AD_____

Award Number: DAMD17-99-1-9367

TITLE: EGF-Receptor Signaling in Endocytosis Deficient Cells

PRINCIPAL INVESTIGATOR: Brian Ceresa, Ph.D.
Sandra Schmid, Ph.D.

CONTRACTING ORGANIZATION: The Scripps Research Institute
La Jolla, California 92037

REPORT DATE: July 2000

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2000	Annual Summary (1 Jul 99 - 30 Jun 00)

4. TITLE AND SUBTITLE
EGF-Receptor Signaling in Endocytosis Deficient Cells

5. FUNDING NUMBERS
DAMD17-99-1-9367

6. AUTHOR(S)
Brian Ceresa, Ph.D. Sandra Schmid, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Scripps Research Institute
La Jolla, California 92037

E-MAIL:
ceresa@scripps.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Award DAMD17-99-1-9367 seeks to understand the role of membrane trafficking in Epidermal Growth Factor Receptor (EGFR) signal transduction. We have used a tissue culture model system (HeLa cells) to isolate the activated EGFR at a distinct stage in the endocytic pathway, namely the early endosome. Stable expression of a constituitively active form of the small molecular weight guanine nucleotide binding protein rab5, we recapitulated a previously described phenotype of enlarged, functional endosomes. When we biochemically explored the consequence of the enlarged endosome, we found unaltered EGFR trafficking though the endocytic pathway. A more thoroughly examination revealed that the originally reported accumulation of transferrin receptors in the early endosomal compartment was not occurring. We next did a series of detailed studies using a transient expression system in HeLa cells in which the level and time of constituitively active rab5 overexpression were varied. From these studies, we discovered that while under virtually all conditions we could obtain the previously described enlarged endosomal phenotype, under no conditions did we observe changes in membrane trafficking. Thus, we conclude that changes in endosomal morphology (i.e. enlarged endosomes) in HeLa cells do not accurately forecast changes in transferrin receptor or EGFR membrane trafficking.

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

BC Where copyrighted material is quoted, permission has been obtained to use such material.

N/A Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

N/A Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

BC In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

BC In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

BC In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Brian P. Cerisa 7/13/00

PI - Signature Date

Table of Contents

Introduction:

The signal transduction specificity that underlies Epidermal Growth Factor (EGF) Receptor (EGFR) physiology is an important component of tissue growth and development . Identifying the basic molecular mechanisms that regulate this signaling network is an important step in distinguishing the difference between positive (normal growth) and negative (uncontrolled cell proliferation) EGFR cell biology. A well described phenomena that accompanies EGFR signaling is the entry of the activated EGFR into the cell via clathrin coated vesicles (1). Inhibition of EGFR internalization results in the selective inhibition of some, but not all, signaling pathways (2-5). The purpose of this research is to further explore the spatial and temporal components of EGFR signal transduction by examining the signaling properties of the activated EGFR when it is trapped in an early endosomal vesicle. The scope of this research includes developing a model system to examine early endosomal signaling, assessing the biochemical properties of EGFR, and determining the signaling properties of the endosomally localized EGFR as well as those of other members of the EGFR family, namely ErbB2.

Body:

Growth Factors and their corresponding cell surface receptors are important components in the maintenance and growth of normal epithelial cells. Overexpression and unregulated signaling of members of the ErbB growth factor receptor family is associated with many mammary carcinomas and a poor prognosis for recovery (6,7). This proposed research seeks to better understand the coordinated regulation of signaling by the ErbB family of cell surface receptors, and more specifically, ErbB1 or the Epidermal Growth Factor (EGF) Receptor (EGFR). The EGFR, like many cell surface receptors, undergoes ligand mediated endocytosis. This internalization process has historically been thought to be merely a mechanism for attenuating receptor signaling by removing the activated receptor and ligand from the cell surface. It is now appreciated that the role of the endocytic pathways plays a more complex role in EGFR signaling (2-5). Our approach utilizes the introduction of mutant forms of proteins involved in membrane trafficking. It is expected that overexpression of these proteins will disrupt normal endocytic trafficking of the EGFR allowing for analysis of EGFR signaling at distinct cellular locations.

Rab5 is a small molecular weight GTP-binding protein that has been well described as an important regulator in the formation of the early endosome (8,9). A point mutation of rab5 (glutamine to lysine mutation at residue 79 – denoted Q79L) reduces the ability of the to hydrolyze GTP thus, leaving the protein in the constituitively active state. The consequence of this mutation is an enlarged early endosome enriched in transferrin receptors (9,10). Our model system incorporated HeLa cells stably transfected with wild type (WT) and activated mutant (Q79L) rab5 under a tetracycline regulated system.

Specifically, in the induced rab5 (Q79L) HeLa cells we observed the characteristic enlarged endosomal morphology as well as immunofluorescent localization of rab5, transferrin and transferrin receptors. These data indicated that we had successfully recapitulated our desired model system. When the endocytic trafficking of the EGFR was examined, quite surprisingly, we discovered there was no change in the rate of EGFR uptake, degradation or recycling.

As experiments progressed and the rab5 (Q79L) HeLa cells were subjected to a more rigorous biochemical characterization, we discovered that in our stably transfected HeLa cells the endosomal morphology and cellular localization of rab5 alone did not accurately predict changes in the kinetics of transferrin receptor enocytosis and/or recycling. We postulated that the levels of rab5 expression were not sufficient to give the desired biochemical effect. To eliminate this problem, we generated and characterized tetracycline inducible adenoviruses that encoded for both the WT and Q79L rab5 proteins. This new model system allowed rapid and uniform introduction of high levels of the rab5 proteins. We began our studies with the morphological and immunofluorescent characterization of the cells. Once again, these data were consistent with those reported in the literature, however upon biochemical analysis of transferrin receptor uptake and recycling, we failed to see the reported phenotype.

A second explanation for the inability to see the expected phenotype was that the cells had compensated for the rab5(Q79L) overexpression, allowing the EGFR receptor to utilize other mechanisms for membrane trafficking. To test this hypothesis, we developed a strategy to rapidly express the rab5 proteins at high levels (approximately 50-fold over endogenous protein) and in a relatively short period of time (1 hour).

Analysis of rab5 expression under these conditions yield the same result: the morphological phenotype but no change in EGFR membrane trafficking.

At this point, we began to suspect that the originally reported data were misinterpreted. Initial publications suggested that rab5 played a role in coordinating the membrane trafficking of the transferrin receptor by regulating fusion to the early endosomal compartment. Thus, when a constituitively activated mutant of rab5 is present, transferrin receptor endocytosis increases, receptor recycling decreases, and the early endosome becomes enlarged. While we were able to see the enlarged endosomes, we did not see the accompanying changes in endosomal trafficking; therefore, the enlarged endosomes were not sufficient to predict biochemical changes in membrane trafficking. In a through analysis of our results, we confirmed that the enlarged endosomes appropriately localized with various markers of cellular compartments (i.e. EEA1, rab7, rab9, LAMP-1, mannose-6- phosphate receptor, and the trans golgi network). The enlarged endosomes could effectively traffic the transferrin receptor through them as demonstrated by the appearance and disappearance of Alexa-Transferrin in them. Kinetic examination of markers of membrane trafficking – uptake and recycling of transferrin, fluid phase uptake (a non-specific marker of membrane trafficking), and lipid recycling (a non-specific marker of membrane recycling).

From these experiments, we have concluded that while rab5(Q79L) can induce the formation of an enlarged early endosome, this endosome is not an accurate predictor of changes in the kinetics of transferrin receptor uptake and/or recycling. These data are being written up and will be submitted as a manuscript to The Proceedings of the National Academy of Science (U.S.A.).

The above described work has provided a "hands on" approach to learning membrane trafficking. As indicated in my proposal, I joined the laboratory of Dr. Sandra Schmid to learn the science of membrane trafficking to complement my existing expertise receptor tyrosine kinase signal transduction. Although these results were unexpected, the rigorous analysis of membrane trafficking has been an excellent training. With this project under my belt as well as the co-authoring a review for Current Opinions in Cell Biology (11), I am very well prepared to study the second and third specific aims.

Appendix to Summary:

1) **Key Research Accomplishments**:

- Characterization of stable rab5(WT) and rab5(Q79L) HeLa cellslines.
- Generation of tetracycline regulatable adenoviruses encoding for rab5(WT) and rab5(Q79L).
- Demonstration that the rab5(Q79) formed enlarged endosome is retains its functionality
- Demonstration that the rab5(Q79) formed enlarged endosome is not an accurate predictor of changes in membrane trafficking.

2) Reportable Outcomes

Manuscripts:

- B. P. Ceresa and S. L. Schmid, Regulation of signal transduction by endocytosis (2000), Current Opinion in Cell Biology Vol 12 (2) p. 204-210. (Appendix A)
- B. P. Ceresa and S. L. Schmid, Endocytic trafficking occurs normally through rab5Q79L-induced enlarged endosomes. (Manuscript in preparation and to be submitted to PNAS).

Employment:

- 6/1/00 – Assistant Professor of Cell Biology at Oklahoma University Health Sciences Center, Oklahoma City, OK.

References

1. Lamaze, C., and Schmid, S. L. (1995) *J Cell Biol* **129,** 47-54
2. Emlet, D. R., Moscatello, D. K., Ludlow, L. B., and Wong, A. J. (1997) *J Biol Chem* **272,** 4079-4086
3. Haugh, J. M., Huang, A. C., Wiley, H. S., Wels, A., and Lauffenburger, D. A. (1999) *J Biol Chem* **274,** 34350-34360
4. Haugh, J. M., Schooler, K., Wells, A., Wiley, H. S., and Lauffenburger, D. A. (1999) *J Biol Chem* **274,** 8958-8965
5. Viera, A. V., Lamaze, C., and Schmid, S. L. (1996) *Science* **274,** 2086-2089
6. Huang, S. H.-J., Nagane, M., Klingbeil, C. K., Line, H., NIshikawa, R., Ji, X.-D., Huang, C.-H., Gill, G. N., Wiley, H. S., and Cavenee, W. K. (1997) *J Biol Chem* **272,** 2927-2935
7. Liu, E., Thor, A., He, M., Barcos, B. M., Ljung, B. M., and Benz, C. (1992) *Oncogene* **7,** 1027-1032
8. Rybin, V., Ullrich, O., Rubino, M., Alexandrov, K., Simon, I., Seabra, M. S., Goody, R., and Zerial, M. (1996) *Nature* **383,** 266-269
9. Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., and Zerial, M. (1994) *EMBO J* **12,** 1287-1296
10. Bucci, C., Parton, R. G., Mather, I. H., Stunnernerberg, H., Simons, K., Hoflack, B., and Zerial, M. (1992) *Cell* **70,** 715-728
11. Ceresa, B. P., and Schmid, S. L. (2000) *Curr Opin Cell Biol* **12,** 204-210

Appendix A:

Published Manuscripts:

Ceresa, B. P., and Schmid, S. L. (2000) *Curr Opin Cell Biol* **12,** 204-210

Regulation of signal transduction by endocytosis

Brian P Ceresa and Sandra L Schmid

Endocytosis of ligand-activated receptors has generally been considered a mechanism to attenuate signaling. There is now a growing body of evidence suggesting that this process is much more sophisticated and that endocytic membrane trafficking regulates both the intensity of signaling and the co-localization of activated receptors with downstream signaling molecules.

Address
The Scripps Research Institute, Department of Cell Biology, IMM-11, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Current Opinion in Cell Biology 2000, **12**:204–210

0955-0674/00/$ – see front matter

Abbreviations

β_2AR	β_2 adrenergic receptor
EBP50	ERM-binding phosphoprotein-50
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ERK 1/2	extracellular regulated kinases 1 and 2
ERM	ezrin–radixin–moesin
G protein	guanine-nucleotide-binding protein
GPCR	G-protein-coupled receptor
IRS-1	insulin receptor substrate-1
NDF	Neu differentiation factor
PI3-K	phosphatidyl inositol 3-kinase
PLD	phospholipase D
RTK	receptor tyrosine kinase
TGF-α	transforming growth factor-α

Introduction

Cell surface receptors are the molecules through which changes in the extracellular environment are communicated within the cell. Among the diverse cellular responses to ligand-mediated signaling events are the uptake of nutrients and ions, the regulation of protein and DNA synthesis, and decisions about the proliferation or death of the cell. These responses are triggered when intracellular signaling molecules are activated or generated through signaling pathways initiated by ligand-bound cell surface receptors. However, it remains poorly understood how these resultant signaling pathways are regulated to form a co-ordinated, receptor-specific response. A cell can express a variety of cell surface receptors, which utilize a limited number of directed and regulated signaling pathways; yet each receptor produces a distinct response in cell physiology. The interactions of activated cell surface receptors with downstream effectors needed to amplify and transduce biochemical signals are governed by such diverse cellular processes as membrane trafficking, compartmentalization and regulated protein expression. This review focuses on the interplay between membrane trafficking and signaling by cell surface receptors. We discuss how membrane trafficking regulates signal transduction and how signaling events, in turn, regulate distinct steps in membrane trafficking.

Caveolae as coordinators of signaling molecules

One well-studied example of compartmentalized signaling occurs from caveolae, which are morphologically defined as 'omega'-shaped invaginations of the plasma membrane. Biochemically, these membrane domains are defined by their association with a family of cholesterol-binding proteins called caveolins, which function to establish and/or maintain these structures. Caveolae constitute microdomains of the plasma membrane that are enriched in cholesterol, glycosphingolipids and lipid-anchored membrane proteins. With over 30 membrane receptors, signaling molecules and membrane transporters localized to caveolae, these lipid- and protein-dense cell surface microdomains are natural candidates for centers of signaling activity (reviewed in [1]).

Recent compelling evidence that caveolae are signaling centers comes from analysis of the direct consequences of modulating endogenous levels of caveolin or interfering with caveolin function in signaling. For instance, using an antisense strategy to inhibit the expression of caveolin-1 in NIH-3T3 cells causes their transformation by facilitating anchorage-independent growth and hyperactivation of extracellular regulated kinases 1 and 2 (ERK 1/2) [2]. Signaling through these pathways was restored when caveolin-1 returned to endogenous levels. Correspondingly, overexpression of caveolin-1 suppresses ERK 1/2 signaling [3]. Caveolin overexpression does not have an inhibitory effect on all signaling pathways. The expression of recombinant caveolin-1 in NIH-3T3 cells causes an increase in phospholipase D1 (PLD1) activity [4].

A more striking example of the specificity of caveolae-dependent signaling events is the finding that the disruption of caveolin function or caveolae structure, caused either by overexpression of dominant-negative caveolin-3 mutants or by depletion of cellular cholesterol, interferes with the activation of the protein kinase raf by activated H-ras without affecting its activation by the almost identical isoform K-ras [5•]. The difference between these two ras isoforms is their localization at the plasma membrane: H-ras, but not K-ras, is palmitylated, a modification that targets it to the cholesterol-rich lipid microdomains associated with caveolin. A functional relationship between caveolin expression and cholesterol was also observed in *Caenorhabditis elegans*, in which either reduction of caveolin expression by RNA interference or depletion of cholesterol perturbs ras signaling through the MAP kinase pathway [6•].

The dramatic and diverse effects that caveolin function has on regulating signaling molecules are probably the result of using extreme measures to manipulate a system

blocks cholesterol translocation from lysosomes. *J Biol Chem* 1992, **267**:23797-23805.

75. Roff CF, Goldin E, Comly ME, Cooney A, Brown A, Vanier MT, Miller SP, Brady RO, Pentchev PG: **Type C Niemann-Pick disease: use of hydrophobic amines to study defective cholesterol transport.** *Dev Neurosci* 1991, **13**:315-319.

76. Lange Y, Steck TL: **Cholesterol homeostasis. Modulation by amphiphiles.** *J Biol Chem* 1994, **269**:29371-29374.

77. Underwood KW, Andemariam B, McWilliams GL, Liscum L: **Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport.** *J Lipid Res* 1996, **37**:1556-1568.

78. Liscum L, Faust JR: **The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-beta-[2-(diethylamino)ethoxy]androst-5-en-17-one.** *J Biol Chem* 1989, **264**:11796-11806.

79. Yoshikawa H: **Effects of drugs on cholesterol esterification in normal and Niemann-Pick type C fibroblasts: AY-9944, other cationic amphiphilic drugs and DMSO.** *Brain Dev* 1991, **13**:115-120.

80. Taylor AM, Nakano Y, Mohler J, Ingham PW: **Contrasting distributions of patched and hedgehog proteins in the *Drosophila* embryo.** *Mech Dev* 1993, **42**:89-96.

81. Capdevila J, Pariente F, Sampedro J, Alonso JL, Guerro I: **Subcellular localization of the segment polarity protein patched suggests an interaction with the wingless reception complex in *Drosophila* embryos.** *Development* 1994, **120**:987-998.

82. Tabata T, Kornberg TB: **Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs.** *Cell* 1994, **76**:89-102.

83. Bellaiche Y, The I, Perrimon N: **Tout-velu is a *Drosophila* homologue of the putative tumour suppressor EXT-1 and is needed for Hh diffusion.** *Nature* 1998, **394**:85-88.

84. Goldstein JL, Brown MS: **Regulation of the mevalonate pathway.** *Nature* 1990, **343**:425-430.

85. Braverman N, Lin P, Moebius FF, Obie C, Moser A, Glossmann H, Wilcox WR, Rimoin DL, Smith M, Kratz L *et al.*: **Mutations in the gene encoding 3 beta-hydroxysteroid-delta 8, delta 7- isomerase cause X-linked dominant Conradi-Hunermann syndrome.** *Nat Genet* 1999, **22**:291-294.

86. Derry JM, Gormally E, Means GD, Zhao W, Meindl A, Kelley RI, Boyd Y, Herman GE: **Mutations in a delta 8-delta 7 sterol isomerase in the tattered mouse and X-linked dominant chondrodysplasia punctata.** *Nat Genet* 1999, **22**:286-290.

87. Kelley RI, Wilcox WG, Smith M, Kratz LE, Moser A, Rimoin DS: **Abnormal sterol metabolism in patients with Conradi-Hunermann-Happle syndrome and sporadic lethal chondrodysplasia punctata.** *Am J Med Genet* 1999, **83**:213-219.

88. Liu XY, Dangel AW, Kelley RI, Zhao W, Denny P, Botcherby M, Cattanach B, Peters J, Hunt SP, Mallon AM *et al*: **The gene mutated in bare patches and striated mice encodes a novel 3beta-hydroxysteroid dehydrogenase.** *Nat Genet* 1999, **22**:182-187.

89. Lange Y, Duan H, Mazzone T: **Cholesterol homeostasis is modulated by amphiphiles at transcriptional and post-transcriptional loci.** *J Lipid Res* 1996, **37**:534-539.

90. Vanier MT, Rodriguez-Lafrasse C, Rousson R, Mandon G, Boue J, Choiset A, Peyrat MF, Dumontel C, Juge MC, Pentchev *et al*: **Prenatal diagnosis of Niemann-Pick type C disease: current strategy from an experience of 37 pregnancies at risk.** *Am J Hum Genet* 1992, **51**:111-122.

91. Gaffield W, Keeler RF: **Craniofacial malformations induced in hamsters by steroidal alkaloids.** *J Nat Toxins* 1996, **5**:25-38.

92. Lange Y, Ye Y, Rigney M, Steck TL: **Regulation of endoplasmic reticulum cholesterol by plasma membrane cholesterol.** *J Lipid Res* 1999, **40**:1-7.

93. Ruvkun G, Hobert O: **The taxonomy of developmental control in Caenorhabditis elegans.** *Science* 1998, **282**:2033-2041.

94. Stone DM, Hynes M, Armanini M, Swanson TA, Gu Q, Johnson RL, Scott MP, Pennica D, Goddard A, Phillips H *et al.*: **The tumour-suppressor gene patched encodes a candidate receptor for Sonic hedgehog.** *Nature* 1996, **384**:129-134.

95. Chen Y, Struhl G: ***In vivo* evidence that Patched and Smoothened constitute distinct binding and transducing components of a Hedgehog receptor complex.** *Development* 1998, **125**:4943-4948.

96. Murone M, Rosenthal A, de Sauvage FJ: **Sonic hedgehog signaling by the patched-smoothened receptor complex.** *Curr Biol* 1999, **9**:76-84.

97. • Burke R, Nellen D, Bellotto M, Hafen E, Senti KA, Dickson BJ, Basler K: **Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells.** *Cell* 1999, **99**:803-815.

Recent data suggest that even the release of Hh may be regulated by cholesterol. A gene called *dispatched* was identified in a screen for mutations with a Hedgehog-like phenotype. Like patched, it has sterol-sensing domain but seems to be required for the release of Hh from the cells that synthesize it. Intriguingly, dispatched does not facilitate the release of GPI-anchored Hh, suggesting that the release of normal cholesterol-modified Hh is not simply related to raft association.

that is, under normal conditions, more delicately balanced by much smaller changes in caveolin function. In NIH-3T3 and Rat1 cells, mutationally activated c-erbB2 (c-neu) causes the downregulation of caveolin-1, but not caveolin-2 [7]. These decidedly more subtle changes are probably part of a complex feedback mechanism to regulate transformation potential under prolonged growth factor stimulation.

The exact role of caveolin and caveolae remains enigmatic. There is good evidence that they play direct roles in regulating plasma membrane cholesterol levels [8]. Although caveolae are clearly regions enriched with signaling proteins, it remains uncertain whether these structures function to spatially coordinate signaling events or whether there exists a more direct role for cholesterol and membrane subdomain composition itself in controlling the activity of signaling complexes.

Endocytosis as a regulator of signal transduction

Ligand-mediated endocytosis is characteristically an early response in the signaling pathways triggered by a diverse group of cell surface receptors, including heterotrimeric guanine-nucleotide-binding protein (G protein)-coupled receptors (GPCRs), receptor tyrosine kinases (RTKs) and cytokine receptors [9,10]. Upon binding of the ligand (hormone, neuropeptide, growth factor, odorant, etc.) to its cognate cell surface signaling receptor, the activated receptors are targeted to clathrin-coated membrane invaginations, which, through a series of highly regulated, yet still not fully understood, biochemical events, eventually pinch off to form a clathrin-coated vesicle. Subsequent membrane fusion and budding reactions deliver the contents of the vesicle through sequential endosomal compartments. During progression along the endocytic pathway, the endosomes are modified in protein composition and pH, and their contents are sorted for shipment to the appropriate cellular destination. Among these fates are retention in the endosomal compartment, recycling back to the plasma membrane and delivery toward a lysosomal degradation pathway.

Ligand-mediated endocytosis plays at least two functions in receptor signaling. First, it can serve as a biophysical mechanism for attenuating the signaling of an activated cell surface receptor. Discussed in this review is the evolving story of the ErbB RTK family, which illustrates how controlled receptor trafficking regulates the potency of mitogenic signaling. Second, endocytosis plays a role in placing the activated cell surface receptor in the appropriate cellular location to interact with downstream signaling molecules. Signaling to ERK 1/2 by the internalized β_2 adrenergic receptor (β_2AR) and the epidermal growth factor (EGF) receptor (EGFR) not only demonstrates this phenomena but also provides insight into how these interactions might be regulated.

Endocytosis as a means of regulating receptor activity

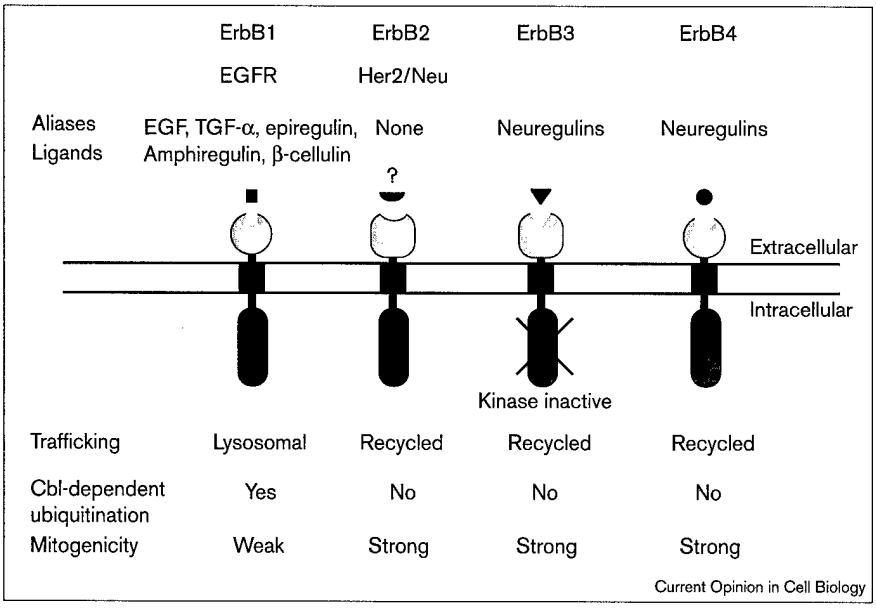
An excellent example of how endocytic membrane trafficking can regulate signaling comes from studies on the ErbB family of RTKs. ErbB family members, including the EGFR (ErbB1), are activated upon dimerization induced by binding their ligands, which are EGF, transforming growth factor-α (TGF-α) and Neu differentiation factor (NDF). The specific ligand determines the composition of the dimeric receptor (Figure 1).

It has been appreciated for some time that different ErbB receptor ligands invoke different signaling potencies, particularly in terms of their mitogenic potential [11]. This has been convincingly demonstrated and mechanistically explored by stably transfecting cells lacking ErbB family members with ErbB1 alone or in combination with either ErbB2 or ErbB3 [12•]. Cells expressing only ErbB1 were less proliferative in response to EGF than in response to TGF-α. When either ErbB2 or ErbB3 was co-expressed with ErbB1, the cells became significantly more responsive to EGF, without altering their response to TGF-α. These increases in mitogenic response correlate with the increased recycling and decreased downregulation of ErbB1 homodimers that occur in the presence of TGF-α or when ErbB2- or ErbB3-containing heterodimers are activated by either EGF or TGF-α.

As implied by these results, endosome sorting is regulated by interaction with both lumenal and cytoplasmic domains of the ErbB family members. Two lines of evidence suggest that endosome acidification and the pH-dependent dissociation of receptor–ligand complexes are central in controlling this sorting decision. First, TGF-α and NDF dissociate from their receptors at a relatively higher pH than EGF, suggesting that this dissociation would occur in early endosomal compartments, which are involved in receptor recycling. Second, treating cells with the ionophore monensin, which increases the pH of endosomal sorting compartments, leads to increased downregulation of receptors activated with TGF-α and NDF [12•,13•]. Studies with chimaeric receptors encoding the ligand-binding extracellular domain of ErbB1 and the intracellular domain of ErbB2 [14] or other ErbB family members [15] indicate that sorting signals in the ErbB1 cytoplasmic domain are required for receptor degradation.

One candidate molecule that may recognize sorting determinants in ErbB1 is c-Cbl or its *C. elegans* ortholog Sli-1. c-Cbl has been shown to be a downstream substrate and negative regulator of a number of cell surface receptors, although its mechanism has been poorly understood [16,17]. Levkowitz *et al.* [18] found that c-Cbl is recruited to endosomes in cells transiently expressing the lysosomally directed ErbB1, but not the recycling ErbB3. c-Cbl is required for ligand-dependent ubiquitination of ErbB1 in endosomes, a modification that may target the protein to the lysosome degradative pathway. Importantly, mutants of either the ErbB1 RTK or c-Cbl

Figure 1



Structural and signaling properties of the ErbB receptor tyrosine kinase family (see text for details).

that disrupt their interactions [18,19,20••] result in decreased ErbB1 degradation and increased mitogenic signaling [19] (Figure 2a). Results using other cell surface signaling receptors suggest a general role for ubiquitination in regulating endocytic membrane trafficking [21].

Pathophysiological consequences of receptor tyrosine kinase trafficking

The physiological significance of differential RTK trafficking has been demonstrated by studies utilizing cultured breast cancer cell lines expressing varying ratios of endogenous ErbB1 and ErbB2 RTKs. Through a series of biochemical and immunocytochemical studies, Wang *et al.* [22•] demonstrate that, despite auto-tyrosine-phosphorylation of both family members upon EGF treatment, endocytosis of dimeric receptor complexes is inversely proportional to ErbB2 expression. The physiological consequence of the differential cellular trafficking is increased mitogenic signaling owing to the prolonged activation state of ligand–receptor complexes involving ErbB2 or ErbB3 subunits [12•,13•,23].

This selective degradation of only ErbB1 receptors may explain the more carcinogenic nature of other ErbB family members. For instance, expression of ErbB2 has long been correlated with many carcinomas and poor prognosis [24,25]. One plausible explanation for ErbB2's carcinogenic effect is its ability to increase the ratio of ErbB1–ErbB2 heterodimers over ErbB1 homodimers. In doing so, alterations in membrane trafficking would result in enhanced mitogenicity.

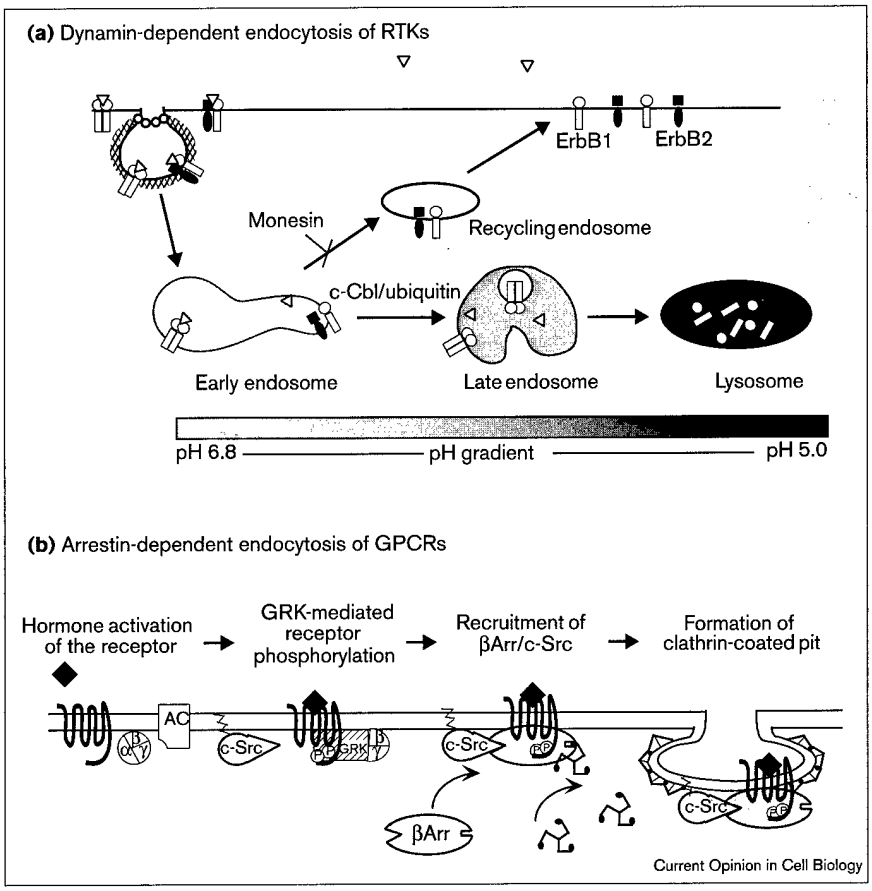
Signaling from within the endosome

The concept that activated receptors interact with downstream signaling molecules at discrete endocytic locations has been postulated for years [26,27]. Previously, testing this hypothesis was limited by the inability to trap an activated receptor at unique endocytic locales without significantly altering the receptor structure and/or impairing signaling pathways. Now, with a clearer understanding of the initial stages of endocytosis and receptor desensitization, less invasive cell biological methods for disrupting endocytosis have been developed. Thus, there has been a plethora of data examining receptor signaling prior to entry into the endocytic pathway.

The two most commonly used tools are dominant-negative constructs of the GTPase dynamin and the GPCR-binding protein arrestin. Overexpression of dominant inhibitory forms of dynamin (those that can not bind or hydrolyze GTP) blocks clathrin-mediated endocytosis [28], causing the retention of many, but not all, receptors at the cell surface. Overexpression of dominant-negative forms of arrestin [10,29•] specifically blocks the endocytosis of GPCR. Arrestin binds activated GPCRs after they become phosphorylated on serine residues within their carboxyl termini through a GPCR-kinase-mediated feedback loop. Arrestin binding prevents activated GPCR further interacting with heterotrimeric G proteins and transducing signals. In addition, arrestins serve as adapter molecules that target activated GPCR to endocytic coated pits [30]. Together, these methods have proven effective in a direct comparison of the signaling of receptors retained on the cell surface with that of those allowed to enter the endocytic pathway.

Not surprisingly, there are some signaling pathways that are completely unaffected or enhanced by retaining activated receptors at the cell surface. These include the most proximal events in receptor signaling, such as the intramolecular kinase activity of RTKs, activation of heterotrimeric G proteins,

Figure 2



Endocytosis of signaling receptors. **(a)** Differential endocytic trafficking of homodimeric and heterodimeric ErbB1 and ErbB2 RTKs. Ligand binding induces receptor dimerization and targeting to clathrin-coated pits, which pinch off in a dynamin-dependent manner. The coated vesicles form, then shed their clathrin coats and fuse with the mildly acidic early endosome, where the RTKs are sorted for recycling or degradation. Ligands dissociate from ErbB2-containing dimers, which are then sorted on a monesin-sensitive step to the recycling endosome and returned to the cell surface. ErbB1 dimers retain bound ligand until encountering the lower pH or later endosomal compartments. Within endosomes, ErbB1 dimers associate with c-Cbl, are ubiquitinated and targeted for degradation in the lysosome. **(b)** Formation of the β_2AR–β-arrestin–c-Src complex as a mechanism to form the β_2AR-containing endosome. Upon hormone stimulation, the α and βγ subunits of the activated heterotrimeric G protein dissociate to cause activation of downstream effectors such as adenylyl cyclase (AC). The βγ subunits facilitate the G-protein-coupled receptor kinase (GRK)-mediated phosphorylation of ligand-bound β_2AR. β-Arrestin (βArr) binds both the phosphorylated β_2AR and c-Src, causing formation of the β_2AR–β-arrestin–c-Src complex at the plasma membrane. The β-arrestin interaction with clathrin targets the β_2AR to coated pits. Its subsequent internalization is a prerequisite for signaling to the ERK 1/2 pathway.

phosphorylation of insulin receptor substrate-1 (IRS-1) by the insulin receptor, regulation of adenylyl cyclase activity and stimulation of phospholipase Cγ. Given the plasma membrane location and/or the kinetics of activation of these effectors, it seems unlikely that receptor endocytosis would be a prerequisite for their activity.

More intriguing are those events that are attenuated when receptor endocytosis is inhibited — namely the activation of phosphatidyl inositol 3-kinase (PI3-K) and ERK 1/2 [31–36]. In the case of insulin receptor signaling, PI3-K associates with IRS-1 in an insulin-dependent manner as a prerequisite for signaling. When insulin receptor endocytosis is blocked by dominant-negative dynamin, there is a significant reduction in insulin-dependent PI3-K activity, despite full tyrosine phosphorylation of the insulin receptor and IRS-1. The change in kinase activity is reflected in a corresponding decrease in the amount of p85 regulatory subunit of PI3-K associated with IRS-1 [31].

Although there are a number of cell surface receptors that require endocytosis for ERK 1/2 activation, there are an equal number that do not (Table 1). One possible explanation is that the need for endocytosis is receptor-specific and that the role of endocytosis in ERK 1/2 activation may be a mechanism through which signal specificity is conferred. These results are reminiscent of the differential effects on ras signaling through MAP kinase observed upon caveolae disruption (see above) and suggest that multiple mechanisms exist to spatially regulate the MAP kinase signaling pathway.

Interestingly, it has been reported that dominant inhibitory dynamin decreases μ opiod receptor mediated activation of ERK 1/2 in HEK293 cells independent of an effect on the endocytosis of the μ opiod receptor [37]. It is possible this is a consequence of dynamin's role in regulating an intermediate signaling protein whose endocytosis is required for ERK 1/2 activation. Alternatively, these data may suggest a second role for dynamin, in addition to regulating endocytosis. However, expression of dominant-negative dynamin has no effect on ERK 1/2 activation by the α_2 adrenergic receptor, which, like the μ opiod receptor, couples to $G\alpha_{i/o}$ guanine-nucleotide-binding proteins and is internalized in a dynamin-independent manner [38].

Taken together, this selective inhibition of signaling pathways suggests that an activated receptor and requisite

Table 1

Consequences of inhibited endocytosis on ERK activity.

Receptor	Endocytic block	Cell line	ERK activity	References
Receptor tyrosine kinase				
EGFR	Dynamin	HeLa	↓ 50%	[35]
IR	Dynamin	H4IIE	↓ 50%	[31]
G-protein-coupled receptors				
α_{2A}AR	Dynamin*	COS-1, HEK293	No change	[33,38]
α_{2B}AR	Dynamin	COS-1, HEK293	No change	[33,38]
α_{2C}AR	Dynamin	COS-1	No change	[33]
βAR	Dynamin, arrestin	HEK293	↓ 60%	[32,45]
δ opiod	Dynamin	Cos-7, HEK293	↓ 80%	[46]
μ opiod	Dynamin	HEK293	↓ 80%	[46]
κ opiod	Dynamin, arrestin	CHO	No change	[47]
	Dynamin	HEK293	↓ 80%	[46]
$5HT_{1A}$ Serotonin receptor	Dynamin, arrestin	HEK293	↓ 50%	[34]
LPA	Dynamin, arrestin	HEK293	↓ 50–80%	[32]
CXCR2	Dynamin	HEK293	No change	[48]

*Endocytosis is dynamin independent.

signaling molecules are not sufficient to direct appropriate cellular responses — endocytosis is also an essential component.

Signal transduction can regulate endocytosis

Although the role of the endocytic pathway in receptor signal transduction has only recently been appreciated, it has been known for some time that signaling receptors must be active for their endocytosis [39]. Our understanding of the role of receptor signaling in endocytosis has been limited to mutagenesis studies defining the receptor domains that are involved in recruitment to clathrin-coated pits and endocytosis. New studies using endocytosis-deficient cell lines have readdressed this mechanism and the nonreceptor tyrosine kinase c-Src has emerged as an important regulator of endocytosis.

The effect of the endocytic pathway on signaling and *vice versa* has been best characterized using β_2AR as a model. With the first observation of β_2AR internalization upon agonist stimulation came the hypothesis that this was strictly a mechanism by which activated receptors were removed from the cell surface and sequestered within the cell to prevent further activation of a downstream effector, namely adenylyl cyclase. This hypothesis continued to garner momentum with the observation of ligand-dependent phosphorylation and the subsequent association of β-arrestin to prevent further signaling to heterotrimeric G proteins.

More recent results have suggested an additional role for β-arrestin binding. In this work, Luttrell *et al.* [40••] demonstrate that agonist activation of β_2AR results in the formation of a β_2AR–β-arrestin–c-Src complex. This protein complex targets the receptor to a clathrin-coated pit (Figure 2b). It has been shown that kinase-inactive forms of c-Src can inhibit, and constitutively active forms of c-Src can enhance, β_2AR endocytosis [41]. Activation of both β_2AR and the LPA (lysophosphatidic acid) receptor leads to phosphorylation of dynamin, in the former case, through a c-Src-dependent process [41,42]. These data suggest that c-Src is an upstream regulator of dynamin function in β_2AR endocytosis. It has been shown that EGF-stimulated activation of c-Src leads to tyrosine phosphorylation of clathrin and that phosphorylation is required for the recruitment of clathrin to the membrane [43]. Taken together, these data strongly suggest a role for c-Src in receptor endocytosis — the question remains whether c-Src is a regulator of dynamin function or a recruiter of clathrin or both.

Another candidate protein for regulating β_2AR signaling is the ezrin–radixin–moesin (ERM)-binding phosphoprotein-50 (EBP50). EBP50 binds to the G-protein-regulated kinase 5 (GRK5)-phosphorylated cytoplasmic tail of β_2AR via a PDZ domain and to the cortical cytoskeleton through an ERM-binding domain. In HEK293 cells, the disruption of the β_2AR–EBP50–actin interaction results in diminished β_2AR recycling [44•]. Although EBP50 has not been shown to have a direct role in β_2AR signaling, its effects on β_2AR endocytic trafficking are likely to have important implications in signaling [44•], similar to those described above for ErbB family members.

Conclusions

The key to fully understanding the cell physiology mediated by cell surface receptors lies not only in the identification of downstream effectors but also in the exquisite spatial and temporal regulation of the interactions

with these effectors. Clearly, membrane trafficking plays an important role both in controlling the location of signaling interactions and in regulating the cellular degradation and recycling of the activated receptor. From a biomedical prospective, the identification of the sites and knowledge of the kinetics of receptor activation of downstream effectors provides an opportunity to design rational therapeutic strategies to manipulate a given signaling pathway.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Anderson RGW: **The caveolae membrane system.** *Annu Rev Biochem* 1998, **67**:199-225.

2. Galbiati F, Volonte D, Engelman JA, Watanabe G, Burk R, Pestell RG, Lisanti MP: **Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade.** *EMBO J* 1998, **17**:6633-6648.

3. Engelman JA, Chu C, Lin A, Jo H, Ikezu T, Okamoto T, Kohtz DS, Lisanti MP: **Caveolin-mediated regulation of signaling along the p42/44 MAP kinase cascade *in vivo*.** *FEBS Lett* 1998, **428**:205-211.

4. Czarny M, Lavie Y, Fiucci G, Liscovitch M: **Localization of phospholipase D in detergent-insoluble, caveolin-rich membrane domains.** *J Biol Chem* 1999, **274**:2717-2724.

5. • Roy S, Luetterforst R, Harding A, Apolloni A, Etheridge M, Stang E, Rolls B, Hancock JF, Parton RG: **Dominant-negative caveolin inhibits H-ras function by disrupting cholesterol-rich plasma membrane domains.** *Nat Cell Biol* 1999, **1**:98-105.

The authors demonstrate that either dominant-negative caveolin or cholesterol depletion can specifically perturb one signaling pathway, but not another.

6. • Scheel J, Srinivasan J, Honnert U, Henske A, Kurzchalia TV: **Involvement of caveolin-1 in meiotic cell-cycle progression in *Caenorhabditis elegans*.** *Nat Cell Biol* 1999, **1**:127-129.

Using *C. elegans* as a model, this report shows the *in vivo* consequences of reduced caveolin expression or cholesterol depletion on MAP kinase signaling.

7. Engelman JA, Lee RJ, Karnezi A, Bearss DJ, Webster M, Siegel P, Muller WJ, Windle JJ, Pestell RG, Lisanti MP: **Reciprocal regulation of Neu tyrosine kinase activity and caveolin-1 protein expression *in vitro* and *in vivo*.** *J Biol Chem* 1998, **273**:20448-20455.

8. Ikonen E, Parton RG: **Caveolins and cellular cholesterol balance.** *Traffic* 2000, **1**:212-217.

9. DiGuglielmo GM, Drake PG, Baass PC, Authier F, Posner BI, Bergeron JJ: **Insulin receptor internalization and signalling.** *Mol Cell Biochem* 1998, **182**:59-63.

10. Ferguson SS, Dewney WE III, Colapietro A, Barak LS, Menard L, Caron MG: **Role of β-arrestin in mediating agonist-promoted G-protein-coupled receptor internalization.** *Science* 1996, **271**:363-366.

11. Reddy CC, Wells A, Lauffenburger DA: **Comparative mitogenic potencies of EGF and TGF alpha and their dependence on receptor-limitation versus ligand-limitation.** *Med Biol Eng Comput* 1998, **36**:499-507.

12. • Lenferink AEG, Pinkas-Kramarski R, van de Poll MLM, van Vugt MJH, Klapper LN, Tzahar E, Waterman H, Sela M, van Zoelan EJJ, Yarden Y: **Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers.** *EMBO J* 1998, **17**:3385-3397.

See annotation to [13•].

13. • Waterman H, Sabanai I, Geiger B, Yarden Y: **Alternative intracellular routing of ErbB receptors may determine signaling potency.** *J Biol Chem* 1998, **273**:13819-13827.

Through the use of cells that express various combinations of ErbB family members, this paper, together with [12•], provides a model that links differential endocytic trafficking of activated receptor tyrosine kinases with their mitogenic potential.

14. Sorkin A, DiFiore PP, Carpenter G: **The carboxyl terminus of epidermal growth factor/erbB2 chimerae is internalization impaired.** *Oncogene* 1993, **8**:3021-3028.

15. Baulinda J, Kraus MH, Alimandi M, DiFiore PP, Carpenter G: **All ErbB receptors other than the epidermal growth factor receptor are endocytosis impaired.** *J Biol Chem* 1996, **271**:5251-5257.

16. Levkowitz G, Klapper LN, Tzahar E, Freywald A, Sela M, Yarden Y: **Coupling of the c-Cbl protooncogene product to Erb-1/EGF-receptor but not to other ErbB proteins.** *Oncogene* 1996, **12**:1117-1125.

17. Yoon CH, Lee J, Jongeward GD, Sternberg PW: **Vulval induction during *Caenorhabditis elegans* development is mediated by LET-23, a homolog of the mammalian protooncogene c-cbl.** *Science* 1995, **269**:1102-1105.

18. Levkowitz G, Waterman H, Zamir E, Kam Z, Oved S, Langdon WY, Beguinot L, Geiger B, Yarden Y: **c-Cbl/Sli-1 regulates ednocytic sorting and ubiquitination of the epidermal growth factor receptor.** *Genes Dev* 1998, **12**:3663-3674.

19. Waterman H, Lefkowitz G, Alory I, Yarden Y: **The RING finger of c-Cbl mediates desensitization of the epidermal growth factor receptor.** *J Biol Chem* 1999, **274**:22151-22154.

20. •• Joazeiro CAP, Wing SS, Huang H-K, Leverson JD, Hunter T, Liu Y-C: **The tyrosine kinase negative regulator c-Cbl is a RING-type, E2-dependent ubiquitin-protein ligase.** *Science* 1999, **286**:309-312.

This paper presents a novel mechanism of targeting cell surface receptors for ubiquitination.

21. Strous GJ, Govers R: **The ubiquitin-proteasome system and endocytosis.** *J Cell Sci* 1999, **112**:1417-1423.

22. • Wang Z, Zhang L, Yeung TK, Chen X: **Endocytosis deficiency of epidermal growth factor (EGF) receptor-ErbB2 heterodimers in response to EGF stimulation.** *Mol Biol Cell* 1999, **10**:1621-1636.

The authors provide evidence that sorting signals in the ErbB1 cytoplasmic domain direct its interactions with c-Cbl and its trafficking along the lysosomal degradation pathway.

23. Waterman H, Alroy I, Strano S, Seger R, Yarden Y: **The C-terminus of the kinase defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing.** *EMBO J* 1999, **18**:3348-3358.

24. Huang SH-J, Nagane M, Klingbeil CK, Line H, Nishikawa R, Ji X-D, Huang C-H, Gill GN, Wiley HS, Cavenee WK: **The enhanced tumorigenic activity of a mutant epidermal growth factor receptor common in human cancers is mediated by threshold levels of constitutive tyrosine phosphorylation and unattenuated signaling.** *J Biol Chem* 1997, **272**:2927-2935.

25. Liu E, Thor A, He M, Barcos BM, Ljung BM, Benz C: **The Her2(c-erbB-2) oncogene is frequently amplified in *in situ* carcinomas of the breast.** *Oncogene* 1992, **7**:1027-1032.

26. Khan MN, Baquiran G, Brule C, Burgess J, Foster B, Bergeron JJM, Posner BI: **Internalization and activation of the rat liver insulin receptor kinase *in vivo*.** *J Biol Chem* 1989, **264**:12931-12940.

27. Burgess JW, Wada I, Ling N, Khan MN, Bergeron JJM, Posner BI: **Decrease in β-subunit phosphotyrosine correlates with internalization and activation of the endosomal insulin recpetor kinase.** *J Biol Chem* 1992, **267**:10077-10086.

28. Damke H, Baba T, Warnock DE, Schmid SL: **Induction of mutant dynamin specifically blocks endocytic coated vesicle formation.** *J Cell Biol* 1994, **127**:915-934.

29. • Orsini MJ, Benovic JL: **Characterization of dominant negative arrestins that inhibit β_2-adrenergic receptor internalization by distinct mechanisms.** *J Biol Chem* 1998, **273**:34616-34622.

This paper characterizes arrestins and their specificity in blocking G-protein-coupled receptor endocytosis.

30. Goodman Jr, OB, Krupnick JG, Santini F, Gurevish VV, Penn RB, Gagnon AW, Keen JH, Benovic JL: **Beta-arresting acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor.** *Nature* 1996, **383**:447-450.

31. Ceresa BP, Kao AW, Santeler SR, Pessin JE: **Regulation of insulin receptor signaling pathways by clathrin mediated endocytosis.** *Mol Cell Biol* 1998, **18**:3862-3870.

32. Daaka Y, Luttrell LM, Ahn S, Rocca GJD, Ferguson SSG, Caron MG, Lefkowitz RJ: **Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase.** *J Biol Chem* 1998, **273**:685-688.

33. DeGraff JL, Gagnon AW, Benovic JL, Orsini MJ: **Role of arrestins in endocytosis and signaling of α_2-adrenergic receptor subtypes.** *J Biol Chem* 1999, **274**:11253-11259.

34. Rocca GJD, Mukhin YV, Garnovskaya MN, Daaka Y, Clark GJ, Luttrell LM, Lefkowitz RJ, Raymond KR: **Serotonin 5-HT_{1A} receptor-mediated Erk activation requires calcium/calmodulin-dependent receptor endocytosis.** *J Biol Chem* 1999, **274**:4749-4753.

35. Vieira AV, Lamaze C, Schmid SL: **Control of EGF receptor signaling by clatharin-mediated endocytosis.** *Science* 1996, **274**:2086-2089.

36. Vogler O, Nolte B, Voss M, Schmidt M, Jacobs KH, van Koppen CJ: **Regulation of muscarinic acetylcholine receptor sequestration and function by beta-arrestin.** *J Biol Chem* 1999, **274**:12333-12338.

37. Whistler JL, von Zastrow M: **Dissociation of functional roles of dynamin in receptor-mediated endocytosis and mitogenic signal transduction.** *J Biol Chem* 1999, **274**:24575-24578.

38. Schramm NL, Limbird LE: **Stimulation of mitogen-activated protein kinase by G protein-coupled α_2-adrenergic receptors does not require agonist-elicited endocytosis.** *J Biol Chem* 1999, **274**:24935-24940.

39. Carpenter G, Soler C, Baulida J, Beguinot L, Sorkin A: **Interaction of signaling and trafficking proteins with the carboxyterminus of the epidermal growth factor receptor.** *Ann NY Acad Sci* 1995, **7**:44-51.

40. •• Luttrell LM, Ferguson SSG, Daaka Y, Miller WE, Maudsley S, Rocca GJD, Lin F-T, Kawakatsu H, Owada K, Luttrell DK *et al.*: **β-Arrestin-dependent formation of β_2 adrenergic receptor-Src protein kinase complexes.** *Science* 1999, **283**:655-661.

This paper introduces a novel role for β-arrestin as a scaffolding protein that coordinates protein interactions involved in β_2 adrenergic receptor endocytosis.

41. Ahn S, Maudsley S, Luttrell LM, Lefkowitz RJ, Daaka Y: **Src-mediated tyrosine phosphorylation of dynamin is required for β_2-adrenergic receptor internalization and mitogen-activated protein kinase signaling.** *J Biol Chem* 1999, **274**:1185-1188.

42. Kranenburg O, Verlaan I, Moolenaar WH: **G_i-mediated tyrosine phosphorylation of Grb2 (growth-factor-receptor-bound protein 2) - bound dynamin-II by lysophosphatidic acid.** *Biochem J* 1999, **339**:11-14.

43. Wilde A, Beattie EC, Lem L, Tiefthof DA, Liu S-H, Mobley WC, Soriana P, Brodsky FM: **EGF receptor signaling stimulates SRC kinase phosphorylation of clathrin, influencing clathrin redistribution and EGF uptake.** *Cell* 1999, **96**:677-687.

44. • Cao TT, Deacon HW, Reczek D, Bretscher A, von Zastrow M: **A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2-adrenergic receptor.** *Nature* 1999, **401**:286-290.

This paper identifies a new protein intermediate that stabilizes β_2-adrenergic receptor (β_2AR) interactions with the cell's cytoskeleton, suggesting a novel regulatory mechanism of β_2AR signaling.

45. Lin F-T, Miller WE, Luttrell LM, Lefkowitz RJ: **Feedback regulation of β-arrestin1 function by extracellular signal-regulated kinases.** *J Biol Chem* 1999, **274**:15971-15974.

46. Ignatova EG, Belcheva MM, Bohn LM, Neuman MC, Coscia CJ: **Requirement of receptor internalization for opiod stimulation of mitogen-activated protein kinase: biochemical and immunofluorescence confocal microscopic evidence.** *J Neurosci* 1999, **19**:56-63.

47. Li JG, Luo LY, Krupnick JG, Benovic JL, Liu-Chen LY: **U50,488H-induced internalization of the human κ-opiod receptor involves a β-arrestin- and dynamin-dependent mechanism.** *J Biol Chem* 1999, **274**:12087-12094.

48. Yang W, Wang D, Richmond A: **Role of clathrin-mediated endocytosis in CXCR2 sequestration, resensitization, and signal transduction.** *J Biol Chem* 1999, **274**:11328-11333.