time and cost effective. Although numerous rapid and cost effective transfection expression methods have been devised recently (Aricescu et al., 2006; Durocher et al., 2002; Meissner et al., 2001), transient transfection requires time consuming large scale DNA preparation and continued growth and maintenance of cell lines prior to transfection. Automation of maintenance, transient transfection of HEK293T and HEK-293S cell line and protein production has been achieved (Zhao et al., 2011), but availability of such platform has been the limiting factor. On the contrary, stable expression involves one time transfection followed by incorporation of vector into the genomic DNA and propagation to daughter cells during cell division. Such stably transfected cells can be stored in liquid nitrogen and propagated repeatedly. In order to minimize the time, effort and cost of protein production stable transfection was preferred over transient cell line using HEK-293S GnTI$^{-/-}$ cell line.

5.2.2 MATERIALS AND METHODS

Generation of stable cell lines: HEK-293S GnTI$^{-/-}$ were grown in DMEM medium containing Glutamine, Penicillin and Streptomycin until 50-60% confluency in 75 cm^2 falcons. Plasmid constructs of pCDNA-4.0 carrying $LR_{\Delta FNIII}$ and $LR_{CRH2\text{-}Ig}$ were subjected to single digestion using PvuI restriction enzyme by incubating for 4 hr at 37° C and confirmed using Agarose Gel electrophoresis before PCR purification (Qiagen PCR purification Kit). Before transfection, old DMEM medium was replaced with new medium and transfection was carried out using standard Calcium phosphate transfection protocol. After six hours of transfection, medium was replaced with new DMEM medium containing Zeocin, which act as selection agent. In order to achieve monoclonal cell lines, once the transfected cells were grown till 60-70% confluency, cells were detached using EDTA/Trypsin and grown in four new 175 cm^2 falcons in the presence of selection agent Zeocine upon following dilutions: 1/5, 1/10, 1/20 and 1/40. After about two weeks of growth with repeated renewal of medium in 3-4 days interval, falcons were cut open and each individual isolated colonies were blotted onto sterile blotting paper and inoculated into 24-well plates, resuspended and grown until 90% confluency. These monoclonal cell lines were transferred to 75 cm^2 falcons and grown till 90% confluency, the expression was induced using tetracyclin and the expression levels were quantified by Western blot using anti-His tag antibody. A part of cells were stored in liquid nitrogen for future use.

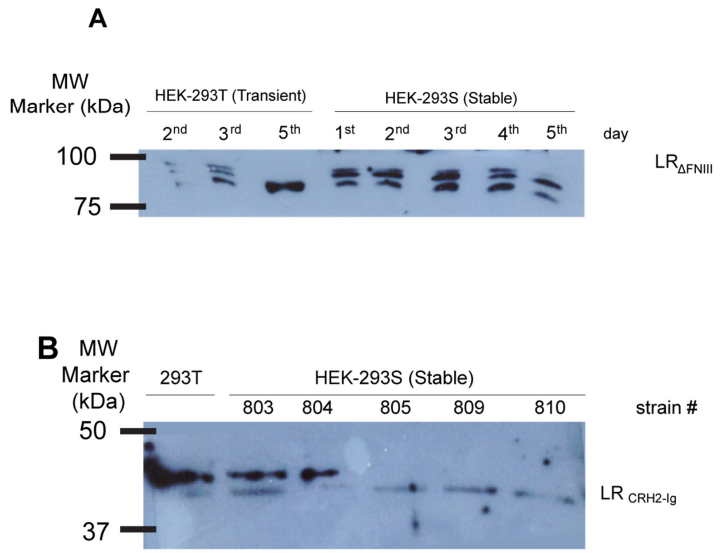

Figure 5.2.2. Transient vs. stable expression of $LR_{\Delta FNIII}$ and $LR_{CRH2\text{-}Ig}$: ***(A)*** Western blot of pCDNA-4/m$LR_{\Delta FNIII}$ using anti-His tag antibody: From left to right, transiently expressed receptor in HEK-293T cells 2nd day, 3rd day, 5th day after transfection, then same construct expressing stably in HEK-293S cell line during 5 days after Tetracyclin induction; ***(B)*** Western blot of pCDNA-4/ $LR_{CRH2\text{-}Ig}$ using anti-His tag antibody: From left to right, transiently expressed receptor in HEK-293T cell line, stably expressing receptor from five monoclonal strains of HEK-293S cells numbered 803, 804, 805, 809 and 810. All the samples are after 3 days of transfection (in case of transient expression) or after 3 days of induction (in case of stable expression).

5.2.3 RESULTS

Comparison of yields from transient vs. stable expression: In case of pCDNA-4/$LR_{\Delta FNIII}$ construct, the yields from transient expression in HEK-293T cell lines did not improve much when stably expressed in HEK-293S cell line (Figure 5.2.1). Also the limited glycosylation in HEK-293S cell line has limited effect on the amount of sugars, as both 293T and 293S expressed receptor run at similar height on SDS-PAGE as detected by Western blotting using anti-His tag. In case of pCDNA-4/$LR_{CRH2\text{-}Ig}$ construct, the yields from stably transfected HEK-293S cells were lower compared to the transient expression in HEK-293T cell line. But in this case there is conspicuous reduction in molecular weight of the receptor due to limited glycosylation in HEK-293S cell line. $LR_{\Delta FNIII}$ produced in HEK-293S ($GnTI^{-/-}$) was further being used for crystallization trial, but without any successful hit so far.

5.3 YIELD OPTIMIZATION OF $LR_{CRH2-Ig}$ and LR_{CRH2}

5.3.1 INTRODUCTION

$LR_{CRH2-Ig}$ construct was initially cloned in pMET7 and the yield was relatively low for crystallization trials. As CRH2 is the leptin binding domain of LR and Ig domain is essential for Leptin-site III mediated oligomerization and activation, this construct would be highly crucial for both structural and functional study. Theoretically, CRH2-Ig should be the minimal unit required for a leptin induced higher order complex. While structural characterization of this quaternary complex would be relatively favorable due to absence of more flexible CRH1 and FNIII domains, the crystal structure will be sufficiently informative about the binding interface. Based on these rational, four CRH2-Ig constructs differing from each other in their N and C-termini were designed.

CRH2 has been shown to be the leptin binding domain. But there was no structural information available of the CRH2 alone or in complex with Leptin, until recently the structure was solved for the complex of CRH2 expressed in *E.coli* with a Fab fragment. We decided to include the CRH2 for optimization of expression by varying the N- and C- termini. Two constructs of CRH2 were planned for construction.

Our earlier plasmids were based on pMET7 vectors utilizing the native secretion signal. We decided to PCR amplify CRH2 and CRH2-Ig fragments with different termini from LR_{ecto} and clone them in pcDNA^{TM}4/TO vector (Invitrogen). This vector has a tetracycline-regulated expression system without any viral transactivator, thus produces higher yield than other regulated mammalian expression system. A highly efficient secretion signal from pHLSec (Aricescu et al., 2006) was fused to the n-terminus of the insert and then ligated to pcDNA^{TM}4/TO.

5.3.2 MATERIALS AND METHODS

Following primers were used for the PCR amplification of CRH2-Ig and CRH2 gene from the pMET7-LR_{ecto} plasmid.

Name	Description	Sequence*
LR_001	Forward Primer for CRH2-Ig	GAA ACC GGT ATT CTG ACT AGT GTT G
LR_002		GAA ACC GGT GTT GTG TAT TTT CCA C
LR_003	Forward Primer for CRH2	GAA ACC GGT GTC AAT ATC AAT ATA TC
LR_004	Reverse Primer for CRH2-Ig and CRH2	G CTT GGT ACC CCA AAA TTC AGG CCC TC
LR_005		G CTT GGT ACC TAC ATC CAT GAC AAG CGT ATA G

*The part of the sequence in yellow will be lost after RE digestion.

Using all possible combinations of forward and reverse primer, total 6 (4 CRH2-Ig and 2 CRH2) fragments were PCR amplified. The termini were digested using KpnI and AgeI restriction enzymes and cloned into predigested pcDNA^{TM}4/TO vector. The cloned vectors were first verified by restriction digestion and then by plasmid sequencing. The cloned vectors were named as LR-14, LR-15, LR-24 and so on based on the forward and reverse primer used.

Small scale expression test was carried out by transfecting each vector to HEK-293T cells grown in 6 well plates either in the presence or absence of Tetracycline, analyzing the supernatant after 3 days by western blot and detecting by anti-His tag antibody.

5.3.3 RESULTS

One out of total four pHLsec:CRH2-Ig constructs analyzed, showed drastic increase in the expression level compared to that of pMET7 vector. All two pHLsec:CRH2 constructs showed higher yield compared to the previous pMET7 variant (Figure 5.3.1). These constructs were used for crystallization trials of these receptor variants.

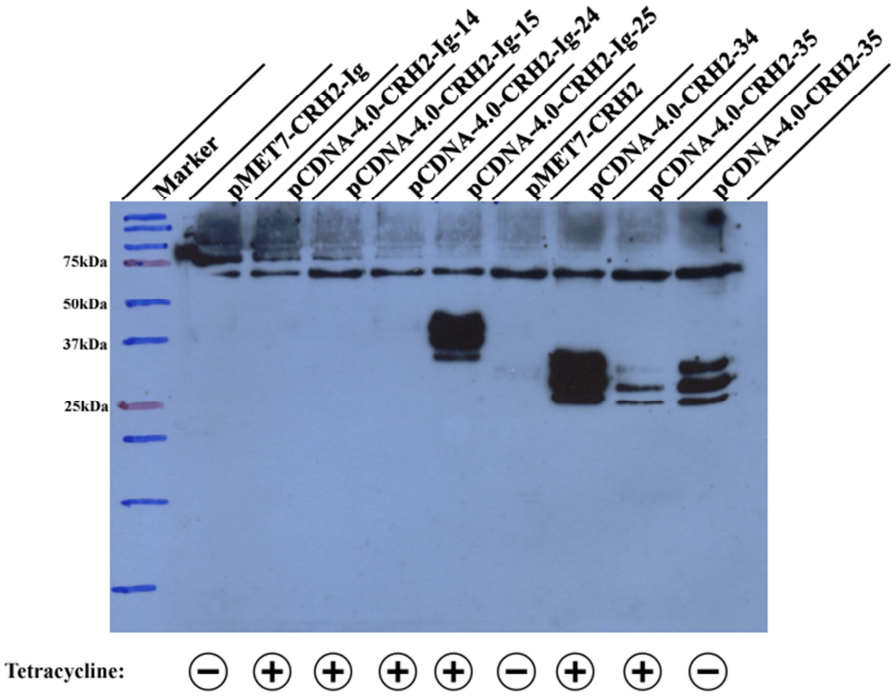

Figure 5.3.1. Western blot from mini-expression test of pCDNA constructs: Mini-expression test in order to quantify the expression levels of different constructs in HEK-293 cell was done by transient transfection followed by western bloting using anti-His_6 antibody.

5.3.4 DISCUSSION

Higher yield of CRH2-Ig and CRH2 will enable the studies of this receptor variants esp. by protein crystallization for X-ray crystallography. Though it would be difficult to explain the exact mechanism behind this increased expression, most scientific reason would be the role of the termini in determining the stability of the receptor both inside cell and outside in the DMEM medium. In case of CRH2-Ig construct, the increase in the yield is very drastic and suggests the domain border optimization is a crucial way to optimize the yield of multi-domain proteins.

5.4 CRYSTALLIZATION TRIAL OF LR_{CRH2}

5.4.1 INTRODUCTION

The open reading frame for CRH2 domain of murine LR has been cloned in pCDNA-4/TO vector with the extrinsic secretion signal from pHLSec (Section 5.2). This domain represent the smallest unit with the leptin binding potential. Thus structural characterization of this domain in complex with Leptin will provide important insights into the binding epitopes and will help in designing of antagonist drugs. However, in theory the absence of Ig-domain will disable the formation of a higher order quaternary complex. Recently, the crystal structure of human CRH2 domain, expressed in *Escherichia coli* has been solved in complex with a competitive Fab fragment (Carpenter et al., 2012). However, the structure of CRH2 domain alone or in complex with cognate ligand is not available yet. So we decided to purify, analyze and set up crystallization trials for the isolated murine CRH2 domain and its complex with wild type as well as antagonist leptins.

5.4.2 MATERIALS AND METHODS

Production of Leptin binding domain CRH2: HEK-293T cells, grown in 175cm^2 Falcons or 850 cm^2 Roller bottles till 90% confluency in DMEM medium containing Penicillin and Streptomycin, were transiently transfected with appropriate transfection agent in fresh DMEM medium containing Penicillin and Streptomycin in the presence of α-mannosidase inhibitor Kifunensine. The transfection agent was prepared by incubating pCDNA-4/TO-LR_{CRH2} plasmid with Polyethyleneimine (PEI) in a 1:1.5 molar ratio for at least 8 minutes at 37° C and a final quantity of 50µg of Plasmid DNA was used for cells grown on 175cm^2 surface area. After 4 days of transfection, the medium was harvested, centrifuged at 10,000g for 30 minutes to remove cell debris and 2 lt of this medium was loaded onto a 20 ml TALON Superflow column (Clontech) overnight. Non-specifically bound proteins to the talon resin was washed by 4mM Imidazole followed by elution of LR_{CRH2} using 300mM Imidazole. Further purification was done by resolving on a size exclusion SD200 column (GE Healthcare) equilibrated with 25mM HEPES (pH=7.4) containing 100mM Sodium Chloride. The eluted fractions were analyzed on SDS PAGE and fractions containing LR_{CRH2} were pooled and stored at -80°C after flash freezing in liquid Nitrogen.

Deglycosylation of LR_{CRH2}: In order to increase the homogeneity, receptor was concentrated to 1mg/ml and incubated with endoglycosidase H (New England Biolabs) enzyme overnight. After deglycosylation, endoglycosidase H was removed by gel filtration chromatography.

Acetylation of Free Cysteines: In order to prevent the non-physiological clustering of free cysteines in LR_{CRH2}, the fractions from size exclusion chromatography containing monomeric receptor were pooled and treated with 5mM Iodoacetamide for 1 hr at room temperature in ambient light. After the carboxymethylation was complete, excess of Iodoacetamide was removed by concentrating the mixture to 1 ml and resolving on a size exclusion column, pre-equillibrated with 25mM HEPES (pH=7.4) containing 100mM Sodium Chloride.

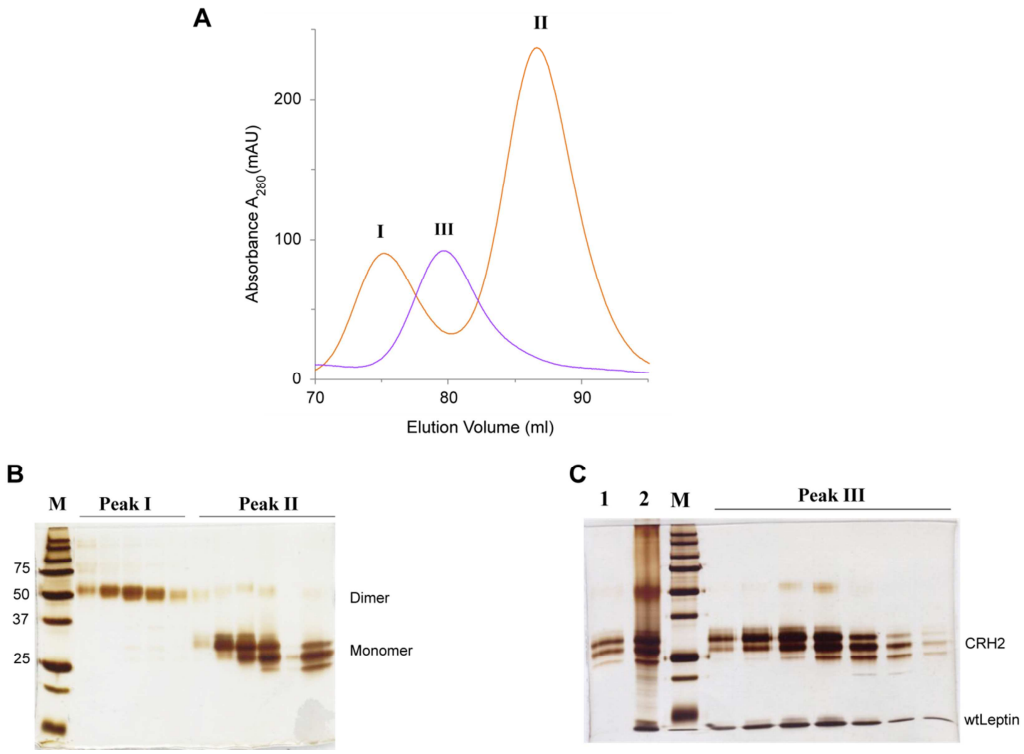

Figure 5.4.1. Production of LR_{CRH2}/Leptin Complex for crystallization: (A) Size exclusion chromatography of LR_{CRH2} after Talon IMAC purification produced two distinct peaks (*orange*) I and II; Complex of wt-Leptin and carboxymethylated LR_{CRH2} resolved on the size exclusion column (*purple*) producing peak III ; **(*B*)** Non-reducing SDS PAGE analysis of fractions from size exclusion chromatography of LR_{CRH2} shows peak 1 to be composed of disulfide linked dimers and peak 2 composed of monomeric CRH2; **(*C*)** Non-reducing SDS PAGE analysis of 1. carboxymethylated LR_{CRH2} before complex formation and gel filtration, 2. Pellet obtained during carboxymethylation with Iodoacetamide, Marker (M) and peak III eluted from size exclusion chromatography of binary complex obtained by incubating LR_{CRH2} incubated with wt-Leptin. Binary complexes of $leptin_{a1}$ and $leptin_{a2}$ have been prepared in similar procedure for crystallization (data not shown).

Isolation of complex of LR_{CRH2} with Leptin variants: Iodoacetamide-treated and deglycosylated receptor and each Leptin variant was concentrated to 5mg/ml and 10mg/ml separately. The receptor was then incubated with 6 times molar excess of Leptin variant for 30 minutes, followed by size exclusion chromatography. Fractions containing CRH2 and Leptin were pooled and used for crystallization trial without storing any further. Similar protocol was also followed with unperturbed receptor, only

Iodoacetamide treated receptor and Iodoacetamide untreated deglycosylated receptor in order to get corresponding complexes.

Crystallization Trial: LR_{CRH2} alone and its complexes with Leptin variants viz. wt-leptin, $leptin_{a1}$ and $leptin_{a2}$ were concentrated to ~ 5 mg/ml and 8 mg/ml and nearly 600 drops were set up with different crystallization conditions from various commercially available viz. Proplex (96) from Molecular Dimensions, Crystal Screen Lite (48), PEG ION 1 & 2 (96), Index Screen (96) and Crystal Screen 1 & 2 (96) from Hampton.

5.4.4 RESULTS

Purification of LR_{CRH2} and isolation of complexes: Size exclusion chromatography of LR_{CRH2} obtained from the elution from Talon IMAC purification, produced two peaks. Reducing and non-reducing SDS PAGE analysis of fractions obtained from size exclusion chromatography showed the composition of those two peaks: (i) The high molecular weight peak contains covalently clustered dimeric LR_{CRH2}; while (ii) The low molecular weight peak comprises monomeric LR_{CRH2} (Figure 5.4.1). The monomeric mLR_{EC}:CRH2, being the physiological form was used for all further analysis.Successful formation of binary complex of LR_{CRH2}/Leptin in 1:1 stoichiometric ratio was achieved when the iodoacetamide treated LR_{CRH2} was incubated with six molar excess of Leptin and resolved on size exclusion column. SDS PAGE analysis further confirmed the composition of the complex. Formaldehyde cross-linking suggested the complex to be a 1:1 complex.

Crystallization Trial: In spite of a very wide approach (~600 conditions) for the crystallization of LR_{CRH2} alone or in complex with different murine Leptin variants has failed. Majority of drops produced amorphous precipitate and brownish precipitate.

5.4.5 DISCUSSION

Crystallization trial of CRH2 domain has not been successful either in isolation or in complex with cognate ligand. The flexible sugar tree on the protein surface may increase the flexibility of this system and reduce homogeneity; but previous works have shown CRH2 domain alone expressed in bacterial host and thus not having any glycans attached not producing any crystal (Carpenter et al., 2012). Thus non-crystallizability of isolated CRH2 could be its inherent property. Similar reasons may exist for the non-crystallizability of its complexes with leptin variants.

5.5 CRYSTALLIZATION TRIAL OF $LR_{CRH2\text{-}Ig}$

5.5.1 INTRODUCTION

The crystal structure of human CRH2 domain in complex with a competitive antibody (PDB ID: 3V6O) is the only available structure of LR (Carpenter et al., 2012) till now. Our attempt to crystallize the leptin binding unit from murine leptin receptor was failed as described in 6.4 section. So we decided to attempt the crystallization trial of $LR_{CRH2\text{-}Ig}$ construct. The expression and yield of this construct has been optimized by subcloning into pCDNA plasmid with the optimized secretion signal from pHLSec plasmid (Section 6.2). The CRH2-Ig can contribute both CRH2-Leptin site II and Ig-Leptin site III interactions, thus has the potential to form the quaternary complex. Crystal structure of such quaternary complex will provide immense information about the binding and activation epitopes on Leptin and its cognate receptor enabling designing of novel antagonist and agonistic molecules with potential therapeutic use in various leptin related pathophysiology.

5.5.2 MATERIALS AND METHODS

Similar methods were followed as for LR_{CRH2} described earlier in 5.3.

5.5.3 RESULTS

Purification of $LR_{CRH2\text{-}Ig}$ and its complexes: IMAC using Talon resin followed by the gel filtration chromatography of eluted peak using Superdex 200 HiLoad 16/600, produced single symmetric peak (Fig. 5.5.1). Upon complex formation with excess of wt-Leptin with subsequent resolution on same gel filtration column showed the existence of Leptin induced higher order oligomerization of $LR_{CRH2\text{-}Ig}$. However, similar analysis for corresponding complex with $leptin_{a1}$ produced only a single peak formed by binary complex suggesting absence of any quaternary $leptin_{a1}/LR_{CRH2\text{-}Ig}$. This behavior supports our initial hypothesis that CRH2-Ig, indeed is the minimal subunit required for the leptin induced receptor oligomerization.

Crystallization Trial: For crystallization trial, $LR_{CRH2\text{-}Ig}$ after purification from gel filtration chromatography, has been carboxymethylated and complexed with $leptin_{a1}$. This

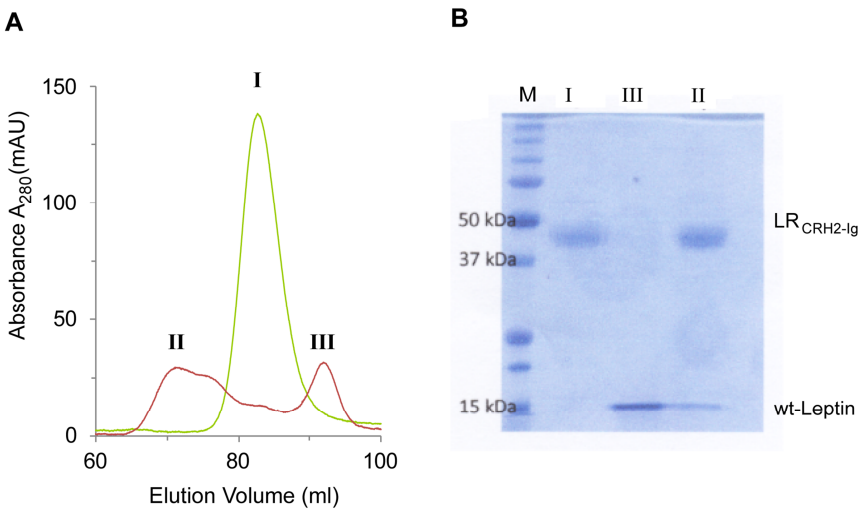

Figure 5.5.1. Purification and crystallization of mLR$_{EC}$:CRH2-Ig and its complexes: ***(A)*** Relative elution profile of mLR$_{EC}$:CRH2-Ig (*green*) and wt-Leptin/mLR$_{EC}$:CRH2-Ig complex (*brown*) ***(B)*** SDS-PAGE analysis of peak fractions I, II and III.

binary complex upon a large-scale crystallization trial involving ~600 conditions, has not produced any hits so far.

5.5.4 DISCUSSION

We were able to successfully express, purify the receptor construct LR$_{CRH2-Ig}$ and isolated its complex with wt- and leptin$_{a1}$. Interestingly, we showed that this receptor construct can form the quaternary complex with wt-Leptin, suggesting redundant role of CRH1 and FNIII domains in ligand induced oligomerization. Although this construct itself or in complex with leptin$_{a1}$ has not produced any crystallization hit in large scale crystallization trials yet, use of antibodies may improve the crystallizability of the receptor as has been observed in the case of Leptin binding domain (Carpenter et al., 2012).

5.6 CRYSTALLIZATION TRIAL OF LR_{ecto}

5.6.1 INTRODUCTION

The biochemical, biophysical and low resolution structural characterization of LR_{ecto} has already been described in chapter 3. This protein construct has also been subjected for extensive crystallization both alone, in complex with cognate ligand.

5.6.2 MATERIALS & METHODS

Similar procedures have been followed as described in Chapter 3 for the production of proteins and isolation of complexes for crystallization trial.

5.6.3 RESULTS AND DISCUSSION

Purification and isolation of LR_{ecto} and its complexes: LR_{ecto} purified from transiently expressing HEK293T cell line in the presence of Kifunensine upon incubation with 6 times molar excess of wild type murine Leptin (wt-Lep) expressed and purified from *E. coli*, for 30 min at room temperature followed by resolution on a Size exclusion column produced two incompletely resolved peaks: a minor high molecular weight (HMW) peak and a major low molecular weight (LMW) peak (Figure 5.6.1A). As the HMW complex is susceptible to dilution-dependent dissociation, this tetrameric complex has been stabilized by Formaldehyde mediated chemical crosslinking (Figure 5.6.2). The purity of LR_{ecto} and leptin has been assessed by SDS-PAGE after purification (Figure 5.6.1B).

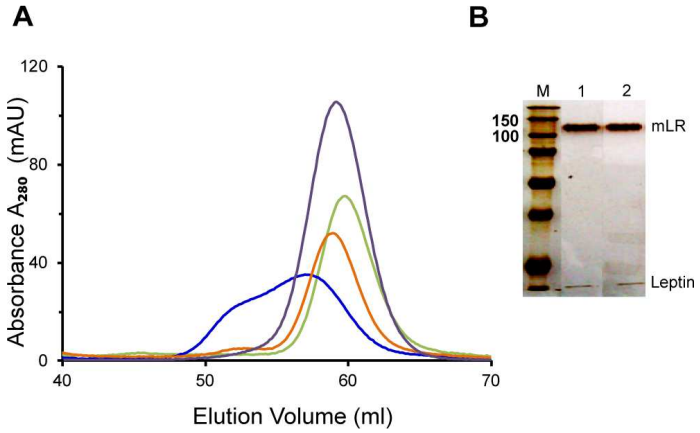

Figure 5.6.1. Isolation of LR_{ecto} and its complexes: (A) Size exclusion chromatography of LR_{ecto} (green), $leptin_{a1}/LR_{ecto}$ binary complex (orange), $leptin_{a2}/LR_{ecto}$ binary complex (violet) and wt-Leptin/LR_{ecto} complex. The wt-Leptin/LR_{ecto} elutes as a two incompletely resolved peaks corresponding to the high molecular quaternary complex and low molecular weight binary complex peaks. **(B)** The purity of the complex of wt-Leptin/LR_{ecto} corresponding to the high molecular weight peak has been assessed by SDS PAGE followed by silver staining. M:molecular weight marker; 1: with reducing agent 2-mercaptoethanol; 2: without reducing agent 2-Mercaptoethanol.

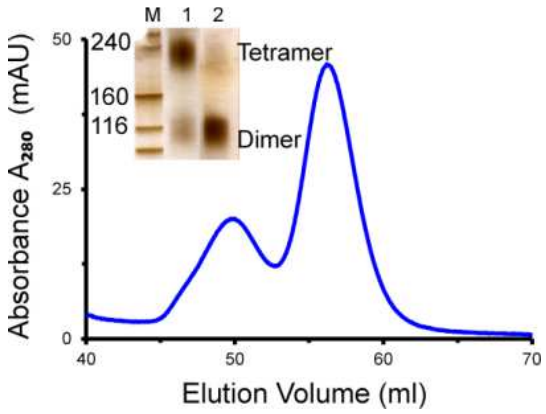

Figure 5.6.2. Chemical crosslinking of quaternary complex of wt-Leptin/LR_{ecto}: The high molecular peak fractions from gel filtration chromatography has been subjected to formaldehyde mediated crosslinking and cross-linked complex was resolved on size exclusion column, producing a high molecular weight and low molecular weight peaks. *Inset:* SDS PAGE analysis of the peak fractions in the presence of reducing agent suggests, successful crosslinking of the 1:1 binary and 2:2 quaternary complexes.

Crystallization Trial: Crystallization trial of unliganded LR_{ecto} with and without endoH mediated deglycosylation and their complexes with wt- and antagonist leptins have been largely unsuccessful. Most of the drops produced brown precipitate. LR_{ecto} treated with iodoacetamide to prevent non-physiological clustering has also been subjected with and without ligands; but no crystallization hit has been obtained yet.

5.7 REFERENCES

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6

Conclusion and Prospects

Ironically, the structural and mechanistic aspects of leptin mediated leptin receptor activation is still an enigma despite of the scientific endeavor during last two decade. Though such endeavor has expanded our knowledge beyond the role of this system in obesity by linking the leptin to multitude of pathophysiologies including autoimmune disease and breast cancer development, our quest surrounding the very first event initiated upon leptin binding to the

receptor and culminated with an active signaling complex is still a black box. The intrinsic complexity of leptin/leptin receptor system reflects from its structural similarity with a family of shared cytokine receptors called gp130 family and yet following an activation paradigm completely different from the members of this family. While gp130 family of receptors share the gp130 coreceptor, Leptin receptor signals on its own by homo-oligomerization. Although G-CSF/G-CSFR system shows the highest degree of sequence homology to this receptor, the existence of multiple non-signaling leptin receptor isoforms and their weak dominant negative repression on leptin receptor signaling indicating a complete novel receptor activation paradigm may underlie this system. Moreover, the lack of structural information on the LR-LR and LR-leptin interactions have obstructed our understanding of this complex system.

This doctoral research work begun in 2009 with an aim to characterize the leptin receptor extracellular domains using various structural biology and biophysical tools. Albeit, unavailability of any structural information about the extracellular part of the receptor and its complex with leptin at that time, provided the logical freedom for an unprejudiced approach, utilizing all the available structural biology tools like X-ray crystallography, small angle X-ray scattering and electron microscopy for simultaneous characterization of the receptor and its complexes. Availability of the antagonist leptin variants complimented this study by dichotomizing the high affinity binding from agonist-induced oligomerization and conformational changes inevitable for receptor activation. A plethora of biochemical, biophysical and structural biology tools deciphered many facets of this system, unexplored earlier.

Various biochemical experiments in conjunction with small angle X-ray scattering could able to show the underlying mechanism of leptin induced LR activation proceeds via the receptor oligomerization. On the contrary, the lack of such dimerizing ability was observed for the two antagonist leptins included for the study, confirming the role of $leptin_{site\text{-}III}/LR_{lg}$ interaction to be the principal driving force for the leptin induced oligomerization. Our observation of the absence of any receptor-receptor homotypic covalent interaction in the leptin induced higher-order complexes disproved a previous proposal of disulfide-linked signaling complex and disproved the proposed role of FNIII domain in such inter-receptor disulfide interaction. The intriguing observation of the monomeric nature of the receptor's complete extracellular part inspired to revisit the assessment of the proposed existence of disulfide clustered receptor dimers on membrane. Inclusion of iodoacetamide in the analysis of membrane receptors confirmed the absence of any disulfide bridge, conclusively suggesting the LR exists on membrane as non-covalent dimer. Truncation of FNIII domains which have been shown to be inevitable for

signaling, did not affect the ligand induced receptor oligomerization, suggesting their redundant role in oligomerization. The solution structure analysis both at high concentrations using small angle X-ray scattering and at low concentrations using negative stain electron microscopy, has provided immense information on the low resolution architecture of the primordial complexes formed at the cell surface upon ligand binding. This 2:2 quaternary complex generated using SAXS although lacks the basic necessity of the >2 receptor for the signaling complex formation, primed to propose an activation model which consolidates available biochemical and cellular paradigms of the signaling complex in literature. Outcome of above experiments together with the available information suggests towards a signaling competent four leptin receptor/four leptin model formed by leptin induced dimerization of the preformed inactive leptin receptor dimers.

Leptin receptor being a connecting link between the unshared cytokine receptors and the gp130 family of shared cytokine receptors, its unique mechanistic behavior may be the reflection of a novel mechanistic paradigm. Although current high resolution structure determination using X-ray crystallography has failed, use of non-competitive antibodies could be a boon for the structure determination of the leptin/leptin receptor complex, providing break-through understanding of the structure and mechanism of this system. Also further research work along the molecular understanding of the receptor activation will widen our horizon in leptin physiology as a pleiotropic hormone and its pathophysiology in autoimmune diseases and breast cancer.

KEDAR MOHARANA

Doctoral Student (Final yr.), L-ProBE, Ghent University

Curriculum Vitae

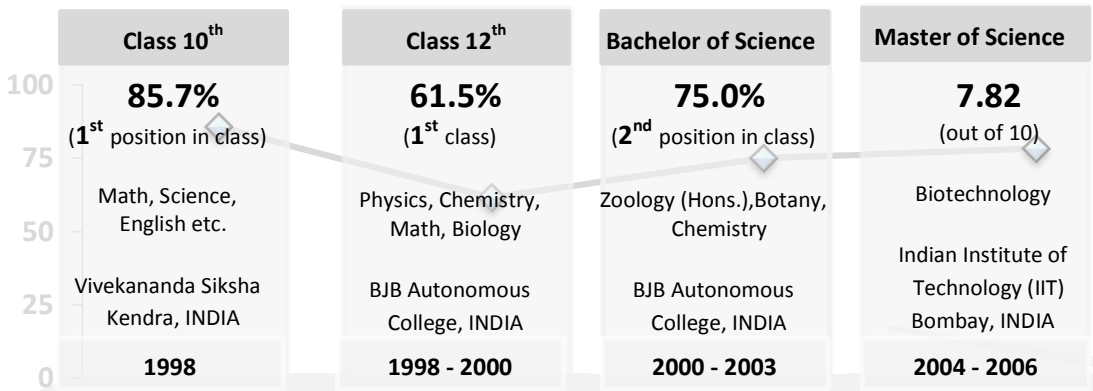
Personal Details

Affiliation	Lab of Protein Biochemistry & Biomolecular Engineering (L-ProBE) Ghent University, Ghent, Belgium 9000		
Phone	+32-486781580	Email	kedar.moharana@ugent.be
Nationality	Indian	Current Residence	Belgium
DOB	4th JUNE 1983	Marital Status	Unmarried

Research Experience

	Title:	Promoter:
Doctoral Study (2008-2013)	Mechanistic Paradigm of Leptin Receptor Activation: A putative supra-quaternary complex as the signaling model	**Prof. Savvas N. Savvides** L-ProBE, Ghent University Ghent, Belgium
Research Assistance (2006-2008)	ClpX – an AAA+ ATPase Component from Mycobacterium tuberculosis: Cloning, expression, purification and functional Characterization	**Dr. Janendra K. Batra** Immuno-Chemistry Lab National Institute of Immunology New Delhi, India
Master's Thesis (2005-2006)	Inferring fold patterns from sequences: How differential physico-chemical classification of amino acids influence structural motif discovery among Globins as a test case	**Prof. Y. U. Sasidhar** Protein Dynamics Lab Indian Institute of Technology (IIT) Bombay, India

Academic Qualification



Technical Skill Sets

Bacterial Protein expression, expression optimization, purification	7 years
Mammalian (HEK293) Protein expression (Transient & Stable), purification	4 years
Protein biochemical characterization, bioassay	7 years
Biophysical techniques (ITC, FRET, GFP-coupled unfoldase assay)	3 years
Multi-angle Light Scattering (MALS)	3 years
Small Angle X-ray Scattering	4 years
PERL Scripting	
Electron Microscopy (Grid Preparation, Image analysis using EMAN2)	
Protein crystallography	

Scientific Publications

1.**Structural and mechanistic paradigm of Leptin Receptor activation revealed by study of its complexes with wild type and antagonist leptins** [***Communicated***]

(Authors: Moharana, K., Zabeau, L., Peelman, F., Ringler, P., Stahlberg, H., Tavernier, J., Savvides, S. N.)

Abstract: The adipocyte-derived cytokine leptin signals via its cognate receptor (LR) to serve as a metabolic switch, thereby regulating body weight and processes with a high metabolic cost such as reproduction and immune responses. The downside of such benevolent pleiotropy has been the possible role of leptin-mediated signaling in autoimmune diseases and breast cancer, which has raised interest in the targeted antagonism of leptin signaling. We here present comparative biochemical and structural studies of the ectodomain of LR (LR_{ecto}) in complex with wild type and antagonist leptin variants to reveal that the basic structural assembly leading to leptin signaling is a 2:2 stoichiometric complex. The quaternary complex is mediated by high-affinity binding of leptin to the CRH2 junction of LR_{ecto}, which is devoid of dimerization propensity, to elicit conformational changes that enable additional interactions with the Ig domain of a second leptin-bound LR_{ecto}. In contrast, antagonist leptin variants carrying mutations at the epitope interacting with LR_{Ig} are only able to establish high affinity 1:1 complexes with LR. Acetylation of free cysteines in LR_{ecto} also abrogates quaternary complexes with leptin suggesting a role for the formation of intra-receptor disulfides upon receptor activation. Together, our studies point to an assembly mechanism at the cell-surface whereby leptin recruits pre-dimerized LR molecules to form a higher-order complex obeying a 4:4 stoichiometry. This mechanistic proposal consolidates a large body of prior studies and establishes a new conceptual framework for future studies of leptin-mediated signaling.

2.Redundant role of FNIII domains in Leptin induced Leptin Receptor oligomerization [***In Preparation***]

(Authors: Moharana, K., Zabeau, L., Peelman, F., Tavernier, J., Savvides, S. N.)

Abstract: Despite two decades of studies on the (patho)physiology of leptin-mediated Leptin receptor (LR activation) our current understanding of the leptin-LR signaling assembly is yet to come to full circle with respect to a mechanistic consensus. As we have previously shown, leptin acts via dimerization of its cognate receptor thereby displaying pleiotropic functionality ranging from energy homeostasis to reproduction, from regulation of immune system to angiogenesis and so on. While administration of leptin in diabetes and congenital leptin deficiency established promising therapeutic applications, its pathophysiology in obesity, autoimmune disease and breast cancer raises unequivocal demand for better understanding of structural and mechanistic paradigm of leptin receptor activation. Here we demonstrate that leptin can induce dimerization of LR lacking membrane proximal fibronectin type III ($LR_{\Delta FNIII}$) domains, thus suggesting a redundant role of these domains in leptin induced LR oligomerization. Earlier studies had shown that the FNIII region is essential for the functionality of LR. The affinity of leptin for this construct was found to be similar to the full length receptor, with nanomolar affinity and stoichiometry of 1:1. Based on light scattering and small-angle x-ray scattering data, we demonstrate that $LR_{\Delta FNIII}$, despite the absence of the membrane proximal domains, can form a quaternary complex with wt-Leptin. In contrast, antagonist leptin bearing S120A/T121A mutation could not dimerize the $LR_{\Delta FNIII}$ receptor. These results evidently suggest a functional dichotomy whereby while FNIII appears to be redundant in the oligomerization of LR it still may be playing a key role in the spatial disposition and orientation of intracellular parts to facilitate a signaling-competent conformation.

Following milestones represent my accomplishments during my Doctoral research work:

- Eukaryotic expression (Transient and Stable) and purification of extracellular LR variants
- Isolation of receptor-ligand complexes for co-crystallization trial
- Establishment of stable cell lines
- Protein production optimization by redesigning constructs
- Biophysical characterization of interactions using Isothermal Titration Calorimetry (ITC)
- Molecular characterization of complexes using Multi-Angle Light Scattering (MALS)
- Low resolution structural characterization using Small angle X-ray scattering (SAXS)
- Low resolution (~5Å) protein crystallography
- Electron microscopic (EM) grid preparation and processing of EM images
- Programming and automation of large-scale SAXS data analysis pipeline using PERL and PYTHON programming

Symposium, Conferences, Workshops

11th International conference Biology and Synchrotron Radiation (2013): Poster presentation entitled "Mechanistic paradigm of Leptin Receptor activation" held at Hamburg, **Germany** between 08-11 September 2013

Electron Microscopy (EM)Training (2012): Sample preparation using Gradient Fixation (GraFix), EM Grid preparation, Image analysis using EMAN2 software package, Center for Cellular Imaging and NanoAnalytics, Biozentrum, University Basel, **Switzerland**

Netherlands-Belgium PHENIX workshop (2011): Workshop on automated determination of macromolecular structure using PHENIX software suite, Utrecht University, **Netherlands**

Belgian Biophysical Society (2011): Poster presentation entitled "Structural Intricacies of Leptin/Leptin Receptor Complex" at 1st Young Scientist Day of the Society, held at 't Pand, Gent, **Belgium** on 31st May 2011

Netherlands Organization for Scientific Research (NWO) (2010): Poster presentation entitled "Structural Intricacies of Leptin/Leptin Receptor Complex " at its Annual meeting of, held at Veldhoven, **Netherlands** on 6th and 7th December 2010

High Performance Computing in Python (2009) : Seminar & workshop organized by Doctoral School of Natural Sciences (High Performance Computing, Parallel computing, Python scripting), Ghent University, **Belgium**

Master Student Thesis Supervision

TA DUY TIEN (2nd yr. Masters)	**2009-10**	Towards structure-function studies of Myristoyl-CoA:Protein N-myristoyltransferase from the malaria parasite *Plasmodium falciparum*
MARIA BORISNOVA (1st yr. Masters)	**2009**	Structural biology of Myristoyl-CoA:Protein N-myristoyltransferase from the malaria parasite *Plasmodium falciparum*
THOMAS MAGGEN (1st yr. Masters)	**2011**	Expression, purification and crystallization of Leptin binding domain of murine Leptin Receptor expressed in mammalian expression system

Academic Achievements

GRE Score (2007): 1330 (Verbal 550/800; Quantitative 780/800; Analytical 3.5/5); **TOEFL** Score (2007): 93/120

Awarded with Research Fellowship in **NET-2006** exam becoming eligible for getting PhD fellowship from Govt. of India.

Awarded twice with Research Fellowship **NET-2007** exam becoming eligible for PhD fellowship from Govt. of India.

Ranked in top 0.2% of examinees in **CBSE-Medical** entrance exam (2004) becoming eligible to join medical school in India.

Secured **2nd position** in BJB autonomous College during B.Sc.

Secured **1st position** in 10th examination in my class.

Elected as Department **student representative** during my B.Sc. at B.J.B. Autonomous College.

Awarded **1st prize** in essay competition titled "50 years of DNA" organized by Dept. of Biotechnology, Govt. of India (2003)

Extracurricular Achievements

One of the four founder members of GenISA, an Indian cultural organization Ghent University and principal organizer of many events during 2010-2012.

Amateur Photographer: Photos have been selected by National Geographic for featuring Ireland and for a world-wide contest.

Amateur web developer: Developed online bioinformatics tool for sequence composition analysis and websites for non-profit organizations (HTML, CSS, CGI).

Amateur android application developer (XML and JAVA).