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Regulation of CXCR4 Intracellular Trafficking by Ubiquitin

Justine Elizabeth Kennedy
Loyola University Chicago

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LOYOLA UNIVERSITY CHICAGO

REGULATION OF CXCR4
INTRACELLULAR TRAFFICKING
BY UBIQUITIN

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

PROGRAM IN PHARMACOLOGY

BY
JUSTINE ELIZABETH KENNEDY
CHICAGO, ILLINOIS
DECEMBER 2015
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I am extremely indebted to my mentor Dr. Adriano Marchese whose guidance and patience has helped me to grow as a scientist. He has provided me with immense opportunities and encouraged me beyond my imagination. I am thankful for his insightful perspective in helping my project move forward and the thoughtful discussions both scientifically and personally. I also admire his strength in encouraging me even when things seemed dismal.

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For my loving husband Domenick Kennedy and our son Niccolo
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

LIST OF TABLES x

LIST OF FIGURES xi

LIST OF ABBREVIATIONS xiii

ABSTRACT xxi

## CHAPTER I: INTRODUCTION

Overview of G protein-coupled receptors 1
Chemokine receptors 4
GPCR activation 5
GPCR signaling 5
Regulation of GPCR signaling 8
  Desensitization 8
  Internalization 12
  Downregulation 13
  Recycling 16
The ubiquitin pathway 16
  Ubiquitin conjugation 16
  Types of ubiquitin modifications 18
  E3 ubiquitin ligases 21
  RING and RING-like domain containing ligases 22
  HECT-domain ligases 23
  HECT domain ligases in GPCR trafficking 24
  Atrophin-interacting protein 4 (AIP4) 25
  Deltex-3-like (DTX3L) 29
  E2 conjugating enzymes 30
  Deubuquitinating enzymes (DUBs) 31
Functional role of GPCR ubiquitination 33
  Ubiquitin in ESCRT-dependent sorting 33
  Recognition 36
  Sorting 37
  MVB formation and ESCRT disassembly 38
CXCR4 38
  Physiological roles of CXCR4 40
CXCR4 in breast cancer 41
CXCR4 in cardiovascular disease 42
CXCR4 in WHIM syndrome 43
CXCR4 in lymphomas (DLBCLs) 44
CXCR4 Signaling 45
   CXCR4 signaling: G protein dependent 46
   CXCR4 signaling: G protein independent 48
Regulation of CXCR4 signaling 49
   CXCR4 desensitization and internalization 49
   CXCR4 ubiquitin dependent downregulation 50
Rationale and research objectives 53

CHAPTER II: METHODS 56
Cell lines and transfection reagents 56
   Transfection of DNA using Polyethylenimine (PEI) 56
   Transfection of small interfering RNA (siRNA) using Lipofectamine® 2000 or 3000 reagents 58
Antibodies and reagents 59
Cloning 59
   Polymerase Chain Reaction (PCR) set up 59
      Generation of FLAG-DTX3L-3C/A mutant 60
   PCR product gel extraction 62
   Restriction digest 62
   Ligation and transformation 64
   Selection of colonies 65
   Screening for inserts 65
   Sequencing 66
   Maxi Prep 67
Sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and Western blot transfer 67
Immunoblot analysis 68
Ligand stimulation 69
CXCR4 degradation assay 69
Confocal fluorescence microscopy 70
Sample preparation 70
   DTX3L co-localization with EEA1 and LAMP2 72
   CXCR4 degradation by IF 73
   Co-localization of DTX3L and AIP4 73
Assessing co-localization with ESCRT-0 73
   YFP-FYVE co-localization studies 74
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image Acquisition and Processing</td>
<td>74</td>
</tr>
<tr>
<td>GST- and His-fusion protein purification</td>
<td>75</td>
</tr>
<tr>
<td>Equilibration of glutathione-sepharose &amp; Talon metal affinity beads</td>
<td>76</td>
</tr>
<tr>
<td>Immobilization of GST- and His-proteins</td>
<td>77</td>
</tr>
<tr>
<td>Quantification of immobilized GST- and His- fusion proteins</td>
<td>77</td>
</tr>
<tr>
<td>Elution of His-tagged proteins</td>
<td>79</td>
</tr>
<tr>
<td>Large scale purification and PreScission protease cleavage of GST-AIP4 and GST-AIP4-C830A</td>
<td>80</td>
</tr>
<tr>
<td>GST- and His-fusion binding assays</td>
<td>82</td>
</tr>
<tr>
<td>Lysate collection</td>
<td>82</td>
</tr>
<tr>
<td>Binding assays</td>
<td>83</td>
</tr>
<tr>
<td>GST-DTX3L truncations</td>
<td>84</td>
</tr>
<tr>
<td>GST-AIP4 truncations</td>
<td>84</td>
</tr>
<tr>
<td>AIP4 and DTX3L binding</td>
<td>84</td>
</tr>
<tr>
<td>Data quantification</td>
<td>85</td>
</tr>
<tr>
<td>Co-immunoprecipitation</td>
<td>85</td>
</tr>
<tr>
<td>Time course co-immunoprecipitation</td>
<td>85</td>
</tr>
<tr>
<td>Co-immunoprecipitation of endogenous proteins</td>
<td>86</td>
</tr>
<tr>
<td>Co-immunoprecipitation data quantification</td>
<td>87</td>
</tr>
<tr>
<td>Ubiquitination assays</td>
<td>87</td>
</tr>
<tr>
<td>HRS and STAM ubiquitination</td>
<td>87</td>
</tr>
<tr>
<td>CXCR4 ubiquitination</td>
<td>89</td>
</tr>
<tr>
<td><em>In vitro</em> ubiquitination assays</td>
<td>90</td>
</tr>
<tr>
<td>AIP4 and DTX3L <em>in vitro</em> ubiquitination</td>
<td>90</td>
</tr>
<tr>
<td>AIP4, DTX3L and Parkin <em>in vitro</em> ubiquitination</td>
<td>90</td>
</tr>
<tr>
<td>Statistical analysis and final figure preparation</td>
<td>91</td>
</tr>
<tr>
<td>CHAPTER III: RESULTS</td>
<td>101</td>
</tr>
<tr>
<td>Role of DTX3L in CXCR4 degradation</td>
<td>101</td>
</tr>
<tr>
<td>CXCR4 activation promote DTX3L distribution to the surface of early endosomes</td>
<td>105</td>
</tr>
<tr>
<td>DTX3L regulates CXCR4 trafficking</td>
<td>107</td>
</tr>
<tr>
<td>AIP4 and DTX3L directly interact</td>
<td>109</td>
</tr>
<tr>
<td>Mapping the AIP4 binding site on DTX3L</td>
<td>115</td>
</tr>
<tr>
<td>Mapping the DTX3L binding site on AIP4</td>
<td>118</td>
</tr>
<tr>
<td>DTX3L regulates ESCRT-0 ubiquitination</td>
<td>120</td>
</tr>
<tr>
<td>DTX3L regulates ESCRT-0 complex stability</td>
<td>122</td>
</tr>
<tr>
<td>DTX3L promotes ESCRT-0 activity on early endosomes</td>
<td>124</td>
</tr>
<tr>
<td>Phosphotidylinositol 3-phosphate is not regulated by DTX3L</td>
<td>127</td>
</tr>
<tr>
<td>DTX3L prevents AIP4 self-ubiquitination <em>in vitro</em></td>
<td>130</td>
</tr>
</tbody>
</table>
DTX3L antagonizes AIP4-mediated ubiquitination of HRS
DTX3L does not prevent agonist-promoted CXCR4 ubiquitination

CHAPTER IV: DISCUSSION
Role DTX3L in CXCR4 degradation
DTX3L regulates CXCR4 trafficking into the degradative pathway
Interaction of DTX3L and AIP4
DTX3L prevents AIP4 E3 ligase activity
DTX3L antagonizes AIP4-mediated ubiquitination of ESCRT-0
DTX3L negatively regulates CXCR4 ubiquitination

Summary and future directions
DTX3L as a general regulator of receptor downregulation
Mechanism of DTX3L inhibition of AIP4 self-ubiquitination
Targeting the DTX3L/AIP4 interaction in CXCR4 pathologies
E2 enzymes in CXCR4 downregulation
Role of UbcH5c in DTX3L-mediated inhibition of AIP4 self-ubiquitination
Conclusion

APPENDIX A: BUFFER AND REAGENT RECIPES

APPENDIX B: PLASMID MAP CONSTRUCTS

REFERENCES

VITA
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Proteins in the ESCRT pathway</td>
<td>35</td>
</tr>
<tr>
<td>2.1 List of Primary Antibodies used in this project</td>
<td>92</td>
</tr>
<tr>
<td>2.2 List of reagents used in this dissertation work</td>
<td>93</td>
</tr>
<tr>
<td>2.3 List of secondary antibodies used in this project</td>
<td>98</td>
</tr>
<tr>
<td>2.4 List of siRNA used for this dissertation work</td>
<td>96</td>
</tr>
<tr>
<td>2.5 DNA constructs used for this dissertation work</td>
<td>97</td>
</tr>
<tr>
<td>2.6 Primers used to generate constructs for this dissertation</td>
<td>98</td>
</tr>
<tr>
<td>2.7 Restriction enzymes used for cloning in this dissertation</td>
<td>99</td>
</tr>
<tr>
<td>2.8 GST- and His-fusion proteins used in this study</td>
<td>100</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 GPCR Activation and Signaling</td>
<td>7</td>
</tr>
<tr>
<td>1.2 GPCR homologous desensitization and internalization</td>
<td>11</td>
</tr>
<tr>
<td>1.3 General model of GPCR endosomal trafficking</td>
<td>15</td>
</tr>
<tr>
<td>1.4 The ubiquitin pathway</td>
<td>17</td>
</tr>
<tr>
<td>1.5 Domain structure of AIP4, Cbl family and DTX family protein structure</td>
<td>28</td>
</tr>
<tr>
<td>1.6 Signaling pathways activated downstream of CXCR4</td>
<td>47</td>
</tr>
<tr>
<td>1.7 Current model of CXCR4 trafficking within the endocytic pathway</td>
<td>52</td>
</tr>
<tr>
<td>2.1 Example of a gel stained with GelCode Blue used to quantify amounts of GST-fusion proteins purified for this study</td>
<td>78</td>
</tr>
<tr>
<td>2.2 Example of a gel stained with GelCode Blue used to quantify amounts of His-fusion proteins purified for this study</td>
<td>80</td>
</tr>
<tr>
<td>2.3 Example of a gel stained with GelCode Blue for cleaved AIP4 and AIP4-C830A purification</td>
<td>82</td>
</tr>
<tr>
<td>3.1 DTX3L regulates CXCR4 degradation</td>
<td>103</td>
</tr>
<tr>
<td>3.2 Cbl proteins in CXCR4 degradation</td>
<td>104</td>
</tr>
<tr>
<td>3.3 DTX proteins in CXCR4 degradation</td>
<td>104</td>
</tr>
<tr>
<td>3.4 CXCR4 activation promotes DTX3L distribution on early endosomes</td>
<td>106</td>
</tr>
<tr>
<td>3.5 DTX3L promotes CXCR4 endosomal to lysosomal degradation</td>
<td>108</td>
</tr>
<tr>
<td>3.6 DTX3L is required for CXCR4 localization to and degradation in lysosomes</td>
<td>109</td>
</tr>
<tr>
<td>3.7 AIP4 and DTX3L directly interact</td>
<td>111</td>
</tr>
</tbody>
</table>
3.8  CXCL12 promotes DTX3L co-localization with AIP4
3.9  DTX3L binds AIP4 via the N-terminal domain
3.10 AIP4 binding to DTX3L involves the C2 and HECT domains
3.11 DTX3L regulates ESCRT-0 ubiquitination and complex formation
3.12 DTX3L regulates ESCRT-0 ubiquitination and complex formation
3.13 DTX3L promotes ESCRT-0 aggregation on early endosomes
3.14 DTX3L localizes with and regulates the aggregation of ESCRT-0 on endosomes
3.15 DTX3L does not reduce phosphatidylinositol 3-phosphate levels
3.16 YFP-FYVE puncta and localization to endosomes is reduced upon reduction in phosphatidylinositol 3-phosphate by wortmannin
3.17 DTX3L inhibits AIP4 ligase activity
3.18 DTX3L counteracts AIP4-mediated HRS ubiquitination
3.19 DTX3L does not prevent agonist-promoted CXCR4 ubiquitination
4.1  Proposed model for the role of DTX3L in CXCR4 endosomal sorting
4.2  Proposed model for DTX3L antagonism of AIP4-mediated ubiquitination of ESCRT-0
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
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</tr>
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<tbody>
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<td>7TM</td>
<td>Seven transmembrane</td>
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<td>A</td>
<td>Angstrom</td>
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<td>AC</td>
<td>Adenyl cyclase</td>
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<td>AIP4</td>
<td>Atrophin interacting protein-4</td>
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<td>ALIX</td>
<td>ALG interacting protein X</td>
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<td>Amp</td>
<td>Ampicillin</td>
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<td>AMSH</td>
<td>Associated molecular with the SH3 domain of STAM</td>
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<td>ANOVA</td>
<td>Analysis of Variance</td>
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<td>AP-3</td>
<td>Adaptor protein complex 3</td>
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<tr>
<td>AP2</td>
<td>Adaptor protein 2</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonia persulfate</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid assay</td>
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<tr>
<td>BME</td>
<td>β-mercaptoethanol</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>C-tail</td>
<td>Carboxyl-terminal tail</td>
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<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CaR</td>
<td>Calcium-sensing receptor</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>CCP</td>
<td>Clathrin-coated pit</td>
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<td>CCV</td>
<td>Clathrin-coated vesicles</td>
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<td>CT</td>
<td>C-terminal</td>
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<td>CXCL12</td>
<td>C-X-C motif chemokine 12</td>
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<td>CXCR4</td>
<td>Chemokine receptor 4</td>
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<tr>
<td>Cys</td>
<td>Cysteine</td>
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<tr>
<td>DAG</td>
<td>Diacyl glycerol</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
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<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>dNTPs</td>
<td>Deoxyribose nucleotide triphosphates</td>
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<td>DOR</td>
<td>delta-opioid receptor</td>
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<td>Dithiothreitol</td>
</tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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<td>Deubiquitinase</td>
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<td>ECD</td>
<td>Extracellular domain</td>
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<td>Acronym</td>
<td>Full Description</td>
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<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EE</td>
<td>Early endosome</td>
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<td>EEA1</td>
<td>Early endosomal antigen 1</td>
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<td>EGF</td>
<td>Epidermal growth factor</td>
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<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<td>ERK</td>
<td>Extracellular signal regulated kinase</td>
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<td>ESCRT</td>
<td>Endosomal sorting complex required for transport</td>
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<td>FBS</td>
<td>Fetal bovine serum</td>
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<td>FL</td>
<td>Full-length</td>
</tr>
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<td>FYVE</td>
<td>Fab1, YOTB, Vac1 and early endosome antigen 1</td>
</tr>
<tr>
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</tr>
<tr>
<td>GABA\textsubscript{B}</td>
<td>gamma-amino-butyric acid type B</td>
</tr>
<tr>
<td>GAIN</td>
<td>GPCR-autoproteolysis inducing</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GLUE</td>
<td>Gram-like ubiquitin-binding in EAP45</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>GPS</td>
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</tr>
<tr>
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</tr>
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</tr>
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<td>-------------</td>
</tr>
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<td>Hour</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin</td>
</tr>
<tr>
<td>HACE1</td>
<td>HECT domain and ankyrin repeat containing E3 ubiquitin protein ligase 1</td>
</tr>
<tr>
<td>HECT</td>
<td>Homologous to the E6-AP Carboxyl Terminus</td>
</tr>
<tr>
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</tr>
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<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>HER2</td>
<td>human epidermal growth factor receptor</td>
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<td>HIV</td>
<td>human immunodeficiency virus</td>
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<td>HRP</td>
<td>Horseradish peroxidases</td>
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<td>HRS</td>
<td>Hepatocyte growth-factor regulated tyrosine kinase</td>
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<tr>
<td>i.e.</td>
<td>id est = “that is”</td>
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<td>IB</td>
<td>Immunoblot</td>
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<td>Intracellular domain</td>
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<td>IP3</td>
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<td>kb</td>
<td>kilobase</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>-------------</td>
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</tr>
<tr>
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<td>MgCl$_2$</td>
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<tr>
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<td>Millimolar</td>
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<td>Multivesicular body</td>
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</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NEB</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>Nedd4</td>
<td>Neuronal precursor cell-expressed developmentally downregulated 4</td>
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<td>NEM</td>
<td>N-Ethylmaleimide</td>
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<tr>
<td>nm</td>
<td>Nanometer</td>
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<tr>
<td>nM</td>
<td>Nanomolar</td>
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<tr>
<td>NT</td>
<td>N-terminal</td>
</tr>
<tr>
<td>PAR1</td>
<td>Protease-activated receptor 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PAR2</td>
<td>Protease-activated receptor 1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
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<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
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<tr>
<td>PI3-K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PI3P</td>
<td>Phosphotidylinositol 3-phosphate</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphotidylinositol 4,5 bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
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<tr>
<td>PLC</td>
<td>Phospholipase C</td>
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<tr>
<td>PLL</td>
<td>Poly-L-lysine</td>
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<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
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<tr>
<td>PRR</td>
<td>Proline rich region</td>
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<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
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<tr>
<td>RGS</td>
<td>Regulators of G protein signalling</td>
</tr>
<tr>
<td>RING</td>
<td>Really interesting new gene</td>
</tr>
<tr>
<td>RLD</td>
<td>Regulator of chromosome condensation</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
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SD  Standard deviation
SDF  Stromal cell derived factor
SDS  Sodium dodecyl sulfate
SDS-PAGE  Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec  Second
SEM  Standard error of the mean
SH3  SRC homology 3
siRNA  Small interfering RNA
SMURF  Smad ubiquitin regulatory factor
STAM  Signal transducing adaptor molecule
STAT  Signal transduction and activator of transcription
TBS  Tris-Buffered Saline
TBS-T  Tris-Buffered Saline plus Tween 20
TEMED  Tetramethylethylenediamine
TM  Transmembrane
Ub  Ubiquitin
UBA  Ubiquitin associate domain
UBC  Ubiquitin conjugating
UEV  Ubiquitin E2 variant
UIM  Ubiquitin interacting motif
USP      Ubiquitin specific protease
V        Volts
V2R      V2 Vasopressin receptor
Vps      Vacuolar protein sorting
WH       Winged helix
WHIM     Warts, Hypogammaglobulinemia, Infections, Myelokathexis
WT       Wild type
β2AR     Beta 2 adrenergic receptor
µg       microgram
µl       microgram
µM       micromolar
ABSTRACT

G protein-coupled receptor (GPCR) sorting into the degradative pathway is important for attenuating signaling. Perturbations in this process can manifest in a variety of diseases. Upon agonist activation of the chemokine receptor CXCR4, a GPCR, it is rapidly ubiquitinated, internalized to endosomes and sorted for degradation in lysosomes via the endosomal sorting complex required for transport (ESCRT) pathway. This process culminates in attenuation of CXCR4 signaling. CXCR4 overexpression and increased CXCR4 signaling have been associated with several pathologies including immune deficiency disorders and over 23 cancers. Yet the mechanisms governing the regulation of CXCR4 signaling remain elusive.

CXCR4 is ubiquitinated by the HECT-domain E3 ligase AIP4 at the plasma membrane. AIP4 is also localized on early endosomes and regulates CXCR4 sorting by modulating the activity of the ESCRT machinery. In particular, ESCRT-0 ubiquitination has been shown to be linked to the efficiency by which CXCR4 is sorted for lysosomal degradation. However, mechanistic insight is lacking and the precise role of AIP4 in these processes remains poorly defined.

The objective of this project is to gain a greater understanding of the mechanisms mediating AIP4 regulation of CXCR4 degradation. AIP4 is known to interact with other E3 ligases, including DTX1 and Cbl-c, but whether these E3 ligases or others are
involved in CXCR4 sorting is not known. Here, we show for the first time that the RING-domain E3 ubiquitin ligase Deltex-3-like (DTX3L) mediates CXCR4 sorting from early endosomes to lysosomes. Using several biochemical and immunochemical techniques including fixed cell confocal immunofluorescence microscopy, co-immunoprecipitation and in vitro ubiquitination assays, we show that upon CXCR4 activation DTX3L localizes to early endosomes where it directly interacts with and inhibits the activity of the AIP4. Thereby, limiting the extent to which ESCRT-0 is ubiquitinated while promoting CXCR4 sorting for lysosomal degradation. Therefore, we have defined a novel role for DTX3L in GPCR endosomal sorting and propose that DTX3L may play a broad role in endosomal sorting. In addition, our data reveal an unprecedented link between two distinct E3 ubiquitin ligases to control the activity of the ESCRT machinery. Overall, these findings may prove beneficial in developing strategies to modulate CXCR4 levels and be broadly applicable to CXCR4-related pathologies.
CHAPTER I
INTRODUCTION

OVERVIEW OF G PROTEIN-COUPLED RECEPTORS

G protein-coupled receptors (GPCRs) comprise a superfamily of proteins that promote changes in cellular response attributable to nearly every physiological process. GPCRs are the largest class of targets for many drugs used to treat a variety of diseases highlighting their role pathophysiologically (Overington, Al-Lazikani, & Hopkins, 2006). Specifically, GPCRs are integral plasma membrane proteins that consist of single polypeptide chains containing seven transmembrane (7TM)-spanning domains that are connected by extracellular and intracellular loops (Pierce, Premont, & Lefkowitz, 2002). There are over 800 functional GPCRs expressed by the human genome that respond to a diverse array of stimuli and control a multitude of physiological processes including vision, smell, pain, neurotransmission, muscle contraction and immune responses (Fredholm, Hokfelt, & Milligan, 2007; Jacoby, Bouhelal, Gerspacher, & Seuwen, 2006; Pierce et al., 2002). The regulation of GPCR signaling is a tightly controlled process and perturbations in GPCR signaling have been linked to several pathologies (Jacoby et al., 2006; Pierce et al., 2002).

According to the International Union of Pharmacology (IUPHAR), GPCRs are classified into five distinct groups based on amino acid sequence similarity: Class-I
rhodopsin-like (family A), Class-II secretin-like (family B), Class-III glutamate (family C), Adhesion and Frizzled/other 7TM families (Hamann et al., 2015).

Class-I receptors comprise the largest group of GPCRs that consists of rhodopsin-like receptors. These receptors all have similarity to the first GPCR identified, the rhodopsin receptor and possess within their transmembrane helix a conserved Asp-Arg-Tyr (DRY) motif. Additionally, there are 273 genes encoding this family of receptors that include 89 orphan receptors for which no known ligands have been identified so far. Members of this family include: β1 and β2 adrenergic receptors, chemokine receptors, opioid receptors and dopamine receptors among many more important receptors. Nearly half of class A GPCRs are olfactory receptors that mediate responses to odor (Buck & Axel, 1991).

The second class of GPCRs include secretin-like receptors and are encoded by 48 genes (Zalewska, Siara, & Sajewicz, 2014). This class of GPCRs bind large glycoproteins and include secretin, calcitonin, corticotropin-releasing factor, growth-hormone releasing hormone and parathyroid hormone receptors (B. Martin et al., 2005). Class II GPCRs contain a large spanning N-terminal region and differ from Class I GPCRs in their amino acid sequence. In particular, these receptors lack the DRY motif found in Class I GPCRs, however, Class II GPCRs display the same heptahelical transmembrane structure.

The third class of GPCRs consists of metabotropic receptors encoded by 22 genes. Receptors in this class bind to relatively small ligands such as glutamate and Ca^{2+}. 
Members of this group include metabotropic gamma-amino-butyric acid type B (GABA_B), calcium-sensing receptor (CaR) and glutamate receptors. Although similar in structure, Class III GPCRs share little sequence identity within their amino acid sequence with Class I and II (Brauner-Osborne, Wellendorph, & Jensen, 2007).

The fourth group of GPCRs is classified as adhesion receptors of which there are 33 members. These receptors are characterized by a long N terminal region containing a GPCR-Autoproteolysis Inducing (GAIN domain) and by a distinct 7TM region displaying little similarity with other families of GPCRs (Arac et al., 2012). Adhesion receptors are required for cell to cell as well as cell to extracellular matrix interactions. An interesting feature of adhesion receptors is that they undergo proteolysis within the N-terminal region mediated at the GPCR Proteolysis Site (GPS) of the GAIN domain. Based on these characteristics, the structure of these receptors is sub-divided into a long spanning extracellular domain (ECD), 7TM domain and intracellular domain (ICD). The majority of adhesion receptors are classified as orphan receptors (Schulte, 2010). However, it has been identified that several of these receptors bind to small molecules or peptides (Hamann, Vogel, van Schijndel, & van Lier, 1996; Stacey et al., 2003; Wandel, Saalbach, Sittig, Gebhardt, & Aust, 2012; T. Wang et al., 2005).

The final group of GPCRs are encoded by 11 genes that include Frizzled receptors involved in Wnt signaling, Smoothened receptors involved in hedgehog signaling and taste receptors that mediate taste responses (Kinnamon, 2012; Schulte, 2010). Taste
receptors are subdivided into two types, T1Rs for sweet and umami stimuli and T2Rs for bitter stimuli (Kinnamon, 2012). Many of these receptors are important in embryonic development and are highly homologous to the second class of GPCRs.

**CHEMOKINE RECEPTORS**

Chemokine receptors belong to the Class A superfamily of GPCRs. In mammals, approximately 19 chemokine receptors have been identified and each can activate a diverse signaling pathway (Murphy et al., 2000). Chemokine receptors bind to shared or specific ligands known as chemokines (Allen, Crown, & Handel, 2007). Chemokines are small proteins (8-10 kDa) whose function is associated with chemotaxis or cell migration. Chemokines are divided into four classes based on the spacing of the N-terminal cysteine residues within the proteins structure (i.e. C, CC, CXC, CX3C) (Allen et al., 2007; Baggioolini, Dewald, & Moser, 1997; Fernandez & Lolis, 2002). Chemokine receptors are named based on the class of chemokine they bind. For example, CXC receptors (CXCR) bind to the class of CXC chemokines; CC receptors (CCR) bind CC ligands, etc. Chemokine receptors regulate a multitude of cellular processes necessary for development as well as hematopoiesis, angiogenesis and inflammation in the adult (Baggioolini et al., 1997; Moser & Loetscher, 2001; Premack & Schall, 1996). Chemokines are known to regulate immune cell trafficking (i.e. homing, extravasion, circulation) among several other biological functions such as cell adhesion and cytokine secretion (Zlotnik, 2006).
GPCR ACTIVATION

Activation of GPCRs allows for the transmission of information from the extracellular environment into the inside of the cell. Classically, GPCR signaling is mediated through associated guanine nucleotide binding proteins (G proteins) that are heterotrimers composed of \( \alpha \) and \( \beta \gamma \) heterodimer (Gilman, 1987). Coupling of the receptor to the associated G proteins allows for the transduction of information from the outside of the cell to the inside. GPCR signaling can also be mediated via mechanisms independent of the associated G protein. These topics are highlighted in the following section.

GPCR SIGNALING

Upon agonist binding, GPCRs undergo a conformational change primarily within the transmembrane regions 3 (TM3) and 6 (TM6) that facilitates the exchange of GDP for GTP on the \( \alpha \)-subunit of associated G protein (Gilman, 1987; Pierce et al., 2002). This event causes dissociation of the heterotrimeric G protein into \( \alpha \) and \( \beta \gamma \) subunits which go on to activate a diverse array of effector proteins to initiate signal transduction of the external stimulus to the internal compartments of the cell (Fredholm et al., 2007; Oldham & Hamm, 2008; Pierce et al., 2002). This signaling culminates in discrete changes in cellular response (Figure 1.1). The \( \text{G} \alpha \)-subunit coupled to a GPCR can vary based on the type of ligand that binds to a particular GPCR. \( \text{G} \alpha \text{i} \) inhibits adenylyl cyclase leading to a reduction in the conversion of to adenosine triphosphate (ATP) into the second
messenger cyclic AMP (cAMP), whereas $G_{\alpha}$ activates adenylyl cyclase to increase cAMP levels. The $G_{\alpha}q$ subunit stimulates phospholipase C (PLC-\(\beta\)) that leads to production of diacylglycerol (DAG) and inositol trisphosphate (IP\(_3\)) from phosphatidylinositol bisphosphate (PIP\(_2\)). The $G_{\alpha}12/13$ subunit is involved in the activation of Rho family GTPases that regulate the actin cytoskeleton. Additionally, the $\beta\gamma$ subunits activate PI3K, PLC- and adenylyl cyclase signaling pathways (Oldham & Hamm, 2008).

Some GPCR signaling does not involve the heterotrimeric G proteins (Figure 1.1). This G protein independent signaling can lead to activation of several signaling pathways including Janus kinase and signal transducer and activator of transcription signaling pathway (JAK/STAT), mitogen-activated protein kinase (MAPK) and p38. Taken together both G protein dependent and independent signaling downstream of GPCR activation leads to changes in cellular responses including migration, proliferation and survival.
Figure 1.1 GPCR Activation and Signaling. GPCR activation can lead to both G protein and G protein independent signaling. Ligand activation of GPCRs results in conformational change in the receptor that promotes the exchange of GDP for GTP on the α-subunit. This results in the dissociation of the heterotrimeric G protein into α and βγ subunits. Certain GPCRs bind preferentially to a subtype of the α-subunit. The Gα-subunit can be further subdivided based on the second messengers they activate. Additionally, the βγ subunits activate particular second messengers and, hence, downstream signaling pathways. G protein independent signaling can also lead to activation of several signaling pathways (Dorsam & Gutkind, 2007).
REGULATION OF GPCR SIGNALING

A key component to the regulation of GPCR signaling involves desensitization, internalization and downregulation or recycling of the receptor. Desensitization is the process by which receptor signaling following initial activation is rapidly terminated even in the continued presence of stimulus. Typically the removal of activated GPCRs from the cell surface occurs via a complex process leading to their internalization and endocytosis onto intracellular compartments known as endosomes (Marchese, Chen, Kim, & Benovic, 2003). On endosomes, GPCRs can be sorted for long-term signaling attenuation by degradation in the highly acidic environment of the lysosome (Marchese, Chen, et al., 2003; Tsao & von Zastrow, 2000). Conversely, GPCRs can be recycled back to the cell surface following dephosphorylation by an endosomal associated phosphatase, therefore, resensitizing the GPCR to further rounds of receptor signaling (Pitcher, Payne, Csortos, DePaoli-Roach, & Lefkowitz, 1995).

DESENSITIZATION

Regulation of GPCR signaling can occur at multiple levels including the level of the signaling proteins and/or the receptor itself. Following the activation of the associated G protein via the exchange of GDP for GTP within the \( \alpha \)-subunit, GPCR signaling can be terminated through GTP hydrolysis. This is mediated by accessory proteins such regulators of G protein signaling (RGS) enzymes. RGS proteins are multifunctional enzymes that facilitate GTP hydrolysis and act to turn off G protein dependent signaling.
pathways (De Vries, Zheng, Fischer, Elenko, & Farquhar, 2000). While effective at deactivating G protein-mediated signaling, this process occurs rather slowly within a time frame of 50 seconds (Ross & Wilkie, 2000). Alternatively, the process of signal termination can occur directly through modification and recruitment of adaptor proteins to the GPCR. Receptors can be desensitized intracellularly by a G protein receptor kinase (GRK) dependent pathway or through the activities of second messenger-dependent kinases including protein kinase A (PKA) and protein kinase C (PKC) (Moore, Milano, & Benovic, 2007).

Stimulation of GPCRs usually results in rapid phosphorylation on serine or threonine amino acid residues located within the third intracellular loop by GRKs (Krupnick, Goodman, Keen, & Benovic, 1997). Mammalian GRKs are subdivided into three groups: The first group is comprised of GRK1 and GRK7, the second GRK2 and GRK3 and the third GRK4, GRK5, and GRK6. Early studies identified that GRK2 regulated agonist-promoted internalization of the M2 muscarinic acetylcholine (M2) receptor (Tsuga, Kameyama, Haga, Kurose, & Nagao, 1994). In particular, overexpression of GRK2 promoted internalization, whereas a catalytically inactive mutant of GRK2 did not. GRK-mediated phosphorylation occurs within a few seconds following receptor activation and provides a binding surface for the adaptor protein β-arrestin, which is required to uncouple the receptor from the associated G protein (Gurevich et al., 1995; Lohse, Benovic, Codina, Caron, & Lefkowitz, 1990; Moore et al., 2007). β-arrestins prevent further coupling to the associated G protein through steric hindrance and bridge the receptor to components of the internalization machinery (Lohse
et al., 1990). β-arrestins can promote signal termination either through degradation of second messengers (Perry et al., 2002) or internalization of the GPCR (Goodman et al., 1996; Laporte et al., 1999). This type of GRK and β-arrestin mediated desensitization is known as homologous desensitization (Figure 1.2).

Alternatively, GPCR signaling can be regulated by heterologous desensitization. In this process, activation of one receptor can lead to the activation of a different receptor through the activities of PKC or PKA. For example, CXCR4 contains many serines (Ser) that are potential PKC phosphorylation sites within its C-terminal tail. Stimulation with the phorbol ester phorbol-12-myristate-13-acetate (PMA) activates PKC and can promote the phosphorylation and internalization of CXCR4 (Haribabu et al., 1997; Signoret et al., 1997; Signoret et al., 1998). Mutational analysis of serine residues within CXCR4 C-terminal tail determined that internalization upon PMA treatment was dependent on either Ser 324 and Ser 325 or Ser 338 and Ser 339 (Signoret et al., 1998). In particular using a phospho-specific antibody, it was demonstrated that PMA treatment could mediate phosphorylation of Ser 339. However, the precise mechanism of PMA promoted CXCR4 phosphorylation and internalization remains to be explored. Overall, PKC regulation of CXCR4 phosphorylation is an important mechanism that regulates also CXCR4 plasma membrane expression.

Additional examples of GPCRs regulated by heterologous desensitization include the dopamine receptor (D3R) (Cho et al., 2007) and metabotropic glutamate receptor 4 (mGluR4) (Mathiesen & Ramirez, 2006). The desensitization of D3R is mediated by a PKC dependent mechanism but independent of GRK/β-arrestin (Cho et al., 2007). In
particular, treatment with PMA induces PKC phosphorylation of Ser 229 and 257 in D3R leading to its downregulation. In the case of mGluR4, its desensitization is also dependent upon PMA activation of PKC (Mathiesen & Ramirez, 2006). Overall, both homologous as well as heterologous desensitization promote downregulation of GPCR signaling albeit by different mechanisms.

Figure 1.2. GPCR homologous desensitization and internalization. (1) GPCRs under basal or resting conditions are associated with heterotrimeric Guanine nucleotide-binding proteins (G proteins) that are heterotrimers composed of $\alpha$ and $\beta\gamma$ heterodimer. Under these conditions the associated $\alpha$-subunit of the G protein is inactive and bound to GDP. (2) Following activation of the receptor upon binding to its cognate ligand, the GPCR undergoes a conformation change that leads to the exchange of GDP for GTP on the $\alpha$-subunit. This results in dissociation of the heterotrimeric G proteins into $\alpha$ and $\beta\gamma$ subunits, which are able to activate downstream signaling pathways through activation of a diverse array of effector molecules. (3) GPCR signaling is typically terminated through G protein receptor kinase (GRK) recruitment to the activated receptor leading to phosphorylation of serine or threonine residues within the C-terminal tail of the GPCR.
(4) Arrestin can bind to the phosphorylated receptor and acts to uncouple the receptor from the associated heterotrimeric G proteins through steric hindrance. This G protein uncoupling promotes receptor desensitization. (5) Desensitized receptors are internalized as a result of the interaction with Arrestin with components of the internalization machinery, clathrin and AP2. (6) Receptor is internalized via clathrin-coated pits.

INTERNALIZATION

One of the major pathways in which a GPCR internalizes is via clathrin-coated pits (CCPs) at the plasma membrane. Two important components of the internalization machinery include clathrin and clathrin-associated protein (AP) complexes (Figure 1.2) (Goodman et al., 1996). Clathrin relies upon adaptor and regulatory proteins to induce formation and invagination of CCPs. In particular, clathrin adaptor protein 2 (AP2) is recruited to the plasma membrane via the phospholipid PIP2. AP2 functions as an adaptor protein and can recognize tyrosine or dileucine-based motifs within the C-terminal tail of some GPCRs to facilitate entry into the forming CCP (as reviewed in Marchese, et al. 2008). Non-visual arrestins (β-arrestin 1 and 2) can interact with both clathrin and AP2 to also link the GPCR into the forming CCP. Some of the first studies to demonstrate a role for β-arrestins in GPCR internalization came from studies of the β2 adrenergic receptor (β2 AR) (Ferguson et al., 1996; Goodman et al., 1996). It was demonstrated that β-arrestins facilitate β2 AR internalization by binding with high affinity directly to clathrin (Goodman et al., 1996). In addition, it has been shown that β-arrestins through discrete elements in the C-terminal tail interact with both heavy chain of clathrin and β-subunit of
AP2 (Y. M. Kim & Benovic, 2002). This interaction of β-arrestins with the assembled CCP promotes recruitment of additional clathrin and clathrin adaptor proteins, culminating in the invagination and release of the CCP from the plasma membrane.

Alternatively, ubiquitin modified GPCRs or those containing short linear peptide sequences can be recognized by alternative clathrin adaptors to mediate entry into CCPs. In addition to binding β-arrestins, AP2 can bind to receptor tyrosine or di-leucine motifs. For example, within its C-terminal tail CXCR2 contains two di-leucine motifs. When these motifs are mutated, CXCR2 can still bind β-arrestins, but is unable to bind AP2 and internalize (Fan, Yang, Wang, Qian, & Richmond, 2001). Thus in the case of CXCR2, β-arrestins do not play a role in internalization. Furthermore, ubiquitin can have an indirect role in GPCR internalization via ubiquitination of β-arrestins. However, the internalization of most GPCRs does not require direct ubiquitination but instead relies upon β-arrestin dependent mechanisms (Kang, Tian, & Benovic, 2014).

DOWNREGULATION

Following internalization, GPCRs can be trafficked to early endocytic compartments where they are sorted for degradation in the lysosome or recycling via recycling endosomes (Figure 1.3). Ubiquitin modification of some GPCRs at the plasma membrane allows for entry into the degradation pathway on early endosomes. This ubiquitin dependent downregulation was first described for β2 AR (Shenoy, McDonald, Kohout, & Lefkowitz, 2001) and the chemokine receptor CXCR4 (Marchese & Benovic, 2001). Upon agonist activation, B2AR was shown to be ubiquitinated, thereby, promoting
its degradation. CXCR4 downregulation is dependent on AIP4-mediated ubiquitination (Marchese, et al., 2003). Other GPCRs that require ubiquitin for their downregulation include PAR2, V2 vasopressin receptor (V2R), and the kappa-opioid receptor (KOR) (Cottrell et al., 2006; Jacob et al., 2005; N. P. Martin, Lefkowitz, & Shenoy, 2003). For these ubiquitin modified receptors, it is thought that ubiquitin acts as a sorting signal that allows for the receptor to be recognized by the ESCRT machinery on early endosomes and subsequently concentrated into the invaginating domain of the endosome that buds off to form multivesicular bodies (MVBs). These MVBs fuse with lysosomes leading to receptor degradation (Figure 1.3).

Some GPCRs are not modified by ubiquitin upon agonist stimulation and, therefore, require additional factors for downregulation as is the case for PAR1. In fact, PAR1 is constitutively ubiquitinated at the plasma membrane and following agonist activation is deubiquitinated promoting its internalization. Once localized onto early endosomes, PAR1 is sorted on endosomes independent of endosomal sorting complex required for transport (ESCRT) machinery components ESCRT-0 (HRS) and ESCRT-I (Tsg101). Instead, the accessory proteins adaptor protein complex 3 (AP-3) and ALG-interacting protein X (ALIX) are required. AP-3 binds to internalized PAR1 on endosomes and facilitates the interaction of PAR1 with ALIX. ALIX binds to the YPX(3)L motif of PAR1 (intracellular loop 2) and recruits ESCRT-III (Dores et al., 2012). Thus, ALIX mediates sorting of PAR1 into MVBs independent of ESCRT-0-II and receptor ubiquitination.
Figure 1.3. General model of GPCR endosomal trafficking. Activation of some GPCRs does not result in receptor ubiquitination (orange receptor). These receptors upon internalization localize to endosomes where they enter the recycling pathway allowing for further rounds of signaling at the plasma membrane. Conversely, some activated receptors (purple receptor) can be modified at the plasma membrane by ubiquitin (Ub) prior to internalization. Internalized receptors localize to the endosome where the ubiquitin modified receptor can be sorted to either degradation or recycling pathways upon deubiquitination by an endosomally associated deubiquitinase (DUB).
RECYCLING

Internalized GPCRs can also be trafficked to early endocytic compartment where they are sorted for recycling via recycling endosomes. Some ubiquitinated receptors can have ubiquitin removed by deubiquitinating enzymes (DUBs) (Figure 1.3). These receptors can traffic back to the plasma membrane by recycling endosomes. Alternatively, GPCRs that are not ubiquitinated prior to internalization and localization to endosomes directly enter the recycling pathway and are returned to the plasma membrane to elicit further signaling.

THE UBIQUITIN PATHWAY

Post-translational modification of protein substrates by ubiquitin dictates the cellular fate of the protein. For example, ubiquitin modification of GPCRs plays a major role in membrane trafficking process on endosomes. In addition, ubiquitin modification of adaptors can regulate signaling downstream of receptors. This section will focus on the ubiquitin pathway and the factors necessary to mediate ubiquitin modification of protein substrates.

UBIQUITIN CONJUGATION

Ubiquitin is a 76 amino acid protein that becomes covalently attached to protein substrates through the formation of an isopeptide bond between the C-terminus of ubiquitin and the lysine residues on target substrates. Specifically, the epsilon amino group of a lysine residue within the protein substrate becomes covalently attached to the
C-terminal glycine residue (Gly-76) of ubiquitin. Ubiquitin conjugation of proteins is carried out by an enzymatic cascade involving the sequential activity of three enzymes: E1, E2 and E3 (Fang & Weissman, 2004; Pickart, 2001). In this process, ubiquitin is first activated in an ATP-dependent manner by the E1-activating enzyme (Figure 1.4). The E1 enzyme forms a thioester bond with activated ubiquitin, which is then transferred to the active site cysteine of the E2-conjugating enzyme. The E2 enzyme helps link E1 activation of ubiquitin to final E3 covalent modification of protein substrates. Final substrate ubiquitination is mediated by the E3 ligase that recruits and binds specific substrates.

Figure 1.4. The Ubiquitin Pathway. Ubiquitin conjugation to protein substrates is dependent upon the activities three enzymes: E1, E2 and E3. The E1 activating enzyme activates ubiquitin in an ATP-dependent fashion leading to the formation of a thioester bond between activated ubiquitin to the active site cysteine (Cys) in the E1 enzymes. The E2 conjugating enzyme accepts the activated ubiquitin forming a thioester linkage
between ubiquitin and the active site Cys in the E2 enzyme. Final substrate ubiquitination is mediated by the activities of one of two classes of E3 ubiquitin ligases. HECT domain E3 ligases accept ubiquitin from the E2 enzyme and lead to direct ubiquitination of the protein substrate. In contrast, RING and RING-like domain E3 ligases facilitate indirect ubiquitination of protein substrates by bridging the E2 enzyme in proximity to the substrate. Ultimately, this process leads to the covalent attachment of C-terminal glycine residue in ubiquitin to epsilon amino group of a lysine residue within the substrate. Substrates can either be modified by one ubiquitin (monoubiquitination) or subject to modification with multiple ubiquitin molecules (polyubiquitination).

**TYPES OF UBIQUITIN MODIFICATION**

Ubiquitin modification of substrates can have multiple outcomes depending on the type of ubiquitin modification. There are several types of ubiquitin modifications that have been identified. The particular type of ubiquitin modification, monoubiquitination versus polyubiquitination, of a substrate will dictate its cellular function. Monoubiquitination or multi-mono ubiquitination have been implicated in receptor internalization and trafficking (Haglund, Di Fiore, & Dikic, 2003; Polo et al., 2002). Ubiquitin itself can act as an acceptor of additional ubiquitin molecules. Within its structure ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, Lys 48 and K63) that can all be linked to ubiquitin to form poly-ubiquitin chains.

The most common types of poly-ubiquitin chains are those formed by linkages at K48 and K63 (Rotin & Kumar, 2009). K48, as well as K29, linkages have been
demonstrated to promote proteolysis of misfolded or damaged substrates mediated by the 26 S proteasome. K48 poly-ubiquitin chains are recognized by the ubiquitin interacting motif (UIM) of proteasomal receptor S5a/Rpn1 (Pickart, 2000; Sparks et al., 2014). Overexpression of a dominant negative form of S5a/Rpn1 lacking it UIMs or siRNA depletion of RS5a/Rpn1 inhibits proteasomal degradation and stabilization of tumor suppressor protein p53 (Sparks et al., 2014). The ubiquitin-associated domain (UBA) containing protein Rad23 binds to K48 chains, thereby, inhibiting their interaction with the 26 S proteasome and proteolysis. This demonstrates that ubiquitin binding domains can both positively and negatively regulate the fate of ubiquitinated proteins (Raasi & Pickart, 2003).

In contrast to K48 linkages, K63 poly-ubiquitin chains play a major role in post-endocytic sorting and localization of substrates for degradation in lysosomes (Erpapazoglou et al., 2012; Lauwers, Jacob, & Andre, 2009). In particular, K63 chains have been linked to MVB biogenesis and UBD containing proteins such as the ESCRT-0 complex recognize K63-modified receptors on endosomes (Erpapazoglou et al., 2012). It has also been suggested that the ESCRT-0 complex itself can be modified by K63 ubiquitination to modulate receptor sorting (Erpapazoglou et al., 2012).

The less common or atypical poly-ubiquitin linkages have been shown to function in several cellular pathways. The function of K6 and K11 poly-ubiquitin chains has been implicated in neurodegenerative disorders (Cripps et al., 2006). For example, loss of the E3 ligase Mahogunin ringer finger-1 (MGRN1) has been implicated in spongiform neurodegeneration. It has been shown that the auto-catalytic activity of the E3 ligase
BRCA1 is dependent upon K6 to promote BRCA functioning with the E3 ligase BARD1 (Wu-Baer, Lagrazon, Yuan, & Baer, 2003). Additionally, K11 poly-ubiquitination plays a major role in endoplasmic reticulum-associated degradation (ERAD) pathway (Xu, P et al., 2009). K27 and K33 poly-ubiquitin chains have been identified in vitro, however, until recently the physiological function of atypical poly-ubiquitin chains linked to K27 and K33 have started to be demonstrated (Birsá et al., 2014; David et al., 2010; Yuan et al., 2014). Recently, it has been shown that K27 poly-ubiquitination of the outer mitochondrial membrane protein Miro1 is dependent upon the E3 ligase Parkin following mitochondrial damage in human dopaminergic neuroblastoma cells (Birsá et al., 2014). Dysregulation of this K27 ubiquitination may play a role in the pathogenesis of Parkinson’s disease. K33 poly-ubiquitin chains have been implicated in trans-Golgi network (TGN) trafficking (Yuan et al., 2014). The ubiquitin ligase Cul30KLHL20 mediates K33 poly-ubiquitination of coronin 7 that is essential for the biogenesis of transport carriers derived from the TGN. Future studies are needed to further shed light on the role of these atypical poly-ubiquitin chains in the regulation of other cellular processes including GPCR trafficking.

Importantly, it has been shown that HECT-(Homologous to the E6-AP Carboxyl Terminus) domain E3 ligases directly contribute to the type of chain linkage formed through its interactions with the E2 enzyme whereas for RING (Really Interesting New Gene) domain ligases the E2 determines the chain linkage (Christensen, Brzovic, & Klevit, 2007; H. C. Kim & Huibregtse, 2009; Li, Tu, Brunger, & Ye, 2007; Schwarz, Rosa, & Scheffner, 1998). For example, the HECT domain of E6AP preferentially
catalyzes K48 poly-ubiquitin chains whereas other HECT ligases such as Rsp5/Itch catalyze K63 poly-ubiquitin chains. The type of E2 does not, however, determine chain specificity given that Rsp5 created similar K63 poly-ubiquitin linkages regardless of the E2 (Ubc1, Ubc5 and Ubc4). Furthermore, a chimera protein of Rsp5 swapped with the C lobe of E6AP conferred Rsp5 the ability to form K48 poly-ubiquitin chains typified by the HECT domain of E6AP. Replacement of the C lobe of Itch/AIP4 with that of E6AP conferred Itch/AIP4 with the ability to form K48 poly-ubiquitin chain formation (Huibregtse, Scheffner, Beaudenon, & Howley, 1995). Thus, the particular type of E3 ubiquitin ligase as well as E2 conjugating enzyme have a major role in dictating the ubiquitin modification of substrates.

**E3 UBIQUITIN LIGASES**

E3 ligases represent a diverse family of over 600 identified proteins in the mammalian genome (Metzger, Hristova, & Weissman, 2012; Metzger & Weissman, 2010). Given the vast number of E3 ubiquitin ligases able to interact with discrete substrates, E3s often provide a level of specificity to the ubiquitin reaction. E3s are generally distinguished by whether they enable indirect or direct modification of protein substrates. E3 ligases are divided into two classes: HECT ligases that facilitate direct ubiquitination of substrates or ligases that act as scaffolds including RING and RING-like F-box domain ligases (Deshaies & Joazeiro, 2009; Metzger et al., 2012). Over 95% of mammalian E3 ligases belong to the RING or RING-like domain family and approximately 30 ligases belong to the HECT domain family (Metzger et al., 2012). Of
the 95% of RING domain ligases, the RING-like family of F-box containing ligases comprise eight members (Petroski & Deshaies, 2005).

RING AND RING-LIKE DOMAIN CONTAINING LIGASES

RING-domain or RING-like domain containing E3s act as scaffolds in the ubiquitination reaction, thereby, facilitating the indirect transfer of ubiquitin from the bound E2 enzyme to the protein substrate. Thus, RING domain and RING-like E3s lack intrinsic catalytic activity and instead act as adaptors. The RING domain motif is characterized by a specific sequence of evenly spaced Cysteines and Histidines residues that are able to coordinate two zinc ions in a crossbrace fashion (Metzger et al., 2012). This enables E2 binding and E2-mediated ubiquitination of substrates.

The crystal structure of the RING domain of c-Cbl in complex with the E2 enzyme UbcH7 and substrate (a ZAP70 kinase peptide) highlights the typical features of a RING domain in mediating indirect transfer of ubiquitin (Zheng, Wang, Jeffrey, & Pavletich, 2000). These studies reveal that three-stranded Beta sheets, an alpha helix and two large loops form the structure of the RING domain, which is stabilized by two zinc ions. UbcH7 binds the RING domain of c-Cbl and is separated by ~60 A from the substrate also bound to the RING domain. Moreover, the specificity of the E2 enzyme for c-Cbl RING domain lies between the interaction of Phe63 of UbcH7 and Trp408/Ile383 of c-Cbl RING domain. Interestingly, comparing RING domain of c-Cbl to the crystal structure of the HECT domain of E6AP bound to UbcH7 reveals a common structural theme between E2 binding to HECT or RING domains even though both are distinct in
their structures (Huang et al., 1999). The crystal structures of the dimeric RING-like domain ligases such as the U-box ligase CHIP display a similar interaction with the yeast E2 Ubc13 and mammalian E2 UbcH5a (Xu et al., 2008; Zhang et al., 2005). UbcH5a binds to the CHIP hydrophobic groove that includes Phe62 whereas Ubc13 binds to CHIP via Met 64, a residue homologous to Ph63. This suggest that cognate E2s for their E3s depends solely on the structural contacts made between the E2 and E3, not the type of E3 domain.

HECT-DOMAIN LIGASES

HECT-domain ligases all possess a highly conserved ~350 amino acid C-terminal HECT domain that directly accepts ubiquitin within a conserved cysteine residue and facilitates substrate ubiquitination directly. The HECT domain is a bilobal domain divided into an N and C Lobe linked by a broad catalytic cleft (Huang et al., 1999). The N-lobe is where E2 enzyme binding occurs while the C lobe contains the catalytic cysteine residue that forms the thioester linkages with ubiquitin. The two lobes are linked by a flexible hinge region that is crucial for E2 transfer of ubiquitin chains to the HECT domain catalytic cysteine (Huang et al., 1999; Verdecia et al., 2003). HECT domain ligases have been shown to interact with E2 enzymes UbcH5, UbcH7 and UbcH8 (S. Kumar, Kao, & Howley, 1997; Nuber, Schwarz, Kaiser, Schneider, & Scheffner, 1996; Schwarz et al., 1998). This structure is well demonstrated for HECT domain ligase E6AP and UbcH7 (Huang et al., 1999). Phenylalanine 63 of UbcH7 is bound to E6AP on a hydrophobic groove within the N lobe region. This brings UbcH7 into close proximity to
the active site cysteine within the C-lobe of E6AP. The distance between the active site cysteine of the HECT domain and UbcH7 is 41 Å. Other HECT domain ligases are thought to display a similar structure to E6AP.

Although HECT ligases are highly homologous in their HECT domain, these ligases differ in their N-terminal regions. The binding of substrates occurs in regions outside of the HECT domain. HECT ligases are subdivided into three families: HERC, Nedd4 and other HECTs (Rotin & Kumar, 2009). The HERC family of HECT ligases is distinguished by the presence of regulator of chromosome condensation 1 (RCC1)-like domains (RLDs). RLDs can interact with chromatin and may be a Guanine nucleotide exchange factor (GEF) the GTPase Ran (Garcia-Gonzalo & Rosa, 2005; Rosa, Casaroli-Marano, Buckler, Vilaro, & Barbacid, 1996). Nedd4 family members contain two to four tandemly linked WW domains able to interact with proline-rich sequences and an N-terminal calcium-dependent phospholipid-binding domain. Other HECT ligases contain only HECT domains or various unique domains. For example, E6AP contains only a HECT domain whereas HACE1 (HECT domain and Ankyrin repeat Containing E3 ubiquitin-protein ligase 1) contains ankyrin repeats in addition to the HECT domain (Anglesio et al., 2004; Rotin & Kumar, 2009).

HECT DOMAIN LIGASES IN GPCR TRAFFICKING

Nedd4 HECT ligases have been implicated in the regulation of plasma membrane receptor trafficking (Marchese, Raiborg, et al., 2003; Rotin & Kumar, 2009; Shenoy et al., 2008). The Nedd4 family comprises nine mammalian members: Nedd4, Nedd4L,
AIP4 (Itch), WWP1, WWP2, SMURF1, SMURF2, NEDL1 and NEDL2. The Nedd4 ortholog in yeast, Rsp5, was the first Nedd4-related ligase shown to mediate endocytosis of a receptor (Belgareh-Touze et al., 2008; Dunn & Hicke, 2001). Following agonist activation by alpha-mating factor, it was shown that Ste2p receptor was rapidly ubiquitinlated by Rsp5. Rsp5 mediates monoubiquitination of Ste2p receptor which promotes receptor endocytosis and recognition by UIM containing endocytic adaptors epsin and Eps15 (Dupre, Urban-Grimal, & Hagenauer-Tsapis, 2004; Terrell, Shih, Dunn, & Hicke, 1998). In mammalian cells, atrophin-interacting protein 4 (AIP4) has been shown to mediate ubiquitin dependent downregulation of CXCR4 (Marchese, Raiborg, et al., 2003). Additionally, downregulation and desensitization of B2AR signaling is dependent on Nedd4-mediated receptor ubiquitination (Shenoy et al., 2001).

**ATROPHIN-INTERACTING PROTEIN 4 (AIP4)**

AIP4 was among the first ubiquitin ligases shown to be involved in agonist-promoted ubiquitination of a mammalian GPCR (Marchese, Raiborg, et al., 2003). AIP4 belongs to the NEDD4-family of HECT ligases. This ligase contains four tandemly linked WW domains, N-terminal calcium-dependent phospholipid-binding domain and a conserved carboxyl-terminal HECT domain (Figure 1.5A) (Rotin & Kumar, 2009). A unique feature of AIP4 is that is has a proline-rich region (PRR) that binds a subset of Src-homology-3 (SH3) domains. Studies have demonstrated that AIP4 can mediate substrate ubiquitination through the interaction of the HECT domain with UbcH5 and UbcH7 families of E2 enzymes (H. C. Kim & Huibregtse, 2009; Schwarz et al., 1998;
Interestingly, AIP4 has been shown to interact with other E3 ligases such as Cbl-c, deltex-1 (DTX1) and RNF11. These three ligases all belong to the RING domain family of E3 ligases.

The Cbl family of proteins consists of 3 related proteins: c-Cbl, Cbl-b and Cbl-c (Fig 1.5B with domain structure). Unlike c-Cbl and Cbl-b which are ubiquitously expressed, the expression of Cbl-c is documented as restricted to epithelial cells (Schmidt & Dikic, 2005). These proteins all contain within their structure an N-terminal tyrosine kinase binding domain (TKB) that mediates interactions with phospho-tyrosine modified proteins, a RING E3 ligase domain that leads to substrate ubiquitination and a proline rich region (PRR). In contrast to c-Cbl and Cbl-b, Cbl-c lacks the C-terminal ubiquitin-binding domain known as an ubiquitin-associated leucine zipper (UBA). The Cbl family of ligases has been implicated in the downregulation of epidermal growth factor receptor (EGFR) signaling (Courbard et al., 2002; Ettenberg et al., 1999; Levkowitz et al., 1999; Levkowitz et al., 1998). Using a yeast two-hybrid screen it was identified that Cbl-c interacts with AIP4 (Courbard et al., 2002). In this study, Cbl-c and AIP4 were shown to co-localize in cells upon EGF stimulation, however, the identity of the subcellular compartment where there interaction occurs was not demonstrated. Although is can be speculated that both proteins are localized to microdomains on early endosomes where EGFR is sorted for degradation. Biochemical assays demonstrated that the interaction between Cbl-c and AIP4 is mediated through the proline rich region of Cbl-c and the WW domains of AIP4. Furthermore, it was shown that AIP4 and Cbl-c act synergistically
to downregulate EGFR signaling, although the mechanism by which this occurs remains unknown.

AIP4 interacts directly with DTX1 on endosomes to target DTX1 for degradation in lysosomes, modulating its cellular activity (Chastagner, Israel, & Brou, 2006). The ortholog of AIP4 in Drosophila (Suppressor of Deltex (Su(Dx)), acts as a negative regulator of activated Notch signaling and antagonizes the activity of Deltex by promoting post-endocytic sorting and degradation of Notch (Mazaleyrat et al., 2003). In mammalian cells, it was demonstrated that AIP4 interacts with DTX1 on endosomes where is mediates K29 poly-ubiquitination of DTX1 targeting it for degradation in lysosomes. These studies conclude that AIP4 antagonizes the cellular function of DTX by promoting K29-dependent degradation of DTX in mammalian cells (Chastagner et al., 2006).

Another AIP4-interacting RING ligase is RNF11. RNF11 is overexpressed in several mammalian breast tumors and has been shown to interact with several HECT-domain containing ligases including AIP4, Smurf1 and Smurf2 (Kitching et al., 2003). However, the role of these ligase interactions physiologically remains to be defined.
Figure 1.5. Domain structure of AIP4, Cbl family and DTX family protein structure. A. AIP4 domain structure consists of a N-terminal C2 phospholipid binding domain, a central proline rich region (PRR), four tandemly linked WW domains and the C-terminal HECT E3 ligase domain. B. The Cbl family proteins of ubiquitin ligases are comprised of three members: c-Cbl, Cbl-b and Cbl-c. All three proteins consist of a Tyrosine Kinase Binding domain (TKB), which interacts with specific phosphotyrosines on substrates, RING ligase domain that mediates substrate ubiquitination and PRR. Both c-Cbl and Cbl-b also possess a C-terminal ubiquitin-associated leucine zipper (UBA) domain whereas Cbl-c lacks the N-terminal UBA domain. These proteins play major roles in the regulation of tyrosine kinase signaling pathways through the activities of their TKB domain. C. The deltex (DTX) family of ubiquitin ligases is comprised of three closely related proteins (DTX1-3) in addition to the distantly related protein known DTX3L. DTX ubiquitin ligases have a highly conserved carboxyl-terminal RING domain and members of the DTX family of proteins were first identified for their role in
Notch signaling. DTX1 and DTX2 contain a basic N-terminal region that binds to intracellular Notch ankyrin repeats in addition to a central PRR and. In contrast, DTX3 and DTX3L contain a unique N-terminus and lack the central proline-rich region; however, DTX3 and DTX3L are highly homologous to the other DTX family proteins within the C terminal RING domain.

**DELTEx-3-LIKE (DTX3L)**

The DTX family of ubiquitin ligases comprises three closely related proteins (DTX1-3) and a distantly related DTX3-like (DTX3L) (Figure 1.5C). DTX proteins were first identified to be regulators of Notch signaling (Kishi et al., 2001). DTX proteins form a family based on the highly homologous C-terminal RING domain. DTX1 and DTX2 possess two tandem N-terminal WWE domains that bind to Notch ankyrin repeats whereas the N-terminus of DTX3 and DTX3L lack these WWE domains (Matsuno, Diederich, Go, Blaumueller, & Artavanis-Tsakonas, 1995; Takeyama et al., 2003). In contrast to other DTX members, DTX3L contains an unique N-terminus and lacks the central proline-rich region, however, DTX3L is highly homologous to the other DTX family proteins within the C terminal RING domain (Takeyama et al., 2003). As described above, RING domain ligases bind to E2 enzymes acting as scaffolds to facilitate indirect transfer of ubiquitin to substrates. In particular, DTX3L has been shown to specifically interact with the UbcH5 family of E2 enzymes to mediate substrate ubiquitination, but not UbcH4, UbcH6 or UbcH7. Additionally, DTX3L has been shown to heterodimerize with DTX1 leading to enhanced DTX1 activity (Takeyama et al.,
This interaction is mediated through the unique N-terminal region of DTX3L. The relevance of this interaction and whether DTX3L interacts with other E3 ubiquitin ligases remains to be explored.

E2 CONJUGATING ENZYMES

In the human genome there are nearly 40 identified E2 enzymes (Metzger et al., 2012). The E2 enzymes contain a conserved ubiquitin-conjugating (UBC) domain of ~160 amino acids that has the catalytic cysteine residue able to form a thioester with activated ubiquitin. Recent data on the trafficking of EGFR, a receptor tyrosine kinase (RTK), suggest that the E2 enzymes UbcH5b/c (also known as Ubc4/5) in conjunction with the E3 ligase c-Cbl is required for degradation and ubiquitination of EGFR (Umebayashi, Stenmark, & Yoshimori, 2008). Immunofluorescence studies demonstrated that of the E2 enzymes screened (Ubc2, Ubc3, Ubc4, UbcH5a, UbcH5b, UbcH5c, UbcH6, UbcH8 and UbcH13), UbcH5b and UbcH5c were the only E2 enzymes to co-localize with c-Cbl at the plasma membrane as well as the early endosomes following EGF stimulation. Furthermore, siRNA-mediated knockdown of UbcH5b/c, but not UbcH7, prevented EGF promoted ubiquitination of EGFR (Umebayashi et al., 2008). These data highlight the potential importance of specific subsets of E2 enzymes through their activities with respective E3s ligases in the regulation of receptor trafficking. While it is intriguing that subsets of E2s may regulate RTK ubiquitination and trafficking, whether this idea applies directly or indirectly to GPCR trafficking remains to be investigated.
DEUBIQUITINATING ENZYMES (DUBs)

Modification of protein substrate with ubiquitin is usually transient and reversible by ubiquitin specific proteases (USPs) or deubiquitinating enzymes (DUBs). DUBs mediate cleavage of the isopeptide bond between Ub and its substrates through protease activity. Several studies have identified important DUBs in regulating GPCR trafficking. A role for DUBs in GPCR trafficking was first defined in yeast. The deubiquitinating enzyme Doa4 was found to localize to the pre-vacuolar compartment where it has a role in recycling ubiquitin from cargos destined for degradation in the vacuole (Amerik, Nowak, Swaminathan, & Hochstrasser, 2000).

In mammals, DUBs and USPs modulate the trafficking of several GPCRs. The DUB AMSH has been shown to interact with and regulate ESCRT-0. In particular, both AMSH and USP8 have been shown to regulate the ubiquitination and degradation of the GPCRs protease activated receptor 2 (PAR2) and delta-opioid receptor (DOR) (Hasdemir, Murphy, Cottrell, & Bunnett, 2009; Hislop, Henry, Marchese, & von Zastrow, 2009). Depletion of either AMSH or USP8 using siRNA prevented PAR2 ubiquitin dependent degradation, trapping the receptor in early endosomes (Hasdemir et al., 2009). DOR downregulation is also dependent upon deubiquitination activities of AMSH and USP8. Overexpression of catalytically inactive mutants of either enzyme prevented WT DOR downregulation, whereas downregulation of lysine-less mutant of DOR still downregulated in the presence of mutant AMSH and USP8 (Hislop et al., 2009).
USP8 and AMSH indirectly promote CXCR4 sorting by antagonizing the ubiquitin modification of ESCRT-0 mediated by the E3 ligase AIP4 (Berlin, Higginbotham, Dise, Sierra, & Nash, 2010). Unlike PAR2, both USP8 and AMSH do not regulate deubiquitination of CXCR4 itself. Instead, the DUB USP14 has been implicated as a direct regulator of CXCR4 ubiquitination (Mines, Goodwin, Limbird, Cui, & Fan, 2009). Upon CXCL12 stimulation, USP14 associates with CXCR4 to promote deubiquitination. Depletion of USP14 by siRNA or overexpression of USP14 prevented CXCR4 ubiquitination, thereby, blocking receptor degradation.

USP8 has recently been shown to control the sorting of lysosomal enzymes necessary for lysosomal degradation (MacDonald, Urbe, & Clague, 2014). Lysosomal enzymes/hydrolases are sorted away from the Trans-Golgi network to the endocytic pathway and are ultimately delivered to the lysosome. Ultimately, delivery of acidic hydrolases to the pre-lysosomal compartment allows for degradation of receptors delivered to the lysosome. Thus, USP8 plays a role in both sorting at the endosome as well as delivery of enzymes to the degradative lysosomal compartment.

The highly related DUBs USP20 and USP33 have been implicated as regulators of β2AR recycling. Downregulation and desensitization of β2AR is dependent upon receptor ubiquitination by Nedd4. On early endosomes, deubiquitination of β2AR by USP20 and USP33 promotes receptor recycling (Berthouze, Venkataramanan, Li, & Shenoy, 2009). This results in resensitization of receptor signaling at the plasma membrane. Altogether the balance of ubiquitination versus deubiquitination in GPCR trafficking is important in controlling the magnitude and duration of receptor signaling.
FUNCTIONAL ROLE OF GPCR UBIQUITINATION

GPCR trafficking and endocytosis is also controlled by covalent modification of lysine residues within the receptor or adaptor proteins by ubiquitin. Ubiquitination of GPCRs regulates cell surface expression and, thus, the level of signaling. The ubiquitin moiety can act as a sorting signal that promotes trafficking of the receptor at the endosomal compartment. Ubiquitin modification of GPCRs can occur constitutively or be promoted by agonist stimulation. Typically, agonist dependent ubiquitination of GPCRs occurs at the plasma membrane as is the case for the chemokine receptor CXCR4. Although this ubiquitin-dependent trafficking is not exclusive to all GPCRs. Some GPCRs themselves are not modified by ubiquitin, however, their endocytic sorting is dependent upon additional factors. Proteins can also possess ubiquitin recognition motifs that can bind ubiquitinated substrates. In particular, ubiquitination of plasma membrane receptors can dictate endocytic sorting via interactions of ubiquitin with endocytic sorting machinery. At the endosome the receptor can be recognized by a number of ubiquitin interacting proteins that possess ubiquitin binding domains (UBDs) or ubiquitin interacting motifs (UIMs). Overall, ubiquitin regulation of GPCRs can have pleiotropic effects.

UBIQUITIN IN ESCRT-DEPENDENT SORTING

Ubiquitinated receptors are targeted at the endosome into forming MVBs by the endosomal sorting complex required for transport (ESCRT) machinery, which comprise
four conserved protein complexes (ESCRT0-III) (Table 1.1). The ESCRTs together with the AAA-ATPase Vsp4 complex and other accessory proteins act in a concerted fashion to recognize and concentrate the ubiquitinated receptor into the invaginating membrane of the endosome and facilitate formation of intraluminal vesicles of MVBs (Hurley, 2008; Marchese, Paing, Temple, & Trejo, 2008; Williams & Urbe, 2007). Thus, ESCRT proteins not only concentrate receptors into the invaginating membrane of the endosome, but also facilitate the formation of the MVB. MVBs fuse with highly acidic lysosomes leading to receptor degradation and, hence, desensitization of receptor signaling.

The ESCRT proteins were first identified in yeast Saccharomyces cerevisiae as class E genes of vacuolar protein sorting (Vps) that associate with endosomes. These studies employed indirect immunofluorescence microscopy in which the vacuole was indirectly visualized by antibodies against either the vacuolar integral membrane protein, ALP, or the subunit of the vacuolar H+-ATPase, V-ATPase (Raymond, Howald-Stevenson, Vater, & Stevens, 1992). It was demonstrated that class E Vps mutants change the morphology of the yeast vacuole (the equivalent of the mammalian lysosome) and result in the accumulation of cargoes (i.e. A-ALP and CPY; Ste3p) to a pre-vacuolar organelle. This accumulation of cargo in these mutants was attributed to defects in the delivery cargo from the limiting membrane of the vacuole to the lumen for degradation (Piper, Cooper, Yang, & Stevens, 1995; Raymond et al., 1992).

Further studies in mammalian cells demonstrated that the ESCRT machinery is highly conserved. In mammalian cells, ESCRT comprises four multimeric protein complexes (ESCRT-0 – III) in addition to activities of AAA-ATPase-Vps4 (Table 1.1).
ESCRT complexes are recruited sequentially to the endosome membrane to mediate cargo targeting into the limiting membrane of endosomes leading to the generation of ILVs that comprise the MVB. Three of the four ESCRT complexes (ESCRT-0-II) contain UBDs that recognize/interact with the surface of ubiquitin on cargo to facilitate sorting into the ILVs of MVBs. UBD containing proteins can be ubiquitinated themselves and disruption of individual ESCRT-UBDs results in defective sorting of certain cargoes into the MVBs. It is thought that ubiquitin supports ESCRT function by mediating changes in the intramolecular interactions between ESCRT proteins or facilitates cargo release by changing intramolecular interactions between UBDs and Ubiquitin on cargo.

Table 1.1 Proteins in the ESCRT pathway

<table>
<thead>
<tr>
<th>Complex</th>
<th>Mammalian Protein</th>
<th>Yeast Protein</th>
<th>Domains</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESCRT-0</td>
<td>HRS</td>
<td>Vps27</td>
<td>FYVE, UIM, VHS, PSAP</td>
</tr>
<tr>
<td></td>
<td>STAM-1, STAM-2</td>
<td>Hse1</td>
<td>UIM, SH3, VHS</td>
</tr>
<tr>
<td>ESCRT-I</td>
<td>Tsg101, Vps28, Vps37A, B, C, D, MVB12A, B UBA1</td>
<td>Vps23, Vps28, Vps37, Mvb12</td>
<td>UEV, Coiled-coil, SOUBA</td>
</tr>
<tr>
<td>ESCRT-II</td>
<td>EAP30, EAP25, EAP45</td>
<td>Vps22, Vps25, Vps36</td>
<td>Coiled-coil, WH, PPXY, WH, GLUE, NZF, WH</td>
</tr>
<tr>
<td>ESCRT-III</td>
<td>CHMP2A, B, CHMP6, CHMP3, CHMP4A, B, C, CHMP5, CHMP1A,B</td>
<td>Vps2, Vps20, Vps24, Vps32/Snf, Vps60, Did2</td>
<td>Coiled-coil, Coiled-coil, Coiled-coil, MIR, Coiled-coil, Coiled-coil, Coiled-coil</td>
</tr>
<tr>
<td>Accessory Proteins</td>
<td>ALIX VPS4A, B LIP5 IST1</td>
<td>Bro1, Vps4, Vta1, Ist1</td>
<td>Bro1</td>
</tr>
</tbody>
</table>
RECOGNITION

ESCRT-0, composed of HRS and STAM-1/STAM-2, initially recognize ubiquitinated cargo at the endosome. HRS and STAM exist as a 1:1 complex on endosomes through the association of their coiled coil region, however, HRS and STAM may also form a heterotetrameric complex (Mayers et al., 2011). Localization of ESCRT-0 to endosomes is mediated in part through selective binding of the FYVE (Fab1, YOTB, Vac1 and early endosome antigen-1) domain in HRS to phosphatidylinositol 3-phosphate (PI-3P), an endosomally enriched phospholipid (Raiborg et al., 2001; Stenmark, Aasland, & Driscoll, 2002). In addition, HRS and STAM-1/STAM-2 both contain ubiquitin binding domains (UBDs) (Bilodeau, Urbanowski, Winistorfer, & Piper, 2002; Henne, Buchkovich, & Emr, 2011; Prag et al., 2007; Raiborg et al., 2002; Ren & Hurley, 2010) able to interact with several ubiquitin moieties at once (Mayers et al., 2011). In particular, the ubiquitin interacting motif (UIM) of HRS interacts with and recruits ubiquitinated cargo to be sorted on the endosome (Stringer & Piper, 2011). In addition to interacting with ubiquitinated cargoes, HRS can bind to internalized cargo on endosomes contained in clathrin coated pits via an interaction of clathrin with the clathrin binding box motif (Leu-Ile-Ser-Phe-Asp) within the C-terminus of HRS. The UIM of STAM also recognizes ubiquitinated cargo by binding to both monoubiquitin and polyubiquitin chains. Furthermore, the proline rich (PSAP) motif in HRS acts to recruit and link ESCRT-0 to ESCRT-I through binding to the UEV (ubiquitin E2 variant) domain in the ESCRT-I subunit Tsg101 (Pornillos et al., 2002; Ren & Hurley, 2011). Together HRS
and STAM bind to ubiquitinated cargo and initiate the sorting of proteins at the endosome.

**SORTING**

Following binding and recognition of cargo on endosomes, ESCRT-0 then recruits ESCRT-I via binding of the PSAP motif in HRS to the UEV domain in Tsg101 (Katzmann, Babst, & Emr, 2001). In mammalian cells, ESCRT-I can comprise several subunits including Tsg101, VPS28, VPS37A-D and the recently identified UBAP1 (Agromayor et al., 2012; Chu, Sun, Saksena, & Emr, 2006; Morita et al., 2007; Stefani et al., 2011; Tsunematsu et al., 2010). In addition to interacting with HRS, the UEV domain of Tsg101 is involved in the further sorting and recognition of ubiquitinated cargo (Stuchell et al., 2004). Together with Vps28, Tsg101 recruits ESCRT-II to the endosome. In mammalian cells, ESCRT-II is composed of EAP30, EAP25 and EAP45. These proteins all contain protein-protein interacting winged-helix (WH) domains. In particular, the WH domain of the ESCRT-II subunit EAP25 acts to recruit ESCRT-III to the endosome (Babst, Katzmann, Snyder, Wendland, & Emr, 2002; Teo, Perisic, Gonzalez, & Williams, 2004). The subunit EAP45 possesses a Gram-like ubiquitin-binding in EAP45 (GLUE) domain able to interact with ubiquitin in addition to endosomally enriched phosphoinositides (Hirano et al., 2006; Slagsvold et al., 2005).
MVB FORMATION AND ESCRT DISASSEMBLY

The final step in ESCRT-dependent sorting involves recruitment of ESCRT-III to endosomes by ESCRT-II. This recruitment ESCRT-III facilitates the concentration of receptor into the ILVS of MVBs and the disassembly of the ESCRT-complex by the AAA-ATPase Vps4 (Williams & Urbe, 2007). ESCRT-III is composed of the mammalian subunits CHMP1A-B, CHMP2A-B, CHMP3, CHMP4A-C, CHMP5 and CHMP6 along with the Ist-1 (Rue, Mattei, Saksena, & Emr, 2008). Each subunit of ESCRT-III is recruited to the endosomal membrane from the cytosol. On the endosome, ESCRT-III subunits are able to bind to microtubule interacting and trafficking (MIT) domains of accessory proteins including the deubiquitinating enzyme AMSH and the AAA-ATPase Vps4 to facilitate disassembly of the ESCRT-complex. Together, the subunits of ESCRT-III act together to cleave the endosomal membrane resulting in the formation of intraluminal vesicles. The AAA-ATPase, Vps4, acts to form MVBs. MVBs are composed of many ILVs and are considered vesicular intermediates between early and late endosomes (Gruenberg & Stenmark, 2004; Piper & Katzmann, 2007). In the final sorting step, AAA-ATPase Vps4 disassembles the ESCRT complex, thereby, allowing for recycling of the ESCRT components to initiate additional rounds of ESCRT-mediated sorting.

CXCR4

The chemokine receptor 4 (CXCR4) is a class A rhodopsin-like G protein coupled receptor (GPCR), previously known as CD184 or Fusin (Berson et al., 1996; Moriuchi,
CXCR4 was first identified as one of the co-receptors that together with the T cell receptor CD4 facilitate HIV fusion and entry into T cells (Berson et al., 1996). Later it was identified in the normal cellular context that CXCR4 binds to the chemokine ligand CXCL12 (a.k.a. SDF-1α) and together it was shown that CXCR4/CXCL12 are essential for development of the heart, brain, and vasculature during embryogenesis (Nagasawa, Tachibana, & Kishimoto, 1998; Tachibana et al., 1998). In addition to binding CXCL12, CXCR4 was recently identified to also bind to the highly conserved small molecule ubiquitin as well as to the cytokine MIF (macrophage migrating inhibitory factor) (Bernhagen et al., 2007; Klasen et al., 2014; Saini, Marchese, & Majetschak, 2010). Studies have highlighted that CXCR7 also binds to CXCL12. In contrast to CXCR4, CXCR7 binding to CXCL12 does not promote activation of classical CXCR4 signaling. It is thought that CXCR7 acts as a scavenger by binding to CXCL12 and, thereby, regulates CXCL12 gradients. Taken together, CXCR7 antagonizes CXCR4 activation and cellular responses (Dambly-Chaudiere, Cubedo, & Ghysen, 2007; Naumann et al., 2010). Of the 7 identified CXC-receptors, CXCR4 is the most widely studied and highly conserved phylogenetically among different species (Arnolds & Spencer, 2014; Knaut, Werz, Geisler, & Nusslein-Volhard, 2003; Nagasawa, 2014; Schabath et al., 1999; Willett et al., 1997; Zou, Redmond, Qi, Dooley, & Secombes, 2015). Yet, detailed information on the regulation of CXCR4 is continuing to be delineated.
PHYSIOLOGICAL ROLES OF CXCR4

CXCR4 as well as its ligand CXCL12 have important roles during embryonic development and in the adult. The knockout (KO) animals of both genes display identical phenotypes. Mice deficient in CXCL12 or CXCR4 die at a late stage of embryogenesis (E 17.5) (Nagasawa, Tachibana, & Kishimoto, 1998; Tachibana et al., 1998). The death is due mainly to a defect in formation of the ventricular septum in the heart. In addition to displaying defects in heart development, these mice also have defects in vascularization of the gastro-intestinal tract, cerebellum formation and hematopoiesis (Ara, Tokoyoda, Okamoto, Koni, & Nagasawa, 2005; Q. Ma et al., 1998; Nagasawa et al., 1996; Nagasawa et al., 1998; Tachibana et al., 1998).

In the adult, the CXCL12/CXCR4 signaling axis has a role in tissue repair, hematopoiesis and cell homing. Following myocardial infarction, the ischemia produced within the heart mediates CXCR4/CXCL12 dependent homing of endothelial progenitor cells to initiate cardiac tissue repair (Askari et al., 2003; Heeschen et al., 2004). In hematopoiesis, CXCR4 mediates the homing of immature and mature bone marrow cells to peripheral sites where CXCL12 gradients are established (Broxmeyer et al., 2003; Lataillade, Domenech, & Le Bousse-Kerdiles, 2004; Q. Ma et al., 1998; Moser & Loetscher, 2001; Nagasawa et al., 1996). Aberrant CXCR4 signaling due to dysregulated CXCR4 expression has been implicated pathophysiologically in many diseases including breast cancer, cardiovascular disease, immunodeficiency disorders and lymphomas (Balkwill, 2004; Busillo & Benovic, 2007; Hernandez et al., 2003; Muller et al., 2001;
Walter et al., 2005; Ahn, Seo, Weinberg, & Arber, 2013; Burger & Kipps, 2002; Corcione et al., 2006; Trentin et al., 2004; Zlotnik, 2006).

**CXCR4 IN BREAST CANCER**

Dysregulated CXCL12/CXCR4 signaling has also been implicated in metastasis of a variety of epithelial carcinomas. CXCR4 is overexpressed in at least 23 types of cancer including cancers of the breast, ovary and prostate (Balkwill, 2004; Busillo & Benovic, 2007). Cancer cells with upregulated CXCR4 receptor levels can home in on distance sites where the CXCL12 ligand is expressed. Additionally, CXCR4 is involved in several cellular pathways that regulate invasion, chemotaxis as well as adhesion. As a result of these effects, the overexpression of CXCR4 in many cancer types promotes metastasis and progression (Balkwill, 2004). In particular, breast cancer subtypes that have high expression levels of CXCR4 can metastasize to regions where high levels of CXCL12 are expressed such as the lung, liver, lymph nodes and bone marrow (Muller et al., 2001). In breast cancer cells, CXCR4 mediated signaling promotes both chemotactic and invasive responses. In particular, CXCR4 signaling leads to changes in actin polymerization as well as pseudopodia formation that facilitate migratory responses of breast cancer cells *in vitro*. *In vivo* in a MDA-MB-231 breast cancer xenograft model, treatment with a neutralizing anti-human CXCR4 monoclonal antibody was able to significantly reduce metastasis to the lymph node and lung (Muller et al., 2001). This study overall highlights the importance of CXCR4/CXCL12 in mediating breast cancer
metastasis and was one of the first studies to demonstrate that targeting this signaling pathway can reduce metastasis.

**CXCR4 IN CARDIOVASCULAR DISEASE**

The CXCL12/CXCR4 signaling axis is highly important to the development as well as tissue repair within the heart (Askari et al., 2003; Dong et al., 2012; Nagasawa et al., 1996; Penn, 2009). Conditional CXCR4-endothelium knockout mice show abnormal vascularization demonstrating a requirement for CXCR4 in normal endothelial vascularization (Ara et al., 2005; Tachibana et al., 1998). In the adult, the CXCL12/CXCR4 signaling axis has a role in tissue repair of the damaged myocardium following cardiac ischemia (Penn, 2009; Tang et al., 2009) and in providing protection to cardiomyocytes from myocardial reperfusion injury (Cai et al., 2015). During myocardial infarction, the resulting ischemia promotes release of CXCL12 from the damaged tissue. This leads to an increase in endothelial progenitor cells released from the bone marrow and their homing to the damaged myocardium to initiate tissue repair (Abbott et al., 2004; Askari & Penn, 2003; Askari et al., 2003; Nagasawa et al., 1996). Perturbations in CXCL12/CXCR4 signaling that arise, for example by aberrant CXCR4 expression, may lead to impaired homing of endothelial progenitor cells thereby impairing tissue repair (Walter et al., 2005). The therapeutic value of CXCR4/CXCL12 in mediating cardiac tissue repair remains to be explored.
CXCR4 IN WHIM SYNDROME

Aberrant CXCL12/CXCR4 signaling has been associated with the rare immunodeficiency disorder known as WHIM syndrome (Hernandez et al., 2003). Specifically, WHIM syndrome is caused by an autosomal dominant mutation in the CXCR4 gene. The acronym WHIM denotes the four most common symptoms of this syndrome, which include Warts, Hypogammaglobulinemia, Infections, and Myelokathexis. These patients exhibit warts as a result of increased susceptibility to HPV-infection, a low level of circulating gamma globulins (hypogammaglobulinemia), retention of neutrophils in the bone marrow (myelokathexis) and the resulting immune deficiency leads to increased infections. Additionally, due to the retention of neutrophils in the bone marrow patients exhibit neutropenia (low number of circulating neutrophils) (Hernandez et al., 2003; Kawai & Malech, 2009).

The identified mutations in CXCR4 involve either nonsense (R334X, G336X, S338X, E343X) or frame shift (S339fs342X) mutations (Hernandez et al., 2003; Liu et al., 2012). Any of these mutations result in truncation of the C-terminal tail of CXCR4 preventing its internalization and, thus, desensitization. As a result, the receptor is hyperfunctional in mediating downstream signaling. This results in disruption of chemokine gradients in the bone marrow and results in retention as well as apoptosis of neutrophils and other immune cells. Interestingly, one patient diagnosed with WHIM syndrome did not display a genetic mutation in the CXCR4 gene per se, however, it has been suggested that disruption of downstream regulators of CXCR4 signaling may be involved. Mice deficient in GRK6 and arrestin-3 exhibit symptoms similar to WHIM
syndrome patients such as neutropenia (Fong et al., 2002; Vroon et al., 2004) indicating that defects in CXCR4 desensitization may contribute to WHIM syndrome in the patient lacking the CXCR4 genetic mutation.

Treatment of WHIM syndrome usually involves infusion with gamma globulins (IV-Ig) and/or administration of the mobilizing therapy granulocyte-colony stimulating factor (G-CSF). Additionally, these patients are often treated prophylactically with antibiotics and undergo surgery to remove HPV-associated warts. Unfortunately, these current therapies come at a high cost to the patient and do not prevent recurring infection. Given the identified hyperfunctional CXCR4 genetic mutation in the majority of WHIM patients, current clinical trials are testing the efficacy of low-dose treatment with the CXCR4 antagonist Plerixafor (AMD3100) (McDermott et al., 2014). While directly targeting CXCR4 may prove efficacious in WHIM patients, the adverse side effects that may be associated with direct CXCR4 antagonism may require the development of alternative therapeutic targets to inhibit CXCR4 indirectly.

**CXCR4 IN LYMPHOMAS (DLBCLs)**

Aberrant CXCR4 expression has been correlated several hematological malignancies that lead to improper trafficking and migration of malignant B cells. While CXCR4 has been implicated in several hematological malignancies (Ahn, Seo, Weinberg, & Arber, 2013; Burger & Kipps, 2002; Corcione et al., 2006; Trentin et al., 2004; Zlotnik, 2006), the significance of CXCR4 in the progression of diffuse large B cell lymphomas (DLBCLs) remains unclear. A recent study of 94 biopsies in patients with
DLBCLs has demonstrated that CXCR4 expression in these patients can be used as a prognostic marker (Moreno et al., 2015). In this study, CXCR4 expression correlated to shorter overall survival as well as progression-free survival. Additionally, CXCR4 was shown to mediate dissemination of DLBCL cells in a mouse xenograft model. This dissemination was inhibited by administration of the CXCR4 antagonist, AMD3100. Recently another study of de novo germinal-center B-cell-like (GCB)-DLBCLs has demonstrated that an increase in CXCR4 is also associated with increased dissemination (Chen et al., 2015). Conversely, a study of gastric extranodal DLBCLs originating from mucosa-associated lymphoid tissue (MALT) established that loss of CXCR4 expression is a prognostic marker for the development and progression (Deutsch et al., 2013). Thus, the expression and role of CXCR4 in DLBCLs varies between different subtypes of DLBCLs. Taken together, these studies highlight the therapeutic potential of targeting the CXCR4 pathway in DLBCLs.

**CXCR4 SIGNALING**

Activation of CXCR4 signaling occurs upon binding to CXCL12. The binding region for CXCL12 on CXCR4 lies within the N-terminal domain of CXCR4 (residues 1-36) (Doranz et al., 1999). Upon activation of CXCR4 by CXCL12, the receptor undergoes a conformational change. Ultimately, CXCR4 activation leads to either G protein dependent or independent signaling as described in the below sections.
CXCR4 SIGNALING: G PROTEIN DEPENDENT

Following CXCL12 binding to CXCR4, several signaling pathways can be activated (Figure 1.6). The majority of signaling that occurs following CXCR4 activation is dependent upon the downstream activities of the associated G proteins. CXCR4 couples canonically to the Pertussis toxin (PTX)-sensitive Gαi subunit although coupling to Gq, Gs, and G12/13 have also been demonstrated (A. Kumar et al., 2006; Tan, Martin, & Gutkind, 2006). Although the vast majority of CXCR4 mediated signaling pathways are PTX-sensitive and, therefore, are Gαi dependent.

Binding of CXCL12 to CXCR4 results in the exchange of GDP for GTP in the Gαi subunit and subsequent dissociation of Gαi from the βγ subunits of the heterotrimeric G protein. Gαi functions to inhibit adenylyl cyclase and activate the kinase Src. Inhibition of adenylyl cyclase inhibits the production of cyclic AMP (cAMP) which in turn affects the activity of the PKA (Yang et al., 2007). Activation of Src by Gαi results in MAPK pathway activation (Ganju et al., 1998; Y. C. Ma, Huang, Ali, Lowry, & Huang, 2000) and can regulate the activities of focal adhesion proteins including FAK and paxillin (Dutt, Wang, & Groopman, 1998; J. F. Wang, Park, & Groopman, 2000).

The liberated βγ subunits can activate phospholipase C-β (PLC-β) leading to the cleavage of inositol-4,5-bisphosphate (PIP2) to form inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 increases the levels of intracellular Calcium (Ca2+) while DAG activates protein kinase C (PKC). Additionally, downstream activation of PI3K leads to the formation of PIP3 and subsequent activation of the rho family of GTPases, Rac and Cdc42. Ultimately, the activation of second messengers downstream of CXCR4 lead to
changes in biological processes including migration, adhesion, proliferation and gene transcription.

Figure 1.6. Signaling pathways activated downstream of CXCR4. Binding of CXCL12 to CXCR4 leads to the activation of both G protein dependent and G protein independent signaling pathways. The downstream effects of these signaling pathways regulate several cellular responses including migration, gene transcription, adhesion and proliferation.

A recent study has demonstrated a novel role for AIP4 and ESCRT-0 subunit STAM-1 in regulating CXCR4 signaling (Malik, Soh, Trejo, & Marchese, 2012). Using siRNA against AIP4 and STAM-1, it was shown that CXCR4 activation of ERK-1/2 signaling
was prevented. This phenotype was dependent upon the ability of AIP4 and STAM-1 to interact as well as the catalytic activity of AIP4. Thus, ubiquitination activity of AIP4 is important in mediating ERK signaling in conjunction with STAM-1. It was further shown that AIP4 and STAM-1 activate ERK signaling in microdomains of the caveolar compartment. Overall, this study reveals that ubiquitination of STAM-1 by AIP4 is important in mediating ERK signaling downstream of CXCR4 activation.

**CXCR4 SIGNALING: G PROTEIN INDEPENDENT**

CXCR4 activation can also lead to the activation of signaling pathways independent of the G protein. CXCR4 activates Janus kinase (JAK) and signal transducer and activator of transcription (STAT) signaling pathway in T cell (MOLT4) lysates. Pharmacological inhibition of Gαi by PTX treatment does not prevent JAK/STAT signaling suggesting this signaling is independent of CXCR4 coupling to Gαi (Vila-Coro et al., 1999). Furthermore, in this study it was demonstrated that CXCL12 treatment did not promote association Gαs with CXCR4. Although this study suggest a G protein independent mechanism, these studies do not rule out whether JAK/STAT signaling downstream of CXCR4 activation can be attributed to coupling to other G proteins including Gq or G12/13.

Non-visual arrestins (β-arrestin-1/2) have also been suggested to mediate CXCR4 G protein independent signaling. It has been demonstrated that β-arrestin-1 and β-arrestin-2 can increase MAPK signaling downstream of CXCR4 activation through activation of ERK and p38. In addition, mice deficient in β-arrestin-2 or GRK6 display defective
lymphocyte chemotaxis in response to CXCL12 (Fong et al., 2002). It is thought that the
defective chemotactic response in these mice is attributed to β-arrestin-2-mediated
signaling and is independent of G protein activation. Thus, these study suggest that β-
arrestin-2 not only functions in CXCR4 desensitization but may also play a vital role in
mediating chemotaxis through G protein independent activation of MAPK/p38 signaling
pathway (Y. Sun, Cheng, Ma, & Pei, 2002). While these study suggest an intriguing role
for G protein independent signaling, more comprehensive studies will be required to
determine whether these are truly G protein independent processes.

REGULATION OF CXCR4 SIGNALING

In order to maintain the proper level of CXCR4 signaling both in magnitude and
duration, attenuation of CXCR4 signaling is a highly regulated process (Figure 1.7). Following activation, CXCR4 signaling is tightly controlled by three processes: desensitization, internalization and downregulation as detailed in the following sections.

CXCR4 DESENSITIZATION AND INTERNALIZATION

Activation of CXCR4 by CXCL12 results in a rapid desensitization process. In this process, CXCR4 is first phosphorylated by GRKs on serine and threonine residues within the C-terminal tail (Krupnick & Benovic, 1998). In particular GRK2, GRK3 and GRK6 have all been implicated in mediating this phosphorylation event (Balabanian et al., 2008; Busillo et al., 2010; Jimenez-Sainz et al., 2006). GRK-mediated phosphorylation recruits β-arrestin-1/2, which uncouple the receptor from the associated
G protein preventing further G protein activation (Gurevich et al., 1995; Lohse et al., 1990). In addition to uncoupling the receptor from the associated G protein, β-arrestins promote proximal degradation of cAMP and DAG through the recruitment of the respective phosphodiesterases or DAG enzymes (Nelson et al., 2007; Perry et al., 2002). It has also been demonstrated that β-arrestin-1 (a.k.a Arrestin-2) interacts with AIP4 on endosomes upon CXCR4 activation, however, siRNA depletion of either β-arrestin-1 or AIP4 does not prevent CXCR4 internalization. This data suggest that the interaction between β-arrestin-1 and AIP4 has a role in the post-endocytic sorting of CXCR4 separate from a function at the plasma membrane (Bhandari, Trejo, Benovic, & Marchese, 2007).

**CXCR4 UBIQUITIN DEPENDENT DOWNREGULATION**

CXCR4 is rapidly internalized from the plasma membrane onto early endosomes upon binding to its cognate ligand CXCL12 (Figure 1.7) (Marchese & Benovic, 2001). Prior to internalization, CXCR4 can be ubiquitinated by E3 ubiquitin ligase AIP4. Phosphorylation of two serine residues (S324 and S325) within CXCR4 (Bhandari, Robia, & Marchese, 2009; Marchese & Benovic, 2001; Marchese, Raiborg, et al., 2003) promotes the recruitment of AIP4. This phosphorylation event is mediated by GRK6 and/or PKCδ (Busillo et al., 2010). AIP4 recruitment to CXCR4 promotes monoubiquitination of the CXCR4 at the plasma membrane (Bhandari et al., 2009). Interaction studies have highlighted that the interaction between AIP4 and CXCR4 involves a non-canonical interaction between WW I-II domain of AIP4 with the
phosphorylated S324 and S325 within the C-terminal tail of CXCR4 (Bhandari et al., 2009). Typically, the WW domains of AIP4 interact with proline rich sequences (PPXY or PPPY motifs) (Ingham et al., 2005; Sudol, Chen, Bougeret, Einbond, & Bork, 1995), however, CXCR4 lacks these sequences. Thus, these studies demonstrate a novel interaction between the WW domains of AIP4 with CXCR4.

Ubiquitin modification of CXCR4 acts as a sorting signal that localizes the receptor to early endosomes, however, ubiquitination of CXCR4 is not required for internalization. On early endosomes, ubiquitinated CXCR4 is localized to microdomains containing ESCRT-0 and sorted for trafficking towards lysosomes where the receptor is degraded (Bhandari et al., 2007; Marchese & Benovic, 2001; Marchese, Raiborg, et al., 2003). Studies have shown that expression of ubiquitination-deficient mutants of CXCR4 display a significant reduction in agonist-promoted degradation (Marchese & Benovic, 2001). Agonist-promoted degradation of CXCR4 is a highly efficient process although a small amount is recycled back to the plasma membrane via Rab11-positive recycling endosomes (A. Kumar, Kremer, Dominguez, Tadi, & Hedin, 2011; Malik & Marchese, 2010; Marchese & Benovic, 2001). These studies highlight the importance of CXCR4 ubiquitination in facilitating receptor downregulation.

Not only does AIP4 mediate ubiquitination of CXCR4 at the plasma membrane, it also acts on early endosomes to regulate the sorting machinery, such as ESCRT-0 protein HRS, to control CXCR4 sorting to lysosomes (Malik & Marchese, 2010; Marchese, Raiborg, et al., 2003). Previous studies have demonstrated that the ESCRT-0 protein STAM-1 and Arrestin-2 (β-arrestin-1) act to recruit AIP4 to the endosome where AIP4
ubiquitinates HRS (Malik & Marchese, 2010). Ubiquitination of HRS is thought to inhibit its ability to target CXCR4 for degradation. Thus AIP4 acts at multiple sites to control CXCR4 trafficking, but detailed molecular insight into its function is lacking.

Figure 1.7 Current model of CXCR4 trafficking within the endocytic pathway. Upon CXCL12 binding to CXCR4, the receptor is rapidly phosphorylated on serine residues 324 and 325 within the C-tail by a G protein-coupled receptor kinase (GRK). This promotes direct binding to the E3 ubiquitin (Ub) ligase AIP4, which mediates Ub conjugation of CXCR4 on nearby lysine residues. The ubiquitin moieties on CXCR4 act as a sorting signal recognized by the ESCRT machinery, which is located on endosomes. This machinery recognizes and interacts directly with the ubiquitin moieties attached to
CXCR4 and acts in a sequential and concerted fashion to deliver ubiquitinated CXCR4 into ILVs of MVBs. MVBs fuse with lysosomes where their contents are degraded. In addition the STAM-1/Arrestin-2 complex on endosomes acts to recruit AIP4 which in turn ubiquitinates HRS and limits the extent of CXCR4 that is targeted for degradation.

RATIONAL AND RESEARCH OBJECTIVES

Despite the fact that dysregulated CXCR4 expression contributes to aberrant signaling, very little is known about the molecular processes regulating CXCR4 expression in cells. Understanding how CXCR4 levels are regulated is critical for two reasons. First, understanding CXCR4 regulation will allow future studies to further pinpoint the contribution of CXCR4 and its usefulness as a biomarker in disease. Secondly, understanding the molecular mechanisms regulating CXCR4 levels will provide alternative strategies to target CXCR4. The goal of this dissertation project is to understand the molecular mechanisms regulating CXCR4 expression, with a specific emphasis on understanding how CXCR4 levels are controlled by membrane trafficking within the endocytic pathway.

CXCL12-activation of CXCR4 results in its rapid ubiquitination and internalization from the plasma membrane. Once on endosomes, ubiquitinated CXCR4 is sorted for lysosomal degradation through an ubiquitin-dependent pathway (Marchese, Raiborg, et al., 2003), although the mechanism remains poorly characterized. CXCR4 is ubiquitinated by the HECT-domain E3 ligase AIP4 at the plasma membrane. AIP4 is also localized on early endosomes and regulates CXCR4 sorting by modulating the activity of
the endosomal sorting complex required for transport (ESCRT) machinery (Malik & Marchese, 2010; Marchese, Raiborg, et al., 2003). However, the precise role of AIP4 in these processes remains poorly defined. The objective of this project is to gain a greater understanding of the mechanisms mediating AIP4 regulation of CXCR4. AIP4 is known to interact with other E3 ligases, including DTX1 and Cbl-c (Chastagner et al., 2006; Courbard et al., 2002), but whether these E3 ligases or others are involved in CXCR4 sorting is not known. Our preliminary data indicate that the DTX protein, Deltex-3-like (DTX3L) regulates CXCR4 degradation. We hypothesize that the E3 ubiquitin ligase DTX3L regulates CXCR4 trafficking within the degradative pathway through an interaction with AIP4 and other key proteins involved in the CXCR4 sorting pathway. To achieve the overall objective of this project we addressed two specific aims:

**AIM # 1. To characterize the role of DTX3L in CXCR4 trafficking to lysosomes.** Our preliminary data indicates that DTX3L mediates CXCR4 lysosomal degradation. In this aim we will elucidate the step at which DTX3L functions in the endocytic pathway to regulate CXCR4 targeting to lysosomes.

**AIM # 2. To elucidate the role of DTX3L on CXCR4-induced ubiquitination.**

In this aim we will test the hypothesis that DTX3L interacts with AIP4 and the endocytic machinery on endosomes to modulate CXCR4 lysosomal sorting.
The results from this study will further the knowledge on the regulation of CXCR4 signaling and trafficking. Furthermore, the results of this study can be correlated to CXCR4 related pathologies and will provide valuable information that may lead to the identification of novel pharmacological targets within the CXCR4 pathway.
CHAPTER II
MATERIALS AND METHODS

CELL LINES AND TRANSFECTION REAGENTS

Human cervical cancer (HeLa) cells were obtained from the American Type Culture Collection (Manassas, VA) and human embryonic kidney (HEK) 293 cells were from Microbix (Toronto, ON, Canada). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories (Logan, UT)) supplemented with 4 mM L-glutamine, 4500 mg/L glucose and 10% fetal bovine serum (FBS; Hyclone Laboratories or Fisher Scientific) and incubated in a 37°C humidified chamber containing 5% CO₂.

Transfection of cells with plasmid DNA and siRNA were performed using the transfection reagents Polyethylenimine (PEI) and Lipofectamine® 2000 or 3000 (Invitrogen, Carlsbad, CA), respectively. Transfections with Lipofectamine® 2000 or 3000 were done per the manufacturers instructions and optimized for experiments performed as outline below. Additionally, all transfections were performed within the biological safety cabinet to maintain sterility.

TRANSFECTION OF DNA USING POLYETHYLENIMINE (PEI)

Transfection of cells with DNA was performed on cells cultured in 10-cm, 6-cm or 6-well culture dishes using polyethylenimine (PEI). The cells were typically at 70-80%
confluency at time of transfection. The protocol was kindly provided by Dr. JoAnn Trejo (UCSD) (Grimsey, Lin, & Trejo, 2014). PEI was made as a stock of 1 mg/ml (Polysciences, Inc., Warrington, PA) in 30% ethanol (Decon Laboratories, Inc., King of Prussia, PA)), as described in Appendix I. PEI was used for DNA transfection at a ratio of 2 µl PEI stock: 1 µg of DNA. Specifically, for a 10-cm dish, 10 µg of total plasmid DNA was combined with 20 µl of PEI in 1 mL DMEM supplemented with 20 mM HEPES pH 7.5. For a 6-cm dish, 5 µg of total plasmid DNA was combined with 10 µl of PEI in 1 mL DMEM supplemented with 20 mM HEPES pH 7.5. Lastly, for DNA transfections in 6-well dishes, 2.5 µg of total plasmid DNA was incubated with 5 µl of PEI in 1 mL DMEM supplemented with 20 mM HEPES pH 7.5.

The PEI/DMEM mixture was incubated at room temperature for 5 min and then it was added drop wise to a second microcentrifuge tube containing the DNA. The DNA:PEI mixture was incubated for 20 min at room temperature, during which time the media was aspirated and replaced with fresh DMEM plus 10% FBS (10 ml for 10 cm dish, 4 ml for 6 cm dish and 1 ml for 6-well plate) prior to adding DNA complexes. DNA was then added drop wise to cells. To evenly distribute DNA:PEI complexes, cells were gently rocked back and forth several times before placement into a 37°C incubator. DNA transfections were allowed to incubate at 37°C for 24-48 h depending on particular experiment and amount of DNA transfected.
TRANSFECTION OF SMALL INTERFERING RNA (siRNA) USING Lipofectamine® 2000 or 3000 REAGENTS

Transfection of cells with siRNA were performed on cells cultured in 10-cm, 6-cm and 6-well culture dishes using Lipofectamine® 2000 or 3000 transfection reagents. siRNA transfections were performed at a ratio of 2.5 µl Lipofectamine® 2000/3000 to 50 pmol of siRNA.

For transfection set up in a biological safety cabinet, the desired amount of siRNA was aliquoted into a microcentrifuge tube containing 500 µl of DMEM supplemented with 20 mM HEPES pH 7.5. In a second microcentrifuge tube, 500 µl of DMEM supplemented with 20 mM HEPES pH 7.5 was aliquoted. The corresponding amount of (2.5 – 30 µl) Lipofectamine® was aliquoted directly into this tube. The two microcentrifuge tubes were left in the biological safety cabinet at room temperature for 5 min. After 5 min, the siRNA/DMEM mixture were added drop wise to the tube containing the Lipofectamine®/DMEM mixture and incubated for an additional 20 min at room temperature. In the mean time, media on 70-80% confluent cells was replaced with warm DMEM plus 10% FBS after which siRNA complexes were added drop wise to cells. To evenly distribute siRNA:Lipofectamine® complexes, cells were gently rocked back and forth several times before placement into a 37°C incubator. Cells were allowed to transfect for 24-72 h depending on siRNA used and experiment.
ANTIBODIES AND REAGENTS

Primary antibodies used in this dissertation work are listed in Table 2.1 while reagents are listed in Table 2.2. siRNA sequences are listed in Table 2.4. DNA constructs and primers used in this work are in Tables 2.5 and 2.6, respectively.

CLONING

Basic steps used to clone DNA into desired vectors are listed below in Sections 2.3.1 -2.3.7. Primers used in PCR reactions are listed in Table 2.6 and construct maps of each DNA are in Appendix II.

POLYMERASE CHAIN REACTION (PCR) SET UP

PCR reactions were set up using the Expand High Fidelity PCR system (Roche) as follows. To a sterile PCR tube each of the following ingredients was added: Expand High Fidelity Buffer (10×) containing 15 mM MgCl₂ (2 µl), 10 mM dNTP mixture (1 µl), DMSO (2 µl), Forward primer (1 µl), Reverse primer (1 µl), template DNA (1 µg) and Expand High Fidelity Enzyme mix (1 µl; 3.5 units/µl) and sterile, autoclaved dH₂O to a final volume of 50 µl. Tubes were placed in a GeneAMP® PCR system Thermocycler (Model 9700; Applied Biosystems) using the following parameters: 94°C for 45 sec- 2.5 min), 40-55°C for 45 sec – 10 min and 72°C for 2 min for 30 cycles with a 2 min hold before the start at 94°C and final elongation hold at 72°C for 7 min.
GENERATION OF THE FLAG-DTX3L-3C/A MUTANT

To generate the FLAG-DTX3L-3C/A mutant three conserved cysteine residues (561, 596 and 599) within the catalytic RING domain of DTX3L were mutated to alanine. Mutation of these residues is predicted to inhibit E2 enzyme binding to the RING domain based on previously described RING mutant of DTX1 (Chastagner, Israel, & Brou, 2006). These same residues are conserved in the DTX3L sequence as determined by manual comparison of the DTX1 and DTX3L sequences. To make this mutant we used two-step PCR mutagenesis. Two constructs were generated with the first using DTX3L DNA that was PCR amplified and extended with primers designed to incorporate one mutation (C561A). The second construct was generated using the C561A DNA as the template in our PCR reaction in order to add the second and third mutations (C596A and C599A).

We first generated the single mutant (C561A). Briefly, FLAG-DTX3L-C561A was PCR amplified using sequential PCR. Reaction one amplified FLAG-DTX3L template DNA using FLAG-DTX3L-Forward and FLAG-DTX3L-C561A-Reverse primer in one tube and FLAG-DTX3L-Reverse and FLAG-DTX3L-C561A Forward primer in tube two. The product of PCR reaction one was then used as a template to anneal the C561A mutant strands. PCR conditions used for this annealing step were: denaturation 94°C (2 min), annealing 40°C (10 min) and elongation 72°C (2 min) for 1 cycle. The annealed product was then extended for 30 cycles using FLAG-DTX3L-Forward/Reverse primers at the following PCR conditions: denaturation 94°C (45 sec), annealing 50°C (45 sec) and elongation 72°C (2 min). The forward primer carried a NotI
restriction site and started at amino acid residue Ala2, in frame with the 3xFLAG epitope. The reverse primer carried a XbaI site and an in frame stop codon and ended at E740. The PCR fragment was digested with NotI and XbaI and cloned into the NotI and XbaI sites of 3xFLAG-CMV10.

In the second PCR reaction, FLAG-DTX3L-3C/A was amplified by PCR using FLAG-DTX3L-C561A (single mutant) template DNA extended with FLAG-DTX3L-C596A/C599A-Forward primer in PCR reaction 1. Product of reaction 1 was then subjected to a second round of PCR using the FLAG-DTX3L-C596A/C599A-Reverse primer in tube two. The product of PCR reaction one was used as a template to anneal the DTX3L-3C/A triple mutant strands and extended using FLAG-DTX3L-Forward/Reverse primers using the same PCR conditions as above to generate the single mutant. The forward primer carried a NotI restriction site and started at amino acid residue Ala2, in frame with the 3xFLAG epitope. The reverse primer carried a XbaI site and an in frame stop codon and ended at E740. The PCR fragment was digested with the restriction enzymes NotI and XbaI and sub-cloned into the NotI and XbaI sites of 3xFLAG-pCMV10. Incorporation of the three mutations was confirmed by sequencing using the FLAG-DTX3L forward and reverse primers, essentially following steps described below in Sections 2.3.2-2.3.7. The FLAG-DTX3L-3C/A was also sub-cloned into the His vector (pET-21a (+)) and used in binding (Section 2.10.2) and in vitro ubiquitination assays (Section 2.13)
**PCR PRODUCT GEL EXTRACTION**

Amplified products were separated by agarose gel electrophoresis and purified by gel extraction. Amplified products diluted in 6X loading dye were loaded onto a pre-made 1% agarose gel (containing 10 µl of ethidium bromide) along with a 1 kb DNA ladder (Promega). Gel was electrophoresed at 130 V for approximately 1-2 h or until dye front neared bottom of gel. Samples were viewed under a UV light and the predicted product band was cut out using a sterile razor. The gel fragment was placed in a sterile 1.75 ml microcentrifuge tube. DNA was extracted from the gel using the Qiagen QIAquick Gel Extraction kit according to the manufacturer’s protocol. DNA was eluted in 30 µl EB buffer (10 mM Tris·Cl, pH 8.5) and amount of DNA eluted was quantified using a Nanodrop 8000 spectrophotometer (Thermo Scientific).

**RESTRICTION DIGEST**

Both the vector and amplified DNA insert were individually subject to restriction enzyme digest using the desired restriction sites that were amplified in the DNA product and compatible with the multiple cloning region of the vector. Restriction enzymes used in this project are listed in Table 2.7. Digestion reactions were set up as follows: 10× BSA (2 µl), 10× NEB buffer 1-3 (2 µl), vector and/or insert DNA, Restriction enzyme 1 (1 µl), Restriction enzyme 2 (1 µl) and sterile, autoclaved dH₂O in a final volume of 20 µl. Samples were incubated overnight at 37°C. NEB buffers were utilized due to low cutting efficiency when using Promega buffers and based on parallel analysis that suggested better compatibility of enzymes in a double restriction digest reactions with
NEB buffers than Promega buffers. Samples were then run on 1% agarose gel (containing 10 µl ethidium bromide) and subjected to Gel Extraction using the Qiagen Kit mentioned previously. DNA was eluted in 30 µl of EB buffer and the amount of DNA purified was quantified using a Nanodrop 8000 spectrophotometer (Thermo Scientific).

Specifically to generate DTX3L, the pCMV-SPORT6-DTX3L cDNA (Thermo-Scientific) purified from bacteria using the Qiagen mini-prep kit per manufacturer’s protocol was amplified by PCR and the product was sub-cloned into the Not I and Xba I sites of 3×FLAG-pCMV-10 (Sigma-Aldrich, St. Louis, MO), the BamHI and XhoI sites of pGEX-6P-1 (GE Healthcare) or the Bam HI and XhoI sites of pET-21a (+).

To generate the GST-N-terminal (NT) and GST-C-terminal (CT) truncations of DTX3L, primers were made based on the DTX3L truncations previously published (Takeyama et al., 2003). Briefly, the NT truncation of DTX3L spans amino acids 1-464, while the CT truncation covers amino acids 528-740. Full-length GST(pGEX6p1) - DTX3L was used as a template in the PCR and PCR products were sub-cloned into the BamHI and XhoI sites of pGEX-6P-1.

The DTX3L-3C/A mutant was designed based on the DTX1 mutant previously reported (Chastagner et al., 2006), as detailed in Section 2.4.1.1. Briefly, cysteine residues 561, 596 and 599 within the RING domain were mutated to alanine residues using two-step PCR and sub-cloned into 3×FLAG-pCMV-10 or pET-21a (+) vectors as described for wild-type DTX3L.
The 3×FLAG-pCMV-10-AIP4-C830A mutant was made by digesting myc-AIP4-C830A-pRK5 with XhoI and BamHI and sub-cloning and replacing the equivalent fragment in 3×FLAG-pCMV-10-AIP4.

To make GST-AIP4-C830A, the FLAG-AIP4-C830A cDNA was used as a template for PCR and the product was sub-cloned into the BamHI and NotI sites of pGEX-6P-1.

The yellow fluorescent protein tagged 2×FYVE domain (YFP-2×FYVE) was made by a previous student, Rohit Malik, by amplifying amino acid residues 147-223 from HRS by two-step PCR and sub-cloning in tandem into the XhoI and EcoRI sites of pEYFP-C1 (Clontech).

**LIGATION AND TRANSFORMATION**

Restriction enzyme digested samples were subject to ligation using different molar ratios of vector to insert (i.e. 1:1, 1:3, 3:1, etc.). The desired ratios of vector to insert DNA were combined in a sterile 0.6 ml microcentrifuge tube with 10× T4 ligase buffer (2 µl; Promega), T4 DNA ligase (1 µl; Promega) and sterile, autoclaved dH₂O to a final volume of 20 µl. As a negative control, a ligation reaction using only the vector but no insert (Vector only control) was set up in parallel. The ligation was incubated overnight (15 h) at 4°C. The next day, competent DH5α’ bacterial cells (100 µl) were combined with ligated DNA (5 µl) in a 1.7 ml sterile, microcentrifuge tube and incubated on ice for 30 min. Cells were then placed in a 42°C water bath for 2 min and then back on ice for 2 min. Next, 600 µl of LB broth was added to the transformed bacteria and
incubated for 40-60 min at 37°C. Transformed cells were then plated onto LB agar plates containing 100 µg/mL ampicillin (LB-amp agar). Briefly, transformed cells were centrifuged at 4,000 rpm for 1 min. Excess LB broth was aspirated to the 100 µl mark on the tube and pelleted bacteria were re-suspended. Cells (25-50 µl) were added to the LB-amp agar plate. To spread cells onto plate, a metal cell spreader was dipped in 70% ethanol and flamed using a Bunsen burner. The spreader was cooled for a 1-2 min and then used to spread the bacterial cells back and forth covering the agar dish. Plate was then inverted (lid downward) and placed at 37°C overnight.

**SELECTION OF COLONIES**

Colonies of transformed bacterial cells plated overnight on LB-amp agar were counted manually and compared to vector only control. The amount of colonies selected was based on the ratio between vector-only and vector plus insert transformations. Briefly, individual colonies were selected and inoculated in 3 ml of LB broth containing 100 µg/mL ampicillin (LB-amp broth). Samples were allowed to grow overnight in an orbital shaker (Forma Scientific, Model 4518) set at 250 rpm and a temperature of 37°C.

**SCREENING FOR INSERTS**

Small scale plasmid DNA was isolated from bacterial cultures using the Qiagen QIAprep Spin Miniprep kit, according to the manufacturer’s protocol. DNA was eluted in a volume of 50 µl EB buffer. Plasmid DNA (500 ng - 1µg) was subjected to a restriction enzyme digest using the same restriction enzymes used in the ligation reaction, as
described in Section 2.3.3. Following digestion, 2 μl of 6X loading dye was added to each sample and loaded onto a 1% agarose gel (containing 10 μl of ethidium bromide) along with a 1 kb DNA ladder (Promega). Gel was electrophoresed at 130 V for approximately 1 h or until dye front neared bottom of gel. Samples were viewed under a UV light box (Fisher Scientific) to determine positive clones for insert incorporation. A clone was determined to be positive for the insert if following digestion two bands appeared that migrated in the proper kb range determined for both vector and insert.

SEQUENCING

DNA of positive clones was sent for Single Pass DNA Sequencing off site at ACGT, Inc. (Wheeling, IL). Specifically, 5-10 μl of desired mini-prep DNA was aliquoted into a 0.6 ml sterile, microcentrifuge tube. In a separate tube, desired primers (5-10 μl; forward and reverse) were aliquoted into a 0.6 ml sterile, microcentrifuge tube or alternatively banked primers on the ACGT website were indicated to be used in sequencing reactions. Typically, data from reactions was uploaded within 2-3 days and sequence files were opened in Serial Cloner software (version 1.3-11) to determine whether correct insert was cloned into desired vector. Briefly, sequences obtained were compared manually to original sequence to determine whether the sequences matched or whether a certain mutation was incorporated. Purity of sequence was determined using Finch TV software (version 1.4.0, Geospiza Inc.).
MAXI PREP

Following confirmation of sequence, large scale plasmid DNA was isolated using the Qiagen HiSpeed Plasmid Maxi kit. Specifically, transformed bacterial cells were inoculated in 5 ml of LB-amp broth. Samples were incubated for 8 h in an orbital shaker (Forma Scientific, Model 4518) set at 250 rpm and a temperature of 37°C. The 5 ml culture was then added to 500 ml of LB-amp broth allowed to grow overnight (18 h) in the same orbital shaker set at 250 rpm and a temperature of 37°C. Bacteria were pelleted by centrifugation at 4,000 rpm for 25 min (Beckman Coulter J6-HC centrifuge). DNA was then purified from bacterial pellet as per manufacturer’s protocol and eluted in a volume of 500-750 µl TE buffer. Concentration of DNA was determined using a Nanodrop 8000 spectrophotometer (Thermo Scientific).

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL (SDS-PAGE) AND WESTERN BLOT TRANSFER

Samples were collected in 2× sample buffer (8% SDS, 10% glycerol, 0.7 M beta-mercaptoethanol, 37.5 mM Tris HCl, pH 6.5, 0.003% bromophenol blue) as detailed in each protocol below. Samples were resolved along with pre-stained molecular weight standards (BioRad) on SDS-polyacrylamide gels (containing 7%, 10% or 12% acrylamide, as detailed in the Appendix I). Gel was electrophoresed at 150-160 volts in 1× SDS running buffer (0.25 mM Tris-HCl pH 7.5, 1% SDS and 0.192 M glycine) for 1 h -1.5 h. Separated proteins were transferred by Western blot onto nitrocellulose membrane
(0.45 μM or 0.20 μM, GE Healthcare or BioRad) for 1 h at 100 volts in 1× transfer buffer (0.25 mM Tris-HCl pH 7.5, 0.192 M glycine and 20% (vol/vol) methanol).

IMMUNOBLOT ANALYSIS

Membranes containing Western blot transferred proteins (immunoblots) were incubated in 5% non-fat milk in Tris-buffered saline (20mM Tris pH 7.5, 150 mM NaCl) containing 0.05% TWEEN-20 (TBS-T) for 30 min rocking at room temperature on a rocking platform (VWR, model 200). Blocked immunoblots were incubated with specific primary antibodies (dilution range 1:500 to 1:3000) in the same buffer overnight at 4°C. The following morning, primary was saved and frozen at -20°C for later re-use or discarded. Primary incubated immunoblots were washed three times for 5 min with TBS-T and then incubated with appropriate horseradish peroxidase (HRP) conjugated secondary antibody (Vector Labs) at a dilution of 1:10,000 in blocking buffer (5% non-fat milk dissolved in TBS-T) for 30 min rocking at room temperature. Immunoblots were washed five times with TBS-T with one 5 min quick wash and four subsequent 10 min longer washes at room temperature. Proteins were visualized using enhanced chemiluminescence (ECL) reagents (DURA extended duration substrate, Thermo Scientific or homemade short duration substrate). Short duration substrate was made as detailed in Appendix I. DURA was generally used on immunoblots for samples obtained from co-immunoprecipitation and degradation experiments for endogenous protein or for primary antibodies of low affinity such as DTX3L, HRS and CXCR4. Short duration substrate was used on immunoblots of samples with DNA overexpression, for loading
controls (Tubulin or Actin), using primary antibodies of high affinity such as AIP4 and
STAM-1 or obtained from in vitro ubiquitination assays. Immunoblot membranes were
incubated with 1:1 ratio of ECL for 1-5 min prior to developing. Signal was captured on
autoradiography film (Dot Scientific high contrast blue or MIDSCI Classic blue
autoradiography film BX) and exposed films were developed using an automated film
processor.

LIGAND STIMULATION

CXCL12 was made as a stock of 10 µM in 0.1% BSA in PBS and stored at -
20°C. For stimulation with CXCL12, the amount/concentration of desired ligand made in
0.1% BSA in PBS was diluted in DMEM incomplete supplemented with 20mM HEPES.
For vehicle stimulation, cells were treated with 0.1% BSA in PBS without ligand. Media
was removed from cells by aspiration prior to the addition of pre-mixed, diluted ligand in
DMEM incomplete supplemented with 20mM HEPES. To evenly distribute the ligand,
plates were gently rocked back and forth twice and placed at 37°C for desired time course
(15 min-3 h).

CXCR4 DEGRADATION ASSAY

Agonist promoted degradation of CXCR4 was assessed by immunoblot analysis,
similar to previously described (Malik & Marchese, 2010; Marchese et al., 2003).
Specifically, HeLa cells grown to 70-80% confluency in 6-well plates were transfected
with 50 pmol siRNA directed against DTX family members, c-Cbl, Cbl-b, AIP4 or
GAPDH or Luciferase using Lipofectamine® 2000/3000 (Invitrogen, Carlsbad, CA), as we have previously described (Marchese et al., 2003). The next day after transfection, cells were split 1:2 into 12- well plates and grown for another 24 hours. At the time of experiment cells had grown to 90-100% confluency. Cells were washed with DMEM complete and then incubated with complete media (500 µl) containing 50 µg/mL cyclohexamide to prevent protein synthesis for 15 min at 37°C. Media was aspirated and replaced with media containing vehicle (0.1% BSA in PBS) or 10 nM CXCL12 for total of 3 h in the continued presence of cyclohexamide. After treatment, media was aspirated and replaced with 500 µl PBS at room temperature, followed rapidly by aspiration. Three hundred µl of 2× sample buffer was added to each well and cells were removed by gently scraping with a rubber policeman and transferred to a 1.7 ml microcentrifuge tube. Lysates were sonicated once for 10 sec at 15 % amplitude using a Branson Digital Sonifier (Model 450). Equal amounts (10 µl) of samples were analyzed by 10% SDS-PAGE and immunoblotting with antibodies directed against CXCR4, DTX3L, AIP4, actin or tubulin. We were unsuccessful in determining DTX1-3, c-Cbl and Cbl-b knockdown by immunoblot due to lack of good reagents that could distinguish these proteins. Future study will require qPCR to determine degree of knockdown for these proteins. Receptor degradation was determined by densitometric analysis of similar enhanced chemiluminescent exposures from multiple experiments and calculated as the percent receptor degraded in CXCL12 treated cells compared to vehicle.
CONFOCAL FLUORESCENCE MICROSCOPY: SAMPLE PREPARATION

HeLa cells grown in 6-well plates were transfected with siRNA targeting DTX3L, AIP4 or control Luciferase using Lipofectamine® 2000/3000, as described above in Section 2.1.2. Alternatively, cells were transfected with 1 µg FLAG-AIP4 DNA using PEI, as detailed in Section 2.1.1. Twenty-four hours later cells were plated onto 22 x 22 mm No. 2 coverslips (Fisher) coated with poly-L-lysine (PLL; 0.1 mg/mL; Sigma-Aldrich, St. Louis, MO). To coat coverslips, in a biological safety cabinet coverslips were placed in 6-well plate and incubated with PLL for 10 min. PLL was aspirated and coverslips were incubated in the biological safety cabinet for 40 min at room temperature to dry prior to passing cells directly onto coverslips.

The following day, cells were washed once with DMEM supplemented with 20 mM HEPES pH 7.5. For serum starvation, media was replaced with 1ml DMEM supplemented with 20 mM HEPES pH 7.5 for 3 h at 37 °C before stimulating with ligand. To stimulate cells, the desired volume of CXCL12 was pre-diluted in DMEM supplemented with 20 mM HEPES pH 7.5 to a final concentration of 10 nM. Media was aspirated from cells and media containing vehicle or CXCL12 (10 nM) was added directly to cells. Cells were stimulated at 37°C for various times (15 min-3h). Cells were then washed twice on ice with ice-cold 1× PBS (500 µl - 1000 µl; Hyclone Laboratories). To fix cells, 1 ml of 3.7% paraformaldehyde-PBS was added to each well followed by incubation for 10 min at room temperature. Cells were washed twice again with 1 ml 1×PBS. To permeabilize cells a solution containing 0.05% (w/v) saponin in 1 ml 1×PBS was added for 10 min at room temperature, as described previously (Malik, Soh, Trejo, &
Marchese, 2012). To block non-specific binding sites 1000 µl blocking buffer (1×PBS plus 1% BSA and 0.05% w/v saponin) was added to each well and incubated for 30 min at 37°C. Primary antibodies (1:25 - 1:100 dilution) directed against either CXCR4, DTX3L, EEA1, LAMP2, HRS, STAM-1 or FLAG in 100 µl were aliquoted on parafilm that was placed on top of moistened filter paper to create a moist chamber. Coverslips were inverted onto the aliquoted primary for 1 h at 37°C and followed by washing with 0.05% (w/v) saponin in 1 ml 1×PBS. Briefly, coverslips were placed back into the 6 well dish (cell side up), washed quickly four times at room temperature and then washed for a longer time period for 15 min at 37°C. Samples were then incubated for 30 min at 37°C with Alexa-Fluor-conjugated secondary antibodies (1:100) in a similar fashion as the primary. Secondary antibodies included: Alexa-Fluor 635-conjugated goat anti-mouse, Alexa-Fluor 633-conjugated goat anti-rat, Alexa-Fluor 488-conjugated goat anti-mouse, Alexa-Fluor 555-conjugated donkey anti-goat, and Alexa-Fluor 488-conjugated goat anti-rabbit antibodies (Table 2.3), Molecular Probes (Eugene, OR)). Controls for staining included samples stained with secondary only, primary with opposite (different species) secondary or staining sample with single primary and secondary combination followed by scanning in opposite channel (i.e. stain with 488 nm conjugated secondary and view in 555 nm for any bleed-through). Cells were washed as before and mounted on glass slides using mounting medium containing DAPI (Vector Laboratories, Burlingame, CA or ProLong Gold Invitrogen, Carlsbad, CA). The edges of the slips were sealed using nail polish and stored either at room temperature or 4°C in the dark prior to viewing.
DTX3L CO-LOCALIZATION WITH EEA1 AND LAMP2

To examine co-localization between DTX3L and EEA1 or LAMP2, HeLa cells plated onto coverslips were serum starved in 1ml DMEM supplemented with 20 mM HEPES pH 7.5 for 3 h at 37 °C, treated with 10 nM CXCL12 for 30 min and co-stained for DTX3L and EEA1 or LAMP2.

CXCR4 DEGRADATION BY IF

To characterize the effect of DTX3L silencing on CXCR4 trafficking, HeLa cells plated on coverslips were transfected with DTX3L or control siRNA for a total of 48 hrs. Cells were then serum starved in 1ml DMEM supplemented with 20 mM HEPES pH 7.5 for 3 h at 37 °C prior to stimulation with 10 nM CXCL12 or vehicle for 3 h. Cells were processed as described above and were co-stained for CXCR4 and EEA1 or LAMP2 or DTX3L.

CO-LOCALIZATION OF DTX3L AND AIP4

To examine co-localization of DTX3L with AIP4, HeLa cells plated on coverslips were first transfected with 1 µg of FLAG-AIP4. The following morning cells were serum starved in 1ml DMEM supplemented with 20 mM HEPES pH 7.5 for 3 h at 37 °C and stimulated with 10nM CXCL12 or vehicle for 0-60 min. Cells were processed essentially as stated above and co-stained for DTX3L, FLAG-AIP4 and EEA1.
ASSESSING CO-LOCALIZATION WITH ESCRT-0

To assess the effect of DTX3L on ESCRT-0 components, HeLa cells plated on coverslips were transfected with control or DTX3L siRNA for a total of 48 hrs. Cells were serum starved in 1ml DMEM supplemented with 20 mM HEPES pH 7.5 for 3 h at 37 °C and then stimulated with 10 nM CXCL12 or vehicle for 1 h. Cells were processed as above and co-stained for HRS or STAM-1 and DTX3L or EEA1.

YFP-FYVE CO-LOCALIZATION STUDIES

To determine the effect of DTX3L silencing on endosomal phosphoinositol 3-phosphate (PI-3P) levels, HeLa cells plated on coverslips were co-transfected with 1 µg YFP-2xFYVE and control or DTX3L siRNA for 48 hrs. Cells were serum starved in 1ml DMEM supplemented with 20 mM HEPES pH 7.5 for 3 h at 37 °C. Cells were then pre-treated with DMSO or wortmannin (100 nM) for 30 min before stimulation with 10 nM CXCL12 for 1 h. Cells were processed as described above, however, cells were immunostained for DTX3L and EEA1.

CONFOCAL FLUORESCENCE MICROSCOPY: IMAGE ACQUISITION AND PROCESSING

Slides were viewed using a Zeiss LSM 510 laser-scanning confocal microscope equipped with a Plan-Apo × 63/1.4 numerical aperture oil DIC M27 lens objective. Images were acquired using a 1.4-megapixel cooled extended spectra range RGB digital camera set at 512 × 512 resolution. Specifically, the following parameters detailed below
were used to acquire images and did not vary unless stated otherwise. Scan mode was set at plane, original data, multitrack, 8 bit. Stack size was set at 512 x 512 x 1. Wavelengths used were 633 nm (17%), 561 nm (12%) and 488 nm (4.1%). Channel (Ch) filters were as follows: Ch 1 LP650, Ch 2 BP 505-550 and Ch 3 LP 575. Pinhole was set to 134 μM for each channel and images were scanned at a speed of 8. The software used to view acquired images was Carl Zeiss Laser Scanning System LSM 510. Images were also viewed using Zeiss LSM Image Browser version 4.2.0.121. Equal acquisition settings were used between parallel samples within each experiment. Specifically, control samples were first viewed for each experiment to determine optimal parameters for staining with each primary used and those same settings were used to view subsequent treated samples during the same sitting on the microscope. Gain and intensity values varied based on each primary and secondary combination used, but remained constant between each experiment repetition.

Images were analyzed using ImageJ software (National Institutes of Health, version 1.37v) and processed using Adobe Photoshop (CS4). Pearson product-moment correlation coefficient was determined using the ImageJ plug-in “Colocalization Finder.” Pixels were restrained to the minimum ratio of 75% to reduce noise from channel bleed through. Analysis results in values ranging from 0 to 1, where 0 represents ‘no co-localization’ and 1 represents ‘absolute co-localization’. Puncta analysis was performed using the “Analyze Particles” macro in ImageJ. Images (8-bit) were manually thresholded to minimum (130-150) and maximum (255) values to exclude background noise. Particles counted were restrained to a size of 0.05-1.0 and circularity of 0.5-1.0.
GST- AND His-FUSION PROTEIN PURIFICATION

*Escherichia coli* BL21 cells transformed (similar to protocol used in Section 2.3.4) with cDNA encoding GST-fusion proteins in pGEX-6p1 or His-tagged proteins in pET-21a (+) vectors were inoculated in 5 ml LB-amp broth from glycerol stocks. Constructs used for GST and His purifications are listed in Table 2.8. Specifically, 10 µl of thawed glycerol stock was pipetted into LB-amp using sterile techniques. Sample was grown overnight at 37°C in an orbital shaker (Forma Scientific, Model 4518) set at 250 rpm. The following morning, 30 ml cultures were diluted (3.7% dilution) into a 50 ml conical tube and grown to OD$_{600}$ 0.35–0.40 at 37°C in an orbital shaker (1-3 h). Protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final 0.1-0.3 mM concentration (IPTG; Sigma-Aldrich, St. Louis, MO) for 3 h at 18°C. Following induction, cells were pelleted by centrifugation (Beckman Coulter J6-HC centrifuge set at 4,000 rpm for 20 min at 4°C). Pellets were re-suspended in 0.7 mL lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 10 µg/mL each of leupeptin, aprotinin, and pepstatin-A). For purification of His-tagged proteins DTT was omitted in the lysis buffer. Cells were lysed by sonication (Branson Digital Sonifier Model 450 set at 15% amplitude, 10 sec twice) on ice and clarified by centrifugation at 14,000 rpm for 20 min at 4°C (5417r-Eppendorf microcentrifuge).
EQUILIBRATION OF GLUTATHIONE-SEPHAROSE AND TALON METAL AFFINITY BEADS

For GST-fusion or His-fusion protein purifications, glutathione-Sepharose 4B (GE Healthcare) or Talon Metal Affinity Beads (Clontech, Mountain View, CA) resin were first equilibrated in lysis buffer. This was done by washing three times with 750 μl of lysis buffer. Specifically for each wash, following the addition of wash buffer beads were rocked back and forth three times, subject to centrifugation for 15 sec at 10,000 rpm and excess buffer was aspirated between washes. The volume of the pelleted resin was determined by reading the graduations on the microcentrifuge tube. The resin was re-suspended in an equal volume of lysis buffer to make a 50% slurry.

IMMOBLIZATION OF GST- AND His-PROTEINS

Clarified lysates were incubated with 25 μl of a 50% slurry of equilibrated glutathione-Sepharose 4B (GE Healthcare) or 20 μl of a 50% slurry of equilibrated Talon Metal Affinity Beads (Clontech, Mountain View, CA) resin for His-fusion proteins while rocking at 4°C for 15-17 h. The next day immobilized proteins were washed three times with 750 μl of lysis buffer, pelleted by centrifugation at 10,000 rpm for 15 sec and re-suspended in 100 μl of lysis buffer. For washes, following the addition of wash buffer samples were manually rocked back and forth three times, wash buffer was pelleted by microcentrifugation for 15 sec at 10,000 rpm (Thermo microcentrifuge 5519, model 120) and excess buffer was aspirated between washes.
QUANTIFICATION OF IMMOBILIZED GST- AND HIS- FUSION PROTEINS

To determine the amount of purified protein, the immobilized protein was compared to known amounts of BSA. To accomplish this 10 μl of the total immobilized sample (100 μl) was combined with 10 μl of 2× sample buffer. Bound protein was eluted from the beads by boiling the sample at 100 °C for 5 min. Eluted proteins (10 μl) were resolved by 7% or 10% SDS-PAGE along with bovine serum albumin (BSA, Roche Diagnostics, Indianapolis, IN) standards (0.1 μg- 3 μg). Gel was placed in a tray and washed in 100 ml distilled water for 30 min. Proteins were stained by incubating gel in approximately 50 ml of GelCode Blue stain while rocking at room temperature. GelCode Blue was decanted and the gel was then destained by washing with distilled water 2-3 times for 15-30 min. Protein amounts were estimated by comparing the staining intensities of purified proteins to the BSA standards of known concentration. Concentration was confirmed by analyzing gel bands using densitometry and comparing values of purified proteins to the densities of the BSA standards to determine quantitatively the purified protein concentration. An example of typical purification yield for GST (0.2 μg/ μl), GST-DTX3L (0.1 μg/ μl) and GST-AIP4 (0.1 μg/ μl) is shown in Figure 2.1.
**Figure 2.1 Example of a gel stained with GelCode Blue used to quantify amounts of GST-fusion proteins purified for this study.** Representative image of GelCode Blue staining of a gel ran for GST-protein purification of GST, GST-DTX3L and GST-AIP4. Ten microliters of immobilized protein were eluted in 10 µl of 2x sample buffer. Sample was boiled, centrifuged and 10 µl of each eluted protein was subsequently run on 10% SDS-PAGE gel along with known BSA standards (0.1 µg, 0.5 µg, 1.0 µg and 3.0 µg). The gel was then washed in distilled H₂O, stained with GelCode Blue and briefly destained with distilled H₂O. Fusion protein concentration was then estimated by comparing intensities of fusion protein band to the BSA standards. As shown here, the protein concentration were estimated to be: GST = 0.2 µg/ µl; GST-DTX3L=0.1 µg/ µl and GST-AIP4 = 0.1 µg/ µl.

**ELUTION OF HIS-TAGGED PROTEINS**

His-tagged proteins bound to Talon Metal Affinity resin were eluted by incubating samples in elution buffer containing 100 mM imidazole. Specifically, immobilized proteins (20-50 µl) were eluted in 0.5 mL His purification lysis buffer containing 100 mM imidazole, while rocking overnight at 4°C. The next day, samples were centrifuged for 30 sec at 10,000 rpm (Thermo microcentrifuge 5519, model 120) and supernatant was then subject to buffer exchange. Eluted proteins were dialyzed in 10K MWCO, 20mm SnakeSkin dialysis tubing (Life Technologies) in 2 L of dialysis buffer (50 mM Tris-HCl, pH 7.4) stirring, overnight and concentrated using Spectra/Gel
absorbent (Spectrum Labs, Rancho Dominguez, CA). Twenty µl aliquots were aliquoted into microcentrifuge tubes and samples were immediately snap frozen on a mixture of dry ice plus methanol for 5 min. Samples were stored at -80°C until use in experiments. Samples were quantified by resolving on 10% SDS-PAGE, followed by staining of gel with GelCode Blue. An example of a typical yield for the purification of His-DTX3L (0.1 µg/ µl) and His-DTX3L-3C/A (0.075 µg/ µl) is shown in Figure 2.2.

Figure 2.2 Example of a gel stained with GelCode Blue used to quantify amounts of His-fusion proteins purified for this study. Representative image of GelCode Blue staining of a gel ran for His-protein purification of His-DTX3L and His-3C/A. Ten microliters of imidazole eluted protein was diluted in 10 µl of 2× sample buffer and 10 µl of each sample was subsequently run on 10% SDS-PAGE gel along with known BSA standards (0.5 µg, 1.0 µg and 3.0 µg). The gel was then washed in distilled H₂O, stained with GelCode Blue and briefly destained with distilled H₂O. His-fusion protein concentration was then estimated by comparing intensities of fusion protein band to the BSA standards. Asterisk’s (*) denotes degradation products left over from purification. As shown here, the protein concentration were estimated to be: His-DTX3L=0.1 µg/ µl and His-DTX3L-3C/A = 0.075 µg/ µl.
LARGE SCALE PURIFICATION AND PreScission PROTEASE CLEAVAGE OF GST-AIP4 AND GST-AIP4-C830A

To generate purified cleaved AIP4 or AIP4-C830A, BL21 E. coli cells transformed with GST-AIP4 or GST-AIP4-C830A cDNA were inoculated into 30 ml of LB-amp broth at 37°C overnight. The following morning, cultures were diluted into 500 mL and grown at 37°C for 3hr. Protein expression was induced with 0.3 mM IPTG (Sigma-Aldrich, St. Louis, MO) for 24 h at 18°C. Cells were then pelleted by centrifugation (4000 rpm for 30 min at 4°C using a Beckman Coulter J6-HC centrifuge) and re-suspended in 10 mL of cold GST purification lysis buffer. Lysates were incubated for 30 min, while rocking at 4°C. Samples were sonicated and then pelleted by microcentrifugation (4000 rpm for 30 min at 4°C). To remove any small degradation products, supernatants (~10 ml) were placed in a 30 ml syringe and passed through a 0.2 μM filter and were combined with 600 μl of a 50% slurry of glutathione-Sepharose 4B (GE Healthcare) resin overnight at rocking at 4°C. Samples were washed three times with 1000 μl lysis buffer. For washes, following the addition of wash buffer samples were rocked back and forth three times manually, beads were pelleted by microcentrifugation for 15 sec at 10,000 rpm (Thermo microcentrifuge 5519, model 120) and excess buffer was aspirated between washes. Samples were re-suspended in 1000 μl of lysis buffer.

To cleave the GST (pGEX6P1) tag from GST-AIP4 and GST-C830A, immobilized proteins in lysis buffer (1000 μl) were directly incubated with 10 units of PreScission Protease (GE Life Sciences) for 12 h, while rocking at 4°C. Eluted samples were dialyzed in 10K MWCO, 20mm SnakeSkin dialysis tubing (Life Technologies) in 2
L of dialysis buffer (50 mM Tris-HCl, pH 7.4) stirring, overnight and concentrated using Spectra/Gel absorbent (Spectrum Labs, Rancho Dominguez, CA). Samples were quantified by resolving on 10% SDS-PAGE, followed by staining of gel with GelCode Blue, similar to sections 2.9.3 and 2.9.4. An example of typical purification yield for cleaved AIP4 (0.2μg/μl) and cleaved AIP4-C830A (0.2μg/μl) is shown in Figure 2.3. Twenty-five μl aliquots were made into microcentrifuge tubes and samples were immediately snap frozen on a mixture of dry ice plus methanol for 5 min. Samples were stored at -80°C until used in experiments.

Figure 2.3 Example of a gel stained with GelCode Blue for cleaved AIP4 and AIP4-C830A purification. Representative image of GelCode Blue staining of a gel ran for the purification and PreScission protease cleavage of (A.) GST-AIP4 and (B.) GST-AIP4-C830A. Five microliters of each sample was diluted in 6 μl of 2x sample buffer and 5 μl of each sample was subsequently run on 10% SDS-PAGE gel along with known BSA standards (0.1 μg, 0.5 μg, 1.0 μg and 3.0 μg). ‘Cleaved’ sample represents GST-fusion protein that was cleaved with PreScission Protease and ‘Bound’ sample represents GST-fusion protein that remain in bead volume after cleavage. The gel was then washed in distilled H2O, stained with GelCode Blue and briefly destained with distilled H2O. Protein concentration was then estimated by comparing intensities of fusion protein band to the BSA standards. Asterisk’s (*) denotes degradation products left over from purification. As shown here, the protein concentration of cleaved proteins was estimated to be: AIP4=0.2 μg/μl and AIP4-C830A= 0.2μg/μl.
GST- AND HIS-FUSION BINDING ASSAYS

Binding assays were used to determine protein-protein interactions by affinity purification.

GST- AND HIS-FUSION BINDING ASSAYS: LYSATE COLLECTION

Typically for a binding reaction using HeLa cell lysates, HeLa cells were first plated onto 6-cm dishes. HeLa cells were either left untransfected or transfected with empty vector or desired DNA constructs for 24 h. Cells were washed two times with 3 ml 1×PBS on ice and collected by gently scraping cells in 0.4 ml of binding buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol, 20 mM N-ethylmaleimide (NEM) and 10 µg/mL each of leupeptin, aprotinin, and pepstatin-A). Samples were rocked for 30 min at 4°C and lysed by sonication (15 % amplitude, 10 sec) on ice. Lysates were cleared by microcentrifugation at 14,000 rpm for 20 min at 4°C (5417r-Eppendorf microcentrifuge). Protein concentrations of supernatants were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). A total of 100-500 µg of supernatant was incubated with immobilized protein overnight while rocking at 4°C.

GST- AND HIS-FUSION BINDING ASSAYS: BINDING ASSAYS

For GST-binding assays, immobilized fusion proteins were incubated with cleared HeLa lysates either transiently transfected without or with 1µg of FLAG-AIP4. Between 100 µg -500 µg of cleared lysate was used in binding assays as detailed below. Samples
were incubated at 4°C for 15-17 h while gently rocking. Samples were washed three times with 750 μl of lysis buffer. Specifically, following the addition of wash buffer samples were manually rocked back and forth three times, beads were pelleted by microcentrifugation for 15 sec at 10,000 rpm (Thermo microcentrifuge 5519, model 120) and excess buffer was aspirated between washes. Samples were eluted with 25 μl 2× sample buffer and followed by immunoblot analysis.

**GST-DTX3L TRUNCATIONS**

For binding reaction involving the GST-DTX3L N-terminal and C-terminal truncation mutants, equimolar amounts of GST, GST-DTX3L-full length, -NT or –CT (100 nmol) were incubated with cleared HeLa lysates (100 μg) transiently transfected to express FLAG-AIP4 or empty vector. Samples were incubated overnight and collected as described above. Lysates were subjected to immunoblot analysis for anti-FLAG-HRP (AIP4) and blot was stained with Ponceau to show amount of purified protein used the reaction.

**GST-AIP4 TRUNCATIONS**

For binding reaction involving the GST-AIP4 truncation mutants, equimolar amounts of GST, GST-AIP4, GST-C2, GST-WWI-IV or GST-HECT (200 nmol) were incubated with His-DTX3L (5nM). Samples were incubated overnight and collected as described above. Lysates were subjected to immunoblot analysis for DTX3L and GST.
AIP4 AND DTX3L BINDING

To determine binding between GST-AIP4 and endogenous DTX3L, HeLa lysates (500 µg) were collected as described above and incubated overnight with immobilized GST or GST-AIP4 (100 nmols). DTX3L binding was determined by immunoblot analysis. To determine direct binding between AIP4 and DTX3L, increasing amounts of purified, eluted His-DTX3L (1-10 nM final concentration) were incubated with equal amounts of immobilized GST-AIP4 (100 nmol) in a volume of 100 µl for 1 h while gently rocking at 4°C. Samples were washed three times with 750 µl lysis buffer. Specifically, following the addition of wash buffer samples were manually rocked back and forth three times, beads were pelleted by microcentrifugation for 15 sec at 10,000 rpm (Thermo microcentrifuge 5519, model 120) and excess buffer was aspirated between washes. Samples were eluted with 25 µl 2× sample buffer and followed by immunoblot analysis to determine His-DTX3L binding to GST-AIP4.

GST- AND HIS-FUSION BINDING ASSAYS : DATA QUANTIFICATION

Data were quantified by densitometric analysis for each experiment. Values were averaged between experiments and represented as a fraction of GST or empty vector control.
CO-IMMUNOPRECIPITATION

Co-immunoprecipitation assays were utilized to determine protein-protein interactions in cells using specific antibodies to capture target proteins as detailed in the following sections.

TIME COURSE CO-IMMUNOPRECIPITATION

To determine whether the interaction between DTX3L and AIP4 is dependent on CXCL12 treatment, we performed a time course co-immunoprecipitation. Specifically, HeLa cells plated in 10-cm dishes were serum starved for 4 h and then treated for 0-60 min with 10 nM CXCL12 at 37°C. Cells were placed on ice, washed once with ice-cold PBS and lysed in immunoprecipitation buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 20 mM N-ethylmaleimide (NEM), protease inhibitors (10 µg/mL each of aprotinin, leupeptin and pepstatin A (Roche))]. Lysates were cleared by centrifugation at 14,000 rpm for 20 min in a 5417r-Eppendorf microcentrifuge. Clarified lysates (500 µg) were incubated with an antibody (2 µg) directed against DTX3L or goat IgG control for 16 h at 4 °C. Twenty µl of a 50% slurry of protein A agarose was then added and samples were incubated for an additional 1 h. Samples were then washed three times as follows: following the addition of wash buffer samples were rocked back and forth three times, beads were pelleted by microcentrifugation for 15 sec at 10,000 rpm (Thermo microcentrifuge 5519, model 120) and excess buffer was aspirated between washes. Immunoprecipitated proteins were denatured by the addition of 25 µl 2× sample buffer,
resolved by 7% SDS-PAGE and analyzed by immunoblotting using antibodies directed against AIP4, DTX3L, and actin.

CO-IMMUNOPRECIPITATION OF ENDOGENOUS PROTEINS

For the immunoprecipitation of endogenous proteins, lysates were collected in a similar fashion as Section 2.10.1, however, left untreated. Specifically, to determine the effect of DTX3L depletion on the binding between ESCRT-0 components HRS and STAM-1, HeLa cells were transfected with control siRNA or DTX3L siRNA (25 nM final concentration) for 24 h before immunoprecipitation of cleared lysates (300 μg) with 2 μg of either anti-HRS or mouse IgG control and anti-STAM-1 or rabbit IgG control for 1 h at 4 °C. Twenty μl of a 50% slurry of protein A agarose was then added and samples were incubated for an additional 1 h. Immunoblots were probed with antibodies against AIP4, DTX3L, HRS, STAM1 or actin.

For endogenous DTX3L co-immunoprecipitation experiments lysates were collected as stated above, however, cleared lysates (500 μg) were immunoprecipitated with 3 μg of anti-DTX3L or goat IgG control. Samples were resolved by 7% SDS-PAGE and immunoblots were probed with antibodies against AIP4, DTX3L, HRS, STAM1 or actin.

CO-IMMUNOPRECIPITATION DATA QUANTIFICATION

Data were quantified by densitometric analysis for each experiment. For the time course co-immunoprecipitation analysis, values were averaged between experiments and
vehicle values were set to 1 and treatment times were represented as a fraction of vehicle. In the ESCRT-0 co-immunoprecipitations, the average of control siRNA values were set to 1 and DTX3L siRNA samples were calculated as a fraction of the control siRNA.

UBIQUITINATION ASSAYS

Ubiquitination assays were used to determine the ubiquitination of substrates in cells or in purified *in vitro* systems.

HRS AND STAM UBIQUITINATION

HeLa cells grown on 6-cm dishes were transfected with 1 µg FLAG-ubiquitin, 4 µg T7-HRS or T7-STAM-1 and control siRNA or siRNA directed against DTX3L, AIP4 or a combination (25 nM final concentration for each siRNA) using Lipofectamine® 3000 (Invitrogen, Carlsbad, CA), as described in Section 2.1.2. Specifically, cells were first transfected with siRNA in the morning and then transfected with DNA seven hours later. The following morning, cells were placed on ice, washed twice with ice-cold PBS and collected into 2 mL microcentrifuge tubes with 200 µl of denaturing ubiquitination buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% SDS, 1% Triton X-100, 5 mM EDTA, 20 mM N-ethylmaleimide (NEM), and protease inhibitors [10 µg/mL each of aprotinin, leupeptin and pepstatin A (Roche, Indianapolis, IN)] by gently scraping with a rubber policeman. Collected samples were boiled for 5 min at 100°C on a heat block, sonicated (15%, 10 sec) on ice and diluted with 1.8 mL dilution buffer [20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA]. Cells were allowed to lyse for 1 h while
rocking at 4 °C. Samples were cleared by centrifugation (14,000 rpm, 25 min at 4 °C) and protein concentrations of supernatants were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). A total of 300 µg of supernatant was incubated with 2 µg of an anti-T7 goat polyclonal antibody overnight while rocking at 4 °C. The next day, samples were then incubated with 20 µl of a 50% slurry of Protein G agarose (Roche) for 1 h while rocking at 4 °C. Samples were washed twice with 500 µl dilution buffer as follows. Following the addition of wash buffer samples were rocked back and forth three times, beads were pelleted by microcentrifugation for 15 sec at 10,000 rpm (Thermo microcentrifuge 5519, model 120) and excess buffer was aspirated between washes. Bound proteins were eluted in 25 µl of 2× sample buffer, resolved by 7% or 12% SDS-PAGE and then analyzed by immunoblotting with antibodies directed against the FLAG and T7 epitopes, DTX3L AIP4 or actin.

**CXCR4 UBIQUITINATION**

To examine CXCR4 ubiquitination, HEK293 cells grown on 10-cm dishes were first transfected with siRNA against control, DTX3L or AIP4 (25 nM final concentration) using Lipofectamine® 3000 as described in Section 2.1.2. Seven hours later cells were transfected with 3 µg FLAG-ubiquitin and 7 µg HA-CXCR4. The next morning cells were split 1:2 into 6-cm dishes. Twenty-four hours later cells were serum starved in DMEM incomplete plus 20 mM HEPES for 3 h at 37 °C, followed by stimulation with 30 nM CXCL12 or vehicle for 30 min. Cells were placed on ice and washed twice with 3 ml ice-cold 1×PBS. Samples were collected with 200 µl of denaturing ubiquitination buffer
by gently scraping with a rubber policeman. Samples were then boiled for 5 min at 100°C on a heat block, sonicated (15%, 10 sec) on ice and diluted with 1.8 mL dilution buffer. Cells were allowed to lyse for 1 h while rocking at 4 °C. Samples were cleared by centrifugation (14,000 rpm, 25 min at 4 °C) and protein concentrations of supernatants were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL). Supernatants (300 µg) were incubated with 2 µg of an anti-HA rabbit polyclonal antibody (Covance/Biolegend) while rocking overnight at 4 °C. Samples were then incubated with 20 µl of a 50% slurry of Protein A agarose (Roche) for 1 h while rocking at 4 °C. Samples were washed twice with 500 µl dilution buffer. Bound proteins were eluted in 25 µl of 2× sample buffer, resolved by 10%, 7% or 12% SDS-PAGE and then analyzed by immunoblotting with antibodies directed against the FLAG and HA epitopes, DTX3L, AIP4 or tubulin.

**IN VITRO UBIQUITINATION ASSAYS**

**AIP4 AND DTX3L IN VITRO UBIQUITINATION**

To determine effect of DTX3L on AIP4 ubiquitination, *in vitro* ubiquitination assays were performed using 500 ng of GST-purified/cleaved AIP4 or AIP4-C830A-HECT mutant and 1 µg His-DTX3L or DTX3L-3C/A RING mutant alone or in combination. Briefly, purified E3s were incubated alone or together with a master mix of E1 (0.5 µg), E2 (UbcH5c, 0.5 µg), and ubiquitin (2.5 µg) and ATP in a final volume of 20 µl for 1 h 30 min at room temperature. Reactions were stopped by the addition of 20 µl of 2× sample buffer. Samples were resolved by 7% or 12% SDS-PAGE and then
analyzed by immunoblotting with antibodies directed against the AIP4, DTX3L, ubiquitin or GST.

**AIP4, DTX3L AND PARKIN IN VITRO UBIQUITINATION**

To examine the effect of AIP4, DTX3L and Parkin ubiquitination alone and in combination, purified MBP-Parkin (500 ng), AIP4 (500 ng) and His-DTX3L (1 μg) were allowed to react alone and in combination for 1 h 30 min at room temperature similar to previous section. Samples were separated by 7% SDS-PAGE and analyzed by immunoblotting with antibodies directed against DTX3L, AIP4, Parkin and ubiquitin.

**STATISTICAL ANALYSIS AND FINAL FIGURE PREPARATION**

Data were analyzed by Student's *t*-test, one-way analysis of variance (ANOVA) using GraphPad Prism 4.0 for Macintosh (GraphPad Software, San Diego, CA). Figures were created using Adobe® Illustrator CS4.
### Table 2.1 List of Primary Antibodies used in this project

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**Table 2.2 List of reagents used in this dissertation work**

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<td>X-ray Film, Classic blue autoradiography film BX</td>
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<tr>
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<td>PI-95000</td>
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<td>PI-2000</td>
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### Table 2.4 List of siRNA used for this dissertation work

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<td>Malik <em>et al.</em>, 2011</td>
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<td>Bhandari <em>et al.</em>, 2007</td>
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## DTX3L constructs

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## Other constructs

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### Table 2.6 Primers used to generate constructs for this dissertation

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Table 2.7 Restriction enzymes used for cloning in this dissertation
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<td>GST-DTX3L-CT</td>
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<td>GST-STAM-1</td>
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CHAPTER III

RESULTS

ROLE OF DTX3L IN CXCR4 DEGRADATION

Endosomal sorting of the chemokine receptor 4 (CXCR4) into the degradative pathway is important for controlling both the duration and magnitude of CXCR4 signaling (Malik, Soh, Trejo, & Marchese, 2012; Marchese et al., 2003). Ubiquitin is known to regulate CXCR4 sorting into the degradative pathway, however, the precise mechanisms by which this occurs remains poorly understood (Bhandari, Trejo, Benovic, & Marchese, 2007; Malik & Marchese, 2010; Marchese & Benovic, 2001; Marchese et al., 2003). Agonist activated CXCR4 is rapidly ubiquitinated at the plasma membrane by the E3 ligase AIP4 and once internalized onto endosomes is sorted towards lysosomes by the endosomal sorting complex required for transport (ESCRT) machinery. AIP4 also ubiquitinates ESCRT-0, negatively impacting its sorting function to control the amount of CXCR4 that is targeted for lysosomal degradation (Malik & Marchese, 2010; Marchese et al., 2003). These data indicate that AIP4 is a central player in governing CXCR4 degradation and yet the mechanism regulating AIP4 remains poorly understood.

Given previous literature that suggest that AIP4 can interact with RING domain E3 ligases including Cbl-c and deltex-1 (DTX1), we tested the hypothesis that the DTX and/or Cbl ligases are involved in CXCR4 trafficking (Chastagner, Israel, & Brou, 2006; Courbard et al., 2002). Initially, we performed a preliminary small interfering RNA (siRNA) screen to determine the role of deltex (DTX) and Casita B-lineage Lymphoma (Cbl) proteins in regulating agonist promoted degradation of CXCR4. To determine this
we examined agonist promoted degradation of endogenous CXCR4 in HeLa cells by immunoblot analysis, similar to previously described (Bhandari et al., 2007; Malik & Marchese, 2010; Marchese et al., 2003). HeLa cells were transfected with siRNA against control, AIP4, DTX1-3, DTX3L, c-Cbl or Cbl-b. Twenty-four hours later cells were split into single wells of a 24 well plate and grown overnight at 37°C. The following day, cells were treated with vehicle (0.1% BSA + PBS) or 10 nM CXCL12 for 3 h in the presence of the protein synthesis inhibitor cyclohexamide. Cyclohexamide is used in order to prevent de novo CXCR4 synthesis during the 3 h treatment period. Cells were harvested in 2x sample buffer and subjected to SDS-PAGE followed by immunoblot analysis for CXCR4. As shown in Figure 3.1, siRNA against AIP4 and DTX3L results in depletion of AIP4 and DTX3L, respectively. Similar to our previously published results, siRNA mediated knockdown of AIP4 significantly attenuates CXCR4 agonist-promoted degradation when compared to control (Marchese et al., 2003). (Figures 3.1, 3.2 and 3.3). Additionally, agonist-promoted degradation of CXCR4 was significantly inhibited in cells transfected with siRNA against DTX3L as compared to cells transfected with control siRNA (Figure 3.1).
Figure 3.1 DTX3L regulates CXCR4 degradation. A. HeLa were transiently transfected with control (Ctrl), AIP4 or DTX3L siRNA and treated with vehicle (0.1% BSA + PBS) or 10 nM CXCL12 for 3 h. Whole cell lysates were collected and analyzed for the level of endogenous CXCR4, AIP4, DTX3L or actin by immunoblot (IB) analysis. B. The percent of CXCR4 levels normalized to actin were determined by densitometric analysis. Data are representative of the average percent of CXCR4 degradation following CXCL12 treatment from 8 independent experiments. Data were analyzed by one-way ANOVA (p<0.001), followed by Bonferroni’s post-hoc test for multiple comparison amongst the groups. Compared to control siRNA-treated cells, AIP4 (p<0.001) and DTX3L (p<0.001) siRNA depletion significantly attenuated CXCR4 degradation.

In contrast, depletion of Cbl and DTX1-3 proteins had no effect on CXCR4 degradation (Figures 3.2 and 3.3). Although no phenotype was displayed, we were unable to determine knockdown of DTX1-4 and Cbl proteins due to lack of suitable reagents. Future studies will be required to confirm knockdown either of protein or mRNA by immunoblot or qPCR analysis, respectively. Overall, these data suggest a novel role for the DTX protein DTX3L in mediating CXCR4 degradation.
Figure 3.2 Cbl proteins in CXCR4 degradation. A. HeLa were transiently transfected with Ctrl, AIP4, c-Cbl or Cbl-b siRNA and treated with vehicle or 10 nM CXCL12 for 3 h. Whole cell lysates were collected and analyzed for the level of endogenous CXCR4 and tubulin by IB analysis. B. The percent of CXCR4 levels normalized to actin were determined by densitomeric analysis. Data are representative of the average percent of CXCR4 degradation following CXCL12 treatment from two independent experiments. Data were quantified by densitomeric analysis and analyzed by one-way ANOVA (**p = 0.0067) followed by Bonferroni’s post-hoc test. Error bars represent the standard deviation.

Figure 3.3 DTX proteins in CXCR4 degradation.

A. HeLa were transiently transfected with Ctrl, AIP4, DTX3L or DTX1-3 siRNA and treated with vehicle or 10 nM CXCL12 for 3 h. Whole cell lysates were collected and analyzed for the level of endogenous CXCR4 and tubulin by IB analysis. B. The percent of CXCR4 levels normalized to actin were determined by densitometric analysis. Data are representative of the average percent of CXCR4 degradation following CXCL12 treatment. Data were quantified by densitometric analysis and analyzed by one-way ANOVA.
(***p = 0.0004). Data represent three independent experiments for Ctrl, AIP4 and DTX3L or two independent experiments for DTX1-3. Error bars represent the standard deviation.

CXCR4 ACTIVATION PROMOTES DTX3L DISTRIBUTION TO THE SURFACE OF EARLY ENDOSONES

Given the novel role identified for DTX3L in regulating CXCR4 degradation, we next assessed the subcellular distribution of endogenous DTX3L in HeLa cells following CXCL12 treatment. To determine whether CXCL12 could regulate DTX3L localization to either early endosomes or late endosomes/lysosomes, we employed fixed cell confocal immunofluorescence microscopy. HeLa cells plated onto coverslips were serum starved for 3 h and treated with CXCL12 or vehicle for 30 min. Cells were then co-stained for endogenous DTX3L along with early endosomal and lysosomal markers EEA1 and LAMP2, respectively. As shown in Figure 3.4, endogenous DTX3L staining appears mostly diffuse in vehicle-treated cells with some punctate staining that co-localizes little with the early endosomal marker EEA1 and the lysosomal marker LAMP2 (Figure 3.4C). In contrast, upon CXCL12 treatment, DTX3L puncta were significantly increased (Figures 3.4A and B). Additionally, in contrast to vehicle, CXCL12 treatment significantly increased the degree of co-localization of DTX3L with EEA1-positive early endosomes (Figures 3.4A and C) compared to control whereas DTX3L co-localization to LAMP2-positive lysosomes was only modestly increased (Figures 3.4A and D).
Figure 3.4 CXCR4 activation promotes DTX3L distribution on early endosomes. A. HeLa cells grown on coverslips were serum starved for 3hrs. Media was replaced with media containing vehicle or 10 nM CXCL12 for 30 min placed at 37°C. HeLa cells were treated with vehicle or 10 nM CXCL12 for 30 min.
Cells were fixed, permeabilized and immunostained for endogenous DTX3L and EEA1 or LAMP2. Yellow in the “merge” panel indicates co-localization between DTX3L and EEA1 or LAMP2. Differential interference (DIC) contrast images are shown. B. Quantification of DTX3L puncta in vehicle or CXCL12 treated cells using the ImageJ Function “Analyze Particles.” Threshold was set at a minimum of 130 and maximum of 255. CXCR4 activation significantly increases DTX3L puncta. C,D. Quantification of average co-localization between DTX3L and EEA1 (C) or LAMP2 (D) using the Pearson product moment correlation co-efficient. Correlation was determined using the ImageJ plug-in “Colocalization Finder.” Error bars represent the standard error of the mean. Data are representative of four independent experiments and analyzed by a Student’s t test.

**DTX3L REGULATES CXCR4 TRAFFICKING**

Because CXCR4 is sorted for degradation by trafficking from early endosomes to lysosomes (Bhandari et al., 2007; Marchese & Benovic, 2001; Marchese et al., 2003) and since CXCL12 treatment promotes DTX3L localization on to early endosomes (Figures 3.4A and C), we next sought to determine whether DTX3L regulates the trafficking of CXCR4 from early endosomes to lysosomes. In order to address this question, we used confocal immunofluorescence microscopy to monitor the distribution of CXCR4 in HeLa transiently transfected with control or DTX3L siRNA. Images were acquired under equal acquisition settings between parallel experiments as detailed in Methods Section 2.7. In control siRNA cells, treatment with CXCL12 significantly increased CXCR4 localization to LAMP2-labeled lysosomes with little CXCR4 co-localizing to the early endosomal marker EEA1 (Figures 3.5A and 3.6A) indicating that CXCR4 has trafficked from early endosome to lysosomes. In contrast to control, depletion of DTX3L significantly
increased the amount CXCR4 puncta (Figures 3.5A and 3.6B) and the co-localization of CXCR4 with EEA1-positive early endosomes (Figures 3.5A and 3.6A), indicating that CXCR4 sorting to lysosome is impaired. Overall, these data indicate that DTX3L is important in mediating CXCR4 sorting from early endosomes to lysosomes.

Figure 3.5 DTX3L promotes CXCR4 endosomal to lysosomal degradation. A. HeLa cells grown on coverslips transiently transfected with either control or DTX3L siRNA were serum starved for 3hrs. Media was replaced with media containing vehicle (PBS + 0.1% BSA) or 10 nM CXCL12 for 30 min placed at 37°C. Cells were fixed, permeabilized and co-stained for endogenous CXCR4, DTX3L and EEA1 or LAMP2. CXCR4 is shown in green, DTX3L is shown in blue and EEA1 or LAMP2 are shown in red. Yellow puncta in the “merge” image indicate co-localization between CXCR4 and EEA1 or LAMP2. Images represent four independent experiments, 45-60 cells. Differential interference (DIC) contrast images are shown.
Figure 3.6 DTX3L is required for CXCR4 localization to and degradation in lysosomes. A. Quantification of average co-localization between CXCR4 and EEA1 or LAMP2 using the Pearson product moment correlation co-efficient. Correlation was determined using the ImageJ plug-in “Colocalization Finder.” Data were analyzed by one-way ANOVA (p<0.001), followed by Bonferroni’s post-hoc test. B. Quantification of CXCR4 puncta in control and DTX3L siRNA-treated cells. Puncta were analyzed using the ImageJ function “Analyze Particles.” Data are representative of the average puncta from four independent experiments, 45-60 cells. Threshold was set at a minimum of 130 and maximum of 255. Error bars represent the standard error of the mean. Data were analyzed by a Student’s t test.

AIP4 AND DTX3L DIRECTLY INTERACT

Since DTX3L not only co-localizes with CXCR4 upon activation, but also regulates the extent to which CXCR4 degrades, we next wanted to determine the mechanism by which DTX3L controls CXCR4 endosomal sorting. Given the role of DTX3L in CXCR4 degradation (Figures 3.1 and 3.5) we hypothesize that DTX3L may interact with key CXCR4 regulators, including AIP4 and ESCRT-0. In particular, prior
studies have shown that AIP4 interacts with other E3 ligases including DTX1 and Cbl-c (Chastagner et al., 2006; Courbard et al., 2002). Therefore, we tested whether DTX3L could interact with AIP4 and the ESCRT-0 subunits, HRS and STAM-1 by co-immunoprecipitation. We immunoprecipitated endogenous DTX3L from HeLa cells and assessed whether endogenous AIP4 or ESCRT-0 were present in the immunoprecipitates by immunoblot analysis. As seen in Figure 3.7A, endogenous AIP4 and ESCRT-0 subunits, HRS and STAM-1 immunoprecipitated with DTX3L, but not immunoglobulin G (IgG), used here as an isotype control. These data indicate that DTX3L can interact with AIP4 and ESCRT-0, suggesting that potentially through these interactions DTX3L may regulate endosomal sorting of CXCR4.

In order to determine the mechanism by which DTX3L controls CXCR4 endosomal sorting, we first focused on the interaction between AIP4 and DTX3L. First, we determined whether AIP4 and DTX3L directly interact by performing binding experiments using purified proteins. As shown in Figure 3.7B, recombinant His-DTX3L interacts directly with recombinant GST-AIP4 in a concentration-dependent manner. Second, we tested whether the interaction of AIP4 and DTX3L is promoted by CXCL12 treatment. HeLa cells were treated with or without CXCL12 for various times (0 - 60 min) and endogenous DTX3L was immunoprecipitated, followed by immunoblot analysis using an anti-AIP4 antibody. As shown in Figure 3.7C, AIP4 co-immunoprecipitates with DTX3L at all time-points, with a significant increase in this interaction at 15 min.
Figure 3.7 AIP4 and DTX3L directly interact. 

A. HeLa cells lysates (500 µg) were subject to immunoprecipitation for endogenous DTX3L or immunoglobulin G (IgG) isotype control and co-immunoprecipitation of proteins was determined by immunoblot analysis. Data represent three independent experiments. 

B. Equimolar amounts of purified recombinant GST-AIP4 (100 nmols) were incubated with increasing concentrations of His-DTX3L (1-10 nM). Samples were analyzed by 10% SDS-PAGE and immunoblotting for DTX3L and GST. Bands in the GST-AIP4 purification lower than 100 kDa represent degradation products from the purification. Data were quantified as the fold change in binding to AIP4 using densitometric analysis. Specifically, average values were subtracted from the GST only values and then normalized to the 1 nM His-DTX3L sample. Data show a significant increase in His-DTX3L to AIP4 with increasing concentration. Data represent three independent experiments. Data were analyzed by one-way ANOVA, followed by Bonferroni’s post-hoc test. 

C. HeLa cells were serum starved for 3 h, followed by treatment with 10 nM CXCL12 for 0-60 min. Cleared lysates (500 µg) were subject to immunoprecipitation using the goat polyclonal anti-DTX3L and isotype control antibodies.
Immunoprecipitates and inputs were resolved by SDS-PAGE and immunoblotting to detect endogenous AIP4, DTX3L or actin. AIP4 binding to DTX3L was significantly increased at 15 min of CXCL12 treatment. Immunoblots from four independent experiments were subject to densitometric analysis and the bar graph represents average AIP4 binding. Data were analyzed by one-way ANOVA followed by Bonferroni’s post-hoc test (p<0.05). Error bars represent the standard error of the mean.

Third, we examined the localization of endogenous DTX3L and FLAG-AIP4 upon CXCR4 activation by fixed cell confocal immunofluorescence microscopy. Images were acquired under equal acquisition settings between parallel experiments as detailed in Methods Section 2.7. Overexpression of AIP4 was required in these experiments due to a lack of suitable antibodies directed against AIP4 that could detect endogenous AIP4 by confocal microscopy. As seen in Figure 3.8, FLAG-AIP4 and DTX3L co-localize upon CXCL12 treatment. Pearson analysis for co-localization reveals that AIP4 and DTX3L significantly co-localizes at 15 - 60 min time points compared to cells treated with vehicle (Figure 3.8B). This co-localization persisted up to the 60 min at which time point there was a slight reduction in DTX3L and AIP4 co-localization (Figures 3.8A and B). Additionally, the average amount of FLAG-AIP4 puncta increased upon CXCL12 treatment and decreased back towards vehicle by 60 min time point. (Figure 3.8C). In contrast, DTX3L puncta levels increased upon CXCL12 treatment and persisted above vehicle levels up to the 60 min time point (Figures 3.8D). The return of AIP4 puncta to baseline at 60 min may be due to AIP4 cytosolic re-distribution rather than degradation given that AIP4 levels in inputs at 60 min are not reduced (Figure 3.7C). Altogether these
data indicate that DTX3L and AIP4 directly interact and CXCR4 activation promotes this interaction.
A.  

Vehicle

15 min

CXCL12

30 min

60 min

B.  

FLAG-AIP4 and DTX3L colocalization

Pearson Coefficient

p < 0.001

C.  

FLAG-AIP4 Puncta

Puncta per cell

p < 0.0001

D.  

DTX3L Puncta

Puncta per cell

p < 0.01

p < 0.05

E.  

WCL

siRNA: Ctrl AIP4

AIP4

Actin

IB
Figure 3.8 CXCL12 promotes DTX3L co-localization with AIP4. A. HeLa transfected with 1 µg FLAG-AIP4 were serum starved for 3 h and stimulated with vehicle or 10 nM CXCL12 for 15-60 min. Cells were fixed, permeabilized and co-stained for FLAG epitope, endogenous DTX3L and EEA1. FLAG-AIP4 is shown in green, DTX3L is shown in red and EEA1 in blue. White puncta in the “merge” image represent co-localization between AIP4, DTX3L and EEA1. Differential interference (DIC) contrast images are shown. B. Quantification of average co-localization between DTX3L and FLAG-AIP4 using the Pearson product moment correlation co-efficient. Correlation was determined using the ImageJ plug-in “Colocalization Finder.” Data were analyzed by a Student’s t test and error bars represent the standard error of the mean. (C, D). Quantification of FLAG-AIP4 (C) and DTX3L (D) puncta. Puncta were analyzed using the ImageJ function “Analyze Particles.” Threshold was set at a minimum of 130 and maximum of 255. Data are representative of the average puncta from four independent. Error bars represent the standard error of the mean. Data were analyzed by a Student’s t test. E. Immunoblot depicting the level of FLAG-AIP4 overexpression in lysates from IF in (A).

MAPPING THE AIP4 BINDING SITE ON DTX3L

We have shown so far that DTX3L regulates the lysosomal degradation of CXCR4 at the level of the endosome and that DTX3L interacts directly with AIP4. To gain insight into the role of DTX3L interaction with AIP4 on CXCR4 trafficking, we performed interaction studies to determine the binding regions. First, we performed GST-pull down experiments using GST-AIP4 and HeLa cell lysates endogenously expressing DTX3L. Endogenous DTX3L is able to bind to full-length recombinant GST-AIP4, but not GST-only control (Figure 3.9A).

In order to determine the binding region through which DTX3L binds to AIP4, we generated GST-fusion proteins of the N-terminal (NT) and C-terminal (CT) regions of
DTX3L based on the DTX3L truncation mutants previously characterized by Takeyama et al. 2003. The NT truncation of DTX3L spans amino acids 1-464 of the unique region, while the CT truncation covers amino acids 528-740 which includes the CT RING domain (Figure 3.9B). These truncation mutants were compared along with GST-DTX3L-Full length (FL) for binding to FLAG-AIP4 expressed in HeLa cells. As shown in Figure 3.9, FLAG-AIP4, but not empty vector binds strongly to GST-DTX3L-FL as compared to GST alone. In addition, FLAG-AIP4 binds to a lesser degree to the NT and CT DTX3L truncations when compared to FL DTX3L (Figure 3.9C). However, densitomeric analysis demonstrates that FLAG-AIP4 binds stronger to DTX3L-NT and weakly to the DTX3L-CT truncation (Figure 3.9B and D). Altogether these data demonstrate that DTX3L and AIP4 bind and that the N-terminal unique region of DTX3L binds strongly to AIP4.
Figure 3.9 DTX3L binds AIP4 via the N-terminal domain. A. Equimolar amounts (100 nmols) of immobilized GST or GST-AIP4 were incubated with HeLa lysates (500 µg). Binding of endogenous DTX3L was determined by immunoblot analysis. Ponceau staining of blot depicts the loading of GST and GST-AIP4 used in the binding reactions. B. Schematic representing the DTX3L truncation constructs used in binding experiments described in panels C and D. Summary of AIP4 binding to each truncation mutant is indicated. +++ = high binding; ++ = intermediate binding; + = low binding. C. Equimolar amounts (100 nmol) of immobilized GST, GST-DTX3L-Full length (FL), GST-DTX3L-N-term (NT) or GST-DTX3L-C-term (CT) were incubated with HeLa lysates (100 µg) transiently transfected with FLAG-AIP4 or empty vector control. Binding of FLAG-AIP4 was detected by immunoblotting with the anti-FLAG-HRP antibody. Amount of FLAG-AIP4 binding was determined by densitometric analysis and normalized to FL. Ponceau staining depicts the loading of the GST-fusion proteins. D. Shown are representative data analyzed from a total of three independent experiments. Data is represents fold AIP4 binding to each DTX3L
construct normalized to control (empty vector). Data were analyzed by one-way ANOVA and error bars represent the standard error of the mean.

**MAPPING THE DTX3L BINDING SITE ON AIP4**

In order to gain further insight into the interaction between DTX3L and AIP4, we next sought to determine the region on AIP4 that could be binding to DTX3L. In order to do this, we performed binding experiments using immobilized GST-AIP4-full length (FL) as well as several GST-AIP4 truncation mutants (Figure 3.10C). Equimolar amounts of the GST fusion proteins were incubated with HeLa lysates to determine binding to endogenous DTX3L. As shown in Figure 3.10, endogenous DTX3L bound strongly to GST-AIP4-FL and to a lesser extent to GST-C2 and GST-HECT truncations relative to GST only control. Little to no binding was detected for GST-WW I-IV samples. These data suggest that DTX3L is able to bind strongly to both the N-terminal C2 domain as well as the C-terminal HECT domain of AIP4 and potentially to regions containing the PRR domain. However, future experiments will need to be performed to determine importance of the two AIP4 binding regions in mediating the interaction with DTX3L.
Figure 3.10 AIP4 binding to DTX3L involves the C2 and HECT domains. A. Equimolar amounts (200 nmols) of immobilized GST, GST-AIP4, GST-C2, GST-WW I-IV or GST-HECT were incubated with His-DTX3L (5 nM) overnight. Binding of His-DTX3L was determined by immunoblotting with the anti-T7 monoclonal antibody. His-DTX3L binds strongly to GST-AIP4 and weaker to GST-C2 and GST-HECT. B. Data from two independent experiments were quantified by densitometry. Data represents fold DTX3L binding to each AIP4 construct as a fraction of control (GST). Error bars represent the standard deviation. D. Cartoon depicting the recombinant GST-AIP4 constructs used in binding reactions and summary of the relative binding of His-DTX3L to each construct denoted as follows: +++ = high binding; ++ = intermediate binding; + = low binding. Data represent two independent experiments.
DTX3L REGULATES ESCRT-0 UBQUITINATION

Previous work from our lab has shown that the extent to which CXCR4 is degraded is dependent on both CXCR4 and ESCRT-0 ubiquitination mediated by AIP4. Agonist-stimulation of CXCR4 promotes ubiquitination of both ESCRT-0 components, HRS and STAM-1 (Malik & Marchese, 2010; Marchese et al., 2003). Recent data from our lab has also highlighted that the interaction of STAM-1 with the adaptor arrestin-2 (a.k.a. β-arrestin-1) on early endosomes acts to potentially regulate the ubiquitination status and sorting function of HRS (Malik & Marchese, 2010). In this complex, Arrestin-2 is thought to recruit AIP4 to endosomes where it ubiquitinates HRS and, thereby, triggers a conformational change in HRS structure that prevents its sorting function with STAM-1. Importantly, CXCR4 degradation is enhanced when HRS ubiquitination is inhibited (Malik & Marchese, 2010). Essentially, the extent of CXCR4 degradation is dependent upon the ubiquitination status of ESCRT-0 (Malik & Marchese, 2010; Marchese et al., 2003). We sought to determine whether DTX3L also has an effect on ESCRT-0 ubiquitination. We examined the degree of ubiquitination of ESCRT-0 subunits HRS and STAM in HeLa cells following DTX3L or control siRNA depletion. As seen in Figure 3.11, in DTX3L siRNA-treated cells there was significantly increased amounts of ubiquitinated HRS (Figure 3.11A) and STAM-1 (Figure 3.11B) compared to control. There was also an increase in total ubiquitination in lysates treated with DTX3L siRNA compared to control, indicating that depletion of DTX3L may result in a global accumulation of ubiquitinated proteins and does not result in a reduction in the protein stability of either HRS or STAM-1. Based on these experiments this data suggest that
DTX3L negatively regulates the ubiquitination process. This is surprising given the role that ubiquitin ligases often have in promoting substrate ubiquitination. To the best of our knowledge, this is first study to show that depletion of a particular E3 ubiquitin ligase actually promotes global ubiquitination of substrates and opens the door to idea that E3 ubiquitin ligases may have both positive as well as negative roles in mediating ubiquitination. Taken together, these data suggest that DTX3L regulates CXCR4 degradation through regulation of ESCRT-0 ubiquitination.
Figure 3.11 DTX3L regulates ESCRT-0 ubiquitination and complex formation. A, B. HeLa cells were first transfected with control or DTX3L siRNA, followed by co-transfection with FLAG-ubiquitin and T7-HRS (A) or T7-STAM-1 (B). Lysates were collected under denaturing conditions and subject (300 µg) to immunoprecipitation using the anti-T7 polyclonal antibody. Immunoprecipitates and lysates were resolved by 7% SDS-PAGE and immunoblotted with the indicated antibodies. Data are representative of 3 independent experiments and were quantified by densitometric analysis. Asterisks (*) indicate HRS or STAM attached to a single or multi-mono ubiquitin. The amount of ubiquitination in control siRNA-treated cells was normalized to one and ubiquitination in DTX3L siRNA-treated cells was represented as a fold over control. Data were analyzed by a Student’s t test and error bars represent the standard error of the mean. Data were analyzed by a Student’s t test.

DTX3L REGULATES ESCRT-0 COMPLEX STABILITY

Previous studies have suggested that ubiquitination of HRS can regulate the ability of ESCRT-0 to form a stable complex on early endosomes due to the inability of HRS to adopt an active conformation and be recruited to early endosomes (Hoeller et al., 2006; Malik & Marchese, 2010; Marchese et al., 2003). Specifically, the sorting activity of HRS is predicted to be inhibited upon ubiquitination due to the adoption of an auto-inhibitory conformation in which the ubiquitin moiety on HRS binds to its own ubiquitin binding domain (UBD). This conformation inhibits HRS from binding to STAM-1 and, thus, prevents ESCRT-0 from sorting ubiquitinated cargo on the endosome (Hoeller et al., 2006). In support of this idea, recent data from our lab has demonstrated that when HRS poly-ubiquitination is inhibited, the amount of CXCR4 sorted to lysosomes is increased (Malik & Marchese, 2010). Having determined that DTX3L functions to regulate ESCRT-0 ubiquitination, we next examined the effect that DTX3L has on the
ability of HRS and STAM-1 to form an active sorting complex on early endosomes. In HeLa cells immunoprecipitated for endogenous HRS or STAM-1, DTX3L siRNA depletion resulted in a significant reduction in co-immunoprecipitation of STAM-1 or HRS, respectively, compared to control (Figures 3.12A and B). Therefore, DTX3L positively regulates ESCRT-0 complex integrity. Overall, these data indicate that DTX3L is an important regulator of ESCRT-0 ubiquitination and complex formation as means to regulate the extent of CXCR4 sorting into the degradative pathway.

**Figure 3.12** DTX3L regulates ESCRT-0 ubiquitination and complex formation. A, B. HeLa lysates transfected with control or DTX3L siRNA were subject to immunoprecipitation for endogenous HRS (A) or STAM-1 (B). Immunoprecipitates and lysates were subject to SDS-PAGE and immunoblotting for
indicated antibodies. Data represent 3-4 independent experiments. The amount of co-immunoprecipitation was determined by densitomeric analysis. Briefly, the amount of the associated protein was calculated as a fraction of the total immunoprecipitated protein for each respective siRNA sample. Average control values were normalized to one and then compared to DTX3L siRNA-treated samples. Data were analyzed by a Student’s t test.

**DTX3L PROMOTES ESCRT-0 ACTIVITY ON EARLY ENDOSONES**

Given that ubiquitin modification of ESCRT-0 may prevent the formation of active complex, we hypothesize that DTX3L through regulating the degree of ESCRT-0 ubiquitination could, therefore, also regulate the ability of ESCRT-0 to be recruited to endosomes (Hoeller et al., 2006; Marchese et al., 2003). In order to address this question, we used confocal immunofluorescence microscopy to monitor the distribution of HRS and STAM-1. HeLa cells grown on coverslips transiently transfected with either control or DTX3L siRNA were serum starved for 3h. Media was replaced with media containing vehicle (PBS + 0.1% BSA) or 10 nM CXCL12 for 30 min placed at 37˚C. Cells were then fixed, permeabilized and immunostained for DTX3L and HRS or STAM-1. Images were acquired under equal acquisition settings between parallel experiments as detailed in Methods Section 2.7. In control siRNA-treated cells, DTX3L co-localizes with both HRS (Figures 3.13A and 3.14B) and STAM-1 (Figures 3.13B and 3.14D). Furthermore, siRNA depletion of DTX3L resulted in a reduction in the level of both HRS (Figures 3.13A and 3.14A) and STAM-1 (Figures 3.13B and 3.14C) puncta compared to control. These data indicate that DTX3L modulates the ability of ESCRT-0 to localize onto endocytic structures following CXCR4 activation and, therefore, the degree of CXCR4
endosomal to lysosomal sorting into the degradative pathway. Taken together, these data provide mechanistic insight into DTX3L regulation of CXCR4 degradation through positively regulating ESCRT-0 complex formation and integrity.

Figure 3.13 DTX3L promotes ESCRT-0 aggregation on early endosomes. A, B. HeLa cells were transiently transfected with control or DTX3L siRNA. Cells were serum starved for 3hr and then stimulated
with 10 nM CXCL12 for 1 h and then fixed, permeabilized and co-stained for HRS (A) or STAM-1 (D) and DTX3L. HRS or STAM are shown in green and DTX3L is shown in red. Yellow in the “merge” image represents co-localization of HRS or STAM with DTX3L. Differential interference (DIC) contrast images are shown. Data represent 4 and 3 independent experiments, respectively. For HRS 110-145 cells were analyzed whereas 45-50 cells were analyzed for STAM-1. Data were analyzed by a Student’s t test.

Figure 3.14 DTX3L localizes with and regulates the aggregation of ESCRT-0 on endosomes. A, C. Quantification of the average HRS (A) and STAM-1 (C) puncta per cell using the ImageJ function “Analyze Particles.” Threshold was set at a minimum of 150 and maximum of 255. Data were analyzed by a Student’s t test and error bars represent the standard error of the mean. B, D. Quantification of average co-localization between DTX3L and HRS (B) or STAM-1 (D) using the Pearson product moment correlation co-efficient. Correlation was determined using the ImageJ plug-in “Colocalization Finder.”
represent 4 and 3 independent experiments, respectively. For HRS 110-145 cells were analyzed whereas 45-50 cells were analyzed for STAM-1. Data were analyzed by a Student’s t test.

PHOSPHATIDYLINOSITOL 3-PHOSPHATE IS NOT REGULATED BY DTX3L

The localization of ESCRT-0 to the surface of early endosomes is dependent partly on the interaction of the FYVE (Fab1, YOTB, Vac1 and early endosome antigen-1) -domain of HRS with phosphoinositide 3-phosphate (PI-3P), an endosomally enriched phospholipid localized to the cytosolic surface (Raiborg et al., 2001; Stenmark, Aasland, & Driscoll, 2002). The FYVE domain contains a cysteine rich zinc finger that binds directly with high selectivity to PI-3P compared to other phospholipids. The FYVE-domain is named after four proteins in which it was originally discovered: Fab1, YOTB, Vac1 and early endosome antigen-1 EEA1 (Burd & Emr, 1998; He et al., 2009). We explored the possibility that DTX3L modulates the steady state levels of PI-3P as a mechanism for controlling ESCRT-0 localization to endosomes. In order to determine this, we employed fixed cell confocal immunofluorescence microscopy in HeLa cells transfected with the fluorescent-tagged FYVE domain fusion (YFP-2xFYVE) construct as a means to monitor PI-3P levels similar to published approaches (Gillooly et al., 2000). In particular, we examined the effect DTX3L depletion has on YFP-2xFYVE localization to early endosomes following CXCR4 activation. Images were acquired under equal acquisition settings between parallel experiments as detailed in Methods Section 2.7. To control for binding to endosomes, we utilized wortmannin a potent inhibitor of phosphoinositide (PI) 3-kinase. Wortmannin is a non-selective PI-3K
inhibitor that disrupts the synthesis of PI-3Ps and, therefore, can disrupt the binding of proteins on endosomes that depend on the interaction with endosomally enriched PI-3Ps (Wymann et al., 1996).

In both control and DTX3L siRNA-treated cells, YFP-2xFYVE was highly co-localized to early endosomes following CXCL12 stimulation as determined by Pearson analysis (Figures 3.15A and 3.16B). However, pre-treatment of both control and DTX3L depleted cells with the PI-3P inhibitor, wortmannin, prevented YFP-2xFYVE localization to early endosomes. Additionally, there was a reduction in EEA1 staining upon wortmannin pre-treatment indicating a disruption of EEA1 binding to PI 3-P enriched endosomes (Figures 3.15A). This coincides with previously published literature that demonstrated that wortmannin treatment disrupts EEA1 binding to PI-3P membranes (Patki et al., 1997). Together, these data demonstrate that DTX3L ability to regulate the endosomal localization of ESCRT-0 is not due to a change in steady-state levels of endosomally localized PI-3P.
Figure 3.15 DTX3L does not reduce phosphatidylinositol 3-phosphate levels. A. HeLa cells were transiently co-transfected with YFP-2xFYVE and either control or DTX3L siRNA. Media was aspirated and cells were serum starved for 3h, followed by pre-treatment with either DMSO or wortmannin (100 nM) for 30 min prior to stimulation with CXCL12 (10 nM) for 1 h. Cells were fixed, permeabilized and co-stained for EEA1 and DTX3L. YFP-2xFYVE is in green, EEA1 is in red and DTX3L is in blue. White in the “merge” panel indicates co-localization between YFP-2xFYVE, EEA1 and DTX3L whereas yellow indicates co-localization between YFP-2xFYVE and EEA1. Differential interference (DIC) contrast images are shown.
Figure 3.16 YFP-FYVE puncta and localization to endosomes is reduced upon reduction in phosphatidylinositol 3-phosphate by wortmannin. A. Quantification of the average YFP-2xFYVE puncta per cell using the ImageJ Function “Analyze Particles.” Data represent 3 independent experiments, 35-40 cells. Threshold was set at a minimum of 130 and maximum of 255. Wortmannin treatment significantly prevents YFP-2xFYVE localization to early endosomes in both control and DTX3L siRNA-treated cells in contrast to DMSO control. Data were analyzed by one-way ANOVA (p < 0.0001) and error bars represent the standard error of the mean. B. Quantification of average co-localization between YFP-FYVE and EEA1 using the Pearson product moment correlation co-efficient. Correlation was determined using the ImageJ plug-in “Colocalization Finder.” Data represent 3 independent experiments. Data were analyzed by one-way ANOVA (p < 0.0001) and error bars represent the standard error of the mean.

DTX3L PREVENTS AIP4 SELF-UBIQUITINATION IN VITRO

Given that DTX3L and AIP4 interact directly and have opposing effects on ESCRT-0 ubiquitination (Figures 3.7 and 3.11) (Holleman & Marchese, 2014; Malik & Marchese, 2010; Marchese et al., 2003), we hypothesized that DTX3L may modulate AIP4 E3 ligase activity. In order to determine this, we performed in vitro ubiquitination assays using purified AIP4, His-DTX3L and their catalytically inactive mutants (AIP4-
C830A; His-3C/A). In general, E3 ubiquitin ligases including AIP4 and DTX3L are activated through self-ubiquitination (Takeyama et al., 2003; Scialpi et al., 2008). Thus, these reactions were designed to examine AIP4 and DTX3L self-ubiquitination alone and in combination. The catalytically inactive mutants are mutated within their active site HECT (AIP4-C830A) or RING (His-3C/A) domain and are unable to mediate direct or indirect transfer of ubiquitin to substrates (Chastagner et al., 2006; Marchese & Benovic, 2001). Specifically, the DTX3L-RING-3C/A mutant was designed based on the DTX1 mutant previously reported in which cysteine residues 561, 596 and 599 within the RING domain were mutated to alanine residues. This mutation prevents E2 enzyme mediated transfer of ubiquitin by disrupting the E2 binding site (Chastagner et al., 2006). Therefore, these ‘ligase-dead’ mutants serve as negative controls since they are unable to promote ubiquitination. Incubation of AIP4 and DTX3L alone results in self-ubiquitination, which is common among E3 ubiquitin ligases (Scialpi et al., 2008; Takeyama et al., 2003). Briefly, reactions were performed at room temperature for 1 h 30 min. Reaction conditions were based on previously described in vitro ubiquitin reactions and optimized by titrating amount of ligase used in the reaction as well as performing reactions at different temperatures (RT and 37°C) and/or times (15, 30 or 60 min) in order to determine optimal activity of either ligase (Laney & Hochstrasser, 2011). Ubiquitination of substrates is determined by immunoblot analysis and is represented by either the accumulation or smearing of higher molecular weight bands above the predicted molecular weight of the unmodified substrate as compared to input.
As seen in Figure 3.17, AIP4 self-ubiquitination is more robust than DTX3L with AIP4 accumulating higher molecular species (Figure 3.17, lane 6 vs. 8). In contrast, incubation of either catalytically inactive mutant (AIP4-C830A or His-3C/A) did not exhibit self-ubiquitination (Figure 3.17, lane 7 and 9). Combination of AIP4 and DTX3L resulted in a robust decrease in the level of AIP4 self-ubiquitination when compared to AIP4 alone, indicating that the co-incubation of DTX3L with AIP4 inhibits AIP4 self-ubiquitination (Figure 3.17, lane 10). A small decrease in DTX3L ubiquitination was also apparent in these reactions as seen in the DTX3L immunoblot (Figure 3.17, lane 10). In addition, co-incubation of AIP4 with the catalytically inactive mutant of DTX3L (DTX3L-3C/A) also resulted in a decrease in AIP4 self-ubiquitination demonstrating that the catalytic integrity of the DTX3L RING domain is not necessary to inhibit AIP4 self-ubiquitination (Figure 3.17, lane 11). In contrast, neither co-incubation of AIP4-C830A with DTX3L nor AIP4-C830A with DTX3L-3C/A resulted in AIP4-C830A ubiquitination due to the inability of AIP4-C830A mutant to load ubiquitin (Figure 3.17, lane 12 and 13). Thus, DTX3L-3C/A and AIP4-C830A mutants both act as negative controls since both are unable to mediate ubiquitination. Altogether, these data show that DTX3L, independent of the RING domain activity, inhibits AIP4 self-ubiquitination.
Figure 3.17 DTX3L inhibits AIP4 ligase activity. A. Ubiquitination reactions containing purified AIP4, AIP4-C830A, His-DTX3L or His-3C/A alone or in combination were incubated with ATP, E1, E2 (UbcH5c) and ubiquitin for 1 ½ h at room temperature in a total volume of 20 µl. Following completion of the reaction, samples were diluted with 20 µl of 2x sample buffer. Samples were resolved by 7% SDS-PAGE followed by immunoblotting for AIP4, DTX3L or ubiquitin. Asterisks indicate residual uncleaved, degraded GST-AIP4-C830A left in purified, cleaved sample. This was confirmed using an anti-GST antibody (unpublished results). Data represent six independent experiments. B. Ubiquitination reactions containing purified AIP4, His-DTX3L or MBP-Parkin alone or in combination were incubated with ATP, E1, E2 (UbcH5c) and ubiquitin for 1 ½ h at room temperature in a total volume of 20 µl. Reactions were terminated by the addition of 20 µl 2x sample buffer and samples were resolved by 7% SDS-PAGE. Samples were analyzed by immunoblot analysis for indicated proteins. Data represent four independent experiments.
To further confirm that the inhibition of AIP4 self-ubiquitination is specific to DTX3L acting upon AIP4 and not due simply to non-specific sequestration of ubiquitin by DTX3L, we examined the effect of the E3 ubiquitin ligase Parkin on AIP4 self-ubiquitination. Parkin belongs to the RING-between-RING (RBR) E3 ligases, which possess features of both HECT and RING domain ligases and are termed RING-HECT hybrids. In addition, Parkin shows self-ubiquitination activity with the E2 enzymes UbcH7 and UbcH5c (Wenzel, Lissounov, Brzovic, & Klevit, 2011). As shown in Figure 17B, co-incubation of AIP4 with Parkin robustly promoted AIP4 self-ubiquitination when compared to AIP4 alone (Figure 3.17B, lanes 5 vs. 9). As expected co-incubation of AIP4 and DTX3L reduced AIP4 self-ubiquitination (Figure 3.17B, lanes 5 vs. 8). Parkin co-incubation with AIP4 promoted a different pattern of AIP4 ubiquitination than seen with AIP4 alone indicating that either the presence of Parkin increases the ability of AIP4 to self-ubiquitinate or that Parkin may mediate AIP4 ubiquitination (Figure 3.17B, lane 5 vs. 9). Furthermore, DTX3L self-ubiquitination was robustly reduced in the presence of Parkin, whereas co-incubation with AIP4 slightly reduced DTX3L self-ubiquitination consistent with the previous panel (Figures 3.17B, lane 10 and 8, and 3.17A, lane 10). Moreover, co-incubation of AIP4 or DTX3L with Parkin did not prevent Parkin self-ubiquitination (Figure 3.18B, lane 9 and 10). These results indicate that the decrease in AIP4 ubiquitination in the presence of DTX3L in these reactions is specific. Although both DTX3L and Parkin belong to the RING-type ligase family, either ligase differentially regulates AIP4 self-ubiquitination in vitro. The importance of Parkin in
mediating differential changes in both AIP4 and DTX3L self-ubiquitination will remain to be investigated by future studies.

**DTX3L ANTAGONIZES AIP4-MEDIATED UBIQUITINATION OF HRS**

Given that DTX3L inhibits AIP4 self-ubiquitination *in vitro*, we next wanted to determine whether DTX3L could prevent AIP4 ligase activity toward its substrates. To this end, we examined the effect of DTX3L on inhibiting the ubiquitination of a known AIP4 substrate, HRS, in HeLa cells (Malik & Marchese, 2010; Marchese et al., 2003). HeLa cells were depleted of DTX3L, AIP4 or the combination and the level of HRS ubiquitination was determined by immunoprecipitation followed by immunoblot analysis. As seen in Figure 3.18, depletion of DTX3L results in hyper-ubiquitination of HRS confirming results shown in Figure 3.11A. In contrast, either AIP4 depletion alone or the combination of AIP4 and DTX3L siRNA resulted in a reduction in HRS ubiquitination. Although in these experiments we noted that AIP4 knockdown efficiency was not as great when co-transfected with DTX3L siRNA as seen with AIP4 knockdown alone. Nonetheless given that the degree of HRS hyper-ubiquitination was reduced in the combined lanes than with DTX3L knockdown alone, these data indicate that DTX3L counteracts AIP4 activity in cells namely the hyper-ubiquitination of HRS. Thus, we have defined that not only does DTX3L regulate CXCR4 endosomal to lysosomal degradation but also DTX3L does so by promoting ESCRT-0 activity while counteracting AIP4 activity on early endosomes.
Figure 3.18 DTX3L counteracts AIP4-mediated HRS ubiquitination. A. HeLa cells were first transfected with siRNA against control, DTX3L, AIP4 or the combination, followed by co-transfected with FLAG-ubiquitin and T7-HRS. Lysates were collected under denaturing conditions and subject (300 µg) to immunoprecipitation using the anti-T7 polyclonal antibody. Immunoprecipitates and lysates were resolved by 7% SDS-PAGE and immunoblotted with the indicated antibodies. Data are representative of 4 independent experiments.

DTX3L DOES NOT PREVENT AGONIST-PROMOTED CXCR4 UBIQUITINATION

AIP4 mediates CXCR4 agonist-promoted ubiquitination at the plasma membrane (Marchese et al., 2003). Given that DTX3L counteracts AIP4 mediated ubiquitination of HRS, we next assessed the effect DTX3L depletion has on AIP4 agonist-promoted
ubiquitination of CXCR4. To examine this, HeLa cells were depleted of DTX3L and the level of agonist-promoted ubiquitination of CXCR4 was determined by immunoprecipitation followed by immunoblot analysis. As shown in Figure 3.19, depletion of DTX3L did not prevent CXCR4 ubiquitination. The level of CXCR4 ubiquitination in DTX3L siRNA-treated cells was even greater than in control. These data indicate that DTX3L counters AIP4 agonist-promoted ubiquitination of CXCR4 such that depletion of DTX3L increases AIP4-mediated ubiquitination of CXCR4. Altogether, we have established that DTX3L is a *bona fide* inhibitor of AIP4 modification of CXCR4 and ESCRT-0 in the context of CXCR4 trafficking and degradation.
Figure 3.19 DTX3L does not prevent agonist-promoted CXCR4 ubiquitination. A. HeLa lysates were co-transfected with FLAG-ubiquitin and HA-CXCR4 plus control or DTX3L siRNA. Serum starved cells were treated with 10 nM CXCL12 for 30 min followed by immunoprecipitation with the anti-HA polyclonal antibody under denaturing conditions. Samples were resolved by 7% SDS-PAGE followed by immunoblotting for indicated proteins. Data represent 7 independent experiments.
CHAPTER IV

DISCUSSION

The findings in this current study have provided further mechanistic insight into the regulation of CXCR4 signaling and trafficking. Our study highlights a novel role for the E3 ubiquitin ligase DTX3L in regulating the extent of CXCR4 degradation. We demonstrate that silencing of DTX3L results in accumulation of CXCR4 on early endosomes preventing its lysosomal degradation. We also show that AIP4 interacts with the RING domain ligase Deltex-3L (DTX3L) upon CXCR4 activation. Furthermore, DTX3L interacts directly with AIP4 and inhibits AIP4 self-ubiquitination in vitro. Additionally, DTX3L interacts with the ESCRT-0 components HRS and STAM and regulates ESCRT-0 complex integrity. Our data show that DTX3L regulates AIP4-mediated ubiquitination of ESCRT-0 to regulate the extent of CXCR4 degradation (Holleman & Marchese, 2014). Altogether this study has provided conceptual advances in understanding both the regulation of CXCR4 signaling as well as expands upon current knowledge into GPCR regulation in general.

ROLE OF DTX3L IN CXCR4 DEGRADATION

Deltex-3-like (DTX3L; also known as B lymphoma and BAL associated protein, BBAP) belongs to the RING domain ligase family of Deltex proteins (Takeyama et al., 2003). While DTX1 and DTX2 have been widely studied as regulators of Notch
signaling, the function of DTX3 and DTX3L are less defined in the literature (Mazaleyrat et al., 2003; Takeyama et al., 2003). DTX3L was first identified in a subset of DLBCLs as a binding partner to the gene BAL (B aggressive lymphoma). The interaction of BAL with DTX3L enables BAL nuclear localization and is implicated in regulating the DNA damage response through DTX3L-mediated monoubiquitination of histone H4 (Yan et al., 2009; Yan et al., 2013). Here in this work, we have defined a novel role for DTX3L in regulating the membrane trafficking of CXCR4 (Figure 4.1). We show that siRNA-mediated knockdown of DTX3L significantly prevented CXCR4 degradation, revealing a novel function of DTX3L as a positive regulator of CXCR4 degradation (Figure 3.1). To our knowledge, this present study is the first to define a role for DTX3L in membrane trafficking of a GPCR.
**Figure 4.1 Proposed model for the role of DTX3L in CXCR4 trafficking.** Upon CXCR4 activation, endogenous DTX3L is recruited to early endosomes where it interacts directly with AIP4. On early endosomes, DTX3L antagonizes AIP4 ubiquitin ligase activity and ability to bridge to the ESCRT-0 subunits through binding to Arrestin-2. This limits the extent to which ESCRT-0 subunits (HRS and STAM-1) are ubiquitinated (green arrows). This promotes ESCRT-0 function on early endosomes such that it is able to interact with ubiquitinated CXCR4 and sort it for lysosomal degradation. Taken together our data are consistent with a model whereby DTX3L acts as an inhibitor of AIP4 activity to promote CXCR4 downregulation.
DTX3L REGULATES CXCR4 TRAFFICKING INTO THE DEGRADATIVE PATHWAY

Our study highlights that DTX3L subcellular distribution to early endosomes is influenced upon CXCR4 activation. The distribution of endogenous DTX3L in HeLa cells is mostly diffuse in the cytosol with few puncta aggregating on early endosomes and lysosomes (Figure 3.4). Following CXCR4 activation, DTX3L co-localization onto early endosomes increases significantly whereas DTX3L localization to lysosomes remains relatively unchanged (Figure 3.4A,C and D). The precise mechanisms governing DTX3L recruitment onto endosomes and lysosomes remain to be determined. Yet given the uncharacterized unique N-terminal region of DTX3L, we speculate that perhaps a non-canonical motif within this N-terminal region may be required for recruitment to endosomes and lysosomes. Alternatively, DTX3L through binding to such proteins as AIP4 or ESCRT-0 may act to recruit DTX3L to these structures.

Moreover, we have demonstrated that DTX3L functions to regulate the endosomal to lysosomal trafficking of CXCR4 since siRNA depletion of DTX3L increases the number of CXCR4 puncta that localize to early endosomes following 3 h of CXCL12 treatment in contrast to control (Figure 3.4A and B). Since DTX3L knockdown prevents CXCR4 degradation, we can speculate that in DTX3L CXCR4 may be trafficked through recycling pathways. Preliminary CXCR4 recycling experiments suggest that indeed DTX3L knockdown promotes CXCR4 recycling back to the plasma membrane. Additionally, the receptor that recycles back to the plasma membrane is able to evoke
agonist-promoted signaling responses. Overall the findings in this study and preliminary data suggest that DTX3L functions to promote ESCRT-dependent trafficking of CXCR4 into the degradative pathway and the removal of DTX3L antagonizes CXCR4 degradation in favor of recycling.

Given that a small subset of endogenous DTX3L does localize to early endosomes and lysosomes prior to CXCL12 stimulation, it is plausible that DTX3L may have additional roles in regulating membrane trafficking not exclusive to ESCRT-0. For instance, DTX3L may regulate the recruitment and ubiquitin modification of the other ESCRT proteins to facilitate the concentration of receptors into the limiting membrane of the endosome to form the MVBs. Preliminary binding experiments demonstrated that GST-DTX3L not only interacts with the endosomally localized ESCRT-0 components HRS and STAM-1 but in addition DTX3L can bind to the ESCRT-I component Tsg101, ESCRT-II component EAP45 as well as Arrestin-2 (β-Arrestin-1). Given that previous literature suggests that the STAM1/Arestin-2 complex may recruit AIP4 to endosomes where it can bind ESCRT-0 and ubiquitinate the subunit HRS, it is plausible that DTX3L may inhibit AIP4 from interacting with STAM-1/Arestin-2 to promote ESCRT-dependent targeting of CXCR4 for degradation (Malik & Marchese, 2010). Preliminary data demonstrate that DTX3L can in fact bind to Arestin-2 directly albeit at a lower affinity than that of DTX3L with AIP4 when compared in parallel binding experiments. Thus, DTX3L may antagonize AIP4 activity by inhibiting AIP4 interaction with STAM-1/Arestin-2 complex. DTX3L may play a general role in endosomal to lysosomal
targeting of other ESCRT-dependent GPCRs through general regulation of the ESCRT machinery or function of Arrestin-2. To this end, whether other GPCR ligands can promote increased DTX3L localization to endosomes remains to be determined.

INTERACTION OF DTX3L AND AIP4

Previously our lab demonstrated that siRNA mediated knockdown of AIP4 significantly inhibits CXCR4 degradation to a similar degree seen with DTX3L siRNA treatment in the current study (Marchese et al., 2003). Given the propensity of AIP4 to interact with RING domain ligases (Chastagner, Israel, & Brou, 2006; Courbard et al., 2002; Kitching et al., 2003), we found in the present study that AIP4 could interact with DTX3L by several complementary approaches (Figure 3.7-3.10). Domain mapping studies also reveal that the N-terminal unique region of DTX3L binds stronger than the C-terminal RING domain to AIP4. In addition, the C2 and HECT domains of AIP4 bind strongly to DTX3L. However, it will be important in future studies to further determine the precise amino acid binding regions between DTX3L and AIP4. This information could provide insight into creating a mini-gene to inhibit or mimic this interaction and help establish more precisely the importance of this interaction in cells.

Given that AIP4 belongs to the Nedd4 family of HECT domain ligases that are highly homologous within their HECT domain sequences, it is intriguing to speculate that DTX3L may bind to and regulate the ligase activity of other members of the Nedd4 family. In line with this, preliminary interaction studies reveal that DTX3L can bind to
the Nedd4 family members AIP2, AIP5 and Nedd4L in addition to AIP4 (data not shown). Future studies will be necessary to establish the general importance of DTX3L interactions with Nedd4 ligases.

**DTX3L PREVENTS AIP4 E3 LIGASE ACTIVITY**

Our lab has demonstrated that AIP4 spatially regulates CXCR4 both at the plasma membrane as well as on early endosomes. Given that DTX3L directly interacts and localizes onto early endosomes with AIP4, we hypothesized that the DTX3L and AIP4 interaction could regulate ligase activity. Indeed, we show that DTX3L inhibits AIP4 self-ubiquitination activity *in vitro* independent of its RING domain activity (Figure 3.17). We did not determine in our experiments the predominant ubiquitin linkage be formed on AIP4. However, based on previous literature which demonstrated that AIP4 self-ubiquitination reactions predominately form K63 poly-ubiquitin chains and that this had a non-degradative role in regulating AIP4 function, we can speculate that K63 poly-ubiquitination may be the predominate linkage form in our *in vitro* ubiquitin reactions (Scialpi et al., 2008). Overall, we have defined DTX3L as an inhibitor of AIP4 ubiquitin dependent self-activation.

The mechanism of this interaction is in contrast to previously published interactions of AIP4 with RING domain ligases whereby AIP4 could act as a positive or negative regulator of RING ligase activity. In the case of DTX1, it was shown that AIP4 could mediate K29-polyubiquitination of DTX1 leading to DTX1 lysosomal degradation.
(Chastagner et al., 2006). Therefore, it was concluded that AIP4 acts to inhibit DTX1 function in ligand-independent Notch signaling (Chastagner et al., 2006). Interestingly, DTX3L has also been shown to interact with DTX1, however, the function of this interaction in cells remains to be determined. The interaction of AIP4 with RING domain ligase Cbl-c was demonstrated to act synergistically to regulate ubiquitination and lysosomal degradation of the epidermal growth factor receptor (EGFR) (Courbard et al., 2002). To our knowledge, our study is the first to demonstrate that a RING ligase (DTX3L) is an inhibitor of HECT domain ligase (AIP4) self-ubiquitination. Whether the effect DTX3L has on AIP4 activity regulates other AIP4 substrates remains to be established. For instance, DTX3L by inhibiting AIP4 may also prevent AIP4-mediated ubiquitination of DTX1 in the context of Notch signaling or Cbl-c in the context of EGFR. Thus, the importance of DTX3L in other receptor pathways will be important to address.

**DTX3L ANTAGONIZES AIP4-MEDIATED UBIQUITINATION OF ESCRT-0**

It has previously been shown that CXCR4 is degraded via ESCRT dependent sorting on early endosomes. Following CXCR4 activation, the ESCRT-0 subunits HRS and STAM-1 are ubiquitinated by AIP4 (Malik & Marchese, 2010; Marchese et al., 2003). It was further defined that Arrestin-2 has a role in this AIP4-mediated ubiquitination of HRS. Specifically, AIP4 is recruited by the STAM-1/Arrestin-2 complex to endosomes and, thereby, ubiquitinates HRS. HRS ubiquitination regulates the
extent of receptor degradation. Importantly, recent work from our lab has demonstrated that CXCR4 sorting to lysosomes is enhanced when HRS ubiquitination is blocked (Malik & Marchese, 2010). However, the precise mechanism underlying CXCR4 ESCRT-dependent sorting remains poorly defined. In addition, previous studies have shown that the DUBs USP8 and AMSH indirectly promote the trafficking of CXCR4 by counteracting the AIP4-mediated ubiquitination of ESCRT-0 (Berlin, Higginbotham, Dise, Sierra, & Nash, 2010).

Our current study provides further insight into AIP4 mediated ubiquitination of ESCRT-0 and its implications in targeting CXCR4 for degradation. We show that DTX3L negatively regulates ESCRT-0 ubiquitination by antagonizing AIP4 (Figure 3.11; 4.2). We also show that DTX3L is also an important regulator of ESCRT-0 complex formation on early endosomes following CXCR4 activation (3.12-3.14). Taken into account previous studies, it could be the case that DTX3L also has effects on the function or localization of DUBs, like USP8 and AMSH, to facilitate CXCR4 downregulation. Future studies aimed at characterizing the effect DTX3L has on DUBs will be important to address. Furthermore whether DTX3L inhibits the ability of the STAM-1/Arrestin-2 to recruit AIP4 remains to be determined. Preliminary studies have revealed that DTX3L can bind to both Arrestin-2 and STAM-1. In addition, DTX3L binds to Arrestin-2 directly in a purified binding assay. Thus DTX3L not only counteracts AIP4 activity itself, but may also interact with the STAM-1/Arretin-2 complex preventing the interaction of AIP4 and, hence, promoting CXCR4 downregulation. This
idea will be important to address in the future. Altogether this current study expands our knowledge on the regulation of CXCR4 downregulation by demonstrating that DTX3L by antagonizing AIP4 function promotes ESCRT activity on early endosomes and, hence, sorting of CXCR4 for degradation (Figure 4.2).

Figure 4.2 Proposed model for DTX3L antagonism of AIP4-mediated ubiquitination of ESCRT-0. In model one (1), AIP4 undergoes self-ubiquitination through its interaction with its cognate E2 conjugating enzyme. This self-ubiquitination is required for AIP4 activation and results in the hyperubiquitination of ESCRT-0, which prevents CXCR4 lysosomal degradation. In model two (2), activation of DTX3L by its
cognate E2 promotes DTX3L binding to AIP4, thereby, preventing AIP4 E2-mediated self-ubiquitination. As a result, ESCRT-0 is moderately ubiquitinated and, therefore, functions to sort CXCR4 for lysosomal degradation.

Although ubiquitin modification of ESCRT-0 seems to regulate ESCRT-dependent sorting of CXCR4, the cellular relevance of ESCRT-0 ubiquitination in mediating receptor sorting remains to be confirmed. In yeast, fusion of ESCRT-0 or ESCRT-I to a DUB was shown to prevent their ubiquitination, but does not effect their sorting of ubiquitinated cargoes into the intraluminal vesicles (ILVs) (Stringer & Piper, 2011). These data demonstrate that receptor ubiquitination is required to promote ESCRT activity on endosomes leading to concentration of receptors into ILVs, however, ESCRT modification by ubiquitination is not necessary to the sorting process. Thus, ubiquitin modified cargos play an essential role in ILV formation and cooperation between ubiquitin cargo and ESCRTs help facilitate ILV formation (Shields & Piper, 2011). In contrast to yeast, in mammalian cells ESCRT ubiquitination has been proposed to play a role in receptor trafficking. CXCR4 trafficking for degradation is proposed to be dependent upon ubiquitination of ESCRT-0. AIP4 counteracts ESCRT-0 activity via poly-ubiquitination (Marchese et al., 2003). This is thought to displace HRS and STAM from early endosomes and perhaps result in an auto-inhibitory conformation. In this study we have further identified that the E3 ligase DTX3L antagonizes the effect AIP4 has on ESCRT-0 ubiquitination by preventing AIP4 self-ubiquitination activity (Holleman &
Marchese, 2014). Although there seems to be a clear effect on ESCRT-0 ubiquitination downstream of CXCR4 activation, whether this plays a direct role in receptor trafficking remains to be determined. Moreover, it has yet to be proven that poly-ubiquitination of HRS or STAM1 leads to either protein being placed in an auto-inhibitory conformation. This could be addressed by fusing either ESCRT-0 protein to a DUB similar to a published approach used in yeast (Stringer & Piper, 2011). Expression of a fusion of ESCRT-0 to a DUB in cells should remove any ubiquitin modification of the fusion and, therefore based on our study, should promote ESCRT-dependent downregulation opposite to that seen with DTX3L knockdown. This will be important to establish in future studies. Although previous data highlight that monoubiquitination of ESCRT-0 is predicted to place HRS in auto-inhibitory conformation, thereby, preventing HRS interaction with STAM1 and the ability to sort ubiquitinated receptors (Hoeller et al., 2006). Another recent study suggests that HRS ubiquitination also effects the extent by which the RTK receptor EGFR is degraded (Sun, Hedman, Tan, Schill, & Anderson, 2013).

One reason for this difference between yeast and mammalian cells may be due to that fact that mammalian cells have evolved to be more complex than yeast. For example, mammalian cells are more compartmentalized with mammalian cells requiring both endosomes and lysosomes to sort cargo, whereas in yeast cargos are sorted into the vacuole (the homolog to the mammalian lysosome). Another reason for the difference between yeast and mammalian cells could be due to the necessity of additional adaptor proteins in facilitating the downregulation of mammalian GPCRs. For example, the
downregulation of mammalian CXCR4 is dependent upon the adaptor protein Arrestin-2 (Bhandari, Trejo, Benovic, & Marchese, 2007; Malik & Marchese, 2010). Arrestin-2 can form a complex with STAM-1, negatively regulating the ESCRT-dependent downregulation CXCR4. Additionally, the STAM-1/Arrestin-2 complex binds and localizes AIP4 on early endosomes enabling AIP4 to mediate ubiquitination the ESCRT-0 component HRS, thereby, modulating ESCRT-0 function. Thus, Arrestin-2 may act to bridge AIP4 with ESCRT-0 to regulate the extent of ESCRT-0 ubiquitination and, hence, targeting of CXCR4 for degradation. Given the vast complexity of mammalian cells, it is not surprising that perhaps ESCRT ubiquitination may have a direct role on GPCR trafficking distinct from yeast.

**DTX3L NEGATIVELY REGULATES CXCR4 UBIQUITINATION**

It has been previously demonstrated that AIP4 mediates agonist-promoted ubiquitination of CXCR4 at the plasma membrane to facilitate downstream sorting and degradation (Marchese & Benovic, 2001; Marchese et al., 2003). In this study, we also demonstrate that silencing of DTX3L enhances both basal and agonist promoted CXCR4 ubiquitination (Figure 3.19). These data are consistent with the observation that DTX3L inhibits AIP4 ligase activity. Thus, DTX3L inhibition of AIP4 may not be restricted to early endosome although preliminary data demonstrate that DTX3L is not localized to the plasma membrane at early time points following CXCR4 activation (data not shown). Given that DTX3L and AIP4 complex basally and optimally at 15 min of CXCL12
treatment (Figures 3.7C and 3.8), it is possible DTX3L may sequester a fraction of AIP4 early on from mediating CXCR4 ubiquitination at the plasma membrane. Future studies will be required to determine whether this is indeed the case.

**SUMMARY AND FIGURE DIRECTIONS**

The findings of this present study are summarized in Figures 4.1 and 4.2. We have identified a novel role for the E3 ubiquitin ligase DTX3L in regulating CXCR4 degradation through inhibition of the activity of AIP4 on early endosomes. Our findings demonstrate that upon CXCR4 activation, DTX3L displays enhanced localization to early endosomes where it directly interacts with AIP4. Through its interaction with AIP4, DTX3L inhibits the ubiquitination of ESCRT-0 and, therefore, promotes ESCRT sorting function on early endosomes. We believe this mechanism serves to regulate the extent to which CXCR4 is sorted for degradation in lysosomes.

**DTX3L AS A GENERAL REGULATOR OF RECEPTOR DOWNREGULATION**

In this study we found that DTX3L regulates CXCR4 downregulation, however, whether DTX3L also regulates the ESCRT-dependent downregulation of other GPCRs or RTKs remains unknown. However, preliminary data suggest that DTX3L does not seem to regulate the ESCRT-dependent sorting of the receptor tyrosine kinase receptor EGFR (data not shown). Even though DTX3L may not have role in EGFR degradation, this could be due to the fact that EGFR sorting by ESCRTs is mediated by a different
mechanism. Recently, it has been shown that EGFR trafficking is dependent upon both Smad anchor for receptor activation (SARA) an endocytically enriched FYVE-domain containing protein as well as the RING domain ligase RNF11 (Kostaras et al., 2013). This study demonstrated that both SARA and RNF11 interact with ESCRT-0 to regulate the extent by which EGFR is degraded. In particular, SARA was shown to interact with clathrin and the ESCRT-I subunit Tsg101 similar to HRS indicating that SARA may function with or is a part of an alternative ESCRT-0 in the EGFR pathway. Whether SARA plays a role in GPCR trafficking remains to be studied. However to the best of our knowledge, the need for additional ESCRT adaptors seen for EGFR degradation such as Eps15b appears to be specific to EGFR. EGFR ubiquitination upon EGF stimulation is also dependent upon the RING ligase Cbl-c. Interestingly, AIP4 has been shown to act synergistically to promote Cbl-c mediated ubiquitination and degradation of EGFR (Courbard et al., 2002). Our study demonstrates that CXCL12 activation of CXCR4 promotes DTX3L localization to early endosomes where we believe that DTX3L inhibits AIP4 activity to promote ESCRT function. Although DTX3L does not regulate downregulation of EGFR, DTX3L localization to early endosomes and the effect on AIP4 as well as ESCRT function may be dependent on a specific a ligand (i.e. CXCL12) or additional yet to be identified ligands. Altogether, whether DTX3L plays a role in the ESCRT-dependent sorting of other GPCRs remains to be determined, however, it plausible to suggest that DTX3L may have a general role in GPCR sorting.
MECHANISM OF DTX3L INHIBITION OF AIP4 SELF-UBIQUITINATION

While this study demonstrates that DTX3L antagonizes AIP4 activity in the context of CXCR4 downregulation, the precise mechanism by which DTX3L prevents AIP4 self-ubiquitination remains unclear. Our data show DTX3L inhibition of AIP4 ubiquitination is independent of its ability to bind to the E2 since the RING mutant of DTX3L still prevents AIP4 ubiquitination in vitro (Figure 3.17). Moreover, interaction studies suggest that DTX3L binds to the HECT domain of AIP4 (Figure 3.10) and preliminary competition assays suggest DTX3L does not compete with E2 for binding to AIP4. Therefore based on these observations, we can speculate that DTX3L occludes E2-mediated transfer of ubiquitin to AIP4, but not the ability of the E2 to bind to AIP4. Additionally, it is conceivable that the inability of AIP4 to self-ubiquitinate in the presence of DTX3L may be due to an inability to adopt an active conformation structurally. To confirm this will require further and more rigorous study. Nonetheless, this study establishes DTX3L as a bona fide antagonist of AIP4 activity in the context of CXCR4 trafficking.

TARGETING THE DTX3L/AIP4 INTERACTION IN CXCR4 PATHOLOGIES

The findings from this study leave the possibility of further exploring the DTX3L and AIP4 interaction as a potential therapeutic option in treating CXCR4 pathologies. For instance, the progression of many cancers is correlated to a high expression of CXCR4. Specifically in HER2/neu positive breast cancers, CXCR4 levels are increased due in part
to inhibition of receptor ubiquitination and downregulation (Li et al., 2004). Overexpression of AIP4, but not the catalytically inactive mutant of AIP4 (C830A), could promote downregulation in these cancer cells. However, the status of DTX3L in the HER2/neu subtype of breast has yet to be determined. Based on this study, one strategy to promote AIP4 activity is to increase AIP4 levels in these cells to promote CXCR4 ubiquitination at the plasma membrane. Yet given the dual functions of AIP4 at both the plasma and early endosome, it would important to promote CXCR4 ubiquitination at the plasma membrane while also ensuring a level of endosomal downregulation. Based on our data if DTX3L is overexpressed in these cancers, it should prevent AIP4 activity on endosomes and, thereby, promote CXCR4 downregulation. However if DTX3L is expressed at a low levels, a combinational therapy that promotes a balance of AIP4 as well as DTX3L function may be efficacious. Therapies that could promote function could include gene therapy or delivery (i.e. PEGylation, PLGA microspheres, nanoparticle) of a drug that promotes activity. A recent study described the creation of small bicyclic peptides that could target the E2 binding site that is crucial for ubiquitin transfer within the HECT domains of Nedd4, Smurf2, WWP1 and Mule/Huwe1 (Mund, T. et al. 2014). This approach could inhibit the auto-ubiquitination of these ligases as well as prevent transfer of ubiquitin to substrates. An approach similar to this could be utilized to target the catalytic HECT domain of AIP4, however, it would be necessary to ensure that endosomally localized AIP4 would be specifically targeted in order to promote CXCR4 downregulation. More detailed analysis in the future will be
required to determine whether these strategies would be useful in order to promote CXCR4 downregulation in HER2/neu cancers.

Whether altered CXCR4 ubiquitination is implicated in other cancers where CXCR4 is overexpressed or whether this is specific to HER2/neu cancers remains to be investigated. Though one could speculate, given the importance of CXCR4 ubiquitination in CXCR4 endosomal to lysosomal downregulation, that defective CXCR4 ubiquitination could underlie other CXCR4 related pathologies. Additionally, a defect in ESCRT-dependent targeting of CXCR4 for degradation may promote increased CXCR4 expression. For instance, increased ubiquitination of ESCRT-0 by AIP4 prevents CXCR4 targeting for degradation and, thereby, may promote CXCR4 recycling (Malik & Marchese, 2010; Marchese et al., 2003). Thus, targeting key regulators in the CXCR4 degradative pathway, including those interactions defined in this dissertation, may prove efficacious in treating this subtype of breast cancer where CXCR4 is overexpressed.

In terms of hematological malignancies, both DTX3L and CXCR4 have prominent roles in DLCBLs. Analysis of the gene expression profile from the Brune Lymphoma Dataset (Oncomine database) reveals that the increased expression of DTX3L (fold change above 0) is correlated with a decrease in AIP4 levels (fold change below 0) in 10 out of 11 samples from DLBCLs (Brune et al., 2008). The reduction in AIP4 levels may be attributed to the DTX3L-mediated inhibition of AIP4. However, whether DTX3L also regulates AIP4 expression remains to be defined. Interestingly, in all of these samples HRS levels were increased (fold change between 0.79 – 1.81) while STAM-1
levels were elevated in 9 out of the 11 samples (fold change between $0.07 - 1.7$). The increase in both ESCRT-0 components HRS and STAM-1 seen in these DLBCLs could be correlated to high levels of DTX3L, which we believe promotes ESCRT-0 function. Consistent with our study, this data reveals that high expression of DTX3L may be correlated to increased ESCRT-0 activity possibly through a reduction in AIP4 levels. More variation is seen in the levels of CXCR4 in these DLBCLs samples ranging from a fold change between $-0.54$ to $0.63$. This variation may reflect differential functions of CXCR4 in DLBCLs subtypes.

A recent study has established that loss of CXCR4 expression is a prognostic marker for the development and progression of gastric extranodal DLBCLs originating from mucosa-associated lymphoid tissue (MALT) (Deutsch et al., 2013). While decrease expression of CXCR4 has been implicated in progression of gastric extranodal DLBCLs, an increase in CXCR4 has been associated with increased dissemination in *de novo* germinal-center B-cell-like (GCB)-DLBCLs (Chen et al., 2015). The latter study assessed CXCR4 expression in 743 patient biopsies. Another study demonstrated that in cell lines derived from 94 DLBCL biopsies, high levels of CXCR4 correlated to increased migration and increased engraftment as well as dissemination in a NOD/SCID xenograft mouse model (Moreno et al., 2015). Treatment of these mice with the CXCR4 antagonist AMD3100 significantly reduced dissemination. Thus, CXCR4 expression and role in progression varies between different DLBCLs sub-types.
Overexpression of DTX3L and B-aggressive lymphoma 1 (BAL1) are a risk factor for the host response (HR) subtype of DLBCLs signified by increased IFN-γ production and immune/inflammatory infiltrate (Juszczynski et al., 2006). In particular, DTX3L has been shown to bind to the nuclear protein BAL1. The DTX3L/BAL complex shuttles from the cytoplasm to nucleus where DTX3L mediates monoubiquitination of Histone H4 to modulate the DNA damage response (Yan et al., 2009). It will be interesting to establish within different subtypes of DLBCLs whether CXCR4 expression correlates to DTX3L levels. For example in line with our study, high levels of DTX3L should correlate to an overall reduction in CXCR4 levels due to increased receptor downregulation. Therefore, in DLBCL subtypes where low levels of CXCR4 drive disease progression, reducing DTX3L activity to promote CXCR4 recycling may prove advantageous. One strategy to reduce DTX3L levels therapeutically could be to prevent DTX3L interaction with AIP4 using a mini-gene that would mimic the minimal binding region on AIP4 to occlude DTX3L binding. Based on the present study by preventing DTX3L interaction with AIP4, this should prevent ESCRT-dependent sorting of CXCR4 for degradation and instead promote CXCR4 recycling and resensitization at the plasma membrane. Conversely, in DLBCLs where high levels of CXCR4 are implicated as a prognostic marker, promoting DTX3L activity by gene therapy to drive CXCR4 downregulation could be an ideal therapeutic strategy. Although our study did not focus on the role DTX3L may have on CXCR4-mediated signaling and migration, it will be important in future studies to establish whether DTX3L regulates these processes in
addition to receptor downregulation. Altogether understanding the molecular mechanisms regulating CXCR4 downregulation and signaling may provide insight into treating different sub-types of DLBCLs.

In addition to studying the therapeutic potential of targeting the DTX3L/AIP4 interaction in the context of CXCR4 related pathologies, future studies may highlight DTX3L as general regulator of ESCRT-dependent sorting of GPCRs (i.e. B₂AR, PAR2, DOR) providing further implications for studying DTX3L in the context of a therapeutic treatment. Given that preliminary data (not shown) demonstrate that DTX3L can interact with other Nedd4 E3 ligases in addition to AIP4 raises the possibility that DTX3L may regulate these ligases in the context of other GPCR trafficking pathways.

**E2 ENZYMES IN CXCR4 DOWNREGULATION**

Ubiquitination plays a major role in the lysosomal downregulation of CXCR4. It has been established here and in previous work that the E3 ligases AIP4 and DTX3L are key regulators of this process (Holleyman & Marchese, 2014; Marchese et al., 2003). AIP4 mediates agonist-induced ubiquitination of CXCR4 at the plasma membrane (Marchese et al., 2003). However on early endosomes, AIP4 acts to limit the extent of CXCR4 sorted for degradation by ubiquitin modification of ESCRT-0 subunits HRS and STAM-1 (Bhandari, Trejo, Benovic, & Marchese, 2007; Malik & Marchese, 2010; Marchese et al., 2003). Work from this dissertation has demonstrated that DTX3L acts to antagonize AIP4 activity on early endosomes to promote CXCR4 downregulation. Given that
ubiquitination of substrates is dependent upon three enzymes (E1, E2 and E3), whether specific E2 conjugating enzymes regulate CXCR4 degradation through their interactions with the E3 ligases AIP4 and DTX3L remains unknown. The function of the E2 enzyme is to link the ATP-dependent activation of ubiquitin by the E1 activating enzyme to the final covalent modification of protein substrates through the activities of E3 ubiquitin ligases. Based on phylogenetic analyses, the E2 enzymes are sub-grouped into 17 families (Wenzel, Stoll, & Klevit, 2011). In contrast, there are over 600 E3 ubiquitin ligases sub-grouped into the RING and HECT domain families (Metzger, Hristova, & Weissman, 2012; Metzger & Weissman, 2010; Pickart, 2001). Given the ratio of E2:E3 enzymes, it is often the case that one particular E3 ligase can work with several E2 enzymes and conversely one particular E2 can interact with several E3 ligases.

The role of specific E2 enzymes in conjunction with their E3 ligases in regulating GPCR downregulation remains unknown. Prior data on the downregulation of the RTK receptor EGFR demonstrated that the E2 enzymes UbcH5b and UbcH5c could regulate EGFR ubiquitin-dependent downregulation. This ubiquitination event is mediated through the interaction of UbcH5b and UbcH5c with the RING domain ligase Cbl-c (Umebayashi, Stenmark, & Yoshimori, 2008). This was the first study to our knowledge that provided insight into the specificity of E2 enzymes in the regulation of receptor trafficking. The precise role for specific subsets of E2 enzymes in GPCR regulation remains elusive.
Previous studies have demonstrated that DTX3L activity is dependent upon UbcH5 members (Takeyama et al., 2003) while AIP4 activity is mediated through either UbcH5 or UbcH7 (Kim & Huibregtse, 2009; Schwarz, Rosa, & Scheffner, 1998; Scialpi et al., 2008; Wenzel et al., 2011). Whether CXCR4 activation promotes the binding of particular E2 enzymes to either DTX3L or AIP4 remain to be determined. Future studies aimed at assessing the subcellular localization of these E2 enzymes with AIP4 and DTX3L upon CXCR4 activation can provide further mechanistic insight into E2 and E3 regulation of CXCR4 trafficking. Although previous literature has shown that UbcH5c can localize to both the plasma membrane as well as the early endosome with the E3 ligase Cbl-c following EGF stimulation (Umebayashi et al., 2008). Whether this localization pattern is true following CXCR4 activation remains to be determined. Though given the E2 specificities for DTX3L and AIP4 defined in the literature, we can speculate that UbcH5c and UbcH7 can localize to similar compartments as AIP4 and DTX3L such as the plasma membrane and early endosome. Understanding the particular E2:E3 interactions in the CXCR4 trafficking pathway may help determine differential specificities of these enzymes in regulating ubiquitination of CXCR4, ESCRT-0 as well as the self-ubiquitination of AIP4 and DTX3L, thereby, providing further mechanistic insight into CXCR4 ubiquitin dependent regulation.
ROLE OF UbcH5c IN DTX3L-MEDIATED INHIBITION OF AIP4 SELF UBIQUITINATION

It has been well documented that self-ubiquitination of some ubiquitin ligases like AIP4 is essential to their function and this may also be the case for DTX3L in CXCR4 trafficking (Scialpi et al., 2008). In particular, our in vitro ubiquitination assays utilized the E2 enzyme UbcH5c. Whether UbcH5c has a functional role in the cellular context in mediating DTX3L inhibition of AIP4 self-ubiquitination will be interesting to address in future study. One could speculate that UbcH5c may influence both the activity and subcellular distribution of either AIP4 or DTX3L to promote endosomal trafficking of CXCR4.

A recent study of the E3 ligase Parkin has demonstrated that specific E2 enzymes (UbcH5b, UbcH5c, UbcH7 and UbcH13) are important regulators of Parkin translocation from the cytoplasm to the mitochondria upon mitochondrial stress (Fiesel, Moussaud-Lamodiere, Ando, & Springer, 2014). Depletion of the identified E2 enzymes reduced Parkin localization to the damaged mitochondria and prevented Parkin mediated ubiquitination of substrates at the mitochondria. Additionally, in the Parkin study it was demonstrated that E2 enzymes UbcH5b, UbcH5c and UbcH7 function redundantly to activate and charge Parkin with ubiquitin. However, whether these E2 enzymes regulate Parkin stability and degradation was not assessed. Based on these studies, it is intriguing to speculate that particular E2 enzymes may also regulate the activation and localization of AIP4 and/or DTX3L within the CXCR4 degradation pathway. Yet given the broad role
of E2 enzymes in the ubiquitination process, whether we may see effects attributed directly or indirectly to AIP4-mediated ubiquitination may be one caveat.

CONCLUSION

Overall, this study provides increased knowledge on the ubiquitin-dependent regulation of CXCR4 signaling and downregulation (Figure 4.1). We have discovered the RING domain ligase DTX3L as a novel endogenous antagonist of AIP4 in the context of CXCR4 trafficking. This interaction may prove beneficial in developing strategies in modulating CXCR4 expression. Furthermore, the details of this study can be used in future studies aimed at creating potential therapies for CXCR4 pathologies, such as breast cancer and DLBCLs, and may even be broadly applicable to other GPCRs.
APPENDIX A:

BUFFER AND REAGENT RECIPES
Polyethylenimine (1mg/ml PEI)

In a 50 mL conical tube, dissolve 0.01 grams of PEI (Sigma-Aldrich) in 3 ml of 100% ethanol. Vortex solution and heat in 37°C water bath for 5 -10 min (vortex 2-3 times) until PEI is dissolved. Dilute PEI solution in 7 ml of DEPC treated (RNAase/DNAase free) H$_2$O (Invitrogen; final volume of 30% ethanol). In a biological safety cabinet, syringe filter the solution with a 0.2 µm filter and make 100 µl aliquots. Store aliquots at -80°C.

Short-duration ECL solution

<table>
<thead>
<tr>
<th>Solution 1</th>
<th>Solution 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 mM luminol</td>
<td>0.02% Hydrogen Peroxide</td>
</tr>
<tr>
<td>0.45 mM p-Coumaric Acid</td>
<td>0.1 M Tris pH 8.8</td>
</tr>
<tr>
<td>0.1 M Tris pH 8.8</td>
<td></td>
</tr>
</tbody>
</table>

To make Solution 1, dissolve 112.5 mg of luminol in 2.5 ml of DMSO. Dissolve 18.5 mg p-Coumaric acid in 1.25 ml DMSO. Add 0.1 M Tris-HCl pH 8.8 in final volume of 250 ml. To make Solution 2, combine 167 µl of Hydrogen peroxide to 250 ml of 0.1 M Tris-HCl pH 8.8. Store each solution at 4°C. To use, combine equal volumes of solution 1 and solution 2. Incubate with immunoblot for 1 min before exposure.

Protease Inhibitor Cocktail
Make 10 mg/ml stocks of aprotinin, leupeptin and pepstatin. To 50 mg of aprotinin and leupeptin, add 5 ml of dH₂O on ice and vortex. Make 1 mL aliquots. Add 1 mL DMSO to 10 mg of pepstatin. Aliquot 50 µl of each protease inhibitor into a sterile microcentrifuge tube. Store at -20°C.

**Acrylamide Running Gels**

<table>
<thead>
<tr>
<th>10 % Acrylamide Running Gel</th>
<th>Volume for 2 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, filtered dH₂O</td>
<td>1.54 ml</td>
</tr>
<tr>
<td>0.75 M Tris-HCl, pH 8.8</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>30 % Acrylamide</td>
<td>3.3 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>60 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>7 % Acrylamide Running Gel</th>
<th>Volume for 2 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, filtered dH₂O</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>0.75 M Tris-HCl, pH 8.8</td>
<td>5 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>30 % Acrylamide</td>
<td>2.33 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>60 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>12 % Acrylamide Running Gel</th>
<th>Volume for 2 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, filtered dH₂O</td>
<td>830 µl</td>
</tr>
<tr>
<td>0.75 M Tris-HCl, pH 8.8</td>
<td>5 ml</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>30 % Acrylamide</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>60 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>3 % Acrylamide Stacking Gel</th>
<th>Volume for 2 gels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile, filtered dH₂O</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>0.75 M Tris-HCl, pH 8.8</td>
<td>500 µl</td>
</tr>
<tr>
<td>10 % SDS</td>
<td>45 µl</td>
</tr>
<tr>
<td>30 % Acrylamide</td>
<td>400 µl</td>
</tr>
<tr>
<td>10% APS</td>
<td>50 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 µl</td>
</tr>
</tbody>
</table>
Mix together desired percentage of resolving acrylamide gel ingredients in a 50 ml conical tube (add Acrylamide, APS and TEMED last). Vortex and pipet into glass plates leaving about a ½ inch space from top of short plate. Fill remaining space with isopropanol. Let resolving gel solidify at room temperature for about 20 min. To pour the stacking gel, first rinse away isopropanol from resolving gel with dH$_2$O and then combine stacking ingredients in a 50 ml conical tube (add Acrylamide, APS and TEMED last). Vortex and pipet into remaining space between glass plates. Place comb of desired size (i.e. 10 sample or 15 sample comb) into stacking liquid. Let solidify at room temperature for about 10 min. To load gel, remove comb and place gel into running apparatus (Bio-Rad). Fill tank with 1× SDS Running Buffer prior to loading samples.

**SDS Running Buffer (10×)**

0.25 mM Tris Base

0.192 M Glycine

1% (w/v) SDS

dH$_2$O  4 L to final volume

To 3 L dH$_2$O, combine Tris Base and Glycine. Once dissolved, add SDS and dH$_2$O to 4 L final volume. To make 1× Running Buffer, dilute 400 ml of 10 X SDS Running Buffer with 3.6 L of dH$_2$O.

**Transfer Buffer (10×)**

0.25 mM Tris Base
0.192 M Glycine
dH₂O 4 L to final volume

To 3 L dH₂O, combine Tris Base and Glycine. Once dissolved, add dH₂O to 4 L final volume. To make 1×Transfer Buffer, combine 400 ml of 10 X Transfer Buffer with 800 ml of methanol (20% vol/vol) and 2.8 L of dH₂O. Store at 4°C.

**Western Blot Stripping Buffer**

10% SDS 200 ml
0.75 M Tris-HCl, pH 6.5 83.4 ml
14.3 M β-mercaptoethanol 7 ml
dH₂O 709.6 ml

Combine SDS, Tris HCl and dH₂O. Add β-mercaptoethanol in biological safety cabinet. Store at room temperature. To strip immunoblots, incubate immunoblots with ~100 ml of Stripping Buffer for 30 min at 60°C. After stripping, wash blots 10 X with d dH₂O. Block immunoblots with 5% TBST-milk for 30 min at room temperature and add primary overnight at 4°C. Develop blots as described above.

**0.75 M Tris-HCl pH 8.8 or 6.5**

0.75 M Tris Base 45.41 g
Concentrated HCl Drop-wise to desired pH
dH₂O 500 ml final volume
Stir together Tris Base with 300 ml dH₂O in glass beaker. Add pH monitor (calibrate if necessary). Once Tris Base is dissolved, add HCl drop-wise until desired pH obtained. Add dH₂O to final volume of 500 ml.

1 M Tris-HCl pH 7.5

1 M Tris Base 193.76 g
Concentrated HCl  Drop-wise to pH 7.5
dH₂O 1.6 L

In a 2 L beaker with a stir bar, dissolve Tris Base in 1.2 L of dH₂O. Add pH monitor to beaker (calibrate if necessary). Add HCl drop-wise until pH 7.5 is achieved. Bring solution to final volume of 1.6 L with dH₂O.

TBS-T (20X)

3 M NaCl 2.4 L
1 M Tris-HCl, pH 7.5 1.6 L
Tween 40 ml
dH₂O 4L final volume

In a 4 L beaker with a stir bar, combine NaCl, Tris-HCl and Tween in 4 L of dH₂O (final volume). Store at 4°C. To make 1× TBS-T, combine 200 ml 10× TBS-T with 3.8 L of dH₂O. Store at 4°C until use.
2× Sample Buffer (8% SDS)

8% SDS
10% glycerol
0.7 M β-mercaptoethanol
37.5 mM Tris-HCl, pH 6.5
0.003% bromophenol blue

To a 500 ml flask with a stir bar, combine SDS, Tris-HCl and glycerol. In a biological safety cabinet, add the β-mercaptoethanol. Lastly, add the bromophenol blue. Once dissolved, make aliquots and store at -20°C.

Co-immunoprecipitation Buffer

50 mM Tris-HCl
150 mM NaCl
2 mM EDTA
1% Triton-X 100
20 mM NEM
10 μg/ml Protease Inhibitor cocktail
dH₂O to final volume

Combine all ingredients except protease inhibitor cocktail. Place at 4°C and let NEM dissolve for 30 min. Then add protease inhibitor cocktail on ice before adding to cells.
**GST-fusion Purification Lysis Buffer**

50 mM Tris-HCl, pH 7.5

300 mM NaCl

1% Triton X 100

1 mM DTT

10 µg/ml Protease Inhibitor cocktail

dH₂O to final volume

Combine all ingredients except protease inhibitor cocktail. Store at 4°C. Then add protease inhibitor cocktail on ice before adding to bacterial pellets.

**His-fusion Purification Buffer**

50 mM Tris-HCl, pH 7.5

300 mM NaCl

1% Triton X 100

10 µg/ml Protease Inhibitor cocktail

dH₂O to final volume

Combine all ingredients except protease inhibitor cocktail. Store at 4°C. Then add protease inhibitor cocktail on ice before adding to bacterial pellets.

**Binding Buffer**
50 mM Tris-HCl, pH 7.5  
300 mM NaCl  
1% Triton X 100  
20 mM NEM  
10 µg/ml Protease Inhibitor cocktail  
dH₂O to final volume

Combine all ingredients except protease inhibitor cocktail. Place at 4°C and let NEM dissolve for 30 min. Then add protease inhibitor cocktail on ice before adding to cells.

**Ubiquitination Buffer**

150 mM NaCl  
50 mM Tris-HCl, pH 7.5  
1% SDS  
5 mM EDTA  
1% Triton X 100  
20 mM NEM  
10 µg/ml Protease Inhibitor cocktail  
dH₂O to final volume

Combine all ingredients except protease inhibitor cocktail. Place at 4°C and let NEM dissolve for 30 min. Then add protease inhibitor cocktail on ice before adding to cells.
Ubiquitin Dilution Buffer

150 mM NaCl
50 mM Tris-HCl, pH 7.5
5 mM EDTA
1% Triton X 100
dH₂O to final volume

Combine all ingredients and place at 4°C until use.

Immunofluorescence Reagents:

3.7% Paraformaldehyde (PFA) Fixing solution

Dilute 1 ml of 37 % PFA (Sigma-Aldrich) into 9 ml of cold, 1×PBS

Permeabilization Buffer

Combine 1×PBS plus 0.05% w/v saponin

Blocking Buffer

Combine 1×PBS plus 1% BSA and 0.05% w/v saponin
APPENDIX B:

PLASMID CONSTRUCT MAPS
Plasmid Name: FLAG-DTX3L-Full Length
Vector Backbone: p3xFLAG-CMV-10 (Product No. E4401; Sigma)
Insert Size: ~2.0 kb  Vector Size: 6.4 kb
Constructed by: Justine Holleman
Construction Date: 10-12-2011
Location: Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C
IMAGE clone ID: 4339554
GenBank No.: BC042191.1

Construction Details:
FLAG-Deltex-3-Like (DTX3L) was PCR amplified from pCMV-SPORT6-DTX3L template (Thermo Scientific). Primers used are listed below. Briefly, the forward primer carried a NotI restriction site and started at amino acid residue Ala2, in frame with the 3xFLAG epitope. The reverse primer carried a XbaI site and an in frame stop codon and ended at E740. The PCR fragment was digested with NotI and XbaI and cloned into the NotI and XbaI sites of p3xFLAG-CMV10.

DTX3L-pCMV10 F: 5’ATATGCGGCCGCG GCC TCC CAC CTG CGC CCG CCG TC NotI A S H L R P P
DTX3L-pCMV10 R: 5’ ATATTCTAGA TTA CTC AAT TCC TTT GGC TTT C XbaI stop E I G K A K
Plasmid Name: FLAG-DTX3L-C561A  
Vector Backbone: p3xFLAG-CMV-10 (Product No. E4401; Sigma)  
Insert Size: ~2.2 kb  
Vector Size: 6.4 kb  
Constructed by: Justine Holleman  
Construction Date: 03-12-2012  
Location: Mini Prep DNA Box 1 (4°C); Glycerol Stock -80C

Construction Details:
To generate the single mutant FLAG-DTX3L-C561A we first amplified FLAG-DTX3L template DNA using FLAG-DTX3L-Forward and FLAG-DTX3L-C561A-Reverse primer in one tube and FLAG-DTX3L-Reverse and FLAG-DTX3L-C561A Forward primer in tube two. The product of PCR reaction one was then used as a template to anneal the C561A mutant strands. The forward primer carried a NotI restriction site and started at amino acid residue Ala2, in frame with the 3xFLAG epitope. The reverse primer carried a XbaI site and an in frame stop codon and ended at E740. The PCR fragment was digested with NotI and XbaI and cloned into the NotI and XbaI sites of 3xFLAG-CMV10.

**DTX3L-C561A-F:** 5’ GAAGGAAAAGGGCATCGCTGTCATCTGTATGGACACC

**DTX3L-C561A-R:** 5’ GGTGTCCATACAGATGACAGCGATGCCCCTTTTCTTC

**DTX3L-pCMV10 R:** 5’ ATATTCTAGATTACTCAATTCTTTTGCTTTTC

**DTX3L-pCMV10 F:** 5’ ATATGGGGCCGGCGGCCCTCCACCTGTGCCCCGCCCCGTCC

[176]
Plasmid Name: FLAG-DTX3L-3C/A
Vector Backbone: p3xFLAG-CMV-10 (Product No. E4401; Sigma)
Insert Size: ~2.2 kb     Vector Size: 6.4 kb
Constructed by: Justine Holleman
Construction Date: 03-12-2012
Location: Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C

Construction Details:
FLAG-DTX3L-3C/A was PCR amplified using FLAG-DTX3L-C561A (single mutant) template DNA extended with DTX3L- C596/599A -Forward oligo in PCR reaction 1. Product of reaction 1 was then subjected to a second round of PCR using the DTX3L-C596/599A -Reverse/pcmv10-R in tube two. The product of PCR reaction one was used as a template to anneal the DTX3L-3C/A triple mutant strands and extended using pcmv10-DTX3L-Forward/Reverse primers. The forward primer carried a NotI restriction site and started at amino acid residue Ala2, in frame with the 3xFLAG epitope. The reverse primer carried a XbaI site and an in frame stop codon and ended at E740. The PCR fragment was digested with NotI and XbaI and cloned into the NotI and XbaI sites of 3xFLAG-CMV10.

DTX3L-C596/599A-Forward:
5’ CATGTCATATAAGCCAATCGCTCCACAGCCAGACTTCTATGCTATATC
  C596A   C599A

DTX3L-C596/599A-Reverse:
5’GAATACCATAGGAAGTCTGGCTGAGCGATTGGCTATATGACATG
  C599A   C596A

DTX3L-pCMV10 R: 5’ ATATTCTAGAATTACTCAATTCCTTTGGCTTT
  XbaI

DTX3L-pCMV10 F: 5’ATATGGCGGGCGGCCTCCACCTCGCCCGCCGTC
  NotI
Plasmid Name: GST-DTX3L
Vector Backbone: pGEX-6p-1
Insert Size: ~2 kb   Vector Size: 5 kb
Constructed by: Justine Holleman
Construction Date: 10-24-2011
Location: Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80°C

Construction Details:
GST-DTX3L was PCR amplified from pCMV-SPORT6-DTX3L template (Thermo). The forward primer carried a BamHI restriction site and started at amino acid residue Ala2, in frame with the pGEX6p1 epitope. The reverse primer carried a XhoI site and an in frame stop codon and ended at E740. The PCR fragment was digested with BamHI and XhoI and cloned into the BamHI and XbaI sites of pGEX6p1.

F: 5’-ATAT **GGA TCC** GCC TCC CAC CTG CGC CCG CCG TC
Bam HI

R: 5’-ATAT **CTC GAG** TTA CTC AAT TCC TTT GGC TTT C
XhoI

![Diagram of plasmid](image)
**Plasmid Name:** GST-DTX3L-N-term  
**Vector Backbone:** pGEX-6p-1  
**Insert Size:** ~1.68 kb  
**Vector Size:** 5 kb  
**Constructed by:** Justine Holleman  
**Construction Date:** 10-24-2011  
**Location:** Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C

**Construction Details:**
GST-DTX3L was PCR amplified from pCMV-SPORT6-DTX3L template (Thermo). The forward primer carried a BamHI restriction site and started at amino acid residue Ala2, in frame with the pGEX6p1 epitope. The reverse primer carried a XhoI site and an in frame stop codon and ended at I560. The PCR fragment was digested with BamHI and XhoI and cloned into the BamHI and XbaI sites of pGEX6p1.

**DTX3L-pGEX- F:** 5’ ATATGGATCCGCCCTCCACCTGCGCCCGCGCCGTTC  
**BamHI**

**DTX3L-Nterm-GEX- R:**  
5’ ATATCTCGAGTCAGATGCCCCCTTTTCTTCTTGCCAGTTCAAGC  
**XhoI**  
**STOP**
Plasmid Name: GST-DTX3L-N-term
Vector Backbone: pGEX-6p-1
Insert Size: ~0.6 kb  Vector Size: 5 kb
Constructed by: Justine Holleman
Construction Date: 10-24-2011
Location: Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C

Construction Details:
GST-DTX3L was PCR amplified from pCMV10- DTX3L template. The forward primer carried a BamHI restriction site and started at amino acid residue D529, in frame with the 3xFLAG epitope. The reverse primer carried a XhoI site and an in frame stop codon and ended at E740. The PCR fragment was digested with BamHI and XhoI and cloned into the BamHI and XbaI sites of pGEX6p1.

DTX3L-Cterm-GEX- F: 5’ ATATGGATCCGACATTTGATAGCGATGATTCC
BamHI

DTX3L-pGEX- R: 5’ ATATCTCGAGTTACTCAATTCCCTTGGCTTTCC
XhoI

![Diagram of plasmid with restriction sites]
Plasmid name: His-DTX3L
Vector Backbone: pET-21a(+) (Katherine Knight lab)
Insert size: ~2.1 kb        Vector size: 5.4 kb
Constructed by: Justine Holleman
Construction Date: 08-23-12
Location: Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C

Construction Details:
Full-length DTX3L was cloned into BamHI and XhoI sites pET-21a(+) vector using below oligos and FLAG-DTX3L as a template:

**DTX3L-pGEX- F:** 5' ATATGGATCC GCC TCC CAC CTG CGC CCG CCG TC

\[\text{BamHI}\]

**pET-21a-DTX3L- R:** 5' ATAT CTCGAG CTC AAT TCC TTT GGC TTT CAG CTC

\[\text{XhoI}\]

**pET-21a(+) cloning/expression region**
Plasmid name: His-DTX3L-3C/A
Vector Backbone: pET-21a(+) (Katherine Knight lab)
Insert size: ~2.1 kb  Vector size: 5.4 kb
Constructed by: Justine Holleman
Construction Date: 08-23-12
Location: Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C

Construction Details:
DTX3L-3C/A was cloned into BamHI and XhoI sites pET-21a(+) vector using below primers and Flag-DTX3L-3C/A as a template:

DTX3L-pGEX- F: 5’ ATATGGATCC GCC TCA CTC CGA CCG CCG TTC GC
BamHI

pET-21a-DTX3L- R: 5’ ATAT CTGCAG CTC AAT TCC TTT GGC TTT CAG CTC
XhoI

Construction Details:
DTX3L-3C/A was cloned into BamHI and XhoI sites pET-21a(+) vector using below primers and Flag-DTX3L-3C/A as a template:

DTX3L-pGEX- F: 5’ ATATGGATCC GCC TCA CTC CGA CCG CCG TTC GC
BamHI

pET-21a-DTX3L- R: 5’ ATAT CTGCAG CTC AAT TCC TTT GGC TTT CAG CTC
XhoI

Construction Details:
DTX3L-3C/A was cloned into BamHI and XhoI sites pET-21a(+) vector using below primers and Flag-DTX3L-3C/A as a template:

DTX3L-pGEX- F: 5’ ATATGGATCC GCC TCA CTC CGA CCG CCG TTC GC
BamHI

pET-21a-DTX3L- R: 5’ ATAT CTGCAG CTC AAT TCC TTT GGC TTT CAG CTC
XhoI
Plasmid Name: FLAG-AIP4-C830A ligase mutant  
Vector Backbone: p3xFLAG-CMV-10 (Product No. E4401; Sigma)  
Insert Size: ~656 kb  
Vector Size: 6.4 kb  
Constructed by: Justine Holleman  
Construction Date: 07-14-2011  
Location: Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C

Construction Details:
FLAG-AIP4-C830A was amplified from myc-AIP4-C830A. Primers used are listed below. Briefly, the forward primer carried a BamHI restriction site and started at amino acid residue Gly2, in frame with the 3xFLAG epitope. The reverse primer carried a XhoI restriction site and an in-frame stop codon and ended at E862. The PCR fragment was digested with BamHI and XhoI and cloned into the BamHI and XhoI sites of p3xFLAG-CMV10.

**F:** 5’- ATATGGATCC GGT AGT CTG ACC ATG AAA TCT TC  
**BamHI**

**R:** 5’ - ATATTCTAGA TTA CTC TTG TCC AAA TCC TTC TGT TTC  
**XhoI**
**Plasmid Name:** GST-AIP4-C830A  
**Vector Backbone:** pGEX-6p-1  
**Insert Size:** ~2.6 kb  
**Vector Size:** 5 kb  
**Constructed by:** Justine Holleman  
**Construction Date:** 4-29-12  
**Location:** Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C  

**Construction Details:**  
GST-AIP4-C830A was PCR amplified from FLAG-AIP4-C830A template DNA and sub-cloned into the BamHI-SmaI sites of pGEX6p1 using the below primers:

**F:** 5’-ATAT **GGATCC** GGT AGT CTG ACC ATG AAA TCT TC  
Bam HI

**R:** 5’-ATAT **CCCGGG** TTA CTC TTG TCC AAA TCC TTC TGT TTC  
SmaI

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**BamHI**

GST

AIP4-C830A

\- SmaI

pGEX6p1-AIP4-C830A

~7.6 kb
**Plasmid Name:** FLAG-DTX1-Full Length

**Vector Backbone:** p3xFLAG-CMV-10 (Product No. E4401; Sigma)

**Insert Size:** ~1.9 kb  
**Vector Size:** 6.4 kb

**Constructed by:** Justine Holleman

**Construction Date:** 10-12-2011

**Location:** Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80C

**IMAGE clone ID:** 5931062

**GenBank No.:** BC048216

**Construction Details:**

FLAG-DTX1 was PCR amplified from human Deltex 1 (DTX1) DNA template. Primers used are listed below. Briefly, the forward primer carried a HindIII restriction site and started at amino acid residue S2, in frame with the 3xFLAG epitope. The reverse primer carried a XbaI site and an in frame stop codon and ended at A620. The PCR fragment was digested with HindIII and XbaI and cloned into the HindIII and XbaI sites of p3xFLAG-CMV10.

**DTX1-met-F:** 5’-ATAT **AAGCTT** TCA CGG CCA GGC CAC GGT GGG C

HindIII S2 R P G H G G

**DTX1-stop-R2:** 5’-ATAT **TCTAGA** TCA AGC CTG GTG TCG ACT CCG GC

XbaI stop A620 K A A A E

![Diagram of plasmid construction](image-url)
**Plasmid Name:** FLAG-DTX2-Full Length  
**Vector Backbone:** p3xFLAG-CMV-10 (Product No. E4401; Sigma)  
**Insert Size:** ~1.9 kb  
**Vector Size:** 6.4 kb  
**Constructed by:** Justine Holleman  
**Construction Date:** 10-12-2011  
**Location:** Maxi Prep DNA Box 1 (4°C); Glycerol Stock -80°C  
**IMAGE clone ID:** 4107018  
**GenBank No.:** BC008856

**Construction Details:**
FLAG-DTX2 was PCR amplified from human Deltex 2 (DTX2) DNA template. Primers used are listed below. Briefly, the forward primer carried a HindIII restriction site and started at amino acid residue A2, in frame with the 3xFLAG epitope. The reverse primer carried a XbaI site and an in frame stop codon and ended at Q622. The PCR fragment was digested with HindIII and XbaI and cloned into the HindIII and XbaI sites of p3xFLAG-CMV10.

**DTX2-Met-F:** 5'-ATAT AAGCTT GCC ATG GCC CCA AGC CCT TCC C  
HindIII A2 M A P S P S

**DTX2-stop-R2:** 5'-ATAT TCTAGA TCA CTG CTG CTC CAG GCA GTC  
XbaI stop Q622 Q E L C D

![Diagram of the plasmid](image)


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Sun, Y., Cheng, Z., Ma, L., & Pei, G. (2002). Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem, 277*(51), 49212-49219. doi: 10.1074/jbc.M207294200


VITA

The author, Justine Elizabeth Kennedy was born in Mishawaka, IN on September 20, 1987 to Thomas Holleman and Beverly Acrey. In May 2010, Justine received a B.S. in biology with minor concentration in chemistry at Loyola University Chicago. During her undergraduate studies at Loyola, Justine got her first experience in the lab working as a lab assistant for courses including genetics, immunology and microbiology. In the summer of 2010, she was awarded a Summer Undergraduate Research Fellowship from ASPET and performed research under the guidance of Dr. Saverio Gentile at Loyola University Medical Center.

In August 2010, Justine continued her studies at Loyola Health Sciences Division where she joined the department of Molecular Pharmacology and Therapeutics. She completed her doctoral work in the laboratory of Adriano Marchese, where she studied the ubiquitin dependent regulation of the chemokine receptor CXCR4. She was awarded a pre-doctoral fellowship from the American Heart Association in the summer of 2013. In September 2014, Justine married fellow graduate student Domenick Kennedy.

Upon successful completion of her Ph.D., Justine will begin a post-doctoral fellowship in Global Medical Affairs at Baxter International, Inc., where she will focus on therapeutics to treat Hemophilia.