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The Endosomal Sorting Complex Required for Transport Pathway Mediates Chemokine Receptor CXCR4 Akt Signaling by Promoting Lysosomal Degradation of mTOR Antagonist Deptor

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THE ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT PATHWAY MEDIATES CHEMOKINE RECEPTOR CXCR4

AKT SIGNALING BY PROMOTING LYSOSOMAL DEGRADATION OF MTOR ANTAGONIST DEPTOR

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

PROGRAM IN MOLECULAR BIOLOGY

BY

RITA RAMKARAN VERMA

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DEDICATED TO
TO HER HOLINESS SHRI MATAJI NIRMALA DEVI
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LIST OF ABBREVIATIONS

7-TM  Seven-Transmembrane
3-MA  3- Methyladenine
At1aR  Angiotensin II type 1a
α2AR  α2-adrenergic receptor
AAA-ATPase  ATPases Associated with diverse cellular Activities
AIP4  Atrophin-1-interacting protein 4
ALIX  Apoptosis lined gene-2 interacting protein-X
APS  Ammonium per sulfate
ANOVA  Analysis of Variance
β2AR  β2-adrenergic receptor
BAEC  Bovine Aortic Endothelial Cells
BSA  Bovine Serum Albumin
cAMP  cyclic-Adenosine Monophosphate
CHAPS  3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate
CHMP4A-C  Charged multivesicular body protein 4A,B or C
CHX  Cycloheximide
CXCR4  C-X-C chemokine receptor type-4
CXCL12  C-X-C motif chemokine 12
DAG  Diacylglycerol
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<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
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<tr>
<td>DEPTOR</td>
<td>DEP domain containing-mTOR interacting protein</td>
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<td>DEP</td>
<td>Dishevelled, Egl-10, Pleckstrin</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
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<td>EEA-1</td>
<td>Early Endosomes Antigen-1</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
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<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinases 1/2</td>
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<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<tr>
<td>FAT</td>
<td>FRAP, ATM and TRRAP</td>
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<td>FoxO</td>
<td>Forkhead box O</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<tr>
<td>FYVE</td>
<td>Fab-1, YGL023, Vps27 and EEA-1</td>
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<tr>
<td>Gα</td>
<td>G alpha protein</td>
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<tr>
<td>Gβγ</td>
<td>G- beta and gamma subunits</td>
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<tr>
<td>GABA&lt;sub&gt;B&lt;/sub&gt;</td>
<td>Gamma-Amino Butyric acid</td>
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<td>GASP-1</td>
<td>GPCR associated sorting protein-1</td>
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<td>GDP</td>
<td>Guanosine Diphosphate</td>
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<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<td>GLUE</td>
<td>GRAM-like ubiquitin binding in EAP45</td>
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<td>GPCR</td>
<td>G protein-coupled receptor</td>
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<td>GRAFS</td>
<td>Glutamate, Rhodopsin, Adhesion, Frizzled/taste2 and Secretin</td>
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<td>GRK</td>
<td>G-protein coupled receptor kinases</td>
</tr>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>GSK3β</td>
<td>Glycogen Synthase Kinase -3β</td>
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<td>GTP</td>
<td>Guanosine Triphosphate</td>
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<tr>
<td>HEAT</td>
<td>Huntington, elongation factor 3, PR65/A and TOR</td>
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<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid</td>
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<td>HIF-1α</td>
<td>Hypoxia inducible factor-1α</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>Hrs</td>
<td>Hepatocyte growth factor-regulated tyrosine kinase substrate</td>
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<td>IP3</td>
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<td>LP2000</td>
<td>Lipofectamine 2000</td>
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<td>Lipofectamine 3000</td>
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<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<td>µL</td>
<td>microlitre</td>
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<tr>
<td>µg</td>
<td>micrograms</td>
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<tr>
<td>min</td>
<td>minutes</td>
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<td>mM</td>
<td>millimolar</td>
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mLST8/GβL  Mammalian lethal with Sec13 protein 8/G protein β-subunit-like protein
mTORC1  mechanistic target of rapamycin complex-1
mTORC2  mechanistic target of rapamycin complex-2
MVB12A/B  Multivesicular body subunit 12 A/B
NC-IUPHAR  Nomenclature Committee of the International Union of the Pharmacology
NEM  N-Ethylmaleimide
NHERF  Na⁺-H⁺ exchanger regulatory factor
nM  nanomolar
PAR1/2  Protease activated receptor 1
PBS  Phosphate buffered saline
PDZ  Postsynaptic density protein-95, disk large tumor suppressor protein, zonula occludens-1
PEI  Polyethylenimine
PH  Pleckstrin homology
PHLPP  PH domain leucine-rich repeat protein phosphatase
PI3K  phosphoinositide-3-kinase
PIP2  Phosphatidylinositol 4,5-bisphosphate
PIP3  Phosphatidylinositol 3,4,5-trisphosphate
PI3P  Phosphatidylinositol-3-phosphate
PKA  Protein kinase A
PKB  Protein kinase B
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>P70S6K</td>
<td>70 kDa ribosomal S6 kinase</td>
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<tr>
<td>PLCβ</td>
<td>Phospholipase-C-beta</td>
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<tr>
<td>PRAS40</td>
<td>Proline-rich Akt substrate of 40 kDa</td>
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<tr>
<td>PROTOR</td>
<td>Protein observed with Rictor 1)/PRR5 (proline-rich protein 5</td>
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<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homologue</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
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<tr>
<td>Rheb</td>
<td>Ras-homolog enriched in brain</td>
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<tr>
<td>RT</td>
<td>Room Temperature</td>
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<tr>
<td>RICTOR</td>
<td>Rapamycin-insensitive companion of mTOR</td>
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<td>RAPTOR</td>
<td>Regulatory-Associated protein of mTOR</td>
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<td>SHIP</td>
<td>SH2-domain-containing inositol polyphosphate 5-phosphatase</td>
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<td>STAM1</td>
<td>Signal-transducing adaptor molecule-1</td>
</tr>
<tr>
<td>STAT1</td>
<td>Signal-transducer and activator of transcription</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Sin1</td>
<td>Mammalian stress-activated protein kinase interacting protein 1</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of Mean</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>shRNA</td>
<td>Small Hair pin RNA</td>
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<tr>
<td>TBK1</td>
<td>TANK-binding kinase 1</td>
</tr>
<tr>
<td>TSG101</td>
<td>Tumor susceptibility gene 101</td>
</tr>
<tr>
<td>TSC2</td>
<td>Tuberous sclerosis complex</td>
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<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<td>-------------</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered saline and Tween 20</td>
</tr>
<tr>
<td>UBAP1</td>
<td>Ubiquitin associated protein-1</td>
</tr>
<tr>
<td>UBD</td>
<td>Ubiquitin Binding Domain</td>
</tr>
<tr>
<td>UEV</td>
<td>Ubiquitin Enzyme Variant</td>
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<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UIM</td>
<td>Ubiquitin-interacting motif</td>
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<tr>
<td>VPS</td>
<td>Vacuolar protein sorting</td>
</tr>
<tr>
<td>VIP</td>
<td>Vasoactive intestinal peptide</td>
</tr>
<tr>
<td>WHIM</td>
<td>Warts, Hypogammaglobunemia, Infections and myelokathexis</td>
</tr>
<tr>
<td>XPLN</td>
<td>Exchange factor found in platelets, leukemic and neural tissue</td>
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ABSTRACT

The chemokine receptor CXCR4 is a member of the G protein-coupled receptor (GPCR) family. The cognate ligand for CXCR4 is the C-X-C chemokine known as CXCL12. The CXCL12/CXCR4 signaling axis is essential for a number of developmental processes including organogenesis, vascularization of the GI tract and hematopoiesis. Dysregulated CXCR4 signaling is also implicated in a variety of pathological conditions such as WHIM (Warts, Hypogammaglobunemia, Infections and myelokathexis) syndrome, cardiovascular disease and cancer. Despite its role in several pathologies, the molecular mechanisms mediating CXCR4 signaling are not completely understood. Upon CXCL12 binding to CXCR4, several signaling pathways are activated including the Akt/mTOR pathway, which mediates several cellular responses including survival, proliferation, invasion, and migration. In the current dissertation project, we aim to elucidate the molecular mechanisms regulating CXCR4 promoted Akt signaling.

Akt is a serine/threonine kinase that is fully activated by a complex multistep process involving phosphorylation of two key amino acid residues. Akt is activated by phosphorylation at threonine residue 308 and serine residues 473 by phosphoinositide-dependent kinase (PDK1) and mechanistic/mammalian target of rapamycin complex 2 (mTORC2), respectively. The mechanisms by which GPCRs promote PDK1 mediated
phosphorylation of Akt at T308 are well understood; however the mechanism by which mTORC2 phosphorylates Akt on S473 remain essentially unknown.

mTORC2 is a multi-subunit kinase complex comprised of key units including rictor, Sin1 and mTOR, the catalytic subunit. An important subunit referred to as DEPTOR binds to mTOR and inhibits its catalytic activity. Aberrant expression of DEPTOR has been linked to altered mTORC2 kinase activity and Akt signaling in several cancers. However, the mechanisms that regulate DEPTOR expression remain poorly understood.

We set out to understand the mechanisms that regulate DEPTOR levels in cells and how this impacts the mTORC2/Akt axis in response to CXCR4 activation. We show for the first time, that CXCL12 stimulation leads to rapid degradation of DEPTOR through lysosomes and that the ESCRT (Endosomal Sorting Complex Required for Transport) pathway that sorts ubiquitinated membrane receptors, mediates lysosomal degradation of DEPTOR. Pharmacological inhibition of heterotrimeric G protein Gα, PI3K signaling and siRNA targeting ESCRTs blocks CXCR4 promoted degradation of DEPTOR. We also show that by promoting DEPTOR degradation, the ESCRT pathway mediates Akt signaling promoted by CXCR4. Depletion of ESCRTs by siRNA leads to increased levels of DEPTOR and attenuates CXCR4 promoted, G protein and PI3K dependent Akt activation and signaling, consistent with decreased mTORC2 activity. In addition, ESCRTs likely have a broader role in Akt signaling because ESCRT depletion also attenuates receptor tyrosine kinase promoted Akt activation and signaling.
Collectively, our data reveal a novel role for the ESCRT pathway in promoting intracellular signaling, which may begin to identify the signal transduction pathways that are important in the physiological roles of ESCRTs and Akt.
CHAPTER 1
INTRODUCTION AND BACKGROUND

G PROTEIN-COUPLED RECEPTOR

G protein-coupled receptors (GPCRs) or seven transmembrane receptor (7TMs) receptors are the largest and the most diverse family of cell surface signaling receptor encoded in the mammalian genome containing more than 900 members (Feigin, 2013; Kroeze et al., 2003; Zhou et al., 2012). GPCRs respond to a wide variety of extracellular stimuli including light, biogenic amines, lipids, protein, amino acids, odorants, hormones, nucleotides, chemokines and many others (Fredriksson et al., 2003; Kroeze et al., 2003). The primary function of GPCR is to transduce extracellular stimuli into intracellular signaling, ultimately contributing to a diverse array of physiological processes including vision, smell, taste, growth, homeostasis, neuronal transmission, metabolism, reproduction and so on (Oh et al., 2006). Since GPCR signaling is very important in physiology and homeostasis, dysregulated GPCR signaling leads to wide range of pathophysiological conditions including metabolic, immunological, neurodegenerative cardiovascular diseases and cancer (Heng et al., 2013). GPCRs are considered as key targets in drug discovery and more than 30% of pharmaceutical drugs in the market act by modulating GPCRs (Hopkins and Groom, 2002; Jacoby et al., 2006; Oh et al., 2006; Wilson and Bergsma, 2000).
GPCR STRUCTURE AND CLASSIFICATION

Topologically GPCRs share a similar structural signature of seven anti-parallel hydrophobic transmembrane helices connected by three extracellular, three intracellular loops, with the C-terminus on the cytoplasmic side while the N-terminus is towards the exterior of cell (Baldwin, 1993; Donnelly et al., 1994; Kobilka, 2007). GPCRs have been classified based on different prediction systems, phylogenetic approach and databases. Early on, an A-F classification system was used however with accumulation of more GPCRs, the GRAFS classification system was built (Kolakowski, 1994). In 2003 Fredriksson et al, classified GPCRs using a phylogenetic approach into five families Glutamate, Rhodopsin, Adhesion, Frizzled/taste2 and Secretin (GRAFS) family (Fredriksson et al., 2003). However, according to the nomenclature committee of the International Union of the Pharmacology (NC-IUPHAR), receptors can be arranged in the five groups in a list that is available online (http://www.iuphar-db.org/index.jsp) and is continuously updated (Foord et al., 2005).

Class I

Also known as Family A, is the largest family containing 276 members with Rhodopsin as a prototype receptor (Bockaert and Pin, 1999; Foord et al., 2005). About 50% of the Class I receptors are involved in sensing taste, odor and light. Best examples of this group include Adreno, Chemokine, Dopamine, histamine, opioid receptors and so on. This group also contains a large number of orphan receptors (> 80) that have no known ligands.
Class II

Also known as Family B, contains 53 members, with secretin receptor as the prototype receptor of the group (Bockaert and Pin, 1999; Foord et al., 2005). Best examples of receptors from this group include Calcitonin, Glucagon, vasoactive intestinal peptide (VIP), Corticotrophin-releasing hormone and parathyroid hormone receptors. This class contains more than 30 orphan receptors (Foord et al., 2005).

Class III

Also known as Family C, is a relatively small family containing 19 receptors with metabotropic glutamate receptors GABA_{B} as the prototype receptor. Other members of this group include calcium-sensing receptor and some taste receptors. This group contains 7 orphan receptors. Members of this family have a very large extracellular N-terminus that is essential for ligand binding to receptor (Foord et al., 2005; Pierce et al., 2002).

Class IV

Also known as the adhesion receptor family that contains 33 members. These receptors contain long N-termini with multiple domains. These receptors contain adhesion-like motifs such as EGF-like repeats, leucine-rich repeats and cadherin domains that participate in cell-cell adhesions (Bjarnadottir et al., 2004; Foord et al., 2002; Fredriksson et al., 2003). The Majority of the adhesion receptors class are orphan receptors with no known ligand (Gupte et al., 2012). Many adhesion receptors are shown to undergo proteolytic cleavage post-translationally at GPCR proteolysis site. Recently,
GPR56 and GPR110 are shown to be activated by proteolytic cleavage to expose the 7TM domain that acts as a tethered agonist to activate heterotrimeric G proteins (Stoveken et al., 2015).

**Frizzled/smoothened receptors**

This group only contains 11 members (Frizzled 1-10 and smoothened) and only a few receptors in this family couple to G protein not all. This group more closely resembles class II family, and ligands of the all family members are known. The Frizzled receptors are activated by Wingless/Int-1 (WNT) which are secreted glycoproteins that activates the classical Wnt-β-catenin pathway. On the other hand, the hedgehog proteins indirectly activate the smoothened receptors (Schulte, 2010).

**G PROTEIN COUPLED RECEPTOR SIGNALING**

*Activation of heterotrimeric GTP-binding proteins*

G protein coupled receptors signal via coupling to heterotrimeric G proteins. Heterotrimeric G proteins are composed of Gα, Gβ and Gγ subunits. There are 21 Gα, 6 Gβ and 12 Gγ subunits (Downes and Gautam, 1999; Oldham and Hamm, 2008). Gα subunits can be further subdivided into 4 groups: Gαs, Gαi, Gαq and Gα12 (Oldham and Hamm, 2008; Simon et al., 1991). In a basal state, the inactive Gα subunit is bound to GDP, Gβγ and only weakly associated with the receptor. Ligand binding to the receptor causes a conformational change in the receptor (Ballesteros et al., 2001; Farahbakhsh et al., 1995), which interacts with G proteins and serves as a Guanine nucleotide-exchange
factor (GEF), enabling exchange of GDP for GTP on the Gα subunit. Exchange of GDP for GTP activates the Gα subunit, which now dissociates from Gβγ dimer. The GTP bound α-subunit and the βγ-heterodimer can activate downstream effector molecules (Pierce et al., 2002; Siderovski and Willard, 2005). Gα subunit possess an intrinsic GTPase activity to hydrolyze GTP to GDP leading to signal termination and reassociation of heterotrimeric G protein. However, regulators of G protein signaling (RGS) proteins are shown to act as GTPase-activating protein to increase the GTPase activity of Gα subunit (De Vries et al., 2000; Lambert et al., 2010; McCudden et al., 2005; Pierce et al., 2002; Ross and Wilkie, 2000).

**G protein dependent signaling**

On GPCR activation both the activated Gα subunit and dissociated Gβγ dimer activate several downstream pathways including adenylyl cyclase, ion channels, phospholipases, Mitogen activated protein kinase pathway (MAPK), Phosphatidylinositol-3-kinase (PI3K)-Akt and so on. Typically, Gαs stimulates adenylyl cyclase and increases cyclic AMP levels, while Gαi inhibits adenylyl cyclase and also regulates ion channels (Jones et al., 1990; Sullivan et al., 1986). Gαq is known to bind and activate Phospholipase C (PLC) that cleaves lipid phosphatidylinositol bisphosphate (PIP2) into diacylglycerol (DAG) and inositol triphosphate (IP3) (Wu et al., 1992).

The Gβγ functions as a single unit and is known to activate PLCβ to activate MAPK Erk1/2 pathway (Katz et al., 1992; Murthy et al., 1996). Gβγ is shown to regulate ion channels and also hyperactivate adenylyl cyclase after Gαs mediated activation
(Dolphin, 2003; Gilman, 1995). Gβγ is also known to directly or indirectly activate PI3K that in turn activates the Akt pathway fostering cell survival and proliferation (Brock et al., 2003; Leopoldt et al., 1998).

**G protein independent signaling**

GPCRs also activate signaling pathways independent of G protein activation. GPCRs are known to associate with scaffold proteins like arrestins, PDZ or non-PDZ scaffolds to mediate G protein-independent signaling pathways (Hall and Lefkowitz, 2002). Non-visual arrestins (arrestin-2 and arrestin-3) were initially identified as signal terminators for GPCR (Krupnick and Benovic, 1998). However, arrestins were soon identified as scaffolds for various signaling molecules downstream of GPCR activation. There are a number of GPCRs identified that activate G protein independent and arrestin dependent signaling pathways. Arrestin serves as a scaffold for MAPK Erk1/2 activation downstream of Angiotensin II type 1a (At1aR) (Luttrell et al., 2001), β2-adrenergic (β2AR) (Ahn et al., 2004), Protease-activated receptor 2 (PAR2) (Stalheim et al., 2005), Chemokine receptor CCR7(Kohout et al., 2004), Vassopressin receptor (Tohgo et al., 2003) and so on. Arrestin is also known to activate Akt/mTOR pathway in response to At1aR (Kendall et al., 2014) and protease activated receptor 1 (PAR1) (Goel et al., 2002). On GPCR activation, Arrestins also regulate other signaling components including non-receptor tyrosine kinases (Src)(Luttrell et al., 1999), NF-κB (Gao et al., 2004), JNK (McDonald et al., 2000) and p38 (Miller et al., 2003).
Non-arrestin scaffolds also mediate GPCR signaling independent of G protein activation. For example, on agonist stimulation, (β2AR) receptor associates with Na⁺-H⁺ exchanger regulatory factor (NHERF). NHERF is a PDZ domain containing protein to regulate cell surface transporter Na⁺-H⁺ exchanger (Hall et al., 1998). Janus kinase signal transducers (JAK2) is known to directly interact with At1aR and recruit and phosphorylate STAT1, representing JAK2 as a non-PDZ domain scaffold (Ali et al., 2000; Sayeski et al., 2001).

**GPCR mediated PI3K/Akt pathway**

GPCRs coupling to all four types of Gα subunit Gαs, Gαq, Gαi, and Gα12/13, are known to activate PI3K/Akt pathway by both Gα and Gβγ subunits. Downstream of many GPCRs Gβγ, directly binds PI3K heterodimers containing either p110β or p110γ subunits. Gβγ directly interacts with the catalytic (p110γ) and regulatory subunit p101 of PI3Kγ (Lopez-Ilasaca, 1997; Schwindinger, 2001). Gβγ dimer is also known to interact and activate p110β containing PI3K (Ciraolo et al., 2008; Engelman et al., 2006). For example, on activation of muscarinic and lysophosphatidic acid (LPA) receptors, PI3Kβ and PI3Kγ respectively are activated by Gβγ dimer (Murga et al., 2000). So far Gα subunit is not known to directly activate PI3K, but Gα subunit can indirectly activate PI3K via transactivation of RTKs. For example, constitutively active Gα12 subunit is known to activate PI3K/Akt pathway via transactivating PDGFRα (platelet derived growth factor receptor α)(Kumar et al., 2006). Intracellular Ca²⁺ and Src family kinases
are also important for Akt activation downstream of some GPCRs. $G_{\alpha_q}$ in response to PAR-2 receptor activation, activates protein kinase C and mobilizes intracellular Ca$^{2+}$ to induce complex formation containing src-family kinases, focal adhesion kinases, pyk2 and PI3K (Wang and DeFea, 2006). β-arrestins also play an important part in PI3K/Akt activation downstream of GPCR. For example, arrestins recruits src family kinases that phosphorylate $G_{\alpha_q}$ leading to activation of endothelin A receptor and in turn PI3K/Akt activation (Imamura et al., 2001).

**REGULATION OF GPCR SIGNALING**

GPCR signaling is under tight spatial and temporal regulation to ensure signals are of the appropriate magnitude and duration. Although this regulation can occur at multiple steps in the signaling pathway, events that occur at the level at the receptor are essential. Three mechanistically distinct processes occur and can be separated based on their temporal and spatial requirements.

**Desensitization of receptor:** It is an important step in attenuation of receptor signaling that occurs within minutes of ligand binding, where the receptors are uncoupled from the G proteins to stop further signaling.

**Internalization:** After initial desensitization, GPCRs redistributed away from the cell surface towards the interior of the cells by sequestration or internalization.

**Downregulation:** Receptors are sorted and targeted for degradation via the lysosomal pathway. This leads to long-term termination of receptor signaling.
**Desensitization of receptor**

This is the first step in attenuation of receptor signaling, that starts within seconds of ligand binding to the receptor. On ligand binding, receptor is rapidly inactivated by phosphorylation leading to functional uncoupling of the receptor from the G proteins even in presence of the ligand. Desensitization only affects the functional activity of receptor and does not affect the number of receptors in cells. Receptors can be desensitized by homologous or heterologous desensitization that is executed by three types of regulatory proteins; second messenger kinases Protein Kinase A (Hausdorff et al., 1990) or Protein Kinase C (Feng et al., 2011), G protein coupled receptor kinases GRKs and arrestins (Sterne-Marr and Benovic, 1995; Zhang et al., 1997).

Serine or threonine kinases PKA and PKC are activated downstream of G\(\alpha_s\) and G\(\alpha_q\) receptors respectively (Feng et al., 2011; Hausdorff et al., 1990; Rittiner et al., 2014). On activation these kinases phosphorylate the C-tail of the receptors containing consensus PKA and/ or PKC phosphorylation sites. Phosphorylation induces a conformation change in the receptor leading to directly uncoupling receptors from respective G proteins. This leads to attenuation of signaling of receptor and also other receptors in cells that contain consensus PKA and/ or PKC phosphorylation sites. This type of receptor desensitization is called non-agonist-specific or heterologous desensitization (Lefkowitz, 1998).

A general mechanism of agonist-specific, homologous desensitization of receptor involves a 2 step process; 1) Phosphorylation of ligand-bound receptor by specific GRKs and 2) binding of arrestin to GRK phosphorylated receptor which sterically prevents
further GPCR and G protein interaction (Inglese et al., 1993; Lefkowitz, 1993; Pitcher et al., 1998; Sterne-Marr and Benovic, 1995; Zhang et al., 1997). There are 7 known members of the GRK family (1-7) shown to phosphorylate GPCRs. GRKs are cytosolic and recruited to the membrane by interaction with Gβγ subunit (Pitcher et al., 1992) or GRKs are farnesylated and palmitoylated for membrane association (Pierce et al., 2002; Stoffel et al., 1994) Arrestin then binds GRK phosphorylated GPCR and mediate GPCR internalization by binding components of internalization machinery.
Figure 1: Regulation of GPCR Signaling

After activation of receptor by the ligand, receptor is rapidly desensitized and redistributed away from the plasma membrane into the interior of the cell. Few receptors are ubiquitinated and these plasma membrane microdomains are pinched off forming vesicles containing receptors that fuse with early endosomes. Although receptors are ubiquitinated, this step is not required for receptor internalization. On the endosomes receptors are either sorted into a recycling pathway or degradative pathway. For recycling pathway, ubiquitin moiety is removed by deubiquitinating enzymes (DUBs) on the
endosomes and receptors are transported back to the plasma membrane. For degradative pathway, ubiquitin is used as a sorting signal to sort and cluster receptors into multivesicular bodies. These multivesicular bodies bud off and fuse with the lysosomes where receptors are finally degraded.

**Internalization**

Agonist treatment leads to redistribution of GPCRs away from the plasma membrane towards the interior of the cell via a process of endocytosis, sequestration or internalization. After phosphorylation and uncoupling from the G proteins, GPCRs are internalized or sequestered by various processes including clathrin coated pits, Caveolae or uncoated vesicles (Chini and Parenti, 2004; Claing et al., 2002; Pierce et al., 2002; Stoffel et al., 1994). Caveolae are caveolin and cholesterol-containing membrane invaginations that are not the major pathway for GPCR internalization. Many GPCR receptors are shown to localize to caveolae including β₁-AR (Rapacciuolo et al., 2003), CXCR4 (Malik et al., 2012), serotonin 5HT2 (Dreja et al., 2002), somatostatin SST2 (Kirsch et al., 1998), Thyrotrophin TRH (Drmota et al., 1999), Angiotensin At₁ (Ishizaka et al., 1998; Ushio-Fukai et al., 2001), Bradykinin B2 (de Weerd and Leeb-Lundberg, 1997; Haasemann et al., 1998), Spingpsine EDG-1 (Igarashi and Michel, 2000), Endothelins ET₆ (Teixeira et al., 1999) and vasoactive intestinal peptide (VIP) (Claing et al., 2000). However, only a few receptors including SST2, VIP and ET₂ are known to internalize via caveolae dependent pathways. Also receptors may be modified to enter the caveolae, for example PKA-mediated phosphorylation is known to localize β₁-AR to
caveolae (Rapacciuolo et al., 2003). However, the mechanisms of localization of other receptors in caveolae are still not entirely clear. For most receptors, caveolae microdomains are sites for activation of different signaling cascades (Barnett-Norris et al., 2005; Chini and Parenti, 2004; Malik et al., 2012; Smart et al., 1999).

Internalization through the clathrin coated pits/vesicles is by far the major pathway for most GPCRs. This pathway is also known as the GRK/β-arrestin dependent pathway. GPCRs are phosphorylated by GRK and phosphorylated receptors are scaffolds for recruitment of β-arrestin (Shenoy and Lefkowitz, 2003). In addition to binding to ligand-activated and phosphorylated receptor (Lohse et al., 1990), β-arrestin also interacts with components of the clathrin-coat pit machinery including clathrin heavy chain (Caplan et al., 1977) and clathrin adaptor protein AP-2 (Laporte et al., 1999). β-arrestin is also shown to interact with phosphoinositide lipid of the plasma membrane (Gaidarov et al., 1999). This manifold role of β-arrestin, targets GPCR into clathrin coated invaginations, which then pinch off with the help of the GTPase dynamin (Hill et al., 2001). Receptors are either rapidly recycled back to the plasma membrane or internalized into the endosomes.

**Downregulation of receptor**

Receptors are internalized onto the endosomal membrane where they are sorted into the recycling pathway or lysosomal degradation pathway. Receptors are sorted into the lysosomal degradation pathway for final signal termination and downregulation of the receptor. After the sorting, receptors are sequestered into Multivesicular bodies (MVBs).
MVBs are vesicular invaginations formed on the limiting membrane that bud off into the lumen of late endosomes. These vesicles containing receptors bud off and fuse with the lysosomes where the content is degraded by hydrolytic enzymes (Futter et al., 1996).

On the endosomal membrane, receptors are sorted by either

1. Endosomal Sorting Complex Required for Transport (ESCRT) machinery or
2. GPCR associated sorting protein-1 (GASP-1) pathway.

In case of lysosomal sorting mediated by the ESCRT machinery, GPCR is ubiquitinated and ubiquitin serves as a sorting signal (Katzmann et al., 2001; Shenoy et al., 2001; Verma and Marchese, 2015). And five complexes of the ESCRT machinery sorts ubiquitinated GPCR. In GASP-1 mediated lysosomal sorting, GASP-1 proteins and some components of the ESCRT machinery mediate sorting of receptors that may not be ubiquitinated (Dores and Trejo, 2012; Dores and Trejo, 2014). In this theses we will focus on ESCRT mediated sorting of proteins. The ESCRT machinery is described in detail in section 1.9.

**CHEMOKINE RECEPTORS**

Chemokine or chemoattractive cytokines are a large family of 8-10 kDa, secreted proteins that function through binding to G protein coupled receptors called Chemokine receptors. Chemokines were first discovered as proteins attracting immune cells to the site of inflammation (Oppenheim et al., 1991). Later on chemokine signaling was shown to function in processes including migration of neurons, germ cells during embryonic development, metastasis and so on (reviewed in (Wang and Knaut, 2014). So far, there
are 18 chemokine receptor and more than 50 chemokine ligands discovered. Chemokine ligands are grouped in four subfamilies according to four conserved cysteines that form two disulfide bonds CC, CXC, CXXXC, XC (Bachelerie et al., 2014). The GPCR chemokine receptors are named according to the ligand they bind. For instance, CXCR4 binds CXCL12 ligand (Bleul et al., 1996).

**C-X-C CHEMOKINE RECEPTORS TYPE-4**

CXCR4 or Fusin or LESTR or CD184 was first cloned and considered as an orphan receptor (Feng et al., 1996; Loetscher et al., 1994). SDF-1 (Stromal cell-derived factor-1) or chemokine CXCL12 was subsequently discovered as a cognate ligand for CXCR4 (Bleul et al., 1996; Oberlin et al., 1996). CXCR4 is constitutively expressed and present on most blood cells and tissues including monocytes, lymphocytes, NK cells, dendritic cell, heart, gastrointestinal tract, astrocytes, microglia, neurons, vascular smooth muscle cells and endothelial cells (Balkwill, 2004; Caruz et al., 1998; Wegner et al., 1998). CXCR4 and its ligand CXCL12 are very important during embryogenesis in development, hematopoiesis, organogenesis and vascularization therefore CXCR4 or CXCL12 knockout mice die in utero or shortly after birth. These mice show ventricular septum formation defects in the heart, poor vasculature development in the gastrointestinal tract, defective bone marrow myeloid formation and defective cerebellum development (Nagasawa et al., 1996; Tachibana et al., 1998; Zou et al., 1998). In normal adult life, CXCL12/CXCR4 is important for stem cell homing, immune cell trafficking and tissue regeneration (Richard and Blay, 2008). Therefore, dysregulated
CXCL12/CXCR4 signaling axis leads to a number of pathological conditions as listed below.

**PATHOPHYSIOLOGICAL ROLE OF CXCL12/CXCR4 AXIS**

**WHIM Syndrome**

WHIM (Warts, Hypogammaglobulinemia, Infections and Myelokathexis) is a rare autosomal dominant immunodeficiency disorder caused by heterozygous mutations in the CXCR4 gene. WHIM syndrome is characterized by human pappilomavirus infection induced warts and carcinoma, recurrent bacterial infection, Hypogammaglobulinemia, lymphopenia, neutropenia and Myelokathexis (retention of neutrophils in the bone marrow). Six different CXCR4 frameshift or nonsense mutations (R334X, G336X, S338X, S339FSX342, E343X, G323FSX343) are described so far that leads to truncation of the 10-19 amino acids in the C-terminus of the receptor (Bachelerie, 2010). Deletion of the C-terminus eliminates the phosphorylation sites important for proper internalization and desensitization of the receptor (Haribabu et al., 1997; Signoret et al., 1997). Defective desensitization of the receptor leads to efficient activation of receptor signaling, like enhanced chemotaxis, F-actin polymerization, increased calcium mobilization and MAPK-Erk1/2 signaling (Balabanian et al., 2005; Balabanian et al., 2008; Gulino et al., 2004). CXCR4 undergoes GRK and arrestin-mediated desensitization, hence defects in the desensitization machinery may also lead to WHIM syndrome, as reported in patients with the wild type CXCR4 gene with selectively decreased GRK3 transcript levels (Balabanian et al., 2008). CXCR4 antagonist plerixafor
is considered as a promising therapy, as clinical trials with short term treatment with plerixafor is shown to correct neutropenia and other cytopenia in WHIM syndrome patients (McDermott et al., 2014).

**Role of CXCR4 in Cancer**

CXCR4 is upregulated and has been described as a prognostic marker in different types of cancer including leukemia (Burger et al., 1999), lung (Burger et al., 2003), prostate (Wang et al., 2005), ovarian (Barbolina et al., 2010), breast (Li et al., 2004), colorectal cancer (Zeelenberg et al., 2003). The CXCR4 positive cancer cells migrate towards lung, liver, lymph node and bone marrow expressing high levels of CXCL12. In case of chronic lymphocytic leukemia (CLL), neoplastic B cells expressing CXCR4 migrate towards bone marrow stromal cells expressing high levels of CXCL12. CXCR4 antagonists such as AMD3100, plerixafor and T140 are considered as a possible therapy for CLL (Burger, 2010; Burger et al., 1999). Plerixafor is also considered as a treatment in CXCR4-expressing epithelial ovarian cancer and melanoma. siRNA or monoclonal antibody for CXCR4 are also known to inhibit migration of breast cancer cells towards lymph nodes and lung in nude mice (Barbolina et al., 2010; Liang et al., 2005). Depletion of CXCR4 inhibit the PI3K/AKT pathway and MMP9 expression that is crucial for bone metastasis of breast cancer cells (Ping et al., 2011; Zeng et al., 2014; Zheng et al., 2007). CXCR4 is also upregulated in Small cell lung cancer (SCLC) and these cells are shown to migrate to bone marrow (Li et al., 2012). CXCR4 is also a prognostic marker in
prostate cancer and higher levels of CXCR4 correlates with poor survival in patients (Akashi et al., 2008).

**Role of CXCR4 in HIV-infection**

CXCR4 acts as a co-receptor for HIV entry in host immune cells (Azevedo-Pereira and Santos-Costa, 2008; Didigu and Doms, 2012). On an HIV infection, the virus enters the target cell by binding CD4 and co-receptor, CXCR4 or CCR5 on the immune cells. Viral coat protein gp120 interacts with CD4, allowing the gp120 V3 loop to interact with the ligand-binding region in CXCR4 (Wu et al., 2010). This leads to a pH-dependent fusion of the virus with the host cell membrane by a conformational change in the viral gp41 protein (Cervia and Smith, 2003; Grande et al., 2008; Seibert and Sakmar, 2004; Wilen et al., 2012). The virus uses CCR5 as a co-receptor in the early stage of HIV infection, while CXCR4 serves as a co-receptor in the late stages of the disease (Connor et al., 1997). CXCR4 utilizing HIV strain is also associated with a fast rate of disease progression (Sucupira et al., 2012).

**Role of CXCR4 in CXCR4 in Ischemic Heart**

The CXCR4/ CXCL12 axis is very important in proper development and functioning of the heart, as knockout mice of either CXCR4 or CXCL12 show defective ventricular septum formation in heart (Tachibana et al., 1998). This axis is also implicated in neovascularization and repair of heart after an ischemic insult (Zhang et al., 2008). CXCL12/SDF-1α is upregulated in the heart after an episode of coronary artery
occlusion, to recruit progenitor or stem cells to the infarct site for repair (Wang et al., 2006). In a rabbit ischemia model, hypoxic conditions at the infarct site is also shown to upregulate factors, like HIF-1α (Hypoxia inducible factor-1α) and G-CSF (granulocyte colony stimulating factor) which causes upregulation of CXCL12, that ultimately recruits CXCR4 positive cells to the infarct site for repair (Ceradini et al., 2004; Misao et al., 2006; Tang et al., 2009). CXCL12 overexpression mediated by adenoviral delivery or direct injection in the peri-infarct is shown to improve cardiac function. This effect is reversed by treatment with AMD3100 a CXCR4 antagonist (Saxena et al., 2008). Further studies have shown that CXCL12 levels are upregulated for a week and these levels drop due to CD26/dipeptidyl peptidase IV (DPP-IV) enzyme (Zaruba et al., 2009). Treatment techniques are being developed for stabilizing CXCL12 levels in infarct area to maximize CXCL12 mediated repair.

REGULATION OF CXCR4 SIGNALING

Like other GPCRs, CXCR4 signaling is also regulated by 3 main processes: Desensitization, Internalization and Degradation.

Desensitization

On CXCL12 mediated CXCR4 and G protein activation, CXCR4 is rapidly desensitized by phosphorylation and functional uncoupling of the receptor from the G proteins. CXCR4 is also known to undergo homologous or heterologous desensitization. The CXCR4 C-tail region S324-S333 is very important for CXCR4 desensitization and downregulation (Marchese and Benovic, 2001). Alanine or truncation mutagenesis of this
region is known to inhibit ligand-induced CXCR4 lysosomal degradation (Marchese and Benovic, 2001). Further studies show that CXCR4 can be phosphorylated by kinases GRK2 (Cheng et al., 2000; Orsini et al., 1999), GRK6 (Busillo et al., 2010; Busillo and Benovic, 2007; Fong et al., 2002; Vroon et al., 2004) and PKC (Busillo et al., 2010). Phosphorylation of CXCR4 by GRK2 between Ser 346 and 352 and GRK6 on residues Ser 324/325, S330/339 is known to recruit arrestin-3 to CXCR4 and thereby functionally uncouples the receptor from the G proteins (Busillo et al., 2010). This leads to homologous desensitization of the receptor. Phorbol ester (PMA) activated PKC is also known to phosphorylate CXCR4 on Ser 324/325 and mediate arrestin-independent heterologous desensitization of the receptor (Busillo et al., 2010).

**Internalization**

On ligand binding CXCR4 is known to internalize, recycle back to the plasma membrane, or sorted into a lysosomal degradation pathway. CXCR4 is known to recycle back to plasma membrane after PKC mediated desensitization (Signoret et al., 1997). However, CXCR4 barely recycles on CXCL12 mediated desensitization (Tarasova et al., 1998). CXCR4 is known to be ubiquitinated on the plasma membrane by an E3 ubiquitin ligase. GRK6-mediated phosphorylation of CXCR4 on residues Ser 324/325 serves as a binding site for the E3 ubiquitin ligase AIP4. AIP4 or ITCH (mouse), is a Nedd4-like E3 ubiquitin ligase (Hustad et al., 1995; Ingham et al., 2004; Perry et al., 1998). Nedd4-like E3s interact with their substrates via WW domains. AIP4 is known to directly interact
with S324/325 phosphorylated C-terminus of CXCR4 and monoubiquitinate lysine residues K321, K333 and K327 (Bhandari et al., 2009).

**Degradation**

Once internalized CXCR4 is sorted into the lysosomal degradation pathway using ubiquitin as the sorting signal. Ubiquitination of CXCR4 by AIP4 is essential for lysosomal degradation of receptor, as mutation of the three lysine residues to Arginine inhibits CXCL12 promoted lysosomal degradation, but did not effect CXCR4 internalization. This suggest that ubiquitination of CXCR4 is not required for internalization, but is required for degradation. The ESCRT complexes, via their ubiquitin interacting motifs or domains recognize ubiquitinated CXCR4, cluster and sort CXCR4. Others, and our lab, have shown that CXCR4 follows a canonical ESCRT sorting pathway, as siRNA depletion of ESCRT-I, II, III and Vps4 AAA ATPase inhibits CXCR4 lysosomal degradation (Malerod et al., 2007; Malik and Marchese, 2010; Marchese et al., 2003; Valiathan and Resh, 2008; Verma and Marchese, 2015).

**ENDOSOMAL SORTING COMPLEX REQUIRED FOR TRANSPORT**

The ESCRT machinery was first discovered in *Saccharomyces cerevisiae* as vesicular protein sorting (Vps) proteins. These studies show that mutation of Vps protein could no longer sort proteins into vacuoles (yeast lysosome) (Banta et al., 1988; Raymond et al., 1992; Robinson et al., 1988). Further studies discovered different complexes of this pathway and this led to discovery of a ubiquitin-dependent protein
sorting pathway in yeast (Asao et al., 1997; Babst et al., 2002; Babst et al., 1997). These highly conserved protein complexes were then identified in mammals and are known to sort GPCRs and Receptor tyrosine kinases (RTKs) (Bache et al., 2003a; Bache et al., 2003b; Lu et al., 2003; Raiborg et al., 2002). There are five distinct ESCRT protein complexes ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III, AAA-ATPase Vps4 complex and accessory proteins. These complexes identify a ubiquitin moiety on the receptor and are sequentially recruited on the endosome membrane to sort receptors.

**ESCRT-0**

The sorting pathway starts with recruitment of ESCRT-0 to the endosomal membrane. ESCRT-0 is comprised of two subunits Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) or Vps27 (yeast) and STAM1/2 (signal transducing adaptor molecule-1/2) or Hse (Yeast). The two subunits occur in 1:1 ratio via an interaction with the coiled-coil GAT domain (Asao et al., 1997; Prag et al., 2007). The Hrs subunit has a FYVE (Fab-1, YGL023, Vps27 and EEA-1) zing finger domain that has affinity for phosphatidylinositol-3-phosphate (PI3P) lipid rich on endosomal membrane. FYVE-PI3P interactions recruit the ESCRT-0 to the endosomal membrane (Raiborg et al., 2001), where it recognizes and clusters ubiquitinated membrane cargo by the ubiquitin interacting motif (UIM) present in both Hrs and STAM1 (Bache et al., 2003a; Mizuno et al., 2003; Raiborg et al., 2002). Hrs also interacts with the UEV domain on the ESCRT-I complex subunit Tsg101 (Vps23) via its PTAP-like motif (Katzmann et al., 2003; Lu et al., 2003). Recruitment of ESCRT-0 and ESCRT-I is
necessary for starting the MVB sorting pathway. The Physiological importance of ESCRT-0 is demonstrated by knockout studies in mice. ESCRT-0 null mice (Hrs -/- or STAM1/2-/-) die early at embryonic stage (E) E11 of severe developmental and morphogenesis defects. Hrs null mice die due to significant apoptosis and abnormally enlarged early endosomes (Komada and Soriano, 1999; Yamada et al., 2002)

**ESCRT-I**

ESCRT-I complex is recruited to the endosomes by ESCRT-0 to further cluster the ubiquitinated cargo and also to recruit ESCRT-II complex on the endosomes. ESCRT-I complex is a stable heterotetramer of Tsg101 (Tumor susceptibility gene 101) or Vps23 (yeast), Vps28, Vps37 and Mvb12 (Multivesicular body 12) present in 1:1:1:1 ratio (Chu et al., 2006; Curtiss et al., 2007; Katzmann et al., 2001). There are four isoforms of Vps37 (A-D) and three isoforms of Mvb12 (Mvb12A, Mvb12B and UBAP1) that can assemble in distinct ESCRT-I complexes (Bache et al., 2004; Bishop and Woodman, 2001; Eastman et al., 2005; Morita et al., 2007). Tsg101 and Mvb12 have UEV (Ubiquitin Enzyme Variant) and ubiquitin binding (UBD) domain respectively to interact with ubiquitinated cargo (Shields et al., 2009; Sundquist et al., 2004). ESCRT-I complex protein Vps28 also interacts with the GLUE (GRAM-like ubiquitin binding in EAP45) domain of ESCRT-II protein Vps36 to recruit ESCRT-II complex to the endosomes (Gill et al., 2007; Teo et al., 2006). Tsg101 is also known to interact with accessory protein ALIX (Segura-Morales et al., 2005). Physiologically, Tsg101 is also necessary during embryogenesis, as knockout mice of Tsg101 die as soon as E6.5.
Tsg101 was found to be essential for proliferation before gastrulation (Brown et al., 2009).

**ESCRT-II**

Like ESCRT-I, ESCRT-II is also a stable heterotetrameric complex composed of three proteins with one subunit of each Vps22 (EAP30), Vps36 (EAP45) and 2 subunits of Vps25 (Babst et al., 2002; Langelier et al., 2006). ESCRT-II is recruited to the endosomes by interaction with ESCRT-I and also by ESCRT-II/lipid interactions. ESCRT-II protein Vps36 has a GLUE domain that binds endosomal membrane lipid PI3P with high affinity (Slagsvold et al., 2005). Like ESCRT-0 and ESCRT-I, ESCRT-II is also shown to bind ubiquitin. ESCRT-II subunit Vps25 interacts with the ESCRT-III subunit Vps20/CHMP6 (Charged multi-vesicular body protein 6) to recruit and initiate ESCRT-III complex formation on the endosomes (Yorikawa et al., 2005).

![Figure 2: The Endosomal Sorting Complex Required for Transport pathway](image-url)
ESCRT-0 subunit Hrs recognizes ubiquitinated cargo via its ubiquitin binding motifs and is recruited to the PI3P rich endosomal membrane via its FYVE domain. ESCRT-0 recruits ESCRT-I via its interaction with ESCRT-I. ESCRT-I also clusters ubiquitinated cargo via UEV domain in Tsg101. ESCRT-II is recruited by an interaction with ESCRT-I and also by direct interaction of GLUE domain of ESCRT-II with PI3P on endosomal membrane. ESCRT-III is recruited by ESCRT-II to deubiquitinate the cargo and cluster the cargo in membrane buds formed by ESCRT-III. Finally Vps4 AAAATPase is recruited to provide the energy for membrane scission and disassembling the ESCRT machinery from the endosomes.

**ESCRT-III**

The main function of ESCRT-III complex is membrane scission, pinching the neck of membrane invagination to form ILVs. ESCRT-III complex do not have ubiquitin binding domains but recruit deubiquitinating enzyme to deubiquitinate the cargo. ESCRT-III complex does not form a stable complex, but only transiently assembles on endosomes. ESCRT-III is composed of four core proteins: Vps20/CHMP6, Snf7/CHMP4(A-C), Vps24/CHMP3 and Vps2/CHMP2(A,B) and three accessory proteins Did2/CHMP1(A,B),Vps60/CHMP5 and Ist1(Babst et al., 2002). ESCRT-III core subunits exist in a stable autoinhibitory, or closed monomer state, by interaction between negatively charged carboxy terminus and a positively charged N-terminus of each subunit (Shim et al., 2007). ESCRT-II protein Vps25 interacts with Vps20 and activates and recruits the ESCRT-III complex and promotes ESCRT-III assembly on endosomes (Teo
et al., 2004). Once Vps20 is on the endosomes, it recruits Snf7, which forms homooligomers. Vps24 recruitment is supposed to cap these Snf7 polymers (Saxena et al., 2008; Teis et al., 2010). Snf7 polymers are also stabilized by ESCRT-III adaptor protein Bro1/Alix (apoptosis lined gene-2 interacting protein-X), which then recruits deubiquitinating enzyme Doa4 essential for removing ubiquitin moiety of cargo protein (Luhtala and Odorizzi, 2004; Odorizzi et al., 2003). Vps2 is finally recruited to complete the ESCRT-III complex assembly. The role of the accessory proteins Ist1, Vps60 and Did2 is to mediate ESCRT-III and Vps4 interaction and promote ATP hydrolysis (Azmi et al., 2008; Yu et al., 2008). ESCRT-III complex subunits are also important during embryogenesis as CHMP4B/mSnf7-2 and CHMP5 knockout mice die early in embryonic day E8 and E11 respectively.

**Vps4-AAATPase complex**

The main function of this complex is catalyzing the dissociation of the ESCRT complexes from the endosomal membrane for the next cycle of cargo sorting. The Vps4 complex is composed of type-I AAA-ATPase Vps4 and its associated co-factor Vta1. ESCRT-III and accessory proteins recruit the inactive Vps4 complex to endosomes where it binds Vta1 and is activated. Vta1 binding enhances the AAA-ATPase activity of enzyme that uses ATP to dissociate ESCRT-III from the membrane (Babst et al., 1998; Scott et al., 2005; Yu et al., 2008).
OTHER BIOLOGICAL ROLES OF ESCRT MACHINERY

Apart from MVB biogenesis and sorting ubiquitinated membrane proteins, ESCRT machinery is also involved in other topologically similar membrane deforming and scission processes like cytokinesis and retroviral budding. ESCRT machinery also functions in cell signaling and is also associated with pathological condition like cancer.

Role of ESCRTs in Cytokinesis

ESCRT machinery is required for executing membrane scission, the final step of cytokinesis. ESCRT-I protein Tsg101 and accessory protein Bro1/Alix are known to be recruited to the midbody in the late stage of cell division by Cep55, a multimeric cell division protein necessary for late stage cell division. This facilitates recruitment of ESCRT-III and Vps4 for abscission of two daughter cells (Carlton and Martin-Serrano, 2007; Spitzer et al., 2006). An important step during cytokinesis is microtubule disassembly; studies show that ESCRT-III complex subunit CHMP1B recruits another AAA-ATPase spastin that severs the microtubules (Yang et al., 2008). Also for constriction during abscission, protein filaments are formed and they spiral around the constriction zone, Tsg101 and CHMP4B localize with and are important for formation of these protein filaments (Guizetti et al., 2011). Recently the role of ESCRT-II in cytokinesis was also elucidated. These studies show that ESCRT-II complex subunits Vps22 and Vps36 localize with CHMP6 on the intracellular bridge during the abscission step in cytokinesis (Goliand et al., 2014). In all, all the ESCRT-I, II, III and Vps4 complexes are involved in cytokinesis except ESCRT-0. Although ESCRT-0 is shown to
localize to the midbody, the exact role of ESCRT-0 in this process is not determined (Mukai et al., 2008).

**Role of ESCRTs in Retroviral budding**

ESCRTs are also implicated in release of many enveloped viruses from the host cell. Several viruses including Human Immunodeficiency Virus (HIV), filovirus, rhabdovirus contain short peptide motif essential for release of viruses from the cell membrane at the late stage called late domain or L domain. Mutation of this domain results in fully formed virus attached to the plasma membrane (Craven et al., 1999; Harty et al., 2000; Harty et al., 1999). ESCRT-I, III and accessory proteins are known to be important for viral budding. ESCRT proteins directly interact with late domain containing proteins of viruses (Carpp et al., 2011; Martin-Serrano et al., 2001; Urata et al., 2007; Wirblich et al., 2006). For example, ESCRT-I protein Tsg101 is known to directly interact with HIV PTAP late domain containing protein P6 (Demirov et al., 2002; Garrus et al., 2001). ESCRT-II is not involved in viral budding, therefore ESCRT-I protein Tsg101 binds and recruits accessory protein Bro1/Alix, which then recruits ESCRT-III and Vps4 to viral budding sites at the plasma membrane (Jouvenet et al., 2011; Odorizzi et al., 2003). Viral budding also does not involve the ESCRT-0 complex, although ESCRT-0 protein Hrs overexpression inhibits HIV budding, possibly by sequestering ESCRT-I Tsg101 (Bouamr et al., 2007). In all, viral budding is dependent on ESCRT-I, III, Vps4 and Alix and independent of ESCRT-0 and II complexes.
**Role of ESCRTs in Cancer**

Different ESCRT complexes subunits are either upregulated or downregulated in different cancer types. ESCRT-0 complex protein Hrs is upregulated in cancer types including liver, cervix, colon, stomach and melanoma. In these studies, Hrs is known to regulate levels of E-cadherin and β-catenin signaling to regulate growth and metastasis of tumor (Toyoshima et al., 2007). As for ESCRT-I, Tsg101 was initially considered as a tumor suppressor gene, but these studies were soon refuted. Nevertheless, Tsg101 is upregulated and is also associated with poor prognosis in ovarian cancer (Young et al., 2007). Tsg101 was also found to be upregulated in breast and lung cancer (Liu et al., 2010; Oh et al., 2007). On the other hand, ESCRT-I complex subunits Vps37A and UBAP1 are known to be downregulated in hepatocellular and nasopharyngeal carcinoma respectively (Qian et al., 2001; Xiao et al., 2006; Xu et al., 2003). Screening of human cancers indicating that, ESCRT-III complex subunit CHMP1A is deregulated in skin and pancreatic tumor. On the other hand, CHMP4C is overexpressed in ovarian cancer (Li et al., 2008; Nikolova et al., 2009). Vps4B is known to be downregulated in stage IV breast tumors and is associated with high levels EGFR in these tumors (Lin et al., 2012). To summarize, ESCRT-0 (Niehrs and Acebron), ESCRT-I (Tsg101) and ESCRT-I (Vps37A), ESCRT-III (CHMP4C), Vps4B are found upregulated and downregulated respectively in cancers.
**Role of ESCRTs in Cell signaling**

Apart from the primary function of ESCRT machinery in sorting ubiquitinated GPCR or RTKs, ESCRTs also functions in cell signaling. ESCRTs sort the receptor into the degradative pathway and negatively regulate the signaling of the receptor. Studies have shown that depletion of components of ESCRT-0 (Niehrs and Acebron) or ESCRT-I (Tsg101) complexes causes EGFR accumulation and prolonged MAPK/Erk1/2 pathway activation (Malerod et al., 2007; Raiborg et al., 2008). ESCRT machinery is also known to be important for signaling downstream of GPCR Frizzled receptor. Ligand Wnt binding to Frizzled activates β-catenin by inhibition of GSK3 protein kinase that phosphorylates and inhibits β-catenin. The ESCRT proteins Hrs and Vps4 are known to sequester GSK3 into multivesicular bodies to allow β-catenin signaling (Taelman et al., 2010). Other studies show that, ESCRT proteins Tsg101 and Vps4 are important for localization of active src at the focal adhesions (Tu et al., 2010). ESCRT-0 complex protein (STAM1) is also shown to regulate MAPK Erk1/2 activation in caveolae on CXCR4 activation (Malik et al., 2012). In this dissertation, we highlight a novel role of ESCRT machinery in positively regulating the Akt/mTOR pathway downstream of CXCR4 receptor. We show that the ESCRT machinery promotes degradation of the mTOR antagonist DEPTOR via lysosomes to allow mTORC2 signaling (Verma and Marchese, 2015).
ROLE OF ESCRT MACHINERY IN SORTING CYTOSOLIC PROTEINS

ESCRT machinery primarily sorts ubiquitinated transmembrane proteins into the lysosomes for degradation (Goh and Sorkin, 2013; Marchese et al., 2008; Shields and Piper, 2011). However, a few cytosolic proteins like Jun and GSK3 are also known to be sorted by the ESCRT pathway (Ikeda and Kerppola, 2008; Taelman et al., 2010). Jun is a nuclear transcription factor that is known to be ubiquitinated and localize to lysosomes. Ikeda et al shows that, Tsg101 and Hrs are localized to lysosomes with ubiquitinated Jun and mediate lysosomal degradation of ubiquitinated Jun (Ikeda and Kerppola, 2008). Taelman et al demonstrate another example of cytosolic protein sorting by the ESCRT machinery. This group shows that on activation of Frizzled receptor by Wnt, GSK3 binds the receptor ligand complex along with other proteins and is internalized and sequestered into the multivesicular bodies with the help of ESCRT proteins Hrs and Vps4. GSK3 is a protein kinase that phosphorylates and inhibits β-catenin. Therefore, ESCRT mediated sequestering of Frizzled-wnt-GSK3 complex into multivesicular bodies allows β-catenin signaling (Taelman et al., 2010). We here describe yet another cytosolic protein that can be sorted and degraded by the ESCRT machinery. We show that siRNA depletion of ESCRT-0 (STAM1), ESCRT-I (UBAP-1) and ESCRT-III (CHMP4C) inhibit lysosomal degradation of DEPTOR, an mTOR antagonist. We also show that ESCRT (Niehrs and Acebron) and ESCRT-I (Vps28) proteins interact with DEPTOR to degrade DEPTOR via lysosomes (Verma and Marchese, 2015).
CXCR4 SIGNALING

G protein dependent CXCR4 signaling

Like most chemokine receptors, CXCR4 also couples to $G_{\alpha_i}$ (inhibitory G protein), however CXCR4 coupling to other $G_{\alpha}$ proteins such as $G_{\alpha_q}$, $G_{\alpha_o}$, and $G_{\alpha_s}$ is also described in literature (Rubin, 2009). Ligand binding to CXCR4 activates numerous cell signaling pathways and the majority of these signaling events are found to be G protein dependent by the use of Pertussis Toxin (PTX). PTX inhibits $G_{\alpha_i}$ subunit activation and downstream signaling, thus serving as a helpful tool in defining CXCR4 signaling pathways (Busillo and Benovic, 2007; Kucia et al., 2004; Mangmool and Kurose, 2011). On CXCR4 activation $G_{\alpha_i}$ is activated and active $G_{\alpha_i}$ dissociates from the $G_{\beta\gamma}$ dimer. Active $G_{\alpha_i}$ inhibits adenyl cyclase and also activates Src, while the dissociated $G_{\beta\gamma}$ activates MAPK-Erk1/2, Focal adhesion proteins (Pyk2, FAK, paxillin), PI3K/Akt, PKC, PLC-γ and NF-κB (Ganju et al., 1998; Helbig et al., 2003; Neuhaus et al., 2003; Tilton et al., 2000; Wang et al., 2000). Within seconds of CXCL12 stimulation, robust phosphorylation of ERK1/2, Akt and focal adhesion components is observed (Libura et al., 2002; Majka et al., 2000; Malik et al., 2012; Verma and Marchese, 2015).
Figure 3: CXCR4 mediated signaling

CXCL12 agonist binding activates a number of G protein dependent (PLC-b, PI3K/AKT/mTOR, pERK1/2,) and G protein independent pathways (p38, pERK1/2) that ultimately regulates cellular phenotypes including migration, apoptosis and protein synthesis.
**G protein independent CXCR4 signaling**

G protein independent signaling pathways are also activated upon CXCR4 activation. CXCL12 binding to CXCR4 leads to transient interaction between CXCR4 and JAK2 and JAK3 leading to their activation by transphosphorylation. Activation of JAK2/3 is $G_\alpha$ independent and leads to activation and nuclear translocation of number of STAT proteins. This suggests that JAK-STAT activation is not $G_\alpha$-protein dependent. However, CXCR4 can couple with Gq protein, and whether JAK STAT is Gq independent is not known (Rubin, 2009; Vila-Coro et al., 1999). CXCR4 homodimerization is also shown to activate JAK/STAT pathway in a G protein independent fashion (Mellado et al., 2001). Arrestins are also important for activation of G protein independent signaling downstream of CXCR4. Arrestin-3 is important for CXCL12 mediated migration and p38 activation (Sun et al., 2002).

**CXCR4 mediated ERK1/2 activation.**

MAP kinase ERK1/2 pathway is known to be activated by both G protein dependent and independent events. Previous studies from the laboratory show a novel G protein dependent activation of ERK1/2 in response to CXCL12 stimulation in HeLa cells. These studies show that ESCRT-0 protein STAM1 and the CXCR4 E3 ubiquitin ligase, AIP4 reside with CXCR4 in caveolar microdomains to mediate CXCR4 promoted ERK1/2 phosphorylation. This is the first report of regulation of MAPK ERK1/2 pathway by the ESCRT-0 complex proteins (Malik et al., 2012). In addition to this, the current study also shows that the other ESCRT complex (I-III) proteins do not regulate the
ERK1/2 pathway. However, we show for the first time that the whole ESCRT machinery (0-III) regulates the CXCR4 promoted Akt/mTOR pathway (Verma and Marchese, 2015).

**CXCR4 MEDIATED AKT/mTOR PATHWAY**

**AKT**

Akt or protein kinase B (PKB) is a ser/thr protein kinase that is a key regulator of many cellular processes, including cell survival, metabolism, development and migration (Downward, 2004; Dummler and Hemmings, 2007; Stambolic and Woodgett, 2006). Akt belongs to the AGC (Protein kinase A,G and C) family of kinases and exists in three isoforms of Akt that are products of distinct genes- Akt1, Akt2 and Akt3. Each of the three isoforms share a conserved domain structure of N-terminal pleckstrin homology domain (PH), a kinase domain and a regulatory hydrophobic motif (Hanada et al., 2004). Akt plays pivotal role downstream of the Class 1A phosphoinositide-3-kinase (PI3K) signaling pathway. Class I PI3K can be activated by activation of cell surface receptors including GPCRs, intergrins and Growth factor/ Receptor tyrosine kinases (RTKs). PI3K activation leads to phosphorylation of phosphatidylinositol (4,5)-bisphosphate to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Hawkins et al., 1992; Stephens et al., 1991). PIP3 can be dephosphorylated by enzymes phosphatase and tensin homologue (PTEN) and SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) (Damen et al., 1996; Maehama and Dixon, 1998). Phosphoinositide-dependent kinase-1 and Akt are recruited to the membrane by an interaction between their PH domain and
PIP3 lipid (Anderson et al., 1998; Klippel et al., 1997). On the membrane, PDK1 phosphorylates Akt on residue Thr308 in its T-loop. Phosphorylation of Akt on residue T308 seems to be important for dissociation of Akt from the membrane, as studies with a T308 phosphomimetic mutant showed diminished membrane binding (Ananthanarayanan et al., 2007). Akt is also phosphorylated by mTORC2 on C-tail turn motif site T450 and hydrophobic motif S473 (Facchinetti et al., 2008; Ikenoue et al., 2008; Jacinto et al., 2006; Sarbassov et al., 2005b). Phosphorylation of Akt on S473 stabilizes Akt in an active conformational state (Yang et al., 2002). mTORC2 is the primary kinase that phosphorylates Akt on S473, as described by studies showing disrupted Akt-S473 phosphorylation in MEFs lacking mTORC2 subunits rictor, Sin1, or mLST8 (Guertin et al., 2006; Jacinto et al., 2006). However, Akt can also be phosphorylated on S473 by other kinases including TANK-binding kinase 1(TBK1) (Xie et al., 2011), integrin-linked kinase (Persad et al., 2001)(ILK), PKCα (Partovian and Simons, 2004) and ATM (Viniegra et al., 2005).

Once fully active, Akt can phosphorylate up to 100 downstream targets to regulate many cellular process such as cell mass, growth, proliferation and apoptosis.

1. Akt is a key controller of cell mass through regulating mTORC1, a machinery involved in protein translation initiation and ribosome biosynthesis. Akt regulates mTORC1 activation by phosphorylating and inhibiting two inhibitors of mTORC1, TSC2 and PRAS40. Akt phosphorylates TSC2 and inhibits the TSC1/2 complex to inhibit the small GTPase Rheb (Viniegra et al., 2005). Rheb (Ras-homolog enriched in brain) then activates mTORC1 by inhibiting negative regulator of mTORC1 called FKBP38 (Bai et
Akt also phosphorylates and inhibits PRAS40 (proline-rich Akt substrate of 40 kDa) that in turn inhibits mTORC1 by competing with Rheb (Kovacina et al., 2003; Vander Haar et al., 2007).

2. Akt inhibits apoptosis by phosphorylating and inhibiting key pro-apoptotic proteins. Akt phosphorylates BAD creating a binding site for 14-3-3 leading to sequestration of BAD (Datta et al., 1997; del Peso et al., 1997). Akt phosphorylates and inhibits FoxO mediated transcription of pro-apoptotic and cell cycle arrest genes (Biggs et al., 1999; Brunet et al., 1999). Akt is also known to phosphorylate procaspase-9 \textit{in vitro}, leading to a decrease in activity of caspase-9 (Cardone et al., 1998). Akt promotes cell survival by promoting degradation of p53 by phosphorylating the E3 ubiquitin ligase MDM2 (Mayo and Donner, 2001).

3. Akt induces cellular proliferation by inhibiting p27 (inhibitor of cyclin dependent kinases) and phosphorylating and inhibiting GSK3 induced inhibition of myc and cyclin D1 (Cross et al., 1995; Fujita et al., 2002; Guertin et al., 2006).

4. Finally, Akt also regulates cell growth and metabolism by inhibiting AS160, which induces localization of glucose transporter Glut4 to the plasma membrane, and inhibits FoxO to inhibit phosphoenol pyruvate carboxykinase and glucose-6-phosphatases leading to an increase in glucose catabolism (Burgering, 2008; Sano et al., 2003). Akt also regulates metabolism by phosphorylating and inhibiting glycogen synthase kinase 3\(\beta\) (GSK3\(\beta\)) to regulate glycogen synthesis (Cross et al., 1995).

A number of Akt binding proteins can regulate Akt activity, including phosphatases like PP2A and PH domain leucine-rich repeat protein phosphatase
PP2A and PHLPP can directly dephosphorylate S473 and T308 on Akt (Andjelkovic et al., 1996; Gao et al., 2005). Apart from phosphorylation, Akt activity can also be regulated by another posttranslational modification called ubiquitination. Two groups have shown that Akt levels can be regulated by ubiquitination and degradation. Yang et al showed that Akt undergoes a K-63 ubiquitination by the E3 ubiquitin ligase TRAF6. This group also showed that TRAF6 mediated ubiquitination of Akt is central for membrane localization and phosphorylation of Akt (Yang et al., 2009). Another study suggests that mTORC2 mediated phosphorylation of Akt on S473 targets Akt for proteasomal degradation by lysine-48 linked polyubiquitination (Wu et al., 2011).

**mTOR**

A large 290 kDa protein mTOR (The mammalian/mechanistic target of rapamycin) is an structurally and functionally conserved serine/threonine protein kinase that belongs to the phosphatidylinositol 3- kinase related protein kinase (PIKK) family (Hanks and Hunter, 1995; Miranda-Saavedra and Barton, 2007). In the 1970s, TOR was first discovered using an anti-fungal drug Rapamycin in *Saccharomyces cerevisiae* (Reiling and Sabatini, 2006; Sehgal et al., 1975; Vezina et al., 1975) and subsequently mammalian homolog of mTOR was also discovered using the yeast two hybrid system (Brown et al., 1994; Chiu et al., 1994).

Structurally mTOR consists of HEAT (Huntington, elongation factor 3, PR65/A and TOR) domain followed by FAT (FRAP, ATM and TRRAP) domain at N terminus. Between the C-terminal another FAT domains lies the kinase domain of mTOR (Huang
and Houghton, 2003). mTOR is very important in physiology as knockout mice of mTOR are primordially embryonic lethal (Gangloff et al., 2004; Martin and Sutherland, 2001; Murakami et al., 2004).

Functionally, mTOR exist in two distinct multisubunit complexes mTORC1 and mTORC2 that respond to growth factors, nutrient stresses and regulates cellular phenotypes like cell mass, growth, survival and proliferation (Guertin and Sabatini, 2007; Sarbassov et al., 2005a). As a part of mTORC1, the mTOR catalytic subunit exists with proteins like Raptor (regulatory-associated protein of mTOR), PRAS40 (proline-rich Akt substrate of 40 kDa), mLST8/GβL (mammalian lethal with Sec13 protein 8/G protein β-subunit-like protein) and DEPTOR (Peterson et al., 2009; Sabatini, 2006). On the other hand the mTORC2 complex is composed of six components, including mTOR, Rictor (rapamycin-insensitive companion of mTOR), PROTOR (protein observed with Rictor 1)/PRR5 (proline-rich protein 5), mLST8/G β L, mitogen activated protein kinase–associated protein 1 (also known as mSIN1) and DEPTOR (Sabatini, 2006). The functional significance of each subunit in mTORC1 and mTORC2 complex is not entirely understood. In the mTORC1 complex, mLST8 has been suggested to work as a signal receiver (Kim et al., 2003), whereas raptor is shown to act as a scaffold to recruit mTORC1 substrates like 4E-BP1. Also Raptor is important for amino acid sensing and sub-cellular localization of mTORC1 through regulation of RagGTPase (Hara et al., 2002; Sancak et al., 2008). In mTORC2, mLST8 is shown to be essential for integrity and kinase activity of mTOR (Guertin et al., 2006). Rictor and mSin1 that interact with each other and with mTOR are required for structural integrity and kinase activity of mTORC2.
(Jacinto et al., 2006; Wullschleger et al., 2005). The function of Rictor interacting protein Portor is yet to be studied (Pearce et al., 2007).

Both mTORC1 and mTORC2 complexes also contains DEPTOR, as a recently discovered endogenous inhibitor (Peterson et al., 2009). Apart from DEPTOR, mTORC1 complex also contains PRAS40, another endogenous inhibitor. PRAS40 inhibits mTORC1 kinase activity by directly inhibiting substrate binding to the complex (Wang et al., 2007). DEPTOR on the other hand binds directly to mTOR and inhibits its kinase activity in both mTORC1 and mTORC2 complexes. In platelets, leukemic and neural tissue, mTORC2 also contains another endogenous inhibitor called XPLN (exchange factor found in platelets, leukemic and neural tissue). XPLN interacts with Rictor and is mTORC2 specific and not present in mTORC1 (Khanna et al., 2013).

Figure 4: The mTOR complexes

mTOR exist in 2 multi subunit complexes mTORC1 (A)and mTORC2 (B). Both the complexes share common subunits like mTOR, mLST8 and DEPTOR. mTORC1 contains distinct subunits like Raptor and PRAS40, whereas, Rictor, mSIN1 and PROTOR are unique to mTORC2.
mTORC1 signaling

mTORC1 can be activated by both extracellular signals such as nutrients, hormones and growth factors like insulin and Insulin like Growth Factor (IGF-I) and intracellular nutrients such as amino acids (Fingar and Blenis, 2004; Hay and Sonenberg, 2004). Upon activation, IGF-1 phosphorylates IRS1-4 (insulin receptor substrates 1-4), which leads to activation of PI3K (Valentinis and Baserga, 2001). PI3K catalyzes formation of PIP3 from PIP2 on the plasma membrane (Hawkins et al., 1992; Stephens et al., 1991). PIP3 recruits PH domain containing proteins like PDK1 and Akt on the membrane. On the membrane Akt is phosphorylated on T308 by kinase PDK1 followed by phosphorylation of Akt on residues S473 by mTORC2 for full activation (Sarbassov et al., 2005b). Fully active Akt activates mTORC1 by phosphorylating and inhibiting a mTOR repressor TSC1/2 complex (Huang and Manning, 2009; Tee et al., 2002). Upon activation, mTORC1 regulates cell growth, survival, proliferation and motility by phosphorylating and activating two best characterized effectors S6K1 and 4E-BP1. Active S6K1 phosphorylates 40S ribosomal subunits and promotes translation initiation (Jefferies et al., 1994; Terada et al., 1994). Active 4E-BP1 causes release of eIF4E from 4E-BP1, which then associates with eIF4G and other relevant factors to enhance translation (Marcotrigiano et al., 1999; Pause et al., 1994).

Localization of mTORC1 to different sub-cellular locations is also important for it’s signaling. Recent studies show that in response to amino acids, mTORC1 localizes on lysosomes. High levels of amino acids recruit mTORC1 on the lysosomes, where mTOR
is activated by GTP bound small GTPase Rheb (Ras homolog enriched in brain) (Saucedo et al., 2003). Recruitment of mTOR on lysosomes in response to amino acids may also require the presence of Rag GTPase on lysosomes. Meanwhile, the TSC1/2/TBC1 complex on the lysosomes that regulates Rheb GTPases is inhibited in the presence of growth factors thus promoting mTORC1 activity (Huang et al., 2008; Sancak et al., 2010; Sancak et al., 2008). In one study, mTORC1 is shown to sense amino acids independent of TSC2/Rheb axis. In these studies hVps34, a class III PI3K, is shown to signal amino acid availability to mTORC1 (Byfield et al., 2005; Nobukuni et al., 2005). Thus, different input signals may regulate activation of mTORC1 in cells.

**mTORC2 signaling**

Unlike mTORC1, signaling pathways that activate mTORC2 are not well characterized. mTORC2 is known to be a rapamycin-insensitive companion of mTOR, although studies in some cell lines show mTORC2 inhibition upon prolonged treatment with Rapamycin (Sarbassov et al., 2006). Growth factor and insulin stimulation can activate mTORC2, which then directly phosphorylates hydrophobic motif site S473 of an important survival kinase Akt (Hresko and Mueckler, 2005; Sarbassov et al., 2005b). Like Akt, mTORC2 is also shown to phosphorylate other protein kinase of the AGC family, serum glucocorticoid-induced kinases SGK1 on hydrophobic motif site S422 (Garcia-Martinez and Alessi, 2008) and PKCα on hydrophobic motif site S657 (Ikenoue et al., 2008). mTORC2 also constitutively phosphorylates site T450 on Akt during translation upon growth factor stimulation and PI3K activation. mTORC2 is known to
localize to translating ribosomes on endoplasmic reticulum on PI3K activation and phosphorylate Akt on T450 (Oh et al., 2010; Zinzalla et al.).

mTORC2 regulates cellular process like cell survival, proliferation, metabolism by regulating the phosphorylation status of Akt. Fully active Akt phosphorylates a number of downstream targets like FoxO1/3a. FoxO1/3a enters the nucleus and promotes transcription of pro-apoptotic genes like Bim1 (Gilley et al., 2003). Phosphorylation by Akt, promotes 14-3-3 binding proteins to bind FoxO, resulting in translocation of FoxO from nucleus to the cytoplasm leading to transcriptional inactivation. Hence, Akt promotes cell survival by phosphorylating and inhibiting FoxO1/3a (Xie et al., 2012). mTORC2 also regulates the actin cytoskeleton, possibly by promoting phosphorylation of PKCα and paxillin and its localization to focal adhesions (Jacinto et al., 2004; Sarbassov et al., 2004). However, the exact mechanism by which mTORC2 components regulate actin cytoskeleton is yet to be studied.

**DEPTOR**

DEPTOR, also known as DEPDC6 (DEP Domain Containing protein-6), is a 48 kDa mTOR interacting protein that is present only in vertebrates. DEPTOR protein contains two N-terminal DEP (Dishevelled, Egl-10, Pleckstrin) domains (Chen and Hamm, 2006) and a PDZ (post synaptic density 95, Discs large, Zonula) (Jemth and Gianni, 2007) domain at the C-terminal. DEPTOR interacts with mTOR via its PDZ domain, while the function of the two DEP domains in DEPTOR is still not studied. DEPTOR interacts specifically with the C-terminal FAT domain of mTOR and exist as a
part of both mTORC1 and mTORC2 complexes. shRNA Depletion of DEPTOR increases both mTORC1 and mTORC2 signaling and *in vitro* kinase activity of both complexes. Also DEPTOR overexpression inhibits only mTORC1 signaling and activates mTORC2 mediated signaling via suppression of the mTORC1/S6K pathway. This suggests that DEPTOR is an endogenous inhibitor of mTOR that inhibits mTOR kinase activity in both mTORC1 and mTORC2 complexes (Gao et al., 2011; Peterson et al., 2009).

*Regulation of DEPTOR*

DEPTOR levels are regulated by proteasomal degradation. It has been shown that in response to serum stimulation, DEPTOR is degraded via proteasomes, as treatment of cells with proteasome inhibitor inhibits serum induced DEPTOR degradation. Serum induced DEPTOR degradation relieves an inhibition from mTORC1 and mTORC2. This was elucidated by increase in phosphorylation of S6K and Akt, downstream substrates of both mTORC1 and mTORC2 respectively upon shRNA depletion of DEPTOR (Peterson et al., 2009). Peterson et al also show that DEPTOR levels can be negatively regulated at both transcriptional and post transcriptional level by mTORC1 and mTORC2 complexes. These studies show that shRNA depletion of either mTORC1 or mTORC2 complexes elevate DEPTOR mRNA and protein levels even in presence of serum. Also by mass spectroscopy analysis of DEPTOR from serum stimulated cells show that DEPTOR contains 13 possible mTOR phosphorylation sites and that mutation of these sites prevents DEPTOR degradation in response to serum treatment. This suggests that mTOR
negatively regulates DEPTOR levels both at mRNA levels and by phosphorylation at protein level (Peterson et al., 2009).

Recent studies from three groups have highlighted molecular mechanisms that regulate DEPTOR degradation upon serum stimulation. These studies show that DEPTOR levels in cells are controlled by (SKP1, Cullins, F-box proteins) SCF^{Cullins-β-TrCP} E3 ubiquitin ligase (Duan et al., 2011; Gao et al., 2011; Zhao et al., 2011). β-TrCP is a F-box family protein that forms a multi-subunit SCF-type of E3 ubiquitin ligase complex (Deshiaies and Joazeiro, 2009; Nakayama and Nakayama, 2006). Substrates that are ubiquitinated by β-TrCP typically have a DSGxxS degron sequence however, DEPTOR has a variant of the typical β-TrCP degron sequence DpSGxxpS that are also reported in other β-TrCP targets (Frescas and Pagano, 2008). These studies report that, DEPTOR is phosphorylated by kinases like mTOR, CKI (Casein Kinase I), RSK1 and S6K1 on the degron motif and that phosphorylation of the degron motif is important for DEPTOR interaction with β-TrCP, its ubiquitination and subsequent degradation through the proteasome (Duan et al., 2011; Gao et al., 2011; Zhao et al., 2011). Regulation of DEPTOR in response to GPCR stimulation is not ventured yet. We show for the first time that upon GPCR activation, DEPTOR is degraded through the lysosomes as soon as 3 hr. We show that in response to GPCR CXCR4 activation, the ESCRT machinery that targets CXCR4 for degradation also regulates DEPTOR degradation.
Biological role of DEPTOR

Role of DEPTOR in cancer

DEPTOR is considered as a tumor suppressor, as it inhibits mTOR signaling that is frequently hyperactivated in many human cancers. Therefore DEPTOR is downregulated in many cancer types (Duan et al., 2011; Gera and Lichtenstein, 2011). However, DEPTOR is upregulated in human multiple myelomas (Peterson et al., 2009). DEPTOR is localized on chromosome locus 8q24, genomic region in this locus is found to be upregulated and is associated with poor prognosis in breast cancer, prostate cancer, chronic myeloid leukemia and lung cancer (Chin et al., 2007; Duan et al., 2011; Joos et al., 2003; van Duin et al., 2005a; van Duin et al., 2005b). DEPTOR is also known to be upregulated in thyroid cancer and DEPTOR expression is also associated with distant metastasis of thyroid cancer. Thyroid cancer patients with overexpressed DEPTOR also show poor prognosis, earlier recurrence and poor survival (Noske et al., 2011). In a study with 581 human multiple myeloma (MM) samples, mRNA level of DEPTOR was found to be upregulated in 28% of multiple myelomas. Multiple myelomas are grouped in hyperdiploid MMs or non-hyperdiploid MMs. Hyperdiploid MMs mostly contain trisomies of chromosome 8 and non-hyperdiploidal MMs are associated with translocation involving Cyclin D1/D2 or c-MAF/MAFB transcription factors (Bergsagel et al., 2005). DEPTOR mRNA was found to be upregulated in non-hyperdiploid MMS with c-MAF or MAFB translocation. Upregulated DEPTOR levels inhibit mTORC1 signaling and promote cell survival in these cells via activation of PI3K/Akt pathway (Peterson et al., 2009). A recent study also highlights an alternative mTORC1 independent cell survival
pathway adapted by multiple myelomas overexpressing DEPTOR. Depletion of DEPTOR in these cells lead to induction of caspase and PARP dependent apoptosis by upregulation of inhibitors of cyclin dependent kinases p21 and p27. Depletion of DEPTOR is also known to inhibit miRNA for p21 and p27 (Yang et al., 2014). These studies present DEPTOR as a prognostic marker in different cancer types.

*Role of DEPTOR in Epithelial to mesenchymal transition*

EMT is an important process for cancer metastasis characterized by loss of epithelial features and adaptation of elongated fibroblast features. EMT involves increased cell migration and invasion by actin reorganization and loss of cell-cell adhesion and polarity (Larue and Bellacosa, 2005). EMT shows decreased epithelial markers like E-cadherin and increased expression of mesenchymal markers including fibronectin, snail, slug and vimentin (Larue and Bellacosa, 2005). DEPTOR is known to play a role in EMT of human lung, breast and prostate cancer cells. These studies show that DEPTOR depletion induces EMT by increasing expression of mesenchymal markers snail and N-cadherin. DEPTOR depletion also reduces E-cadherin epithelial markers and increased Akt/GSK3β pathway (Chen et al., 2012b).

*CXCR4 mediated PI3K/Akt pathway*

CXCR4 is known to activate PI3K/Akt pathway and regulate cell growth, proliferation and chemotaxis in different cell types (Barbero et al., 2003; Fox et al., 2002). However, the exact mechanism by which CXCR4 activates the PI3K/Akt/mTOR
pathway is still not entirely clear. CXCR4 is known to activate Akt via PI3Kβ, as the TGX-221 inhibitor that specifically inhibits p110β subunit in PI3Kβ in completely abrogates CXCR4 promoted Akt activation in MNK-45 gastric cancer cells. In these studies, receptor CXCR4 was also shown to interact with p110β subunit upon 20s of CXCL12 stimulation in MNK45 cells, suggesting that the receptor itself activates PI3K upon CXCL12 stimulation (Chen et al., 2012a). However, others and we show that PI3K/Akt activation on CXCL12 stimulation requires Gα and Gβγ subunits. As Gα and Gβγ subunit inhibitors PTX and gallein/βArk-CT respectively inhibit CXCR4 mediated Akt activation. In mature dendritic cells, CXCR4 is shown to activate PI3Kα or PI3Kγ, as inhibitors inhibiting only PI3Kα or PI3Kγ resulted in inhibition of CXCL12 promoted Akt activation. In these cells, CXCR4 is shown to not to involve PI3Kβ for Akt activation (Delgado-Martin et al., 2011). mTOR is a major effector of PI3K/Akt pathway and CXCR4 activation is shown to activate mTORC1 and mTORC2, as measured by phosphorylation of TSC2, mTOR, S6K, 4EBP-1 and Akt on CXCR4 activation (Delgado-Martin et al., 2011; Dillenburg-Pilla et al., 2015; Dutt et al., 1998; Sotsios et al., 1999). CXCR4 promoted mTORC1 activation is shown to be important in CXCR4 mediated chemotaxis in gastric carcinoma cells. However, the exact mechanism of CXCR4 promoted PI3K/Akt/mTOR activation is not clear. In current theses, we define a novel ESCRT machinery mediated Akt/mTOR pathway activation.
Figure 5: CXCR4 mediated Akt signaling

Agonist CXCL12 binding to CXCR4, leads to activation and dissociation of active Gα subunit from the Gβγ dimer. Gβγ dimer activates PI3K that produces PI3P on the plasma membrane. Akt is recruited to the membrane and is fully activated by phosphorylation by PDK1 and mTORC2. Active Akt phosphorylates downstream targets TSC2, GSK3β and FoxO1/3a. Akt mediated phosphorylation of TSC2, inhibits it and relieves an inhibition from mTORC1, leading to mTORC1 activation. Akt pathway is regulated by DEPTOR, which is an endogenous inhibitor of mTORC1 and mTORC2.
RATIONALE

CXCR4 signaling is very important during development of organ, vasculature and immune system. Deregulated CXCR4 signaling is also implicated in pathological conditions including cancer. CXCR4 promoted Akt signaling is also implicated in tumor cell invasion, migration and angiogenesis. CXCR4 mediated Akt signaling is also responsible for migration of bone marrow stromal cells towards the infarcted myocardium. However, molecular mechanisms regulating CXCR4 promoted signaling is not entirely understood.

Akt is a serine threonine kinase that is important for many cellular functions, including cell survival, proliferation and cell motility. To be fully activated Akt is phosphorylated on T308 and S473 by kinase PDK1 and mTORC2 respectively. mTORC2 is a multi-subunit complex containing an endogenous inhibitor of mTOR called DEPTOR. Molecular mechanisms regulating DEPTOR and thus mTOR signaling in response to CXCR4 are currently not known. In response to serum, DEPTOR is phosphorylated, ubiquitinated and degraded via the proteasomes in response to serum treatment. We set out to understand mechanisms that regulate DEPTOR and mTORC2/Akt pathway in response to CXCR4 using the following aims.

1. Regulation and mechanism of DEPTOR degradation in response to CXCR4 activation

2. To understand link between CXCR4 promoted DEPTOR degradation and Akt activation

3. Role of ESCRT machinery in regulation of CXCR4 Akt signaling
4. Role of ESCRT machinery in regulation of Akt signaling in response to GPCR and RTK activation.

This study will help us understand mechanisms that regulate Akt/mTOR pathway downstream of CXCR4. This will help us better understand diseases caused by dysregulated CXCR4 signaling.
CHAPTER 2
MATERIALS AND METHODS

CELL LINES
The human cervical cancer cell line (Jacoby et al.) was from the American Type Culture Collection (Manassas, VA). The bovine aortic endothelial cell line was kindly provided by Dr. Matthias Majetschak. The human embryonic kidney cell line HEK293) was from Microbix (Toronto, ON). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 4500 mg/L glucose and lacking sodium pyruvate and antibiotics (HyClone Laboratories) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories) at 37°C and 5% CO₂. Cells were grown to 90-95% confluency and passed every second day. All cell culture work was performed using a Forma class II, A2 Biological safety cabinet (Biosafety Hood) from Thermo Electron Corporation (Waltham, MA). For passaging, cells grown to 90-100% confluency in 10-cm dishes were removed from the incubator and medium was aspirated and replaced with 10 mL warm sterile phosphate buffered saline (PBS) (Hyclone). PBS was immediately aspirated followed by adding 2 mL of warm 0.05% trypsin-EDTA (1x; Gibco) and incubating at 37°C for 5 min (minutes). To terminate trypsin reactions, 4 mL of warm complete-DMEM media was added and quickly mixed. Cells were collected and divided
equally into three 10-cm tissue culture petri dishes containing 8 mL of warm complete DMEM. Dishes were rocked back and forth to evenly disperse cells followed by incubation at 37°C.

For long-term storage, cells grown to 100% confluency on 10-cm dishes were detached from the dish surface, as described in the preceding paragraph, followed by the addition of 4 mL complete DMEM to terminate the trypsin reaction. Cells were carefully mixed and transferred to a 15-mL conical tube, followed by centrifugation using Jouan GR412 centrifuge at room temperature at 1000 rpm for 5 min to pellet cells. The supernatant was carefully aspirated and cells were carefully resuspended in 1 mL warm complete DMEM containing 5% DMSO by gently pipetting up and down 5 times. Cells were transferred to a cryogenic vial, which was then immediately placed on a bed of dry ice for approximately 30 min to allow contents to freeze. Frozen vials of cells were placed in cardboard boxes and stored in liquid nitrogen.

**ANTIBODIES, REAGENTS AND DNA CONSTRUCTS**

A list of all the reagents, antibodies, siRNA and DNA constructs used in this study is arranged in Tables (3-10)

*Reconstitution of siRNA*

Table 8 lists all the siRNA purchased for this dissertation project. Stock siRNA purchased with 10 nmol or 20 nmol concentration was resuspended in 1× siRNA buffer. First, 100 µL of 5× siRNA buffer was diluted in 400 µL RNase free water (Dharmacon). 500 µL and 1000 µL of 1× siRNA buffer was then added to 10 nmol and 20 nmol stock
siRNA tube respectively to achieve a final concentration of 20 µM. Tube was placed on the multipurpose rotator (Barnstead Lab line) for 1 hr at RT (room temperature) with occasional vortexing. After thorough mixing, 20 µL of siRNA was aliquoted in 0.5 mL microcentrifuge tubes. Tubes were labeled with siRNA name, date and stored at -20°C.

**Preparation of Plasmid DNA**

Plasmid DNA purchased from Addgene was received as DNA transformed bacterial stabs in Luria Bertani agar (LB). Bacteria from the stabs was streaked on the LB agar plate containing appropriate antibiotic (100 µg/mL ampicillin or 50 µg/mL kanamycin). Plates were incubated at 37°C for 16 hr. Isolated colonies were picked and inoculated into 3 mL LB broth with appropriate antibiotic for 5-6 hr at 37°C at 225 rpm speed shaker incubator. After 6hr, 3 mL of culture was added to 250 mL LB broth with antibiotic for 16-18 hr at 37°C in a 225 rpm speed shaker incubator. Culture was transferred to sterile 500 mL centrifuge bottles and bacteria were pelleted by centrifugation at 4°C for 20 min at 3500 rpm using Beckman JS4.2 centrifuge.

Media was carefully decanted and the bottle containing pellet was placed on ice and plasmid DNA was extracted using Qiagen HiSpeed plasmid Maxi kit protocol. Briefly, genomic DNA is sheered and cells were lysed using buffers (P1, P2 and P3) followed by pouring the lysate in QIAfilter Cartridge for 10 min. Cleared lysate was then applied to HiSpeed filter tip followed by elution of DNA. Eluted DNA was precipitated with isopropanol and applied to QIAprecipitator module followed by washing the module twice with 70% ethanol. DNA is eluted into microcentrifuge tubes using TE DNA elution
buffer provided in the kit. DNA concentration was measured using Nanodrop. Typically the 260/280 absorbance was approximately 1.8. Microcentrifuge tubes were labeled with construct name, date and concentration in µg/ mL and stored at 4°C in cardboard boxes.

**Preparation of glycerol stocks**

For long term storage of plasmid DNA, bacterial glycerol stocks in 20% glycerol were prepared. 800 µL of overnight culture used for plasmid DNA extraction, was mixed with 200 µL of 100% autoclaved glycerol in a cryogenic tube. After mixing the tube 4-5 times, tube was placed on ice and immediately stored at -80°C.

**TRANSFECTION OF PLASMID DNA USING PEI TRANSFECTION REAGENT.**

**Preparation of transfection reagent Polyethylenimine (PEI)**

1 mg/mL stock of Polyethylenimine (PEI) from Polysciences.Inc was made by dissolving 10 mg PEI in 3 mL of 100% ethanol by vortexing and incubating at 37°C for 10 min. A final volume of 10 mL was achieved by adding 7 mL of RNAse free water (Dharmacon). PEI was filtered using 0.2 µM syringe filter (Fischer brand) and 100 µL aliquots were made and stored at -80°C. An aliquot of PEI was thawed before use and re-frozen after use at -20°C. Re-frozen aliquots were only used one more time.
Transfecting cells using Polyethylenimine (PEI)

DNA transfections were carried out in 6-well, 6-cm or 10-cm dishes. In each case, 2 µL of PEI was used for every 1 µg of DNA. In the biosafety hood, DNA was aliquoted into microcentrifuge tubes, followed by adding 250 µL of warm Opti-MEM (Reduced Serum Minimum Essential Media, Gibco) and incubating for 5 min at RT. In another microcentrifuge tube, appropriate volume of PEI was aliquoted into microcentrifuge tube followed by adding 250 µL of Opti-MEM. This PEI/Opti-MEM mixture was incubated for 5 min at RT. After the incubation, the DNA-Opti-MEM mixture was added slowly and drop-wise to the microcentrifuge containing the PEI and Opti-MEM mixture and incubated for 20 min at RT. During this incubation, media on cells to be transfected was aspirated and replaced with warm complete-DMEM. After 20 min, the DNA-PEI-Opti-MEM mixture was added slowly to the cells drop wise. Plates were incubated at 37˚C until experiment.

TRANSFECTION OF SIRNA USING LIPOFECTAMINE TRANSFECTION REAGENT

Transfecting cells using Lipofectamine 2000

Lipofectamine 2000 (LP2000) was purchased from Invitrogen (Carlsbad, CA). As per the manufacturer’s protocol, for every 10 pmol of siRNA 1 µL of LP2000 was used. In the biosafety hood, siRNA was aliquoted into a microcentrifuge tube followed by addition of 250 µL of Opti-MEM for 5 min at RT. In another microcentrifuge tube, an appropriate volume of LP2000 was incubated with 250 µL of Opti-MEM for 5 min at
RT. After 5 min, the siRNA-Opti-MEM mixture was added slowly and dropwise to the microcentrifuge tube containing the LP2000-Opti-MEM mixture and incubated for 20 min at RT. During this incubation, the media on cells to be transfected was aspirated and replaced with warm complete-DMEM. After 20 min, the DNA-LP2000-Opti-MEM mixture was added slowly to the cells drop wise. Plates were incubated at 37˚C for 48 hr for a typical signaling experiment.

**Transfecting cells using Lipofectamine 3000**

Lipofectamine 3000 (LP3000) transfection kit containing p3000 and LP3000 was purchased from Invitrogen (Carlsbad, CA). LP3000 was used to transfect siRNA in 6-well or 6-cm dishes. As per the manufacturer's protocol, for every 1 µg of DNA 2 µL of P3000 was used. Typically for transfection in 6-well dishes, siRNA was aliquoted in sterile microcentrifuge tubes followed by addition of 2 µL of p3000 and 125 µL of Opti-MEM. In a separate microcentrifuge tube, 3.75 µL of LP3000 was aliquoted followed by addition of 125 µL of Opti-MEM and incubation for 5 min at RT. In the mean time, media on the cells to be transfected was replaced by warm complete DMEM. After 5 min incubation, the siRNA-P3000-Opti-MEM mixture was added slowly drop wise to microcentrifuge tube containing LP3000-Opti-MEM mix and incubated for 5 min at RT. Post incubation the whole mixture was added to the cells drop wise followed by incubation of cells at 37˚C for until before experiment. For transfections in 6 cm dishes, 6 µL of LP3000 was used.
STIMULATION WITH LIGAND

Several ligands were used for stimulation of cells including CXCL12 (10 nM), Norepinephrin (10 mM), EGF (100 ng/mL), Insulin (50 nM) and FBS (10%) (Table 6). To stimulate cells with CXCL12, 1 mL of ligand from a stock solution of 10 mM made in 0.1% BSA in PBS was directly added to wells of a 6-well plate containing 1 mL of media to give a final concentration of 10 nM. Plates were gently rocked back and forth to evenly spread the ligand throughout the plate. Plates were incubated at 37°C for different time points with the ligand depending on the experiment. Typically, cells were incubated with the ligand for 5 min or 3 hr for a signaling or DEPTOR degradation experiment, respectively. Similarly, to achieve a final concentration of 100 ng/mL of EGF, 1 mL of ligand from a stock solution of 100 mg/mL made in 0.1% BSA in PBS was directly added to wells of a 6-well plate containing 1 mL of media. 100 mL of FBS from a stock of 100% FBS was added directly to 1 mL of media in a 6-well plate to achieve a 10% FBS concentration. Similarly, 50 nM final concentration of Insulin was achieved by adding 5 mL from the 10 mM stock tube.

SDS-PAGE AND IMMUNOBLOTTING

Preparation of SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

Samples were analyzed by SDS-PAGE followed by immunoblotting. First, 10% SDS-PAGE gels were custom made with a 10% resolving and 3% stacking gels using the recipe listed in Tables 1 and 2. Bio-Rad MiniProteon 3 gel casting system containing 0.75
mm/ 1 mm spacer and small plate was used for all SDS-PAGE. Typically, 0.75 mm spacer plate and small plates are assembled using clamp assemblies and gasket in a gel-casting stand. The 1 mm spacer plates were used to run gels that required larger sample amounts (up to 30 µL) for analysis. After mixing all the reagents in the order listed in Table 1, the resolving gel was slowly pipetted in the gel cassette followed by adding 1 mL of isopropanol to ensure a flat surface for the resolving gel. The resolving gel was typically polymerized within 20 min upon casting. The isopropanol was washed out with water and residual water was removed using filter paper. The 3% stacking gel was carefully pipetted on top of the resolving gels and immediately a 10- or 15-well comb was set in place for 20 min. Once polymerized the comb was removed and gel was placed in a buffer tank containing 1× SDS Running buffer (25 mM Tris Base, 192 mM Glycine, 0.1% SDS).

Table 1: 10 % SDS-PAGE separating gel recipe.

<table>
<thead>
<tr>
<th>10% separating gel</th>
<th>1 gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75M Tris HCl pH 8.8</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>10% SDS</td>
<td>150 µL</td>
</tr>
<tr>
<td>30% Acrylamide-0.8%Bisacrylamide(@ 4°C)</td>
<td>1.67 mL</td>
</tr>
<tr>
<td>10% Ammonium per sulfate (@ 4°C)</td>
<td>30 µL</td>
</tr>
<tr>
<td>Sterile filtered water</td>
<td>0.75 mL</td>
</tr>
<tr>
<td>TEMED (Tetramethylethylenediamine) (@ 20°C)</td>
<td>5 µL</td>
</tr>
</tbody>
</table>

Table 2: 3% SDS-PAGE stacking gel recipe.

<table>
<thead>
<tr>
<th>3% stacking gel</th>
<th>1 gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.75M Tris HCl pH 6.5</td>
<td>250 µL</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>10% SDS</td>
<td>22.5 µL</td>
</tr>
<tr>
<td>30% Acrylamide-0.8%Bisacrylamide(@ 4°C)</td>
<td>200 µL</td>
</tr>
<tr>
<td>10% Ammonium per sulfate (@ 4°C)</td>
<td>25 µL</td>
</tr>
<tr>
<td>Sterile filtered water</td>
<td>1000 mL</td>
</tr>
<tr>
<td>TEMED (Tetramethylethylenediamine) (@ 20°C)</td>
<td>2.5 µL</td>
</tr>
</tbody>
</table>

**Sample preparation for SDS-PAGE**

Samples were prepared differently depending upon the experiment. Typically, for sample preparation following an immunoprecipitation (IP) experiment, proteins were eluted by adding 20 µL of 2× sample buffer (8% SDS, 10% glycerol, 0.7 M β-mercaptoethanol, 37.5 mM Tris-HCl, pH 6.5, 0.003% bromophenol blue) to IP samples. Samples are incubated for 5 min at 100°C followed by centrifugation for 30 sec at 13,000 rpm using a Spectrafuge 24D microcentrifuge (Labnet International, Inc.).

In signaling or degradation experiments, whole cell lysates were prepared by lysing cells in 300 µL of 2× sample buffer. Samples were either stored at -20°C or immediately processed for gel electrophoresis. Samples were sonicated using a Branson 450 digital sonifier containing a convertor and microprobe set at 11% amplitude for 10 sec. Samples were then centrifuged for 30 sec at 13,000 rpm using a Spectrafuge 24D microcentrifuge (Labnet International, Inc.). After sample preparation, 10 µL of sample was loaded onto a 10% SDS-PAGE gel. To monitor electrophoretic separation and molecular weight sizing protein standard (Bio-rad laboratories.Inc) was diluted 1 µL in 9 µL of 2× sample buffer and loaded along with the samples. The samples were electrophoresed at 160V for 1 h in the Mini-PROTEAN 3 system (Bio-Rad).
**Electrophoretic Transfer of proteins onto nitrocellulose membrane**

Gel was removed from the SDS-PAGE set up and placed in 1× Transfer buffer (0.025M Tris-Base, 0.192M Glycine, 20% (vol/vol) methanol and water). Gel-membrane sandwich was made using fiber pads, 0.45 μM nitrocellulose membrane (Bio-rad), gel and whatman filter paper. In the transfer cassette, fiber pads are placed first on the grey side of the cassette followed by placing the whatman filter paper. Gel is then placed on the filter paper followed by placing the nitrocellulose membrane and fiber pads on the gel. Cassette is closed carefully by removing bubbles to ensure efficient transfer of proteins. Cassette is then placed in transfer tank containing 1× transfer buffer, ice-pack and magnetic needle with gel side facing the negative electrode. Transfer tank was placed on the stir plate and transferred for an hour at 100V. After the transfer, the membrane was removed and incubated with ponceau for 5 min to stain proteins. Membranes were quickly rinsed in approximately 50 mL water to remove background binding and to determine the efficiency of protein transfer. Membrane was labeled with ethanol free marker with name and date of experiment.

**Immunoblotting**

Membrane was rinsed once with approximately 50 mL of 1× TBST (Tris-Buffered Saline and Tween 20) (50 mM Tris HCl pH 7.4, 150 mM NaCl, 0.1% Tween 20) to remove the bound ponceau staining. Membrane was then incubated with 5% milk made in 1× TBST for 30 min on a rocking platform VWR (04033ID). In order to immunoblot with pAkt-T308 antibody, membrane was incubated with western blot
blocking reagent from Roche (Mannheim, Germany). After 30 min of blocking, membranes were then incubated with primary antibodies made in 5\% milk made in 1× TBST or blocking reagent based on the experiments for 1 hr or 12-16 hr at 4˚C on the rocking platform. After the incubation, membrane was washed 3× with 1× TBST on the rocker for 5 min each at RT. 5\% milk containing species-specific Horse-Radish Peroxidase (HRP) conjugated secondary antibody was added to the membrane for 30 min followed by 5× washes with 1× TBST for 10 min each at RT.

**Preparation of chemiluminescent substrates**

To analyze the signals on the membrane, we used super signal- west –DURA enhanced chemiluminescent substrate (Thermo Scientific). To detect tagged transfected proteins or loading control proteins such as actin or tubulin, a chemiluminescent substrate prepared in the laboratory was used. The in house chemiluminescent substrate was prepared by mixing solution 1 (2.5mM 3-Aminophthal Hydrazide and 0.45mM p-Coumaric acid in 0.1mM Tris pH 8.8) and solution 2 (0.02\% Hydorgen peroxide in 0.1M Tris pH 8.8) in 1:1 ratio. After the final 1× TBST wash, membranes were then removed from the 1× TBST, dabbed to remove excess buffer and incubated with DURA or lab made chemiluminescent substrate for 5 min. In the dark room, protein bands were visualized by exposing the autoradiography films on the membrane for various exposure times followed by developing films in a SRX-101A automated medical film processor (Konica Minolta Medical and Graphic, Inc).
CO-IMMUNOPRECIPITATION

In order to immunoprecipitate Myc-tagged ESCRT subunits, HeLa cells grown on 6-cm dishes were transfected with either myc-Empty vector, myc-Hrs, myc-Tsg101, myc-Vps28 or myc-Vps36 using PEI transfection reagent protocol as described in section 2.3b. Twenty-four hour post transfection, 6-cm dishes were placed on ice, medium was aspirated, followed by adding 10 mL ice-cold PBS, which was then quickly aspirated. To the cells 300 μL of chilled immunoprecipitation (IP) buffer was added. The recipe for IP buffer is as follows: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton-X-100, 5 mM NEM (N-ethylmaleimide), 25 mM Sodium fluoride, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate deca-hydrate and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, and 10 μg/ml pepstatin-A). Cells were carefully scrapped using a rubber cell scraper and samples were transferred into a microcentrifuge tube on ice. The samples were then incubated at 4°C for 30 min on a speci-mix Barnstead Thermolyne rocker. Samples were placed on ice and then subjected to sonication while tubes were on ice using the Branson digital sonifier with the following settings: 1× at 11% amplitude for 10 sec. Samples were subject to centrifugation at 14,000 rpm at 4°C for 20 min using a 5417r-Eppendorf centrifuge. The supernatant represents the cleared cell lysate and was transferred to a fresh microcentrifuge tube and the pelleted debris was discarded.

The protein concentration of the cleared cell lysate was determined by using the BCA Protein Assay Kit (Pierce® Rockford, IL), according to the manufacturer's instructions. Briefly, BCA reagent was prepared by mixing 10 mL of solution A with 200
µL of Solution B and adding 200 µL of this mixture to 96-well plate containing 10 µL of cleared lysate and BSA standard aliquoted in duplicate. Plate was then incubated for 30 min at 37°C followed by reading the plate at 562 nm in a Spectramax plus spectrophotometer. Protein concentration was determined by comparison to standard curve prepared by known BSA amounts. 200 µg of cleared lysate in ~ 300-400 µL was incubated with either an anti-myc (1 µL) mouse monoclonal or mouse IgG1 isotype control antibody for 12-16 hours at 4°C while rocking. After incubation with antibody, 20 µL slurry of equilibrated protein G agarose beads (Roche, Indianapolis, IN). For equilibration of beads, protein G beads were washed 3× with 700 µL of IP buffer for 10 min on rocker at 4°C. After the final wash supernatant was aspirated and equal amounts of buffer was added to beads and 20 µL of beads is added to each sample for an hour on the rocker at 4°C. Samples were washed 3× with 700 µL IP Buffer and proteins were eluted in 20 µL of 2× sample buffer followed by SDS-PAGE.

For immunoprecipitating myc-Raptor (mTORC1) from ESCRT-0 (STAM1) depleted cells, HeLa cells in 6-cm dishes were co-transfected with 50 nM siRNA directed against luciferase or STAM1 and 1 µg of each FLAG-DEPTOR and myc-Raptor using Lipofectamine 3000 as described in section 2.4b. Forty-eight hour later cells were washed once with ice-cold PBS, and lysed in 300 µL CHAPs-Immunoprecipitation buffer [40 mM HEPES, pH 7.4, 0.3% CHAPS, 2 mM EDTA, 50 mM sodium fluoride, 10 mM sodium pyrophosphate decahydrate, 10 mM β-glycerophosphate and protease inhibitors (10 µg/mL each of aprotinin, pepstatin A and leupeptin)] and incubated on the rocker for 30 min at 4°C. Cleared cell lysates were prepared by centrifugation at 13, 000 rpm for 20
min at 4°C using a 5417r-Eppendorf microcentrifuge. The cleared supernatants were collected and protein concentrations were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). A total of 300-400 μg of supernatant in a volume of 200-300 μL was incubated with either anti-myc (1 μL) mouse monoclonal antibody for 12-16 hr. Protein-G agarose beads were equilibrated as described in paragraph above and samples were then incubated with 20 μL of 50 % slurry of protein G agarose for 1 hr at 4°C while rocking. Samples were washed 3× with 700μL of immunoprecipitation-buffer and finally buffer was aspirated and bound proteins were eluted in 2× sample buffer lacking β-mercaptoethanol, followed by 10% SDS-PAGE and immunoblotting.

For Co-IP experiment of endogenous Rictor, Hela cells were lysed in CHAPs-Immunoprecipitation buffer as described above and a total of 300-400 μg of cleared lysate prepared from untransfected HeLa cells were incubated with either anti-mouse IgG or anti-Rictor mouse monoclonal antibodies. For immunoprecipitating endogenous Rictor from ligand stimulated cells, HeLa cells were serum starved for 3 hr and treated with the ligand for 0.5 or 30 min with CXCL12 followed by co-immunoprecipitation procedure described above.

CXCR4 AND DEPTOR DEGRADATION ASSAYS

**CXCR4 degradation assay**

CXCR4 degradation in response to CXCL12 was assessed by protocol essentially as previously described (Bhandari et al., 2007; Malik and Marchese, 2010; Marchese and Benovic, 2001). Briefly, HeLa cells grown to 100% confluency on a 10-cm dish were
dissociated from the plate surface with trypsin, as described above in section 2.1. To
determine the cell number, a 10 µL aliquot of cells was mixed with 10 µL of Trypan blue
and counted using a countess automated cell counter from Invitrogen (C10227). 300,000
cells were plated per well in a 6-well plate in a volume of 2 mL complete DMEM.
Twenty-four hour later cells were transfected with 50 nM final concentration of siRNA
directed against ESCRT-I (UBAP-1, Tsg101, Mvb12A or Mvb12B), ESCRT-II (Vps22
or Vps36), ESCRT-III (CHMP4A, B or C) or luciferase (control) using Lipofectamine
2000 transfection reagent as described in section 2.4a. Forty-eight hour post transfection,
media on the cells was replaced with 1 mL warm complete-DMEM (DMEM containing
serum) followed by directly adding 10 µL of cyclohexamide 50 µg/mL from a stock of 5
mg/mL cyclohexamide to each well for 15 min at 37°C. After 15 min, 1 µL of 10 µM
CXCL12 (final 10 nM) or vehicle (0.1% BSA in PBS) control was directly added to the
wells and placed in the incubator at 37°C for 3 hr. After incubation, plates were removed
from the incubator and placed on ice and media as aspirated followed by 1× wash with 2
mL ice-cold 1× PBS. PBS was immediately aspirated and cells were lysed in 300 µL of
2× sample buffer. Lysed samples were collected by scraping off cells using a cell scraper
from the wells into microcentrifuge tubes. Samples were then sonicated 1× using Branson
digital sonifier at 11% amplitude for 10 sec. 10 µL sample was loaded onto a 10-well
10% SDS-PAGE gel and analyzed by immunoblotting as described in section 2.6 using
antibodies for CXCR4, UBAP-1, Tsg101 and actin.
**Western Blot and statistical analysis**

After developing the western blots, amount of CXCR4 degraded was quantified. Multiple CXCR4 and actin western blot exposures from 3 experiments were scanned using an EPSON scanner and saved as a JPEG file. These files were opened in VisionWorksLS analysis Software for densitometric analysis by manually drawing a box and placing the same box on each band followed by analyzing each band. Background reading was also taken by placing the boxes randomly on the blot. Raw data was collected and exported as excel files. In a separate excel file, raw data for CXCR4 and actin from different experiments was collected. Background reading was subtracted from CXCR4 and actin readings and a ratio of CXCR4/Actin was calculated and each reading was subtracted from 100. Reading in vehicle treated in luciferase transfected condition was considered as 100% and each reading was calculated as a percent of 100% reading. These reading from 3 experiments were copied in a GraphPad Prism 6.2b for Mac OS X (GraphPad Software, San Diego, CA) and data was analyzed by either student-\(t\)-test or one-way Anova and a \(p<0.05\) was considered significant.

**DEPTOR degradation assay**

For DEPTOR degradation, HeLa cells (300,000) were passaged onto 6-well plates, as described above. The following day, cells were washed 2× with warm DMEM without serum but containing 20 mM HEPES. After the second wash 1 mL of DMEM + 20mM HEPES was added to the plate followed by directly adding 10 \(\mu\)L of cyclohexamide 50 \(\mu\)g/mL from a stock of 5 mg/mL cyclohexamide to each well for 15
min at 37°C. After 15 min, 1 µL of vehicle (0.1% BSA in PBS) was added to one well and 1 µL of CXCL12 (10 μM stock) was added to another well to give a final concentration of 10 nM for CXCL12. The 6-well dish was placed at 37°C for a total of 3 hr. After 180 min, 1 µL of CXCL12 was added to another well. This was repeated 60 min later, followed by 30, 15 and 10 minutes later.

For DEPTOR degradation in response to various ligands, DEPTOR degradation assay was set up as described in paragraph above. Cells were treated with 50 μg/mL cyclohexamide for 15 min at 37°C. Cells were then treated with vehicle (0.1% BSA in PBS), 10 nM CXCL12, 100 ng/ml EGF, 10μM norepinephrine or 50-200 nM insulin for 3 hr at 37°C. To examine the effect of inhibitors on CXCR4 promoted DEPTOR degradation, cells were washed with warm DMEM supplemented with 20 mM HEPES, then treated with the same media containing 50 μg/mL cyclohexamide for 15 min at 37°C, followed by pretreatment with dimethylsulfoxide (DMSO), 200 μM chloroquine, 10 μM lactacystin, 50 ng/ml pertussis toxin, 50 mM gallein, 50-100 nM wortmannin and 0.5-5 mM 3-methyladenine for 1 hr. Cells were then treated with vehicle or 10 nM CXCL12 for 3 hr.

For CXCR4 induced DEPTOR degradation in cells transfected with ESCRT-I siRNA, cells were transfected with siRNA for either luciferase (control) or ESCRT-I (UBAP-1) for 48 hr using Lipofectamine 2000 transfection reagent as described in section 2.4a. Cells were washed with DMEM without serum and containing 20 mM HEPES and then treated with 50 μg/mL cyclohexamide for 15 min at 37°C.
then treated with either 1 µL of vehicle (0.1% BSA in PBS) or 1 µL of 10 nM CXCL12 directly to the 6-well containing 1 mL of media for 3 hr.

For all DEPTOR degradation assays, after ligand treatment, plates were removed from the incubator and placed on ice followed by 1× wash with ice-cold 1× PBS. After aspirating the residual PBS, cells were lysed in 300 µL of 2× sample buffer. Lysed sample was collected by scraping off from the wells and sonication at 11% amplitude for 10 sec using a Branson 450 digital sonifier. 10µL sample was loaded onto a 10 well 10% SDS-PAGE gel and analyzed by immunoblotting as described in section 2.6 with using antibodies for DEPTOR, mTOR, Rictor, Sin1, CXCR4, actin and UBAP-1.

**Western Blot and statistical analysis**

After developing the western blots, amount of DEPTOR remaining was quantified and expressed as a percentage of the vehicle treated samples. Multiple DEPTOR and actin western blots from 3 experiments were scanned and quantified as described in section 2.8b. DEPTOR/Actin ratio in vehicle treated in luciferase transfected condition was considered as 100% and each reading was calculated as a percent of 100% reading. These reading from 3 experiments were copied in a GraphPad Prism 6.2b for Mac OS X (GraphPad Software, San Diego, CA) and data was analyzed by two-way Anova and a p<0.05 was considered significant.
DETECTING AKT AND ERK-1/2 PHOSPHORYLATION BY IMMUNOBLOTTING

HeLa cells were counted, plated as described in section 2.8a and transfected with 50 nM siRNA directed against individual components of ESCRT-0 (STAM1), ESCRT-I (UBAP1 and Tsg101), ESCRT-II (Vps22), ESCRT-III (CHMP4C) or control (luciferase or GAPDH) siRNA using Lipofectamine 2000 as described in section 2.4a. After 48 hr of transfection, cells were trypsinized, counted and 300,000 cells were plated onto 6-well dishes. The next day, cells were washed 2× with warm DMEM containing 20 mM HEPES and after the second wash 1 mL of the same media was added to cells for 3 hr for serum starvation. After serum starvation for 3 hr, cells were treated for 5 min at 37 °C with 10 nM CXCL12, 100 ng/ml EGF, 10μM norepinephrine, 50-200 nM insulin or vehicle (0.1% BSA in PBS).

In a time course Akt signaling experiment, cells were serum starved as above and stimulated with vehicle (0.1% BSA in PBS) or 10 nM CXCL12 for 60 min, followed by 10 nM CXCL12 stimulation at 45 min. Cells were then stimulated again with 10 nM CXCL12 at 30, 20, 10, 5 and 2 min. In Akt signaling experiments with inhibitors, cells were serum starved for 3 hr and treated with DMSO (control) or inhibitors (Wortmanin, gallein, Pertussis toxin or 3-Methyladenine) for 1 hr followed by stimulation with vehicle or 10 nM CXCL12 for 5 min at 37°C.

For all signaling assays, after ligand treatment, cells were taken out of the incubator and placed on ice and washed once with ice-cold 1× PBS. After aspirating any residual PBS, cells were lysed in 300 μL of 2× sample buffer. Lysed sample was
collected by scraping off from the well with a cell scraper and sonicated 1× at 11% amplitude for 10 sec using a Branson digital sonifier. Equal amounts of samples were loaded onto a 10% SDS-PAGE followed by immunoblotting as described in section 2.6 with antibodies for Akt, pAkt-S473, pAkt-T308, Fox01/03a, pFox01/03a-Thr25/32, TSC2, pTSC2-T1462, GSK-3β, pGSK-3β, P70S6K, pP70S6K-T389 and actin.

**Western Blot and statistical analysis**

After developing the western blots, phosphorylation levels of Akt and FoxO-1/3a, TSC-2, GSK3β was quantified. pAkt-S473, Akt, pFoxO-1/3a-T24/32, FoxO-1/3a, pTSC2-T1462, TSC2, pGSK-3β–S9 western blots from 3 experiments were scanned and quantified as described in section 2.8b. Data was analyzed using GraphPad Prism 6.2b for Mac OS X (GraphPad Software, San Diego, CA) and data was analyzed by two-way ANOVA followed by a Bonferroni’s post hoc test and a probability (p) value < 0.05 was considered significant. Data are represented as the mean ± standard error of at least three experiments or determinations.
### Table 3: Antibodies

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<td>T5701 Sigma (St. Louis, MO)</td>
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<td>UBAP-1</td>
<td>Rabbit</td>
<td>12385-1-AP Proteintech Group (Chicago, IL)</td>
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<td>STAM-1</td>
<td>Rabbit</td>
<td>12434-1-AP, ProteinTech (Chicago, IL)</td>
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<td>Actin</td>
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<td>FLAG</td>
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<td>F3165 Sigma-Aldrich (St. Louis, MO)</td>
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<td>Myc</td>
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<td>MMS-150R Covance (Berkeley, CA)</td>
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<tr>
<td>Tubulin</td>
<td>Mouse</td>
<td>T5293, Sigma-Aldrich (St. Louis, MO)</td>
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<td>CXCR4 (2B11)</td>
<td>Rat</td>
<td>551852, BD Biosciences (San Jose, CA)</td>
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<td>Vps22 –SNF8</td>
<td>Rabbit</td>
<td>Aas49579C, Antibody Verify.Inc (Las Vegas,NV)</td>
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<td>Goat IgG</td>
<td>Goat</td>
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<td>DC01L, Calbiochem</td>
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### Table 4: mTOR complex antibodies

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<td>mTOR</td>
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<td>Sc-1549-R, Santa Cruz (Santa Cruz, CA)</td>
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<td>SIN1</td>
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<td>Raptor</td>
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**Table 5: Akt activation and signaling antibodies**

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<td>Akt-1</td>
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<td>pAkt-S473</td>
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<td>pAkt-T308</td>
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P70S6K | Rabbit | 9202, Cell Signaling Technologies (Danvers, MA)

Table 6 Ligands

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<td>PeproTech (Rocky Hill, NJ)</td>
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<td>Norepinephrine</td>
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<td>EGF (Epidermal growth Factor)</td>
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<td>Insulin</td>
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<td>Fetal Bovine Serum</td>
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Table 7 Inhibitors

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<td>Chloroquine</td>
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<td>Lactacystin</td>
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<td>Wortmanin</td>
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<td>3-methyladenine</td>
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<td>Gallein</td>
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<td>Dimethylsulfoxide (DMSO)</td>
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<td>Cyclohexamide</td>
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<td>STAM1</td>
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<td>ESCRT-I</td>
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**mTOR complex siRNA/shRNA**

| RICTOR     | HSC.RNAI.N152756.12.4, Integrated DNA Technologies (Coralville, IA) |
| pLKO human | Addgene (Cambridge, MA) plasmid # 21335 created and deposited by Dr. David Sabatini |

**Table 9: DNA constructs**

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CHAPTER 3
RESULTS

REGULATION OF DEPTOR IN RESPONSE TO GPCR ACTIVATION

DEPTOR was discovered by Peterson et al in 2009 as an endogenous antagonist of mTOR kinase activity in both mTORC1 and mTORC2 complexes. Peterson et al and three other groups (Duan et al., 2011; Gao et al., 2011; Peterson et al., 2009; Zhao et al., 2011) have shown that DEPTOR is stabilized and degraded in absence and presence of serum respectively. GPCRs also activate mTORC1 and mTORC2 signaling; however how GPCRs regulate DEPTOR levels in cells is yet to be determined. We asked if DEPTOR is degraded in response to activation of GPCR CXCR4.

**DEPTOR is degraded upon CXCR4 activation**

In an attempt to understand regulation of DEPTOR in response to CXCR4 activation, we examined DEPTOR protein levels by immunoblotting in cells stimulated with CXCL12 for different time points. HeLa cells grown in 6-well dishes were placed in incomplete DMEM with 20 mM HEPES. Cells were then treated with 50 µg/mL
cycloheximide for 15 min to inhibit *de novo* synthesis of DEPTOR followed by treatment with 10 nM CXCL12 for 5, 15, 30, 60, 120 or 180 min. The vehicle control (0.1% BSA in PBS) was incubated for 180 min (Figure 6A) in the continued presence of cycloheximide. As shown in Figure 6B, we observed that, CXCL12 treatment induces degradation of endogenous DEPTOR within 1 hr. Levels of DEPTOR from 4 experiments was calculated and graph was plotted as in Figure 6C. This was specific to DEPTOR because CXCL12 treatment only induced time and CXCL12 dependent degradation of DEPTOR, but not other mTORC2 complex proteins. (mTOR, Rictor, Sin1; Figure 6B). The mTORC2 target Akt or control actin did not change on CXCR4 activation for over a period of 3 hr. On activation, endogenous CXCR4 itself degrades in 3 hr (Figure 6B), as shown previously (Bhandari et al., 2007; Malik and Marchese, 2010; Marchese and Benovic, 2001). These data indicate a rapid degradation and regulation of DEPTOR in response to CXCR4 activation.
Figure 6: DEPTOR degrades in response to CXCR4 activation

(A) A schematic representation of experimental design of DEPTOR degradation in response to CXCR4 activation. (B) HeLa cells were treated with cycloheximide for 15 min and then stimulated with either 0.1% BSA in PBS as vehicle (0) for 3 hr or 10 nM CXCL12 for 5, 15, 30, 60, 120 and 180 min. Cells were lysed and immunoblotted for DEPTOR, Rictor, Sin1, mTOR, Akt, CXCR4 and actin. (C) Experiment was repeated for 4 times and DEPTOR levels were normalized to actin and 0 (Vehicle) time point was set at 100% and graph was plotted with SEM (Standard Error of Mean) as error bars.
DEPTOR is degraded upon GPCR and not RTK activation

Given that GPCR CXCR4 activation induces degradation of DEPTOR within 3 hr (Figure 6), we next asked if DEPTOR could also be degraded in response to activation of other GPCRs or RTKs. HeLa cells express GPCRs such as α2-adrenergic receptors (α2AR), which are activated by the agonist norepinephrine (Gibson and Gilman, 2006) and RTKs, such as the epidermal growth factor (EGFR) and insulin receptors (IR) (Guertin et al., 2006; Jacinto et al., 2006). To examine whether DEPTOR levels are regulated by activation of α2AR, EFG or IR, HeLa cells were set up for a DEPTOR degradation assay, as described in Figure 6. Cells were treated with 10 nM CXCL12, 10 μM Norepinephrine, 100 ng/mL EGF, 50 nM Insulin or vehicle (0.1% BSA in PBS) for 3 hr in the presence of cycloheximide. As shown in Figure 7A, α2AR activation, like CXCR4, also induces DEPTOR degradation within 3 hr. However, activation of EGF or Insulin receptors did not induce DEPTOR degradation. The levels of DEPTOR were quantified by densitometric analysis and the percentage degradation of DEPTOR averaged over 5 independent experiments was quantified by one-way ANOVA using Bonferroni’s multiple comparison. Significant DEPTOR degradation was observed in cells stimulated with CXCL12 and norepinephrine as compared to Vehicle p<0.05 (Figure 7B).
Figure 7: GPCRs promote DEPTOR degradation

(A) HeLa cells were set for a DEPTOR degradation experiment as in Figure 6. Cells were stimulated with vehicle (VEH- 0.1% BSA in PBS), 10 µM Norepinephrine, 10 nM CXCL12, 100 ng/mL EGF, 50 nM Insulin for 3 hr. Samples were immunoblotted for DEPTOR and actin. (B) Experiment was repeated 5 times and DEPTOR levels were normalized to actin and quantified using one-way ANOVA followed by Bonferroni’s post hoc test p=0.00072.
MECHANISM OF DEPTOR DEGRADATION IN RESPONSE TO CXCR4 ACTIVATION

DEPTOR is degraded through the lysosomes and not proteasomes

We next set out to determine the mechanism of DEPTOR degradation in response to CXCR4 activation. The two main pathways that mediate protein degradation in mammalian cells are lysosomal proteolysis pathway and ubiquitin-proteasomal pathway (Ciechanover, 2005; Martinez-Vicente et al., 2005). Previous studies indicate that in response to serum stimulation DEPTOR is targeted for proteasomal degradation (Duan et al., 2011; Gao et al., 2011; Peterson et al., 2009; Zhao et al., 2011). Since, we now show that CXCR4 activation induces rapid degradation of DEPTOR (Figure 6&7) (Verma and Marchese, 2015), we asked if DEPTOR follows a proteasomal or lysosomal pathway for degradation on CXCR4 activation. We performed DEPTOR degradation assay in presence of pharmacological inhibitors lactacystin and chloroquine. Lactacystin specifically inhibits the proteasomal pathway (Fenteany and Schreiber, 1998) and a lysoosmotropic agent chloroquine specifically inhibits the lysosomal degradation pathway (Seglen and Gordon, 1979).

HeLa cells were set up for a DEPTOR degradation assay as in Figure 6, followed by treatment with either DMSO (Ctrl), 10 µM lactacystin or 200 µM chloroquine for 1 hr. Cells were then stimulated with 10 nM CXCL12, 10% FBS (Fetal bovine serum) or vehicle (0.1%BSA in PBS) for 3 hr. Cells were lysed and samples were analyzed for DEPTOR, Rictor, Sin1, mTOR, Akt, CXCR4 and actin. We noticed that DEPTOR degrades normally in response to CXCL12 stimulation in DMSO and
lactacystin treated cells, however DEPTOR degradation was completely inhibited in cells pretreated with chloroquine (Figure 8A). This highlights that upon CXCR4 activation, DEPTOR is degraded through the lysosomes and not proteasomes as described in response to serum treatment (Duan et al., 2011; Gao et al., 2011; Zhao et al., 2011). In our experiments we also noticed that serum treatment did not induce DEPTOR degradation in 3 hr. We also immunoblotted to detect CXCR4 levels; CXCR4 is shown to degrade through the lysosomes (Bhandari et al., 2007; Malik and Marchese, 2010; Marchese and Benovic, 2001) and therefore CXCR4 degradation is only inhibited in the presence of chloroquine and not lactacystin. These experiments were repeated 4-7 times and DEPTOR levels were quantified by densitometry and normalized to actin. Data was analyzed by two-way ANOVA followed by Tukey’s post-hoc test (p<0.05) Figure 8B.
Figure 8: DEPTOR is degraded through the lysosomes on CXCR4 activation

(A) HeLa cells were set up for a DEPTOR degradation assay as described in Figure 6 and cells were treated with vehicle, 200 µM chloroquine or 10 µM lactacystine for an 1 hr. Cells were then stimulated with 0.1% BSA in PBS as vehicle (-), 10 nM CXCL12 or 10% Fetal bovine serum (FBS)(+) for 3 hr. Cells were lysed and samples were immunoblotted for DEPTOR, Rictor, Sin1, mTOR, Akt, CXCR4 and actin. (B) Graph was plotted after analyzing data using two-way ANOVA p < 0.05.
ESCRT machinery regulates degradation of DEPTOR through the lysosomes

ESCRT pathway is the major pathway for sorting ubiquitinated membrane proteins including GPCRs and RTKs into the lysosomes for degradation (Goh and Sorkin, 2013; Marchese et al., 2008). Recently, the ESCRT machinery was shown to target cytosolic proteins into intraluminal vesicles of MVBs (Niehrs and Acebron, 2010; Taelman et al., 2010). Since, we show that CXCR4 activation induces lysosomal degradation of DEPTOR we asked if the ESCRT machinery targets DEPTOR for lysosomal degradation. As described earlier, ESCRT machinery consists of five complexes ESCRT-0, I, II, III and Vps4 and each complex is a multi-subunit complex.

To determine the role of ESCRT machinery on DEPTOR degradation, HeLa cells were transfected with siRNA targeted against luciferase (Control) or ESCRT-I subunit UBAP-1 for 48 hr and then treated with 10 nM CXCL12 or vehicle (0.1% BSA in PBS) for 3 hr followed by immunoblotting for DEPTOR. We observed that siRNA depletion of ESCRT-I not only leads to upregulation of DEPTOR in basal conditions but also inhibits CXCL12 induced DEPTOR degradation (Figure 9A).

We also immunoblotted for Rictor, Sin-1 and mTOR and the levels of these proteins did not change in ESCRT-I depleted cells or by CXCL12 treatment. These experiments were repeated 4 times and DEPTOR levels were normalized to actin. DEPTOR levels in vehicle treated cells in control transfected cells were set to 100% and percent DEPTOR remaining was quantified. Data was analyzed by two-way ANOVA with p<0.05 (Figure 9B).
Figure 9: Effect of siRNA depletion of ESCRT-I subunit UBAP-1 on DEPTOR degradation

(A) HeLa cells were transfected with siRNA for either luciferase (Ctrl) or ESCRT-I (UBAP-1) for 48 hr. Cells were kept in incomplete DMEM+HEPES media and treated with cycloheximide followed by stimulation with 0.1% BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 3 hr. Samples were immunoblotted for DEPTOR, Rictor, Sin1, mTOR, UBAP-1, Akt and actin. (B) DEPTOR levels were normalized to actin and analyzed by two-way ANOVA.
siRNA depletion of ESCRTs stabilizes DEPTOR levels in mTORC2 complex

Effect of siRNA depletion of ESCRT on DEPTOR levels in mTORC1 complex

Although ESCRT-I depletion increases DEPTOR levels in cells and inhibits CXCL12 promoted degradation of DEPTOR (Figure 9), the status of DEPTOR in complex with mTOR was not addressed. To address this, we examined the amount of DEPTOR associated with mTORC1 and mTORC2 complex by co-immunoprecipitation in ESCRT depleted cells. For immunoprecipitation of mTORC1 in ESCRT depleted cells, HeLa cells were co-transfected with either luciferase (control) or STAM1 (ESCRT-0) siRNA and FLAG-DEPTOR and myc-Raptor or pcDNA. Fortyeight hour later cells were lysed and equal amounts of lysates were incubated with an anti-myc antibody to immunoprecipitate mTORC1 followed by immunoblotting to detect DEPTOR in the immunoprecipitates. As shown in Figure 10B, although DEPTOR levels were increased in cells depleted of STAM1 (ESCRT-0), the amount of DEPTOR associated with mTORC1 did not change compared to control.
Figure 10: siRNA depletion of ESCRT-I did not stabilize DEPTOR in mTORC1 complex. (A) A schematic representation of different subunits of mTORC1 complex and anti-myc antibody is used to immunoprecipitate Raptor and other mTORC1 complex subunits. (B) HeLa cells were transfected with siRNA directed against luciferase (Ctrl) or ESCRT-0 (E-0) subunit STAM1. Cells were also co-transfected with FLAG-DEPTOR and pcDNA or myc-Raptor. Samples were subjected to immunoprecipitation (IP) as in section 2.7 using anti-myc antibody. IP samples and lysates were immunoblotted for presence of DEPTOR, Raptor, mTOR and STAM1. Experiment was repeated 3 times and representative blots are shown.
Effect of siRNA depletion of ESCRT on DEPTOR levels in mTORC2 complex

For immunoprecipitation of mTORC2, HeLa cells were transfected with either luciferase (control) or STAM1 and equal amounts of lysates were incubated with an anti-Rictor antibody or IgG control to immunoprecipitate mTORC2. Immunoprecipitates were analyzed for the presence of DEPTOR. As shown in Figure 11B, DEPTOR levels were increased in cells depleted of STAM1 (ESCRT-0) and this corresponded to greater amount of DEPTOR associated with mTORC2 compared to controls. Samples were also immunoblotted for mTOR, STAM1 and Rictor. These data suggest that ESCRTs regulates mTORC2 and not mTORC1 by regulating DEPTOR levels.

Figure 11: siRNA depletion of ESCRT-I stabilizes DEPTOR in mTORC2 complex. (A) A diagram showing different subunits of mTORC2 complex and anti-Rictor antibody is used to immunoprecipitate mTORC2 complex. (B) HeLa cells were transfected with siRNA directed against luciferase (Ctrl) or ESCRT-0 (E-0) subunit STAM1. Endogenous
Rictor was immunoprecipitated as in section 2.7 and Samples were subjected to immunoblotting for presence of DEPTOR, Rictor, mTOR and STAM1. Experiment was repeated 3 times and representative blots are shown.

**DEPTOR interacts with ESCRT machinery**

We show that ESCRTs mediate lysosomal degradation of DEPTOR (Figure 8&9) and one possible mechanism by which ESCRTs target DEPTOR into intraluminal vesicle of MVBs is by an interaction with DEPTOR. We asked if DEPTOR interacts with subunits of the ESCRT machinery. HeLa cells plated in 6 cm dishes were transfected with myc-tagged subunits from ESCRT-0 (Hrs, STAM1), ESCRT-I (Tsg101, Vps28) and ESCRT-III (Vps36) or empty vector and FLAG-DEPTOR. Myc-tagged proteins were immunoprecipitated and samples were analyzed for presence of FLAG-DEPTOR. As shown in Figure 12, FLAG-DEPTOR was detected in the immunoprecipitates of myc tagged Hrs (ESCRT-0) and Vps28 (ESCRT-I), but not the other ESCRT proteins, suggesting that DEPTOR interacts with ESCRT-0 and ESCRT-I. Although, FLAG-DEPTOR does not interact with ESCRT-II subunit Vps35, we can not rule out that it interacts with ESCRT-II because we have not examined the interaction with other ESCRT-II subunits.
Figure 12: myc-tagged ESCRT-0 and –I subunits interacts with FLAG-DEPTOR.

HeLa cells were transfected with myc-tagged Empty vector, ESCRT-0 (E-0) (Hrs, STAM1), ESCRT-I (E-I) (Tsg101, Vps28) or ESCRT-II (E-II) (Vps36) and FLAG-DEPTOR. Immunoprecipitation was performed using anti-myc or anti-IgG antibody and samples were analyzed for FLAG-DEPTOR and myc. Experiment was repeated 2 times and representative images are presented.
DEPTOR DEGRADATION IS LINKED TO CXCR4 PROMOTED AKT SIGNALING

To determine if CXCR4 promoted DEPTOR degradation is linked to CXCR4 promoted Akt activation, we first describe the process of Akt activation downstream of CXCL12 stimulation. We then determine the role of DEPTOR expression on CXCR4 promoted Akt activation.

Activation of Akt pathway downstream of CXCR4 receptor activation

Activation of CXCR4 promoted Akt signaling pathway

We first looked at the time course of Akt activation in HeLa and BAE cells in response to CXCR4 activation. To be activated, Akt is phosphorylated on residue T308 by kinase PDK1 and S473 by kinase mTORC2 (Sarbassov et al., 2005b; Vivanco and Sawyers, 2002). We looked at levels of phosphorylation of Akt on these residues in response to CXCL12 treatment for different time points by immunoblotting. HeLa cells grown in 6-well plates were serum starved followed by treatment with 10 nM CXCL12 for various times. Cells were lysed and analyzed by immunoblotting for pAkt-S473, pAkt-T308 and Akt. As shown in Figure 13A, Akt was phosphorylated on S473 and T308 as soon as 2 min with maximum activation at 5 min of CXCL12 treatment and phosphorylation of Akt returns to basal levels in 60 min of CXCL12 treatment. These experiments were also repeated using Bovine Aortic Endothelial cells (BAEC). As indicated in Figure 13B Akt was maximally phosphorylated on residue S473 at 10 min of CXCL12 treatment in BAE cells.
Figure 13: Time course of Akt activation in response to CXCR4 activation.

Akt activation in HeLa (A) and BAE cells (B). HeLa cells were serum starved for 3 hr and stimulated with either 0.1% BSA in PBS as vehicle (0) for 5 min or stimulated with 10 nM CXCL12 for 2, 5, 10, 20, 30, 45 and 60 min. pAkt-S473 levels were normalized to Akt and vehicle treated sample was set to 100% and a graph was plotted by setting 5 min (HeLa cells) and 10 min (BAE cells) time point as 100% and standard error of mean as the error bars.
Akt is phosphorylated on residue T308 by PDK1 and is thought to be phosphorylated at S473 by mTORC2 (Sarbassov et al., 2005b; Vivanco and Sawyers, 2002). However, more than one kinase can phosphorylate Akt at S473 (Partovian and Simons, 2004; Persad et al., 2001; Viniegra et al., 2005; Xie et al., 2011). We asked if mTORC2 is the kinase that phosphorylates Akt on S473 in HeLa cells in response to CXCR4 activation. Cells transfected with siRNA for GAPDH (control) or Rictor a component of mTORC2 complex were serum starved followed by treatment with 10 nM CXCL12 or vehicle for 5 min. Samples were analyzed for pAkt-S473, pAkt-T308, Akt, Rictor, DEPTOR and actin by immunoblotting. We noticed that phosphorylation of Akt on S473 was significantly attenuated in Rictor siRNA transfected cells as compared to control. On the other hand, siRNA depletion of Rictor had no change on phosphorylation of Akt on T308 as reported before (Guertin et al., 2006) (Figure 14B). This suggests that on CXCR4 activation, Akt is phosphorylated on S473 by kinase mTORC2 in HeLa cells.
Figure 14: mTORC2 kinase phosphorylates Akt on Serine 473

(A) Schematic representation of mTORC2 kinase complex mediated phosphorylation of Akt on S473. (B) HeLa cells were transfected with siRNA for GAPDH (Ctrl) or mTORC2 subunit Rictor for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1% BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Experiment was repeated for 3 times and samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, Rictor, DEPTOR and actin. * indicates the non-specific band identified by the pAkt-T308 antibody.
As is typical for GPCRs, CXCL12 stimulation of the Akt/mTOR pathway is dependent on G protein and PI3K activation, as shown recently in gastric cancer and mature dendritic cells (Chen et al., 2012a; Delgado-Martin et al., 2011). To confirm this in HeLa cells, we tested the effect of G protein inhibitors on CXCR4 promoted activation of Akt signaling. Pertussis toxin (PTX) and gallein were used to inhibit Gαi and Gβγ subunits, respectively. PTX ADP ribosylates the Gαi subunit and inhibits Gαi mediated signaling events (Mangmool and Kurose, 2011). Gallein is a small molecule that binds to Gβγ with high affinity and inhibits Gβγ-dependent signaling (Lehmann et al., 2008).

HeLa cells were serum starved for 3 hr followed by treatment with, vehicle (0.1%BSA in PBS) or 50 ng/mL pertussis toxin (PTX) for 16 hr and DMSO (Control) or 10 µM gallein for 1 hr. Next, CXCL12 (10 nM final concentration), EGF (100 ng/mL) or vehicle (0.1%BSA in PBS) was added directly to well for 5 min. Samples were analyzed for levels of pAkt-S473, pAkr-T308, Akt, pFoxO1/O3a-T24/T32, FoxO3a pERK-1/2 and ERK-1/2 by immunoblotting. As expected, (Chen et al., 2012a; Delgado-Martin et al., 2011) CXCR4, but not EGF, promoted Akt and ERK-1/2 activation was completely abrogated in PTX treated cells (Figure 15A). In addition, Akt signaling, as assessed by immunoblotting for pFoxO-1/3a-T24/T32, was also abolished in PTX treated cells. Similarly, only CXCR4 induced Akt activation and signaling was attenuated in gallein treated samples as compared to samples treated with EGF (Figure 15B).
Figure 15: CXCR4 mediated Akt signaling is G protein dependent

HeLa cells were serum starved for 3 hr and treated with vehicle or 50 ng/mL PTX for 16 hr (A) DMSO or 50 µM Gallein (B) for an 1 hr followed by stimulation 0.1% BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Lysates were immunoblotted for presence of pAkt-S473, pAkt-T308, Akt, pFoxO1/O3a-T24/T32, FoxO3a, pERK1/2 and ERK1/2. * Indicates the non-specific bands identified by the pAkt-T308 and pFoxO1/3a-T24/T32 antibodies.
Role of PI3K activation on CXCR4 mediated Akt signaling

Released Gβγ G proteins subunits on CXCR4 activation activate phosphatidylinositol-3-Kinases (PI-3Ks). We tested the effect of so-called selective Class-I and class-III PI3K inhibitors wortmannin and 3-methyladenine (3-MA) on CXCR4 mediated Akt activation (Figure 16). HeLa cells were serum starved for 3 hr and treated with DMSO, 50-100 nM wortmanin or 5 mM 3-MA for 1 hr followed by stimulation with 10 nM CXCL12 or vehicle (0.1%BSA in PBS) for 5 min. Samples were analyzed with pAkt-S473, pAkt-T308, Akt, pFoxO1/O3a-T24/T32 and FoxO3a by immunoblotting. We confirmed that CXCR4 promoted Akt phosphorylation and downstream signaling was completely abrogated in Wortmanin and 3-MA treated cells, as compared to DMSO (Figure 16). This suggests that phosphorylation of Akt on both T308 and S473 is dependent on activation of both Class-I and III PI3Ks.
Figure 16: CXCR4 mediated Akt signaling is PI3K dependent

Effect of Wortmannin and 3-Methyladenine (3-MA) two pan-PI3K inhibitors on CXCL12 promoted Akt activation and signaling were tested. HeLa cells were serum starved for 3 hr and treated with DMSO, 50-100 nM Wortmannin or 5-10 mM 3-MA for an hour followed by stimulation with 0.1% BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Lysates were immunoblotted for presence of pAkt-S473, pAkt-T308, Akt, pFoxO1/O3a-T24/T32, FoxO3a, pERK1/2 and ERK1/2. * indicates a non-specific band identified by the pAkt-T308 antibody.
Role of G proteins and PI3K activation on CXCR4 promoted DEPTOR degradation

To determine if CXCR4 promoted DEPTOR degradation is linked to Akt activation, we examined if DEPTOR degradation is G protein and PI3K dependent. Pertussis toxin (PTX) and gallein were used to inhibit Goα and Gβγ subunits, respectively. We also tested the effect of so-called selective Class-I and class-III PI3K inhibitors wortmannin and 3-methyladenine (3-MA) on DEPTOR degradation.

HeLa cells were set up for a DEPTOR degradation assay as in Figure 6, followed by treatment with 50 µg/mL cycloheximide for 15 min. Cells were then treated with DMSO, 50 ng/mL PTX, 10 µM gallein, 100 nM wortmannin and 5 mM 3-MA for 1 hr followed by treatment with vehicle (0.1%BSA in PBS) or 10nM CXCL12 for 3 hr. Cells were lysed and samples were analyzed by western blotting for levels of DEPTOR. As shown in Figure 17, DEPTOR degradation is completely inhibited in cells treated with PTX, wortmannin and 3-MA but not with gallein. This suggests that DEPTOR degradation is dependent on activation of G protein and PI3K and is linked to Akt activation. Samples were also immunoblotted for CXCR4 and actin. Class-III PI3K is important for production of lipid PI3P on endosomes that is important for lysosomal sorting of proteins to lysosomes for degradation (Petiot et al., 2003). CXCR4 is degraded through the lysosomes and therefore Class-III PI3K inhibitor 3-MA slightly inhibits CXCR4 degradation.
Figure 17: DEPTOR degradation is a G protein and PI3K activation dependent event

HeLa cells were treated with 50 µg/mL cycloheximide for 15 min and then treated with DMSO, 50 ng/mL PTX, 100 nM wortmannin, 5 mM 3-MA and 50 µM gallein for an hour. Cells were then stimulated with either 0.1% BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 3hr. Samples were lysed and immunoblotted for DEPTOR, actin and CXCR4. Experiments were repeated 2 times and representative blots are presented.
**CXCR4 activation depletes DEPTOR from mTORC2 complex**

In response to CXCL12 stimulation Akt is phosphorylated and activated as soon as 5 min and returns to basal phosphorylation level in 30-45 min (Figure 13). Also, in time course DEPTOR degradation assay we show that DEPTOR starts degrading in 30-60 min of CXCL12 stimulation (Figure 6B). We asked if DEPTOR is targeted for degradation in 30 min of CXCL12 stimulation, leading to depletion of DEPTOR from the mTOR complexes (Figure 18A). To address this we performed immunoprecipitation studies. mTORC2 complex is a multi-subunit complex containing Rictor, Sin1, mTOR and mLST8. Immunoprecipitation of Rictor has been shown to immunoprecipitate the entire mTORC2 complex (Peterson et al., 2009). HeLa cells grown in 6 cm dishes were serum starved for 3 hr stimulated with 10 nM CXCL12 for 5 min or 30 min and vehicle (0.1%BSA in PBS) for 30 min. Endogenous Rictor was immunoprecipitated following the protocol described in section 2.7. Bound proteins were analyzed by immunoblotting. We observed less DEPTOR associated with Rictor in CXCL12 stimulated cells as compared to vehicle treated cells (Figure 18B). This suggests that CXCR4 activation targets DEPTOR for degradation leading to dissociation of DEPTOR from the mTORC2 complex. This time of DEPTOR depletion from the mTORC2 complex also correlates with the time of maximal Akt activation, suggesting that DEPTOR degradation is probably linked to Akt activation.
Figure 18: CXCR4 activation promotes DEPTOR depletion from mTORC2 complex

(A) Schematic representation showing CXCR4 activation induced dissociation of DEPTOR from the mTORC2 complex. (B) HeLa cells were stimulated with 0.1% BSA in PBS as vehicle (-) for 5 min or 10 nM CXCL12 for 5 and 30 min followed by immunoprecipitating Rictor and immunoblotting for presence of DEPTOR, Rictor and mTOR in the immunoprecipitates. Experiment was repeated 2 times and representative blots are shown.
Role of DEPTOR expression on CXCR4 promoted mTORC1 and mTORC2 signaling

To directly assess the role of DEPTOR on CXCR4 promoted Akt activation and signaling, Akt signaling assays were performed in cells depleted of DEPTOR or overexpressing DEPTOR.

Effect of overexpression of DEPTOR on CXCR4 promoted mTORC1/2 Signaling

We looked at mTORC1 and mTORC2 signaling in cells overexpressing DEPTOR. HeLa cells were transfected with either pCMV or FLAG-DEPTOR and serum starved for 3 hr. Cells were then treated with 10 nM CXCL12 or vehicle (0.1% BSA in PBS) as control for 5 min. Cells were lysed and samples were analyzed by SDS-PAGE and western blotting for pAkt-S473, pAkt-T308, Akt, FoxO1/3a, pFoxO1/3a-T24/32, P70S6K, pP70S6K-T389, pERK1/2, ERK1/2 and FLAG-DEPTOR. As shown in Figure 19A, overexpression of DEPTOR inhibited CXCR4 promoted and mTORC2 mediated phosphorylation of Akt on S473, as compared to control transfected cells. Active Akt phosphorylates downstream target FoxO1/3a on T24/T32, we noticed that overexpression of DEPTOR also attenuates phosphorylation of FoxO1/3a on T24/T32.

To measure the effect of overexpression of DEPTOR on CXCR4 promoted mTORC1 signaling, samples were immunoblotted for phosphorylation of P70S6K on T389. We noticed that DEPTOR overexpression also inhibited phosphorylation of P70S6K on T389. We also observed that FLAG-DEPTOR expression did not have a
global effect on cells as assessed by phosphorylation of ERK1/2 (Figure 19A). These experiments were repeated 3 times and representative blots are presented. Immunoblots were analyzed by densitometry and pAkt-S473 levels were normalized to Akt. Data was quantified by two-way ANOVA using Bonferroni’s multiple comparison test and graph was plotted with p<0.05 (Figure 19B). This data suggests that overexpression of DEPTOR inhibits both mTORC1 and mTORC2 activity.
Figure 19: Effect of DEPTOR overexpression on CXCR4 promoted mTORC1 and mTORC2 activity

(A) HeLa cells transfected with either FLAG-DEPTOR or pCMV were subjected to Akt signaling assay. Cell lysates were immunoblotted for pAkt-S473, pAkt-T308, Akt, pFOXO1/O3a-T24/T32, FOXO3a, pERK-1/2, ERK-1/2, pP70S6K-T389, and P70S6K. (B) Phosphorylation levels of Akt in was normalized and analyzed by two-way ANOVA followed by Bonferroni’s post hoc test. (p = 0.0041).
Effect of shRNA depletion of DEPTOR on CXCR4 promoted mTORC1/2 Signaling

mTORC1 and mTORC2 activity was also assessed in cells depleted of DEPTOR. HeLa cells were transfected with either pcDNA or shRNA for DEPTOR (Figure 20) and serum starved and treated with 10 nM CXCL12 or vehicle (0.1% BSA in PBS) for 5 min. Cells were lysed and samples were analyzed by western blotting for pAkt-S473, Akt, P70S6K, pP70S6K-T389, pERK1/2, ERK1/2, FoxO1/3a, pFoxO1/3a-T24/32 and DEPTOR. As shown below, shRNA depletion of DEPTOR not only increased CXCR4 promoted phosphorylation of Akt on S473, but considerably increased phosphorylation of downstream Akt target FoxO3a. However, depletion of DEPTOR had no effect on mTORC1 mediated pP70S6K-T389 phosphorylation. Depletion of DEPTOR did not affect other signaling pathways, as assessed by phosphorylation of ERK1/2. These experiments were repeated 3 times and representative blots are presented (Figure 20).
Figure 20: Effect of shRNA mediated depletion of DEPTOR on CXCR4 promoted mTORC1 and mTORC2 activity.

HeLa cells transfected with pcDNA or shRNA for DEPTOR-1 were serum starved and stimulated with 0.1% BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min followed by cell lysis. Cell lysates were immunoblotted for pAkt-S473, pAkt-T308, Akt, pERK-1/2, ERK-1/2, DEPTOR, pFoxO1/O3a-T24/T32, FoxO3a, pP70S6K-T389 and P70S6K.
ROLE OF ESCRT MACHINERY IN REGULATION OF CXCR4 AKT SIGNALING

We now show that CXCR4 activation induces ESCRT mediated lysosomal degradation of mTOR antagonist DEPTOR (Figure 8&9). We also show that the ESCRT complexes controls the amount of DEPTOR associated with mTORC2 (Figure 11B), but not mTORC1 (Figure 10B). These data suggest that by regulating DEPTOR levels in cells the ESCRT complexes regulate the activity of mTORC2. We here tested the role of each of the four complexes ESCRT-0, I, II& III on CXCR4 promoted Akt activation and signaling.

Effect of siRNA depletion of ESCRT-0 on CXCR4 promoted Akt activation and signaling

We first tested the role of ESCRT-0 complex on CXCR4 promoted Akt activation and signaling. To address this HeLa cells transfected with siRNA targeting luciferase (Control) or ESCRT-0 (STAM1) were serum starved and stimulated with 10 nM CXCL12 or vehicle (0.1%BSA in PBS) for 5 min. Samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, STAM1 and DEPTOR. As shown in Figure 21A, mTORC2 mediated phosphorylation of Akt at S473 was attenuated in cells depleted of STAM1 in response to activation of CXCR4, as compared to control. In confirmation with previous studies, depletion of ESCRT-0 subunit STAM1 attenuated CXCR4-promoted ERK-1/2 activation, that likely occurs via a discrete pool of STAM1 that is localized to caveolae, possibly unrelated to its functional role as part of ESCRT-0
In addition, phosphorylation of Akt at T308 by PDK1 was also attenuated in STAM1 depleted cells compared to control. The reason for this remains unknown, but it could be that PDK1 mediated phosphorylation of Akt at T308 is dependent upon initial phosphorylation of S473 (Feldman et al., 2009; Garcia-Martinez et al., 2009; Sarbassov et al., 2005b), although there may be other explanations.

We next examined whether Akt signaling was also impacted by ESCRT-0 depletion. Once fully active, Akt is known to phosphorylate over a 100 downstream substrates (Manning and Cantley, 2007) including TSC2 on T1462, FoxO1/3a on T24/32 and GSK3β on S9 (Guertin et al., 2006; Jacinto et al., 2006). We immunoblotted the signaling experiments in cells transfected with siRNA for ESCRT-0 for TSC2, pTSC2-T1462, FoxO1/3a, pFoxO1/3a-T24/32, GSK3β and pGSK3β-S9.

We observed that siRNA depletion of ESCRT-0 inhibits phosphorylation of FoxO1/3a on T24/32 but not TSC2 on T1462 or GSK3β on S9 (Figure 22B). This can be explained by previous studies that show Akt phosphorylation attenuated by mTORC2 depletion only attenuates phosphorylation of FoxO1/3a on T24/32 but not TSC2 on T1462 or GSK3β on S9. This suggests differential regulation of different substrates by active Akt as described previously (Guertin et al., 2006). These experiments were repeated 4 times and representative blots are shown. Immunoblots were analyzed by densitometry and pAkt-S473 and FoxO1/3a-T24/32 levels were normalized to Akt and FoxO1/3a respectively (Figure 21B & 22B respectively). Data was quantified by two-way Anova using Bonferroni’s multiple comparison test and graph was plotted with p<0.05.
Figure 21: Effect of siRNA depletion of ESCRT-0 subunit STAM1 on CXCR4 promoted Akt activation

(A) HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-0 (STAM1) for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Experiment was repeated 3 times and samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, STAM1, pERK1/2, ERK1/2, DEPTOR and actin. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.
Figure 22: Effect of siRNA depletion of ESCRT-0 subunit STAM1 on CXCR4 promoted Akt signaling

(A) HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-0 (STAM1) for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Experiment was repeated 3 times and samples were immunoblotted pFoxO1/O3a-T24/T32, FoxO3a, pTSC2-T1462, TSC2, pGSK3β-S9 and GSK3β. (B) Phosphorylation of FoxO1/O3a-T24/T32 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.
Effect of siRNA depletion of ESCRT-I on CXCR4 promoted Akt activation and signaling

We then tested the role of ESCRT-I complex on CXCR4 promoted Akt activation and signaling. To address this, Akt phosphorylation experiments were performed in ESCRT-I depleted cells. BAE cells were transfected with siRNA targeted for either GAPDH or ESCRT-I subunit Tsg101 for 48 hr. Cells were serum starved for 3 hr and stimulated with 10 nM CXCL12 or vehicle (0.1%BSA in PBS) for 10 min. Samples were immunoblotted for pAkt-S473, Akt, Tsg101 and DEPTOR (Figure 23A).

In another set of experiments, HeLa cells were transfected with siRNA targeted for either luciferase (Control) or a subunit in ESCRT-I (UBAP-1) and serum starved followed by stimulation with 10 nM CXCL12 or vehicle (0.1%BSA in PBS) for 5 min. Samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, UBAP-1 and DEPTOR (Figure 24A). As shown in Figure 23A & 24A, siRNA depletion of Tsg101 or UBAP-1 attenuated CXCR4 promoted Akt phosphorylation on both S473. Depletion of ESCRT-I did not have a global effect on other cell signaling pathways, as observed by no change in phosphorylation of ERK1/2 in ESCRT-I depleted cells (Figure 24A). Like ESCRT-0, ESCRT-I depletion also attenuated T308 phosphorylation of Akt. Data from both BAE and HeLa cells suggests that ESCRT-I complex is also important for CXCL12 promoted Akt activation. These experiments were repeated 4 times and representative blots are shown. Immunoblots were analyzed by densitometry and pAkt-S473 levels were normalized to Akt. Data was quantified by two-way ANOVA using
Bonferroni’s multiple comparison test and graph was plotted with $p<0.05$ (Figure 23B & 24B).

Samples were also analyzed for phosphorylation of downstream Akt substrates. We noticed that siRNA depletion of UBAP-1 inhibits phosphorylation of FoxO1/3a on T24/32 but not TSC2 on T1462 or GSK3β on S9 as observed by siRNA depletion of ESCRT-0 (Figure 25A, 22A). These experiments were repeated 4 times and representative blots are shown. Immunoblots were analyzed by densitometry and FoxO1/3a-T24/32 levels were normalized to FoxO1/3a. Data was quantified by two-way ANOVA using Bonferroni’s multiple comparison test and graph was plotted with $p<0.05$ Figure 25B.
Figure 23: Effect of siRNA depletion of ESCRT-I subunit Tsg101 on CXCR4 promoted Akt activation.

(A) HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-I subunit Tsg101 for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 10 min and 5 min. Experiment was repeated 4 times and samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, Tsg101 and DEPTOR. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p<0.05.
Figure 24: Effect of siRNA depletion of ESCRT-I subunit UBAP-1 on CXCR4 promoted Akt activation.

(A) HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-I subunit) and UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Experiment was repeated 4 times and samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, DEPTOR, UBAP-1 and DEPTOR. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.
Figure 25: Effect of siRNA depletion of ESCRT-I subunit UBAP-1 on CXCR4 promoted Akt signaling.

(A) HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-I subunit UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Experiment was repeated 4 times and samples were immunoblotted for pFoxO1/O3a-T24/T32, FoxO3a, pTSC2-T1462, TSC2, pGSK3β-S9 and GSK3β. (B) Phosphorylation of pFoxO1/O3a-T24/T32 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.
Effect of siRNA depletion of ESCRT-II on CXCR4 Akt activation and signaling

We also tested the role of the ESCRT-II complex on CXCR4 promoted Akt activation and signaling. To address this Akt phosphorylation experiments were performed in ESCRT-II depleted cells. HeLa cells were transfected with siRNA targeted for either luciferase (Control) or a subunit in ESCRT-II (Vps22) and serum starved and stimulated with 10 nM CXCL12 or vehicle (0.1%BSA in PBS) for 5 min. Samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, Vps22 and DEPTOR. As shown in Figure 26A, mTORC2 and PDK1 mediated phosphorylation of Akt at S473 and T308 respectively, was attenuated in cells depleted of a subunit from ESCRT-II complex in response to activation of CXCR4. Like ESCRT-I depletion, ESCRT-II depletion also had no effect on phosphorylation of ERK1/2 (Figure 24A & 26A).

Samples were also analyzed for phosphorylation of downstream Akt substrates. We noticed that siRNA depletion of Vps22 inhibits phosphorylation of FoxO1/3a on T24/32 but not TSC2 on T1462 or GSK3β on S9 as observed by siRNA depletion of ESCRT-0 and –I (Figure 27A, 25A &22A). These experiments were repeated 4 times and representative blots are shown. Immunoblots were analyzed by densitometry and pAkt-S473 (Figure 26B) and FoxO1/3a-T24/32 (Figure 27B) levels were normalized to Akt and FoxO1/3a respectively. Data was quantified by two-way ANOVA using Bonferroni’s multiple comparison test and graph was plotted with p<0.05.
Figure 26: Effect of siRNA depletion of ESCRT-II on CXCR4 promoted Akt activation

(A) Effect of siRNA depletion of ESCRT-II on CXCR4 promoted Akt activation was assessed. HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-II subunit Vps22 for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1% BSA in PBS as vehicle (V) or 10 nM CXCL12 (S), 100 ng/mL EGF (E) or 50 nM Insulin (I) for 5 min. Experiment was repeated 4 times and samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, Vps22, pERK1/2, ERK1/2, DEPTOR and actin. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.
**Figure 27:** Effect of siRNA depletion of ESCRT-II on CXCR4 promoted Akt signaling

(A) Effect of siRNA depletion of ESCRT-II on CXCR4 promoted Akt signaling was assessed. HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-II subunit Vps22 for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (V) or 10 nM CXCL12 (S), 100 ng/mL EGF (E) or 50 nM Insulin (I) for 5 min. Experiment was repeated 4 times and samples were immunoblotted for pFoxO1/O3a-T24/T32, FoxO3a, pTSC2-T1462, TSC2, pGSK3β-S9.
and GSK3β. (B) Phosphorylation of pFoxO1/O3a-T24/T32 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.

**Effect of siRNA depletion of ESCRT-III on CXCR4 promoted Akt activation and signaling**

Effect of siRNA depletion of ESCRT-III on CXCR4 promoted Akt activation and signaling was also assessed. HeLa cells were transfected with siRNA targeted for either luciferase (Control) or a subunit in ESCRT-III (CHMP4C) and serum starved and stimulated with 10 nM CXCL12 or vehicle (0.1%BSA in PBS) for 5 min. Samples were immunoblotted for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, Vps22 and DEPTOR. As shown in Figure 28A, mTORC2 and PDK1 mediated phosphorylation of Akt at S473 and T308 respectively, was attenuated in cells depleted of a subunit from ESCRT-III complex in response to activation of CXCR4. Like ESCRT-I and –II depletion, ESCRT-III depletion also had no effect on phosphorylation of ERK1/2 (Figure 28A).

Samples were also analyzed for phosphorylation of downstream Akt substrates. We noticed that siRNA depletion of CHMP4C inhibits phosphorylation of FoxO1/3a on T24/32 but not TSC2 on T1462 or GSK3β on S9 as observed by siRNA depletion of ESCRT-0, -I and –II Figure 29A. These experiments were repeated 4 times and representative blots are shown. Immunoblots were analyzed by densitometry and pAkt-S473 (Figure 28B) and FoxO1/3a-T24/32 (Figure 29B) levels were normalized to Akt
and FoxO1/3a respectively. Data was quantified by two-way ANOVA using Bonferroni’s multiple comparison test and graph was plotted with p<0.05.

Figure 28: Effect of siRNA depletion of ESCRT-III on CXCR4 promoted Akt activation.

(A) Effect of siRNA depletion of ESCRT-III on CXCR4 promoted Akt activation and Akt signaling was assessed. HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-III subunit CHMP4C for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min. Experiment was repeated 3 times and samples were immunoblotted for
pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, DEPTOR and actin. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p < 0.05.

Figure 29: Effect of siRNA depletion of ESCRT-III on CXCR4 promoted Akt signaling.

(A) Effect of siRNA depletion of ESCRT-III on CXCR4 promoted Akt signaling was assessed. HeLa cells were transfected with siRNA directed against either luciferase (Ctrl) or ESCRT-III subunit CHMP4C for 48 hr. Cells were serum starved for 3 hr and stimulated with 0.1%BSA in PBS as vehicle (-) or 10 nM CXCL12 (+) for 5 min.
Experiment was repeated 3 times and samples were immunoblotted for pFoxO1/O3a-T24/T32, FoxO3a, pTSC2-T1462, TSC2, pGSK3β-S9 and GSK3β. (B) Phosphorylation of pFoxO1/O3a-T24/T32 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05

ESCRT MACHINERY REGULATES GPCR AND RTK MEDIATED AKT ACTIVATION AND SIGNALING

siRNA depletion of ESCRT-I and ESCRT-II inhibits α2AR, EGF and Insulin mediated Akt activation and signaling

Because ESCRTs regulate DEPTOR levels in cells, it is possible that ESCRTs have a broad role in Akt signaling. As described earlier, HeLa cells endogenously express α 2-adrenergic receptors (α 2AR), epidermal growth factor and insulin receptors. We asked whether ESCRTs regulate Akt signaling induced by activation of these receptors. HeLa cells were transfected with siRNA for either luciferase (Control) or ESCRT-I (UBAP-1) or ESCRT-II (Vps22) for 24 hr. Post transfection, cells were serum starved for 3 hr and stimulated with norepinephrine (10 µM), EGF (100 ng/mL) or insulin (50 nM) or vehicle (0.1%BSA in PBS) for 5 min. Samples were analyzed for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, UBAP-1, Vps22 and DEPTOR.

We noticed that siRNA depletion of UBAP1 attenuated Akt phosphorylation at S473 promoted by norepinephrine (Figure 30A), EGF (Figure 32A) or insulin (Figure 34A). As observed before, ESCRT depletion did not have a global effect on cell signaling, as measured by activation of ERK1/2. Activation of EKR-1/2 by
norepinephrine, EGF or insulin, was not impacted by depletion of UBAP1 (Figure 30A, 32A & 34A, respectively). In addition, depletion of ESCRT-II subunit Vps22 also attenuated Akt phosphorylation at S473, but not activation of ERK-1/2, promoted by EGF or insulin (Figure 26A). Experiments were repeated 4 times and representative blots are presented. Phosphorylation of pAkt-S473 was analyzed by densitometry and analyzed by two-way ANOVA and plotted as described above (Figure 30B, 32B & 34B).

To determine if Akt signaling downstream of norepinephrine, EGF or insulin was impacted by ESCRT depletion we examined the phosphorylation status of Akt substrates FoxO1/O3a, TSC2 and GSK-3β. Similar to CXCL12, norepinephrine, EGF or insulin promoted phosphorylation of FoxO1/O3a at T24/T32 was attenuated in UBAP1 (Figure 31A, 33A & 35A respectively)) and Vps22 (Figure 27A) depleted cells compared to control, while phosphorylation of TSC2 at T1462 and GSK-3β at S9 was not noticeably impacted.
Figure 30: Effect of siRNA depletion of ESCRT-1 on α2AR promoted Akt activation.

(a) Effect of siRNA depletion of ESCRT-I on GPCR α2AR promoted Akt activation was measured. HeLa cells were transfected with either luciferase (Ctrl) or ESCRT-I subunit UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with either 30 mM ascorbic acid (control) (-) or 10 μM Norepinephrine (+). Cells were lysed and analyzed by immunoblotting for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, UBAP-1, DEPTOR and actin. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s *post hoc* test p<0.05.
Figure 31: Effect of siRNA depletion of ESCRT-I on α2AR promoted Akt signaling.

(A) Effect of siRNA depletion of ESCRT-I on GPCR α2AR promoted Akt activation was measured. HeLa cells were transfected with either luciferase (Ctrl) or ESCRT-I subunit UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with either 30 mM ascorbic acid (control) (-) or 10 μM Norepinephrine (+). Cells were lysed and analyzed by immunoblotting for pFoxO1/O3a-T24/T32, FoxO3a, pTSC2-T1462, TSC2, pGSK3β-S9, GSK3β and UBAP-1. (B) Phosphorylation pFoxO1/O3a-T24/T32 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p<0.05.
Figure 32: Effect of siRNA depletion of ESCRT-I on EGF promoted Akt activation

(A) Effect of siRNA depletion of ESCRT-I on Receptor tyrosine kinase promoted Akt activation was measured. HeLa cells were transfected with either luciferase (Ctrl) or ESCRT-I subunit UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with either vehicle (-) or 100 ng/mL EGF (+) for 5 min. Cells were lysed and analyzed by immunoblotting for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, UBAP-1, DEPTOR and actin. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.
Figure 33: Effect of siRNA depletion of ESCRT-I on EGF promoted Akt signaling

(A) Effect of siRNA depletion of ESCRT-I on Receptor tyrosine kinase promoted Akt signaling was measured. HeLa cells were transfected with either luciferase (Ctrl) or ESCRT-I subunit UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with either vehicle (-) or 100 ng/mL EGF (+) for 5 min. Cells were lysed and analyzed by immunoblotting pFoxO1/O3a-T24/T32, FoxO3a, pTSC2-T1462, TSC2, pGSK3β-S9 and GSK3β. (B) Phosphorylation of pFoxO1/O3a-T24/T32 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05
Figure 34: Effect of siRNA depletion of ESCRT-I on Insulin promoted Akt activation.

(A) Effect of siRNA depletion of ESCRT-I on Receptor tyrosine kinase promoted Akt activation was measured. HeLa cells were transfected with either luciferase (Ctrl) or ESCRT-I subunit UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with either vehicle (-) or 50 nM Insulin (+) for 5 min. Cells were lysed and analyzed by immunoblotting for pAkt-S473, pAkt-T308, Akt, pERK1/2, ERK1/2, UBAP-1, DEPTOR and actin. (B) Phosphorylation of pAkt-S473 was analyzed by two-way ANOVA using Bonferroni’s post hoc test p< 0.05.
Figure 35: Effect of siRNA depletion of ESCRT-I on Insulin promoted Akt signaling

(A) Effect of siRNA depletion of ESCRT-I on Receptor tyrosine kinase promoted Akt signaling was measured. HeLa cells were transfected with either luciferase (Ctrl) or ESCRT-I subunit UBAP1 for 48 hr. Cells were serum starved for 3 hr and stimulated with either vehicle (-) or 50 nM Insulin (+) for 5 min. Cells were lysed and analyzed by immunoblotting pFoxO1/O3a-T24/T32, FoxO3a, pTSC2-T1462, TSC2, pGSK3β-S9 and GSK3β. (B) Phosphorylation of pFoxO1/O3a-T24/T32 was analyzed by two-way ANOVA using Bonferroni’s *post hoc* test p< 0.05.
**ESCRT machinery does not regulate mTORC1 activity**

DEPTOR is a part of mTORC1 and mTORC2 complex, so far we show that ESCRT depletion attenuates mTORC2 mediated Akt activation and signaling. We also show that ESCRT depletion stabilizes DEPTOR only in mTORC2 and not in mTORC1 complex. In sync with this data we also show that ESCRT depletion had modest effect on phosphorylation of TSC-2, an upstream regulator of mTORC1 activity. Over all, this suggests that ESCRT depletion probably does not regulate mTORC1 mediated signaling. To explore the effect of ESCRT depletion on mTORC1 signaling, we looked at phosphorylation status of mTORC1 target protein p70S6K-T389 in ESCRT depleted cells.

HeLa cells were transfected with luciferase (control) or ESCRT-I subunit UBAP-1 and serum starved for 3 hr followed by stimulation with vehicle (0.1%BSA in PBS), 10 nM CXCL12, 10 µM norepinephrine, 100 ng/mL EGF or 50 nM Insulin for 5 min. Samples were analyzed for P70S6K and pP70S6K-T389. We observed that siRNA depletion of UBAP-1 had no effect on mTORC1 signaling mediated by CXCR4 (Figure 36A), α2AR (Figure 36B), EGF (Figure 36C) or Insulin (Figure 36D) receptor activation.

Effect of siRNA depletion of other ESCRT complexes on mTORC1 activity was also tested. HeLa cells were transfected with luciferase (control) or subunits from ESCRT-0 (STAM1) (Figure 37A), ESCRT-II (Vps22) (Figure 37B) or ESCRT-III (CHMP4C) (Figure 37C) complexes followed by serum starvation for 3 hr. Cells were then stimulated with 10 nM CXCL12 or vehicle (0.1%BSA in PBS) for 5 min. For Vps22 siRNA transfected experiments, cells were also stimulated with EGF and Insulin (Figure
Samples were analyzed for P70S6K and pP70S6K-T389. As shown below, siRNA depletion of either ESCRT-0, II or III had no effect on phosphorylation of P70S6K (Figure 37 A-C respectively). Experiments were repeated 3 times and representative blots are shown.

**Figure 36: Effect of siRNA depletion of ESCRT-I on mTORC1 signaling.**

Effect of siRNA depletion of ESCRT-I on CXCR4 mediated mTORC1 signaling was also tested. HeLa cells were transfected with either luciferase (Ctrl) or ESCRT-I subunit UBAP-1 for 48 hr. Cells were serum starved for 3 hr and stimulated with either vehicle (control) or 10 nM CXCL12 (A), 10 µM Norepinephrine (B), 100ng/mL EGF (C), 50 nM Insulin (D) for 5 min. Cells were lysed and analyzed by immunoblotting for phosphorylation of pP70S6K-T389 and P70S6K an mTORC1 target protein (A-D).
Figure 37: Effect of siRNA depletion of ESCRT-0, II or III on mTORC1 signaling.

HeLa cells were transfected with either luciferase (Ctrl) or subunits from (A) ESCRT-0 (STAM1), (B) ESCRT-I (Vps22) or (C) ESCRT-III (CHMP4C) for 48 hr. Cells were serum starved for 3 hr and stimulated with either vehicle (control) or 10 nM CXCL12 for 5 min. For Panel B, cells were also stimulated with 100 ng/mL EGF and 50 nM Insulin. Cells were lysed and analyzed by immunoblotting for phosphorylation of pP70S6K-T389 and P70S6K and mTORC1 target protein (A-C).
CHAPTER 4
DISCUSSION

The current study provides novel insights into GPCR CXCR4 mediated Akt signaling. To tightly regulate the Akt pathway, mTORC2 and mTORC1 exist upstream and downstream of Akt respectively. DEPTOR is identified an endogenous antagonist of mTOR in both mTORC1 and mTORC2 complexes. Few studies have highlighted mechanisms that regulate DEPTOR in response to serum. However molecular mechanism regulating DEPTOR in response to GPCR activation are unknown. We show for the first time that GPCR activation induces rapid degradation of DEPTOR. In particular, we show that activation of CXCR4 induces rapid degradation of DEPTOR through the lysosomes. We then show that the ESCRT machinery that normally targets ubiquitinated membrane receptors for lysosomal degradation is mediating lysosomal degradation of DEPTOR. In addition, we also show that the ESCRT machinery positively regulates CXCR4 mediated Akt signaling by targeting the mTOR antagonist DEPTOR for lysosomal degradation. ESCRT machinery normally downregulates receptors and negatively regulates signaling. We for the first time show a novel role of ESCRT machinery in positive regulation of Akt/mTOR pathway in response to not only GPCR but also RTK activation.
REGULATION OF DEPTOR IN RESPONSE TO GPCR ACTIVATION

Previous studies show that DEPTOR is targeted for proteasomal degradation in response to serum stimulation for 4-24 hr (Peterson et al., 2009). We show that GPCR (CXCR4, α2AR) activation induces rapid degradation of DEPTOR within 3 hr of ligand stimulation (Figure 6&7). DEPTOR degradation was assessed in response to only two GPCRs; CXCR4 and α2AR. Both CXCR4 and α2AR are known to couple to Gαi subunit of the heterotrimeric G proteins. There are three subtypes of α2AR; α2α2AR, α2β2AR & α2γ2AR and in most cell types α2ARs are known to couple to Gαi subunit except in CHO cells, where α2AR is shown to couple to both Gs or Gαi subunits (Eason et al., 1992; Gibson and Gilman, 2006). In addition, other studies show that in HeLa cells α2AR couple to Gαi subunit (Gibson and Gilman, 2006). This suggests a possibility that, this rapid pathway of DEPTOR degradation is unique to GPCRs that couple to Gαi subunit of heterotrimeric G proteins. Further studies testing the role of activation of different GPCRs on DEPTOR degradation is required to make a conclusive statement. Also DEPTOR was only shown to be degraded through the lysosomes in response to CXCR4 activation. Whether α2AR activation promotes lysosomal degradation of DEPTOR is yet to be tested. The degradation pathway adapted by DEPTOR in response to other GPCR activation also needs to be tested.

In our experiments we also stimulated the cells with EGF, Insulin and serum and we did not see significant degradation of DEPTOR in 3 hr of EGF, Insulin or serum stimulation (Figure 7&8). This highlights a difference in regulation of DEPTOR in response to different extracellular stimuli. It is possible that EGF and Insulin stimulation
over a long time for 4-24 hr like serum might target DEPTOR for degradation. It can possibly be explained by different degradation pathways adapted by DEPTOR in response to different stimuli. Serum treatment is known to degrade DEPTOR by proteasomes, while GPCR CXCR4 seems to degrade DEPTOR via the lysosomes. Whether EGF and Insulin receptor activation induces DEPTOR degradation and whether DEPTOR degrades through lysosomes in response to these stimuli is yet to be tested.

**MECHANISM OF DEPTOR DEGRADATION IN RESPONSE TO CXCR4 ACTIVATION**

*DEPTOR is degraded through the lysosomes in response to CXCR4 activation*

We show that DEPTOR is degraded through the lysosomes in response to CXCR4 activation. We show that DEPTOR degradation was inhibited in presence of a lysosomal inhibitor and not a proteasomal inhibitor. To support this data we also show that 3-MA Class-III PI3K inhibitor inhibits DEPTOR degradation. 3-MA is known to inhibit Vps34/Vps15 complex mediated production of PI3P on endosomes. PI3P production on endosomes is known to be required for lysosomal degradation of proteins (Petiot et al., 2003). Inhibition of DEPTOR degradation in 3-MA treated cells confirms that DEPTOR indeed degrades through the lysosomes. Although we show lysosomes mediate degradation of DEPTOR, we do not show localization of DEPTOR to either the endosomes, MVBs or lysosomes on CXCL12 stimulation. Confocal immunofluorescence
microscopy or subcellular fractionation experiments to show DEPTOR in endosomal or lysosomal compartments will be supportive data.

While, we show that DEPTOR degrades through the lysosomes on GPCR activation, the exact mechanism that targets DEPTOR to the lysosomes is still not clear. It is possible that DEPTOR is post-translationally modified upon CXCR4 activation and then targeted to lysosomes for degradation. DEPTOR is known to be phosphorylated and ubiquitinated before proteasomal degradation in response to serum stimulation (Peterson et al., 2009). Whether DEPTOR is phosphorylated or ubiquitinated before lysosomal degradation in response to CXCR4 is yet to be determined.

mTOR complex proteins mTOR (Mao et al., 2008), Rictor (Koo et al., 2015), Raptor (Choi et al., 2014) and DEPTOR (Gao et al., 2011; Peterson et al., 2009; Zhao et al., 2011) are known to be degraded through the ubiquitin proteasome system. However, regulation of mTOR complex proteins has not been studied in response to GPCR activation. We identified a rapid mechanism of DEPTOR degradation in response to GPCR activation. We show that in response to CXCL12 stimulation for 3 hr, DEPTOR is degraded through the lysosomes with no effect on levels of other mTOR complex proteins (Figure 8A).

ESCRTs mediate lysosomal degradation of DEPTOR in response to CXCL12 stimulation

We show that on CXCL12 treatment DEPTOR is degraded through the lysosomes and that the ESCRT machinery targets DEPTOR for lysosomal degradation (Figure 8 &
9). However, the exact mechanism of DEPTOR recruitment to the endosomes is still unclear. To partly support DEPTOR recruitment to endosomes we show that DEPTOR interacts with ESCRT-0 and ESCRT-I complex subunits in co-immunoprecipitation experiments. However, whether DEPTOR/ESCRT interaction is direct still needs to be determined. There are a few possibilities for DEPTOR recruitment to the endosomes.

1. ESCRTs sort ubiquitinated membrane cargo and based on previous studies it’s possible that DEPTOR is ubiquitinated. Ubiquitinated DEPTOR may be recognized by the ubiquitin-binding domain of ESCRT-0 (Niehrs and Acebron) and ESCRT-I (Tsg101) to be sorted into the multivesicular bodies for degradation. This suggests that DEPTOR may interact with the ESCRT complexes via ubiquitin. The E3 ubiquitin ligase SCFβ-Trcp is known to ubiquitinate DEPTOR and targets it to proteasomal degradation in response to serum stimulation (Zhao et al., 2011). It would be interesting to know the role of this E3 ubiquitin ligase in CXCR4 mediated DEPTOR degradation. This also raises a possibility of lysosomal pathway associated E3 ubiquitin ligases to ubiquitinate DEPTOR. These ligases regulate cargo sorting by interacting and ubiquitinating the ESCRT complexes and the cargo. For example, AIP4, Mahogunin and Tal (Amit et al., 2004; Kim et al., 2007; Malik and Marchese, 2010). The role of siRNA depletion of these E3 ubiquitin ligases on CXCR4 promoted DEPTOR degradation may be studied.

2. Another possibility is that the ESCRT pathway sorts DEPTOR in a complex with receptor CXCR4. The ESCRT pathway is known to sort cytosolic proteins in a complex with other transmembrane proteins. For example, on activation of Frizzled
receptor by Wnt, GSK3 binds the receptor ligand complex along with other proteins and is internalized and sequestered into the multivesicular bodies (Taelman et al., 2010). CXCR4 is known to be ubiquitinated and sorted by the canonical ESCRT pathway into the lysosomes for degradation (Malerod et al., 2007; Malik and Marchese, 2010; Marchese and Benovic, 2001; Valiathan and Resh, 2008; Verma and Marchese, 2015). It is possible that DEPTOR interacts direct or indirectly with the receptor CXCR4 on CXCL12 stimulation on the endosomes and the ESCRT machinery sorts DEPTOR/CXCR4 complex by recognizing the ubiquitin moiety of CXCR4.

3. It is possible that DEPTOR is recruited to the endosomes as part of the mTORC2 complex as soon as 5 mins of CXCL12 treatment, where DEPTOR is dissociated from the mTORC2 complex to enter into the ESCRT pathway. mTORC2 complex protein Rictor is known to partially localize to endosomes, which can bring the whole complex to the endosomes (Boulbes et al., 2011). Alternatively, lipid PI3P on the endosomes can also recruit the mTORC2 complex on the endosomes. mTORC2 complex protein Sin1 contains PH domain that is known to bind PI3P with high affinity (Schroder et al., 2007). To somewhat support this idea, we show that Class-III PI3K inhibitor 3-MA that inhibits Vps34/Vps15-mediated production of PI3P inhibits only DEPTOR degradation and not CXCR4 degradation. Lipid PI3P is important for recruiting the ESCRT complexes on the endosomes for proper lysosomal sorting. We noticed that CXCR4 degradation is not inhibited in the presence of 3-MA, suggesting that basal level of PI3P is enough for recruitment and ESCRT mediated lysosomal sorting of CXCR4. On the other hand 3-MA treatment completely inhibited lysosomal
degradation of DEPTOR. This suggests that PI3P on endosomes functions not only to recruit DEPTOR (as a mTORC2 complex) but also for ESCRT mediated degradation of DEPTOR. This also suggests that DEPTOR probably does not enter the ESCRT pathway along with the receptor. However, more studies with respect to time of DEPTOR/mTORC2 recruitment to the endosomes is required. This can be addressed by confocal immunofluorescence microscopy experiments to show DEPTOR and mTORC2 complex proteins Sin1 and Rictor localize to the endosomes on CXCR4 activation.

4. Finally, DEPTOR may enter the ESCRT pathway by direct interaction with an unknown protein. Arrestins are shown to serve as scaffold for signaling molecules downstream to GPCR activation (Kendall et al., 2014; Luttrell et al., 2001). Arrestins also play an important role in lysosomal degradation of receptors via arrestin/ESCRT-0 interaction (Malik and Marchese, 2010). This makes arrestin as a likely scaffold for DEPTOR recruitment and entry into the ESCRT pathway for degradation.

Table 11: DEPTOR degradation in response to serum and CXCR4

<table>
<thead>
<tr>
<th></th>
<th>SERUM</th>
<th>CXCR4 (GPCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPTOR degradation</td>
<td>Proteasomes</td>
<td>Lysosomes (Figure 8)</td>
</tr>
<tr>
<td>Degradation time</td>
<td>4-24 hr</td>
<td>3 hr (Figure 6,7,8&amp;9)</td>
</tr>
<tr>
<td>DEPTOR phosphorylation</td>
<td>Yes, RSK1 and S6K1 phosphorylates DEPTOR on S286, S287 and S291</td>
<td>Not determined</td>
</tr>
<tr>
<td></td>
<td>CK1 phosphorylates DEPTOR on S286 and</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>S287</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>DEPTOR ubiquitination</td>
<td>Yes</td>
<td>Not determined</td>
</tr>
<tr>
<td>DEPTOR E3 ubiquitin ligase</td>
<td>SCF$^{β,TrCP}$ (Skp1-Cullins-F box protein)</td>
<td>Yet to be determined</td>
</tr>
<tr>
<td>Kinases phosphorylating DEPTOR</td>
<td>RSK1, S6K1 and CKI</td>
<td>Yet to be determined</td>
</tr>
<tr>
<td>ESCRT mediated DEPTOR degradation</td>
<td>No</td>
<td>Yet to be determined</td>
</tr>
<tr>
<td>DEPTOR dissociates from mTOR complex</td>
<td>Starting at 4.5 hr of serum stimulation</td>
<td>Within 5-30 min of CXCL12 treatment</td>
</tr>
<tr>
<td>DEPTOR mRNA levels</td>
<td>Decreased on Serum treatment</td>
<td>Yet to be determined</td>
</tr>
<tr>
<td>DEPTOR degradation mTOR dependent</td>
<td>Yes mTORC1 and mTORC2 both regulate DEPTOR mRNA and protein level</td>
<td>Yes, DEPTOR levels are elevated in cells depleted of mTORC2 complex subunit Rictor (Figure 14B)</td>
</tr>
<tr>
<td>DEPTOR degradation</td>
<td>Yes, Akt and P70S6K are</td>
<td>Yes 5-30 mins of DEPTOR</td>
</tr>
</tbody>
</table>
CXCR4 PROMOTED DEPTOR DEGRADATION IS LINKED TO AKT ACTIVATION

Within minutes of CXCL12 stimulation, Gαi subunit is activated and active Gαi dissociates from the Gβγ dimers, which in turn activates PI3K leading to Akt activation. We show that CXCL12 promoted Akt activation is dependent on both Gαi and Gβγ dimer as both pertussis toxin and gallein inhibits Akt activation and signaling on CXCL12 stimulation. We also show that in response to CXCL12 stimulation, DEPTOR degradation is sensitive to pertussis toxin, suggesting that Gαi subunit activation is required for targeting DEPTOR for degradation (Figure 17). In contrast, an inhibitor of
Gβγ subunit, gallein, did not inhibit DEPTOR degradation. This suggests that Gβγ dimer is not involved in DEPTOR degradation. It is also possible that gallein is not a potent inhibitor of Gβγ. We show that 50 μM gallein inhibits Akt activation and 100 μM gallein completely abrogates Akt activation (data not shown). DEPTOR degradation assays were performed using 50 μM gallein, using higher concentration of gallein (100 μM) may inhibit DEPTOR degradation. Alternatively, a more potent peptide inhibitor of Gβγ subunit BARKct (c-terminus of β-adrenergic receptor kinase-2) may be used.

Gβγ dimer is important for activation of class I PI3K, that is required for Akt activation. We show that DEPTOR degradation is inhibited in presence of pan Class I and III PI3K inhibitors; wortmannin and 3-MA (Figure 17). This data further supports our hypothesis that Gβγ dimer is required for DEPTOR degradation, at least via activation of PI3K. This also suggests that DEPTOR degradation is linked to G protein, PI3K and Akt activation. As activation of G proteins and PI3K occur within minutes of ligand binding, DEPTOR is probably also targeted for degradation within this time. We support this data by showing that DEPTOR dissociates from mTORC2 complex as soon as 5 min of CXCL12 stimulation, which is the time of maximal Akt activation (Figure 13 &18). Although, we show that in 5-30 min of CXCR4 stimulation, DEPTOR dissociates from the mTORC2 complex (Figure 18B), DEPTOR levels/dissociation still needs to be assessed in mTORC1 complex from CXCL12 stimulated cells.

Also, DEPTOR degradation is shown to be mTOR dependent in response to serum stimulation. siRNA depletion of subunits from both mTORC1 and mTORC2 increases both DEPTOR protein and mRNA level (Peterson et al., 2009). DEPTOR
degradation in response to CXCR4 also seems to be dependent on mTORC2 complex. As shown in Figure 14B, siRNA depletion of the mTORC2 complex protein Rictor leads to accumulation of DEPTOR, indicating that mTORC2 complex is important for DEPTOR degradation in response to CXCR4. Whether mTORC1 complex is essential for DEPTOR degradation in response to CXCR4 is yet to be examined. Nevertheless, this suggests that G protein; PI3K activation and mTOR somehow target DEPTOR for degradation. Further studies are needed to know that exact mechanism by which DEPTOR is degraded in a G protein, PI3K and mTOR dependent manner.

It is possible that PI3K/mTOR kinases phosphorylate DEPTOR or induce DEPTOR phosphorylation through other kinases. Studies from Peterson et al and two other groups show that DEPTOR is phosphorylated by kinases S6K1 (p70 Ribosomal S6 kinase), RSK1 (p90 Ribosomal S6 kinase) and CKI (Casein Kinase I) (Gao et al., 2011; Peterson et al., 2009; Zhao et al., 2011). Phosphorylation of DEPTOR is required for its subsequent ubiquitination and proteasomal degradation. Whether DEPTOR is phosphorylated before dissociation from the mTORC2 complex is yet to be determined. Analysis of DEPTOR isolated from CXCL12 stimulated cells for different time points using mass spectroscopy for presence of phosphorylated residues will supportive this idea.

**ROLE OF ESCRTS IN CXCR4 PROMOTED AKT SIGNALING**

The primary function of ESCRTs is to target ubiquitinated membrane receptors for lysosomal degradation and thereby downregulating receptor signaling. We now show that CXCR4 activation induces ESCRTs mediated lysosomal degradation of
not only CXCR4 (data not shown), but also the mTOR antagonist DEPTOR (Figure 8 and 9).

This suggests that ESCRTs probably regulate Akt signaling in two possible ways

1. ESCRTs may negatively regulate CXCR4 Akt signaling by targeting CXCR4 for degradation or

2. ESCRTs may positively regulate CXCR4 Akt signaling by targeting mTOR antagonist DEPTOR for degradation

We show that siRNA depletion of ESCRT-I, II and III complex inhibit CXCR4 degradation and our data also shows that the ESCRT machinery positively regulates Akt signaling. This suggests that ESCRT pathway does not regulate CXCR4 promoted Akt signaling by promoting CXCR4 degradation.

Role of ESCRTs in regulation of CXCR4 promoted Akt activation

We show that the ESCRT machinery promotes DEPTOR degradation and relieves an inhibition from mTORC2 complex that phosphorylates Akt on S473. We show that siRNA depletion of subunits from either ESCRT-0, I,II and III complex attenuates CXCR4 promoted and mTORC2 mediated phosphorylation of Akt on S473 (Figure 21, 23, 24, 26 & 28). This suggests that the ESCRT machinery positively regulates CXCR4 promoted and mTORC2 mediated Akt signaling. In addition we also show that siRNA depletion of ESCRT complexes also attenuates PDK1 mediated Akt phosphorylation T308 (Figure 21, 23, 24, 26 & 28).
On CXCL12 stimulation, PI3K catalyzes the formation of PIP3 from PIP2 at the plasma membrane, which recruits PDK1 and Akt via their respective PH domain (Filippa et al., 2000; Hirsch et al., 2000). In some studies, PDK1 is also shown to be constitutively associated with the plasma membrane and hence PDK1 is considered to be constitutively activated in cells (Currie et al., 1999; Filippa et al., 2000; Mora et al., 2004). We think ESCRTs do not directly regulate PDK1 activity. It could be that PDK1 mediated phosphorylation of Akt at T308 is dependent upon initial phosphorylation of S473 (Feldman et al., 2009; Garcia-Martinez et al., 2009; Sarbassov et al., 2005b), although there may be other explanations. It is possible that ESCRT depletion could alter the localization of PDK1, Akt or some other factor that regulates the phosphorylation status of Akt, but this remains to be examined.

Since the ESCRT machinery assemble on the endosomes to target DEPTOR for degradation. It is possible that other signaling molecules like PDK1 and mTORC2 and Akt also are recruited to the endosomal membrane. Many GPCRs induce signaling on the endosomes. For example, activation of angiotensin II receptor recruits Akt and Akt activation kinases on the endosomes (Nazarewicz et al., 2011). PDK1 and mTORC2 complexes are also shown to localize on the endosomes (Boulbes et al., 2011). To partly support this data, we see that on CXCL12 stimulation, DEPTOR is not only dissociated from the mTOR complex but Akt is also fully activated in 5 mins. This suggests that DEPTOR, mTOR complexes, Akt and PDK1 are localized in same cellular compartment. The exact mechanism by which ESCRTs regulate Akt full activation needs more examination. This can be examined by confocal immunofluorescence microscopy and
subcellular fraction studies to examine the localization of mTOR, PDK1, Akt and DEPTOR in ESCRT depleted and CXCL12 stimulated cells as compared to control.

**Role of ESCRTs in regulation of CXCR4 promoted Akt signaling**

We show that siRNA depletion of subunits from either ESCRT-0, I,II and III complex not only attenuates CXCR4 promoted Akt activation, but also downstream Akt signaling (Figure 22, 25, 27& 29). The effect of ESCRT depletion on Akt activation or signaling is likely not because of an indirect effect linked to other roles that ESCRTs have in other membrane related functions, such as cytokinesis, viral budding or plasma membrane repair, because to the best of our knowledge, ESCRT-0 and ESCRT-II are not involved in these processes (Jimenez et al., 2014; Langelier et al., 2006; Morita et al., 2007; Pornillos et al., 2003). Further work will be required to thoroughly understand how ESCRTs govern signaling downstream of Akt activation.

We also show that Akt signaling is impacted by ESCRTs. In ESCRT depleted cells phosphorylation of Akt substrates FoxO1/3a is substantially impaired, while phosphorylation of other Akt substrates GSK-3β and TSC2 is only moderately impaired (Figure 22A, 25A, 27A & 29A). These data are consistent with other studies that have shown that silencing of mTORC2 mediated phosphorylation of Akt at S473 impacts only phosphorylation of T24/T32 on FoxO1/O3a, but not phosphorylation of T1462 on TSC2 or S9 on GSK-3β (Guertin et al., 2006; Jacinto et al., 2006). One possible explanation is that there was not a complete abrogation of Akt phosphorylation on both residues S473 and T308 in cells depleted of ESCRTs. This remaining active Akt can possibly
phosphorylate substrates like TSC2 and GSK3-β. It is also possible that phosphorylation of these sites is less sensitive to perturbations in Akt activity or a redundant kinase is able to phosphorylate these proteins when Akt activity is attenuated or absent (Laplante and Sabatini, 2012). For example, mTORC2 also phosphorylates another kinase SGK1 that also phosphorylates FoxO1/O3a on T24/T32. Loss of activation of two kinases may explain why there is significant attenuation in phosphorylation of FoxO1/O3. It will be interesting to know the phosphorylation status of SGK1 in ESCRT depleted cells. It also raises a possibility of a redundant kinase that can phosphorylate TSC-2, p90 Ribosomal S6 Kinase 1 activated downstream of pERK1/2 is shown to phosphorylate multiple sites on TSC2 including T1462 (Huang and Manning, 2009; Roux et al., 2004; Tee et al., 2002). Although T1462 is a weak p90RSK1 phosphorylation site on TSC2, an unperturbed pERK1/2 signaling in ESCRT depleted cells can contribute to TSC2- T1462. Alternatively, differences in subcellular localization may account for differences in Akt mediated phosphorylation of its substrates (Schenck et al., 2008). Different Akt substrates are known to localize to different cellular compartments. For example, GSK3β and not TSC2 is known to localize with Akt to endosomes, while TSC2 is known to localize on lysosomes (Menon et al., 2014; Schenck et al., 2008). Whether, these Akt substrates localize to different subcellular localization or if there exists a redundant kinase for TSC2 and GSK3β in response to GPCR is yet to be examined.
Role of ESCRTs in Akt signaling downstream of other GPCRs

We propose that ESCRTs may play a general role in GPCR promoted Akt signaling. We show that not only CXCL12, but stimulation with norepinephrine also promotes DEPTOR degradation. In addition we also show that downstream of norepinephrine stimulation ESCRTs regulate Akt activation and signaling (Figure. 26-27 & 30-31A).

This ESCRT mediated regulation of Akt signaling may not extend to all GPCRs, for example, in contrast to CXCR4, activation of the AT1A receptor by angiotensin II does not activate Akt via mTORC2 (Nazarewicz et al., 2011). This suggests that AT1AR promoted Akt signaling may occur via an ESCRT-independent mechanism. Therefore, it is possible that ESCRTs mediate Akt signaling of only a subset of GPCRs. It is also possible that the ESCRT mediated Akt regulation via promoting DEPTOR degradation is only unique to GPCRs that couple to Gαi subunit of heterotrimeric G proteins. Akt signaling in ESCRT depleted cells were assessed in response to GPCRs CXCR4 and α2AR and α2AR can couple to Gαi and Gαs but in HeLa cells α2AR couple to Gαi subunit (Gibson and Gilman, 2006).

Finally, whether this is a general mechanism used by GPCRs to regulate Akt signaling via ESCRT pathway remains to be determined. HeLa cells express a number of GPCRs including muscarinic, β-adrenergic, lysophosphatidic acid receptor and so on (Tallman et al., 1977; Woclawek-Potocka et al., 2014) (Schonbrunn A. and Steffen D., “Endogenous GPCR list in common cell lines”, http://www.tumor-
Further studies are required to test Akt activation in ESCRT depleted cells in response to various GPCRs.

**Role of ESCRTs in Akt signaling downstream of RTKs**

Our study highlights an important distinction between GPCRs and RTKs in that GPCR activation promotes rapid degradation of DEPTOR, whereas activation of EGF or insulin receptors does not (Figure. 7A and quantification in Figure. 7B). Serum treatment of cells has been shown to promote DEPTOR degradation, but it occurs over long treatment times (4-24 hr) and via the proteasome (Peterson et al., 2009). In contrast, CXCR4 promotes DEPTOR degradation through the lysosomes in 3 hr CXCL12 treatment. We also show that serum (10% FBS) treatment, at least under our experimental conditions, did not promote DEPTOR degradation (Figure. 8A&B). Even though EGF and Insulin stimulation did not induce DEPTOR degradation, we found that siRNA depletion of ESCRTs still did attenuate Akt signaling induced by EGF or insulin (Figs. 27, 33 & 35A, respectively). This can be explained by an overall increase in cellular complement of DEPTOR in ESCRT depleted cells that may impact Akt signaling induced by cell signaling receptors in general. Alternatively, RTKs are probably not inducing DEPTOR degradation, but could selectively promote DEPTOR sequestration into ILVs without leading to its degradation, similar to what occurs with GSK-3β (Taelman et al., 2010). Whether growth factor or RTK drives DEPTOR sequestration into ILVs will require further investigation.
ESCRTs REGULATE ONLY MTORC2 AND NOT MTORC1 SIGNALING

We show that ESCRT depletion inhibits DEPTOR degradation and attenuates Akt signaling. We also show that siRNA depletion of ESCRT stabilizes DEPTOR in mTORC2 complex but not in mTORC1 complex. Due to lack of antibody to efficiently immunoprecipitate mTORC1 complex, mTORC1 immunoprecipitation experiments were performed by overexpressing myc-Raptor and FLAG-DEPTOR and it is possible that CXCL12 induced degradation of endogenous DEPTOR may not be efficiently seen in cells expressing overexpressed DEPTOR. More experiments looking at endogenous DEPTOR levels in endogenous Raptor immunoprecipitates is required to support the above data.

It is also possible that ESCRTs indeed do not stabilize DEPTOR levels in mTORC1 complex. This suggests that siRNA depletion of ESCRTs should not effect mTORC1 signaling. Indeed, we show that siRNA depletion of ESCRT complexes only inhibits mTORC2 mediated Akt activation and signaling but did not affect TSC2 phosphorylation and downstream mTORC1 signaling (Figure 36 &37). mTORC1 activity was only measured by phosphorylation status of p70S6K. To assess if mTORC1 activity was indeed not affected in ESCRT depleted cells, phosphorylation of mTOR and other mTORC1 substrates needs to be measured. To be activated, Akt directly phosphorylates mTOR on S2448 and mTORC1 can phosphorylate other targets like 4E-BP1 on S65. ESCRT depletion experiments needs to be analyzed for phosphorylation of mTORC1 at S2448 and 4E-BP1 at S65 to make a conclusive statement.
Another reason, why ESCRT depletion does not affect mTORC1 signaling, is activation of mTORC1 activity by other signaling pathway. Akt signaling pathway is not the only pathway that can activate mTORC1. mTORC1 is shown to be activated downstream of MAPK ERK1/2 pathway, where downstream p90RSK1 is shown to phosphorylate TSC-2 and relieve an inhibition from mTORC1 (Tee et al., 2002). To support this we show that siRNA depletion of ESCRT I, II or III does not affect CXCR4 promoted MAPK ERK1/2 pathway (Figure 24, 26 & 28). Whether, MAPK ERK1/2 pathway activates mTORC1 downstream of CXCR4 activation needs to be examined.

Localization of mTORC1 to different sub-cellular locations is also important for it’s signaling. Recent studies show that in response to amino acids, mTORC1 localizes on lysosomes. High levels of amino acids recruit mTORC1 on the lysosomes, where mTOR is activated by GTP bound small GTPase Rheb (Ras homolog enriched in brain) (Saucedo et al., 2003). Whether, mTORC1 localizes to the lysosomes in response to CXCR4 activation is not known and needs to be examined.

**ESCRTS MAY REGULATE CELL SURVIVAL**

Akt signaling is involved in several cellular processes including cell survival and cell motility (Pearce et al., 2007). Phosphorylation of FoxO1/O3a transcription factors by Akt prevents them from translocating to the nucleus and enhancing the expression of genes that promote apoptosis, thereby facilitating cell survival (Delgado-Martin et al., 2011; Greer and Brunet, 2008; Zoncu et al., 2011). Interestingly, ESCRT-0 or ESCRT-I knock-out mice die early during embryogenesis and the embryos show a high degree of
cellular death in part due to apoptosis (reviewed in (Rodahl, 2009), which is consistent with a decrease in Akt signaling. Akt also inhibits apoptosis by phosphorylating and inhibiting key pro-apoptotic proteins. Akt phosphorylates BAD creating binding site for 14-3-3 leading to sequestration of BAD (Datta et al., 1997; del Peso et al., 1997). Further studies are required; to examine the level of Akt mediated BAD phosphorylation in ESCRT depleted cells. We recently showed that siRNA targeting ESCRT-0 subunit STAM1 does not affect CXCR4-induced proliferation or survival of HeLa cells (Malik et al., 2012). The role of ESCRTs on cell survival remains to be examined further, by assessing the levels of apoptotic proteins in ESCRT depleted cells but it is also possible that ESCRTs have other roles.

Akt signaling has been linked to CXCR4-induced directed migration of HeLa cells (Peng et al., 2005) and directed cell migration is an important developmental and pathophysiological function of CXCR4 (Li et al., 2004; Muller et al., 2001; Tachibana et al., 1998; Zou et al., 1998). Also ESCRTs have been linked to cell polarity and controlling focal adhesion dynamics (Lobert and Stenmark, 2012; Tu et al., 2010). Therefore it is possible that ESCRTs and Akt signaling have important physiological roles in directed cell migration. In conclusion, our findings begin to explain the signal transduction pathways important in the physiological roles of ESCRTs and Akt.

**EFFECT OF DEPTOR EXPRESSION ON CXCR4 PROMOTED MTORC1 AND MTORC2 ACTIVITY AND SIGNALING**

Peterson et al show that, overexpression of FLAG-DEPTOR inhibits mTORC1
activity and increases mTORC2 activity (Peterson et al., 2009). In these experiments, the authors not only overexpressed FLAG-DEPTOR but also GST-HA-Akt. Phosphorylation of GST-HA-Akt on S473 was shown to be increased in cells overexpressing FLAG-DEPTOR. These studies did not show the effect of DEPTOR overexpression on phosphorylation of endogenous Akt. mTORC1 signaling pathway is shown to inhibit Akt signaling pathway by a negative feedback loop (Breuleux et al., 2009). The mTORC1/Akt negative feedback loop is well defined in response to receptor tyrosine kinase activation. Peterson et al explain the increase in Akt activation as a result of inhibition of mTORC1 negative feedback loop in cells overexpressing DEPTOR.

We show that overexpression of DEPTOR inhibits both mTORC1 and mTORC2 signaling (Figure 19A). We observed that phosphorylation of both endogenous and overexpressed GST-HA-Akt was inhibited in cells overexpressing FLAG-DEPTOR. This suggests that in response to CXCR4 activation, mTORC1/Akt feedback loop is not present. To somewhat support this data, mTORC1 inhibitor Rapamycin is shown to inhibit Akt-S473 in CXCL12 stimulated gastric carcinoma cells (Chen et al., 2012a). mTORC1/Akt negative feedback loops are not well studied in response to GPCR activation and more experiments are needed to define this loop.
CHAPTER 5
CONCLUSION

The present study provides mechanistic insights into CXCR4 promoted Akt/mTOR pathway. Ligand CXCL12 binding activates the CXCR4 receptor leading to activation of G proteins. Activated G proteins in turn activate lipid kinase PI3K that is crucial for activation of Akt. We show for the first time that CXCR4, G protein and PI3K activation is essential for targeting DEPTOR for degradation as inhibition of either G protein or PI3K activation completely blocks CXCL12 induced DEPTOR degradation. G protein and PI3Ks are activated within minutes of receptor activation, suggesting that DEPTOR is also targeted for degradation within minutes of receptor activation. To support this idea we show that DEPTOR dissociates from the mTORC2 complex within 5 minutes of CXCR4 activation possibly to be targeted for degradation. The exact mechanism of DEPTOR dissociation from the mTORC2 complex is not yet assessed. From the all the possibilities listed in the discussion, I believe that DEPTOR probably undergoes post-translational modification by a phosphorylation or ubiquitination event, leading to its dissociation from the mTOR complex. Akt probably does not phosphorylate DEPTOR as DEPTOR lacks the Akt consensus phosphorylation site. However, mTOR and S6K1 are shown to phosphorylate DEPTOR previously
(Peterson et al., 2009; Zhao et al., 2011). Whether these kinases phosphorylate DEPTOR in response to CXCR4 activation is yet to be tested. G protein and PI3K activation are also essential for phosphorylation and activation of Akt. We noticed that Akt is also maximally activated at 5 min of CXCL12 stimulation. This correlates with the time of DEPTOR dissociation from the mTORC2 complex. These experiments suggest that CXCR4 promoted DEPTOR degradation and Akt activation events are linked, however, an in detail study is required with respect to timing of these events.

After dissociation from the mTORC2 complex, DEPTOR is then sequestered in the ESCRT pathway for its lysosomal degradation. The exact timing and mechanism of DEPTOR entry into the ESCRT pathway is not entirely clear, however interaction between DEPTOR and ESCRT-0 and –I subunits provides a possible mechanism. DEPTOR may directly interact with the ESCRT machinery to enter the ESCRT pathway for degradation. Alternatively DEPTOR may interact with CXCR4 to indirectly enter the ESCRT pathway, however, this needs further examination.

Mechanisms that recruit DEPTOR to endosomes also needs further analysis, however I believe that mTORC2 complex containing DEPTOR is recruited to endosomes within 5 min of CXCR4 activation. mTORC2 complex protein Sin1 is shown to bind the lipid PI3P via its PH domain (Schroder et al., 2007). This may possibly recruit mTORC2 along with DEPTOR to the endosomal membrane. On the endosomes DEPTOR is dissociated from the mTOR complex probably by phosphorylation or ubiquitination events. These modifications possibly promote interactions between DEPTOR and the
ESCRT complexes. ESCRT machinery then targets only DEPTOR for lysosomal degradation, whereas other mTORC2 complex proteins are not. It is possible that the mTORC2 complex transiently associates with the endosomes, which prevents it from entering the ESCRT pathway for degradation. We predict that mTORC2 is recruited to endosomes via its interaction with PI3P lipid on the endosomes and proteins that interact with endosomal membrane lipids including ESCRT complexes are normally excluded from the degradation (Mageswaran et al., 2014).

We also show that by mediating DEPTOR degradation, the ESCRT machinery also regulates mTORC2 mediated phosphorylation of Akt on S473. As siRNA depletion of ESCRT subunits not only inhibits DEPTOR degradation, but also inhibits CXCR4 promoted phosphorylation of Akt on S473. ESCRT machinery not only regulates Akt activation but also attenuates downstream Akt signaling as measured by phosphorylation of FoxO1/3a. Akt activation promotes cell survival and migration, however whether ESCRT complexes regulate these cellular phenotypes by activating Akt is yet to be tested. Surprisingly, depletion of ESCRT machinery did not have any effect on mTORC1 activity. It can possibly be explained by different sub cellular localization of mTORC1. mTORC1 is shown to localize on lysosomes along with TSC2 where mTORC1 is activated (Saucedo et al., 2003). This ESCRT mediated DEPTOR degradation and regulation of Akt signaling is possibly a general pathway adapted by GPCR. As we show that activation of another GPCR, α2AR also induces rapid degradation of DEPTOR and the ESCRT machinery regulates Akt activation promoted by α2AR. In line with this data,
we also show ESCRTs mediate Akt activation downstream of RTKs including EGFR and the Insulin receptor.

The ESCRT machinery primarily targets ubiquitinated membrane cargo including CXCR4 for degradation. The ESCRT machinery is rarely shown to degrade cytosolic protein (Ikeda and Kerppola, 2008). However, we show that the ESCRT sorting machinery sequesters and degrades the mTOR antagonist DEPTOR to promote CXCR4 mediated Akt signaling. ESCRTs are shown to sequester an antagonist of signaling pathway before. In response to activation of Frizzled receptor, ESCRT components Hrs and Vps4 are shown to sequester GSK3β to allow β-catenin signaling (Taelman et al., 2010). These studies define an upcoming role of ESCRT machinery in signaling.

**BIOLOGICAL SIGNIFICANCE**

*ESCRTs may regulate cellular survival or migration via Akt/mTOR pathway.*

CXCR4 signaling is tightly regulated however, dysregulation of CXCR4 signaling leads to pathological conditions like cancer. Dysregulation in CXCR4 mediated Akt/mTOR pathway is implicated in survival and migration of different cancer types (Chen et al., 2012a; Dubrovska et al., 2012). CXCR4 mediated PI3K/Akt/FoxO pathway is very crucial for survival and tumorigenicity of prostate cancer cells. It is reported that CXCR4 promotes survival of prostate cancer progenitor cells by activating PI3K/Akt/FoxO pathway. Inhibition of either CXCR4 or PI3K/Akt pathway significantly attenuates prostate cancer progenitor cell population (Dubrovska et al., 2012). In the
current thesis, we show that siRNA depletion of ESCRT complexes significantly attenuates Akt activation and FoxO phosphorylation. Once phosphorylated by Akt, transcription of pro-apoptotic genes by FoxO is inhibited (Biggs et al., 1999; Brunet et al., 1999). This suggests that ESCRT machinery not only regulates Akt signaling but also probably regulates cell survival. It will be interesting to know the effect of siRNA depletion of ESCRT complexes on CXCR4 promoted cell survival. Previous studies from our laboratory suggest that the ESCRT-0 complex does not regulate cell survival (Malik et al., 2012). However role of other ESCRT complexes on cell survival and proliferation was not tested.

CXCR4 promoted cellular migration is also implicated in dissemination of tumor cells, trafficking and homing of cancer stem cells (Mukherjee and Zhao, 2013). Moreover, CXCR4 promoted Akt/mTOR pathway is also shown to mediate migration in different cancer types (Chen et al., 2012a; Dubrovska et al., 2012; Phillips et al., 2005). This suggests that the ESCRT machinery may also regulate cellular migration by regulating Akt/mTOR pathway. The kinase mTORC2 and Akt are shown to regulate cellular migration via regulating actin cytoskeleton via different mechanisms. mTORC2 regulates actin cytoskeleton via interaction with P-Rex1, a Rac specific guanine nucleotide exchange factor (Hernandez-Negrete et al., 2007). Interestingly, CXCR4 promoted cell migration and angiogenesis of endothelial cells also involves P-Rex1 and Rac activation (Carretero-Ortega et al., 2010). This suggests that ESCRT machinery may promote mTORC2/P-Rex1 interaction to promote cell migration. Analysis of
mTORC2/P-Rex1 interaction from CXCL12 stimulated and ESCRT depleted cells will be helpful. On the other hand mTORC2 also regulates actin cytoskeleton via phosphorylation of Akt. Akt in turn phosphorylates diverse proteins involved in cell migration including PAK (p21-activated kinase), Integrin β3 and Girdin (Kitamura et al., 2008; Somanath et al., 2006). It will be interesting to know the phosphorylation status of these protein in cells depleted of ESCRT complexes.

Alternatively, ESCRTs may directly regulate cellular migration by involving Src kinase. ESCRT complex proteins Tsg101 and Vps4 are shown to localize active Src to focal adhesions that is essential for cell migration (Tu et al., 2010). Src is upstream and active Src is shown to active PI3K/Akt pathway in many instances (Haynes et al., 2003; Lei and Ingbar, 2011). This suggests that ESCRTs probably regulates CXCR4 promoted migration via first activating Src followed by activation of Akt/mTOR pathway, that in turn regulates actin cytoskeleton. Further studies are required to specifically assess the role of Src, ESCRTs, Akt, mTOR and DEPTOR in CXCR4 mediated cellular migration.
Figure 38. Proposed Model for the role of ESCRTs in regulating GPCR-promoted Akt activation.
Figure 38. Proposed model for the role of ESCRTs in regulating GPCR-promoted Akt activation.

Binding to agonist leads to GPCR-promoted activation of G protein signaling, whereby GDP is exchanged for GTP leading to an active Gα subunit (Gα-GTP) and dissociation of the Gβγ heterodimer. Free Gβγ activates PI3K, which leads to the recruitment of PDK1 and Akt to the plasma membrane where Akt is phosphorylated at Thr-308 by PDK1 and by mTORC2 at Ser-473. Gβγ and PI3K signaling drive DEPTOR degradation likely by promoting DEPTOR targeting into the ILV of the MVBs) via the ESCRT pathway. MVBs eventually fuse with lysosomes where DEPTOR degradation occurs. DEPTOR degradation may facilitate phosphorylation of Akt at Ser-473 by mTORC2. GPCRs can also be targeted into ILVs via ubiquitination and direct interactions with ESCRTs. How DEPTOR is targeted into ILVs of MVBs remains to be determined.
CHAPTER 6
FUTURE DIRECTIONS

We show that in response to CXCR4 activation, the ESCRT pathway positively regulates CXCR4 mediated Akt signaling by mediating DEPTOR degradation. Although, a new role of ESCRT machinery in CXCR4 trafficking and signaling is now defined, there are a few questions that are yet to be answered.

AKT SIGNALING ON THE ENDOSONMES

Although, we show that the ESCRT machinery mediates DEPTOR degradation and Akt signaling, we need to address the exact mechanism of Akt activation by the ESCRT machinery with respect to localization of Akt, receptor and other signaling components.

Localization of Akt signaling

We show that the ESCRT machinery not only regulates mTORC2 mediated S473 but also PDK1 mediated T308 phosphorylation (Figure 21A, 24A, 26A & 28A). T308 phosphorylation generally occurs at the plasma membrane. This suggests that downstream of CXCR4, the ESCRT subunits are required for Akt phosphorylation on the plasma membrane. It is also possible that Akt is phosphorylated on the endosomes where
the ESCRTs assemble for sorting proteins. It also raises a possibility that the ESCRT containing endosomes move to plasma membrane where Akt phosphorylation starts. Further study is required to know the exact localization of Akt activation. It is possible that Akt interacts with the ESCRT complex proteins and is recruited to the endosomes where Akt is activated.

**Localization of the receptor**

We show that siRNA depletion of ESCRTs not only inhibits CXCR4 but also DEPTOR degradation in 3 hr of CXCL12 stimulation. ESCRT depletion probably accumulates DEPTOR as well as CXCR4 on the endosomes. We also show that siRNA depletion of ESCRTs also attenuates CXCR4 promoted Akt activation and signaling. This suggests that attenuation of Akt signaling in ESCRT depleted cells is due to accumulation of mTORC2 inhibitor DEPTOR. We believe that accumulated CXCR4 on endosomes in ESCRT depleted cells is not actively signaling. This raises the question, if Akt signaling is receptor dependent. More experiments are required to address if internalization of receptor is required for ESCRT mediated DEPTOR degradation and Akt signaling. DEPTOR degradation and Akt signaling experiments in cells expressing CXCR4 internalization defective mutants will be helpful experiments.

**Localization of other signaling components**

We show that CXCR4 mediated Akt signaling is G protein and PI3K activation dependent (Figure 15). In 5 min of CXCL12 stimulation, siRNA depletion of ESCRTs
inhibits Akt phosphorylation of both residues, suggesting that Akt is recruited and phosphorylated on the endosomes. Further studies are needed to show that kinase PDK1 and mTORC2 are also recruited to the endosomes. PDK1 is recruited via the lipid PIP3 on the membrane, previous studies show that PI3K, PDK1 and Gβγ heterodimers may localize to the endosomes for signaling (Chamberlain et al., 2004; Garcia-Regalado et al., 2008; Slagsvold et al., 2006). This raises a question if PI3K and Gβγ heterodimers are recruited to the endosomes on CXCR4 activation?
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