HOMEOSTASIS OF ENDOCYTIC AND AUTOPHAGIC SYSTEMS: INSIGHTS FROM THE HOST-PATHOGEN INTERACTION

By

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*We also certify that written approval has been obtained for any proprietary material contained therein.
Dedication

For my wife and my parents.
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<td>ERGIC</td>
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Hr  hour

HRP  horseradish peroxidase

I  isoleucine

IB  immunoblot

IL-8  interleukin-8

IM  integral membrane

IP  immunoprecipitation

L  late

L  leucine

LBPA  lysobisphosphatidic acid

LDL  low density lipoprotein

LDLR  LDL receptor

LE  late endosome

LSO  lysosomal storage organelle

LXR  liver X receptor

Ly  lysosome

M  molar
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Homeostasis of Endocytic and Autophagic Systems:
Insights from the Host-Pathogen Interaction

Abstract
by
NICHOLAS L. CIANCIOLA

The host-pathogen interaction provides a unique system offering insight into both the mechanisms by which pathogens subvert host cellular processes, and the biological processes themselves. The aim of this study was to understand the molecular basis of action of the adenovirus protein RIDα, which was originally identified by its ability to divert constitutively recycling EGF receptors to lysosomes during an adenovirus infection, and also when expressed in cells devoid of other adenovirus proteins. We report that RIDα usurps host cell machinery to exit the trans-Golgi network by an AP-1-dependent mechanism. A tyrosine motif located in the carboxyl tail of RIDα mediates this interaction, and mutation to alanine blocks biosynthetic export and inhibits RIDα function. We also found that RIDα mimics GTP-Rab7 by binding two Rab7 effector proteins, RILP and ORP1L, which coordinately recruit the motor proteins necessary for movement of late endosomes along microtubules. As RIDα shares no sequence homology with Rab7 and has no intrinsic enzymatic activity, we asked how RIDα is regulated to mimic Rab7. It was discovered that RIDα is post-translationally modified by the addition of the lipid palmitate to the lone cysteine residue in the carboxyl tail.
Mutation of this site did not affect the localization of RIDα, as both wild-type and mutant proteins localized to a unique perinuclear “autophagy-like” compartment. However, palmitoylation does affect protein function, as expression of the RIDα palmitoylation-deficient mutant altered the morphology of LAMP1-positive late endosomes. These enlarged compartments were loaded with cholesterol and other lipids, similar to the phenotype observed in the cholesterol storage disease Niemann-Pick type C (NPC). While mutant RIDα disrupted sterol regulated gene regulation, wild-type RIDα was found to rescue the defects created by the mutations that cause NPC disease. This novel function of RIDα was found to be dependent on the class III phosphatidylinositol 3-kinase that regulates the expansion of autophagosomal membranes. We conclude that RIDα activates an autonomous cholesterol egress mechanism that is independent of the NPC disease gene products. As a model system, RIDα provides insight into the coordination and homeostasis of endocytic and autophagic systems, and the role of cholesterol in these pathways.
Chapter 1

Introduction

1.1 Overview

The interactions between a pathogen and its host are complex and dynamic. The outcome of such interactions is a reflection of the properties of the microbial agent and the ability of the host to respond to infection. This outcome may range from disease to microbe elimination or even asymptomatic carriage. Microbial pathogens have evolved complex adaptations to secure their own replication and survival, and the study of the cell biology and immunology of host-pathogen interactions is emerging as a key area of research in the post-genomic era. The study of host-pathogen interactions illuminates not only the mechanisms by which pathogens subvert host cellular processes, but also brings unique insight into the cell processes themselves. “Indeed, time and again, we have seen how the study of the strategies used by these microbial pathogens to interact with their host cells have given us remarkable insight into the inner workings of the cells themselves” [1]. There are myriad examples of sophisticated strategies evolved by pathogens to modulate host cell function, which in turn have expounded host cell function and highlighted their potential as tools to study basic cell biology.

In the introduction I will present two examples of host-pathogen interactions that have helped to elucidate novel aspects of cell biology: *Listeria monocytogenes* and the actin-based motility machinery; and mammalian viruses and host endocytic systems. The
pathogen that is the focus of my thesis is adenovirus, specifically the adenoviral protein RID\(\alpha\), and this topic will be covered in detail. Since the sub-cellular distribution and trafficking of RID\(\alpha\) is an important part of this thesis, protein transport in the biosynthetic, endocytic, and autophagic pathways will be discussed. Also, cholesterol homeostasis, which plays a role in membrane dynamics and protein trafficking, will be covered as well.

1.2 Host-pathogen interactions

*Listeria monocytogenes*

One of the most instructive in providing clues about basic aspects of cell biology has been the study of *Listeria monocytogenes* and their use of the host actin-based motility machinery to move within the cell and to invade neighboring cells. *L. monocytogenes* is a Gram-positive bacterium that causes listeriosis, a disease characterized by severe gastroenteritis, encephalitis, meningitis, or septicemia [2]. The main outcome of intracellular movement is cell-to-cell spread, whereby bacteria push the plasma membrane of the host cell into an invagination in a neighboring cell, which then takes up the bacteria by phagocytosis [3]. By avoiding extracellular exposure, the bacteria escape the humoral immune system of the host. Researchers discovered that the bacterial protein ActA mimics host actin filament nucleation-promoting factors and recruits actin-binding proteins needed to assemble the actin-based force-producing machinery [4]. ActA forms a chimeric mimic of the functions of the host cell zyxin-
vinculin proteins, which recruit enabled-vasodilator-stimulated phosphoproteins (Ena-VASP) that drive actin filament elongation; and the Wiskott Aldrich Syndrome Proteins (WASP)-WASP verprolin (Wave) proteins, which recruit the actin-related proteins 2 and 3 (Arp2/3) complex that nucleates new branches of actin filaments from existing filaments [5]. Identifying the role of the Arp2/3 complex in bacterial intracellular motion has provided the groundwork for the current model of lamellipodial outgrowth [6], and *L. monocytogenes*-derived biometric motility assays are being used to elucidate the role of proteins in lamellipodial actin dynamics [5].

**Mammalian viruses**

Another example of the host-pathogen interaction shedding light on cell biological concepts is the use of mammalian viruses to study host endocytic systems. Because of their simplicity, viruses depend on host cell machinery for nearly all stages of their life cycle, including internalization of the virus and escape into the cytosol. Viruses utilize many forms of endocytosis to enter the host cell, including classical clathrin-coated pit (CCP)-mediated internalization, the most common pathway of virus entry [7]. However, knowledge of alternative endocytic routes has greatly increased from the study of viruses [8]. Studies of simian virus 40 (SV40) have revealed endocytosis from lipid rafts or caveolae followed by transport to the smooth endoplasmic reticulum (ER) [9, 10]. Many viruses, including Semliki Forest virus, utilize the low pH of endosomes to trigger escape into the cytosol, and research with this virus has delineated the kinetics of endosome acidification [11]. Thus, analysis of virus-host interactions has provided
insights not only into the infectious cycle, but also into the biology of the cell, and further research may highlight potential antiviral strategies.

1.3 Protein transport in the biosynthetic, endocytic, and autophagic systems

Biosynthetic sorting

Synthesis of new proteins and transport of those proteins to their correct location in the cell is a process that begins in the ER. The ER is a vast network of interconnected tubules, vesicles, and cisternae contiguous with the nucleus, which functions in the facilitation of protein folding and transport of proteins to the Golgi complex after budding from coatamer protein complex II (COPII) vesicles through an ER-Golgi intermediate complex (ERGIC) [12]. The ER also serves a quality control function by way of chaperone mediated protein folding and targeted retention and degradation of improperly folded proteins [13]. The Golgi is a network of cisternae stacks that is integral in modifying, sorting, and packaging proteins for excretion or transport throughout the cell, with the trans-Golgi network (TGN) as the endpoint for biosynthetic sorting.

The TGN is an important biosynthetic sorting station to direct the transport of newly synthesized secretory and membrane proteins to their correct sub-cellular destinations (Fig. 1.1). Appropriate sorting of transmembrane proteins is achieved by the recognition of specific sorting motifs in the cytosolic portion of a protein that are recognized by cytosolic adaptors and coat proteins. These sorting motifs include
consensus tyrosine-based motifs (NPXY, where X is any amino acid; YXXØ, where Ø is an amino acid with a bulky hydrophobic side chain) and di-leucine-based motifs ([DE]XXXL[L,I], and DXXLL) which bind to specific adaptor or coat proteins to mediate proper targeting [14-16]. For example, the clathrin adaptor protein (AP) complex AP-1 recognizes YXXØ or [DE]XXXL[L,I] motifs, while DXXLL motifs interact with Golgi-localized γ-ear containing ARF-binding proteins (GGAs) to mediate sorting of select protein cargo out of the TGN in CCPs [15]. The function of these interactions is highlighted by the mannose 6-phosphate receptor (MPR), which transports newly synthesized acid hydrolases from the TGN to endosomes where the cargo is delivered and the receptor recycles back to the TGN [17]. Mutations in the AP-1 or GGA binding sites of MPR results in impaired sorting of lysosomal enzymes [18-21], providing an example of the importance of proper TGN sorting for protein function and cell physiology.

**Endocytic sorting**

The endocytic pathway is a complex system of vesicular structures that routes cargo to and from different sub-cellular destinations (Fig. 1.2). Vesicles are formed after internalization of select cargo by a number of different mechanisms, including clathrin-mediated endocytosis. Vesicles release their protein coat and fuse to form early endosomes (EEs), and the lumen begins to acidify thru the action of H⁺-ATPases. Maturation of EEs occurs with the invagination of the limiting membrane to produce internal vesicles. These structures, termed multivesicular bodies (MVBs), have a number
of specialized functions including sorting of protein cargo, attenuation of receptor
signaling, and retrograde transport in neurons [22]. MVBs continue to mature and
acquire additional luminal vesicles as they are transported along microtubules (MTs)
towards the microtubule organizing center (MTOC), eventually becoming late endosomes
(LEs), which then fuse with the lysosome (Ly) to degrade cargo in internal vesicles. The
endocytic pathway is a dynamic process with the capability to recycle as well as degrade
protein cargo depending on the physiology of the cell. There are a number of proteins
that regulate vesicular trafficking and cargo selection throughout the endocytic system,
including the endosomal sorting complex required for transport (ESCRT) and the Rab
family of small guanine triphosphatases (GTPases).

ESCRT machinery

The protein products of the yeast Class E subset of vacuolar protein sorting (Vps)
genes comprise the ESCRT machinery. These genes are required for proper sorting into
the yeast vacuole or mammalian Ly. An abundance of genetic and biochemical data has
been collected to support a role for these proteins in the biogenesis of MVBs and the
sorting of ubiquitinated cargo into internal vesicles of MVBs [23, 24]. The ESCRT
machinery consists of three multisubunit protein complexes; ESCRT-I, ESCRT-II, and
ESCRT-III, each comprised of multiple protein subunits, in addition to a number of
ESCRT-associated proteins that regulate their activity (Fig. 1.3, Table I). ESCRT-I
interacts with a complex of Vps27-Hse1 (sometimes referred to as ESCRT-0) to mediate
cargo selection and membrane interaction. The ubiquitin interacting motif (UIM) domain
of Vps27 recognize and bind mono-ubiquitinated proteins, including ligand activated epidermal growth factor (EGF) receptor (EGFR) for targeting to Ly [25]. Membrane and cargo bound ESCRT-0 then recruits ESCRT-I, which facilitates recruitment of ESCRT-II, which can then recruit ESCRT-III, passing the cargo from complex to complex. ESCRT-III forms a lattice on the limiting membrane of MVBs and sorts select cargo into internal vesicles, after which the complex disassembles. In addition to regulating endosomal sorting, the ESCRT machinery is hijacked by a number of enveloped retroviruses, including HIV, to facilitate in the life cycle of the virus [26-28]. Moreover, a number of ESCRT proteins are required to restrict the growth of *Mycobacterium tuberculosis*, a pathogen that is able to survive within macrophages by blocking the maturation of endosomes [29]. The significance of the ESCRT machinery in the sorting of endocytic cargo and the ability of pathogens to usurp their functions highlights the importance of these proteins in the physiology of the cell and the pathophysiology of disease.

**Rab function in endocytosis**

The Rab family of small GTPases is another group of proteins that play a major role in protein trafficking in both endocytic and exocytic systems [30-32]. Rabs regulate a number of sorting steps, including vesicle formation, motility along microtubules, vesicle tethering, and fusion, and function as molecular switches, being active in their guanine triphosphate (GTP)-bound state and inactive in their guanine diphosphate (GDP)-bound state. Furthermore, Rabs are believed to determine organelle identity by
the presence of specific Rab proteins on the membrane of each organelle [33-35]. For example, Rab5 localizes to EEs, while Rab7 localizes to LEs, and vesicles abruptly convert from Rab5 identity to Rab7 as they mature [36]. Rabs are cytosolic proteins that require post-translational lipid modification to mediate membrane binding, by way of dual prenylation of carboxy-terminal cysteine motifs. Once associated with the membrane, Rab GTPases cycle between inactive and active states, facilitated by a number of accessory proteins including guanine nucleotide exchange factors (GEFs), GTPase activating proteins (GAPs), Rab escort proteins (REPs), and GDP dissociation inhibitors (GDIs). In the active state, Rabs interact with effector proteins that aid in carrying out the diverse functions of specific Rabs in endocytic sorting [37].

Of interest to my research, Rab7 regulates sorting through LEs [35]. Transport of proteins through LEs is carried out by a set of effector proteins that interact with Rab7 and mediate its function. These proteins include Rab7 interacting lysosomal protein (RILP) [38], Rabring7 [39], hVPS34/p150 phosphatidylinositol 3-kinase (PI3K) complex [40], the α-proteasome subunit XAPC7 [41], and oxysterol binding protein related protein 1 long (ORP1L) [42]. Rab7 regulates LE trafficking by forming a tripartite complex along with RILP and ORP1L, that recruits dynein-dynactin motors necessary for minus-end directed transport of vesicles along microtubules towards the MTOC [43] (Fig. 1.4). Aside from their interactions with Rab7, RILP and ORP1L have other significant functions in the cell. RILP also interacts with Rab34 and may play a role in lysosome positioning [44], as well as the ESCRT-II proteins VPS22 and VPS36 to potentially regulate MVB biogenesis [45-47]. As its name would suggest, ORP1L is part of a family of oxysterol binding proteins (OSBP) that was shown to bind cholesterol and
25-hydroxycholesterol (25-HC) \textit{in vitro} [48]. However the role of sterol binding by ORP1L is at this time unknown.

The importance of Rab7 is evidenced by mutations that cause the peripheral neuropathy Charcot-Marie-Tooth (CMT) disease (specifically CMT2B) [49]. Furthermore, a number of bacterial pathogens impair Rab7 function for their survival, including but not limited to \textit{Coxiella burnetii}, \textit{Helicobacter pylori}, and \textit{Salmonella enteric} [50]. Aside from its role in the endocytic pathway, Rab7 is also an important player in other membrane sorting events such as melanosome trafficking [51, 52], phagosome maturation [53], and autophagy [54-56].

\textbf{Autophagy}

Autophagy (also referred to as macroautophagy) is a normal degradative pathway involving the sequestration of portions of the cytoplasm or organelles in characteristic double-membraned vesicles termed autophagosomes, which fuse with the lysosome to degrade the sequestered material (Fig. 1.5). Autophagy is an important process for cell survival during nutrient deprivation, and for the turnover of long-lived proteins and the degradation of cytoplasmic aggregates [57-59]. In most settings, autophagy is induced by the de-repression of the mammalian target of rapamycin (mTOR) Ser/Thr kinase, which inhibits induction of autophagy by targeted phosphorylation of the autophagy gene (Atg) protein Atg13 [60]. One of the initial steps of autophagy involves membrane vesicle nucleation, which requires the activation of Vps34, a class III PI3K, and the formation of a multiprotein complex that includes Beclin 1 and Atg14 [61]. Elongation of the
Autophagic vesicles occur by an ubiquitin-like conjugation system, and by the addition of phosphatidylethanolamine (PE) to LC3/Atg8. LC3-I is a cytosolic protein that, upon lipiddation (LC3-II), is recruited to the isolation membrane or pre-autophagosomal structure (PAS). The function of LC3-II is unknown, however it does form the basis of two widely used autophagy assays: fluorescence microscopy, and biochemical analysis by SDS-PAGE since PE addition to form LC3-II increases its electrophoretic mobility on SDS-PAGE gels compared to LC3-I. Autophagosomes mature and eventually fuse with the lysosome, facilitating the degradation of luminal content. Rab7 is believed to function in the maturation of autophagosomes, potentially by transporting the vesicles towards lysosomes along microtubules [55]. Interestingly, the PI3K Vps34 complex containing Beclin 1 functions in both endocytosis (UVRAG) and autophagy (Atg14) [61, 62], revealing the crosstalk of these components with both membrane trafficking systems. Furthermore, the autophagy machinery can be hijacked by poliovirus to mediate RNA replication, which occurs on LC3-II positive, double-membrane vesicles [63]. It has also been shown that infection of cells with oncolytic adenoviral mutants induces autophagy as a mechanism of host cell survival [64].

Autophagy can be selective or, as described above, non-selective. Selective autophagy was originally identified in the yeast cytoplasm-to-vacuole targeting (Cvt) pathway in which precursor hydrolases are delivered to the vacuole (analogous to mammalian Ly) where they carry out their function [65, 66]. Newly synthesized hydrolases oligomerize in the cytosol and are recognized by their receptor Atg19, which is then recruited to the PAS by additional Atg proteins including LC3/Atg8 [67]. After autophagosome formation is complete, the autophagosome fuses with the vacuole where
the receptor is degraded. Selective autophagy also occurs in mammalian cells, for example when invading bacteria escape into the host cytosol. Intracellular bacteria, including *Streptococcus pyogenes* are targeted for autophagy, likely mediated by an unknown bacterial factor that is recognized by the host autophagic machinery [68]. Another example of mammalian selective autophagy is the targeted degradation of ubiquitinated protein aggregates. The polyubiquitin-binding protein p62/SQSTM1 mediates incorporation of ubiquitinated protein aggregates into autophagosomes through an interaction with LC3 [69].

1.4 Adenovirus

Adenovirus (Ad) has also played a role in understanding the fundamentals of cell biology. There are six groups of Ad, designated A through F, based on a number of criteria including restriction endonuclease digestion of the virus hexon capsid gene [70]. Ad is a non-enveloped double-stranded DNA virus with an icosahedral protein shell composed of 240 trimers of the hexon protein along with 12 pentamers of the penton base protein that create the vertices of the icosohedron, with fibre-like projections protruding from each penton [71]. Infection of humans with Ad causes several diseases of the respiratory, gastrointestinal and ocular systems. Approximately 5-10% of acute respiratory disease in children under the age of 5 is due to group C Ad infection [72]. Although acute infections that are rapidly cleared are more common, latent Ad infections where the virus persists is also an outcome.
The classical adenovirus infection pathway can be roughly divided into five stages: binding, entry, escape, translocation, and nuclear import (summarized in Fig. 1.6). Group C Ad binds to the cell surface through a high affinity interaction of the fiber knob with the coxsackie and adenovirus receptor (CAR) [73], and a lower affinity interaction between the fiber shaft and heparan sulfate proteoglycans [74]. A third interaction of the penton base with various integrins is believed to initiate internalization [75-78]. Entry of the virus occurs by classical CCP-mediated endocytosis which is dependent on dynamin [79] and the presence of free cholesterol in the plasma membrane (PM) [80]. After CCP separation, the endosome acidifies through the activity of vacuolar H⁺-ATPases thus triggering changes in the viral capsid that results in lysis of the endosomal membrane [81], where cholesterol is also required for escape [82]. Escape of the viral capsid to the cytosol is followed by capsid binding to dynein-dynactin motors and transport along microtubules toward the MTOC [83]. Finally, the viral capsid interacts with the nuclear pore complex through multiple protein interactions, allowing for import of the viral genome and the initiation of transcription [84-86].

Group C Ad possesses a linear, double-stranded DNA genome comprised of multiple transcription units (Fig. 1.7 A). Both ends of the genome contain a region of inverted terminal repeats [87] that when separated, anneal to form panhandle structures believed to be DNA replication intermediates [88]. The remaining Ad genome is divided into regions corresponding to both function and time of expression post-infection, including 6 early region (E) transcription units (E1A, E1B, E2A, E2B, E3, E4) and one late region (L1) unit. Each unit encodes multiple mRNA transcripts that come about from differential splicing and alternate start codon usage [89]. Interestingly, the
discovery of mRNA splicing arose from the study of the L1 transcription unit [90, 91], indicating another case where the study of host-pathogen interactions provided clues to the inner workings of the cell itself.

As previously mentioned, the Ad genome is divided into early and late regions based on function and time of expression after infection. The E1A and E1B transcripts activate transcription of the other early genes [92, 93], are capable of transforming rodent cells [94], and have been used to generate commonly used tissue culture cell lines including HEK293 [95]. The E2 region (E2A and E2B) encodes DNA binding proteins and a DNA polymerase necessary for viral genome replication [96]. The E3 region is dispensable for replication, but serves an immunosuppressive function, and will be discussed later in further detail. The E4 region transcripts have a very diverse set of functions, including RNA stabilization and transport, DNA replication, and cell transformation [97]. Finally, the late region is a family of five transcripts (L1-L5) that codes for the proteins of the viral capsid [98, 99].

Dissection of the Ad genome has proven to be important for the construction of Ad-based gene therapy vectors. The main goal of gene therapy is to treat loss-of-function genetic disorders by delivering correcting therapeutic DNA sequences into the nucleus of a cell, allowing its long-term expression at physiologically relevant levels, and human Ads were initially evaluated as vectors to treat cystic fibrosis [100]. Ad vectors are one of the most widely used vector systems for gene transfer studies, and have been used in one-quarter of all clinical gene therapy trials to date (Gene Therapy Clinical Trials Worldwide database, updated September 2008; http://www.wiley.co.uk/genmed/clinical). Deletion of Ad regions E1 and E3 to make room for the gene of interest has progressed to
removal of all Ad genes [101]. However, a major disadvantage of these Ad vectors lies in the activation of both the innate [102-104] and adaptive [105, 106] immune response of the recipient. Infection with Ad vectors containing an E3 deletion causes increased lymphocyte and macrophage/monocyte inflammatory responses compared to full-length Ad [107]. Although none of the E3 region gene products are essential for replication, they do play a role in regulating the host immune response, warranting the re-introduction of this region into Ad-based vectors for gene therapy.

E3 region gene products

The E3 region is of interest for its role in modulating the host immune response. Transcription of the E3 region yields a common mRNA that undergoes differential splicing to make eight overlapping mRNAs which are translated into six known proteins (Fig. 1.7 B). These include E3-gp19k, E3-14.7, E3-11.6k, E3-6.7, RIDα (formerly known as E3-13.7 and E3-10.4), and RIDβ (formerly known as E3-14.5) which were named originally based on their molecular weight. Several reviews have been published detailing the function of the region and each individual protein [108-116]. To parse out the functions of these proteins, researchers have studied the phenotype of Ad deletion mutants, as well as ectopic expression of individual genes in tissue culture cells. Furthermore, various methods to discover protein-protein interactions have been utilized, including yeast-two hybrid and glutathione S-transferase (GST)-pull down assays.

E3-gp19k is a type I integral membrane protein that resides in the ER due to a carboxy-terminal ER retention motif [117]. This E3 protein protects infected cells by
interacting with and retaining major histocompatibility complex (MHC) class I antigens in the ER, thereby preventing cell lysis by cytotoxic T lymphocytes (CTLs) [118, 119]. In addition, E3-gp19k binds a second protein, transporter of antigenic peptides (TAP), which functions in the maturation of MHC class I molecules. The outcome of this interaction is that E3-gp19k causes a delay in the expression of antigens on the surface of infected cells [120].

E3-14.7 modulates the host immune response by inhibiting tumor necrosis factor alpha (TNFα) induced apoptosis, doing so without affecting the surface expression of the TNF receptor (TNFR1) [121, 122]. E3-14.7 is a cytoplasmic protein that has been shown to associate with the nucleus [122], and can block TNF-mediated activation of phospholipase A2 and release of arachidonic acid [123]. Furthermore, it was discovered that E3-14.7 interacts with FIP-1, a novel member of the low-molecular-weight GTP-binding protein family [124]. Using a yeast two-hybrid system, the authors identified two additional proteins (FIP-2 and FIP-3), where FIP-3 modulates the activity of nuclear factor (NF)-kappaB [125, 126]. It was further shown that E3-14.7 blocks that ability of the NF-kappaB p50 homodimer from binding to DNA to promote transcription of anti-inflammatory genes [127], and also by inhibiting ligand-induced TNFR1 internalization [128].

E3-11.6k is an atypical E3 protein in that the majority of the protein is synthesized at very late stages of an infection from the major late promoter, but minimally in early stages of infection from the E3 promoter [129]. E3-11.6k is a type III integral membrane protein that localizes to the nuclear membrane [130] and has been renamed adenovirus death protein (ADP) because it is required for efficient lysis of Ad-
infected cells and subsequent Ad release [129]. Although the mechanism of ADP-mediated cell death is not known, it was found to interact with mitotic arrest deficiency 2B (MAD2B) protein by a yeast-two hybrid screen [131]. When overexpressed in A549 cells, MAD2B countered the lytic function of ADP, suggesting the interaction is biologically relevant.

E3-6.7 is a type III integral membrane protein found in the ER [132], and functions in conjunction with two other E3 proteins, RIDα and RIDβ, which form the receptor internalization and degradation (RID) complex [133]. The RID complex will be discussed in greater detail below. Independent of the RID complex however, E3-6.7 functions to maintain calcium levels in the ER, and to prevent induction of apoptosis by the release of arachidonic acid [134].

**RIDα and the RID complex**

RIDα is the focus of this study, and was originally identified by its ability to downregulate the EGFR [135] by a mechanism independent of ligand activation [136], EGFR tyrosine kinase activity [137], and receptor ubiquitination [138], and also independent of other Ad genes [139]. RIDα is a type II integral membrane protein that has a short amino-terminal cytosolic domain, followed by two transmembrane domains separated by an exocytic loop region, and a carboxy-terminal cytoplasmic domain of 29 amino acids [140]. RIDα is cleaved in the exocytic loop after the first transmembrane domain creating a shorter isoform of 11.3 kDa [141]. A critical cysteine residue in the loop domain mediates dimerization through post-translational addition of a disulfide
bond, producing homo- and heterodimers of the protein [140] (Fig. 1.8). RIDβ is a type I integral membrane protein oriented with an exocytic amino-terminus and a cytoplasmic carboxy-terminal tail [142]. RIDβ is also serine and threonine phosphorylated, and O-glycosylated [143, 144].

As stated above, one of the functions of RIDα is to divert constitutively recycling EGFR to Ly for degradation [135]. We have demonstrated that RIDα alone can mediate this activity [139, 145], although there are conflicting reports that RIDβ is also required to downregulate the EGFR [146]. RIDα does cooperate with RIDβ to form the RID complex, which is composed of two α subunits and one β subunit. The RID complex mediates cell surface removal of TNFR1 [147], and Fas [148, 149]. Furthermore, together with E3-6.7, the RID complex activates the internalization and degradation of TNF related apoptosis inducing ligand receptor (TRAIL-R1) [133] and TRAIL-R2 [150]. Downregulation of these receptors blocks the ability of cells to initiate pro-inflammatory and pro-apoptotic signaling pathways, thus suppressing the host immune response [147-149, 151, 152]. Though the outcome of RIDα-mediated EGFR downregulation in regards to host immunomodulation is unknown, one possibility may be to decrease the production and release of the chemokine interleukin-8 (IL-8) downstream of a well characterized TNFα-TNFR-EGFR signaling cascade [153-155; Shah AH, Rubio ML and Carlin CR unpublished data].

RIDα was initially reported to localize to the PM based on sucrose gradient fractionation, which required co-expression of RIDβ [156]. However, these data were likely misinterpreted because the researchers did not colocalize the RIDα/RIDβ fraction with any known PM markers. Furthermore, we have found that RIDα PM localization
can only be achieved by severe overexpression of the protein [141]. Studies by our laboratory in Ad-infected cells have indicated that RIDα is present on the limiting membrane of MVBs as assayed by cryo-electron microscopy [157], and also colocalizes with markers for EEs and MVBs by confocal microscopy and Percoll gradient cell fractionation [145, 157]. We have also demonstrated that RIDα localizes to perinuclear vesicles when expressed in cells separately from other Ad proteins, and that proper RIDα localization is dependent on microtubules. Disruption of microtubule filaments with nocodazole caused the dispersal of RIDα positive vesicles throughout the cytosol, while disruption of actin filaments with cytochalasin D had no effect [145].

While we have shown that RIDα interacts with the EGFR by co-immunoprecipitation [157], the mechanism of RIDα-mediated EGFR downregulation has not been elucidated. We have recently determined that RIDα mimics GTP-Rab7, raising the possibility that RIDα has a direct effect on LE trafficking [145]. As stated above, Rab7 regulates sorting of late endocytic cargo by binding multiple effector proteins including RILP, and ORP1L [38, 42]. Though RILP and ORP1L have independent functions beyond their interaction with Rab7, a RILP-Rab7-ORP1L tripartite complex is thought to function in the recruitment of dynein-dynactin motor complexes to initiate minus-end directed transport of endosomes along microtubules towards the MTOC [43]. RIDα mimics Rab7 by binding these same two effector proteins, which were identified by a yeast-two hybrid screen, and verified by a GST pull down assay [145]. Interestingly, a functional RILP interaction is necessary for RIDα-mediated downregulation of EGFR and Fas, illustrating the importance of this protein in the activity of RIDα. In the future it will be interesting to test the ability of RIDα to
concomitantly bind RILP and ORP1L to form a tripartite complex similar to Rab7 to facilitate the recruitment of microtubule motors.

1.5 Cholesterol homeostasis

As previously stated, membrane cholesterol plays an important role in the internalization and escape of Ad into the cytosol. Cholesterol homeostasis is a vital aspect of cell physiology and membrane dynamics, and is regulated by a complex set of molecules. At the cellular level, cholesterol levels are controlled by the influx of exogenous cholesterol in the form of low density lipoprotein (LDL), de novo synthesis of cholesterol, catabolism of cholesterol to bile acids or other metabolites, or efflux of cholesterol out of cells. The LDL receptor (LDLR) was first identified by the team of Brown and Goldstein from the study of familial hypercholesterolemia [158], and the receptor-ligand complex is internalized through clathrin-mediated endocytosis. As the pH of EE decrease, LDL is released from the LDLR and the receptor recycles back to the PM to be reutilized for more rounds of LDL delivery. LDL is then delivered to LE/Ly where cholesterol esters in LDL are hydrolyzed to free cholesterol, which is exported out of this compartment by a mechanism that is not fully characterized. Cholesterol is delivered to different membranes in the cell, with the highest concentration in the PM and low levels of cholesterol in the ER. Cholesterol synthesis is regulated by sterol-sensing proteins in the ER and is under tight feedback control that is sensitive to small changes in cholesterol concentration. The sterol regulatory element binding protein (SREBP) is an ER resident transmembrane protein that is cleaved in the Golgi to yield active
transcription factors that activate all of the genes required for cholesterol synthesis and LDL uptake [159, 160]. During periods of low sterol load, SREBP cleavage-activating protein (SCAP) binds to SREBP, clustering it in COPII-coated vesicles and escorts it to the Golgi for cleavage [161]. SREBP is cleaved to form a transcription factor that translocates to the nucleus. As a consequence, target gene transcription increases and cholesterol levels rise. When sterol levels are high, cholesterol binds SCAP and alters its conformation allowing another protein termed Insig to bind the complex and occlude SCAP from interacting with COPII proteins, thus blocking the transport of SREBP to the Golgi for processing [162, 163]. Cholesterol levels then decrease due to the inhibition of gene transcription. Cholesterol levels are also sensed by oxysterols, which are metabolites of cholesterol created by hydroxylation of the different cholesterol side chains. 25-HC binds to Insig, allowing it to bind to SCAP/SREBP and block the transport and processing of SREBP, resulting in a decrease in cellular cholesterol levels [164, 165].

As a reward for their work in the discovery of the LDLR and its role in the control of cholesterol metabolism, Brown and Goldstein received the Nobel Prize in 1985, and this group has continued to illuminate the mechanisms of the regulation of cholesterol homeostasis including the elucidation of the SREBP pathway.

Cholesterol biosynthesis occurs in the ER after SREBP-dependent expression of the appropriate genes. Cholesterol is synthesized from acetate in a series of about 30 enzymatic reactions, with hydroxymethylglutaryl CoA reductase (HMGR) as the rate-limiting enzyme in the pathway. Cholesterol levels are also regulated by liver X receptors (LXRs), a family of sterol-sensing transcription factors. LXRs are activated by a variety of oxysterols, and act to lower cellular cholesterol levels by expression of
proteins involved in cholesterol metabolism and by conversion of cholesterol into bile acids in the liver [166].

As stated above, the egress of cholesterol out of LE/Ly is not fully characterized, however two proteins implicated in this action are NPC1 and NPC2. A major clue to the mechanism of cholesterol egress from LE/Ly came from studies in cells from patients with the autosomal recessive disease Niemann-Pick type C (NPC) [167]. NPC1 is a polytopic membrane protein located in LE/Ly [168], while NPC2 is a soluble protein localized to Ly [169]. Mutations in either gene produce the same disease phenotype, suggesting these two proteins function in the same pathway for endosomal cholesterol egress [170]. Both NPC1 and NPC2 possess sterol binding domains that mediate interaction with cholesterol and sterol derivatives [171-175], with NPC1 and NPC2 binding to opposite ends of the cholesterol molecule [176]. Since LDL-derived cholesterol egress out of LE/Ly to the ER is unidirectional, it has been proposed that NPC2 shuttles cholesterol to NPC1, which then inserts cholesterol into the membrane [176]. This would allow for the transport of cholesterol to the ER by an as yet unidentified carrier protein or vesicle.

**NPC disease**

The mechanism of NPC1 and NPC2 function will prove to be important not only for the understanding of the biology of cholesterol transport, but also for the treatment of NPC. As stated above, NPC is an autosomal recessive disorder that manifests as progressive neurodegeneration in afflicted patients, an estimated 1:150,000. NPC is one
of a number of single gene disorders that affects cholesterol transport, a list that includes familial hypercholesterolemias, Wolman disease, Tangier disease, sitosterolemia, Smith-Lemli-Opitz syndrome, cerebrotendinous xanthomatosis, congenital lipoid adrenal hyperplasia, and hyperbetalipoproteinemias [177]. Mutations to either NPC1 or NPC2 cause the accumulation of cholesterol and other lipids, including sphingolipids (like GM₁ ganglioside) and lysobisphosphatidic acid (LBPA), in aberrant Ly termed lysosomal storage organelles (LSOs). As a result, cholesterol is not transported to the ER and sensed by the machinery that regulates cholesterol homeostasis, and synthesis of new cholesterol is activated along with uptake of exogenous LDL. Increasing amounts of cholesterol then become trapped in LSOs, compounding the disease phenotype [178]. Further complications occur because of defects in the trafficking of select protein cargo that are normally routed through LE/Ly but persist in LSOs due to the increase in membrane cholesterol in this compartment. As detailed above, MPR normally traffics through LE to the TGN after delivering its cargo. However, MPR is redistributed to LSOs in fibroblasts with a mutation to NPC1 [179]. The transferrin (Tf) receptor (TfR) is another protein that is mis-sorted in NPC fibroblasts. The TfR internalizes exogenous Tf and the receptor recycles back to the PM after delivering its cargo, similar in trafficking to the LDLR. In NPC cells, a portion of the TfR pool is redirected to LEs and accumulates in cholesterol- and LBPA-rich LSOs [180, 181]. What effect these altered trafficking itineraries have on NPC pathophysiology is unknown, however NPC patients have been reported to have reduced levels of ferritin, a protein involved in the sequestration of Fe^{+3} [182]. Hydrolase sorting and lysosomal function are normal in NPC cells, likely due to the two distinct MPRs, the cation-dependent MPR (MPR46) and
cation-independent MPR (MPR300). MPR300 is believed to be transported from the TGN to EEs and back, without becoming trapped in LSOs [183], thus allowing for the delivery of lysosomal enzymes. Along with lipid and protein mis-sorting phenotypes, NPC is associated with increased levels of autophagy, which could potentially be involved in the degradation of these aggregates [184]. Failure of autophagy, which is particularly important for the maintenance of long-lived post-mitotic cells such as neurons, is thought to be a major cause of neurodegeneration that is ultimately fatal.
1.6 Figures

**Figure 1.1. Protein sorting through the biosynthetic pathway.**

Proteins are synthesized in the ER and are exported to the ERGIC through COPII coated vesicles, then transported to the Golgi complex. Protein cargo is trafficked through the Golgi stacks where they can be modified by glycosylation or other post-translational modifications. Proteins then enter the TGN and are trafficked to their appropriate subcellular destination. Some protein cargo are exported out of the TGN through CCPs through interactions with clathrin adapters including AP-1, which recognize tyrosine and di-leucine based sorting motifs in the protein cargo and direct them to CCPs. In some cases, protein cargo is transported back to the TGN from endosomes. Retrograde transport also occurs from the Golgi to the ER, and is mediated by COPI. Abbreviations: AP, adapter protein; CCP, clathrin-coated pit; COP, coatamer protein; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate complex; PM, plasma membrane; TGN, trans-Golgi network.
Figure 1.2. Protein sorting through the endocytic system.

Ligand activated receptors are concentrated in CCPs and internalized from the PM. The clathrin coat disassembles and vesicles fuse to form EEs, which are positive for Rab5. EEs mature and switch to a Rab7 identity and become MVBs/LEs. MVBs are characteristic for multiple internal vesicles which occur by internal invagination of the limiting membrane. Cargo is sorted into internal vesicles by the action of the ESCRT machinery which recognizes ubiquitinated protein cargo (see Fig. 1.3), and MVBs are transported along MTs through Rab7 mediated recruitment of MT motor proteins (see Fig. 1.4). MVBs/LEs eventually fuse with the lysosome to degrade internal vesicles and cargo. Cargo can also be recycled back to the PM from EEs and the limiting membrane of MVBs through REs. Abbreviations: AP, adaptor protein; CCP, clathrin-coated pit; EE, early endosome; ESCRT, endosome sorting complex required for transport; LE, late endosome; MT, microtubule; MVB, multivesicular body; PM, plasma membrane; RE, recycling endosome; PI3P, phosphatidylinositol-3-phosphate; Ub, ubiquitin.
Figure 1.3. Schematic of ESCRT machinery in MVB sorting.

Vps27 binds PI3P on endosomal membranes resulting in the membrane docking of the Vps27-Hse1 complex (also referred to as ESCRT-0). Vps27 also mediates recruitment of ubiquitinated cargo through interaction with its UIM domain for sorting into MVB internal vesicles. Vps27-Hse1 then recruits ESCRT-I through an interaction of Vps27 with Vps23 of ESCRT-I. Protein cargo marked for degradative sorting is passed to ESCRT-I, as Vps23 binds ubiquitinated cargo. Vps28 of ESCRT-I recruits ESCRT-II through an interaction with Vps36 of ESCRT-II. Vps36 mediates interactions with ubiquitinated cargo and membrane interaction through PI3P binding. Vps25 of ESCRT-II recruits and interacts with Vps20 of ESCRT-III, which forms a lattice on endosomal membranes. ESCRT-III facilitates cargo sorting into internal vesicles after deubiquitination of cargo by Doa4 (through an interaction with Bro1). The AAA-ATPase Vps4 then mediates disassembly of the ESCRT-III complex and recycling of its components. Abbreviations: ESCRT, endosome sorting complex required for transport; MVB, multivesicular body; PI3P, phosphatidylinositol-3-phosphate; Ub, ubiquitin; UIM, ubiquitin interacting motif.
Table 1.1. Components of the ESCRT machinery.

The ESCRT machinery plays an important role in biogenesis of MVBs and the sorting of ubiquitinated cargo into internal vesicles of MVBs [23, 24]. The ESCRT machinery consists of three (or four) multisubunit protein complexes; ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III. Each complex is comprised of multiple protein subunits, in addition to a number of ESCRT-associated proteins that regulate their activity. Abbreviations: PI3P, phosphatidylinositol-3-phosphate.
Table 1.1. ESCRT machinery components.

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<th>Mammalian</th>
<th>Function</th>
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<td>EAP30, SNF8</td>
<td>Assembly with ESCRT-III (Vps20)</td>
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<td></td>
<td>Vps25</td>
<td>EAP20</td>
<td>Interaction with cargo and PI3P, assembly with ESCRT-I</td>
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<td></td>
<td>Vps36</td>
<td>EAP45</td>
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<tr>
<td>ESCRT-III</td>
<td>Vps20</td>
<td>CHMP6</td>
<td>Assembly with ESCRT-II (Vps25)</td>
</tr>
<tr>
<td></td>
<td>Vps32/Snf7</td>
<td>CHMP4A, B, C</td>
<td>Membrane deformation, vesicle invagination</td>
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<td></td>
<td>Vps2/Did4</td>
<td>CHMP2A, B</td>
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<td>Vps24</td>
<td>CHMP3</td>
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<td>Vps4</td>
<td>VPS4, B/SKD1</td>
<td>ESCRT disassembly and recycling</td>
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<td>Vps31/Bro1</td>
<td>ALIX/AIP1</td>
<td>Doa4 recruitment, interaction with ESCRT-III</td>
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<td>Vps60/Mos10</td>
<td>CHMP5</td>
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<td>Vps46/Did2</td>
<td>CHMP1A, B</td>
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<td>Vta1</td>
<td>LIP5</td>
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Figure 1.4. RILP-Rab7-ORP1L tripartite complex involved in vesicle transport along microtubules.

(1) Rab7 is activated by GDP-GTP exchange and is prenylated to mediate attachment to LE. (2) GTP-Rab7 interacts with RILP and ORP1L to form a tripartite complex. (3) RILP then recruits the dynein-dynactin microtubule motor complex by interacting with the p150\textsuperscript{Glued} projecting arm of dynein. (4) ORP1L mediates the transfer of the dynein-dynactin complex to a βIII spectrin coat to facilitate minus-end directed transport of the endosome along microtubules. (5) Rab7 is inactivated by GTP hydrolysis and deprenylation to allow for the cycle to start again. Abbreviations: GDI, GDP dissociation inhibitor.
**Figure 1.5. Autophagic protein sorting.**

Autophagy is induced by a number of cellular stresses, and begins with the engulfment of cytoplasmic material (cytoplasm, organelles, protein aggregates, etc.) by the phagophore, or PAS. The initial nucleation of the phagophore is mediated by PI3K complex I, which is composed of the PI3K Vps34/p150, the adaptor protein Beclin-1, and Atg14. The phagophore is also marked by the localization of cytosolic LC3 to the membrane after ubiquitin-like modifications and the addition of PE to mediate membrane insertion. The phagophore elongates to form the autophagosome, which can degrade its contents by two ways. (1) The autophagosome can dock and fuse with the lysosome to form an autolysosome, or (2) the autophagosome can fuse with an MVB/LE to form an amphisome, which then fuses with the lysosome to mediate degradation of autophagic and endocytic cargo. Interestingly, PI3K complex II, composed of Vps34/p150, Beclin-1, and UVRAG, has been shown to function in endocytic sorting [61]. Abbreviations: 3-MA, 3-methyladenine; EE, early endosome; LE, late endosome; MVB, multivesicular body; PAS, pre-autophagosomal structure; PE, phosphatidylethanolamine; PI3K, phosphatidylinositol 3-kinase.
Figure 1.6. Schematic of human group C adenovirus entry.

Ad binds the surface of a host cell through a high affinity interaction of the Ad fiber knob (blue) with CAR, and another interaction of the penton base with integrins (1). These interactions mediate Ad internalization through CCPs (2), which pinch off to form endosomes. As the pH of endosomes becomes more acidic, changes in the viral capsid result in lysis of endosomal membranes allowing escape of the virus into the cytosol (3). The viral capsid then interacts with dynein-dynactin motors for minus-end directed transport of the virus along microtubules towards the nucleus (4). Finally, the viral capsid interacts with the nuclear pore complex (5) and delivers its dsDNA into the nucleus to facilitate transcription of early Ad genes (6). Abbreviations: Ad, adenovirus; dsDNA, double-stranded DNA; CAR, coxsackie and adenovirus receptor; CCP, clathrin-coated pit; MT, microtubule; PM, plasma membrane.
Figure 1.7. Map of group C Ad2 genome and transcription map of E3 region.

(A) Schematic of the 36 kb, dsDNA Ad type 2 genome, which is divided into early (E1-E4) and late (L1-L5) regions based on time of gene expression post-infection. Both DNA strands encode for mRNA transcripts as depicted by the directionality of the arrows, with the E3 region highlighted in light blue. (B) Transcription map of the E3 region illustrating the 8 known mRNA transcripts (a-h) and their protein products (blue), and host targets (green). All mRNA transcripts arise from a single promoter and by alternative splicing as indicated by the grey bars. The protein that is the focus of this study, RIDα, is starred. Abbreviations: Ad, adenovirus; dsDNA, double-stranded DNA; E, early; L, late.
Figure 1.8. Membrane topology schematic of RIDα.

RIDα is a 13.7 kDa transmembrane protein with a short cytosolic amino-terminus, followed by two transmembrane domains separated by an exocytic loop region, and a cytosolic carboxy-terminus of about 29 amino acids. For convenient detection of the protein by various techniques, we added a FLAG epitope tag to the amino-terminus of the protein (red triangle) [185, 186]. RIDα is cleaved in the exocytic loop between A22 and A23 by signal peptidase, creating a shorter isoform of 11.3 kDa [140, 141]. A C residue at position 31 mediates dimerization through post-translational addition of a disulfide bond, producing homo- and heterodimers of the protein [140]. The cytosolic carboxy-terminus includes amino acids important for palmitoylation (C67) [186], TGN exit through interaction with AP-1 (Y72) [185], GTP-Rab7 mimicry through interactions with RILP (di-histidine motif at H75) and ORP1L (77-PQYR) [145], and a di-leucine EGFR homology domain necessary for RIDα-mediated EGFR downregulation at L87 [138].

Abbreviations: A, alanine; AP, adaptor protein; C, cysteine; D, aspartic acid; EGFR, epidermal growth factor receptor; H, histidine; I, isoleucine; L, leucine; P, proline; Q, glutamine; R, arginine; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
Chapter 2

A tyrosine-based signal plays a critical role in the targeting and function of adenovirus RID\(\alpha\) protein

2.1 Summary

E3 genes of human adenoviruses contribute to the virus life cycle by altering trafficking of cellular proteins involved in adaptive immunity and inflammatory responses. The ability of E3 genes to target specific molecules suggests they could be used to curtail pathological processes associated with these molecules and treat human disease. However this approach requires genetic dissection of the multiple functions attributed to E3 genes. The purpose of this study was to determine the role of targeting on the ability of the E3-encoded protein RID\(\alpha\) to downregulate the EGFR. A fusion protein between the RID\(\alpha\) cytoplasmic tail and GST was used to isolate clathrin-associated AP-1 and AP-2 complexes from mammalian cells. Deletion and site directed mutagenesis studies showed that residues 71-AYLRH of RID\(\alpha\) are necessary for \textit{in vitro} binding to both adaptor complexes and that Tyr72 has an important role in these interactions. In addition, RID\(\alpha\) containing a Y72A point mutation accumulates in the TGN and fails to downregulate the EGFR when it is introduced into mammalian cells as a transgene. Altogether our data suggest a model where RID\(\alpha\) is trafficked directly from
the TGN to an endosomal compartment where it intercepts EGFRs undergoing constitutive recycling to the plasma membrane and redirects them to the Ly.
2.2 Introduction

Ads are non-enveloped DNA viruses that replicate and assemble in the host cell nucleus [187]. Ads are responsible for approximately 5% of acute upper respiratory tract [72] and 15% of lower respiratory tract [188] infections in infants and children. Similar to other DNA viruses, Ads are also capable of establishing persistent infections because they have evolved numerous strategies to evade host antiviral surveillance mechanisms. Some of these same mechanisms also facilitate survival of the virus during an acute infection. Thus a thorough understanding of viral genes that control host immune and inflammatory responses is fundamental to our ability to prevent and treat virus-induced disease at multiple levels. These same issues have also influenced strategies for designing Ad-based vectors for gene therapy [189].

The E3 transcription genes of human Ads encode several proteins that exploit the intracellular trafficking machinery to modify host immune or inflammatory responses [115, 116]. Thus E3 proteins have served as novel probes of membrane transport mechanisms in addition to providing insights to Ad pathophysiology. The most abundant E3 protein, E3/19K, suppresses the host adaptive immunity response by retaining class I MHC molecules in the endoplasmic reticulum. Other E3 proteins affect the functions of molecules involved in proliferation and apoptosis, intracellular cell signaling events linked to NF-κB, and secretion of pro-inflammatory chemokines. Two E3 proteins, RIDα (also called E3-10.4 and E3-13.7) and RIDβ (also called E3-14.5), have been identified that act to remove several receptors from the surface of host cells, including death receptors FAS and TRAIL, TNFR1, and EGFR. These two E3 proteins have
independent functions, and are also capable of forming a molecular complex that has been named the RID complex. However, mutations that affect the ability of the RID complex to downregulate one receptor molecule has no effect on expression on a second class of receptors, suggesting that regulation of different targets by the RID complex involves multiple mechanisms [190].

RIDα is a type II membrane protein expressed by all human Ad serotypes [141]. Its basic structural organization consists of a short N-terminal cytosolic domain (~4 residues), two transmembrane domains connected by an exocytic loop domain (~17 residues), and a C-terminal cytosolic tail domain (~28 residues). We have shown previously that RIDα is sufficient to downregulate the EGFR [139], and that it localizes to the endocytic pathway where it diverts EGFRs in a constitutive recycling loop to lysosomes [157]. However, other laboratories have reported that RIDα only functions in the context of the RID complex that localizes to the plasma membrane where it regulates EGFR internalization [156]. Although the precise role of EGFR downregulation during an Ad infection is not completely understood, the EGFR has been linked to TNFα-dependent NF-κB activity [191], and release of pro-inflammatory chemokines [192] in other biological settings.

The RIDα cytosolic tail has several consensus sorting signals that likely engage the host cell machinery necessary for its localization and function. The goal of this study was to identify these sorting signals and to disable them by site-directed mutagenesis. These studies focused on potential interactions with clathrin AP complexes involved in membrane protein sorting at the level of the TGN (AP-1) and the plasma membrane (AP-2) [193]. Our results indicate that RIDα residues 71-AYLRH bind to both classes of
APs. In addition, we show that a Y72A point mutation traps RIDα in the TGN but not the plasma membrane suggesting the Ad protein encounters AP-1 first in the biosynthetic pathway. Similar to many other membrane cargoes with overlapping AP binding sites [15], our data also support a model where AP-2 serves as a quality control function to capture RIDα molecules that leak to the plasma membrane and redirect them back to endosomes. These studies were carried out with RIDα transgenes, confirming the original Ad mapping studies demonstrating the ability of this protein to modulate EGFR trafficking independent of other E3 proteins [135].
2.3 Materials and methods

Cell lines, Ad stocks, and transfections

Human hepatocellular carcinoma-derived N-PLC-PRF/5 cells [194] and fetal lung-derived WI-38 cells [195] were maintained in minimal essential medium (MEM). Human epithelial-derived A431 carcinoma cells [196], and HEK-293 cells [95] were maintained in Dulbecco’s modified MEM (DMEM). Chinese hamster ovary (CHO) cells [197] were maintained in MEM-α medium. Human lung carcinoma-derived A549 [196] cells were maintained in Ham’s F12 medium. All media were supplemented with 10% fetal bovine serum and 2 mM glutamine. Cells were grown at 37°C with 5% CO₂ and were sub-cultured with 0.25% trypsin-0.1% EDTA in PBS.

Adenovirus stocks were grown in human embryonic kidney 293 cells, and titers were determined by plaque assay, using standard techniques. The Ad2-Ad5 recombinant adenovirus mutant that overproduces the RIDα protein has been described elsewhere [135]. Cells were acutely infected with approximately 200 plaque-forming units per cell according to established protocols [137]. CHO cells were transiently transfected using TransIt-CHO (Mirus, Madison, WI) as directed by the manufacturer, and HEK-293 cells were transfected using Fugene 6 (Roche, Basel, Switzerland) following the manufacturer’s instructions.

Antibodies
The following antibodies were used: α-adaptin, γ-adaptin, FLAG M2, and FLAG-BioM2 monoclonal antibodies from Sigma (St. Louis, MO); transferrin receptor monoclonal antibody from Zymed (San Francisco, CA); human-specific EGF receptor EGF-R1 monoclonal antibody [198]; RIDα rabbit polyclonal antibody produced with a synthetic peptide corresponding to residues 76 to 91 [141]; furin convertase rabbit polyclonal antibody from ABR (Golden, CO); GST goat polyclonal antibody from Amersham-Pharmacia (Piscataway, NJ); Golgi gp125 antibody was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies were purchased from Amersham-Pharmacia (HRP) or Jackson ImmunoResearch (fluorescent and HRP; West Grove, PA).

**Cloning and mutagenesis**

Wild-type and modified RIDα carboxyl terminal (C-term) sequences were subcloned into pGEX-3X (Amersham-Pharmacia) as follows. RIDα residues Asp64 to Leu91 (see Fig. 1A) were subcloned in the M13mp18 single-stranded DNA phage vector (New England BioLabs, Ipswich, MA). Y72A and Y79A point mutations were introduced using the M13mp18 RIDα template, a Sculptor In Vitro Mutagenesis System (Amersham-Pharmacia) and the following mutagenic primers to introduce tyrosine-to-alanine substitutions (in **bold**): 5’-TGTGCGCATTGCGGCCCTCAGGCACCAT-3’ for Y72A, and 5’-GCACCATCCGCAAAGCCAGAGACGACT-3’ for Y79A. Wild-type and mutated sequences were amplified by PCR using a forward primer 5’-
CAUCAUCAUCAUGATATCGACTGGTTTGTGTCGATTGC-3’ designed to anneal to the codon for Asp64 (in **bold**) and a reverse primer 5’-CUACUACUACUAGATATCTTTCGGAACCACCATCAAAAACAGGATTTTCG-3’ to anneal to M13mp18 vector sequences 3’ to the polylinker region. Both primers incorporate EcoR V restrictions sites (**underlined**) and also have non-complementary dU repeats on the 5’ ends (**italics**). The dU repeats in the PCR products were depurinated with uracyl DNA glycosidase to expose single-stranded 3’ ends and enable base-pairing to complementary ends in the linear shuttle vector pAMP (Invitrogen, Gaithersburg, MD). EcoR V fragments were sub-cloned in frame at the Sma I site in pGEX-3X, which reconstituted the RIDα Ile63 codon. The GST fusion plasmid encoding RIDα carboxyl terminal sequences truncated to residues Tyr70 or Tyr79 were prepared using PCR and full-length RIDα cloned in the pcDNA1/Amp eukaryotic expression vector (Clontech, Mountain View, CA) as a template. Sequences were amplified with a forward primer 5’-CTAAGTCTAGAGAACCCACTGC-3’ designed to anneal to vector sequences 5’ to the RIDα coding region, and reverse mutagenic primers 5’-AGTGGGATCCTAGTATTGCCGGTTGTCGCTGA-3’ to incorporate an A71Stop codon, or 5’-CCTAGGATCCCTAAATGCACACACAAACCA-3’ to incorporate an R80Stop codon, respectively (both stop codons in **bold**). The reverse mutagenic primers also incorporated BamH I sites (**underlined**) compatible with the pcDNA1/Amp polylinker. The cytoplasmic tail sequences were amplified using forward 5’-GTTTCCCCGGGATTGACTGGTTTGTG-3’ and reverse 5’-GAGTCCCCGGGTGAGGTGACGACGATAGC-3’ primers complementary to Ile63 and sequences in pcDNA1/Amp 3’ to the RIDα coding region, respectively. Both primers
incorporate Sma I sites (underlined) to facilitate in-frame cloning at the Sma I site in the pGEX-3X polylinker region. The GST fusion plasmid encoding RIDα carboxyl terminal sequences truncated to residue His75 was created using an overlap PCR extension technique, the RIDα/pcDNA1/Amp template, and these four primers: primers 1 and 4 are forward and reverse flanking primers that anneal to unique restriction enzymes in the polylinker region at the 5’ and 3’ ends of the RIDα coding region that were already described; and primers 2 and 3 are forward 5’-ACCTCAGGCATAACCGAATACA-3’ and reverse 5’-CTGTATTGCGGTATTAGTCGCTGAG-3’ mutagenic primers that replace His76 with a stop codon (in bold). The first round of PCR reactions were carried out with primers 1 and 3 or primers 2 and 4, creating two overlapping PCR products. Both fragments were gel-purified and used as templates for a second PCR reaction with the flanking primers to generate a 627-bp product with the H76Stop mutation. This fragment was inserted into pcDNA1/Amp using restriction enzymes incorporated in the ends of the product compatible with restriction sites in the polylinker region. Carboxyl terminal sequences encoding the H76Stop were amplified and subcloned in pGEX-3X exactly as described for the A71Stop and R80Stop constructs.

The cDNAs encoding full-length wild-type RIDα and RIDα with a single Y72A or Y79A amino acid substitution for expression in eukaryotic cells were constructed as follows. Wild-type RIDα cloned in pcDNA1/Amp was used as template for a PCR reaction using forward 5’-ATCGTAAAGATCTTGATTTCTGAGTCTTTTATTTATTG-3’ and reverse 5’-CTAAGATCTCTCTAAAGAATTCTGAGAAGATCAGCTATAGTCCTG-3’ primers to amplify the RIDα ORF and incorporate flanking Bgl II restriction sites (underlined).
PCR products were digested with Bgl II and ligated to an amino terminal FLAG epitope in the polylinker region of the pExchange2 plasmid (Stratagene, La Jolla, CA) digested with the same restriction enzyme. The Y72A point mutation was incorporated into pExchange2/ RIDα using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene), and forward 5’-GTGTGCATTGCGGCCCTCAGGCACCATC-3’ and reverse 5’-GATGGTCCTGAGGGCCGCAATGCACACAC-3’ PCR primers (Y72A substitution in bold), or forward 5’-AGGCACCATCCGCAAGGCCAGAGACAGGGACTATAG-3’ and reverse 5’-CTATAGTCTCTGCTCTGGCTTGGGATGTGGCTC-3’ (Y79A substitution in bold) according to the manufacturer’s instructions.

PCR primers were designed using the DNASTAR software package (DNASTAR, Inc., Madison, WI). PCR amplifications were carried out using a RoboCycler 40 Temperature Cycler (Stratagene, La Jolla, CA). All PCR products and re-ligated recombinant products were sequenced by automated DNA sequencing (Cleveland Clinic Foundation Genomics Core, Cleveland, OH).

GST-pull down assay

GST fusion proteins were purified from BL21 cells that were freshly transformed with pGEX-3X plasmids. Bacteria were cultured at 37°C to an O.D.₆₀₀ of approximately 0.6, induced with 0.1 mM IPTG for 16 hr at room temperature, and collected by low-speed centrifugation. Cells were subjected to one freeze-thaw cycle, resuspended in a solution of 50 mM Tris, pH 7.7, 0.1 M NaCl, 0.2 mM EDTA, and protease inhibitors (0.2
mM PMSF and 1 µM leupeptin), and then digested with lysozyme (0.1 mg/ml) for 1 hr at room temperature. MgSO$_4$ was added to a final concentration of 3 mM, and bacterial lysates were digested for an additional 1 hr at room temperature with 0.02 mg each of DNase and RNase. Lysates were adjusted to pH 7.4, and incubated with 1.5% L-lauryl sarcosine to solubilize inclusion bodies for 15 min on ice followed by centrifugation at 12,000 × g for 10 min at 4°C. Supernatants were adjusted to 3% Triton X-100 and incubated with glutathione-Sepharose beads (Amersham-Pharmacia) for 20 min with rotation at 4°C. Beads with attached fusion proteins were washed 3 times with a solution of 50 mM Tris, pH 7.4, 10 mM MgCl$_2$, and 1% Triton X-100 supplemented with 0.4 M NaCl (high salt wash), and twice with the same solution supplemented with 0.15 NaCl (low salt wash). Beads with attached fusion proteins were incubated with crude sub-cellular fractions enriched for clathrin adaptors using the method described in the next paragraph for 20 min at 4°C, and the beads were washed 3 times with the high salt solution and then twice with the low salt solution. Fusion protein complexes were solubilized with Laemmli buffer and resolved by SDS-PAGE for immunoblotting using standard methods.

**Cell fractionation**

Crude sub-cellular fractionation was carried out essentially as described in [199]. Briefly, cells were rinsed twice and then scraped in PBS supplemented with 2 mM EDTA, 5 mM EGTA, and protease inhibitors. Cells were resuspended in 0.1 M MES, pH 6.5, 0.2 M EDTA, 0.5 mM MgCl$_2$, 0.02% NaN$_3$, 10 mg/ml BSA, and protease inhibitors,
and then lysed with 1% NP-40 for 5 min at room temperature. Post-nuclear supernatants were centrifuged at 60,000 × g for 30 min at 4°C in an Optima TL Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) to collect supernatant corresponding to crude cytosol (Cyt). The resulting membrane pellet was resuspended in the NP-40 lysis buffer and incubated with 0.5 M Na₂CO₃ for 5 min on ice to release peripheral (Peri) membrane proteins. The mixture was then centrifuged at 50,000 × g for 20 min at 4°C, and the Peri-enriched supernatant was incubated with 0.5 M KH₂PO₄ for 1 hr on ice. Integral membrane (IM) proteins were solubilized by incubating membrane pellets with RIPA detergent (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) in 50 mM Tris, pH 8.0, supplemented with 150 mM NaCl, 2 mM EDTA, 5 mM EGTA, and protease inhibitors, for 30 min on ice.

Cell homogenates were also fractionated on Percoll (Amersham-Pharmacia) gradients essentially as described in [200]. Briefly, cells were rinsed twice with PBS supplemented with 2 mM EDTA and 5 mM EGTA, and then scraped in ice-cold homogenization buffer (HB) consisting of 10 mM HEPES, pH 7.5, 0.25 M sucrose, 1 mM EDTA, and protease inhibitors. Cells were collected by centrifugation, resuspended in HB buffer, and homogenized with 22 strokes of a Dounce homogenizer. The homogenate was diluted with an equal volume of fresh HB, and centrifuged at 400 × g for 10 min at 4°C to pellet unbroken cells and nuclei. Post-nuclear supernatants were adjusted to a final concentration of 27% Percoll in 0.25 M sucrose using a 90% Percoll stock solution, and then layered over a 1 ml sucrose cushion consisting of 10× HB. Gradients were centrifuged for 90 min at 25,000 × g in an SS34 fixed-angle rotor (Sorvall Instruments, Newtown, CT) without braking. A total of nine 1.2 ml fractions were
collected manually starting from the top of the gradient. Fractions were analyzed by comparing equal aliquots of total cellular protein determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), or by immunoprecipitation after membranes were solubilized with the RIPA lysis buffer and centrifuged at 100,000 × g for 30 min at 4°C in a TL 100.3 fixed angle rotor (Beckman Instruments) to precipitate Percoll. Alternatively, fractions were assayed for β-hexosaminidase activity by incubating 20 µg of protein in a solution of 0.1 M MES, pH 6.5, 1 mM p-nitrophenyl-β-D-glucosaminide, and 0.2% Triton X-100 for 90 min at 37°C. The reaction was quenched with 0.5 M glycine, pH 10, and absorbance was read at 405 nm using a Model 3550 automatic microplate reader (Bio-Rad). Gradients were also collected from cells that had been incubated with 125I-EGF (50 ng/ml, 150 µCi/µg; PerkinElmer-NEN) or 125I-transferrin (200ng/ml, 0.5 µCi/µg; PerkinElmer-NEN) to label plasma membrane receptors. Fractions from these gradients were analyzed by gamma counting (Cobra Auto-Gamma Counter; Packard Instruments, Meriden, CT).

**Immunoprecipitation and immunoblotting**

Immunoprecipitations were carried out using antibodies adsorbed to protein A-Sepharose CL-4B beads (Sigma), or Streptavidin agarose (Pierce, Rockford, IL). Immune complexes were centrifuged at 14,000 × g for 15 min at 4°C, washed 5 times with lysis buffer, solubilized with Laemmli buffer, and resolved by SDS-PAGE using standard methods. Immunodepleted supernatants were centrifuged a second time to ensure removal of immune complexes before they were added to GST fusion protein
pull-down assays. Gels with radioactive proteins were treated with En3Hance (PerkinElmer-NEN, Wilmington, DE) for fluorography. Immunoblotting was carried out using proteins transferred to nitrocellulose membranes by standard methods. Nitrocellulose filters were incubated with primary antibodies and appropriate HRP-conjugated secondary antibodies (Amersham Life Sciences, Inc., Arlington, IL; Jackson ImmunoResearch Laboratories, Inc.) for detection by enhanced chemiluminescence (ECL) (Amersham Life Sciences). For quantification, immunoblots were incubated with 125I-labeled goat anti-mouse secondary antibody (1 x 10^6 cpm/ml; 8.07 µCi/µg; PerkinElmer-NEN) for 1 hr at room temperature. Blots were air-dried after extensive washing, and radiolabeled proteins were quantified by phosphorstorage autoradiography. Digitized images were analyzed using the ImageQuant™ software package (Molecular Dynamics, Sunnyvale, CA), which averages 5 measurements of light emission for each pixel location, to give a pixel value that is proportional to the amount of stored radiation.

**Confocal microscopy**

HEK-293 cells or CHO cells transiently transfected with RIDα expression plasmids were prepared for staining essentially as described in [201]. Briefly, cells were permeabilized with 0.5% β-escin in a solution of 80 mM PIPES, pH 6.8, supplemented with 5 mM EGTA and 1 mM MgCl₂ for 5 min, and then fixed with 3% paraformaldehyde-PBS for 15 min. Cells were stained with primary antibody or secondary antibodies and DAPI for 45 min at room temperature. Antibodies were diluted in a solution containing 0.5% β-escin and 3% RIA-grade bovine serum albumin, and
were blocked with a solution containing 1% normal serum from the host animal used to generate the secondary antibody. Cells were examined with a Leica TCS SP2 with AOBS confocal microscope (Leica, Mannheim, Germany) using the 405 nm wavelength line of a UV laser and the 488/568 lines of an argon-krypton laser. Image resolution using a Leica 100X 1.4 numerical aperture oil immersion lens and Leica LCS software was 512 x 512 pixels. Some cells were pre-treated with nocodazole (100 μM; Sigma) or vehicle (DMSO) for 30 min prior to staining. Phase contrast images were collected and the outline of the cells was drawn using Metamorph (Molecular Devices, Sunnyvale, CA) and overlaid onto the respective confocal image.

**Surface reduction of extracellular disulfide bonds**

Cells were pulse-labeled for 1 hr with L-[³⁵S]cysteine (50 mCi/ml; 1075 Ci/mmol; PerkinElmer-NEN) in cysteine-free MEM supplemented with 10% dialyzed fetal bovine serum and 0.2% BSA, and then incubated in chase medium (complete Dulbecco’s MEM supplemented with 500 μM non-radioactive cysteine) for 3 hr to allow proteins to achieve steady-state localization. Radio-labeled cells were incubated twice (25 min/incubation) with an ice-cold solution of 80 mM L-cysteine, 75 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.5 N NaOH, and 1% fetal bovine serum to reduce external disulfide bonds of surface proteins [202]. Cells were rinsed twice with PBS containing iodoacetamide (1 mg/ml), and then incubated with the RIPA lysis buffer supplemented with iodoacetamide (1 mg/ml) and protease inhibitors for analysis by immunoprecipitation. Immunoprecipitates were solubilized in Laemmli buffer supplemented with 1 mg/ml
iodoacetamide and separated by SDS-PAGE under reducing [0.1 M dithiothreitol (DTT)] or non-reducing (no DTT) conditions.

**EGFR protein stability assay**

CHO cells, which lack endogenous EGFRs, were co-transfected with plasmids encoding human EGFR [203] and FLAG-tagged wild-type or Y72A or Y79A RIDα plasmids or empty vector. Cells were radiolabeled with [35S]cysteine for 30 min starting at 48 hr post-transfection as described previously. Cells were either harvested immediately or following a 5 hr chase in media supplemented with a 10-fold excess of nonradioactive cysteine. Cells were lysed with RIPA buffer and analyzed by immunoprecipitation, SDS-PAGE and fluorography.
2.4 Results

The C-terminal cytoplasmic tail of the RIDα interacts with AP complexes

We have shown previously that RIDα localizes to the endocytic pathway where it redirects constitutively recycling EGFRs to lysosomes in adenovirus-infected cells [137, 157]. The RIDα cytoplasmic tail contains a number of putative sorting motifs that may regulate these sorting properties [15], including a dileucine motif at residues 87-LL, and two possible tyrosine-based sorting motifs containing Tyr72 and Tyr79, respectively (Fig. 2.1). To determine whether the RIDα cytoplasmic tail interacts with clathrin AP complexes involved in membrane protein sorting, we utilized a fusion protein consisting of the 30-amino acid cytoplasmic domain of RIDα fused to GST (characterized in Fig. 2.1 B) to pull down complexes from mammalian cell extracts. The mammalian cell fractionation scheme involved purification of a peripheral membrane protein fraction to enrich for functional AP complexes according to the method described in [199]. Fig. 2.1 C validates this purification scheme, showing that α-adaptin and γ-adaptin (subunits of AP-2 and AP-1, respectively) are both enriched in the peripheral membrane fraction, but not integral membrane fractions that include both of the known molecular weight forms (e.g., 13.7-kDa and 11.3-kDa) of the RIDα protein. A small amount of each of these adaptin molecules was also present in crude cytosol, as expected for AP complexes that cycle on and off membranes. Thus the crude cytosol and peripheral membrane protein fractions were mixed with GST or the C-Term fusion protein, precipitated with glutathione agarose, washed, and subjected to SDS-PAGE and immunoblot analysis with
monoclonal antibodies to \( \alpha \)-adaptin and \( \gamma \)-adaptin. As shown in Fig. 2.1 D, \( \alpha \)-adaptin and \( \gamma \)-adaptin from a human hepatocellular cell line were both co-precipitated with the C-Term fusion protein in contrast to GST alone. Similar results were obtained using peripheral membrane protein fractions from a number of other cell lines including epithelial carcinoma-derived A431 cells and normal human diploid WI-38 fibroblasts (Fig. 2.1 E), and human lung carcinoma-derived A549 cells and human embryonic kidney HEK cells (data not shown).

**Analysis of RID\( \alpha \) cytoplasmic tail mutants**

Our next goal was to identify the sequence(s) within the cytoplasmic tail of RID\( \alpha \) that interacts with each of the AP complexes identified in the first set of experiments. As already noted, RID\( \alpha \) contains putative dileucine and tyrosine motifs that could be involved in adaptor complex formation. Thus we constructed a series of fusion proteins with cytoplasmic tail truncations that are shown in Fig. 2.2 A-B to assess a role for each of these putative signals. The fusion protein with an R80Stop substitution has the carboxyl terminal dileucine motif deleted, the H76Stop has the distal tyrosine residue Tyr79 deleted, and the A71Stop has all putative sorting motifs deleted. *In vitro* pull down assays with these fusion proteins revealed that RID\( \alpha \) residues 71-AYLRH are primarily responsible for binding both types of AP complexes (Fig. 2.2 D-E). These data also show that the carboxyl terminal dileucine motif does not bind APs directly, contradicting published reports from another laboratory [204]. Consistent with the results shown here, we have also observed that the conserved 679-LLRIL sequence localized to the EGFR
The juxtamembrane region that regulates ligand-induced post-endocytic sorting to lysosomes [205, 206] does not bind APs in vitro (data not shown).

The AP binding site identified in the in vitro mapping studies has one tyrosine at residue Tyr72. To assess its role in AP binding, fusion proteins were constructed with Y72A as well as Y79A point mutations incorporated into the full-length cytoplasmic tail (Fig. 2.2 C). These fusion proteins were incubated with peripheral membrane protein fractions, and adaptin binding was quantified by immunoblotting with 125I-labeled secondary antibodies and phosphor storage autoradiography after the bound proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters. Results from one set of experiments are summarized in Table 2.1, and similar results were obtained in three separate determinations. We found that the Y72A substitution was associated with reduced binding to both AP-1 and AP-2 although its effect on AP-2 binding was consistently greater than AP-1. Interestingly, the Y79A substitution adjacent to the primary AP binding site identified in Fig. 2.2 was associated with increased binding to AP-2 but reduced binding to AP-1, suggesting that residues outside of the binding motif may be important for AP interactions.

**AP-1 and AP-2 bind to the RIDα cytoplasmic tail with distinct properties**

Results presented to this point suggest that AP-1 and AP-2 bind overlapping but distinct regions in the RIDα cytoplasmic tail. To further test this hypothesis, studies were carried out to determine whether there is competition between AP-1 and AP-2 for binding to the C-Term fusion protein. These studies were possible because the γ-adaptin antibody immunoprecipitates intact AP-1 complexes comprised of all four of the protein
subunits (Fig. 2.3 A). Thus peripheral membrane protein fractions were immunodepleted for AP-1 prior to incubation with GST or the C-Term fusion protein (Fig. 2.3 B). AP-2 binding was essentially identical when the C-Term fusion protein was incubated with cell fractions that were pre-cleared with an isotype-matched non-specific IgG versus the γ-adaptin specific antibody, compared to AP-1 binding that was substantially reduced in the immunodepleted samples in the same experiment (Fig. 2.3 C). Although the reverse experiment was not possible since immunoprecipitations with the α-adaptin antibody must be carried out under denaturing conditions, these results strongly suggest that AP-1 and AP-2 binding to the RIDα cytoplasmic tail is non-competitive.

Fig. 2.3 D provides further evidence that AP-1 and AP-2 bind to RIDα by distinct mechanisms. This experiment was carried out by incubating cell fractions with increasing amounts of the C-Term fusion protein, followed by quantitative immunoblotting for α-adaptin and γ-adaptin binding exactly as described for Table 2.1. Data are represented as the percent of total adaptin protein detected in combined crude cytosol and peripheral membrane fractions that were also quantified by immunoblotting with 125I-labeled secondary antibodies and phosphor storage autoradiography. This experiment demonstrates that α-adaptin binding is rapidly saturated compared to γ-adaptin. To evaluate the relative importance of ionic interactions in the binding of AP complexes to the cytosolic tail of RIDα, the in vitro binding assay was carried out in the presence of increasing concentrations of salt or by changing the pH of the binding solution. Ionic interactions are highly sensitive to changes in salt or pH, while hydrophobic interactions are not. Binding of α-adaptin and γ-adaptin to RIDα cytosolic tail sequences was not appreciably affected by changes in salt concentration (Fig. 2.3 E)
or in pH (data not shown), suggesting these protein-protein interactions are enabled primarily by hydrophobic amino acids with non-polar side-chains. Thus Ala71 and/or Leu73 in the 71-AYLRH motif may be important for AP binding, in addition to hydrophobic residues in the surrounding tail sequences that may not be necessary but that nevertheless contribute to the overall strength of AP binding.

The Y72A point mutation alters sub-cellular localization of RIDα in mammalian cells

The Y72A and Y79A substitutions were engineered into the full-length viral protein to study their expression and localization in mammalian cells. Wild-type and mutant proteins were transiently expressed in HEK-293 cells, and sub-cellular localization was determined by collecting confocal images of cells co-stained for the viral protein and furin, or the transferrin receptor, which are well-characterized markers of the biosynthetic and endocytic compartments, respectively. The wild-type protein was largely excluded from the biosynthetic compartment defined by furin that is enriched in the TGN (Fig. 2.4 A) and was found to co-localize with the TfR (Fig. 2.4 B) a marker for endocytic vesicles as previously shown [157]. The Y72A mutant, however, almost exclusively co-localized with the TGN marker furin (Fig. 2.4 A), and did not co-localize with the TfR (Fig. 2.4 B), consistent with the idea that the Y72A mutation blocks exit from the biosynthetic compartment. Similar to wild-type, the Y79A mutant did not co-localize with furin (Fig. 2.4 A), but partially co-localized with the TfR (Fig. 2.4 B). This
indicates that while the Y79A mutation does affect AP-1 binding in vitro (Table 2.1), the protein is able to efficiently exit the biosynthetic compartment.

The sub-cellular distribution of wild-type and mutant proteins was confirmed by cell fractionation of membrane compartments on Percoll gradients. Fractions were initially characterized by quantifying radioactivity in fractions from cells that were incubated with $^{125}$I-EGF or $^{125}$I-transferrin to label plasma membrane receptors, and by assaying fractions for β-hexosaminidase activity enriched in lysosomes (Fig. 2.5 A); and by immunoblotting with antibodies to well-characterized markers of biosynthetic (gp125) and endocytic (TfR) compartments (Fig. 2.5 B). Based on these analyses, we concluded that biosynthetic compartments are concentrated in fractions 2 through 5; plasma membrane is enriched in fractions 1 and 2; and TfR-positive endosomes are concentrated in fractions 2 and 3. Both molecular weight forms of the wild-type protein exhibited a sub-cellular distribution that is most similar to TfR in light fractions 2 and 3, and distribution of the mutant protein was most similar to the Golgi glycoprotein gp125 (Fig. 2.5 C). Thus these data are consistent with the images obtained by confocal microscopy showing that the wild-type protein localizes to an endocytic compartment compared to the mutant protein localized to the biosynthetic pathway.

In contrast to results shown here and published elsewhere [136, 137, 157, 207], other laboratories have reported that RID$\alpha$ is localized to the plasma membrane [156, 204]. Thus the observation that the wild-type protein is enriched in fraction 2 could also be interpreted as plasma membrane localization based on distribution of radioactivity in gradients obtained from cells labeled with $^{125}$I-labeled ligands (Fig. 2.5 A). In addition, disabling a critical AP-2 binding site could result in plasma membrane accumulation of
the Y72A mutant protein. Additional experiments were therefore carried out to exclude the possibility that RIDα is targeted to the plasma membrane. RIDα exists in two molecular weight forms: one corresponding to the full-length molecule, which has two membrane-spanning α-helices and cytosolic amino- and carboxy-termini; and a lower molecular weight species lacking the amino terminal α-helix which is cleaved by signal peptidase in the endoplasmic reticulum (see Fig. 2.1 A) [141]. Sequences connecting the α-helices should be exposed at the cell exterior in either form of the molecule located at the plasma membrane. However, this region cannot be labeled by conventional surface biotinylation, since it lacks residues with free amino groups. We therefore took advantage of the fact that both RIDα molecular weight species form disulfide-linked dimers at Cys31 (see Fig. 2.1 A) [141]. Hence disulfide bonds on proteins located at the cell surface should be reduced if cells are exposed to an external reducing agent such as cysteine, while intracellular proteins are protected [202, 208]. Transfected cells were subjected to pulse-chase metabolic labeling, and then incubated with cysteine-containing medium and lysed in the presence of iodoacetamide to prevent de novo disulfide bond formation. When RIDα immune complexes were resolved under non-reducing conditions (i.e., in the absence of DTT), radio-labeled proteins migrated as high molecular dimers, regardless of whether or not cells were exposed to extracellular cysteine (Fig. 2.5 D). Introduction of the Y72A mutation had no effect on resistance of high molecular dimers to surface reduction. This was in contrast to disulfide-linked TfRs that were partially reduced by external cysteine (Fig. 2.5 E), consistent with approximately 20%-45% of this molecule exhibiting plasma membrane localization with the remainder in endocytic compartments [209, 210]. These data confirm that the
majority of wild-type and mutant proteins are localized to intracellular membrane compartments at steady-state. Endosomal compartments are dispersed throughout the cytoplasm following a brief exposure to the microtubule-depolymerizing agent nocodazole [211]. We have shown previously that wild-type RIDα displays this same behavior consistent with the viral protein’s localization to endocytic compartments [145]. We therefore reasoned that a similar treatment should have little or no effect on localization of the RIDα protein with the Y72A substitution if the mutant protein accumulates in a biosynthetic compartment as indicated by the results shown in (Fig. 2.4, 2.5). Results in Fig. 2.6 indicate that nocodazole has a very modest effect on the distribution of the Y72A mutant protein compared to vesicles with wild-type RIDα that were redistributed from the perinuclear region the peripheral cytosol. Altogether these data suggest that the mutant protein is retained in the biosynthetic pathway and mostly excluded from a nocodazole-sensitive endocytic pathway.

The Y72A and Y79A point mutations prevent EGFR downregulation by RIDα

Although many functions have now been associated with this adenovirus protein, RIDα was first identified because of its ability to specifically reduce EGFR plasma membrane expression [135]. Thus studies were carried out to determine the effect of the Y72A and Y79A mutations in RIDα on EGFR metabolic stability in CHO cells expressing both proteins. Cells were co-transfected with viral protein and human EGFR plasmids, and harvested for total cellular protein or pulse-labeled with [35S]cysteine for
30 min to measure protein stability. When viral protein immune complexes were examined by immunoblotting, we observed that wild-type and mutant viral proteins were expressed at similar levels at 48 hr post-transfection (Fig. 2.7 A). The EGFR acquires 7 to 9 N-linked high-mannose oligosaccharides co-translationally that are processed to complex carbohydrates during Golgi maturation, resulting in retarded migration by SDS-PAGE [212]. A molecular weight species corresponding to the high mannose oligosaccharide EGFR precursor was detected in cells co-transfected with an empty vector control or plasmids encoding wild-type or mutant viral protein in cells that were harvested immediately after a 30-min pulse label (Fig. 2.7 B), showing that the EGFR is synthesized to similar extents in all four populations of transfected cells. The molecular weight species corresponding to the mature EGFR protein with complex carbohydrates was detected in cells transfected with the empty vector control or with plasmids encoding the Y72A or Y79A mutant proteins after a 5 hr chase with non-radioactive amino acid precursors. This was in contrast to cells expressing wild-type RIDα where mature EGFR protein was not detected, consistent with the known ability of RIDα to target EGFR to lysosomes for degradation in the absence of other adenovirus proteins [139]. Thus the Y72A mutation appears to block the ability of the viral protein to facilitate EGFR degradation. RIDα with the Y79A mutation also does not downregulate the receptor even though these mutants appear to localize to endosomes (Fig. 2.4 B). The loss-of-function phenotype associated with the Y79A mutant can likely be attributed to the fact that this region mediates protein-protein interactions with ORP1L [145], a Rab7 effector that links late endocytic vesicles to minus end-directed microtubule motor complexes [43].
2.5 Discussion

The E3 region is not required for viral replication, however it nevertheless plays a critical role in Ad pathogenesis [213]. The importance of this region is underscored by the fact that the first generation of Ad gene therapy vectors which contained large E3 deletions were ultimately deemed unsafe [189]. E3 genes encode integral membrane proteins that regulate a variety of host cell functions involved in innate immunity and inflammatory responses. The ability of these proteins to modify host cell function is due in part to cytosolic tail sequences that interact with sorting machinery and target membrane proteins to specific intracellular compartments. The E3 protein RIDα was originally identified because of its ability to downregulate the EGFR [135] and other related receptor tyrosine kinases [214]. In this study we have demonstrated that RIDα residues 71-AYRLH comprise a binding site for AP complexes, and that Tyr72 is required for RIDα localization to endosomes and its ability to downregulate the EGFR. These findings support previous studies concluding that RIDα acts by targeting EGFRs undergoing constitutive recycling to the plasma membrane [136, 137, 157]. The fact that 71-AYLRH is precisely conserved in all Ad serotypes that have been sequenced except for Ad12 suggests its fundamental importance in a majority of Ad-induced diseases [135].

Although mutation of Tyr72 to an alanine residue leads to a clear reduction in AP binding in vitro (Table 2.1), 71-AYLRH does not conform to classical tyrosine-dependent YXXØ motifs that have a preference for hydrophobic residues with bulky side chains at the Ø position [215]. Instead it has a histidine residue that is mildly basic and
hydrophilic. However, many other factors contribute to sorting signal recognition, including the position of the signal relative to the membrane and to the carboxyl terminus, and amino acid residues in areas adjacent to the signal. We have reported previously that 71-AYLRH exists in an amphipathic helix that is stabilized by interactions with a membrane mimetic phospholipid micelle surface based on data obtained using NMR spectroscopic methods [216]. This close degree of membrane association could have an important role in regulating availability of the signal for interaction with APs. Thus 71-AYLRH may be masked when the cytoplasmic tail is intimately associated with membrane, while cellular events that result in its translocation into the cytosol could make it available for binding APs. Some examples that could bring about such a change include modulation by another membrane protein, changes in the endosomal pH or ionic environment as RIDα traverses different cellular compartments, post-translational modification, or a dramatic shift in the tilt or membrane placement of the adjacent transmembrane helix. For example, we have recently demonstrated that RIDα function is highly dependent on reversible palmitoylation at a residue in the cytosolic tail [186], suggesting association of the RIDα cytosolic tail with membranes is tightly regulated in cells [217]. The possibility that 71-AYLRH availability is regulated by a transmembrane mechanism is particularly intriguing since the RIDα loop domain connecting its two membrane spanning domains resides in compartmental lumens. Thus the transmembrane domain could act as a conduit to fine-tune 71-AYLRH recognition at specific sub-cellular organelles.

Even though 71-AYLRH recognizes two different classes of APs, we have demonstrated that AP-1 and AP-2 do not compete for binding to RIDα in vitro (Fig. 2.3
C), and that mutation of adjacent residue Tyr79 leads to increased binding of AP-2 but diminished binding to AP-1 (Table 2.1). Thus it seems likely that AP-1 and AP-2 recognize distinct but overlapping sets of tyrosine-based sorting signals in RIDα. The Y72A mutation traps RIDα in the TGN (Fig. 2.4 B) but not the plasma membrane (Fig. 2.5 D) suggesting the Ad protein encounters AP-1 first in the biosynthetic pathway. AP-2 is known to interact with a majority of tyrosine-based signals identified in other molecules, in agreement with studies showing that most naturally occurring signals mediate internalization [218]. The broad specificity of AP-2 recognition implies it serves a quality control function to retarget membrane proteins to their correct intracellular location that escape to the plasma membrane [15]. This would suggest that the plasma membrane may not be an obligatory membrane transport destination for RIDα, and that a majority of RIDα is delivered directly to endosomes (see summary model in Fig. 2.8).

Accordingly, even though RIDα can be detected on the plasma membrane, this localization correlates with high levels of protein expression and constitutes a relatively minor fraction of the total protein in the cell [157]. This is entirely consistent with the quality control role for AP-2 that has been proposed for membrane proteins that leak to the plasma membrane.

In addition to the Tyr72-based sorting motif, RIDα also has a potential dileucine-based motif located at residues 87-LL (see Fig. 2.1 A). Although another laboratory has published that these residues constitute an AP-2 binding site [204], those results were not substantiated in the present study. We did observe that AP binding was remarkably insensitive to pH or salt (Fig. 2.3 D), supporting a role for hydrophobic interactions either within the signal itself or in adjacent regions. It is possible that the dialanine substitution
at 87-LL analyzed in [204] lowers the overall strength of AP binding. Interestingly, 87-LL is part of a larger motif that is precisely conserved in the EGFR [137], suggesting its involvement in cargo selection and/or targeting EGFRs to lysosomes. This conjecture is supported by evidence that this sequence in the EGFR is necessary for ligand-induced trafficking to lysosomes [205, 206], and also RIDα mediated diversion of recycling EGFRs to lysosomes [138]. Thus although 87-LL may not be directly involved in AP recognition, it undoubtedly has an important role in RIDα function at least as it relates to EGFR downregulation. The 87-LL motif shared with the EGFR is found in group C Ad2 and Ad5, however, this region is not precisely conserved in other serotypes [135]. Thus different Ads may vary in their ability to specifically target the EGFR.

RIDα has been associated with other activities besides EGFR downregulation. For example, the RID complex (comprised of RIDα and RIDβ) downregulates death receptors including TNFR1 and FAS [190]. However, mutagenesis studies support a model where the RID complex acts on TNFR1 at the plasma membrane, in contrast to FAS where the functional interaction occurs intracellularly. These seemingly paradoxical results are best understood by considering the many steps involved in receptor downregulation. These include cargo selection, sorting to specific endocytic compartments involved in transport to lysosomes, and coupling to microtubules necessary for transporting MVB intermediates to the perinuclear region [219]. We have already discussed that the molecular basis for RIDα-mediated EGFR cargo selection likely involves the dileucine motif that is conserved in EGFR and RIDα encoded by Ad2 and Ad5. In addition, we have recently discovered that RIDα interacts with Rab7 effectors including RILP and ORP1L, which are necessary for microtubule-dependent
transport [145]. Thus it is likely that RID$\alpha$ regulates EGFR downregulation at multiple levels. Other cargoes could require accessory molecules to deliver specific receptors to endosomes once the maturation process is underway. Thus RID$\beta$ may promote TNFR1 uptake to endosomes, whereupon they are then sorted to lysosomes by a mechanism involving RID$\alpha$-dependent endosomal maturation. This conjecture is consistent with the observation that RID$\beta$ binds AP-2 and that AP-2 is required for RID-mediated downregulation of TNFR1 but not FAS [190]. The ability to “mix-and-match” different aspects of RID function may have evolved to allow Ads to fine-tune RID activity in different cell types, or during acute versus persistent infections.
2.6 Figures

**Figure 2.1. Binding of RIDα cytosolic tail to AP complexes.**

(A) GST or the C-Term fusion protein were purified from *E. coli* by glutathione affinity chromatography and resolved by SDS-PAGE for detection by Coomassie staining, or by immunoblotting with GST or RIDα-specific antibodies. (B) Adenovirus-infected cells were fractionated into crude Cyt, and Peri and IM proteins as described in Materials and methods. Equal aliquots of total cell equivalents were resolved by SDS-PAGE and immunoblotted with antibodies to RIDα, α-adaptin, or γ-adaptin. (C) GST or C-Term fusion proteins were incubated with crude cytosol or peripheral membrane proteins isolated from N-PLC-PRF/5 cells, and bound proteins were immunoblotted with adaptin-specific antibodies. (E) Fusion proteins were incubated with peripheral membrane proteins from A431 or WI-38 cells and analyzed as described in part (E). Molecular weight standards: β-galactosidase, 116,300; phosphorylase B, 97,400; carbonic anhydrase, 30,000; lysozyme, 14,400; aprotinin, 6,000. Abbreviations: A, alanine; C, cysteine; Cyt, cytosol; C-Term, carboxy-terminus; D, aspartic acid; GST, glutathione S-transferase; H, histidine; I, isoleucine; IB, immunoblot; IM, intergral membrane; L, leucine; P, proline; Peri, peripheral; Q, glutamine; R, arginine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T, threonine; V, valine; W, tryptophan; Y, tryosine.
Figure 2.2. Mapping the sites of interaction of RIDα with AP complexes using truncation mutants.

(A) Schematic showing sequences of cytoplasmically truncated GST fusion proteins used in the mapping studies. Critical residues in putative sorting signals are highlighted in black. (B and C) Proteins were purified from *E. coli* by glutathione affinity chromatography and resolved by SDS-PAGE for detection by Coomassie staining. Proteins in (B) were used in part (D-E) of this figure, and those in (C) were used to obtain data in Table 2.1. (D and E) Fusion proteins shown in (B) were incubated with peripheral membrane proteins isolated from N-PLC-PRF/5 cells, and bound proteins were immunoblotted with adaptin-specific antibodies. Abbreviations: A, alanine; C, cysteine; C-Term, carboxy-terminus; D, aspartic acid; GST, glutathione S-transferase; H, histidine; I, isoleucine; IB, immunoblot; L, leucine; P, proline; Q, glutamine; R, arginine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; T, threonine; V, valine; W, tryptophan; Y, tyrosine.
Table 2.1. Quantitation of adaptin binding to fusion proteins.

<table>
<thead>
<tr>
<th>Fusion protein&lt;sup&gt;1&lt;/sup&gt;</th>
<th>α-adaptin&lt;sup&gt;3&lt;/sup&gt;</th>
<th>% difference&lt;sup&gt;5&lt;/sup&gt;</th>
<th>γ-adaptin</th>
<th>% difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-term&lt;sup&gt;2&lt;/sup&gt;</td>
<td>657 ± 33&lt;sup&gt;4&lt;/sup&gt;</td>
<td>- - -</td>
<td>618 ± 54</td>
<td>- - -</td>
</tr>
<tr>
<td>Y72A</td>
<td>140 ± 8</td>
<td>−79</td>
<td>222 ± 19</td>
<td>−64</td>
</tr>
<tr>
<td>Y79A</td>
<td>1,009 ± 97</td>
<td>+53</td>
<td>373 ± 23</td>
<td>−40</td>
</tr>
</tbody>
</table>

<sup>1</sup>Peripheral membrane proteins were incubated with 100 µg of fusion protein attached to glutathione beads.

<sup>2</sup>Fusion proteins tested had wild-type RIDα C-term sequences, or C-term sequences with Y72A or Y79A substitutions.

<sup>3</sup>Bound proteins were immunoblotted with adaptin-specific antibodies followed by a <sup>125</sup>I-labeled secondary antibody, and blots were exposed to a phosphor screen.

<sup>4</sup>Volume integration of pixel values in a defined area with background subtracted. Pixel values are arbitrary units that are proportional to the amount of radiation stored and re-emitted from the phosphor screen. Values in the table represent the mean of three independent determinations for each band ± SEM, and have been multiplied by 10<sup>3</sup>. Similar results were obtained in two independent determinations.

<sup>5</sup>Relative to amount of protein bound to C-term fusion protein which is set to 100%.

Abbreviations: A, alanine; C-Term, carboxy-terminus; SEM, standard error of the mean; Y, tyrosine.
Figure 2.3. Lack of competition between AP-1 and AP-2 for RIDα C-term binding.

(A) Peripheral membrane proteins from cells that were metabolically labeled with L-[35S]cysteine for 16 hr were incubated with the γ-adaptin monoclonal antibody which immunoprecipitates intact AP-1 complexes comprised of β-, μ-, and σ-subunits in addition to γ-adaptin. (B and C) Peripheral membrane fractions were pre-incubated with non-specific mouse IgG or with the γ-adaptin monoclonal antibody, and the immunodepleted samples were then incubated with GST or C-Term fusion proteins. Aliquots of the immunodepleted samples (B), and proteins bound to glutathione beads (C), were analyzed by immunoblotting with adaptin-specific antibodies. (D) Equal aliquots of peripheral membrane proteins were added to increasing concentrations of the C-Term fusion protein indicated on the X-axis. Bound proteins were transferred to nitrocellulose filters, which were incubated with 125I-labeled secondary antibodies after an initial incubation with primary antibodies to α-adaptin (solid squares) or γ-adaptin (solid circle), for quantification by phosphorstorage autoradiography. The amount of bound protein was calculated as a percentage of total adaptin proteins in the peripheral membrane fractions based on immunoblots of total protein quantified using the same method. This experiment was carried out twice with similar results. (E) C-Term/adaptin complexes were washed 5 times with wash buffers supplemented with various NaCl concentrations indicated in the figure. Abbreviations: AP, clathrin adaptor protein; C-Term, carboxy-terminus; GST, glutathione S-transferase; IB, immunoblot; IP, immunoprecipitation; M, molar.
Figure 2.4. Colocalization of wild-type, Y72A, and Y79A RIDα with membrane compartment markers.

HEK-293 cells transiently expressing wild-type, Y72A, or Y79A mutant proteins were co-stained with antibodies to the FLAG-tagged viral protein (red channel) and to the TGN marker furin (A) or the endocytic marker TfR (B) (green channel) and DAPI (blue channel) for analysis by confocal microscopy. Red and green channels were merged and “yellow” indicates the overlap of red and green fluorescence. Higher magnification images of outlined areas are shown to the right of each set of panels. Size marker: 10 µm. Abbreviations: A, alanine; TfR, transferrin receptor; TGN, *trans*-Golgi network; WT, wild-type; Y, tyrosine.
Figure 2.5. Sub-cellular distribution of wild-type and Y72A RIDα proteins.

(A) CHO cells were fractionated on Percoll gradients and individual fractions were assayed for β-hexosaminidase activity (open circles). Cells were also fractionated after they had been incubated with $^{125}$I-transferrin for 30 min on ice (open squares), or after they were transfected with a human EGFR expression plasmid and incubated with $^{125}$I-EGF for 30 min on ice 48 hr later (open triangles). Enzyme activity or radioactivity was plotted as a percentage of total activity or radioactivity detected across the entire gradient. 

(B) Equal aliquots of total cellular protein from individual cell fractions were immunoblotted with antibodies to well characterized markers of the Golgi (gp125), and plasma membrane and early endosomes (TfR). 

(C) CHO cells were transfected with wild-type (WT) or Y72A RIDα plasmids and immune complexes from individual Percoll gradient fractions were analyzed by immunoblotting with a RIDα-specific antibody. 

(D and E) CHO cells transfected as in (C) and non-transfected CHO cells were pulse-labeled and then switched to chase medium to allow proteins to reach their steady-state localization. Proteins were immunoprecipitated and analyzed by SDS-PAGE under standard reducing conditions (+ DTT), or were immunoprecipitated from cells subjected to surface reducing (+) or sham (−) conditions followed by SDS-PAGE under non-reducing conditions (− DTT). 

Abbreviations: A, alanine; CHO, Chinese hamster ovary; EGF, epidermal growth factor; EGFR, EGF receptor; hr, hour; IB, immunoblot; IP, immunoprecipitation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TfR, transferrin receptor; WT, wild-type; Y, tyrosine.
Figure 2.6. Effect of nocodazole on RIDα protein sub-cellular localization.

CHO cells transiently expressing wild-type or Y72A mutant proteins were pre-treated with DMSO vehicle or nocodazole (100 μM) for 30 min prior to staining with antibody to the FLAG-tagged viral protein (red channel) and with DAPI (blue channel). Cell outlines (in green) were made with the MetaMorph program using phase contrast images as a guide. Size marker: 10 μm. Abbreviations: A, alanine; CHO, Chinese hamster ovary; Noc, nocodazole; Veh, vehicle; WT, wild-type; Y, tyrosine.
Figure 2.7. Effect of Y72A and Y79A point mutations on RIDα expression and function in mammalian cells.

CHO cells were co-transfected with plasmid expressing the human EGFR along with a plasmid expressing wild-type RIDα (WT), plasmids expressing RIDα with Y72A (Y72A) or Y79A (Y79A) substitutions, or an empty vector (Sham). (A) Equal aliquots of total cell protein were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with a RIDα-specific antibody. (B) The cells were radio-labeled with [35S]cysteine for 30 min starting at 48 hr post-transfection, and either harvested immediately or following a 5 hr chase in media with an excess of non-radioactive cysteine. EGFR immune complexes were resolved by SDS-PAGE and detected by autoradiography. Abbreviations: A, alanine; CHO, Chinese hamster ovary; EGFR, EGF receptor; Hr, hour; IB, immunoblot; IP, immunoprecipitation; Min, minute; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; WT, wild-type; Y, tyrosine.
Figure 2.8. Summary model.

Data presented in this study suggest newly synthesized RID\(\alpha\) is delivered directly from the TGN to endosomes (open arrows) where it encounters EGFRs undergoing constitutive recycling to the plasma membrane (dashed arrows). Since RID\(\alpha\) proteins with a defective AP binding site accumulate in the Golgi and TGN, we conclude that newly synthesized RID\(\alpha\) proteins encounter AP-1 necessary for incorporation into clathrin-coated vesicles (CCVs) necessary to exit the biosynthetic compartment. Data also shows that RID\(\alpha\) is delivered directly to endosomes and not the plasma membrane, suggesting the ability of the viral protein to bind AP-2 serves a quality control function in which proteins that "leak" to the plasma membrane are quickly endocytosed and retargeted to their appropriate intracellular locations. Although the EGFR and RID\(\alpha\) are both transported to late endosomes (LE) via multivesicular body (MVB) transport intermediates (solid arrows), only EGFR is degraded. Abbreviations: AP, clathrin adaptor protein; CCV, clathrin-coated vesicle; EE, early endosome; EGFR, EGF receptor; LE, late endosome; MT, microtubule; MVB, multivesicular body; RE, recycling endosome; TGN, \textit{trans}-Golgi network.
Chapter 3

Adenovirus RIDα activates an autonomous cholesterol regulatory mechanism that rescues defects linked to Niemann-Pick disease type C

3.1 Summary

Host-pathogen interactions are important model systems for understanding fundamental cell biological processes. Here we describe a cholesterol trafficking pathway induced by the adenovirus membrane protein RIDα that also subverts the cellular autophagy pathway during early stages of an acute infection. A palmitoylation-defective RIDα mutant deregulates cholesterol homeostasis and elicits lysosomal storage abnormalities similar to mutations associated with Niemann-Pick type C (NPC) disease. Wild-type RIDα rescues lipid sorting defects in cells from patients with this disease by a mechanism involving a class III phosphatidylinositol-3-kinase. In contrast to NPC disease gene products that are localized to late endosomes/lysosomes, RIDα induces the accumulation of autophagy-like vesicles with a unique molecular composition. Ectopic RIDα regulates intracellular cholesterol trafficking at two distinct levels: egress from endosomes, and transport to endoplasmic reticulum necessary for homeostatic gene regulation. However RIDα also induces a novel cellular phenotype suggesting it
activates an autonomous cholesterol regulatory mechanism distinct from NPC disease gene products.


3.2 Introduction

Lysosomal storage diseases (LSDs) comprise more than 40 human genetic disorders [220]. Although a majority of LSDs involve mutations in lysosomal acid hydrolases, others such as Niemann-Pick type C (NPC) disease have underlying defects in intracellular trafficking [221]. NPC is a fatal autosomal recessive disorder caused by mutations in the polytopic membrane protein NPC1 located in late endosomes (LE) and lysosomes (Ly) in 95% of cases, or more rarely the soluble protein NPC2 that is concentrated in Ly [222]. NPC1 and NPC2 coordinate egress of unesterified cholesterol from LE/Ly and mutations in either protein causes cholesterol overload in these organelles. As a result, elevated cholesterol levels are not counterbalanced by sterol homeostatic mechanisms in the endoplasmic reticulum (ER), and cholesterol and other lipids continue to accumulate causing formation of abnormal lysosomal storage organelles (LSOs) [163]. NPC cholesterol dysfunction also increases basal levels of autophagy [223, 224], indicating a possible role for sterol trafficking in this pathway as well. Perturbed autophagy has been implicated in cell death associated with NPC and other neuropathies including Alzheimer’s and Huntington’s disease, suggesting a common molecular basis for disorders with extensive endocytic-autophagic-lysosomal neuropathology [225].

In contrast to the endocytic-lysosomal pathway that degrades extracellular and plasma membrane proteins, autophagy mediates turnover of cytosolic constituents [226, 227]. Although autophagy occurs at low basal levels in virtually all cells, multiple stimuli including nutrient depletion, accumulation of protein aggregates, and organelle
obsolescence up-regulate this pathway. Autophagy is controlled by a unique set of autophagy-related (Atg) proteins that sequester cytosolic components in double-membrane vesicles known as autophagosomes [226, 227]. One of these proteins, LC3 (the mammalian homologue of yeast Atg8), is lipidated by an Atg8 ubiquitin-like conjugation system facilitating its insertion into nascent autophagic membranes [228]. Although the functional significance of this modification is unknown, LC3 translocation provides a convenient means of identifying autophagy-derived membranes [228].

Despite differences in substrates and compartmental structure, cellular homeostasis requires coordinated activity of endocytic-autophagic-lysosomal pathways. Some of the key molecules linking these pathways include the class III phosphatidylinositol-3-kinase (PI3K) Vps34 that regulates early endosome (EE) biogenesis as well as autophagosome membrane expansion [229], and the small GTPase Rab7 [54, 230]. In mammalian cells, autophagosomes have also been shown to fuse with endosomes en route to lysosomes, resulting in intermediate structures known as amphisomes [231]. Recently, mutations in components of the ESCRT machinery responsible for sorting ubiquitinated endocytic protein cargo in multivesicular bodies (MVBs) have been shown to block autophagy by inhibiting autophagosome-endosome fusion [232-234]. Furthermore, autophagy is impaired by loss of COPI coatamer necessary for normal early endosome function [235].

Despite recent progress, however, there are relatively few mechanistic insights to how endocytosis and autophagy are coordinated. Continued examination of the molecular basis for connectivity between these two degradative pathways is crucial in order to identify common therapeutic targets for LSDs and other disorders where accumulation of undegraded substrates is a prominent feature.
Adenovirus (Ad) is a non-enveloped DNA virus internalized by receptor-mediated endocytosis that escapes to cytosol by lysing endosomal membranes (Fig. 3.1a) [236, 237]. Although the majority of Ad infections are subclinical, approximately half of Ad serotypes are associated with human diseases [238]. The molecular basis for Ad-induced disease involves signaling pathways elicited during viral uptake and early Ad gene products that modulate host innate immune responses [213, 239]. RIDα (formerly E3-13.7), which is encoded by an early 3 region (E3) Ad transcript, was first identified by its ability to redirect constitutively recycling EGF receptors (EGFR) to Ly independent of ligand and EGFR tyrosine kinase activity or ubiquitination (Fig. 3.1b) [136-138]. Subsequent studies demonstrated RIDα cooperates with a second E3 protein, RIDβ, to downregulate pro-apoptotic receptors TNFR1, TRAIL-R1, and Fas [116]. Recently we discovered that RIDα regulates protein sorting by mimicking GTP-Rab7 through binding of two Rab7 effectors, RILP and ORP1L [145]. However, RIDα shares no sequence homology with Rab7 and has no intrinsic catalytic activity, providing a unique example of how a viral protein mimics the function of an endogenous protein to hijack an intracellular trafficking process. In this study, we report that RIDα coordinates trafficking in endocytic and autophagic pathways by activating a cholesterol homeostasis mechanism that operates independent of NPC1 and NPC2 by a class III PI3K-dependent mechanism.
3.3 Materials and methods

Antibodies and reagents

The following antibodies were used: actin and FLAG-BioM2 mouse monoclonal antibodies (mAb), Sigma (St. Louis, MO); β-COP mouse mAb, Novus Biologicals (Littleton, CO); E1A mouse mAb, BD Biosciences (San Jose, CA); E1B rat mAb, Calbiochem (Gibbstown, NJ); furin rabbit polyclonal antibody (pAb), Affinity Bioreagents (Golden, CO); LBPA mouse mAb kindly provided by J. Gruenberg (University of Geneva, Switzerland); LC3 mouse mAb, MBL (Woburn, MA); LC3 rabbit pAb, Abgent (San Diego, CA); cation-dependent MPR chicken pAb, Chemicon (Billerica, MA); human-specific IL2Rα mAb, ATCC (Manassas, VA); ORP1L goat pAb, Imgenex (San Diego, CA); Rab5 mouse mAb, Transduction Laboratories (San Jose, CA); Rab7 goat pAb and CD73 rabbit pAb, Santa Cruz (Santa Cruz, CA); RIDα rabbit pAb produced with a synthetic peptide corresponding to residues 76 to 91 [141]; p62/SQSTM1 mouse mAb, Abnova (Taipei, Taiwan); TlrR mouse mAb, Zymed (San Francisco, CA); hamster- and human-specific LAMP1 mouse mAbs, Developmental Studies Hybridoma Bank developed under the auspices of NICHD and maintained by The University of Iowa Department of Biological Sciences (Iowa City, IA). Fluorescent- and HRP-tagged secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA), filipin, MDC, U18666A, and 3-MA from Sigma, LY294002 from Calbiochem, and Alexa-674 CT-B and Dil-LDL from Molecular Probes (Carlsbad, CA).
EGFP-Rab7 mammalian expression plasmids were a kind gift from C. Bucci (University of Helsinki, Helsinki, Finland).

**Mutagenesis and cloning**

Wild-type RIDα with an amino-terminal FLAG epitope (FLAG-RIDα) was amplified by PCR from pExchange2/RIDα template [185] using forward 5’-GCGGAGCTAGCATGGATTACAAGGATG-3’ and reverse 5’-GATCAGCTAGCCCTAAAGAATTCTGAGAAGATCA-3’ primers incorporating flanking Nhe I restriction sites (underlined), and sub-cloned in pCR2.1 supplied with the TOPO® TA Cloning Kit (Invitrogen, Carlsbad, CA). FLAG-RIDα was excised from pCR2.1 and ligated to the Nhe I restriction site in multiple cloning site (MCS) A of the bicistronic mammalian expression vector pIRES (BD Biosciences). A cDNA encoding human IL2Rα subunit excised from pBK-IL2Rα [138] was ligated to Bam HI and Not I restriction sites in pIRES/RIDα MCS B. A RIDα C67S point mutation was incorporated into pIRES/FLAG-RIDα/IL2Rα using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), and forward 5’-CAGTTCAATTGACTGGGTATGCTCCGACATTGCTAC-3’ and reverse 5’-GTACGCAATGCGCACACTAACCCAGTCATAGGACT-3’ primers (C67S substitution in boldface). FLAG-RIDα (C67S) was amplified using forward 5’-TTACAGCTTTCGGGCAGCAGTACTTAAATACGACTC-3’ and reverse 5’-ATGCTCGACGCAGCAGCAGCTCGAGGCTAG-3’ primers incorporating 5’ Not I and 3’ Bsi WI restriction sites (underlined) and sub-cloned into pCR2.1. FLAG-
RIDα (C67S) excised from pCR2.1 was ligated to the retroviral bicistronic expression vector pQCXIN (BD Biosciences) digested with Not I and Bsi WI. PCR primers were designed using the DNASTAR software package (DNASTAR, Inc., Madison, WI) and purchased from Operon (Huntsville, AL). PCR amplified sequences were verified by automated DNA sequencing.

**Cell lines and Ad stocks**

To create a permanent CHO cell line expressing FLAG-RIDα (referred to as CHO-RIDα), parental CHO cells were transfected with pIRES/FLAG-RIDα/IL2Rα using Trans-IT CHO transfection reagent (Mirus Bio, Madison, WI) and selected for drug-resistance in media supplemented with G418 (200 µg/ml; Calbiochem). Drug-selected cells were further enriched for IL2Rα surface expression by sterile sorting on a flow cytometer (BD Aria, BD Biosciences) after surface staining with an IL2Rα mAb and FITC-conjugated secondary antibody. To create a permanent CHO cell line expressing FLAG-RIDα (C67S) [referred to as CHO-RIDα (C67S)], GP2-293 retrovirus packaging cells were transfected with pQCXIN/FLAG-RIDα (C67S) using Trans-IT 293 transfection reagent (Mirus Bio). Pantropic retrovirus was generated upon subsequent transfection of drug-selected packaging cells with pVSV-G plasmid. Retrovirus-containing media was collected 48 hr later and added to CHO cells followed by G418 selection. Stable RIDα expression was verified by immunoblotting and immunostaining with FLAG and RIDα antibodies.
GP2-293 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) and CHO cells in MEMα. A549 cells were grown in Ham’s F12 media, and normal (GM05659) and NPC (GM03123) fibroblasts (Coriell Institute, Camden, NJ) in MEM. All media were supplemented with 10% FBS (unless otherwise noted) and 2 mM glutamine. For cholesterol loading, cells were cultured in media with 10% LPDS obtained commercially (Sigma) followed by LPDS-containing media supplemented with freshly prepared LDL-cholesterol (50 µg/ml; kindly provided by R. Morton, Cleveland Clinic Foundation, Cleveland, OH). For 25-HC loading, cells were cultured in media with 10% DS that was prepared by incubating FBS with 20 mg/ml Cab-O-Sil® (Sigma) that is known to deplete lipoproteins [240] for 16 h at 4°C with rotation, clarified by centrifugation (15,000 rpm × 1 h, 4°C), and sterilized sequentially with 0.45 and 0.22 micron filters [241, 242]. The cells were incubated for 24 h in the DS-containing media with or without supplemented 25-HC (5 µg/ml; diluted from a 2.5 mg/ml ethanol stock; Sigma) for the final 24 h.

Wild-type Ad2 was obtained from ATCC. Mutant Ads with an internal RIDα deletion or a RIDα C67S substitution are described elsewhere [135, 141]. Ad stocks were grown in HEK293 cells, and titers were determined by plaque assay using standard techniques. Cells were acutely infected with approximately 200 plaque-forming units per cell [137].

Cell harvesting
Cells were lysed under denaturing conditions using 1% SDS in 10 mM Tris, pH 7.4, preheated to 100°C to analyze total cellular protein, and under non-denaturing conditions using RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EGTA, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) to recover immune complexes on protein A Sepharose beads [243].

Differential detergent extractability experiments were carried out according to previously established methods [244]. Briefly, cells were washed twice with PBS supplemented with 1 mM MgCl₂ and 1 mM CaCl₂, scraped in the same buffer, and centrifuged for 3 min at 3200 rpm. Cells were lysed with extraction buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 0.2 mM PMSF, 1 μM leupeptin) supplemented with 1% Triton X-100, 1% Brij 98, or 60 mM OG. Cells were passed through a 22-gauge needle 10 times and incubated at 4°C or 37°C for 30 min, followed by 5 passages through a 22-gauge needle. Lysates were centrifuged at high speed for 10 min at 4°C, and supernatants (detergent soluble fraction) were adjusted to 0.1% SDS. Membrane pellets were washed once with appropriate extraction buffer, resuspended in 100 μl of solubilization buffer (50 mM Tris, pH 8.8, 1% SDS, 5 mM EDTA), passed through a 22-gauge needle 12 times, brought up to 1 ml with appropriate extraction buffer and centrifuged at high speed for 10 min at 4°C, to generate a detergent insoluble fraction. Detergent soluble and insoluble fractions were subjected to immunoprecipitation and immune complexes were washed extensively, solubilized with Laemmli buffer, resolved by SDS-PAGE, and transferred to nitrocellulose filters for immunoblotting using standard methods.
Cell fractionation

Cells were homogenized and fractionated on Percoll™ (Amersham-Pharmacia, Piscataway, NJ) gradients as previously described [185]. Briefly, cells were rinsed twice with PBS supplemented with 2 mM EDTA and 5 mM EGTA, and then scraped in ice-cold homogenization buffer (HB) consisting of 10 mM HEPES, pH 7.5, 0.25 M sucrose, 1 mM EDTA, and protease inhibitors. Cells were collected by centrifugation, resuspended in HB buffer, and homogenized with 22 strokes of a Dounce homogenizer. The homogenate was diluted with an equal volume of fresh HB, and centrifuged at 400 × g for 10 min at 4°C to pellet unbroken cells and nuclei. Post-nuclear supernatants were adjusted to a final concentration of 27% Percoll in 0.25 M sucrose using a 90% Percoll stock solution, and then layered over a 1 ml sucrose cushion consisting of 10× HB. Gradients were centrifuged for 90 min at 25,000 × g in an SS34 fixed-angle rotor (Sorvall Instruments, Newtown, CT) without braking. A total of nine 1.2 ml fractions were collected manually starting from the top of the gradient. Fractions were analyzed by comparing equal aliquots of total cellular protein determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA), or by immunoprecipitation after membranes were solubilized with RIPA buffer and centrifuged at 100,000 × g for 30 min at 4°C in a TL 100.3 fixed angle rotor (Beckman Instruments, Inc., Palo Alto, CA) to precipitate Percoll.

Metabolic labeling
For metabolic labeling with $^{3}$H-palmitate, cells were pretreated with 100 µM 2-BP or DMSO vehicle for 24 h and labeled with 1 mCi $^{3}$H-palmitate (American Radio-labeled Chemicals, Inc., St. Louis, MO) in 3 ml of complete media supplemented with 2-BP or DMSO for 3 h. Cells were lysed with RIPA buffer and lysates were immunoprecipitated with anti-FLAG BioM2 antibody. Following SDS-PAGE, gels were incubated for 16 h in 1 M hydroxylamine, pH 7, or a control solution of 1 M Tris, pH 7, and prepared for fluorography. For metabolic labeling with $^{35}$S-amino acids, cells were pre-incubated in cysteine and methionine-free medium for 1 h. Amino acid-starved cells were pulse-labeled for 30 min with $^{35}$S-Express Protein Labeling Mix (PerkinElmer Life Sciences, Boston, MA) diluted in amino acid-deficient medium supplemented with 10% dialyzed FBS. Radio-labeled cells were incubated with medium supplemented with 10% dialyzed FBS and a 10-fold excess of non-radioactive cysteine and methionine (chase medium) for 2 h prior to Ad infection, followed by additional incubation with chase medium for up to 12 h.

Confocal microscopy

Normal and NPC fibroblasts were mock treated or transfected with 3 µg plasmid DNA using the Normal Human Dermal Fibroblast nucleofector kit and the Amaxa Nucleofector Device (Amaxa, Gaithersburg, MD). CHO cells were transfected using Trans-It CHO transfection reagent (Mirus Bio). All cells were seeded on poly-L-lysine treated coverslips, perforated with 0.5% β-escin, fixed with 3% paraformaldehyde, incubated with appropriate primary antibodies for 1 h at room temperature and
fluorochrome-conjugated secondary antibodies for 16 h at 4°C, and mounted on glass slides using SlowFade® (Molecular Probes). For PI3K inhibitor studies, cells were incubated with 10 mM 3-MA (diluted from a 200 mM stock made in water), or 100 µM LY294002 (diluted from a 10 mM stock made in DMSO) for 3 h prior to staining. Some cells were incubated with Dil-LDL (10 µg/ml) for 15 min at 4°C, or Alexa 674 CT-B (5 µg/ml) for 30 min at 4°C, followed by a 2 h chase in serum-free media at 37°C, prior to staining. Other cells were incubated with 50 µM MDC diluted in PBS for 10 min at 37°C prior to staining. Fixed cells were incubated with filipin (50 µg/ml) for 16 h at 4°C to detect free cholesterol. Confocal images were acquired with a Zeiss LSM 510 Meta laser scanning microscope (Carl Zeiss MicroImaging, Jenna, Germany) and accompanying software using diode (excitation 405 nm), Argon (excitation 488 nm), and HeNe (excitation 543 and 633 nm) lasers, and 63x or 100x Plan Apo, NA 1.4 objectives. Filipin was excited with the diode laser, and emissions collected between 411 and 486 nm. When necessary, phase contrast images were collected, and cell and nucleus boundaries were drawn using MetaMorph software (Molecular Devices, Sunnydale, CA) and overlaid onto respective confocal images. All images were processed with Adobe Photoshop CS3 and Illustrator CS3 software.

**Quantification of total cellular cholesterol**

CHO cell lines were treated with 500 nM U18666A for 8 h, washed twice with PBS, scraped in 1 ml PBS, and homogenized by 10 passages through a 22-gauge needle. Aliquots were analyzed for cholesterol content using the Amplex Red cholesterol assay.
(Molecular Probes) following the manufacturer’s instructions and absorbance was read at 568-nm using a SpectraMax M2 plate reader and SoftMax Pro v5 software (Molecular Devices). Cholesterol content was calculated using cholesterol standards supplied with the kit and normalized to total denatured cellular protein as determined by Bradford assay.

**Quantification of filipin fluorescence intensity**

Confocal images were collected using a 40x C-Apo, NA 1.2 objective and a 6 µm optical slice. Data were collected for untransfected and transfected cells that were discriminated based on RIDα staining. Free cholesterol content was determined using Metamorph software to quantify the average filipin intensity of individual cells. The highest value obtained for all the untransfected cells in a single microscopic field was set to 100 and the data for cells expressing RIDα in the same field were normalized to this value.

**Autophagy induction**

Prior to staining, cells were washed twice with PBS and incubated with Earl’s Balanced Salt Solution (EBSS) for 6 h, with the addition of pepstatin A and E64d (both at 10 µg/ml; Sigma) for the final 3 h.

**Gene expression studies**
RNA extracted using the ToTALLY RNA Kit (Ambion, Austin, TX), was reverse-transcribed using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Target gene mRNA levels were analyzed by real-time PCR using standard hydrolysis probe (TaqMan) techniques and a GAPDH internal control. The mRNA expression levels for cells receiving various sterol treatments were plotted as fold-change relative to mRNA levels for control cells cultured in standard media containing 10% FBS for each individual cell line. PCR was carried out on a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA), and products were analyzed using 7500 System SDS Software version 1.3. Probe and primer pairs listed in Table 3.1 were designed using the Universal ProbeLibrary Assay Design Center (Roche, Indianapolis, IN). Probes and PCR master mix were purchased from Roche and primers from Operon.

**Statistical analysis**

Statistical analyses for Amplex Red cholesterol quantification were performed using the Student’s \( t \) test (SigmaStat, San Jose, CA). Data are expressed as mean ± SEM from three independent experiments. A \( P \) value of <0.01 was considered statistically significant. Statistical analyses for real-time PCR were performed using a one-way repeated measures analysis of variance (ANOVA) and Bonferroni post-hoc testing (SigmaStat). Values obtained from three independent experiments are expressed as mean ± SEM. A \( P \) value of <0.001 was considered statistically significant. Statistical analyses for filipin fluorescence quantification were performed using the Student’s \( t \) test. Data are
expressed as mean ± SEM from three independent experiments. A $P$ value of $<0.001$ was considered statistically significant.
3.4 Results

**RIDα counteracts Ad induced autophagy**

To gain further insight into RIDα function, Ad-infected cells were examined with antibodies to early (EEA1) and late (LAMP1) endocytic markers. Cells were also stained with filipin, an intrinsically fluorescent antibiotic that binds free cholesterol. Intracellular cholesterol levels vary greatly among different organelles, and specific sorting mechanisms are required to establish and maintain these distinct compositions [245]. Since Ad triggers endosome membrane lysis, we hypothesized this might lead to altered sterol balance in infected cells. Human A549 cells were infected with wild-type Group C Ad2 and a mutant Ad2 virus with an internal deletion in the RIDα ORF that both produce equivalent levels of major early region (E1) proteins (Fig. 3.1c, d). Similar to mock-infected cells (Fig. 3.1e), EEA1 and LAMP1 vesicles were dispersed throughout the cytosol in cells infected with wild-type Ad2 (Fig. 3.1f). In addition, intracellular distributions of free cholesterol were essentially identical in mock-treated and Ad2-infected cells. In contrast, cells infected with the RIDα-null Ad2 virus displayed relatively few enlarged LAMP1 compartments that were concentrated in a perinuclear cluster. These cells were essentially devoid of typical cytosolic EEA1 vesicles, and EEA1 was sequestered in the enlarged LAMP1 compartments (Fig. 3.1g). The LAMP1 structures were also enriched for free cholesterol (Fig. 3.1g), and the autophagosomal membrane protein LC3 (Fig. 3.1h). Altogether these data suggest Ad infection *per se* induces an autophagic response characterized by a pronounced redistribution of
intracellular cholesterol and EEA1 that is offset by RIDα expression. This hypothesis was further investigated by monitoring levels of the LC3-binding protein p62/SQSTM1 that regulates formation of protein aggregates and is selectively degraded by autophagy [246]. In contrast to mock- and Ad2-infected cells where levels were essentially unchanged, cells infected with RIDα-null Ad2 virus exhibited significant p62/SQSTM1 turnover by 24 h p.i. compared to a control protein (actin) which is unaffected by autophagy (Fig. 3.1i). Interestingly, although EEA1 was sequestered in enlarged LAMP1/LC3-positive autophagosomes in RIDα-null Ad2 infected cells, EEA1 protein levels were essentially unchanged (Fig. 3.1i) consistent with recent reports that fusion with early endosomes is required for efficient autophagy [235]. These data suggest RIDα regulates homeostatic balance between endocytosis and autophagy to facilitate endosome-lysosome degradation of select membrane cargo in Ad-infected cells.

**RIDα is palmitoylated at Cys67**

Despite a functional relationship to Rab7 [145], RIDα lacks intrinsic GTPase activity raising questions as to how this viral protein is regulated in cells. The RIDα carboxyl tail has a cysteine residue located 6 amino acids from its second transmembrane domain that could modulate RIDα function by undergoing reversible palmitoylation (Fig. 3.2a). Since RIDα downregulates EGFR independent of other Ad proteins [139, 185], the following studies were carried out in CHO cells stably expressing amino-terminal FLAG-tagged RIDα to facilitate biochemical analysis. We found that RIDα was labeled with 3H-palmitate, and that radioactive incorporation was blocked in cells incubated with
the palmitoylation inhibitor 2-bromopalmitate (2-BP; Fig. 3.2b). Furthermore, as expected for thioester linkages, the $^3$H radiolabel was significantly reduced when RIDα immune complexes were incubated with hydroxylamine after SDS-PAGE (Fig. 3.2b). $^3$H-palmitate incorporation was also completely blocked by introducing a serine mutation at position 67 (C67S; Fig. 3.2b). Although palmitoylation can have an important role in sorting proteins lacking transmembrane peptide sequences to detergent resistant membranes (DRMs), this modification is less likely to affect DRM partitioning of integral membrane proteins [217, 247]. This notion was tested for RIDα by extracting cell membranes with Triton X-100 at different temperatures to determine whether the viral protein partitions into classic Triton X-100 insoluble DRMs [248]. Results in Fig. 3.2c indicate that wild-type RIDα and mutant RIDα (C67S) proteins are completely solubilized by Triton X-100 at low temperature, in contrast to the GPI-anchored protein CD73 found exclusively in the Triton X-100 insoluble fraction (Fig. 3.3a). Similar to transferrin receptor (TfR) which is a non-raft protein, both of the viral proteins also exhibited modest insolubility with Brij 98 and essentially complete solubility with octyl glucoside (OG; Fig. 3.2c, Fig. 3.3b) [249]. Altogether these data suggest the viral protein is found in bulk membrane independent of palmitoylation status.

**RIDα induces accumulation of an autophagy-like compartment**

Previous studies showed that RIDα binds GTP-Rab7 effectors, and that at least one of these interactions is necessary for RIDα biological activity in infected cells [145]. To determine whether Rab7 and RIDα are in the same or different membrane
compartments, cell membranes were fractionated on iso-osmotic Percoll gradients. Wild-type and RIDα (C67S) mutant proteins were enriched in relatively light fractions overlapping EE markers as well as the TGN marker furin (Fig. 3.2d). RIDα sedimentation profiles were in sharp contrast to Rab7 in parental CHO cells which co-sedimented with LAMP1 in a relatively dense fraction (Fig. 3.2d). Similarly, confocal microscopy revealed that EGFP-Rab7 but not wild-type RIDα co-localized with LAMP1-positive LE/Lys (Fig. 3.2e). Instead, wild-type RIDα was associated primarily with a discrete population of perinuclear vesicles (Fig. 3.2e). To determine whether RIDα compartments were accessible to endocytic probes, cells with ectopic RIDα expression were incubated with LDL conjugated to a highly fluorescent lipophilic dye (Dil-LDL) and then fixed and stained for the viral protein and LAMP1. LDL co-localized with LAMP1 but not RIDα (Fig. 3.4a), indicating that this ligand is largely excluded from RIDα compartments. In contrast to the endocytic probe, however, the RIDα compartment was readily accessible to the autofluorescent compound monodansylcadaverine (MDC) that is a specific in vivo marker for autophagic vesicles (Fig. 3.4b) [250]. Furthermore RIDα was extensively co-localized with the autophagic membrane marker LC3 (Fig. 3.4b, Fig. 3.5a, b). The LC3 staining pattern in RIDα-expressing cells is striking for two reasons. First, RIDα/LC3-positive compartments are devoid of LAMP1 suggesting they do not fuse with lysosomes. Second, relatively little LC3 is membrane-associated in parental CHO cells unless the cells are cultured under autophagy-inducing conditions to trigger formation of LC3-positive autophagosomes (Fig. 3.4c, left and middle panels). Interestingly, LC3-positive vesicles retained the same perinuclear distribution in autophagy-induced cells expressing ectopic RIDα (Fig. 3.4c,
right panel) that we had originally observed under basal conditions (Fig. 3.4b) suggesting 
RIDα modulates starvation-induced autophagic machinery.

Several other cellular proteins were enriched in RIDα compartments including the 
COPI coatamer subunit β-COP (Fig. 3.4d, Fig. 3.5c) involved in retrograde Golgi-to-ER 
transport, endocytosis, and autophagy [251]; and the Rab7 effector protein ORP1L (Fig. 
3.4e, Fig. 3.5d) normally present on LEs but which also binds directly to the RIDα 
carboxyl tail [42, 145]. In addition, we demonstrated that some RIDα compartments 
contained β-COP and ORP1L (Fig. 3.4f), or LC3 and ORP1L (Fig. 3.4g), suggesting the 
presence of all three marker proteins in a common set of vesicles. Contrary to the pattern 
of staining in RIDα-expressing cells, β-COP and ORP1L were found primarily on non- 
overlapping small vesicles dispersed throughout the cytosol in parental CHO cells (Fig. 
3.4h). Introduction of the C67S mutation had no apparent effect on accumulation of 
RIDα-positive compartments enriched for LC3, β-COP, and ORP1L (Fig. 3.4i-k, Fig. 
3.5e-g). Previous studies showed that RIDα exhibits only minimal overlap with other 
early endosomal markers (EEA1, Rab5) and none with the Golgi [157, 185]. Together 
with the new data presented here, RIDα compartments do not correspond to any well 
defined intracellular organelle. In addition assembly of the organelle is unaffected by 
RIDα palmitoylation status.

**Palmitoylation regulates RIDα function**

In contrast to membrane partitioning and intracellular localization, our data 
indicate palmitoylation does influence RIDα function. This conclusion is based on
confocal images showing that RIDα (C67S) alters morphology of LAMP1-positive LE/Ly in the absence of other Ad proteins compared to parental cells or cells expressing wild-type RIDα (Fig. 3.6a). LAMP1-postive structures are enlarged and also accumulate high levels of several molecules, including cholesterol (Fig. 3.6a, b), the atypical LE/Ly lipid LBPA (Fig. 3.6b, c), and cholera toxin subunit B (CT-B) which binds GM₁ ganglioside (Fig. 3.6c). These data suggest that RIDα controls a cholesterol egress mechanism unmasked in cells with normal NPC1/NPC2 machinery by a mutation affecting its ability to undergo palmitoylation. The RIDα (C67S)-induced phenotype is similar to the phenotype produced by U18666A that impairs intracellular cholesterol biosynthesis and perturbs cholesterol egress from endosomes by an unknown mechanism (Fig. 3.6d) [252]. Interestingly, ectopic expression of wild-type RIDα substantially reduced the U18666A-induced phenotype compared to parental CHO cells, in contrast to RIDα (C67S) that appeared to exacerbate U18666A-induced accumulation of free cholesterol in enlarged LAMP1-positive LE/Lys (Fig. 3.6d). Furthermore ectopic RIDα was associated with a statistically significant reduction in intracellular levels of free cholesterol compared to parental CHO cells following U18666A treatment (Fig. 3.6e). Similarly RIDα (C67S) produced a statistically significant increase in intracellular free cholesterol following U18666A treatment, compared to parental CHO cells (Fig. 3.6e).

Aberrant LAMP1-positive, cholesterol loaded vesicles were also found in A549 cells infected with a RIDα (C67S) Ad2 mutant virus (Fig. 3.6f). Similar to results obtained in stable RIDα-expressing CHO cell lines RIDα undergoes palmitoylation at Cys67 in Ad2-infected cells (Fig. 3.6g). Cells infected with RIDα (C67S) mutant Ad2 nevertheless degrade EGFR similar to cells infected with wild-type Ad2, and in contrast
to cells infected with a RIDα-null virus (Fig. 3.6h). This result is not unexpected since LEs contain lysosomal enzymes that are active at the low pH of the compartment [253]. Altogether these data indicate that RIDα (C67S) affects lipid trafficking but not EGFR degradation suggesting these two RIDα functions are dissociable.

Analysis of GTP-Rab7 function has been greatly aided by design of mutant proteins that are either constitutively active or dominant inhibitory because of defective GTP-GDP cycling [254]. To determine whether RIDα (C67S) has constitutively active or dominant-inhibitory properties, its effects on LE morphology were compared to those elicited by mutant Rab7 proteins. Although constitutively active Rab7 and RIDα (C67S) both induce formation of enlarged LAMP1-positive vesicles, constitutively active Rab7 co-localizes with the enlarged vesicles while RIDα (C67S) does not (Fig. 3.6i). Dominant-inhibitory Rab7 disperses LAMP1-positive vesicles throughout the cytosol (Fig. 3.6j) by interfering with microtubule (MT)-dependent MVB/LE motility [230]. RIDα (C67S) co-expression counteracts the effect of dominant-inhibitory Rab7 and alters the morphology of LAMP1 compartments (Fig. 3.6j) similar to results in cells with normal levels of wild-type Rab7 (Fig. 3.6a). These results suggest RIDα (C67S) alters LE morphology similar to constitutively active Rab7, and that wild-type RIDα regulates a pathway that parallels Rab7-dependent trafficking or acts upstream of GTP-Rab7 in a common pathway.

**RIDα regulates cholesterol homeostatic gene expression**
Cholesterol levels are tightly regulated by classic end-product feedback mechanisms that control SREBP-dependent gene expression at the level of the ER (Fig. 3.7a) [255]. These mechanisms are set by rapid cholesterol flux between the ER and endosomes, and they become exaggerated or erratic if this dynamic flux is perturbed. If RIDα regulates cholesterol trafficking to the ER, it should also modulate SREBP-dependent gene transcription. In addition, results in the previous section showing LSO-like structures in CHO cells expressing RIDα (C67S) predicts this mechanism should be highly dependent on RIDα palmitoylation. These hypotheses were tested using real-time PCR to examine expression of two SREBP-dependent genes products: LDL receptor (LDLR), responsible for endosomal transport of LDL-cholesterol; and HMG-CoA reductase (HMGR), the rate-limiting enzyme in de novo cholesterol synthesis (Fig. 3.7a). Homeostatic responses were assayed by comparing mRNA levels in sterol-depleted CHO cell lines without and with supplemental sterol (see Materials and methods). In addition to LDL-cholesterol, levels of 25-hydroxycholesterol (25-HC), an ER cholesterol metabolite and another potent regulator of the SREBP pathway (Fig. 3.7a) [163], were manipulated. Similar to parental CHO cells, LDLR and HMGR mRNA levels declined following LDL-cholesterol loading in CHO-RIDα cells (Fig. 3.7b, c). CHO-RIDα (C67S) cells exhibited exaggerated LDL-cholesterol homeostatic responses compared to the other two cell lines that were statistically significant for both genes. In contrast to HMGR (Fig. 3.7c), LDLR mRNA was not reduced by LDL-cholesterol loading (Fig. 3.7b), suggesting these two genes respond differently to fluctuations in ER cholesterol pool size in CHO-RIDα (C67S) cells. Parental CHO and CHO-RIDα cells both exhibited elevated LDLR and HMGR gene expression after sterol depletion that sharply
declined with addition of 25-HC (Fig. 3.7b, c). Although CHO-RIDα (C67S) cells displayed a similar pattern, the homeostatic response for both genes was significantly exaggerated suggesting RIDα (C67S) also perturbs the 25-HC ER pool. These results support the hypothesis that RIDα regulates the SREBP pathway by a cholesterol trafficking mechanism that is phenotypically silent unless it is blocked by the C67S RIDα mutation. They also suggest the RIDα-dependent cholesterol trafficking pathway is the prevalent mechanism in CHO cells with functional NPC proteins.

In addition to the SREBP pathway, oxysterols are potent activators of nuclear liver X receptors (LXRs) that regulate genes involved in sterol storage, transport, and catabolism (Fig. 3.7a) [256]. Thus cells were also examined for expression of the gene encoding CYP7B, a P450 enzyme that is transcriptionally suppressed by activated LXRs [256]. Whereas LDL-cholesterol loading provoked a modest homeostatic response in CHO parental and CHO-RIDα (C67S) cells, CHO-RIDα cells displayed an exaggerated 7-fold increase in CYP7B mRNA expression in sterol depleted cells that declined with LDL-cholesterol loading (Fig. 3.7d). In contrast, CHO-RIDα cells exhibited a CYP7B homeostatic response comparable to parental cells following 25-HC loading (Fig. 3.7d). Since LXRs are activated by multiple oxysterols, these data suggest RIDα regulates LXR gene transcription by perturbing a different oxysterol pool (Fig. 3.7a). For example, NPC fibroblasts have reduced levels of the cholesterol metabolite 27-hydroxycholesterol (27-HC) produced in mitochondria indicating the NPC1/NPC2 machinery also regulates cholesterol egress to this compartment [257]. In contrast to LDL-cholesterol, 25-HC loading did not elicit a CYP7B homeostatic response in CHO-RIDα (C67S) cells (Fig. 3.7d). These results are consistent with data obtained for the SREBP pathway indicating
that RIDα regulates 25-HC-dependent gene transcription by a palmitoylation-dependent mechanism.

**RIDα reduces LSOs and intracellular cholesterol levels in NPC cells**

Production of LSO-like structures in CHO-RIDα (C67S) cells with functional NPC1 and NPC2 proteins suggests RIDα (C67S) either disables the NPC1/NPC2 machinery or deregulates a novel NPC1/NPC2-independent pathway. We determined whether or not the viral protein complements genetic defects in NPC cells to distinguish between these two possibilities. Despite some cell type-specific variability in compartment morphology, our data indicate that RIDα induces accumulation of a compartment that contains the autophagic membrane protein LC3 in normal fibroblasts (Fig. 3.8a) and fibroblasts from an NPC patient (Fig. 3.8b) similar to results obtained in stable CHO cell lines (Fig. 3.4). By comparison, membrane-associated LC3 was virtually undetectable in either cell type following mock transfection under basal conditions (Fig. 3.8a, b). Similarly, RIDα co-localized with β-COP and ORP1L in both normal and NPC fibroblasts (data not shown). However, in contrast to normal fibroblasts where LBPA was present in dispersed cytosolic vesicles with or without ectopic RIDα expression (Fig. 3.8c), LBPA was highly concentrated in RIDα compartments in NPC cells expressing the viral protein (Fig. 3.8d). By comparison, LBPA was present in a few enlarged vesicles that presumably correspond to LSOs (arrowheads in Fig. 3.8d) in addition to smaller dispersed vesicles in mock-transfected NPC cells. These data imply RIDα vesicles are
dynamic compartments that may be remodeled in response to intracellular cholesterol levels or other abnormalities associated with NPC proteins.

We next determined whether RIDα offsets NPC cholesterol dysfunction. Cholesterol-enriched LSOs were virtually undetectable in NPC cells expressing RIDα (Fig. 3.9a) compared to mock-transfected NPC cells (Fig. 3.9b). The finding that RIDα and LC3 co-localize suggests RIDα-dependent lipid trafficking subverts the autophagy machinery. This hypothesis was tested by treating cells with the class III PI3K inhibitor 3-methyladenine (3-MA) that prevents autophagic sequestration following LC3 membrane insertion [231]. 3-MA substantially reduced the ability of the viral protein to clear cholesterol-filled LSOs in NPC cells (Fig. 3.9c) compared to mock-transfected NPC cells where it had no apparent effect (Fig. 3.9d). Similar results were obtained using a second PI3K inhibitor, LY294002 (Fig. 3.9e, f). Morphological data were corroborated by quantification of filipin fluorescence which revealed that cholesterol levels were reduced by approximately 33% in NPC cells expressing RIDα versus mock-transfected cells in the same microscopic field (Fig. 3.9g). Furthermore, cholesterol levels were essentially identical in NPC cells treated with 3-MA or LY294002 with and without ectopic RIDα expression (Fig. 3.9g).

NPC mutations also adversely affect trafficking of protein cargo such as mannose 6-phosphate receptors (MPR) [258]. MPRs deliver newly synthesized acid hydrolases to the endocytic pathway where hydrolases are subsequently transferred to Ly and MPRs recycle back to TGN [17]. MPRs may also reach the cell surface followed by rapid uptake from clathrin-coated pits. RIDα expression caused MPRs to accumulate at PM (Fig. 3.9h), in contrast to mock-transfected NPC cells where MPRs are trapped in LSOs.
due to lipid imbalance imposed by cholesterol accumulation (Fig. 3.9i). Expression of RIDα in normal fibroblasts had no discernable effect on intracellular MPR distribution (Fig. 3.9j). These data suggest RIDα does not reconstitute MPR EE-to-LE transport enabling increased cell surface expression and/or it prevents MPR re-internalization from PM. Similar to results obtained for cholesterol, 3-MA blocked MPR re-routing to PM in RIDα-expressing NPC cells (Fig. 3.9k), in contrast to mock-transfected cells where 3-MA had no apparent effect on intracellular MPR distribution (Fig. 3.9l).
3.5 Discussion

In this report we demonstrate that RIDα is a potent regulator of intracellular cholesterol in gene transfer experiments as well as during acute Ad infections. Our data indicate RIDα facilitates two distinct steps in cholesterol trafficking: egress from endosomes, and transport to ER necessary for homeostatic gene regulation (Fig. 3.10). RIDα rescues the cholesterol storage phenotype in NPC fibroblasts suggesting the viral protein regulates a molecular mechanism that operates independent of NPC1 and NPC2. This supposition is further supported by evidence obtained with a palmitoylation-defective RIDα (C67S) mutant that alters LE morphology to produce NPC-like LSOs and deregulates ER homeostatic mechanisms in CHO cells with functional NPC proteins [169, 259]. Even though the viral protein binds GTP-Rab7 effectors [145], RIDα is not present in LAMP1-positive LEs but influences endosome function at a distance from a membrane compartment with characteristics of autophagic vesicles. Furthermore, RIDα is excluded from cholesterol-rich DRMs suggesting its function is not restricted by NPC mutations in contrast to Rab7 that is inhibited by high levels of cholesterol [258]. The RIDα (C67S) mutant also abrogates the cellular phenotype associated with a dominant-negative form of Rab7, providing further evidence that RIDα and GTP-Rab7 are not necessarily interchangeable. Previous studies indicate newly synthesized RIDα is sorted at the level of TGN via AP-1 clathrin adaptors [185], and the working model in Fig. 3.10 suggests that RIDα regulates cholesterol trafficking in endosomes following TGN exit. A similar mechanism has been reported in T cells of the adaptive immune system where antigen-loading compartments for major histocompatibility complex class II molecules
receive continuous input from autophagosomes [260]. However we cannot exclude the possibility that RIDα facilitates retrograde cholesterol transport to ER via a TGN intermediate [261]. The presence of β-COP in RIDα compartments is consistent with either role since COP1 coatamer regulates endocytosis and autophagy as well as Golgi-to-ER retrograde transport [251]. It is also conceivable RIDα regulates transport in both directions depending on sterol load and cell physiology.

Our results indicate the RIDα-containing membranes do not correspond to any well defined intracellular organelle. Although the origin of these compartments remains unclear, it is possible RIDα arrests maturation of a physiologically short-lived early autophagic compartment. Alternatively RIDα could create a virus-specific compartment by remodeling intracellular membranes. Our data favor the first possibility since RIDα vesicles are enriched in LC3 recruited to nascent autophagic membranes but devoid of LAMP1 found in mature autophagosomes. This is not the first example of a pathogen hijacking host autophagic machinery to carry out a novel function, as poliovirus triggers formation of unique LC3-positive compartments [63]. However in contrast to poliovirus that utilizes virally-induced autophagosomal membranes to facilitate viral RNA replication, RIDα regulates innate immune responses [213, 239]. We have also demonstrated that RIDα promotes endosome-to-lysosome transport of select membrane cargo at the expense of enhanced autophagic flux in acutely infected cells. It is conceivable RIDα compartments sequester key proteins required for autophagosome-lysosome fusion, or alternatively provide a surplus of rate-limiting molecules required for efficient endocytosis. Evidence that RIDα regulates endosome sterol balance independent of NPC1/NPC2 favors the latter hypothesis. However it is conceivable
RIDα integrates endocytosis and autophagy by a combination of these two mechanisms. The finding that RIDα function requires class III PI3K activity necessary for trafficking in both pathways [229] also supports a coordinating role for RIDα.

Another molecule enriched in RIDα compartments is ORP1L, a member of the family of oxysterol binding proteins implicated in a variety of cellular functions [262]. ORP1L is a GTP-Rab7 effector linked to LE MT-dependent motility and lipid trafficking that also binds RIDα [42, 43, 145]. ORP1L is comprised of multiple amino terminal ankyrin repeats, a pleckstrin homology domain, and a carboxyl terminal oxysterol regulatory domain (ORD) capable of binding cholesterol and 25-HC \textit{in vitro} [48]. Contrary to GTP-Rab7 that recognizes ORP1L ankyrin repeats, RIDα interacts with ORD sequences [145] suggesting it may directly regulate sterol binding. While palmitoylation does not alter RIDα membrane partitioning or intracellular compartmentalization, this modification does influence RIDα function presumably by regulating conformation of the RIDα carboxyl tail and its ability to interact with protein binding partners [263]. Thus failure of RIDα to undergo palmitoylation may sequester ORP1L and/or inhibit its sterol binding properties leading to deregulated cholesterol homeostasis. RIDα also binds a second GTP-Rab7 effector RILP and this interaction is required for RIDα biological activity in Ad2-infected cells [145]. It will be interesting in the future to determine whether RILP is also recruited to RIDα-induced compartments since RILP binds ESCRT components involved in dual regulation of endocytosis and autophagy independent of GTP-Rab7 [45, 46, 264].

In contrast to CHO cells and normal fibroblasts, RIDα compartments in NPC fibroblasts are also enriched for LBPA which is known to regulate cholesterol clearance.
from endosomes [265]. The NPC phenotype is partially reversed by addition of exogenous LBPA, suggesting LBPA is a limiting factor contributing to disease pathology [265]. It is therefore possible RIDα restores NPC cholesterol trafficking by upregulating a basal mechanism involved in LBPA transport to the endocytic system. The finding that LBPA is only detectable in RIDα compartments in cells with inherent cholesterol imbalance reveals the plasticity of RIDα-based mechanisms.

Although its role in controlling innate-immune responses is well known, our studies have identified at least three new RIDα-associated functions during acute Ad infections. First, RIDα subverts virus-induced autophagy. While the molecular basis for Ad2-induced autophagy is currently unknown, it is conceivable this pathway is activated to remove endosomes damaged by membrane lysis immediately after Ad internalization (Fig. 3.1a). Ad-induced endosome membrane lysis may also deplete a critical rate-limiting factor sufficient to disrupt trafficking throughout the endocytic pathway that is reintroduced to the system via a RIDα-dependent mechanism (Fig. 3.10). Second, in contrast to receptors involved in innate-immune responses, RIDα may divert MPRs to the PM where they fulfill distinct functions compared to their normal role in shuttling acid hydrolases to Lys [17]. Third, RIDα regulates endosome cholesterol egress in acutely infected cells. Cholesterol is required for Ad2 internalization from PM and endosome escape, suggesting Ads may be taken up to specialized cholesterol-enriched domains that execute membrane lysis [82]. It is therefore conceivable RIDα restores cholesterol balance that is perturbed during the early stages of an acute Ad infection. RIDα-induced cholesterol trafficking may also be important in latent infections where E3 proteins are thought to have a prominent role [152]. In contrast to NPC1 and NPC2, however, RIDα
selectively regulates cholesterol trafficking to ER and consequently could trigger cholesterol imbalance in other intracellular compartments during an acute infection.

These studies highlight several new areas for future investigation. First, given its capacity to reconstitute cholesterol trafficking in cells with defective NPC proteins, it will now be of interest to fully characterize the newly identified RIDα-induced pathway. Second, these studies raise the possibility that the NPC1/NPC2 machinery is impaired during acute Ad infections. Third, RIDα-containing vesicles may shed new light on the intracellular membrane origin of autophagic vesicles.
3.6 Figures

Figure 3.1. RIDα counterbalances Ad-induced autophagy.

(a) Ad2 internalized by clathrin-mediated endocytosis escapes from early endosomes by membrane lysis followed by MT-dependent transport to the nucleus and viral gene expression. (b) RIDα, which is expressed within the first few hours of an acute infection, facilitates targeted degradation of EGFR (blue) and pro-apoptotic receptors (yellow). (c) Confocal images of A549 cells infected with wild-type or RIDα-null Ad2 viruses stained with E1B and RIDα antibodies. Arrowheads indicate RIDα-positive compartments. (d) Equal aliquots of total cellular protein from mock- and Ad-infected A549 cells resolved by SDS-PAGE and immunoblotted with E1A antibody. (e - g) Confocal images of (e) mock, (f) Ad2, and (g) RIDα-null Ad2 infected A549 cells stained with EEA1 and LAMP1 antibodies and filipin; magnified images of single and merged channels shown on right. (h) Confocal images of RIDα-null Ad2 infected A549 cells stained for LC3 and LAMP1. (i) Equal aliquots of total cellular protein from mock- and Ad-infected cells immunoblotted with antibodies to p62/SQSTM1, EEA1, and actin as a function of time post-infection (p.i). Size bars, 10 µm. Abbreviations: CCV, clathrin-coated vesicle; IB, immunoblot; MVB, multivesicular body; Nu, nucleus; P.I., post-infection; PM, plasma membrane.
Figure 3.2. RIDα is palmitoylated at Cys67.

(a) Schematic of RIDα membrane topology showing amino-terminal FLAG epitope (red arrowhead), luminal signal peptidase cleavage and disulfide bond formation sites, and carboxyl-tail sequence with palmitoylation and protein interaction sites and EGFR homology domain [138, 141, 145, 185, 216]. (b) CHO-RIDα and CHO-RIDα (C67S) cells treated with 2-BP or DMSO and radio-labeled with 3H-palmitate. FLAG immune complexes separated by SDS-PAGE and gels incubated with Tris or hydroxylamine solutions prior to fluorography. Bottom panel shows FLAG immune complexes from duplicate samples analyzed by RIDα immunoblotting for loading control. (c) CHO cell lines extracted with indicated detergents, and RIDα (left and middle panels) or TfR (right panel) immune complexes from detergent soluble and insoluble fractions immunoblotted with antibodies to the same protein. (d) Membranes from stable CHO cells expressing wild-type RIDα or RIDα (C67S) fractionated on 27% Percoll gradients and equal aliquots of total membrane protein immunoblotted with antibodies to RIDα (top two panels). Equal aliquots of total membrane protein from parental CHO cells immunoblotted with antibodies to marker proteins for intracellular membrane compartments listed in the figure (bottom five panels). (e) Confocal images of CHO cells transfected with EGFP-Rab7 plasmid and stained for LAMP1, or CHO-RIDα cells stained for LAMP1 and RIDα. Size bars, 10 μm. Abbreviations: 2-BP, 2-bromopalmitate; A, alanine; C, cysteine; D, aspartic acid; EE, early endosome; H, histidine; I, isoleucine; IB, immunoblot; IP, immunoprecipitation; L, leucine; LE, late endosome; Ly, lysosome; OG, octyl glucoside; P, proline; PM, plasma membrane; Q, glutamine; R, arginine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel
electrophoresis; T, threonine; TfR, transferrin receptor; TGN, trans-Golgi network; TX-100, Triton X-100; V, valine; W, tryptophan; Y, tyrosine.
Figure 3.3. Detergent extraction controls and quantification.

(a) GPI-anchored protein CD73 is insoluble in TX-100 at low temperature. Cells were extracted with TX-100 at the indicated temperatures and CD73 immune complexes from detergent soluble and insoluble fractions were immunoblotted with an antibody to the same protein. (b) Detergent extraction results for wild-type and C67S RIDα proteins from 3 independent experiments were scanned on a GS-800 Densitometer (Bio-Rad) and bands were quantified using NIH ImageJ software. Data are expressed as percent detergent-insoluble relative to total RIDα indicated as mean ± SD. Abbreviations: GPI, glycosyl phosphatidylinositol; SD, standard deviation; TX-100, Triton X-100.
b

Percent insoluble after extraction

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<th>Detergent</th>
<th>CHO-RID\alpha</th>
<th>C67S</th>
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<td>5.6 ± 1.6</td>
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<tr>
<td>TX-100 (37°C)</td>
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<td>1.7 ± 0.5</td>
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<tr>
<td>Brij 98</td>
<td>18.6 ± 1.4</td>
<td>20.6 ± 3.8</td>
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<tr>
<td>Octyl Glucoside</td>
<td>3.3 ± 1.8</td>
<td>2.7 ± 0.4</td>
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Figure 3.4. RIDα induces formation of a hybrid organelle with characteristics of both endocytic and autophagic vesicles.

(a) CHO-RIDα cells stained with LAMP1 and RIDα antibodies following incubation with Dil-LDL.  (b) CHO-RIDα cells stained for LC3 and RIDα following incubation with MDC.  (c) Parental CHO or CHO-RIDα cells stained for LC3, or LC3 and RIDα under basal conditions (left) or following 6 h incubation in EBSS to induce autophagy (middle and right panels).  (d, e) CHO-RIDα cells stained for RIDα and (d) β–COP, or (e) ORP1L.  (f, g) Magnified images of single and merged channels of CHO-RIDα cells triple stained for RIDα and ORP1L, and either (f) β–COP or (g) LC3.  (h) Parental CHO cells stained for β-COP and ORP1L.  (i - k) CHO-RIDα (C67S) cells stained for RIDα and either (i) LC3, (j) β-COP, or (k) ORP1L.  Size bars, 10 µm.  Abbreviations: LDL, low density lipoprotein; MDC, monodansylcadaverine; Nu, nucleus.
Figure 3.5. RIDα induces formation of a hybrid organelle with characteristics of both endocytic and autophagic vesicles (supplementary).

(a) Wide-field confocal image of CHO-RIDα cells stained for LC3 and RIDα, with asterisks indicating that all cells in the field have RIDα/LC3 positive vesicular compartments. (b-d) Multiple confocal images of CHO-RIDα cells stained for RIDα and either (b) LC3, (c) β-COP, or (d) ORP1L. (e-g) Multiple confocal images of CHO-RIDα (C67S) cells stained for RIDα and either (e) LC3, (f) β-COP, or (g) ORP1L. Size bars, 10 µm.
Figure 3.6. RID\(\alpha\) (C67S) induces formation of enlarged lipid-filled LAMP1 structures.

(a) Confocal images of CHO cell lines stained with LAMP1 antibody and filipin. (b, c) Magnified images of single and merged channels from CHO-RID\(\alpha\) (C67S) cells stained with (b) LBPA antibody and filipin, or (c) LBPA antibody after incubation with Alexa 647 CT-B. (d) CHO cell lines treated with U18666A for 8 h and stained with LAMP1 antibody and filipin. (e) Cholesterol quantification in CHO cell lines treated with DMSO (vehicle) or U18666A for 8 h using the Amplex Red Cholesterol Assay Kit. Values were normalized to total cellular protein and displayed as mean ± SEM, \(*P < 0.01\). (f) Confocal images of A549 cells infected with a mutant RID\(\alpha\) (C67S) Ad2 virus and stained with LAMP1 antibody and filipin 24 h p.i. (g) A549 cells infected with wild-type or RID\(\alpha\) (C67S) Ad2 viruses radio-labeled with \(^3\)H-palmitate and RID\(\alpha\) immune complexes separated by SDS-PAGE for fluorography. (h) A549 cells radio-labeled with \(^35\)S-Express Protein Labeling Mix and mock-infected or infected with wild-type or mutant Ad viruses, and EGFR immune complexes analyzed by SDS-PAGE and fluorography at times indicated. (i) Parental CHO cells transfected with constitutively active (Q67L) EGFP-Rab7 and stained for LAMP1, or CHO-RID\(\alpha\) (C67S) cells stained for LAMP1 and RID\(\alpha\). (j) Parental CHO or CHO-RID\(\alpha\) (C67S) cells transfected with dominant-negative (T22N) EGFP-Rab7 and stained for LAMP1 (parental), or LAMP1 and RID\(\alpha\) [CHO-RID\(\alpha\) (C67S)]. Arrowheads indicate examples of co-stained vesicles. Size bars, 10 \(\mu\)m. Abbreviations: CT-B, cholera toxin subunit B; EGFP, enhanced green fluorescent protein; EGFR, EGF receptor; IB, immunoblot; IP, immunoprecipitation;
LBPA, lysobisphosphatidic acid; Nu, nucleus; P.I., post-infection; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean.
Table 3.1. Gene, probe, and primer details for real-time PCR.

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<th>Gene name, UPL probe</th>
<th>Primer</th>
<th>Sequence</th>
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<td>GAPDH (X52123)</td>
<td>Forward</td>
<td>5’-ATCTACTGGCGTCTTCACCAC-3’</td>
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<td>Roche UPL probe #133</td>
<td>Reverse</td>
<td>5’-GGAGATGATGACCCCTCTTTGG-3’</td>
</tr>
<tr>
<td>HMGR (X00494)</td>
<td>Forward</td>
<td>5’-CACGTCTCTCTGTGACC-3’</td>
</tr>
<tr>
<td>Roche UPL probe #50</td>
<td>Reverse</td>
<td>5’-CAGAGGCTCCCCGTCTACAAC-3’</td>
</tr>
<tr>
<td>CYP7B (L04690)</td>
<td>Forward</td>
<td>5’-GGCAAAACACTATTCTGCAAC-3’</td>
</tr>
<tr>
<td>Roche UPL probe #50</td>
<td>Reverse</td>
<td>5’-CAGTCAAATTGCTATCCACCTG-3’</td>
</tr>
<tr>
<td>LDLR (M94387)</td>
<td>Forward</td>
<td>5’-ACCATTGGAGATGGAAGC-3’</td>
</tr>
<tr>
<td>Roche UPL probe #133</td>
<td>Reverse</td>
<td>5’-CGTCCAAAATACTTTGTCTCA-3’</td>
</tr>
</tbody>
</table>

Target gene (with Genbank accession number), probe, and primer sequences used for real-time PCR. Abbreviations: GAPDH, glyceraldehydes 3-phosphate dehydrogenase; HMGR, hydroxymethylglutaryl CoA reductase; LDLR, LDL receptor; UPL, universal probe library.
Figure 3.7. RIDα modulates sterol-regulated gene expression.

(a) Transcriptional mechanisms controlling expression of target genes LDLR, HMGR, and CYP7B described in text. (b) LDLR, (c) HMGR, and (d) CYP7B mRNA levels quantified by real-time PCR. Values expressed as relative units after internal normalization to GAPDH mRNA levels, and compared to control samples from the same cell lines cultured in 10% FBS from 3 independent experiments. Data presented as mean ± SEM, *P < 0.001. Abbreviations: FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HC, hydroxycholesterol; HMGR, hydroxymethylglutaryl CoA reductase; LDL, low density lipoprotein; LDLR, LDL receptor; LXR, liver X receptor; SEM, standard error of the mean.
Figure 3.8. RIDα induces formation of dynamic hybrid organelles with characteristics of both endocytic and autophagic vesicles in NPC fibroblasts.

(a, b) Confocal images of (a) normal or (b) NPC cells stained for RIDα and LC3 after mock transfection or transfection with a RIDα expression plasmid. (c, d) Confocal images of (c) normal or (d) NPC cells stained for RIDα and LBPA after mock transfection or transfection with a RIDα expression plasmid. Arrowheads in (d) show presumptive LBPA-positive LSOs. Size bars, 10 μm. Abbreviations: LBPA, lysobisphosphatidic acid; LSO, lysosomal storage organelle; NPC, Niemann-Pick type C; Nu, nucleus.
**Figure 3.9. RIDα expression rescues cholesterol trafficking and associated defects in NPC cells by a class III PI3K-dependent mechanism.**

(a-f) NPC cells transfected in the absence or presence of PI3K inhibitors as indicated in the figure and stained for (a, c, e) LAMP1, filipin and RIDα or (b, d, f) LAMP1 and filipin. (g) Quantification of normalized filipin fluorescence intensity in cells treated similarly to panels a-f as described in Materials and methods. Data presented as mean ± SEM, *P < 0.001. (h-l) NPC and normal cells transfected in the absence or presence of PI3K inhibitors as indicated in the figure and stained for (h, j, k) LAMP1, MPR and RIDα or (i, l) LAMP1 and MPR. Cells in (h-l) were cultured in 10% LPDS media for three days followed by 50 µg/ml LDL for 24 h prior to staining. Size bars, 10 µm. Abbreviations, 3-MA, 3-methyladenine; LDL, low density lipoprotein; LPDS, lipoprotein deficient serum; NPC, Niemann-Pick type C; MPR, mannose 6-phosphate receptor; PI3K, phosphatidylinositol 3-kinase; SEM, standard error of the mean.
Figure 3.10. Model of the role of RIDα as a coordinator of endosome trafficking.

LDL cholesterol esters are internalized via PM LDLR (1a), unesterified, and trafficked to MVB/LEs (1b). Cholesterol is egressed out of these organelles by coordinated action of NPC1 and NPC2, and transported to the ER where it is sensed by cholesterol and 25-HC homeostatic machinery (2a), and to mitochondria where it is converted to other oxysterols (2b). NPC1 and NPC2 mutations block cholesterol egress to both these compartments, deregulate cholesterol homeostasis, reduce oxysterol production, and induce LSO formation (3). Although RIDα vesicles resemble autophagic vesicles they also have distinct molecular properties that distinguish them from bona fide autophagosomes (4a). Specialized RIDα compartments that sequester the MDC indicator dye from bulk cytosol may supply an unknown rate-limiting factor important for endocytic maintenance and/or cholesterol homeostasis. RIDα activates an autonomous class III PI3K-dependent cholesterol egress mechanism that restores ER cholesterol trafficking in NPC1-defective cells (4b). RIDα also suppresses Ad-induced autophagy (5a) and facilitates endosome-to-lysosome targeting of select membrane protein cargo (5b). Positive and negative RIDα mediated actions are highlighted in red.

Abbreviations: Chol, cholesterol; EE, early endosome; ER, endoplasmic reticulum; HC, hydroxycholesterol; LE, late endosome; LDL, low density lipoprotein; LDLR, LDL receptor; LSO, lysosomal storage organelle; MDC, monodansylcadaverine; MVB, multivesicular body; NPC, Niemann-Pick type C; PI3K, phosphatidylinositol 3-kinase; PM, plasma membrane; TGN, trans-Golgi network.
Chapter 4

Discussion and future directions

4.1 Discussion

RIDα is an Ad protein encoded by the E3 transcript. The proteins expressed from this transcript function to modulate host innate-immune responses during infection. RIDα was originally identified by its ability to direct constitutively recycling EGFRs to the Ly for degradation [135]. Furthermore, it was demonstrated that RIDα downregulates the EGFR independent of ligand activation [136], kinase activity [137], or receptor ubiquitination [138]. It was also shown that RIDα downregulates the EGFR independent of other Ad genes [139, 145, 185, 186]. Later it was found that RIDα interacts with another E3 protein, RIDβ, to form the RID complex involved in downregulation of apoptotic receptors including TNFR1, TRAIL-1/2, and Fas [113]. Despite this information, however, very little was known about the molecular basis of RIDα function. The work of my thesis has provided new information on the trafficking and function of RIDα (summarized in Fig. 4.1). First, we determined that RIDα utilizes host cell machinery to exit the biosynthetic compartment, and that proper TGN exit is necessary for EGFR downregulation [185]. Second, our laboratory revealed that RIDα mimics GTP-Rab7 and regulates endosome trafficking by binding two Rab7 effectors
(Appendix I; Shah, 2007 #1261). Finally, we discovered that RIDα is palmitoylated, and that palmitoylation regulates a novel function for RIDα in cholesterol homeostasis. We also determined that RIDα localizes to a unique intracellular compartment where it mimics GTP-Rab7.

Although the exact identity of the RIDα compartment was unknown, it had been previously reported that RIDα partially colocalizes with the EE marker TfR and the MVB marker RhoB [157]. What remained to be elucidated were the amino acid-based sorting signals present in RIDα that mediate its proper localization. The carboxyl tail of RIDα contains both tyrosine and dileucine-based sorting signals that could potentially interact with AP complexes. Therefore, the RIDα carboxyl tail was fused to GST to test potential interactions in vitro. AP-1 and AP-2 were shown to bind RIDα, and truncations of the carboxyl tail determined the sequences responsible for AP-1 and AP-2 interaction. The interaction site contained Tyr72, and mutation of this amino acid to alanine reduced the interactions with both adaptors. As described previously, AP-1 is involved in the export of newly synthesized proteins from the TGN. Accordingly, while wild-type RIDα localizes to perinuclear vesicles, RIDα Y72A colocalized predominantly with the TGN marker furin. AP-1 binding serves an important function through mediating proper TGN exit of RIDα to direct the viral protein to its proper localization so that it can carry out its functions of regulating LE transport and downregulation of the EGFR. RIDα with a Y72A mutation is unable to exit the TGN and subsequently cannot downregulate the EGFR. AP-2 is involved in incorporation of cargo into CCPs during endocytosis, and likely serves a quality control mechanism to internalize RIDα that missorts to the PM.
This work elegantly illustrates how a viral protein utilizes the machinery of the host cell to carry out its function.

RIDα mimics GTP-Rab7 by interacting with two Rab7 effectors. However RIDα shares no sequence homology with Rab7 and has no intrinsic enzymatic activity. Therefore, we asked how RIDα is regulated to mimic Rab7. We established that RIDα is post-translationally modified by the addition of the lipid palmitate to the lone cysteine residue in the carboxyl tail. We hypothesized that palmitoylation of RIDα regulates the conformation of the carboxyl tail to mediate reversible interactions with RILP and ORP1L. Evidence for palmitoylation regulating RIDα function ensued from the study of a palmitoylation-deficient Cys67Ser mutant. With ectopic expression or when cells were infected with a virus containing the C67S RIDα Ad mutant, cells displayed enlarged LAMP1-positive compartments which were filled with cholesterol, LBPA, and a marker for GM1 ganglioside. This phenotype is similar to that found in fibroblasts cultured from patients afflicted with NPC disease. In these cells, mutation in either NPC1 or NPC2 involved in endosomal egress of cholesterol causes buildup of cholesterol and other lipids in LE/Ly and interrupts sterol regulated gene expression, inducing formation of lysosomal storage organelles (LSO).

Classically, palmitoylation regulates targeting of cytosolic proteins to detergent resistant membranes, or lipid rafts. However, this modification does not play as great a role in the trafficking of transmembrane proteins like RIDα. Hence, we found that palmitoylation does not affect localization of RIDα, as both wild-type and the palmitoylation-deficient mutant were found in a similar population of perinuclear vesicles. As opposed to Rab7, RIDα does not colocalize with LAMP1-positive LE/Ly.
Instead, RIDα colocalizes with the autophagosome marker LC3, the COPI coatamer component β-COP important for Golgi-to-ER retrograde transport, endocytosis and autophagy, and the Rab7 effector ORP1L that directly interacts with RIDα. RIDα also colocalizes with another autophagosome marker MDC, which is taken up by mature autophagosomes. Since the RIDα compartment does not correspond to any well-defined intracellular organelle, and since RIDα colocalizes with some (LC3, MDC) but not all (LAMP1) markers for mature autophagosomes, we describe the RIDα compartment as “autophagy-like.” We hypothesize that the RIDα vesicles correlate to an early autophagic compartment and that RIDα could exert positive or negative regulatory effects on the progression of autophagy or modulate a selective autophagic response.

Further studies with the C67S RIDα mutant delineated insight into the function of RIDα palmitoylation. Since RIDα C67S blocks egress of cholesterol out of LAMP1-positive LEs in the presence of functional NPC1/2, we asked whether wild-type RIDα could stimulate cholesterol transport in cells with a mutation in NPC1. Fibroblasts from an NPC patient were transiently transfected with wild-type RIDα, and stained for LAMP1 and free cholesterol. We found that RIDα could rescue the LSO phenotype in NPC cells, and that RIDα expression reduced total free cholesterol by 33%. Due to the colocalization of RIDα with the autophagy markers LC3 and MDC, we studied the effect of autophagy inhibitors on RIDα function in these cells. Inhibition of the PI3K necessary for induction of autophagy with 3-MA or LY294002 blocked the ability of RIDα to rescue the LSO phenotype. Furthermore, cholesterol content was unchanged in mock transfected cells or cells expressing RIDα treated with either inhibitor. Thus we
hypothesize that RIDα is activating an autonomous cholesterol egress mechanism that is independent of NPC1/2 and dependent on class III PI3K activity.

While my thesis has delineated a number of important aspects of RIDα localization and function, it has also raised several new questions. For example, what is the function of the “autophagy-like” RIDα compartment? Does RIDα modulate the classical autophagy pathway or Rab7 function? What role does autophagy play in the ability of RIDα to rescue the LSO phenotype in NPC cells, and how does the palmitoylation-deficient RIDα mutant induce the NPC-like phenotype? Is ORP1L involved, and if so, what is its role in endosomal egress of cholesterol? What role does RIDα play in regulating cholesterol homeostasis during an adenovirus infection and how might this impact pathophysiology? Further studies of this interesting viral protein will help answer these questions and more, and offer novel insights into the cell biology of the cell.
4.2 Future directions

RIDα and autophagy

As discussed earlier, autophagy is a degradative process by which portions of the cytosol are engulfed by double-membraned vesicles termed autophagosomes that eventually fuse with the Ly. Autophagy is important during times of cellular stress, but also occurs at basal levels in every cell. As opposed to non-selective autophagy induced by multiple stressors, cells also possess forms of selective autophagy that cause the degradation of specific targets. A classic example is the Cvt pathway in yeast in which precursor hydrolases are taken up by autophagosomes and delivered to the vacuole. However, little is known about the molecular mechanisms that activate selective autophagic responses. There is also a great deal of crosstalk between the autophagic and endocytic systems, and a number of proteins that can function in either pathway depending on the physiology of the cell. Proper balance between these two systems is necessary to maintain homeostasis, and RIDα provides a unique model for understanding the molecular basis of endocytic and autophagic homeostasis.

My studies have determined that RIDα colocalizes with two markers for autophagosomes: the cytosolic protein LC3 which becomes lipidated and localizes to autophagosomal membranes, and the autofluorescent dye MDC which is engulfed by autophagy isolation membranes. Furthermore, we have preliminary data showing RIDα co-localization with Beclin 1, which is an important protein involved in the regulation of Vps34, the class III PI3K required for activation of the early steps in autophagy.
progression. However, the RIDα vesicles do not co-stain with LAMP1, indicating that they do not fuse with LE/Ly enriched for this marker protein. Furthermore, when cells expressing wild-type RIDα are stressed by nutrient deprivation to induce non-selective autophagy, we do not observe characteristic large, LC3-positive autophagosomes that are seen in parental cells during autophagy activation. Taken together, these data indicate that the RIDα vesicles correspond to a mature "autophagy-like" compartment that cannot fuse with the LAMP1-positive compartments. We hypothesize that RIDα may be activating a selective autophagic response that can be tailored to the physiology of the cell, whether it is during an Ad infection or the high cholesterol load of NPC cells (Fig. 4.1). A second hypothesis is that RIDα may be sequestering a component(s) necessary for the maturation of classical autophagosomes. Likely candidates are the Rab7 effectors RILP and ORP1L since Rab7 is involved in the translocation of autophagosomes along MTs towards the Ly during the final steps in autophagy [55]. RIDα may be sequestering one or both of these effector proteins and inhibiting Rab7 activity to halt the progression of autophagy or activate an alternative endocytic process. On the other hand, RIDα may be priming cells to rapidly respond to cellular stress by inducing the formation of multiple early autophagic compartments that undergo rapid maturation under certain conditions. Further experiments will be needed to delineate the role of RIDα in the selective and non-selective autophagy pathways.

As stated above, increased autophagy is a hallmark of NPC, and is likely involved in the neuronal cell death that accompanies this disease [184]. NPC cells display increases in LC3-II and have higher levels of Beclin 1 protein leading to increased activation of Vps34. However, induction of autophagy is not met with a similar increase
in autophagic flux, as NPC tissues accumulate ubiquitinated proteins. This leads to autophagic stress and cell death. We have shown that RIDα is able to rescue the cholesterol storage phenotype in NPC cells, and that this function is blocked by treatment of cells with autophagy inhibitors. We hypothesize that RIDα is activating an alternative trafficking route to bypass the defects that cholesterol imposes on the endocytic system. As Rab7 activity is blocked with high cholesterol, RIDα can compensate for loss of Rab7 function and potentially induce an alternative transport pathway that may rely on membrane originating in the autophagic system (Fig. 4.1). Alternatively, RIDα may be increasing autophagic flux to balance the induction of autophagy and allow the cell to handle the increased autophagic load.

Although much is known about the activation and progression of autophagy, there is still much to learn about the onset of autophagy, specifically the origin of autophagic membrane and how autophagosomes are formed. Recently, evidence was provided that autophagosomes initiate from cup-shaped protrusions derived from the ER [266]. A second study indicated that the PAS fuses with vesicles derived from the TGN and LE by an Atg5/Atg7-independent pathway [267]. Therefore, it is possible that RIDα-positive vesicles fuse with the PAS upon exit from the TGN, or after recycling from another compartment or domain (Fig. 4.1). RIDα may offer insight into the molecular basis of directed transport to the PAS during the membrane expansion step of autophagy. We found that RIDα colocalizes with β-COP, a component of COPI coatamer important for Golgi-to-ER retrograde transport. It follows that β-COP could act as a scaffold to direct RIDα vesicles originating in the TGN to fuse with the ER-derived
PAS. In the future it will be of interest to study the trafficking route of RIDα as it exits the TGN and travels to its steady-state localization in the “autophagy-like” compartment.

**Role of ORP1L in cholesterol homeostasis**

As previously discussed, ORP1L is a Rab7 effector protein that, along with RILP, coordinates recruitment of dynein-dynactin motors for transport of LEs along MTs [43]. ORP1L also binds directly to the carboxyl tail of RIDα, but unlike Rab7, RIDα interacts with ORP1L through its oxysterol binding domain (ORD) as determined by yeast 2-hybrid assay [145]. ORP1L is a member of the family of oxysterol binding proteins, and can bind cholesterol and 25-OH through its ORD *in vitro* and *in vivo* [48]. However, it remains to be determined what role sterol binding has on ORP1L function. A recent paper provides insight for the role of cholesterol sensing by ORP1L in the positioning of LEs. Rocha et al. determined that ORP1L adopts different conformations in cholesterol bound or unbound states [268]. When LE cholesterol levels are low (i.e. when ORP1L is not bound to cholesterol), the FFAT motif of ORP1L is exposed and can interact with the ER protein VAP, producing contact sites between the LE and ER. VAP binds in *trans* to the Rab7-RILP-ORP1L tripartite complex and disconnects the p150Glued arm of dynein-dynactin from the complex, preventing minus-end directed movement along MTs. In the cholesterol bound form of ORP1L, or when LE cholesterol levels are high, the FFAT motif of ORP1L is occluded, inhibiting contact with the ER and causing the clustering of LEs around the MTOC. Therefore, cholesterol regulates LE positioning through ORP1L
and VAP interactions resulting in the scattering of cholesterol poor LEs and clustering of cholesterol loaded LSOs, as in NPC disease.

ORP1L normally localizes to LE membranes, but colocalizes extensively with RIDα when the viral protein is expressed ectopically in cells. Assuming that palmitoylation of RIDα acts as a switch to promote the release of ORP1L, we hypothesize that RIDα C67S binds ORP1L and sequesters it from Rab7. Therefore, Rab7 activity is blocked and LEs enlarge and accumulate cholesterol. By competing for and sequestering Rab7 effectors, RIDα C67S induces the formation of LSOs from a distance. In the case of wild-type RIDα, RIDα bound to ORP1L may block cholesterol sensing by the ORD and induce a conformation that would promote an interaction with VAP. The generation of RIDα-mediated ER contact sites may promote endosome-to-ER cholesterol transport simply from the close proximity of these compartments. In the future it will be of interest to determine the ability of ORP1L to bind VAP in cells expressing wild-type or C67S RIDα, and the consequences of this interaction on cholesterol homeostasis.

**New tools to study RIDα**

It is evident from our research that ability to follow the trafficking of RIDα in a live cell would give us a technical advantage to better understand its myriad functions. Standard techniques for live cell imaging include labeling a protein of interest with a fluorescent tag such as green fluorescent protein (GFP). However GFP is a large protein of ~27 kDa when compared to RIDα which is only 13.7 kDa. Therefore, constructing a
chimera of RIDα and GFP could present some problems in the ability of RIDα to properly fold, localize correctly, or function efficiently. There are at least three possible sites to incorporate a GFP tag into RIDα: the amino terminus, the exocytic loop, or the carboxy terminus. While there are drawbacks to each site for GFP addition, it is conceivable that one of the chimeras would produce functional RIDα, and this would provide a valuable resource to further study the trafficking of RIDα. Alternatively, there are a number of post-translational labeling methods that can overcome the shortcomings of traditional genetic fusion with fluorescent proteins [269]. These methods include peptide tags that are as small as 1-2 kDa that can interact with a fluorophore probe that is added to cells. As we have used RIDα with an amino terminal FLAG (12 amino acid peptide) tag with much success, the addition of one of these peptide sequences to the amino terminus could give us the perfect tool to study the trafficking of RIDα in live cells.

One caveat to future studies of RIDα function in NPC cells is the low transfection efficiency we achieve using nucleofection. This inability to express RIDα in a greater number of NPC fibroblasts inhibits us from performing numerous biochemical experiments needed to further our research. One possibility to increase ectopic expression of RIDα in these cells is the use of retrovirus or lentivirus-mediated gene transfer techniques. We currently have a pantropic retrovirus that expresses wild-type RIDα, and researchers have used lentivirus to effectively deliver siRNA into the NPC mouse [270]. This approach comes with its own pitfalls, as we do not know how the NPC cells would react to infection, even with a replication-defective virus like retrovirus or lentivirus. An alternative system to study the role of RIDα in NPC1
function is the NPC1-null CHO cell line designated M12 [271].

4.3 Conclusions

We have determined that RIDα uses the host cell machinery to exit the TGN, and that RIDα vesicles may fuse directly with the autophagosomal isolation membrane, thus providing the novel membrane needed to complete the autophagosome. Data showing colocalization of β-COP with RIDα supports a role for COPI coatamer in the retrograde transport of TGN-derived vesicles with the ER-derived isolation membrane. We have also shown that RIDα is a potent regulator of endocytic and autophagic trafficking through the mimicry of GTP-Rab7, and that RIDα plays a role in the regulation of cholesterol homeostasis. Based on these studies, we hypothesize RIDα activates a novel mechanism for egress of cholesterol out of LEs that is independent of NPC1/2. Three results reinforce our hypothesis: 1) expression of the palmitoylation-deficient RIDα mutant alters the morphology of LEs and induces an NPC-like phenotype even in the presence of functional NPC1/2; 2) both wild-type and RIDα C67S alter the expression of genes involved in the regulation of cholesterol homeostasis in cells with high or low sterol load; and 3) ectopic expression of wild-type RIDα in NPC1 mutant fibroblasts rescues the cholesterol sorting defect seen in these cells. This proposed mechanism for cholesterol egress may act upstream of NPC1/2 or be highly saturable, and modulation of its function may predominate over NPC1/2. Little is known about the transport of cholesterol out of endosomes to the ER or other membranes in the cell, and RIDα can be used as a model system for insight into this important trafficking step.
The function of RIDα during an Ad infection is also of interest. We hypothesize that RIDα induces the degradation of the EGFR to subvert EGFR-induced chemokine production through TNFα transactivation, since RIDα blocks the production of IL-8 through an EGF and TNFα dependent pathway (Shah, unpublished data). However, it is possible that RIDα does not induce targeted downregulation of EGFR, but rather RIDα-mediated EGFR downregulation is a product of reengaging the endocytic system after Ad infection. Our work has also indicated a role for RIDα in the regulation of cholesterol homeostasis and autophagy during an Ad infection. As previously discussed, cholesterol is important during the internalization of Ad and the exit of Ad from EEs. The lysis of EE membranes may deplete this compartment of a critical factor in cholesterol homeostasis. Lysed EEs seem to be taken up by autophagosomes during an Ad infection, and RIDα functions to subvert this response and repair the defects to the endocytic system caused by the escape of Ad into the cytosol. Previous work has identified a role for autophagy during the exit of Ad from the cell during a lytic infection, while our results describe for the first time an induction of autophagy during an Ad infection per se. Ongoing research will seek to further identify the role of RIDα in autophagy regulation during an Ad infection. Interestingly, Group B Ad may provide a useful tool in these studies due to the divergent sequence of RIDα from these viruses. RIDα expressed by Ad7 has a phenylalanine at position 67 and cannot be palmitoylated, along with other sequence variations in the carboxyl tail that may affect protein-protein interactions. Therefore, nature may have already provided us with the appropriate mutations in RIDα to study the role of RILP and ORP1L in RIDα function.
The study of the host-pathogen interaction provides insights into the pathophysiology of the pathogen, and also into the biology of the host cell itself. Our studies of RIDα have served both functions, as we have uncovered novel roles for RIDα during an Ad infection, and also discovered the potential for novel mechanisms of host cell regulation. RIDα has proved to be a useful tool in providing insight into host cell biology, and future studies of RIDα as a model system will hopefully continue this trend.
4.4 Figures

Figure 4.1. Integrated model of RIDα transport and function.

In normal cells, endocytic cargo traffics through the EE where it can be recycled back to the PM or incorporated into internal vesicles in MVBs destined for degradation after fusion of LE with Ly. Cholesterol can impact this pathway if disrupted by mutations to NPC1 or NPC2, which block the egress of LDL-derived cholesterol out of LE/Ly to the ER. Therefore the cell cannot maintain cholesterol homeostasis through gene regulation and cholesterol levels increase, causing buildup of lipids in LE/Ly termed LSOs. Elevated endocytic cholesterol levels inhibit the function of Rab7 and also block the sorting of various proteins including MPR which accumulates in LSOs. Newly synthesized RIDα exits the TGN by an AP-1-dependent mechanism and is likely transported directly to the “autophagy-like” compartment. This transport mechanism may involve targeting by COPI involved in retrograde transport to the ER-derived isolation membrane of nascent autophagic vesicles, where the RIDα vesicles may provide the membrane necessary for completion of this compartment. The model also predicts that the RIDα “autophagy-like compartment is a double-membraned vesicle similar to classic autophagosomes. RIDα mimics GTP-Rab7 and can regulate sorting through LEs, but likely does so through a specialized LAMP1-negative compartment that can rescue the endocytic perturbations caused by NPC1/2 mutants. This endocytic compartment is likely expanded by incoming membrane from the RIDα “autophagy-like” vesicles which may be positive for the PI3K product PI3P important for incorporation of EGFR into internal vesicles. RIDα also facilitates egress of cholesterol out of endosomes.
to the ER for reengagement of the cholesterol homeostatic machinery likely through ORP1L function. Abbreviations: LBPA, lysobisphosphatidic acid; LDL, low density lipoprotein; LE, late endosome; LXR, liver X receptor; MDC, monodansylcadaverine; MPR, mannose 6-phosphate receptor; MVB, multivesicular body; PI3P, phosphatidylinositol-3-phosphate; SREBP, sterol regulatory element binding protein.
References


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