THE INTERPLAY BETWEEN DYNEIN, ACCESSORY PROTEINS AND THE ENDOCYTIC PATHWAY

A thesis submitted to The University of Manchester for the degree of Doctorate of Philosophy in the Faculty of Life Science

2013

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<td>Trans Golgi Network</td>
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Abstract

Elizabeth Granger
The University of Manchester
The interplay between dynein, accessory proteins and the endocytic pathway
Doctor of Philosophy, Organelle Function
25-09-13

Cytoplasmic dynein 1 (dynein) is a multi-subunit complex that transports cargo along microtubules towards their minus ends. These microtubule minus ends are normally located toward the centre of the cell. Dynein is involved in transport of endocytic and autophagic membranes and is tightly regulated by interactions between dynein subunits and by dynein-accessory proteins. Dynein accessory proteins that are involved in a wide range of dynein-driven transport events include dynactin, Lis1 and the paralogues Nde1 and Nde1. Lis1 and Nde1/Ndel1 interact with each other and are involved in the recruitment of dynein to cargo and in regulating dynein activity. Although much is known about the specific interactions of dynein and accessory proteins, the interplay between dynein and its network of regulators in living cells is not well defined.

This project used RNAi to investigate how the dynein subunits light intermediate chain (LIC) and intermediate chain (IC) as well as Lis1 and Nde1/Ndel1 influence the endocytic pathway, autophagy and cargo recruitment. Biochemical analysis of bulk membrane preparations showed that IC is important for dynein and dynactin association with intracellular membranes. In addition, dynein and dynactin recruitment to Rab interacting lysosomal protein (RILP)-positive membranes was shown to require LIC and there was redundancy between LIC1 and LIC2 in this role. Lis1 was also needed for dynactin-dynein recruitment to these membranes, in a context that was Nde1/Ndel1-independent.

Loss of LIC, IC, Lis1 and Nde1 had differing effects on endocytic compartment size and distribution, but they all led to mislocalisation of early endosomes and lysosomes and caused lysosomes to become enlarged. Loss of LIC led to a specific phenotype whereby cells formed lamellipodia-like regions in which early endosomes and lysosomes accumulated. Loss of Lis1 prevented traffic from the early endosome to late endosomes and caused a striking enlargement of late endosomes and lysosomes. These enlarged lysosomes were LC3-positive, indicating that they were autophagic. In addition, loss of IC and LIC also led to an increase in LC3 puncta, but the LC3 did not colocalise specifically with lysosomes.

In summary, the results from this project show that i) dynactin recruitment to intracellular membranes, including RILP-postive membranes, requires dynein, ii) Lis1 and LIC1 or LIC2 are necessary but not sufficient, individually, to recruit dynein and dynactin to RILP-positive membranes iii) LIC, IC, Lis1 and Nde1/Ndel1 influence endocytic progression in specific ways, which may in turn affect autophagic flux.
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Acknowledgements

I would like to thank the BBSRC for funding my PhD. I would especially like to thank Professor Viki Allan for allowing me to do a PhD in her lab and provide a huge amount of support and guidance. It has been a wonderful experience and the last four years have been really enjoyable. I am very grateful for being given this opportunity. I would like to thank Professor Martin Lowe for being my advisor and the many insightful and helpful conversations. Thank you to Professor Phil Woodman for the helpful advice in the lab and during lab meetings.

I would like to thank everyone in the Allan and Woodman lab, without whom I would have fallen at the first hurdle. Every member of the two labs has helped me at some point and they are one of the reasons this PhD has been so much fun. I would particularly like to thank, Dr. Gavin McNee who not only helped me enormously but also taught me old people can be fun, Dr. Peter Ruane who also helped a great deal, Dr. Ling Zhang for the electron microscopy, Dr. Lydia Wunderley for conversations and advice, Dr. Nick Johnson and Louise Walker for advice, support and camaraderie. I would like to thank Dr. Mark Holden for collaborating in the production of magnetic particles and enduring several days of ‘Liz does Chemistry’. Thank you to the members of the High and Lowe lab for the kindly lending reagents and offering advice throughout my PhD.

Thank you to my family, who are the reason I ever found my way to university in the first place. Thank you to Kathy and Vickie for providing adequate company for the last 8 years. Finally, thank you James for the support in the rough times, sharing the good times and all the bits in between.
Chapter 1

Introduction
1. Introduction

Every living thing on the planet uses the cell as a basic unit for life. Complex multi-cellular organisms alongside the single-celled life forms are all made out of cells and even a virus, the only type of organism not composed of cells, needs to infect a host in order to survive. All cells are self-contained finite structures that are enclosed by an outer membrane and in the case of bacteria, plants and some fungi, a cell wall. Prokaryotic cells only contain a cell surface plasma membrane and they are filled with soluble proteins, DNA, RNA, sugars and other molecules. When eukaryotes diverged from prokaryotes, they evolved intracellular membranes that allow different parts of the cell to be partitioned and this gave rise to organelles that can be devoted to specific functions within the cell. This division of labour within eukaryotic cells allowed them to become larger and more complex, but with this complexity and space comes a challenge in terms of controlling movement of these organelles within the cell. The organelles need to be able to move within the cytoplasm in a directed and regulated fashion, in order to interact and deliver components to each other. In order to achieve directed movement, a transport system within the cell exists consisting of two different networks of filaments: actin and microtubules (MTs). Motor proteins are able to move along these filaments and carry cellular cargo to and fro. This allows organelles to move and be positioned within the cell in an efficient manner compared with random diffusion. Importantly, the regulation of this movement also provides a means for the trafficking of proteins, lipids and other molecules in a controlled fashion (Alberts et al., 2007).

Intracellular transport is achieved in mammalian cells using three families of motor proteins: myosins that travel along actin filaments and kinesins and dyneins that move along MTs (Vale, 2003). With few exceptions mammalian kinesins transport cargo to the plus ends of MTs, whereas dynein moves towards the minus end. Dynein is a large multi-subunit complex with ATPase activity, the energy from which can be harnessed to produce conformational changes within the dynein complex that allows it to move in a step-like motion along MTs (see section 1.1). There are seven families of dynein, the majority of which are axonemal, meaning they are involved in the movement of flagella and cilia. Unlike the kinesin super-family that has many different members able to transport cargo, only two forms of dynein, cytoplasmic dynein 1 and cytoplasmic dynein 2, are able to attach to and transport intra-cellular cargo (Hirokawa et al., 2009; Hook and Vallee, 2006). Cytoplasmic dynein 2 is predominantly found in flagellated and ciliated cells, where it plays
an important role in cargo transport along the flagella and cilia (Mikami et al., 2002).
Cytoplasmic dynein 1 (herein referred to as dynein) is evolutionarily conserved from yeast,
some algae and fungi through to animals (but not higher plants) (Mikami et al., 2002;
Pfister et al., 2006; Wickstead and Gull, 2007). Dynein is a large multi-subunit complex and
has many functions including a role in proper MT arrangement during both interphase and
mitosis, transport of mRNAs during development, cell polarisation, cell migration and the
proper transport and positioning of proteins and cell organelles. Due to this wide range of
functions, dynein is tightly regulated through post-translational modifications as well as via
a network of dynein-interacting accessory proteins (Allan, 2011).

This project focuses on how dynein interacts with membranes and acts as both an effector
in membrane dynamics and a regulator. Specifically, it will look at how dynein interacts
with the endocytic pathway and how the loss of key dynein regulators affects the size and
position of membranes within this pathway. In order to understand these interactions it is
first essential to be aware of how membranes are trafficked within the cell as well as
having an understanding of how dynein functions within these pathways. The trafficking of
proteins and lipids would not be possible without organelle movement, which is dependent
on motor proteins. In turn, these motor proteins would not be able to move around inside
the cell without the cytoskeleton, thus if we are to understand the movement of
membranes and their trafficking we must first look at the cytoskeleton as a basis for
transport. In the case of dynein-driven transport the cytoskeleton filament that is of
importance is the MTs.

1.1 Microtubules
MTs are polarised structures consisting of polymerised α- and β-tubulin heterodimers. The
tubulin dimers align with an α subunit in one dimer interacting with the β of another, to
form proto-filaments. 13 of these protofilaments join to form the hollow tubular structure
of a MT, with α-tubulin facing the minus end and β-tubulin exposed at the plus end (Figure
1.1 A) (Desai and Mitchison, 1997). Tubulin can switch between polymerisation and
depolymerisation, which allows MTs to grow and shrink in a dynamically unstable manner
(Mitchison and Kirschner, 1984). After polymerisation, β-tubulin has the ability to hydrolyse
GTP to GDP and when in a polymerised state as part of the MT, GDP on the β-tubulin
cannot be exchanged for GTP (Amos, 2011). A layer of tubulin at the plus end of MTs is
thought to contain GTP bound β-tubulin, forming the ‘stable cap’, which does not
depolymerise. When the GTP-tubulin at the plus end of a MT is hydrolysed to GDP, the MT becomes unstable and tubulin readily depolymerises (David-Pfeuty et al., 1977; Mitchison and Kirschner, 1984; O’Brien et al., 1987; Purich and MacNeal, 1978). When GDP-bound there is a conformational change in the tubulin that alters the way protofilaments associate with one and other and this causes the tubulin to dissociate laterally and bend outwards, leading to depolymerisation. The GTP cap prevents this bending of protofilaments by keeping the structure linear (Wang and Nogales, 2005).

In most interphase non-polarised mammalian cells, MTs are arranged in a radial fashion, with the minus ends of MTs often anchored to the MT organising centre (MTOC). The MTOC is usually located close the nucleus and plus ends of MTs extend out towards the cell periphery (Figure 1.1 B). The MTOC is a roughly spherical structure containing two centrioles and has a cytoplasmic-facing surface decorated with rings of γ-tubulin from which MTs are nucleated and anchored (Figure 1.1 C) (Kollman et al., 2011). During mitosis the mitotic spindle is formed out of MTs. The minus ends are anchored to the spindle poles, which are formed from centrioles that duplicate prior to mitosis and move to the opposite ends of the dividing cell. The plus ends extend toward the cell centre ready for chromosome attachment (Karsenti and Vernos, 2001; Nigg and Stearns, 2012). During mitosis astral MTs form, which are anchored to the spindle pole and extend a shorter distance to the plasma membrane. These astral MTs and their interaction with dynein are essential in spindle positioning (McNally, 2013; Raaijmakers et al., 2013). The dynamic nature of MTs, whereby they can grow, shrink and redistribute within the cell, allows them to carry out cellular functions in both mitotic and interphase cells.

This project focuses on dynein-driven movement of membranes in interphase cells and therefore the rest of this introduction will concentrate on dynein in this context. The function of dynein is intrinsically linked to the structure and interactions of proteins within the complex. As such, the next section (1.2) will explore the proteins that comprise the dynein complex and look at their structure and function.
Figure 1.1: A. A cartoon of MT polymerisation. Growing MT (above) and shrinking MT (below). Yellow circles represent Alpha tubulin, red circles represent GTP-bound beta-tubulin and green circles represent GDP-bound beta tubulin. B. Representation of typical MT arrangement in a non-polarised interphase mammalian cell.
1.2 Structure of Dynein

Dynein is a complex with several subunits that work together to coordinate movement, MT association and cargo interactions. The major dynein subunit is the dynein heavy chain (DHC), with which the light intermediate chains (LIC), intermediate chains (IC) interact. The light chains (LCs) associate with the complex via IC (Allan, 2011). In the next section there will be a focus on the structure of these subunits, how they interact with each other and the mechanism by which dynein can move along MTs in a directed fashion.

1.2.1 Dynein heavy chain (DHC)

The DHC protein is encoded by the DYNC1H1 gene. The protein forms a homodimer that contains the functional regions, which enable dynein to move along MTs and provides a backbone to which other subunits of dynein can associate. At 530kDa, DHC is the largest subunit of the dynein complex and has several distinct functional regions (Mikami et al., 1993). The motor head region contains a ring of 6 ATPases, which belong to the AAA ATPase family of related ATP-binding proteins that contain a two or more highly conserved regions of around 230 amino acids termed the AAA box (Frohlich, 2001; King, 2000). Each AAA ATPase domain has been assigned a number. AAA1 is attached to a linker region that connects the motor domain and the N-terminal tail region and AAA6 is adjacent to the C-terminal domain. A coiled-coil stalk region with the MT binding domain (MTBD) at its tip, descends from AAA4 and is connected to AAA5 by a buttress region (Figure 1.2) (Carter et al., 2011; Carter et al., 2008; Hook et al., 2009; Kon et al., 2012).

The ATPase activity of AAA1 is thought to lead to conformational changes within the linker region that gives rise to dynein’s force-producing movement, often termed the power stroke (Burgess et al., 2003). When AAA1 is ADP-bound the MTBD is associated with MTs and upon ATP binding it dissociates. This is followed by ATP hydrolysis, which leads to the stalk and linker region undergoing conformational changes that give rise to the step-like motion of dynein (Carter and Vale, 2010; Gibbons et al., 2005; Kon et al., 2005; Kon et al., 2012). Prior to the power stroke the linker region associates with AAA2, whereas post-power stroke it is thought to move and to lie over AAA4 or AAA5 (Carter et al., 2011; Kon et al., 2012; Schmidt et al., 2012). AAA1 is now ADP-bound and the MTBD re-associates with MTs (Burgess et al., 2003). This cycle repeats in a coordinated manner to create the stepping motion of dynein.
The AAA2-AAA6 domains are not thought to contribute directly to the conformational changes within the motor domain, but AAA2-AAA4 have been shown to interact with nucleotides and may play a role in dynein processivity (i.e. the ability of dynein to move continuously along a MT in a single direction) (Schmidt et al., 2012). *In vitro* studies have shown that in the absence of dynactin, dynein is non-processive in low ATP concentrations but is processive in higher ATP concentrations (Walter et al., 2010). In yeast, the dynein from mutants lacking the ability to hydrolyse ATP in AAA3 and AAA4 domains show increased minus-end processivity (Cho et al., 2008). These results have led to a suggested model whereby ATP-binding to AAA2-AAA4 increases dynein-MT processivity.

The N-terminal tail domain emerges from the linker region on DHC. It is the site for cargo attachment and association with LIC and IC homodimers. Three LC homodimers (Tctex-1, LC7 and LC8) associate with IC at distinct binding sites (Figure1.2) (Allan, 2011). Mice that have the *legs at odd angles* (LAO) mutation possess DHC with a point mutation (F580Y) within the tail domain. Purified dynein from these mice has impaired processivity and its stepping is less coordinated. This suggests the tail may play a role in coordinating the movement of the two motor domains in a dynein complex (Ori-McKenney et al., 2010).

The smaller subunits that associate with DHC have many functional roles in terms of cargo and dynein-regulator interactions. An overview of their structure and interaction with other dynein components is given in the next subsections (1.2.2-1.2.4) before some of their roles in membrane trafficking are discussed further (Sections 1.5-1.8).
Figure 1.2: A cartoon of dynein subunits. AAA ATPases of Dynein heavy chain (blue) are labelled 1-6 and the C-terminal domain is labelled C. Abbreviations: Light intermediate chain (LIC); Intermediate chain (IC): Light chain (LC).
1.2.2 Dynein Intermediate Chain (IC)

There are two IC genes encoding the 74kDa proteins, DYNC1I1 (IC1 protein) and DYNC1I2 (IC2 protein). In overexpression experiments, the two proteins can form both homo- and heterodimers (Lo et al., 2006). There are many tissue specific IC isoforms created through alternate splicing (Kuta et al., 2010; Nurminsky et al., 1998). Analysis of IC1 and IC2 levels shows that IC1 is not widely expressed and in HeLa cells only IC2 is detectable (Palmer et al., 2009). IC binds to the N-terminal base of DHC as observed by electron microscopy with immuno-gold labelling and this interaction has been mapped to the amino acids 406-701 on DHC (Habura et al., 1999; Steffen et al., 1996; Tynan et al., 2000a). It has been shown in Dictyostelium that the N-terminal region of IC (amino acids 1-278) is needed for this DHC binding (Ma et al., 1999). The light chains interact with IC in its natively unstructured N-terminal region and their interaction here is thought to induce structural changes to IC and dictate the area of IC that associates to induce a homodimer formation (Hall et al., 2010; Lo et al., 2001; Makokha et al., 2002; Nyarko and Barbar, 2011; Nyarko et al., 2004). Dynein IC can be phosphorylated and this is particularly important for an interaction with the dynein regulator dynactin (discussed in section 1.3), with phosphorylation leading to dissociation of IC from the p150 subunit of dynactin (Vaughan et al., 2001). Both IC and dynactin p150 have been shown to be cleaved during apoptosis, which leads to dissociation of dynein from membranous cargo (Lane et al., 2001). More of the functions of IC in terms of membrane interactions are discussed in sections 1.5 and 1.7.

1.2.3 Light chains (LCs)

Unlike ICs and LICs, the LCs, (LC7, LC8 and Tctex-1) have activities outside of the dynein complex (Allan, 2011). They also play roles in many dynein functions including membrane transport and interaction with the cell cortex (Allan, 2011). Studies in which dynein subunit function was assessed using siRNA depletion showed that the LCs are important for many dynein-driven processes including maintaining the structure of endoplasmic reticulum golgi intermediate compartments (ERGIC) and Golgi apparatus, trafficking between the Golgi apparatus and endoplasmic reticulum (ER), recycling of transferrin receptor (TFR), centrosome anchoring during prophase, the focusing of mitotic poles during prometaphase, localization of dynein to the kinetochore (KT), chromosome alignment on the metaphase plate, pushing apart MTs during mitosis and the completion of cytokinesis (Palmer et al., 2009; Raaijmakers et al., 2013).
LC8 homodimers interact with the IC via a (K/R)XTQT motif and phosphorylation of LC8 leads to dissociation of its two homodimeric regions resulting in a loss of interaction between LC8 and IC (Lo et al., 2001; Song et al., 2007). When part of the dynein complex, LC8 is thought to increase IC self-association (Nyarko and Barbar, 2011; Nyarko et al., 2004). LC8 has also been shown to interact with the actin-based motor, myosin V (Espindola et al., 2000; Naisbitt et al., 2000). In terms of specific functions, LC8 has essential roles in retrograde intraflagella transport (movement of cargo toward the minus end of MTs in flagella) and nuclear migration (Beckwith et al., 1998; Pazour et al., 1998).

Tctex-1 has a similar tertiary structure to LC8 despite the two proteins sharing virtually no amino acid sequence homology (Mok et al., 2001). Similarly, phosphorylation of TcTex-1 also results in its dissociation from IC, but the mechanism of this dissociation differs from that of LC8. In this instance phosphorylation masks the IC binding region on Tctex-1 (Song et al., 2007). Interestingly, when bound to IC, Tctex-1 increases the affinity for the LC8-IC interaction and if LC8 is IC bound, it too increases the affinity for a Tctex-1-IC interaction (Hall et al., 2009).

LC7 (also referred to as roadblock) binds IC around 20 amino acids upstream of Tctex-1 and LC8 (Susalka et al., 2002). NMR studies have shown that binding of LC7 causes a shift in the region of IC that dimerises. When Tctex-1 and LC8 are bound to IC, one IC protein interacts with another between the amino acids 222-231, but when all three light chains are attached IC associates near amino acid residue 204 (Nyarko and Barbar, 2011). LC7 has been shown to interact with a protein, Rab6, that is associated with membranes (discussed in section 1.5.2), which has implicated LC7 in dynein association with these membranes (Wanschers et al., 2008).

1.2.4 Light Intermediate Chains (LIC)
LIC binds to DHC between amino acids 649-674, overlapping somewhat with the IC binding site (Tynan et al., 2000a). LIC and IC do not interact directly with each other, so although their DHC binding sites overlap a little, it is thought they interact with DHC independently. This may be achieved via different orientations on the DHC tertiary structure (Tynan et al., 2000a). However, despite their independent interactions with DHC, studies in fungi suggest that LIC may be important for strengthening the interaction between DHC and IC (Zhang et al., 2009). There are two LIC genes in vertebrates, DYNC1LI1 and DYNC1LI2, which encode the LIC1 and LIC2 proteins, respectively (Allan, 2011). LIC1 and LIC2 homodimerise...
and associate with DHC in a mutually exclusive manner, giving rise to two distinct pools of dynein containing either LIC1 or LIC2 (Tynan et al., 2000b). There is also a third LIC gene encoding the LIC3 protein, which exclusively associates with cytoplasmic dynein-2 and is important during intra-flagella transport (Mikami et al., 2002). LIC1 and LIC2 have been shown to have specific roles, for example only LIC1 is able to interact with pericentrin and LIC2 only is able to interact with Par3 (Schmoranzer et al., 2009; Tynan et al., 2000b). Aside from these two examples of direct interactions, the published data regarding LIC1- and LIC2-specific roles are conflicting. One study shows that LIC1 is important for ER and Golgi apparatus membrane structure and trafficking and that LIC2 is important for regulating endocytic recycling (Palmer et al., 2009). However, a different set of studies showed that both LIC1 and LIC2 have roles in endocytic recycling (Horgan et al., 2010a; Horgan et al., 2010b). Partial depletion of either LIC1 or LIC2 using RNAi leads to disruption in late endosome and lysosome localisation suggesting a level of redundancy but this study also implicated LIC1 specifically in a dynein recruitment mechanism to Rab7 membranes (discusses further in section 1.7.4) (Tan et al., 2011). LIC1 and LIC2 have recently been shown to have independent roles on early endosome (EE) membranes, whereby LIC1 and LIC2 associate with different membrane domains and this helps drive protein sorting at this organelle (discussed further in section 1.7.2) (Hunt et al., 2013). Experiments using siRNA depletion show that LIC1 and LIC2 both play roles in processes that are needed for mitotic progression (Raaijmakers et al., 2013).

The dynein complex has been shown to have different combinations of ICs, LCs and LICs (Tynan et al., 2000a; Vallee et al., 2000). It is possible that these different dynein formations may give rise to differing dynein complexes to have functionally distinct roles. The functions of dynein and how dynein is able to interact with specific cargo will be discussed in later sections but in order to understand fully how dynein interacts with these cargos and regulates membrane trafficking pathways, it is first important to know how dynein is regulated by accessory proteins. There are several accessory proteins that can interact with, and regulate dynein. Different combinations of accessory proteins regulate dynein during different cellular processes leading to a tight control network with both cross-talk and specificity (Kardon and Vale, 2009). This project mainly focuses on four accessory proteins that are known to regulate dynein in a wide range of contexts. These are dynactin, Lis1, Nde1 and Ndel1. The next sections will give an overview of these proteins’ structure and explore some of the mechanisms they use to directly regulate dynein function.
1.3 Dynactin

Dynactin is a large multi-subunit complex that was originally discovered as a dynein activator in vesicular transport and is an accessory protein required for most dynein functions \textit{in vivo} (Gill et al., 1991; Schroer, 2004). Dynactin can be divided into two main regions, the Arp1 rod and the projecting arm. Arp1, Arp11, p62, p27, and p25 subunits can all be found in the Arp1 rod. The projecting arm is composed of the subunit p150, p50 and p24/p22 (Figure 1.3) (Schroer, 2004). The association between the projecting arm and Arp1 rod can be blocked by over-expressing the p50 subunit and this subsequently prevents dynactin regulating dynein, leading to a loss of dynein function characterised by a scattering of cytoplasmic organelles and disruption of mitotic spindle assembly (Burkhardt et al., 1997; Echeverri et al., 1996; Melkonian et al., 2007). The coil-coiled 1 (CC1) region of p150 interacts directly with dynein via two amino acid stretches on IC (1-41 and 46-75) (King et al., 2003; Morgan et al., 2011; Vaughan and Vallee, 1995). The p150 subunit can also interact with MTs via its N-terminal region (Waterman-Storer et al., 1995). Despite having many specific roles regulating different aspects of dynein-driven transport, the function of dynactin is not well understood. Although it is needed for most dynein functions \textit{in vivo}, dynein is able to move along MT without dynactin \textit{in vitro} (King and Schroer, 2000). This section will detail some of the specific roles dynactin has been shown to play in regulating dynein and discuss how these may contribute to the overall function of dynactin.

1.3.1 The role of dynactin during mitosis

Dynactin is important during mitosis and overexpression of p50, which breaks apart the dynactin complex, slows mitotic progression, leads to abnormal multi-polar spindles and causes dynein to be displaced from the KT (Echeverri et al., 1996; Starr et al., 1998). During prophase dynactin is important for dynein association with the nuclear envelope and for centrosome anchoring (Raaijmakers et al., 2013). Individual loss of the p50, p150, Arp1, p62 or p22/p24 subunits leads to a reduction of dynein located at KTs (Raaijmakers et al., 2013). There is a complex set of accessory protein interactions at the KT which regulate dynein recruitment and these are discussed below in section 1.4.3.
Figure 1.3: A cartoon of dynactin subunits. The shoulder region is composed of Arp1, Arp11, p62 and p25/p27. The p150 subunit can interact with dynein IC and MTs and is linked to the shoulder region by the p50 subunit.
1.3.2 The role of dynactin in recruitment of dynein to membranes

Dynactin has been proposed to act as a cargo adapter, which can recruit dynein to intracellular membranes, although there are conflicting data in the literature regarding the ability of dynactin to carry out this role. The Arp1 rod can bind βIII-spectrin found on the surface of membranes and this implicates dynactin in the recruitment of dynein to spectrin-coated organelles (Holleran et al., 2001). However, there is a lot of evidence to suggest that this model, in which dynactin is a generic regulator able to recruit dynein to a wide range of membranes, may be flawed. Overexpression of p50 leads to the scattering of Golgi apparatus membranes (Burkhardt et al., 1997), which does indeed seem to support the idea that Arp1 is recruiting dynein to membrane because p50 overexpression prevents p150, and therefore dynein, associating with Arp1. Surprisingly however, overexpression of p50 does not displace dynein or p150 from the membranes of fractionated cells, indicating p150 does not need to be associated with Arp1 for most dynein and p150-cargo interactions to take place (Flores-Rodriguez et al., 2011). Additionally, Drosophila mutants lacking Arp1 show perturbed bidirectional movement of neuronal transport vesicles but biochemical analysis shows normal membrane recruitment of dynein (Haghnia et al., 2007). Taking these studies into account, although Arp1 is able to interact with βIII-spectrin it does not seem likely that this provides a general dynein-membrane recruitment mechanism.

Irrespective of the ability of dynactin to act as a general membrane adaptor for dynein via Arp1, there are various specific dynactin-cargo subunit interactions that have been characterised. Dynactin plays a role in proper dynein recruitment to the nuclear envelope during the transition through the S/G2 stage of the cell cycle, with siRNA depletion of p150, p62 or Arp11 resulting in a reduction of dynein on this membrane (Yeh et al., 2012). The C-terminal region of p150 has been shown in HeLa and Vero cells to interact directly with Sec23 of COPII vesicle coats, which are involved in transport between the ER and Golgi apparatus (Watson et al., 2005). Dynactin has also been implicated in the transport between sorting endosomes (SEs) and the trans golgi network (TGN). The p150 subunit has been shown to associate with membrane bound sorting nexins SNX5 and SNX6, which are sub-complexes of retromer (discussed further in section 1.6 and 1.7), which is thought to drive traffic between SEs and the TGN EE movement and p25 has been shown to be important for dynein recruitment to EEs (Yeh et al., 2012; Zhang et al., 2011). Dynactin is also involved in the recruitment of dynein to Rab7-positive melanosomes, phagosomes, late endosomes and lysosomes through interactions with Rab interacting lysosomal protein...
(RILP), which is discussed further in section 1.7.4 (Harrison et al., 2003; Johansson et al., 2007; Jordens et al., 2001; Ohbayashi et al., 2012; Rocha et al., 2009).

1.3.3 Dynactin in bidirectional transport

Dynactin is thought to play an important role in a bidirectional transport along MTs and has been shown to associate with several plus end-directed kinesins (Blangy et al., 1997; Deacon et al., 2003; Kodani et al., 2013). Dynactin can interact with c-Jun N-terminal kinase (JNK)-interacting protein (JIP), JIP1, and this inhibits kinesin-1 mediated transport of neuronal vesicles (Fu and Holzbaur, 2013). Additionally, p150 has been visualised moving in a plus-end directed and minus-end directed fashion in mouse cells and is needed for kinesin-II-driven movement of melanosomes derived from Xenopus (Deacon et al., 2003; Ross et al., 2006). As discussed in section 1.3.2, loss of Arp1 results in disruption to both minus end-directed and plus end-directed transport (Haghnia et al., 2007). There is evidence that an interaction with the Huntingtin (Htt) protein can regulate dynactin in neuronal cells by mediating a switch between dynactin-based association and activation of either kinesin-1 or dynein on brain-derived neurotrophic factor-positive vesicles (Colin et al., 2008). These studies suggest dynactin may play a fundamental role in switching membrane transport between plus-end-directed kinesin-driven transport and minus-end-directed dynein-driven transport. This switching of directions is likely to be used in order to overcome obstacles that motors and the vesicles they are attached to, might encounter during transport.

1.3.4 Dynactin’s interaction with +TIP proteins

Dynactin has been implicated in a role whereby it can load cargo onto the plus ends of MTs ready for transport by dynein. The plus ends of MTs have proteins associated with them that have been termed +TIP proteins, which include CLIP-170, EB1 and EB3. Both CLIP-170 and EB1 can interact with each other and p150, however the three proteins cannot interact simultaneously (Berrueta et al., 1999; Goodson et al., 2003; Ligon et al., 2006; Vaughan et al., 1999). The role dynactin plays at MT +TIPs is controversial, with a great deal contradictory evidence. It has been proposed that the interaction of dynactin with these proteins mediates the loading of vesicles onto MTs, mediating a way for MTs to ‘capture’ vesicles at the cell periphery (Valetti et al., 1999; Vaughan et al., 1999). Other evidence, however, shows that loss of CLIP-170 leads to a loss of dynactin from +TIPs but does not affect dynein-driven transport of dynactin-positive membranes or cause peripheral
accumulation of vesicles (Watson and Stephens, 2006). Two recent studies in neuronal cells show that p150 is enriched at distal neurites, which is dependent on the MT-interacting region of p150 as well as kinesin-1, EB1 and EB3. Loss of p150 from this region does not affect the general movement of vesicles but it does prevent transport initiation of vesicles from the +TIP regions (Moughamian and Holzbaur, 2012; Moughamian et al., 2013). Based on the evidence from these studies, it is possible that dynactin is needed for initiation of vesicle transport from +TIPS, but loss of this mechanism does not prevent vesicle transport in general. If this is the case, then vesicles that are not loaded onto MTs at the +TIPs are likely to associate with dynein at another location on MTs and be transported, therefore the +TIP vesicle loading mechanism may indeed occur, but has functional redundancy with other transport initiation mechanisms.

1.3.5 Processivity
It has been suggested that the ability of dynactin to interact with MTs can increase dynein processivity and there is in vitro evidence that shows dynein in the presence of dynactin can move for greater distances along MTs than dynein alone, but with similar velocity (Culver-Hanlon et al., 2006; King and Schroer, 2000; Ross et al., 2006). However, the loss of the MT binding region of p150 does not affect organelle movement in vivo (Kim et al., 2007). Studies in yeast show that dynactin increases dynein MT processivity but that loss of the MT binding region does not reduce dynein and dynactin MT run lengths (Kardon et al., 2009). Additionally, although the loss of the MT-interacting region of p150 reduces the flux of vesicle transport from distal neurites, the processivity of vesicle movement down the length of MTs is not affected (Moughamian and Holzbaur, 2012). Together these data would suggest that although dynactin can increase dynein processivity in vitro, its ability to interact with MTs has no effect on dynein processivity in vivo.

1.3.6 An overview of dynactin function
The current understanding of dynactin function is fraught with contradiction as it is crucial for dynein function in vivo and is involved in many specific processes yet it appears to be essential in few. It can increase dynein processivity (Culver-Hanlon et al., 2006; King and Schroer, 2000), but this does not appear to involve MT association (Kardon et al., 2009; Moughamian and Holzbaur, 2012). Arp1 can associate with the βIII-spectrin that covers membrane surfaces (Holleran et al., 2001), yet disrupting the interaction of dynein with this subunit does not affect its membrane recruitment (Flores-Rodriguez et al., 2011; Haghnia
Dynactin can associate with +TIPs and does seem to play a role in transport initiation here (Ligon et al., 2006; Moughamian and Holzbaur, 2012; Moughamian et al., 2013; Valetti et al., 1999; Vaughan et al., 1999), yet the same vesicles can be transported in the absence of these +TIP interactions (Moughamian and Holzbaur, 2012; Moughamian et al., 2013; Watson and Stephens, 2006). Dynactin does seem to play a role in regulating bidirectional transport, but this alone is unlikely to account for the striking disruption to organelle positioning in the cell when dynactin is disrupted.

In the absence of cargo and the MT-binding domain, dynactin can still increase dynein run lengths (Kardon et al., 2009). The fact that dynactin can have an upregulatory effect on dynein function in the absence of both cargo and the ability to bind MTs suggest that dynactin may in fact function in the way it was originally described; as a dynein activator (Gill et al., 1991; Kardon et al., 2009). It is also possible that dynactin may play many other specific roles in the cell via its numerous protein interactions. These interactions are part of a complex dynein regulatory network that involves many other proteins (three of which are Lis1, Nde1 and Ndel1 discussed in the next section, 1.4) and thus there may be a level of redundancy in some dynactin functions. Whatever the level of regulation dynactin plays in dynein-driven transport, it is clearly essential as reflected by its loss or inactivation preventing most dynein-driven processes taking place in vivo.

1.4 Lis1, Nde1 and Ndel1

Lis1, Nde1 and Ndel1 are dynein accessory proteins that regulate dynein in a wide variety of contexts and as such are considered ubiquitous cofactors (Kardon and Vale, 2009). Lis1 was originally termed platelet activating factor acetylhydrolase 1B1 (PAFAH1B1), Pac1 in budding yeast and NudF in fungi, whilst Nde1 is sometimes referred to as Nde1 or NudE and Ndel1 is sometimes referred to as NudEL. Lis1 was first discovered as a potential dynein regulator in fungi, where it is important for dynein-driven nuclear positioning and this led to the subsequent identification of Nde1 and Ndel1 as its interactors (Kardon and Vale, 2009; Niethammer et al., 2000; Xiang et al., 1994; Xiang et al., 1995). Lis1, Nde1 and Ndel1 are important in brain development, with loss of their function leading to developmental defects in neuronal cell migration, cerebral development and lamination of the CNS (Feng et al., 2000; Feng and Walsh, 2004; Hirotsune et al., 1998). Loss of one copy of the LIS1 gene results in the disease Lissencephaly, whilst loss of both copies is embryonically lethal (Dobyns et al., 1993).
1.4.1 Interactions of Nde1, Ndel1 and Lis1 with each other, dynein and dynactin

Nde1 and Ndel1 are considered paralogues due to their structural similarity, shared ability to regulate dynein in complex with Lis1 and functional redundancy in many processes (Kardon and Vale, 2009). Nde1 and Ndel1 both have a coiled-coil in their N-terminal region that homodimerises and an unstructured C-terminal region that interact with Lis1 (Derewenda et al., 2007; Efimov and Morris, 2000; Sasaki et al., 2000). Both Nde1 and Ndel1 can interact with dynein via a 40 amino acid stretch in the unstructured C-terminal domain and via their N-terminal coil-coiled dimerisation region, upstream of the Lis1 binding site (Stehman et al., 2007; Zylkiewicz et al., 2011). Ndel1 can be phosphorylated and has been shown to be a target for Cdk5 (Niethammer et al., 2000). Additionally, point mutations that mimic phosphorylation in the C-terminal region of Ndel1 positively regulated association with Lis1 and dynein (Zylkiewicz et al., 2011). It has been shown that Nde1 competes with p150 for binding to the same region of IC, suggesting that the dynein in complex with Nde1 or dynactin may have functionally distinct roles (McKenney et al., 2011). Ndel1 can also interact with LC8, but does so when LC8 is not in complex with dynein, indicating that LC8-Ndel1 may have a role outside of dynein complex (McKenney et al., 2011).

Lis1 is a 45kDa protein that homodimerises via a region in its N-terminus (Mateja et al., 2006). It has 7 WD40 domains at its C-terminus, which form a β-propeller that is able to interact with the AAA1 of the DHC motor domain (Huang et al., 2012; Kim et al., 2004; Tai et al., 2002; Tarricone et al., 2004). Lis1 is also able to interact with the p50 subunit of dynactin (Tai et al., 2002).

1.4.2 A role for Lis1 outside the Nde1/Ndel1 complex

Lis1 has a role that is independent of dynein and Nde1/Ndel1. The β-propeller region of Lis1 can interact with homo- or heterodimers of α1 and α2 to form the PAFAH complex, which is involved in maintenance and tubulation of Golgi membranes, tubulation of endosomal membranes and is a platelet activating factor acetylhydrolase (Arai et al., 2002; Bechler and Brown, 2013; Bechler et al., 2009; Bechler et al., 2010; Tarricone et al., 2004). When Lis1 is part of the PAFAH complex it does not associate with Nde1/Ndel1, as α1/α2 compete with Nde1/Ndel1 for Lis1 binding (Tarricone et al., 2004). It is unclear whether Lis1 in the PAFAH complex has any effect on dynein activity, although most evidence to date would suggest there is no connection between these two complexes.
1.4.3 Functions of the Lis1-Nde1/Ndel1 Complex

The Lis1-Nde1/Ndel1 complex is involved in many of the functions of dynein including mitotic MT aster formation, neuronal migration, neuronal axon growth, mitotic progression, recruitment of dynein to the KTs, mitotic spindle orientation, the recruitment of dynein to membranes and maintaining proper organelle positioning (Faulkner et al., 2000; Lam et al., 2010; Liang et al., 2004; Raaijmakers et al., 2013; Siller and Doe, 2008; Smith et al., 2000; Stehman et al., 2007; Vergnolle and Taylor, 2007; Zylkiewicz et al., 2011).

The ability of Lis1, Nde1 and Ndel1 to act as ubiquitous dynein regulators and their involvement in so many dynein-driven processes is likely due to their ability to directly regulate the dynein complex in two ways, which are the ability to influence dynein recruitment to cargo and regulation of the force-producing ability of dynein.

One dynein cargo that has been well studied is the KT which attaches chromosomes to MTs during mitosis. This cargo presents one of the few examples in which Nde1 and Ndel1 are known to have differing roles in recruiting dynein to cargo. Both Nde1 and Ndel1 can interact with CENPF on KTs and are able to recruit Lis1 to these structures. However, only Nde1 is required for dynein recruitment (Figure 1.4A) (Stehman et al., 2007; Vergnolle and Taylor, 2007). Displacement of Lis1 does not affect dynein recruitment to KTs, but displacement of dynein leads to a loss of Lis1, indicating that Lis1 is recruited to KTs via the Nde1/Ndel1-dynein complex (Tai et al., 2002). Lis1 is, however, needed to recruit the MT +TIP protein CLIP-170 to KTs (Figure 1.4 A) (Coquelle et al., 2002; Tai et al., 2002). There also appears to be an additional pathway for dynein recruitment to the KTs via a protein called ZW10, which is part of the RINT1-ZW10-Zwilch (RZZ) complex, which is associated with KTs (Figure 1.4 A) (Liang et al., 2007; Stehman et al., 2007; Varma et al., 2006). It is possible that ZW10 may recruit Nde1 and Ndel1 to the KTs via dynein/dynactin, as ZW10 can interact directly with dynactin p50, and p50 overexpression results in loss of Lis1 from the KT (Inoue et al., 2008; Starr et al., 1998; Tai et al., 2002). The interactions of dynein accessory proteins are complex and it is likely that there is cross-talk between proteins in the potentially differing dynein recruitment pathways. The recruitment of dynein to organelle membranes is more complex still, with different organelles having differing subsets of accessory proteins that are involved in recruiting dynein to their surface. What is apparent, though, is that Lis1, Nde1 and Ndel1 are needed for the vast majority of these
recruitment events as shown by their ability to affect dynein membrane recruitment and organelle positioning (Lam et al., 2010).

The role of Lis1-Nde1/Ndel1 in dynein recruitment to the membranes of organelles during interphase differs from the KT in two main ways: Lis1 is needed for dynein recruitment and Nde1 and Ndel1 have redundancy in both the recruitment of dynein and Lis1 (Lam et al., 2010). This redundancy between Nde1 and Ndel1 in dynein recruitment may be due to the involvement of Lis1, with which they both interact. Individually, depletion of Nde1 or Ndel1 does not lead to a substantial loss of Lis1 and dynein from cellular membrane fractions, but when both are depleted dynein and Lis1 are displaced from membranes, indicating redundancy in this role (Figure 1.4B) (Lam et al., 2010). Loss of Lis1 does not affect the role of Nde1/Ndel1 in associating with membranes but in contrast to the role of Lis1 in dynein recruitment to the KT, Lis1 knock down (Kd) leads to a reduction of membrane associated dynein (Lam et al., 2010). Although Lis1-Nde1/Ndel1 are involved in dynein membrane association, there are no known membrane proteins that Lis1-Nde1/Ndel1 interact with. Nde1 and Ndel1 have been shown to undergo palmitoylation in vivo, but the single lipid that is added is not thought to be sufficient to induce membrane interaction (Shmueli et al., 2010). There are many examples of Lis1 and Nde1/Ndel1 being involved in the movement and positioning of specific organelles, but as this sections aims to give an overview of their general function. These specific examples will be discussed in sections 1.5 and 1.7.

Another important role for Lis1 and Nde1/Ndel1 in dynein regulation is their ability to affect dynein force production. Lis1 has been shown to activate dynein’s ATPase activity (Mesngon et al., 2006), and in vitro studies demonstrate that Nde1 and Lis1 increases the force generated by dynein (McKenney et al., 2010). Lis1 is also proposed to act as ‘clutch’ allowing dynein to continue to hydrolyze ATP while remaining attached to MTs. This is thought to increase the load-bearing ability of dynein (Huang et al., 2012). This model is further supported by previous in vitro studies that show Lis1 can break the mechano-chemical coupling of dynein, which is rectified by addition of Ndel1 (Yamada et al., 2008). These data suggest that Lis1 and Nde1/Ndel1 can work together to regulate the activity, force production and load bearing activity of dynein, which may be needed for larger cargo (Figure 1.4B). It has been suggested that Lis1-Nde1/Ndel1 regulate dynein in this way on KTs, whilst transporting chromosomes that have a large mass (Raaijmakers et al., 2013).
This may go some way to explaining why Lis1 and Nde1/Ndel1 show differing mechanisms and interaction hierarchies in their roles of recruiting dynein to KT versus organelle membranes (Figure 1.4).

Depletion of Lis1 in neuronal cells leads to a lack of mobility of larger endocytic vesicles but has no effect on smaller vesicles (Yi et al., 2011). This, taken with data showing competitive binding of Nde1 and dynactin to IC, has lead to the suggestion that dynactin might be used to regulate dynein in fast/low load transport and Lis1-Nde/Ndel1 might regulate dynein in slow/high load transport (Figure 1.4 B) (McKenney et al., 2011). Contradicting this model, studies in fungi show that Lis1 is needed for fast minus end-directed transport of small endosomes at MT plus ends (Egan et al., 2012; Lenz et al., 2006). The loss of Lis1 does not slow transport but decreases frequency of movements. Moving cargo will associate with dynein and dynactin but not Lis1, which correlates with biochemical analysis that shows a large proportion of Lis1 is found in the cytosolic fraction of cells rather than being membrane bound (Egan et al., 2012; Lam et al., 2010). This implies that Lis1 is an initiator of endosome transport but is not required for ongoing transport of these vesicles. Data from neuronal cells further support this model. Dynactin is needed for initiation of vesicle transport specifically at the +TIPs of MTs, but Lis1 can initiate transport events down the entire MT (Moughamian et al., 2013). It is possible that the ‘clutch’-like properties of Lis1-Nde1/Ndel1 and the ability to increase force production of dynein, may be used to create the force needed for ongoing transport of larger cargo, such as chromosomes, and provides the initial force needed to set in motion the movement of smaller cargo, like endosomes.

After this initial burst of movement Lis1-Nde1/Ndel1 might dissociate from cargo and the dynein might be ‘handed over’ to the dynactin complex to help continue transport. Depletion of Lis1 leads to a reduction of dynein in the membrane fraction but not to the same extent as Nde1/Ndel1 Kd (Lam et al., 2010). It is possible that this could be due to loss of Lis1 leading a failure in transport initiation and therefore a dynein population that will more readily dissociate from the membrane.

Another possible role for Lis1 in the regulation of dynein activity has been proposed, where Lis1 can essentially inhibit dynein so that it can be transported to the plus ends of MTs ready for organelle transport. Lis1 alone has been shown to prevent dynein activity and promote transport of the complex to the plus ends of MTs via interactions with kinesin-1 and a protein called NudC (Yamada et al., 2010; Yamada et al., 2008). It is thought that this
could provide a means to replenish dynein at the cell periphery, ready for cargo transport to the cell centre. This also provides another potential model that might account for the role of Lis1 as a transport initiator. In the absence of Lis1, dynein may not be being transported to the cargo it needs to move and therefore fewer minus end-directed transport events are taking place.

The precise roles Nde1/Ndel1 and Lis1 play in regulating dynein activity and initiating transport are presently not at all clear. They can directly regulate the force-producing and load-bearing ability of dynein but there is still much work to be done in order to understand how this plays out in vivo and influences dynein-driven transport of cargo and interactions with other dynein-regulating accessory proteins. Whatever the precise mechanisms for Lis1-Nde1/Ndel1 mediate dynein regulation, they are very important for proper dynein regulation, demonstrated by their requirement for positioning of organelles, mitotic progression and dynein-membrane association. Dynein and its interaction with Lis1-Nde1/Ndel1 and dynactin influence many membrane trafficking events. In the next sections (1.5-1.8) some of the membranous cargo dynein can move will be described and the ways in which dynein interacts with these organelles will be explored.
Figure 1.4 Dynein, dynactin Lis1 and Nde1 Ndel1 interaction with cargo. A: Interactions at the kinetochore during mitosis. B: Proposed model for accessory protein interactions with cargo in different contexts requiring different levels of force production.
1.5 Regulation of membrane traffic at the endoplasmic reticulum and Golgi apparatus

1.5.1 The endoplasmic reticulum and Golgi apparatus

This project focuses on how dynein interacts with endocytic membranes but dynein is important in many other aspects of membrane trafficking outside of the endocytic pathway. Dynein is known to transport mitochondria, the motility of which is very important in neuronal cells, but relatively little is known about dynein-mitochondria interactions (Frederick and Shaw, 2007; Rintoul and Reynolds, 2011). Dynein cargoes that have been well studied are the ER and Golgi apparatus and the membranes that traffic between them. The ER and Golgi apparatus are important in protein synthesis, folding, modification and sorting. The ER membrane is continuous with the nuclear envelope and the organelle stretches out into the cell periphery, associating with MTs. It has a reticular structure, with ER tubules that form three-way junctions (Chen et al., 2013). A third of all mammalian proteins will be imported into the ER, where chaperones ensure correct protein folding and they often undergo N-linked glycosylation. Following modification in the ER, proteins in the secretory pathway are trafficked to the Golgi apparatus (Braakman and Bulleid, 2011; Hirschberg and Snider, 1987).

In the Golgi apparatus N-linked sugars are trimmed and proteins are sorted ready for transport to their various cellular destinations (Helenius and Aebi, 2001; Hirschberg and Snider, 1987). In mammalian cells the Golgi apparatus is composed of cisternae that form stacks resembling ribbons, which are located close to the MTOC, through dynein mediated MT interaction (Rios and Bornens, 2003). Vesicles bringing protein from the ER interact and fuse with the cis-Golgi cisternae while proteins exit the Golgi apparatus from the TGN. The membranes physically located between these two subsets are the medial and trans cisternae. The different types of cisternae can be characterised by resident protein content, the modification status of proteins being sorted and the cisternae location, with the cis-Golgi cisternae usually being located nearest the MTOC followed by medial- and trans-Golgi cisternae (Goud and Gleeson, 2010).

1.5.2 Endoplasmic reticulum-Golgi intermediate compartments

Transport between the ER and Golgi apparatus occurs via a distinct organelle compartment called the ERGIC and membrane traffic occurs in both directions. Compartments moving from the Golgi apparatus to the ER are often characterised by the presence of a COPI
membrane coat and membranes moving from the ER to Golgi apparatus often have a COPII coat (Brandizzi and Barlowe, 2013; Duden, 2003). The membranes cycling between the Golgi apparatus and ER can also be characterised by the Rab proteins present on their surface. Rab proteins are small GTPases that play an essential role in membrane trafficking and transport by interacting with many effectors including motor proteins (Zerial and McBride, 2001). Rab proteins found on membranes moving from the ER to Golgi apparatus include Rab1, Rab13, Rab30 and Rab43. Those associated with membranes moving from the Golgi apparatus to the ER include Rab2, Rab6 and Rab33 (Sandoval and Simmen, 2012).

Maintenance of the Golgi apparatus and the ER as separate organelles is dependent on traffic between the two. The fungal metabolite Brefeldin A inhibits Sec7 which is needed to activate Arf1 and Arf1 in turn is needed for COPI recruitment to membranes. Treatment with Brefeldin A leads to protein accumulation in the ER and induces retrograde transport from the Golgi apparatus to the ER. Ultimately this leads to the collapse of the Golgi apparatus with Golgi resident enzymes relocating the ER (Donaldson et al., 1992; Sciaky et al., 1997).

1.5.3 The role of dynein in endoplasmic reticulum to Golgi traffic

MT-associated motor proteins are essential for traffic between the ER and Golgi apparatus but also play important roles in maintaining the position of these compartments within the cell. ER tubules align with MTs and MT depolymerisation prevents ER extending out into the cell periphery (Terasaki et al., 1986; Wozniak et al., 2009). Although much of the observed movement of ER is driven by kinesins, the ER also undergoes minus end-directed movement, which is dynein-dependent (Lane and Allan, 1999; Lane et al., 2001; Terasaki et al., 1986; Wozniak and Allan, 2006; Wozniak et al., 2009). The peri-nuclear positioning of the Golgi apparatus is MT-dependent and depolymerisation of MTs leads to the breakdown of the Golgi cisternae into mini stacks, which scatter throughout the cell and this can be rescued with re-polymerisation of MTs (Cole et al., 1996; Ho et al., 1989; Rogalski and Singer, 1984). Dynein can associate with Golgi membranes via a direct interaction between IC and the CC7 domain of Golgin-160. During mitosis, Golgin-160 dissociates from the Golgi membrane mediating a loss of dynein, which correlates with the Golgi apparatus forming smaller fragmented membranes (Yadav et al., 2012). Kd of DHC, IC, LIC, Tctex-1 and LC7 all lead to scattered Golgi membranes and DHC, LIC and IC are needed for maintaining proper ERGIC structure (Palmer et al., 2009). Lis1 and Nde1/Nde1 are also very important for
dynein mediated Golgi positioning and the loss of Lis1 or Nde1 and Ndel1 leads to scattered Golgi membranes, which can be rescued when the proteins are reintroduced (Lam et al., 2010).

Inward transport of membranes from the ER to the Golgi apparatus requires dynein, and the LIC1, Tctex-1 and LC8 subunits have been shown to be particularly important for this transport (Palmer et al., 2009; Presley et al., 1997; Roghi and Allan, 1999). When cells are partially depleted of DHC or kinesin-1 light chain, the ERGIC forms more tubules than under control conditions (Tomas et al., 2010). Interestingly, the tubules in dynein depleted cells are highly mobile whereas kinesin depleted cell tubules are static. It is difficult to interpret this study though, because the DHC is not completely depleted from cells and so these tubules may still have some DHC present on them, allowing bidirectional movement to take place (Tomas et al., 2010). These studies show that the ERGIC is a highly dynamic and motile organelle and the association of dynein is important to maintain its movement and thus mediate trafficking events between the ER and Golgi apparatus.

As mentioned previously, membranes within the cell can be characterised by their Rab protein content. One Rab protein that is important in traffic between the Golgi apparatus and the ER as well as membranes moving from the TGN to the plasma membrane (PM) is Rab6 (Jones et al., 1993; Ladinsky and Howell, 1992; Martinez et al., 1997; Starr et al., 2010). In its role driving retrograde intra-Golgi membrane and Golgi apparatus to ER transport, Rab6 has overlapping function with Rab33 (Starr et al., 2010). Both COPI and clathrin coated vesicle formation at the Golgi apparatus requires Rab6, but it is also involved in COPI–independent transport between the Golgi apparatus and the ER (Matanis et al., 2002; Storrie et al., 2012). Rab6 is interesting in terms of dynein-driven transport as it has been shown to interact with dynein and dynein-interacting proteins. Dynein LC7 can interact with Rab6 although it is not at all clear whether LC7 is part of the dynein complex when this interaction takes place (Wanschers et al., 2008). Rab6 is able to interact with ZW10, the protein found in the RZZ complex that can help recruit dynein to KTs discussed in section 1.4.3. ZW10 can localise to ER via the RINT1 and syntaxin-18 complex (Arasaki et al., 2006). ZW10 also associates with Golgi membranes and contributes to proper Golgi apparatus to ER transport in a Rab6 regulated manner (Hirose et al., 2004; Inoue et al., 2008; Sun et al., 2007). ZW10 may be involved in dynein-driven membrane trafficking as blocking its function or its depletion leads to scattering of Golgi membranes (Varma et al.,
Another mechanism for Rab6-membrane transport is the well characterised interaction that links Rab6 to dynein via Bicaudal D (BicD). BicD is a dynein- and dynactin-interacting protein that can bind Rab6 and is needed for dynein-driven COPI-independent transport between the Golgi apparatus and the ER (Hoogenraad et al., 2001; Matanis et al., 2002). Lis1 is needed for BicD/dynein/dynactin-driven cargo transport but, surprisingly, GTP-bound Rab6 causes Lis1 to dissociate from dynein, which has an upregulatory effect on transport (Splinter et al., 2012; Yamada et al., 2013). As discussed before, it is thought that Lis1 can initiate transport (Egan et al., 2012; Lenz et al., 2006; Moughamian et al., 2013; Yamada et al., 2013), therefore it may initiate Rab6-positive membrane transport and dissociate soon after.

There are many different mechanisms that dynein is involved with in transporting membranes between the ER and Golgi apparatus and it is still unclear to what extent these different pathways overlap. What is clear, however, is that dynein is essential for driving the minus-end directed transport between these two organelles and it is regulated in a complex network of protein-protein interactions. Loss of dynein function leads to misregulation and mislocalisation of ER, Golgi and ERGIC membranes and therefore dynein is essential for the correct regulation of these organelles.

1.6 The endocytic pathway

Endocytosis is the process in which cells can take in extra-cellular materials by invagination of the PM. The process is very important for intake of nutrients and for responding to extra-cellular signals. There are several routes through the cell that newly endocytosed materials can take. They can be recycled back to the PM either directly or via recycling endosomes (REs), sent to TGN for sorting or be targeted for degradation in the lysosome (Figure 1.5) (Jovic et al., 2010). Different compartments in the endocytic pathway can be distinguished by their resident proteins and lipids associated with their membranes, which not only characterise specific organelles but carry out functional roles in the sorting and trafficking of endocytosed materials (Mellman, 1996; Scita and Di Fiore, 2010; van Weering et al., 2010). The next sections will give an overview of these different organelles, the proteins that characterise them and describe their functions.
1.6.1 The roles of early endosomes and recycling endosomes and the proteins that characterise them

The first step in endocytosis is the invagination of the PM to form pits that surround the material being engulfed. Coat proteins such as clathrin and caveolin that associate with the newly forming vesicle along with actin filaments are important in this process and help create the physical force that shapes the newly emerging vesicle (Doherty and McMahon, 2009; Engqvist-Goldstein and Drubin, 2003; Galletta and Cooper, 2009). Once the roughly spherical compartment has formed, a protein called dynamin wraps around the narrowing connection with the PM and pinches off the now fully formed vesicle. The coat proteins are shed from the membrane and the vesicle begins to associate with EE proteins (Doherty and McMahon, 2009). Proteins in EEs can be sent to several places within the cell. They can be recycled back to the PM by ‘fast’ recycling directly from EEs or ‘slow’ recycling via REs. EEs mature into SEs that can sort and traffic proteins to REs or to the TGN. Additionally, the SEs can retain protein destined for protein degradation in the lysosome (Jovic et al., 2010; Scita and Di Fiore, 2010). The SEs form intra-luminal vesicles (ILVs), which give SEs (and the late endosomes (LEs) they mature into) a multi-vesicular body (MVB) morphology (Babst, 2011). SEs retain the resident proteins of EEs and can therefore be thought of as a subset of EEs. Rab5 is found on EEs and SEs (Figure 1.5) (Bucci et al., 1992; Gorvel et al., 1991) whereas Rab4 associates with EEs and REs (along with Rab11 on REs) (Figure 1.5) (Cormont et al., 1996; Sheff et al., 1999; Trischler et al., 1999; van der Sluijs et al., 1992; Van Der Sluijs et al., 1991). Additionally, there are several other Rab proteins that have been found to localise to EEs including Rab10, Rab14, Rab21 and Rab22 (Jovic et al., 2010).

EEs can also be characterised by their membrane lipid content and are enriched with Phosphatidylinositol-3-Phosphate (PtdIns(3)P), in part due to the fact Rab5 recruits PI3-kinase to the EE membrane (Christoforidis et al., 1999b). Early endosome antigen 1 (EEA1) is a Rab5 effector protein and is recruited exclusively to EE membranes via PtdIns(3)P and Rab5 (Figure 1.5) (Christoforidis et al., 1999a; Lawe et al., 2000; Simonsen et al., 1998). EEA1 is essential for endosomal fusion and recruits Syntaxin-13 and -16, part of a SNARE complex needed for membrane fusion, to the surface of EE (McBride et al., 1999; Mills et al., 2001; Simonsen et al., 1998). APPL1 and APPL2 are homologous Rab5 effectors, which are found on a subset of EEs and are essential for EE-mediated cell signalling. In response to endocytosed epidermal growth factor receptor (EGFR), they translocate from EEs to the nucleus where they induce transcriptional changes in response to EGF (Figure 1.5) (Miaczynska et al., 2004). One of the most important features of EEs and SEs is their ability
to sort proteins by separating cargo into functionally distinct regions within the vesicle. The next three sections will discuss how some of these processes take place.

1.6.2 Intra-luminal vesicle formation and the endosomal sorting complex required for transport (ESCRT) machinery

The formation of ILVs provide a means to sequester proteins destined for lysosomal degradation and represent a process by which EEs are able to sort proteins by producing spatially and functionally distinct regions within the organelle. Proteins such as EGFR are marked as destined for degradation by the addition of ubiquitin and the EE removes them from the cytosolic face of its membrane into ILVs and this give rise to the MVB morphology of SEs, and the LEs found later in the pathway (Babst, 2011; Jovic et al., 2010; Mosesson et al., 2003). The formation of these ILVs is driven by the endosomal sorting complex required for transport (ESCRT) proteins (Henne et al., 2011). There are several classes of ESCRT proteins, which are assigned numbers 0-III and together recognise ubiquitinated cargo and sequester the proteins into ILVs in a step-wise manner (Henne et al., 2011). ESCRT-0 proteins include Hrs and STAM-1, which recognise ubiquitinated protein, localise to the EE membrane and initiate the ILV formation process (Bache et al., 2003a; Bache et al., 2003b; Bishop et al., 2002; Raiborg et al., 2002; Raiborg et al., 2001). ESCRT-I proteins include UBAP, TSG101, Vps28, Vps38 and Mvb12 (Bishop and Woodman, 2001; Doyotte et al., 2005; Henne et al., 2011; Stefani et al., 2011). The ESCRT-I complex interacts with ESCRT-0 proteins and recruit ESCRT-III proteins. ESCRT-I and ESCRT-III also interact with the ESCRT-II complex (Henne et al., 2011; Slagsvold et al., 2005). The ESCRT-III complex is composed of the CHMP proteins (CHMP2, CHMP3, CHMP4 and CHMP6) and they physically drive the process of sequestering cargo into ILV (Henne et al., 2011). HD-PTP couples deubiquitination and the dissociation of ESCRT-0, ESCRT-I and ESCRT-II with the transfer of cargo to ESCRT-III and invagination into ILVs (Ali et al., 2013; Doyotte et al., 2008). The SEs containing the proteins sequestered into ILVs, eventually mature into LEs and undergo a switch from association with Rab5 to the LE-associated Rab7 and this process requires the Rab7 GEF, homotypic fusion and vacuole protein sorting (HOPS) (Rink et al., 2005). During this transition some of the PtdIns(3)P is converted to PtdIns(3,5)P₂ (Figure 1.5) (Gruenberg, 2003; Ikonomov et al., 2006). The LEs retain this MVB morphology and go on to fuse with lysosomes, delivering the cargo for degradation (discussed in section 1.6.5)
1.6.3 Sorting proteins for endosomal recycling

Many proteins, for example TFR, that have been endocytosed need to be recycled back to the PM (Jovic et al., 2010; Scita and Di Fiore, 2010). Rab4 is associated with RE membranes that are recycling directly back to the PM or are recycling slowly via the Rab11-positive RE (Figure 1.5) (Sheff et al., 1999; van der Sluijs et al., 1992; Van Der Sluijs et al., 1991). One Rab4 effector that is important for recycling is Rabaptin-5 (it also acts as an effector for Rab5) and it acts as a linker between Rab4 and AP-1, the clathrin adaptor protein (Deneka et al., 2003). It is possible that in the absence of ubiquitin, cargo goes through the recycling pathway as a ‘default’, but the process of recycling has been shown to be Arf1-dependent indicating there is some level of regulation. Arf1 activating protein, ACAP1, has been shown to interact with TFR and increase its recycling from endosomes to the PM (Dai et al., 2004). Recycling of proteins can also be regulated by SNX proteins which are discussed in the next section (1.6.4).

1.6.4 The role of SNX proteins in endocytic trafficking

The SNXs are a group of proteins that contain a SNX-PX domain, which can interact with PtdIns(3)P, and some of these proteins contain BAR domains (van Weering et al., 2010). BAR domains allow proteins to induce membrane curvature and thus promote tubule formation (Suetsugu et al., 2010). SNX can associate with endosomal membranes in similar manner to Rab proteins, whereby different SNXs are found associated with different organelles in the pathway. SNXs act in a way that allows proteins to be sorted by sequestering them into tubules or domains in the membrane ready for trafficking to their target organelle (van Weering et al., 2012a; van Weering et al., 2012b). Motor proteins can interact with many SNX proteins and this will be discussed in section 1.7.3 but here the role of SNX proteins in terms of membrane association and cargo trafficking will be explored.

Some of the most studied SNX proteins are those that are part of the retromer complex. The retromer complex is composed of a trimer containing Vps26, Vps29 and Vps35 along with a dimer of SNXs (Cullen and Korswagen, 2012). A retromer complex containing SNX3 can associate with EEs and sort proteins destined for the TGN (Harterink et al., 2011). SNX3 does not contain a BAR domain and therefore is not involved in membrane tubulation but still associates with EEs via its PX domain, while the Vps26/29/35 proteins of this complex can associate with cargo (Cullen and Korswagen, 2012; Xu et al., 2001). One cargo this SNX3 retromer can sort is Wntless, a protein that is needed for Wnt secretion and thus needs to
be trafficked back to the TGN after reuptake by endocytosis (Franch-Marro et al., 2008; Harterink et al., 2011). Another well characterised retromer complex contains Vps26/29/35 with SNX1 or SNX2 and SNX5 or SNX6 (Cullen and Korswagen, 2012). Once again Vps26/29/35 can associate with cargo but in this context it can also bind to GTP Rab7 (Rojas et al., 2008). The SNX proteins can interact with the remaining PtdIns(3)P as well as PtdIns(3,5)P$_2$ found on SEs that are undergoing maturation into LEs (Cozier et al., 2002; van Weering et al., 2012b). The SNX1/2 and SNX5/6 contain BAR domain proteins that sense and enhance membrane curvature (van Weering et al., 2010) and tubules form from the EE-LE transitional compartments that are eventually pinched off and sent to the TGN (Figure 1.5) (Cullen and Korswagen, 2012; Seaman, 2004; Seaman, 2007; Seaman et al., 1998; van Weering et al., 2012b). Mannose-6-phosphate receptor (M6PR) is needed to transport newly synthesised lysosomal hydrolases from the TGN to EE (discussed further in section 1.6.5) (Saftig and Klumperman, 2009). In the EEs hydrolases dissociate from M6PR and M6PR is isolated by the SNX1/2 and SNX5/6 retromer and trafficked back to the TGN (Arighi et al., 2004; Mari et al., 2008).

Not all SNX proteins are part of the retromer complex. One example is SNX4, which plays a role in recycling TfR back to the PM. It has been shown to direct TfR to ACAP1-positive REs for transport back to the PM (Figure 1.5) (Traer et al., 2007). It is not clear whether SNX8 interacts with the retromer but it does localise to the same membranes and is needed for transport of Shiga and Ricin toxins from EEs to TGN, which the SNX1 associated retromer has been shown to be important for too (Bujny et al., 2007; Dyve et al., 2009).

SNX proteins, Rab proteins (and their effectors) and the ability of EEs and SEs to produce ILVs provide a complex and highly regulated set of mechanisms that endosomal vesicles use in order to sort cargo by producing functionally distinct regions within the same membrane. Many of the proteins that are endocytosed are sent to lysosomes for degradation and the next section will look at the regulation of LE and lysosome fusion events and how lysosomes function.

1.6.5 Late endosome and lysosome fusion events and lysosome function

LEs contain the proteins that have been sorted in EEs and SEs for lysosomal degradation and are characterised by the presence of Rab7 on their membranes (Rink et al., 2005). The switch of Rab5 associated with EEs and SEs, to Rab7 is regulated by the HOPS protein
complex, has also been shown to mediate the tethering of LE that takes place prior to homotypic and lysosomal fusion (Brocker et al., 2012; Cabrera et al., 2009; Ostrowicz et al., 2010; Rink et al., 2005; van der Kant et al., 2013). Other proteins that are important in LE and lysosome fusion are soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs), which allow membranes within the cell to tether and fuse. A docking membrane possesses v-SNAREs that interact with t-SNAREs on the membrane targeted for fusion (Ungar and Hughson, 2003). The SNAREs can also be classified as r-SNAREs and q-SNAREs based on the amino acid residue that contributes to the SNARE-interaction (Fasshauer et al., 1998). LEs can fuse with other LEs using the q-SNARE proteins, Syntaxin7, Vti1b and Syntaxin 8 on one LE docking with the r-SNARE VAMP8 on another LE (Pryor et al., 2004). The LEs fuse with lysosomes to deliver their contents for degradation and this process requires the same qSNARE proteins used in LE homotypic fusion, but in this case they dock with the lysosome r-SNARE VAMP7 (Mullock et al., 1998; Mullock et al., 1994; Pryor et al., 2004).

Once the contents of LEs have been delivered to lysosomes they are degraded by hydrolases. Lysosomes have a low pH, more acidic than other endocytic compartments, and this is maintained by V-type H⁺ ATPases that pump H⁺ into the lysosome lumen. This acidity provides the ideal environment for the ~50 hydrolases that degrade the lysosomal contents (Eskelinen et al., 2003). Lysosomes contain lysosomal membrane proteins (LMPs), which are needed for lysosome formation, stability and integrity. The most abundant of these are CD63 (also found in LEs), LAMP1 and LAMP2 (Figure 1.5) (Saftig and Klumperman, 2009). LMPs are sorted to the lysosome by the presence lysosome targeting motifs present in their cytosolic tails (Braulke and Bonifacino, 2009). Lysosomal hydrolases and other soluble proteins synthesised in the ER and are modified in the Golgi apparatus so that their N-linked glycans contain a mannose-6-phosphate moiety that is recognised by M6PR (also known as sortillin) and delivered to the EEs (Helenius and Aebi, 2001). The lysosomal proteins are carried through maturing endosomes and are eventually delivered to lysosomes (Braulke and Bonifacino, 2009). When the LEs fuse with lysosomes these hydrolases are able to degrade proteins and produce amino acids that can be reused by the cell.
Figure 1.5: Protein and lipid composition of membranes in the endocytic pathways and their trafficking. Abbreviations: Phosphatidylinositol (PtdIns), Transferrin (Tf), Epidermal Growth Factor (EGF), Clathrin Coated Vesicle (CCV), Early Endosome (EE), Recycling Endosome (RE), Sorting Endosomes (SE) and Late Endosome (LE).
1.7 The role of dynein in the endocytic pathway

The sorting and fusion events discussed above require the different compartments of the endocytic pathways to be transported to the correct place within the cell. These transport events require MT-associated motor proteins, which not only act as effectors in endocytic flux but can also exert regulatory functions.

1.7.1 Dynein and dynactin function in the endocytic pathway

It has been known for many years that dynein-driven transport is needed for movement and maintaining the correct position of EEs (Aniento et al., 1993). There are in vitro data that show dynein inhibition has no significant effect on endosome movement (Nielsen et al., 1999), but this contradicts considerable in vivo data showing dynein is needed for proper movement and distribution of EEs (Driskell et al., 2007; Flores-Rodriguez et al., 2011; Hunt et al., 2013; Lam et al., 2010; Palmer et al., 2009; Valetti et al., 1999). Additionally, subsequent in vitro studies have shown that dynein is present on both purified EEs and LEs and while inhibition of dynein does not have an effect on late endosome movement, it reduces EE movement significantly (Loubery et al., 2008). The in vitro data are conflicting as to whether dynein is needed for transport of LE as well, but in vivo experiments in which dynein function is inhibited generally cause scattering of LEs and lysosomes (Bananis et al., 2004; Burkhardt et al., 1997; Harada et al., 1998; Lam et al., 2010; Loubery et al., 2008; Tan et al., 2011). Furthermore, biochemical analysis of late endosome fractions show that dynein is present on these membranes (Bananis et al., 2004; Loubery et al., 2008; Tan et al., 2011). It is quite possible that LEs and lysosomes require dynein for maintaining correct perinuclear positioning but do not require high levels of dynein-driven motility. Whatever effects dynein has on endocytic membrane movement in vitro, it is clear that, in vivo, dynein is essential for proper progression through the endocytic pathway. The next sections will look at some of the interactions and mechanisms that place dynein and dynactin as regulators within endocytic trafficking.

1.7.2 Dynein and dynactin in cargo sorting at the early endosome, sorting endosome and recycling endosomes

Dynein plays an important role in cargo-sorting in EEs. Depletion of DHC, LIC, IC or LCs leads to peripheral accumulation of TfR-positive endosomes in HeLa cells (Palmer et al., 2009). Dynein has been shown to play a role in sorting cargo for recycling versus degradation. Under control conditions EGF (destined for degradation) and TfR (destined for recycling back to the PM) will be sorted into spatially distinct areas within 30 minutes of uptake, but
after dynein inhibition they still colocalise after this time (Driskell et al., 2007). This may be
due to an inability of SNX proteins to function properly in the absence of dynein function. Recent work shows that SNX1- and SNX4- positive membrane regions in EEs colocalise with LIC2, whilst SNX8-positive membranes colocalise with LIC1. Depleting DHC, and in some cases LIC1 or LIC2, leads to these SNX proteins localising to the same membrane regions, suggesting dynein plays a role in keeping SNX proteins separated, thus creating the functionally distinct domains (Hunt et al., 2013). Dynein is also needed for SNX4-dependent the sorting of TfR at EEs and the transport of TfR via ACAP1- and Rab11-positive REs to the PM (Figure 1.6) (Traer et al., 2007). There is a potential interaction between SNX4 and dynein via KIBRA a protein which interacts with both SNX4 and LC8, although it is worth noting that it has not been shown whether LC8 is in complex with dynein when it interacts with KIBRA (Rayala et al., 2006; Traer et al., 2007). Dynein has been further implicated in regulating the recycling of proteins via REs through the ability of LIC1 and LIC2 to interact with the Rab11-effector FIP3. Blocking this interaction prevents transport between EEs and REs (Horgan et al., 2010a; Horgan et al., 2010b). Another interaction that has provides a mechanism for dynein regulated EE sorting is the interaction between SNX5/SNX6 and p150, which can recruit dynein to EE membranes. Disrupting these interactions prevents proteins being trafficked from EEs to the TGN (Figure 1.6) (Hong et al., 2009; Wassmer et al., 2009)

1.7.3 **The role of dynein in early endosome and sorting endosome maturation**
Dynein is also needed for proper endosomal maturation. Around 40 minutes after EGF uptake, the EGF-containing compartments have usually lost Rab5, but after dynein inhibition this process is significantly slowed (Driskell et al., 2007). Another way dynein may contribute to endosomal maturation is by facilitating EE homotypic fusion, indicated by the inhibition of dynein leading to smaller EEs (Driskell et al., 2007). LICs may be important for dynein-specific roles in sorting protein for degradation in the endocytic pathway as partial depletion of LIC1 leads to a reduction and slowing of EGF degradation (Tan et al., 2011). Inhibition of dynein function via dynactin p50 overexpression perturbs long range minus-end-directed movements of Rab5-positive endosomes (Flores-Rodriguez et al., 2011), and a study in fungi show that dynactin is needed for EE movement and correct localisation (Lenz et al., 2006). Although breaking apart the dynactin complex does not appear to affect dynein recruitment when looking at a bulk membrane fraction (Flores-Rodriguez et al., 2011), another study in fungi showed the dynactin p25 subunit is needed for dynein-EE association (Zhang et al., 2011). Reductions in p25 and p27 subunit protein levels lead to a
reduction in EE movements and p25 loss prevents dynein interacting with EE membranes (Figure 1.6) (Yeh et al., 2012).

1.7.4 Dynein and dynactin recruitment to late endosomes and lysosomes

Rab7, found on LE and lysosomes, is thought to recruit dynein to these compartments via RILP. Cells either microinjected with or overexpressing RILP form large CD63-positive membranes clustered at the cell centre (Johansson et al., 2007; Jordens et al., 2001). The N-terminal region of RILP can interact with the first 25 amino acids of dynactin p150 and overexpression of a C-terminal truncated region of RILP (ΔN-RILP) leads to scattering of ΔN-RILP-positive membranes that lack dynactin (Johansson et al., 2007; Jordens et al., 2001). Overexpressing p50 does not affect the colocalisation of p150 on RILP-positive membranes but does prevent them from clustering centrally indicating that dynactin or dynein function is needed for their central localisation (Johansson et al., 2007). The oxysterolbinding-related protein 1L (ORP1L) can interact with the C-terminal region of RILP via its N-terminal ANK repeat region and forms a complex with GTP-bound Rab7. Depletion of ORP1L has no effect on RILP colocalising with p150 or Rab7 but does cause dispersion of the structures indicating ORP1L may be needed for proper transport (Johansson et al., 2007). When cholesterol is present, ORP1L has a closed conformation that can interact with RILP and the dynein/dynactin complex, but in the absence of cholesterol ORP1L unfolds, dissociates from dynactin and interacts with VAP, an ER membrane protein and this mediates LE-ER contacts (Rocha et al., 2009). Dynein has also been implicated as a regulator of LE homotypic and lysosomal tethering because RILP can interact with HOPS (involved in the Rab5/Rab7 switch and LE tethering) in the presence of closed ORP1L, and p150 strengthens the RILP-HOPS interaction (van der Kant et al., 2013).

LIC is also needed for dynein-RILP association (Tan et al., 2011). LIC1 is proposed to play a more prominent role than LIC2, based on stronger LIC1 antibody staining of RILP-positive membranes compared to LIC2 staining and biochemical analysis of a membrane fraction enriched with LAMP1 from LIC1Kd cells that does not contain detectable dynein (Tan et al., 2011). Surprisingly, in this study overexpression of myc-p50 displaces p150, which is contrary to previously published data, when p50 overexpression did not affect p150 association with RILP-positive membranes (Johansson et al., 2007; Tan et al., 2011). This study led to the suggestion that there are two separate dynein recruitment mechanisms to RILP-positive membranes: dynactin-dependent and LIC1-dependent.
Another potential role for dynein in the regulation of lysosome transport comes from neuronal cells where LIC has been implicated as an interactor of JNK scaffold protein, JIP3, in the retrograde transport of lysosomes (Drerup and Nechiporuk, 2013). Dynein has also been implicated in endocytic membrane traffic via interactions with JIP1 and JIP4 (Fu and Holzbaur, 2013; Montagnac et al., 2009).

Further complexity in the possible routes for dynein recruitment to LE membranes is provided by Snapin, which is part of the biogenesis of lysosomal organelle-like complex (BLOC). BLOC proteins were discovered to be mutated in Hermansky–Pudlak syndrome diseases and the BLOC-1 complex is thought to plays a role in trafficking membrane proteins such as CD63 from the TGN to the lysosome and LE (Di Pietro et al., 2006; Luzio et al., 2007). Snapin is a SNARE-associated protein that is needed for fusion of synaptic vesicle in neuronal cells and has also been identified as a subunit of the BLOC-1 complex (Ilardi et al., 1999; Starcevic and Dell'Angelica, 2004). Snapin can interact with dynein IC and is needed for dynein-driven LE retrograde transport in neurons (Figure 1.6) (Cai et al., 2010).

Dynactin seems to play a role in initiating transport of EEs, LEs and lysosomes at MT +TIPs, but these mechanisms have crossover with Lis1 and as such are discussed in the next section (1.7.5). Much of the in vivo data would suggest that dynein and dynactin are needed for the transport and regulation of LEs and lysosomes, which is reflected by the many interactions characterised between the dynein-dynactin complex and LE and lysosome proteins.

### 1.7.5 The role of Lis1 and Nde1/Ndel1 in the endocytic pathway

Lis1 and Nde1/Ndel1 also play a role in regulating dynein in the endocytic pathway. Lis1 and Nde1/Ndel1 are needed for correct positioning of EE in mammalian cells and Lis1 has been shown to be important for initiating dynein-driven transport of endosomes in fungi (Egan et al., 2012; Lam et al., 2010; Lenz et al., 2006). Lis1 and Nde1/Ndel1 are also needed for correct positioning of LAMP1-positive vesicles (Lam et al., 2010). Evidence from neuronal cells suggests Lis1 plays a role in transporting larger LEs as its loss does not affect motility of smaller LEs (Yi et al., 2011).

Lis1 and dynactin have both been implicated in EE transport initiation due to their ability to interact with proteins found at the plus-end of MTs. CLIP-170, the +TIP protein, was
originally proposed to mediate EE association with the plus ends of MTs (Pierre et al., 1992). Both Lis1 and dynactin have been suggested to play a role in loading dynein-associated cargo on to plus ends of MTs via their interactions with CLIP-170 (Coquelle et al., 2002; Ligon et al., 2006; Valetti et al., 1999; Vaughan et al., 1999). Studies in fungi show that Lis1 and dynactin are indeed needed for the localisation of EE to the plus tips of MTs found in hyphae. There may, however, be a possible redundancy between the fungi homologues of CLIP-170 and Nde1 in the recruitment of Lis1 and dynactin to MT plus ends as loss of CLIP-170 alone has no effect on dynein and Lis1 localisation or endosome transport (Efimov et al., 2006; Lenz et al., 2006). This is further supported by evidence in mammalian cells where loss of CLIP-170 causes the loss of p150 from plus ends and a disruption in MT dynamics, but has no effect on endocytosis or endosome movement (Watson and Stephens, 2006). However, a recent study may account for these differences as it showed that p150 (via its MT-interacting domain) is needed for initiation of Rab5- and LAMP1-positive membrane movement specifically at MT +TIPs, whereas Lis1 is needed for initiation of LAMP1-positive membrane transport along the whole length of the MT (Moughamian et al., 2013). It is possible both Lis1 and p150 can initiate membrane transport at the +TIPs but if this does not occur Lis1 may initiate transport at other locations on the MTs.

It is not completely clear how Lis1 and Nde1/Ndel1 regulate the initiation of transport or regulate motility of endocytic membranes, but they do seem to play a role regulating dynein-driven transport of these organelles and this is reflected by the effects their depletion has in vivo on motility of larger lysosomes, in addition to initiating transport and positioning EE and lysosomes (Egan et al., 2012; Lam et al., 2010; Lenz et al., 2006; Moughamian et al., 2013; Yi et al., 2011).

1.7.6 The role of Huntingtin in the endocytic pathway

Two dynein-regulating proteins that have been shown to influence dynein-driven trafficking of endosomes are Htt and Huntingtin associated proteins 1 (HAP1). The Htt protein has been studied extensively due to the fact mutations in the gene can cause a hugely expanded poly-glutamate repeat tail, which leads to the neurodegenerative Huntington’s disease (Ross and Tabrizi, 2011). Htt can interact directly with dynein IC and HAP1 can interact with dynactin p150 (Caviston et al., 2007; Engelender et al., 1997). Loss of Htt leads to mislocalisation of TFR- and Rab-11 positive REs (Caviston et al., 2010). Htt is needed for
the GEF activity that activates Rab11 and fibroblasts taken from patients with Huntington’s disease show slowed recycling of TfR (Li et al., 2008; Li et al., 2009). Furthermore, Htt-associated protein 40 (HAP40) has also been shown to affect the movement of Rab5-positive endosomes. Elevated protein expression of HAP40 can cause a shift of endosomes from MT-attachment to actin-associated, which reduces their motility (Pal et al., 2006).

Htt and HAP1 play a role in the motility and trafficking of LE and lysosomes. Loss of Htt leads to LAMP1-positive lysosomal clustering at the cell periphery, which can be rescued to near control conditions with the reintroduction of Htt (Caviston et al., 2010). In addition to lysosome positioning, Htt appears to be needed for detachment of lysosomes from MTs. Loss of DHC leads to peripheral lysosomes that are not associated with MTs and appear to enmeshed in cortical actin, whereas the peripheral lysosomes that accumulate in Htt and DHC Kd cells still localise to MT. Reminiscent of the interactions between endosomes, actin and HAP40, this indicates the Htt complex may play a role in LE- and lysosome-actin association at the cell periphery and may affect dynein-endosome offloading from MTs, i.e. Htt helps switch LE association from MT to actin association (Caviston et al., 2010; Pal et al., 2006).
Figure 1.6: Dynein and dynactin recruitment interactions on endocytic membranes. Abbreviations: Clathrin Coated Vesicle (CCV), Early Endosome (EE), Recycling Endosome (RE), Late Endosome (LE), Sorting Endosome, Intermediate Chain (IC), Light Intermediate Chain (LIC) and Trans Golgi Network (TGN).
1.7.7 The role of other dynein regulators in the endocytic pathway

It is possible the dynein regulator BicD may play a role in some stages of transport in the endocytic pathway as it has been shown interact with clathrin heavy chain and has been implicated in proper dynein-driven recycling of synaptic vesicles in Drosophila neuronal cells (Figure 1.6) (Li et al., 2010). ZW10 is another dynein accessory protein that may participate in the regulation of endocytosis as its depletion leads to less motile EEs and lysosomes along with their peripheral mislocalisation (Varma et al., 2006).

The studies discussed 1.7 show dynein and its correct regulation are important to nearly every aspect of protein trafficking through the endocytic pathway and that dynein does not just transport endocytic vesicles, but its recruitment is intrinsic to many regulatory events. Although the full breadth of regulation that takes place in endocytic transport is not entirely known at present, the growing understanding of the intricacies surrounding dynein recruitment and activation on specific membranes is likely to continue to provide further insight into how these endocytic organelle movement events are controlled.

1.8 Autophagy

Autophagy is the process by which cells are able to sequester portions of the cytoplasm in order to degrade proteins and organelles. It occurs in response to low nutrient conditions or as a means to remove old, damaged or aggregated proteins and organelles. Autophagy can occur via several distinct pathways, but non-selective macro-autophagy is the focus here and will be referred to as autophagy. Much has been discovered about the mechanisms of autophagy in recent years, in part due to the fact it has been implicated in many diseases including myopathy, cancer, metabolic diseases, genetic diseases and neurodegenerative diseases (Lamb et al., 2012; Lieberman et al., 2012; Murrow and Debnath, 2012; Wong and Cuervo, 2010). The main focus of this project in terms of autophagy, concerns how it is linked to endocytosis. To be able to understand this though, it first important to know how autophagy is initiated and how autophagic membranes form.

1.8.1 Autophagy induction

A complex series of signalling events take place during autophagy induction. In low nutrient conditions autophagy can be induced by the inhibition of the protein mammalian target of rapamycin complex 1 (mTORC1). In the presence of amino acids mTORC1 inhibits the
serine/threonine kinase ULK-1, but in nutrient poor conditions, mTORC1 is itself inhibited and the now active ULK-1 can in turn activate the PtdIns(3) kinase Vps34. In some cell lines, such as HeLa, it is worth noting that detectable levels of autophagy are present all the time and induction via low nutrient conditions is not required (observations and personal communication with Dr. Sharon Tooze). The initial autophagy membrane, the phagophore, is derived from organelle membranes, predominantly the ER and ERGIC with possible contribution from the mitochondria, PM and Golgi apparatus (Ge et al., 2013; Hailey et al., 2010; Lamb et al., 2012; Tooze and Yoshimori, 2010). The membrane surrounds the targeted protein and organelles and closes to produce an autophagosome (AP), which is characterised by the presence of a double membrane (Lamb et al., 2012). Recent evidence suggests AP are generated near ER-exit sites in a COPII-dependent manner (Graef et al., 2013).

Once activated by ULK-1, Vps34 forms complexes with other proteins (discussed in section 1.8.2) and localises to the phagophore, beginning a cascade of autophagy initiation events (Funderburk et al., 2010; Lamb et al., 2012; Yang and Klionsky, 2010). The Vps34 complex phosphorylates PtdIns3 to produce PtdIns3P on the membranes of the phagophore and two proteins are recruited: DFCP1 and WIPI2 (Levine et al., 2011; Polson et al., 2010). There are four WIPI isoforms but in most cell types WIPI2 is the major form and is downstream of DFCP1 (Levine et al., 2011). After WIPI2 and DFCP1 recruitment the membrane elongates. This is dependent on two conjugated ubiquitin-like proteins, ATG12 and ATG5, in complex with ATG16L (Walczak and Martens, 2013). ATG12 and ATG5 are conjugated by ATG7 and ATG10, which are E1 and E2 enzymes, respectively (Ohsumi, 2001). The complex is important for the lipidation and recruitment of LC3 to the phagophore (Fujita et al., 2008). LC3 is the only known marker so far for the AP compartment that is not found on any other organelle (Moreau et al., 2011; Rubinsztein et al., 2009). Pro-LC3 is found in the cytosol where it is cleaved by ATG4 to form LC3-I. LC3-I interacts with ATG3 prior to its membrane recruitment and ATG3 can interact with ATG12 in the ATG12-ATG5-ATG16L complex localised to the phagophore (Figure 1.7) (Fujita et al., 2008). Upon recruitment to the phagophore, LC3-I is conjugated to phosphatidylethanolamine to form the membrane associated LC3-II (Figure 1.7) (Klionsky et al., 2008; Moreau et al., 2011). LC3 is then able to target ubiquitinated protein destined for AP-driven degradation via interactions with p62 and Nbr1 proteins (Lamark et al., 2009; Shvets et al., 2008; Weidberg et al., 2011). Nbr1 and p62 can interact with polyubiquitinated cargo, which can then be engulfed by the AP.
A Golgi-associated protein, GATE-16 can also be recruited to AP membranes and undergo phosphatidylethanolamine conjugation (Weidberg et al., 2010). GATE-16 is needed for AP formation and is thought to play a role in the final stages where the phagophore membrane closes to form an AP (Weidberg et al., 2011; Weidberg et al., 2010). Once the AP has fully formed it can fuse with endosomes and lysosomes to mature and eventually form the autolysosome, at which point the double membrane is lost via degradation (Figure 1.7) (Lamb et al., 2012; Moreau et al., 2011; Razi et al., 2009).

When the contents of the autolysosome have been degraded, the lysosomal proteins and membranes form tubules and break away from the autophagic membranes, reforming lysosomes (Chen and Yu, 2012). mTORC1 is recruited to the autolysosome surface and reactivated in response to the amino acids and sugars that are produced as a result of autophagy and pumped into the cytosol (Figure 1.7) (Lamb et al., 2012; Rong et al., 2010; Yu et al., 2010). Rag proteins, a family of four GTPases, which are recruited to the autolysosome surface by a protein complex termed the Ragulator, interact strongly with mTORC1 when GTP bound and in presence of high amino acid concentrations (Sancak et al., 2010; Sancak et al., 2008). When the autolysosome is releasing amino acids into the cytosol it is thought these proteins help recruit mTORC1 to the membrane surface so that it is in close proximity to Rheb. Rheb is a Ras-like GTPase that can interact with and activate mTORC1 and this results in a inhibition of ULK-1 and therefore prevents further autophagy induction (Bai et al., 2007; Long et al., 2005). If the degraded contents of autolysosomes are not needed in the cell, for example in erythropoiesis where membrane-bound organelles are depleted from the cell, the degraded contents of the AP can be exocytyosed (Griffiths et al., 2012).
Figure 1.7: Autophagosome (AP) formation and maturation  
A: Protein interactions that promote autophagosome formation  
B: The maturation pathway of APs. Abbreviations: PE = phosphatidy ethanolamine.
1.8.2 The role of endocytic pathway components and dynein in autophagy

As endosomes and lysosomes fuse with the AP they influence the AP maturation and thus its function (Lamb et al., 2012). In the next section the role of different endocytic compartments and the proteins associated with them will be considered in terms of autophagy regulation.

During autophagy initiation the REs are particularly important during AP formation. ATG9 and ATG16, which are both important for AP formation, localise to REs prior to forming APs (Puri et al., 2013). Additionally, ULK-1, which is needed to initiate autophagy, has been shown to localise to Rab11- and Tfr-positive REs (Longatti et al., 2012; Longatti and Tooze, 2012).

Early endosomes can fuse with APs and this contributes to the AP maturation (Lamb et al., 2012). Many of the proteins that are associated with SEs and those that regulate the Rab5 to Rab7 switch during endosome maturation are involved in autophagy regulation. A RabGAP, TBC1D5, can interact with the retromer complex (associated with SEs) and LC3 simultaneously to form a bridge between SEs and APs (Popovic et al., 2012). HOPS is the Rab7 effector, which can interact with RILP and is involved in membrane tethering and mediating the switch between Rab5 and Rab7 on SEs as they mature into LEs (Brocker et al., 2012; Ostrowicz et al., 2010; Rink et al., 2005; van der Kant et al., 2013). HOPS may be involved in regulating mTORC1 (Flinn et al., 2010; Yang and Klionsky, 2010). The activation of mTORC1 has been shown to be dependent on endosome maturation. If the HOPS-mediated switch of Rab5 to Rab7 does not occur, hybrid organelles form that contain EE and lysosome markers. mTORC1 is still recruited to these organelles but Rheb is not and therefore mTORC1 remains inactive thus unable to inhibit autophagy induction (Flinn et al., 2010).

Another autophagy regulation mechanism that involved Rab5, Rab7 and HOPS involves the interaction of two proteins; Rubicon and UVRAG. Rubicon localises to Rab5-positive EEs and can negatively regulate endocytic and autophagic progression (Sun et al., 2010). Rubicon can sequester UVRAG that is able to promote HOPS activity when not in complex with Rubicon. When Rab7 is GTP bound it competes with UVRAG for binding with Rubicon. UVRAG is displaced and can promote HOPS GEF activity, providing a positive feedback loop for more GTP-Rab7 membrane loading (Liang et al., 2008; Sun et al., 2010; Tabata et al., 2010). Overexpression of UVRAG can increase the number of LC3-GFP puncta positive for HOPS and LE markers indicating UVRAG can promote the maturation of autophagosomes.
Beclin-1 is a protein that interacts with and activates the Vp34 PtdIns3 kinase needed for autophagy initiation (Funderburk et al., 2010; Furuya et al., 2005; Kang et al., 2011). It can also interact with UVRAG and Rubicon, which is important during autophagy regulation and progression (Liang et al., 2008; Matsunaga et al., 2009). The Beclin-1-binding protein ATG14 can interact with Snapin and this interaction is needed for endocytic and autophagy progression (Kim et al., 2012; Lu et al., 2009). Snapin has also been shown to be needed for dynein-driven transport of LEs and lysosomes in neurons and loss of Snapin leads to a block in autolysosome clearance, possibly due to impaired lysosomal degradation capacity and inefficient lysosome-AP encounters (Cai et al., 2010).

Lysosomes play a fundamental role in autophagy as they are needed to deliver the hydrolases which degrade proteins in the AP (Lamb et al., 2012). Many of the processes in lysosome movement and fusion also affect autophagy. The LE/lysosome-associated Rab7 has been shown to be important for progression through autophagy, with overexpression of dominant negative mutants causing an accumulation of LC3 and may prevent LE/lysosome-AP fusion (Bains et al., 2011; Gutierrez et al., 2004). Overexpression of a dominant-negative mutant of ATG4 (that sequesters LC3) in erythrocytes, leads to an accumulation of hybrid EE-autophagosome compartments that have not fused with lysosomes, suggesting cells require ATG4 activity for AP-lysosome fusion to occur properly (Betin et al., 2013). As Rab7-interacting protein RILP has been shown to recruit HOPS to LE and promote membrane tethering (van der Kant et al., 2013), it is possible these interactions may be needed for LE/lysosome-AP fusion. RILP has been implicated in the transport of APs, but it is not clear what role it plays. Inducing autophagy in neurons leads to a large increase in RILP expression levels. This endogenous RILP is visible in neuronal processes but only a small proportion of the RILP colocalised with dynein, Rab7 or LC3 (Bains et al., 2011). There are many processes that influence endosome maturation and a lot of these seem to also affect APs and autolysosome formation. This highlights how closely related the endocytic and autophagy pathways are.

1.8.3 Transport of autophagosomes

Formation of APs and their movement requires MTs (Kochl et al., 2006). Dynein can transport AP and is needed for AP-lysosome encounters, which are a prerequisite for fusion (Kimura et al., 2008; Ravikumar et al., 2005). The Kif2 kinesin also plays a role in AP-
lysosome encounters. Mild starvation conditions cause Arl8b (and ARF family G protein) and Kif2 to dissociate from the lysosomal membrane. The resulting perinuclear positioning of lysosomes brings them into close contact with APs and increases fusions events (Bagshaw et al., 2006; Korolchuk et al., 2011). Arl8b has also been shown to regulate Kinesin-1 on lysosomal membranes (Rosa-Ferreira and Munro, 2011), so it is possible that kinesin-1 is also contributing to this process. When they have fused with lysosomes they begin to move in a bidirectional manner and membrane fractions enriched for AP membranes contain dynein, kinesin-1 and kinesin-2 (Maday et al., 2012). The ability of dynein to regulate autophagy progression is of particular importance in diseases where dynein function is lost, such as Huntington’s disease, which results in impaired protein aggregate clearance due to faults in autophagy. For example, drosophila and mouse models for Huntington’s disease show increased LC3 puncta indicative of impaired AP-lysosome fusion (Ravikumar et al., 2005).

1.8.4 A transport-independent role for dynein in autophagy induction

Dynein can influence autophagy progression though transport of endocytic vesicles and AP themselves, but some of the dynein subunits have a role outside of transport in which they can regulate autophagy initiation. During normal conditions dynein interacts with AMBRA-Beclin-1 via LC8 but during starvation ULK-1 phosphorylates AMBRA freeing the Beclin-1 from dynein (Di Bartolomeo et al., 2010). It is thought this dynein-AMBRA interaction sequesters Beclin-1 to MT preventing autophagy initiation events taking place erroneously. A separate mechanism for dynein-mediated Beclin-1 sequestering is via an interaction of LC8 with Bim, which then may be able to recruit Beclin-1 to MTs independently of AMBRA (Luo et al., 2012). In this study Beclin-1 localisation with MT is lost after depletion of LC8 or Bim. This suggests LC8-Bim-Beclin-1 may be in complex with dynein but there is no biochemical analysis to confirm this. Irrespective of this, the combined data from these LC8 studies would suggest that LC8 plays a role in regulating the induction of autophagy.

There is still a great deal to understand in terms of the interconnections between the endocytic pathways and autophagy but there are clearly many links between these two processes. Given dynein’s involvement in both these pathways it would be particularly interesting to consider how dynein-driven transport and trafficking events may influence autophagy.
1.9 Project aims

The proteins and interaction pathways that regulate dynein are highly complex and context-specific (Allan, 2011). Ubiquitous regulators such as dynactin, Lis1 and Nde1/Ndel1 play different roles on diverse organelle membranes during various processes (Kardon and Vale, 2009). The dynein subunits themselves have multiple roles on different organelles and can interact with a wide range of regulating proteins. Another layer of complexity is added by the fact dynein subunits and dynein regulators can be modified post-translationally and this can affect dynein-regulator and dynein-cargo interactions. Additionally, dynein is in functionally distinct pools, defined by subunit composition and the splice variants of these subunits it contains (Allan, 2011; Kardon and Vale, 2009; Pfister et al., 2006).

Although a huge amount has been revealed in the last 40 years in terms of dynein function, there is still much to understand about the fine detail of how its different roles in the cell are regulated. As such, dynein and its interactions with cargo and accessory proteins in the context of the endocytic pathway were investigated in this project. The endocytic pathway itself is extremely complex and proteins are sorted and trafficked during every step of the process in a highly ordered and elegant manner. This project aimed to identify the importance of specific dynein subunits and accessory proteins in endocytic membrane maintenance and trafficking. In order to assess how the individual dynein subunits LIC and IC as well as the accessory protein Lis1 and Nde1/Ndel1 contribute to the cellular positioning and morphology of endocytic vesicles, the proteins were depleted using RNAi. EE, LE and lysosomes were observed using microscopy after these protein depletions. There was a particular focus on assessing the similarities and differences in the endocytic phenotypes resulting from these depletions to help identify whether certain processes were regulated by specific proteins or whether the proteins might work together in the same regulatory pathways to maintain aspects of organelle positioning and size. The role of these proteins was also investigated in terms of dynein recruitment to membranes. LIC and IC Kd experiments were carried out to investigate whether these proteins have a role as general membrane adaptors or whether they may have recruitment roles on specific endocytic membranes. Another aim of this project was to investigate how dynein might regulate the flux through the autophagy pathway. Dynein is known to move autophagic membranes (Kimura et al., 2008; Maday et al., 2012), but the focus here was how dynein’s influence on the endocytic pathway might feed into autophagy flux. Again, the roles of LIC,
IC, Lis1 and Nde1/Ndel1 were investigated using RNAi, to assess how the loss of these proteins affects flux through the autophagy pathway and positioning of autophagic vesicles.

Overall the aim of this project was to gain more insight into how the interactions of dynein subunits and accessory proteins might affect dynein recruitment to membranes, transport of endocytic vesicles and autophagic flux. These processes are interlinked and the results from this project show that LIC, IC, Lis1 and Nde1/Ndel1 all produce different endocytic and autophagic phenotypes which are likely to be a result, at least in part, of the ability of these proteins to recruit dynein to different organelles through specific mechanisms.
Chapter 2

Materials and Methods
2. Materials and methods

Unless otherwise stated all materials used were obtained from Fisher Scientific (Loughborough, UK), Sigma Aldrich (Dorset, UK) or VWR (Leicestershire, UK).

2.1 Media and solutions

Blotting transfer buffer: 25 mM Tris, 0.192 M Glycine, 20% (v/v) Methanol; 0.02% (w/v) SDS, pH 8.3.

BRB80: 80 mM PIPES pH 6.85, 1 mM MgCl₂, 1 mM EGTA.

Complete media: Dulbecco’s Modified Eagle Medium (DMEM), 2 mM L-Glutamine (200 mM in 0.85% NaCl solution), 10% South American Foetal Calf Serum (Hyclone, Thermo Scientific, Leicestershire).

Homogenisation Buffer (HB): 5 mM imidazole, pH 7.4, 5mM MgSO₄, 1 mM EDTA, 0.5 mM EGTA.

ImunoPrecipitation (IP) Buffer: 40 mM NaCl, 25 mM Tris pH7.4, 0.5% w/v Igepal.

Laemmli sample buffer (3x): 10 ml glycerol, 12.5 ml resolving Tris buffer with 3 g SDS made up to 95 ml with ddH₂O, pinch of Bromophenol blue, 5% (v/v) beta-mercaptoethanol.

Lysis Buffer: 150 mM NaCl, 40 mM Tris pH7.4, 1% (v/v) Triton X-100.

Lysogeny Broth (LB) bacterial growth media: 25 g/l granulated LB dissolved in distilled water and autoclaved.

Phosphate Buffered Saline (PBS): 137mM NaCl, 10mM Na₂PO₄, 2.7mM KCl, 1.8mM KH₂PO₂.

Ponceau stain: 0.2% (w/v) ponceau, 3% (v/v) TCA in distilled water.

Protease Inhibitor Cocktail: Leupeptin 10 µg/ml, chymostatin 10 µg/ml, pepstatin 10 µg/ml and aprotinin 10 µg/ml.

Sodium Dodecyl Sulphate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) resolving buffer: 375 mM Tris-HCl, pH 8.8, 0.1% (w/v) SDS.

SDS-PAGE running buffer: 25 mM Tris, 0.025 M Glycine, 0.1% (w/v) SDS, pH 8.3.

SDS-PAGE stacking buffer: 125 mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS.
## 2.2 Primary antibodies

<table>
<thead>
<tr>
<th>Name</th>
<th>Raised Against</th>
<th>Concentration</th>
<th>Dilution IF/(Western Blot)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-DYNC1LI1</td>
<td>Dynein LIC1</td>
<td>1 mg/ml</td>
<td>1/300 (1/2000)</td>
<td>Sigma Prestige, Dorset, UK</td>
</tr>
<tr>
<td>Anti-GFP</td>
<td>GFP</td>
<td>0.4 mg/ml</td>
<td>(1/1000)</td>
<td>Roche, Welwyn Garden City UK</td>
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<tr>
<td>Anti-LC3b</td>
<td>LC3</td>
<td>1 mg/ml</td>
<td>1/200 (1/1000)</td>
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<td>Anti-CD63</td>
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<td>CRM1</td>
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<td>(1/2000)</td>
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<tr>
<td>DCTN2 RabMAb</td>
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<td>Unknown</td>
<td>1/300</td>
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<tr>
<td>EEA1</td>
<td>EEA1</td>
<td>0.25 mg/ml</td>
<td>1/200</td>
<td>BD Transduction Laboratories, Oxford, UK</td>
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<tr>
<td>EEA1</td>
<td>EEA1</td>
<td>Unknown</td>
<td>1/200</td>
<td>Cell Signalling Technologies, Boston, MA, USA</td>
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<tr>
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<td>GM130</td>
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<td>1/200</td>
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<td>H4A3</td>
<td>LAMP1</td>
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<td>Anti-TfR</td>
<td>TfR</td>
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<td>Zymed (Life Technologies, Paisley, UK)</td>
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<td>IC74</td>
<td>Dynein IC2</td>
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<td>Monoclonal Anti-Lis1</td>
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<td>Nde1</td>
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<td>PAFAH α2</td>
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<td>TAT-1</td>
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<td>Hybridoma Cell Line Supernatant</td>
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<td>Keith Gull, FLS, University of Oxford, UK</td>
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<tr>
<td>YL1/2</td>
<td>Tyrosinated alpha tubulin</td>
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<td>1/500</td>
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<td>αKHC</td>
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<td>4.2 mg/ml</td>
<td>(1/5000)</td>
<td>Ron Vale, Howard Hughes Medical Institute, San Francisco</td>
</tr>
</tbody>
</table>

Table 2.1: Primary antibodies and the conditions used during the project.
## 2.3 Secondary antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Anti-Species</th>
<th>Host Species</th>
<th>Concentration</th>
<th>Dilution IF/ (Western Blot)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
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<td>Mouse</td>
<td>Donkey</td>
<td>1.5 mg/ml</td>
<td>1/300</td>
<td>Jackson Immuno Research Laboratories Inc, West Grove, PA, USA</td>
</tr>
<tr>
<td>Cy3 - conjugated AffiniPure anti-mouse IgG (H+L)</td>
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<td>Donkey</td>
<td>1.5 mg/ml</td>
<td>1/400</td>
<td>Jackson Immuno Research Laboratories Inc, West Grove, PA, USA</td>
</tr>
<tr>
<td>AlexaFluor 594 anti-Mouse (H+L)</td>
<td>Mouse</td>
<td>Donkey</td>
<td>2 mg/ml</td>
<td>1/400</td>
<td>Molecular Probes, Eugene, OR, USA</td>
</tr>
<tr>
<td>Cy2 - conjugated AffiniPure anti-rat IgG (H+L)</td>
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<td>Goat</td>
<td>1.5 g/ml</td>
<td>1/300</td>
<td>Jackson Immuno Research Laboratories Inc, West Grove, PA, USA</td>
</tr>
<tr>
<td>AlexaFluor 488-conjugated AffiniPure anti-rabbit IgG (H+L)</td>
<td>Rabbit</td>
<td>Donkey</td>
<td>1.5 mg/ml</td>
<td>1/300</td>
<td>Jackson Immuno Research Laboratories Inc, West Grove, PA, USA</td>
</tr>
<tr>
<td>AlexaFluor 594 anti-Rabbit (H+L)</td>
<td>Rabbit</td>
<td>Donkey</td>
<td>2 mg/ml</td>
<td>1/400</td>
<td>Molecular Probes, Eugene, OR, USA</td>
</tr>
<tr>
<td>Cy5 - conjugated AffiniPure anti-rabbit IgG (H+L)</td>
<td>Rabbit</td>
<td>Donkey</td>
<td>1.5 mg/ml</td>
<td>1/300</td>
<td>Jackson Immuno Research Laboratories Inc, West Grove, PA, USA</td>
</tr>
<tr>
<td>IRDye 680RD</td>
<td>Mouse</td>
<td>Donkey</td>
<td>1 mg/ml</td>
<td>1/2000</td>
<td>LI-COR, Lincoln, UK</td>
</tr>
<tr>
<td>IRDye 680RD</td>
<td>Mouse</td>
<td>Donkey</td>
<td>1 mg/ml</td>
<td>1/2000</td>
<td>LI-COR, Lincoln, UK</td>
</tr>
<tr>
<td>IRDye 680RD</td>
<td>Rabbit</td>
<td>Donkey</td>
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<td>IRDye 800CW</td>
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<td>Donkey</td>
<td>1 mg/ml</td>
<td>1/2000</td>
<td>LI-COR, Lincoln, UK</td>
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</tbody>
</table>

Table 2.2: Secondary antibodies used during this project.

## 2.4 DNA plasmids

The following DNA plasmids were used in transfection experiments: pEGFP C1 (kanamycin resistant), pcDNA3.1 containing c-Myc (ampicillin resistant), pcDNA5 containing Myc-Lis1 (ampicillin resistant) (Vergnolle and Taylor, 2007), pcDNA5 containing Myc-Nde1 (ampicillin resistant) (Vergnolle and Taylor, 2007), pcDNA5 containing Myc -Ndel1 (ampicillin resistant) (Vergnolle and Taylor, 2007), pEGFP C1 containing Lis1 (kanamycin resistant) (Lam et al., 2010), pEGFP C1 containing siRNA resistant Lis1 (SR-GFP-Lis1; kanamycin resistant) (Lam et
al., 2010), pEGFPC1 containing RILP (kanamycin resistant) (Colucci et al., 2005) which was a kind gift from Prof Cecilia Bucci at the University of Salento, Italy, pmCherry (kanamycin resistant) (Wozniak et al., 2009) and pmCherry-chicken p50 (kanamycin resistant) (Wozniak et al., 2009).

To prepare DNA plasmids, Top10 E.coli (Life Technologies, Paisley, UK) were transformed by incubating 50 μl of bacteria with 2 μg of DNA on ice for 20 minutes, followed by a heat shock at 42°C for 30 seconds. Cells were left on ice to recover for 10 minutes then transferred to 1 ml LB and incubated in a shaker incubator at 37°C for 90 minutes. From this culture 100 μl was plated onto a pre-dried 1% agar plates containing either 100 μg/ml ampicillin or 50 μg/ml kanamycin and incubated at 37°C over night. The next day, a colony was picked using a pipette tip and incubated in 5ml of LB containing antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) in a shaker incubator at 37°C for 8 hours. This culture was then transferred to 100ml of LB containing antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) and incubated in a shaker incubator at 37°C over night. The bacteria cells were centrifuged in a Rotina 420 centrifuge (Hettich, Buckinghamshire, UK) at 4000g. The LB was removed and DNA from the bacteria cell pellet was isolated using a NucleoBond Xtra Midi DNA purification kit (Machery-Nagel, Duren, Germany), as per the manufacture’s guidelines.

2.5 Cell culture
Throughout this project HeLaM cells were used (a sub-clone of the HeLa human cervical cancer cell line), which were obtained from Graham Warren, Vienna, Austria. Cells were grown at 37°C and 8% CO₂ in complete media and maintained in T75 culture flasks (Corning, NY, USA). Cells were passaged under sterile conditions using an Auro 2000 BS cell culture hood (BioAir, Pavia, Italy). The cells were washed twice with pre-warmed (37°C) PBS, incubated with trypsin containing 0.2 mg/ml EDTA for 5 minutes at 37°C to induce cell dissociation from the flask. Pre-warmed (37°C) complete media was added to neutralise the trypsin and cells were diluted either 1/10 or 1/5 in pre-warmed complete media and transferred to a fresh T75 flask. During experiments, cells were grown in 12 well plates, 10cm dishes and 15cm dishes (Corning, NY, USA). During microscopy experiments cells were grown on glass cover slips, 13 mm diameter No. 1.5 (Agar Scientific, Essex, USA).
2.5.1 Transfections

Polyethyleneimine max (Polysciences, Eppelheim, Germany) was used for transfections of single DNA plasmids. Polyethyleneimine max was prepared by dissolving in autoclaved MiliQ water and adjusting to pH7.4 using NaOH. The volume was adjusted to give a final concentration of 1mg/ml and the solution was filter sterilised. DNA was added to OptiMem media (Gibco, Paisley, UK), mixed with polyethyleneimine max and incubated for 25 minutes in the following ratios:

<table>
<thead>
<tr>
<th></th>
<th>Complete media</th>
<th>Optimem</th>
<th>DNA</th>
<th>Polyethyleneimine Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 well dish</td>
<td>1 ml</td>
<td>50 μl</td>
<td>0.5 μg</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>(per well)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10cm plate</td>
<td>10 ml</td>
<td>500 μl</td>
<td>5 μg</td>
<td>15 μl</td>
</tr>
</tbody>
</table>

Table 2.3: Ratios of reagents used in single DNA plasmid transfections.

Co-transfections of two DNA-plasmids were carried out using PolyPlus Jet Pei (BioSource, Nottingham, UK) in 12 well plates, as per the manufacturer’s instructions, using reagents in the following ratios:

<table>
<thead>
<tr>
<th></th>
<th>Complete Media</th>
<th>150mM NaCl/DNA (total)</th>
<th>150mM NaCl/JetPei</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ml</td>
<td>50 μl/2 μg</td>
<td>50 μl/ 2 μg</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: Ratios of reagents used per well in a 12-well plate for double DNA plasmid transfections.

2.5.2 RNAi

RNAi was carried out using PolyPlus Interferin (BioSource, Nottingham, UK) as per the manufacturer’s instructions. Cells were treated with 20nM siRNA and the amount of Optimem and Interferin were adjusted from the manufacturer’s recommendations so that for every 1ml of complete growth media present, 100μl of Optimem and 1.5μl of Interferin were used. ON-TARGETplus Non-targeting siRNA #1 (Thermo Scientific Dharmacon, Leicestershire, UK) was used as a control. All other siRNA duplexes were either siMAX (Eurofins MWG Operon, Ebersberg, Germany) or SMARTpools (Thermo Scientific Dharmacon, Leicestershire, UK). The siRNA oligos used are detailed in table 2.5.
<table>
<thead>
<tr>
<th>Target Protein</th>
<th>siRNA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dynein IC1 ON-TARGETplus SMART pool</td>
<td>GGAAGGCACUGUUGAGUUA GGAUUUCGUACUAACAGA CAAGGAAAGUAGUUGUCCUA CGGGAGACGUCAUAACUU</td>
<td>-</td>
</tr>
<tr>
<td>Dynein IC2 ON-TARGETplus SMART pool</td>
<td>GUAAGACUUUGGACAAACUA GAUGUAUGUGGUCACCCUA GCAUUCUGUGGAGGUAA GUGGUUAGUUGUUUGGAUU</td>
<td>-</td>
</tr>
<tr>
<td>Dynein LIC1</td>
<td>AGAUGACAGUGUAGUGUA</td>
<td>(Palmer et al., 2009)</td>
</tr>
<tr>
<td>Dynein LIC2</td>
<td>ACCUCAUCUUGUGUAUAA</td>
<td>(Palmer et al., 2009)</td>
</tr>
<tr>
<td>Lis1</td>
<td>GAAACAAGCAUGCAUGAA</td>
<td>(Lam et al., 2010)</td>
</tr>
<tr>
<td>Nde1</td>
<td>GGACCCAGCUCAAGUUUAACUU</td>
<td>(Lam et al., 2010)</td>
</tr>
<tr>
<td>Ndel1</td>
<td>GGACCAAGCAUCACGAAAUU</td>
<td>(Lam et al., 2010)</td>
</tr>
<tr>
<td>PAFAH α1</td>
<td>AGAAUGGAGAGCGUGGAACAUU</td>
<td>(Bechler and Brown, 2013)</td>
</tr>
<tr>
<td>PAFAH α2</td>
<td>GGGAACUGGAGAAAUAUU</td>
<td>(Bechler and Brown, 2013)</td>
</tr>
</tbody>
</table>

Table 2.5: siRNA oligos used during this project.

### 2.6 Cell fixation

#### 2.6.1 Methanol fixation

Cover slips were dipped in room temperature PBS to wash off complete media and excess PBS was removed using blotting paper (Whatman, Austria). The cover slips were submerged in -20°C methanol for 5 minutes. The cells were washed three times in PBS and stained in the manner described in section 2.7.

#### 2.6.2 Formaldehyde fixation

Cover slips were dipped in room temperature PBS and placed in 3% formaldehyde in PBS for 15 minutes at room temperature. Cover slips were washed in PBS and then placed in PBS with a few drops of 1M glycine (pH8) for 5 minutes to quench any residual formaldehyde. Cover slips were washed twice in PBS for 5 minutes, and then incubated in 0.1% (v/v) Triton X-100 in PBS for 5 minutes to permeabilise cell membranes. Cells were washed three times for 5 minutes in PBS and stained in the manner described in section 2.7.
2.6.3 Formaldehyde and glutaraldehyde fixation

Cells were fixed as in sections 2.6.2, but formaldehyde was supplemented with 0.2% glutaraldehyde and in place of glycine, three 5 minute incubations with 0.5mg/ml sodium borohydride in PBS were used.

2.6.4 Pre-extraction of cytosol

Unfixed cells were incubated with room temperature 0.5% (w/v) saponin in BRB80 supplemented with 2mM sodium orthovanadate and 50mM NaF for 5 minutes at room temperature to extract cholesterol, which is most prevalent in the PM. Cells were then fixed in 3% formaldehyde in BRB80 for 10 minutes. Cover slips were washed in PBS 3 times and incubated in PBS with 0.5% (w/v) saponin and a few drops of 1M glycine (pH8) for 5 minutes. Coverslips were washed twice in PBS and incubated with PBS with 0.5% (w/v) saponin for 20 minutes at room temperature. Cells were washed three times in PBS and stained in the manner described in section 2.7 except antibody incubations were supplemented with 0.5% (w/v) saponin.

2.7 Antibody labelling

For antibody incubation, 25 μl of PBS was used per cover slip in which primary antibody was diluted as described in table 2.1. The excess PBS was removed from the cover slip and it was placed cell side down on the 25 μl of antibody PBS solution on parafilm. Cells were incubated for 20-30 minutes in the antibody solution, at room temperature, in the dark and in the presence of moistened tissue. The cover slips were floated off the parafilm by the addition of 1 ml of PBS at the cover slip edge. The coverslips were washed three times in PBS for 5 minutes. The same method was used for secondary antibody incubation after which cover slips were again washed three times in PBS for 5 minutes. The second wash contained 100 μl of 1 μg/ml DAPI solution, to stain DNA. Finally the cover slips were dipped in distilled water to wash off PBS, the excess water was removed using blotting paper and the cover slips were mounted cell side down in 7 μl of ProLong Gold (Life Technologies, Paisley, UK) mounting medium. The mounting medium was allowed to set overnight at room temperature in the dark.

2.8 Imaging

Images in figures 4.10 and 4.11 were taken using an IX71 microscope (Olympus, Watford, UK) with a x60 oil emersion objective (Olympus, Watford, UK) and a Coolsnap camera.
(Photometrics, Tuscon, AZ, USA). Image stacks were made and deconvolution was performed using softWoRx software (applied precision, Issaquath, WA, USA). All other images were taken on a BX60 microscope (Olympus, Watford, UK) with a x60 or x100 oil immersion objective (Olympus, Watford, UK), a Coolsnap camera and Metamorph or MetaVue imaging software (Molecular Devices, Sunnyvale, CA, USA). Image processing was performed in MacBiophotonics Image J software (McMaster University, Ontario, Canada) and figures were constructed in Illustrator CS2 (Adobe, San Jose, CA, USA).

2.9 Cell lysis

2.9.1 Normal lysate preparation

Unless otherwise stated, cells were lysed in the following manner. Cells were trypsinised, centrifuged in a Rotina 420 centrifuge (Hettich, Buckinghamshire, UK) at 1000g, washed in PBS and spun down again. The pellet volume was estimated and the pellet was resuspended in the same volume of ice cold lysis buffer was added with protease inhibitor cocktail (1/1000 dilution). Cells were incubated in lysis buffer, on ice for 20-30 minute, gently pipetting the lysate after approximately 10 minutes. The lysate was spun at 32000g in a Mikro22 centrifuge (Hettich, Buckinghamshire, UK) for 30 minutes at 4°C to remove insoluble matter. The supernatant was collected and protein concentrations were measured using a Bradford Assay (BioRad, Hertfordshire, UK) and an albumin protein standard. Protein concentrations were normalised across samples from the same experiment. Laemmli sample buffer (3x) was diluted to 1x in lysate and these samples were heated to 100°C for 10 minutes ready for SDS-PAGE analysis.

2.9.2 Whole cell lysate preparation

Cells were scraped in into 1.5x Laemmli sample buffer in PBS that had been heated to 95°C. The scraped cells were intermittently vortexed vigorously and heated to 100°C for 10 minutes.

2.9.3 IP lysate preparation

Dishes of cells (10 cm) transfected with DNA constructs containing GFP were washed in room temperature PBS three times. All the PBS was removed and 850 μl of ice cold IP buffer supplemented with protease inhibitors (protease inhibitor cocktail, 1/1000 dilution) and phosphatase inhibitors (50mM NaF and 1mM sodium orthovanadate), was added to each dish, on ice. The dishes were transferred to a slow rocker for 20 minutes at 4°C. Dishes
were transferred back on to ice and scraped. The scraped cells were collected and spun at 31864g in a Mikro22 centrifuge for 30 minutes at 4°C. The supernatants were collected, the protein concentrations were measured in the same manner described in 2.9.1 and the protein concentrations were normalised across samples from the same experiment. The lysates were kept on ice and used in co-IP experiments.

2.10 GFP Co-Immuno-Precipitations (Co-IPs)

Up until protein denaturing, all steps were carried out on ice or at 4°C. G-protein sepharose beads (GE Health Care Life Sciences, Buckinghamshire, UK), were washed and blocked in 50mg/ml bovine serum albumin in IP buffer for 30 minutes. These beads were used to pre-clear lysates prior to IP. For each IP, 10 μl of blocked beads were resuspended in 30 μl of IP buffer (40 μl total), were added to IP lysates and rotated for 30 minutes. The beads were spun down at 100g in a Mikro22 centrifuge for 5 minutes and the supernatants were collected. Some of the pre-cleared lysate (50 μl) was removed for use as an input during Western blot analysis. To these inputs, 25 μl of Laemmli sample buffer (3x) was added and they were heated to 100°C for 10 minutes. The rest of the pre-cleared lysates were incubated with 15 μg of anti-GFP antibody (a kind gift from the Philip Woodman laboratory, purified from goat serum, raised against GST-GFP) and rotated overnight. The next day, 30 μl of bovine serum albumin blocked beads suspended in 90 μl of IP buffer (120 μl total) were added to each IP lysate and rotated for 2 hours. The beads were then spun down at 100g in a Mikro22 centrifuge for 5 minutes. The beads were washed in 1 ml of IP buffer supplemented with protease and phosphatase inhibitors and centrifuged at 100g for 5 minutes in a Mikro22 centrifuge. This was repeated a further 6 times with IP buffer lacking protease and phosphatase inhibitors. In the final wash, IP buffer lacking Ipegal was used. The final wash was removed and 30μl of Laemmli sample buffer (diluted to 2x in IP buffer lacking Ipegal) was added to the beads and they were heated to 100°C for 10 minutes. The beads were spun at low speed in a Mini Array centrifuge (VWR, Leicestershire, UK) at room temperature and the supernatant was analysed using SDS-PAGE and Western blotting.

2.11 Membrane preparation by centrifuging

Cells were grown on 6 x 15 cm plates per experimental treatment, trypsinised and centrifuged in a Rotina 420 at 1000g. Cells were washed and centrifuged in the same manner, with ice cold HB supplemented with 250 mM sucrose. The cell pellet volume was estimated and resuspended in half that volume in ice cold HB, supplemented with 250 mM
sucrose and additions (1 mM DTT, 10 μg/ml cytochalasin D and protease inhibitor cocktail, 1/1000 dilution). From this point onwards the preparation was carried out at 4°C or on ice. Cells were passed though a ball bearing homogeniser (HGM, Heidelberg, Germany) with an 8.020 mm barrel and an 8.006 mm ball bearing. The cells were typically passed through the homogeniser 10 times, but after 6 passes and each pass thereafter, the cells were observed using a CK40 microscope (Olympus, Watford, UK) to assess homogenisation. When sufficiently homogenised cells were spun in a Mikro22 centrifuge at 1000g for 10 minutes. The supernatant was collected and this step was repeated. The post nuclear supernatant (PNS) that was produced was collected. In an 800μl Thinwall UltraClear centrifuge tube (Beckman Coulter, High Wycombe, UK) a two step sucrose gradient was made by layering 350 μl of 0.5 M sucrose in HB supplemented with additions, over 100 μl of 2 M sucrose in HB supplemented with additions. The PNS (350 μl) was then layered on top of the 0.5 M sucrose phase and the tubes were centrifuged in a SW55 Ti rotor (Beckman Coulter, High Wycombe, UK), using split delrin adaptor with a 5 mm diameter (Beckman Coulter, High Wycombe, UK) at 88,000g in an L-90K centrifuge (Beckman Coulter, High Wycombe, UK) for 40 minutes. The cytosol-containing supernatant above the 0.5 M sucrose phase was collected and most of the 0.5 M phase was removed with care so that the layer of membranes that form above the 2 M sucrose phase were not disturbed. The membranes were collected and their volume was adjusted to 70 μl (approximately 1/5 of the PNS volume loaded) in ice cold HB supplemented with 250 mM and additions. The membranes are adjusted to this small volume to ensure proteins are concentrated enough to allow detection via Western blotting. The concentration of protein in the cytosol fractions were measured using a Bradford assay and an albumin standard. The volumes of the cytosol fractions were adjusted so that the protein concentration was equal across samples in the same experiment. The membrane fractions volumes were adjusted with the same dilution factor as the corresponding cytosol fraction. Laemmli sample buffer (3x) was diluted to 1x in the samples and they were heated to 100°C for 10 minutes. The samples were separated using SDS-PAGE and analysed by Western blotting.

2.12 Magnetic particle synthesis

Magnetic nano-particles were made in collaboration with Dr. Mark Holden (Centre for nano-porous materials, The University of Manchester) in a protocol adapted from published work (Auffan et al., 2006; Bee et al., 1995; Fauconnier et al., 1997; Liang et al., 2006; Wilhelma et al., 2003). Under nitrogen, 1 ml of 2 M Fe(II)Cl$_2$ was dissolved in 2 M HCl
and 4 ml of 1M FeCl$_3$ was dissolved in deionised water. The two solutions were mixed, remaining under nitrogen. After mixing, 40 ml of 1 M ammonia was added and the solution and was mixed for 30 minutes under nitrogen. Magnetite formed and the solution was placed on a strong magnet (kindly loaned by the Centre of nano-porous material, The University of Manchester). The magnetite separated from the non-magnetic solution, which was decanted off. To produce maghemite, 25 ml of 0.34 M Fe(NO$_3$)$_3$ (dissolved in 0.1M HEPES pH7.4), was added to the magnetite and mixed for 30 minutes under nitrogen. The maghemite was placed on a strong magnet and once separated, the non-magnetic solution was decanted off. The maghemite was resuspended in 30 ml of 5% (v/v) tetramethylammonium hydroxide, which induced dispersion of the maghemite into nano-sized particles. The solution was mixed for 5 minutes and then the tetramethylammonium hydroxide was separated from the maghemite using the strong magnet. The tetramethylammonium was decanted off and the maghemite was resuspended in 10 ml of 0.1 M HEPES (pH7.4) containing 5 g of dissolved dimercaptosuccinic acid, which coats the particles allowing them to bind the PM of cells (Auffan et al., 2006). This solution was mixed for 30 minutes, placed on a strong magnet and the excess dimercaptosuccinic acid in solution was decanted off. The nano-particles were washed three times in 0.1 M HEPES (pH7.4) using a strong magnet to separate the nano-particles and decant off the HEPES. Finally, the nano-particles were resuspended in 200 ml of 0.1 M HEPES (pH7.4) ready for use with cells.

2.13 Magnetic endosome preparation

Cells were grown in 6 x 15cm dishes to approximately 80% confluency. In the cell culture hood, cells were washed three times in warm (37°C) PBS. Cells were either incubated with pre-cooled (4°C) L15 containing NP (1/20) at 4°C before being transferred to 37°C for 10 minutes or incubated with pre-warmed (37°C) serum-free DMEM containing NP (1/20) and incubated at 37°C for 15 minutes. The nano-particle-containing media was removed and cells were quickly washed in warm (37°C) PBS in the cell culture hood. Warm (37°C) complete media was added to the dish and the cells were incubated at 37°C for a further 10-15 minutes (for EE preparations) or 80-105 minutes (for LE and lysosome preparations). A PNS was produced in the same manner as described in section 2.11 except the pellet was resuspended in 2 ml of HB (supplemented with 250 mM sucrose and additions) and an 8.004 ball bearing was used, typically with fewer passes through the homogeniser. The PNS (~1.4 ml) was transferred to a 1.5 ml eppendorf tube. Four 10 mm x 6 mm neodymium
magnets (Amazon, Slough, UK) were secured to the side of the tube and it was left upright, overnight at 4°C. The next day the non-magnetic supernatant was collected and the magnetic fraction as washed in ice cold HB (supplemented with 250 mM sucrose and additions). The magnetic fraction was resuspended in 50 μl of HB. Laemmli sample buffer (3x) was diluted to 1x in the magnetic fractions and they were heated to 100°C for 10 minutes. The small volume used was to ensure the proteins in the sample are concentrated enough to detect via Western blotting. The non-magnetic fraction was centrifuged in a thick wall tube, a TLS55 Rotor (Beckman Coulter, High Wycombe, UK) and an Optima TLX centrifuge (Beckman Coulter, Wycombe, UK) at 136000g for 40 minutes at 4°C. The supernatant was collected and the membranes were washed in HB (supplemented with 250 mM sucrose plus additions) and centrifuged at 136000g again. The supernatant was removed and the membranes were resuspended in 200μl of HB buffer. Laemmli sample buffer (3x) was diluted to 1x in the samples and they were heated to 100°C for 10 minutes. All the fractions were then separated using SDS-PAGE and analysed by Western blotting.

2.14 SDS-PAGE
Gels were prepared and run using the MiniProtean 3 system (BioRad, Hertfordshire, UK). For co-IP experiments 4-15% Mini-Protean TGX precast gels (BioRad, Hertfordshire, UK) were used. For all other experiments the gels were prepared to a 1mm thickness. The percentage of acrylamide used in the resolving gel was dictated by the protein being blotted (as detailed in table 2.6). Acrylamide was diluted from a 30% (w/v) ProtoGel acrylamide solution (National diagnostics, Atlanta, GA, USA) in SDS-PAGE resolving buffer and gels were set using 0.015% (w/v) APS and 0.36% (v/v) TEMED. In the stacking gel 10% acrylamide was diluted in SDS-PAGE stacking buffer and the gel was set using 0.06% (w/v) APS and 0.14% (v/v) TEMED. Wells were formed using 10 well- or 15 well-combs (BioRad, Hertfordshire, UK). Gels were run at 100V until proteins reached the resolving gel at which point the voltage was increased to 200V. Broad range (10-230kDa) ColourPlus prestained protein ladder (New England BioLabs, Ipswich, MA, USA) were used to show relative protein size.
2.15 Western blotting
Protein transfers were carried out using buffers described in section 2.1 and Mini Trans-Blot electrophoretic transfer system (BioRad, Hertfordshire, UK). Proteins separated using SDS-PAGE were transferred onto nitrocellulose with a pore size of 0.2 μm (Whatman, Austria). Transfers were carried out in pre-cooled transfer buffer at 100V for 90 minutes. For Western blots where DHC was being probed, transfers were carried out at 100V for 250 minutes at 4°C, using nitrocellulose with a 0.1 μm pore size (Whatman, Austria), which helped prevent protein passing through the nitrocellulose. The nitrocellulose was stained using ponceau in order to assess how well the protein had transferred and the stain was removed with repeated PBS washes. The nitrocellulose was then incubated in 4% (w/v) dried milk (Marvel, Premier Foods, St Albans, UK) in PBS for 30 minutes at room temperature. The nitrocellulose was incubated with primary antibody in 4% (w/v) milk in PBS with 0.5% (v/v) tween overnight on a rocker at 4°C. The next day the blots were washed three times in PBS with 0.5% (v/v) tween. The blots were then incubated with secondary antibody in 4% (w/v) milk in PBS with 0.5% tween for 1 hour at room temperature on a rocker. Blots were washed three times in PBS with 0.5% (v/v) tween, followed by a rinse in distilled water to remove any salts from the PBS. Blots were visualised using the Odyssey Sa infrared imaging system (LI-COR, Lincoln, UK). Blots were analysed and processed in Odyssey Sa software (LI-COR, Lincoln, UK) and figures were made in Illustrator CS2 (Adobe, San Jose, CA, USA).

2.16 EGF pulse chase experiments
Cells were grown on cover slips and serum starved for 12 hours by incubating in serum free media. The coverslips were quickly washed in room temperature PBS to remove serum free media and incubated for 5 minutes in 12 well plates, partially submerged in a 37°C water bath, containing L15 media with 0.1 μg/ml biotinylated EGF conjugated to streptavidin-Alexa 488 (Life Technology, Paisley, UK). The cover slips were transferred to 12 well dishes partially submerged in the same water bath, containing L15 media with 0.1 μg/ml unlabelled EGF for 25 minutes or 180 minutes. Cell were fixed and stained as described in sections 2.6 and 2.7.

2.17 Statistics
Prism 6 (GraphPad Software, La Jolla, CA, USA) was used to draw graphs and perform statistical analysis throughout this project. It was assumed the data had a normal distribution and as such unpaired t-tests were used to compare means and assess whether
data from experimental treatments were significantly different from that of controls. When comparing the means of more than one experimental condition with the same control a one way ANOVA with a Dunnett’s post-hoc test was used to assess whether there was significant difference. The p values throughout this project were denoted as follows: p<0.05=*, p<0.01=**, p<0.001=*** and p<0.0001=****.
Chapter 3

Results: The role of dynein regulation in endocytic organelle positioning
3. Results: The role of dynein regulation in endocytic organelle positioning

Dynein is needed for the correct positioning of organelles and the LIC and IC subunits have been shown to play specific roles in recruitment events to some organelles (Cai et al., 2010; Horgan et al., 2010a; Horgan et al., 2010b; Palmer et al., 2009; Tan et al., 2011; Yadav et al., 2012). Depletion of the dynein regulators Lis1, Nde1 and Ndel1 leads to improper organelle positioning due to loss of dynein from intra-cellular membranes (Lam et al., 2010). As dynein function is essential for proper sorting and trafficking through the endocytic pathway (Driskell et al., 2007), it is likely that its recruitment to the pathway’s constituent organelles may be needed in order for dynein to exert its regulatory function. Initially the aim of this project was to explore the interplay between the IC and LIC dynein subunits and accessory proteins (Lis1, Nde1 and Ndel1) in the context of how they influence the positioning of endocytic vesicles and progression through the pathway. To look at the specific roles these proteins might have in this process, each protein was depleted using siRNA and endocytic organelle positioning, distribution and size was observed.

3.1 Characterisation of knock downs

Prior to assessing the effects of protein depletion on the endocytic pathway it was first important to establish that proteins were being efficiently knocked down (Kd). To assess the efficiency of Kd the morphology of the Golgi apparatus was observed after depletion of proteins since this is a sensitive indirect readout for dynein function. In addition, the corresponding protein levels were assessed using Western blotting.

3.1.1 Characterisation of dynein LIC Knock downs

Previous data from our laboratory (Veillemant et al., manuscript in preparation) show that Kd of LIC leads to a scattering of the Golgi apparatus in addition to the formation of ERGIC-cis Golgi tubules at the cell edge. In line with this, in HeLaM cells that were treated with siRNA for both LIC1 and LIC2, the Golgi apparatus was scattered (Figure 3.1A bottom panel) and tubules could be seen in many of the cells (Figure 3.1 A middle panel). Cells treated with a non-targeting control siRNA had normal perinuclear-positioned Golgi apparatus (Figure 3.1 A top panel). MT staining was used to give an indication of cell shape and size. The image was taken with particular focus on MT at the cell periphery.
The number of cells with normal, fragmented or scattered Golgi apparatus was counted to assess the fraction of cells that had abnormal Golgi apparatus distribution. Kd of both LIC1 and LIC2 led to 89.7% of cells counted having scattered Golgi fragments compared to 0.96% of the control cells (Figure 3.1 B). LIC Kd did not lead to any cells with fragmented Golgi, whereby the Golgi apparatus is still largely central but the ribbon-like formation is disrupted into smaller membranes (see fig 3.2A for an example of this phenotype). A minimum of 100 cells were counted per experiment (n=3). An unpaired t-test shows there is a statistically significant difference (p<0.0001) between the scattering of Golgi in LIC Kd cells compared with control cells (Figure 3.1B).

The level of LIC1 protein in LIC Kd cells lysates compared with control cell lysates was assessed using Western blotting. The anti-tubulin antibody TAT1 was used as a loading control. The amount of LIC1 in LIC Kd cells has been substantially reduced indicating an efficient Kd (Figure3.1C). There was no working stock of anti-LIC2 or pan-LIC antibody available for Western blotting at the these experiments were carried out in order to assess LIC2 levels, but unpublished data from our lab would suggest the Golgi apparatus position and morphology observed after treatment with both LIC1 and LIC2 siRNA is indicative of efficient Kd of both LIC1 and LIC2.

3.1.2 Characterisation of dynein IC knock down

Dynein IC is able to interact with Golgin 160 to mediate dynein-Golgi association and so loss of IC would be expected to prevent dynein-mediated Golgi positioning (Yadav et al., 2012). Additionally, IC2 has been shown to be important for Golgi apparatus morphology and positioning (Palmer et al., 2009). As the cell line used here (HeLaM) is slightly different to the cell type (HeLa) in which it has been established that IC1 is not expressed (Palmer et al., 2009), we could not be completely sure that IC1 was not present. Cells were therefore treated with siRNA for both IC1 and IC2. A mixed phenotype was observed with some cells having a fragmented Golgi apparatus (Figure 3.2A middle panel) and some cells having scattered Golgi membranes (Figure 3.2A bottom panel). The Golgi apparatus in control cells had a normal perinuclear Golgi distribution (Figure 3.2 A top panel).

The number of cells with normal, fragmented or scattered Golgi apparatus was counted to assess the fraction of cells that had abnormal Golgi distribution in the same manner as described in for section 3.1.1. None of the control treated cells counted had scattered Golgi membranes and 7.15% had fragmented Golgi apparatus, whereas 38.9% of IC Kd cells had
scattered Golgi membranes and 54.8% of cells had fragmented Golgi apparatus. Unpaired t-tests show there is a statistically significant difference (p<0.0001) between both the scattering and fragmentation of Golgi apparatus in IC Kd cells compared with control (Figure 3.2B).

The level of IC2 protein in the lysates of IC Kd and control cells was assessed by Western blotting to confirm an efficient depletion (Figure 3.2C). IC74 antibody, which recognises IC2, was used. Due to lack of working anti-IC1 or pan-IC antibodies it was only possible to assess IC2 protein levels. However, it is unlikely that HeLaM cells express IC1 as they are very similar to HeLa and the expression of IC1 across cell types is very limited (Palmer et al., 2009). CRM1 is a protein found in the nucleus and cytosol (Paraskeva et al., 1999), and its expression would not be expected to be affected by dynein IC depletion so CRM1 was used as a loading control in these blots.
Figure 3.1: Characterisation of LIC d. Loss of both LIC1 and LIC2 leads scattering of the Golgi apparatus and the formation of peripheral Golgi tubules. A: HeLaM cells were treated with control or both LIC1 and LIC2 siRNA for 65 hours and methanol fixed. The Golgi apparatus and microtubules (MT) were visualised using anti-GM130 and YL1/2 (anti-tubulin), respectively. The blue staining in the merge is DAPI. Scale bars represent 20μm. B: Mean percentage of cells with scattered or fragmented Golgi apparatus (n=3 independent experiments, minimum of 100 cells counted per siRNA treatment). Error bars represent standard deviation; unpaired t-test **** = p<0.0001. C: Western blots of whole cell lysates, probed with anti-LIC1 and anti-tubulin (TAT-1), used as a loading control.
Figure 3.2: Characterisation of IC d. Loss of IC leads scattering and fragmentation of the Golgi apparatus. A: HeLaM cells were treated with control or IC siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.1. Scale bars represent 20μm. B: Percentage of cells with scattered or fragmented Golgi apparatus (n=3 independent experiments, minimum of 100 cells counted per siRNA treatment). Error bars on graph represent standard deviation, **** = p<0.0001, unpaired t-test. C: Whole cell lysates probed using anti-IC (IC74) and α-ti-CRM1, used as a loading control.
3.1.3 Characterisation of Lis1 and Nde1/Ndel1 knock down

Published data show that depletion of Lis1 or both Nde1 and Ndel1 leads to a scattered Golgi phenotype in HeLaM cells (Lam et al., 2010). To assess the efficiency of these protein depletions in this work, Golgi morphology and positioning was observed in HeLaM cells that had been treated with Lis1 or both Nde1 and Ndel1 siRNA.

Fragmentation and scattering of Golgi apparatus in Lis1 Kd and Nde1/Ndel1 Kd cells differ from IC Kd and LIC Kd cells. When Golgi membranes are fragmented in Lis1 Kd and Nde1/Ndel1 Kd cells the Golgi fragments tend to be smaller but less diffuse than IC Kd and LIC KD cells. Conversely, in cells that have fully scattered Golgi apparatus, the scattering is more diffuse in Lis1 Kd and Nde1/Ndel1 Kd cells compared with LIC Kd and IC Kd cells.

Most of the Lis1 siRNA-treated cells had scattered (Figure 3.3 A bottom panel) or fragmented Golgi apparatus (Figure 3.3 A middle panel) whereas control cells mostly had normal perinuclear Golgi apparatus (Figure 3.3 A top panel). Fragmented Golgi apparatus were rarely observed in Nde1/Ndel1 Kd cells and most cells had scattered Golgi membrane (Figure 3.4A). MTs in some Lis1 Kd cells had odd arrangements as if there were large structures in the cytoplasm that prevent MT penetration (Figure 3.3A, bottom row). When images of MTs were taken, the focus was particularly at the cell periphery, leading to poor resolution of centrally located MTs.

Scoring of Golgi apparatus morphology revealed that under control conditions almost all cells had normal Golgi apparatus, whereas 61.6% of Lis1 Kd cells had scattered Golgi and 25.2% of cells had fragmented Golgi apparatus (Figure 3.3B). This is similar to the phenotype observed in published data where 70% of cells have scattered Golgi after Lis1 Kd and 17% have fragmented Golgi apparatus (termed partial scatter in the published data) (Lam et al., 2010). In Nde1/Ndel1 Kd experiments 3% of control cells had fragmented Golgi membranes and almost none had scattered Golgi apparatus, whereas 89.9% of Nde1/Ndel1 Kd cells had scattered Golgi and 2.9% of cells had fragmented Golgi apparatus (Figure 3.4B). This is a slight increase in Golgi scattering compared to the phenotype observed in published data where ~70% of cells have scattered Golgi but this may be accounted for by the fact in this paper ~25-30% of Golgi were fragmented (termed partial scatter) after Nde1/Ndel1 Kd (Lam et al., 2010), which is much higher than the 2.9% observed in this work.
Western blotting of cell lysates demonstrated an efficient depletion of Lis1 and Nde1/Ndel1 (Figure 3.3C and 3.4C). For Nde1/Ndel1 Kd analysis, an anti-Nde1 antibody that recognises both Nde1 (lower band) and Ndel1 (upper band) was used (see appendix 1 for antibody characterisation).

The Golgi apparatus morphology and Western blot analysis in these Kd experiments show that, IC, LIC, Lis1 and Nde1/Ndel1 are being efficiently depleted. Observing Golgi apparatus morphology and using Western blotting provided two convenient and useful assays for assessing these Kds. All subsequent experiments in this project therefore used either one or both of these assays to assess the efficiency of IC, LIC, Lis1 or Nde1/Ndel1 Kds.
Figure 3.3: Characterisation of Lis1 d. Loss of Lis1 leads scattering and fragmentation of the Golgi apparatus. A: HeLaM cells were treated with control or Lis1 siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.1. Scale bars represent 20μm. B: Mean percentage of cells with scattered or fragmented Golgi apparatus (n=3 independent experiments, minimum of 100 cells counted per siRNA treatment). Error bars on graph represent standard deviation, **** p<0.0001, ** p<0.01, * p<0.05, unpaired t-test. C: Western blots of whole cell lysates using anti-Lis1 and anti-CRM1 used as a loading control.
Figure 3.4: Characterisation of Nde1/Ndel1 Kd. Loss of Nde1/Ndel1 leads scattering and fragmentation of the Golgi apparatus. A: HeLaM cells were treated with control or Nde1 and Ndel1 siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.1. Scale bars represent 20μm. B: Percentage of cells with scattered or fragmented Golgi apparatus (n=3 independent experiments, minimum of 100 cells counted per siRNA treatment). Error bars on graph represent standard deviation, **** = p <0.0001, unpaired t-test. C: Whole cell lysates probed using anti-Nde1 and anti-tubulin (TAT-1), used as a loading control.
3.2 Effect of LIC, IC, Lis1 and Nde1/Ndel1 depletion on early endosome distribution

To investigate the effect loss of these proteins had on the early stages of the endocytic pathway, EEs were visualised using the EE marker EEA1. MTs were also visualised in order to reveal cell shape, although the staining was poor due to the use of formaldehyde fixation, which gave poor preservation of MTs but was necessary for anti-EEA1 to recognise antigen.

3.2.1 Early endosomes distribution in LIC knock down cells

To investigate whether the loss of LIC has an effect on EE distribution both LIC1 and LIC2 were depleted using siRNA. The decision was made to deplete both as observations from our laboratory have shown a level of redundancy between these two homologues in membrane sorting and positioning (Veillemant et al., manuscript in preparation), so depleting both LIC1 and LIC2 together was a logical starting point for exploring the role of LIC in EE positioning.

LIC Kd led to the formation of peripheral arrays of EEA1-positive EE at the cell edge in 64.0% of cells, compared to control cells where this distribution of EE was never observed (Figure 3.5A and D, p<0.05). These arrays appeared more frequently at the edge of cells that were not adjacent to another, i.e. facing an empty area of the cover slip. This indicates the arrays may have been forming in lamellipodia-like structures. HeLaM cells are not known for their migratory properties, but these lamellipodia are likely to have been forming as the cells spread into their surroundings.

LIC depletion also led to EE forming ring-like structures at the cell periphery in 75.2% of cells compared with 11% of control cells (Figure 3.5B2, highlighted with red arrows, Figure 3.5C, p<0.01). Smaller ring structures could be observed in the centre of control cells but they lacked the clear hollow space visible in LIC Kd cells (Figure 3.5B1, highlighted with red arrow).

3.2.2 Early Endosomes distribution in IC knock down cells

In contrast to LIC Kd cells, depletion of IC caused only a slight dispersion of EEs (Figure 3.6A). Strikingly, however, they tend to cluster and aggregate (Figure 3.6 A and B). In 78.7% of IC Kd cells the EE have aggregated compare to 10.5% o (Figure 3.6C, p<0.001). Some of these aggregates had a somewhat ring-like formation, but they were morphologically
distinct from the EE rings seen in LIC Kd cells. EE aggregates were more clustered, have a spherical appearance and are not as large as the ring-like arrangements.

### 3.2.3 Early Endosomes distribution in Lis1 and Nde1/Ndel1 knock down cells

Our laboratory has previously shown that Nde1 and Ndel1 have functional redundancy organelle positioning (Lam et al., 2010). The two proteins were therefore depleted together when studying the morphology and distribution of endocytic vesicles. While loss of either Lis1 or Nde1/Ndel1 led to a profound scattering and fragmentation of the Golgi apparatus (Figure 3.2 and 3.3), their loss only led to a general dispersion of EE throughout the cytoplasm (Figure 3.7A and 3.8A), which is consistent with previously published data (Lam et al., 2010). The general dispersion of EE is seen in 87.6% of Lis1 Kd cells and 89.2% of Nde1/Ndel1 Kd cells compared with 15.1% of control cells (Figure 3.7B, p<0.001 and 3.8D, p<0.01, respectively).

The loss of both Nde1 and Ndel1 also led to the formation of EE that are in a ring structures (Figure 3.8B2, highlighted with arrows), some of which were extremely large (Figure 3.8B3, highlighted with arrows). These rings formed in 72.2% of Nde1/Ndel1 Kd cells compare with 11.0% of control cells (Figure 3.8C, p<0.01). These rings were similar to those seen in an LIC Kd but were often much larger as highlighted in figure 3.8B3.
Figure 3.5: The effect of LIC depletion on early endosome positioning. A: HeLaM cells were treated with control or both LIC1 and LIC2 siRNA for 65 hours. Cells were fixed in formaldehyde. Endosomes were visualised with anti-EEA1 and microtubules were visualised with anti-tubulin antibody, YOL1. Scale bars represent 20μm. The blue staining in the merge is DAPI. B: Enlargement of 20μm x 20μm sections from panel A. Red arrows in these enlargements highlight ring arrangements. C: Mean percentage of cells with early endosomes in ring formations (n=3, independent experiments, minimum of 70 cells counted per siRNA treatment). D: Mean percentage of cells with peripheral early endosomes (n=3 independent experiments, minimum of 70 cells counted per siRNA treatment). Error bars on graphs represent standard, * = p<0.05, ** = p<0.01, unpaired t-test.
Figure 3.6: The effect of IC depletion on early endosome positioning. A: HeLaM cells were treated with control or IC siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.5. Scale bars = 20μm. B: Enlargement of 20μm x 20μm sections from panel A. C: Mean percentage of cells with aggregated early endosomes (n=3 independent experiments, minimum of 70 cells counted per siRNA treatment). Error bars on graphs represent standard deviation, *** = p<0.001, unpaired t-test.
Figure 3.7: The effect of Lis1 depletion on early endosome positioning. A: HeLaM cells were treated with control or Lis1 siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.5. Scale bars = 20μm. B: Mean percentage of cells with dispersed early endosomes (n=3 independent experiments, minimum of 70 cells counted per siRNA treatment). Error bars on graphs represent standard deviation, *** = p<0.001, unpaired t-test.
Figure 3.8: The effect of Nde1 and Ndel1 depletion on early endosome positioning. A: HeLaM cells were treated with control or both Nde1 and Ndel1 siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.5. Scale bars represent 20μm. B: Enlargement of 20μm x 20μm sections from panel A. Red arrows in panel 2 indicate medium-sized early endosome rings. The red arrow in panel 3 indicates a large early endosome rings. C: Mean percentage of cells with endosomes in ring formations. D: Mean percentage of cells with dispersed endosomes. In both C and D, n = 3 independent experiments, with a minimum of 70 cells counted per siRNA treatment. Error bars represent standard deviation, ** = p<0.01, unpaired t-test.
3.2.4 Summary of the effects of LIC, IC, Lis1 and Nde1/Ndel1 depletion on early endosome positioning

The similarity in the peripheral EE rings formed in Nde1/Ndel1 and LIC Kd cells indicates these two proteins may be involved in the same functional pathway in terms of EE function and positioning. Loss of either led to EE ring formation, with Nde1/Ndel1 loss generating a more severe phenotype. Loss of Lis1 and Nde1/Ndel1 led to many of the EEs becoming generally dispersed throughout the cytoplasm. This suggests that the Lis1-Nde1/Ndel1 complex is needed for correct EE localisation but the loss of this complex does not lead to a complete redistribution of EE to the cell periphery. Loss of IC caused EEs to become slightly dispersed and cluster in aggregates, which were not seen in LIC Kd, Lis1 Kd or Nde1/Ndel1 Kd cells. Interestingly a similar EE distribution is seen in DHC cells, with EEs clustering in aggregated structures (Caviston et al., 2010). The peripheral arrays of EEs that form in LIC Kd cells are not seen in any of the other protein depletions. Collectively these data suggest that although there is cross-talk between these proteins in EE transport regulation, individually they may also play specific roles (Table 3.1).

<table>
<thead>
<tr>
<th>Kd</th>
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<th>EE in Peripheral Ring Formation</th>
<th>EE dispersed in Cytoplasm</th>
<th>EE in Aggregates</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIC</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Nde1/Ndel1</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lis1</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>IC</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 3.1: Summary of early endosome distribution phenotypes observed in knock down experiments
3.3 Effect of LIC, IC, Lis1 and Nde1/Ndel1 depletion on late endosome size and distribution

To assess the effect that loss of LIC, IC, Lis1, and Nde1/Ndel1 has on LE, the proteins were depleted using siRNA and CD63 was visualised in cells as a marker for LE, MVB and lysosomes. In IC Kd and Nde1/Ndel1 Kd cells there was no obvious difference in distribution or size of CD63-positive organelles. Distinct phenotypes were observed following LIC and Lis1 depletion, however.

3.3.1 Distribution of CD63 after loss of LIC

Endocytic compartments in control and LIC Kd cells were stained using anti-EEA1 and anti-CD63 antibodies (Figure 3.9A). MTs were stained using YOL1/34 (not shown) to show cell size and shape, which allowed the cell outlines to be traced. In control cells EEA1- and CD63-positive compartments were located in the same area, towards the centre of the cell. Loss of LIC did not cause an obvious redistribution of CD63-positive vesicles but it was striking that a subset of cells in sparsely populated areas, had spatially separated EEA1-positive EE and CD63-positive compartments (Figure 3.9). In these cells the CD63 were located on the same side of the nucleus as the EE peripheral arrays, suggesting some kind of cell polarisation was taking place.

3.3.2 Distribution of CD63 after loss of Lis1

Endocytic compartments in control and Lis1 Kd cells were visualised using anti-EEA1 and anti-CD63 antibodies (Figure 3.10). Lis1 depletion did not lead to an obvious redistribution of CD63-positive compartments but these MVB, LE and lysosomes were noticeably larger than control cells.
Figure 3.9: The effects of LIC depletion on CD63 and EEA1 distribution. A: Cells were treated with control or both LIC1 and LIC2 siRNA for 65 hours. Cells were fixed in formaldehyde and stained using anti-EEA1 and anti-CD63 antibodies. The blue staining in the merge is DAPI. Scale bars represent 20μm. B: Enlargement of 40μm x 40μm sections from panel A.
Figure 3.10: The effect of Lis1 depletion on CD63-positive late endosome and lysosome size. Cells were treated with control or Lis1 siRNA for 65 hours. Cells were fixed in formaldehyde and stained using anti-EEA1 and anti-CD63 antibodies. The blue staining in the merge is DAPI. Scale bars represent 20μm.
3.4 Effect of LIC, IC, Lis1 and Nde1/Ndel1 depletion on lysosome size and distribution

To investigate what effect loss of these proteins has on the final stages of the endocytic pathway, lysosomes were visualised using LAMP1 as a marker to assess their location and size within cells. Whereas CD63 is found in LE and some lysosomes, LAMP1 is found predominantly associated with lysosomes (Eskelinen et al., 2003) so is an ideal marker for lysosomes.

3.4.1 Lysosome distribution and size in LIC knock down cells

Labelling with antibodies against LAMP1 and tubulin revealed that 32.4% LIC Kd cells have lysosomes arranged in peripheral arrays, which is an arrangement almost never seen in control cells (Figure 3.11A and 3.11C, p<0.0001). Similar to the peripheral arrays of EE that formed in LIC Kd cells, these peripheral bands of lysosomes seemed to be located in lamellipodia-like structures. In 36.1% LIC Kd cells the lysosomes clustered at the cell periphery (Figure 3.11A, red arrow highlights a cluster, Figure 3.11 D, p<0.001). The peripheral arrays observed were often located a small distance from the cell edge, whereas clusters tend to be right at the edge of the cell (Figure 3.11A).

Many LIC Kd cells had enlarged lysosomes (Figure 3.11B2), that tended to be located centrally in the cell. Although there is variability in lysosome size in control cells with some large lysosomes (Figure 3.11B1), the enlarged lysosomes in LIC Kd cells had a somewhat hollow appearance (Figure 3.11 B2, red arrows) that was distinct from those seen in control cells. In 35.2% of LIC Kd cells there were enlarged hollow-looking lysosomes compared with only 4.2% of control cells (Figure 3.11D, p<0.01).

The difference between LAMP1 and CD63 staining in LIC Kd cells is interesting because these markers have some cross-over in their organelle staining. LAMP1 acts predominantly as a lysosomal marker whereas CD63 is found in lysosomes, LE and MVB. CD63-positive organelles do not scatter to the cell periphery in the same way some LAMP1-lysosomes do (Figure 3.9A and Figure 3.11A). Although LAMP1-positive lysosomes can accumulate at the cell periphery, they are still present in many central regions of the cell (Figure 3.11A) and these may well colocalise with CD63-positive lysosomes that are not peripheral.
Unfortunately there were no LAMP1 and CD63 antibodies available that could be used simultaneously in fluorescence microscopy to allow co-staining of these compartments.

3.4.2 Lysosome distribution and size in IC knock down cells
After IC Kd, 75.1% of cells that have lysosomes were clustered at the cell edge (Figure 3.12A) compared with 3.9% of control cells (Figure 3.12C, p<0.0001). Although a high proportion of IC Kd cells had peripheral clusters of lysosome this only constituted a subset as many of the lysosomes were still located near the centre of the cell (Figure 3.12A). The lysosomes at the centre of the cell were often enlarged (Figure 3.12B2, highlighted with red arrows) and were similar to the enlarged lysosomes in LIC Kd cells as they appear to be hollow. In 71.0% of IC Kd lysosomes that were enlarged and hollow in appearance were observed compared with 4.8% of control cells (Figure 3.12D, p<0.05).
The effect of LIC depletion on lysosome size and position. A: HeLaM cells were treated with control or both LIC1 and LIC2 siRNA for 65 hours. Cells were fixed in methanol and stained with anti-LAMP1 and anti-tubulin (YL1/2). The blue staining in the merge is DAPI. Red arrow in bottom row highlights a peripheral cluster of lysosomes, the remaining cells in this image have lysosomes arranged in peripheral arrays. Scale bars represent 20μm. B: Enlargement of 20μm x 20μm sections from panel A highlighting enlarged lysosomes. C: Mean percentages of cells with different lysosome distributions. Abbreviations: Mostly central (MC), Peripheral Cluster (PC) and Peripheral Array (PA). D: Mean percentage of cells with enlarged lysosomes. In both C and D, n=3 independent experiments, with a minimum of 100 cells counted per siRNA treatment. Error bars represent standard deviation, **** = p<0.0001, *** = p<0.001, ** = p<0.01, unpaired t-test.
Figure 3.12: The effect of IC depletion on lysosome size and position. A: HeLaM cells were treated with control or IC siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.11. Scale bars represent 20μm. B: Enlargement of 20μm x 20μm sections from panel A highlighting enlarged lysosomes. C: Mean percentages of cells with different lysosome distributions. Abbreviations: Mostly central (MC), Peripheral Cluster (PC) and Peripheral Array (PA). D: Mean percentage of cells with enlarged lysosomes. In both C and D, n=3 independent experiments, with a minimum of 100 cells counted per siRNA treatment. Error bars represent standard deviation, **** = p<0.0001, * = p< 0.05, unpaired t-test.
3.4.3 Lysosome distribution and size in Lis1 knock down cells

Previous data from our lab show that Lis1 Kd leads to accumulation of lysosomes in the cell periphery (Lam et al., 2010). To confirm if the same phenotype was seen in this project and to investigate lysosomes size, the LAMP1-positive lysosome and MT were stained in control and Lis1 Kd cells (Figure 3.13A). Peripheral lysosome clusters were present in 30.5% of Lis1 Kd cells (Figure 3.13A middle row and 3.13B2, p<0.01), which was significantly more than the 4.5% of control cells (Figure 3.13C, p<0.01). Similar to LIC Kd and IC Kd cells that had peripheral clusters of lysosomes, this only represented a subset of lysosomes with many remaining central (Figure 3.13A). The most striking difference between lysosomes in control and Lis1 Kd cells was the large increase in size of most lysosomes following the depletion of Lis1 (Figure 3.13B3). All of the Lis1 Kd cells observed had either a proportion (Figure 3.13A, middle row) or all lysosomes enlarged (Figure 3.13A, bottom row). These lysosomes had a clear hollow appearance (Figure 3.13B3), reminiscent of that seen in LIC Kd and IC Kd, but far more exaggerated. None of the control cells observed had lysosomes that were enlarged in this way.

3.4.4 Lysosome distribution and size in Nde1/Ndel1 knock down cells

In Nde1/Ndel1 the lysosomes appeared to be generally more dispersed but they did not cluster at the cell periphery (Figure 3.14A), which is consistent with observations in previously published work (Lam et al., 2010). It was difficult to quantify this general dispersion phenotype as lysosomes distribution in the cytoplasm of control cells was highly variable. However, 55.7% of Nde1/Ndel1 Kd cells contained enlarged lysosomes, similar to those seen in LIC Kd and IC Kd cells (Figure 3.14 B2, highlighted with red arrows) compared to 4.8% of control cells that had enlarged lysosomes (Figure 3.14C, p<0.05).
Figure 3.13: The effect of Lis1 depletion on lysosome size and position. A: HeLaM cells were treated with control or Lis1 siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.11. Scale bars represent 20μm. B: Enlargement of 20μm x 20μm sections from panel A highlighting peripheral lysosomes (B2) and enlarged lysosomes (B3). C: Mean percentages of cells with different lysosome distributions (n=3 independent experiments, with a minimum of 100 cells counted per siRNA treatment). Error bars represent standard deviation, **= p<0.01, unpaired t-test. Abbreviations: Mostly central (MC), Peripheral Cluster (PC) and Peripheral Array (PA).
Figure 3.14: The effect of Nde1 and Nde1 depletion on lysosome size and position.
A: HeLaM cells were treated with control or Nde1/Ndel1 siRNA for 65 hours. Cells were fixed and stained as described in Figure 3.11. Scale bars represent 20μm. B: Enlargement of 20μm x 20μm sections from panel A highlighting enlarged lysosomes. C: Mean percentages of cells with enlarged lysosomes (n=3 independent experiments, with a minimum of 100 cells counted per siRNA treatment). Error bars represent standard deviation, * = p<0.05, unpaired t-test.
3.4.5 Summary of lysosome phenotypes following depletion of LIC, IC, Lis1 and Nde1/Ndel1

The different lysosome phenotypes that were observed are summarised in table 3.2. There were similarities between lysosome size and distribution when LIC, IC, Lis1 and Nde1/Ndel1 were depleted, which indicated there may be some functional overlap between their roles in regulating the dynein complex in lysosomal traffic and transport. Lis1 and Nde1/Ndel1 both led to a general dispersion of lysosomes but Lis1 Kd had a much more dramatic effect on lysosome size and could cause peripheral clustering that was not seen in Nde1/Ndel1 Kd cells. All these protein depletions led to an enlargement of lysosomes to some degree, which indicates proper regulation of dynein is required to maintain lysosome size. In LIC, IC and Lis1 Kd cells lysosomes were often found clustered at the cell periphery, but these cells always contained some central lysosomes, indicating only a subset of lysosomes were affected by these protein depletions. Similar to the effects of LIC depletion on EEs, LIC Kd caused peripheral arrays of lysosomes to form in lamellipodia-like structures and this was only seen in LIC Kd cells. Together these results indicate there may be several mechanisms for maintaining dynein-driven lysosomal transport, in which IC, LIC, Lis1 and Nde1/Ndel1 play specific roles that may have some functional overlap.

<table>
<thead>
<tr>
<th>Kd</th>
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<th>Greatly Enlarged Lysosomes</th>
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</tr>
<tr>
<td>IC</td>
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<td>No</td>
</tr>
<tr>
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<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Nde1/Ndel1</td>
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</tr>
</tbody>
</table>

Table 3.2: Summary of lysosome distribution and size phenotypes observed after Knock down experiments.

3.5 Conclusions

The results from this chapter show how different dynein subunits and accessory proteins have diverse roles in endocytic vesicle positioning. The EE and lysosome peripheral arrays that formed in LIC Kd cells seem to be linked to cell shape and occurred in cells in sparsely populated areas that had lamellipodia-like structures. The spatial separation of CD63-positive vesicles and EE (Figure 3.9) would indicate the distribution of CD63-positive LE and some lysosomes is not affected by the formation of these lamellipodia. Loss of LIC and Nde1/Ndel1 led to EE forming ring-like structures at the cell periphery, an effect not
observed in Lis1 Kd or IC Kd cells, which suggests that LIC and Nde1/Ndel1 might both be involved in a process that is related to these ring formations. Other than the peripheral arrays that formed in LIC KD cells and the ring-like structures in LIC Kd and Nde1/Ndel1 Kd cells, EEs were not seen at the cell periphery in the Kds carried out here, where dynein function had been perturbed. This might suggest that EEs are not being transported to the plus ends of MTs. IC Kd cells had a specific EE aggregation phenotype, which is not seen in other Kds carried out here but is similar to published work that shows loss of DHC leads to the same phenotype (Caviston et al., 2010). Lis1 Kd and Nde1/Ndel1 Kd cells had dispersed EEs throughout the cytoplasm, which is consistent with published data and shows they may be working together in the same functional capacity to position EE.

The peripheral clustering of LAMP1-positive lysosomes seen in LIC Kd, IC Kd and to some extent Lis1 Kd cells did not occur in Nde1/Ndel1 Kd cells, which would suggest there are some dynein driven transport mechanisms that require IC, LIC and Lis1 but not Nde1/Ndel1. Alternatively, it is possible Nde1/Ndel1 might regulate dynein and kinesins, so when Nde1 and Ndel1 are depleted transport to both plus-ends and minus-ends of MTs is reduced. As only a sub-population of lysosomes cluster at the cell edge in IC, LIC and Lis1 depleted cells, the roles of these proteins may not necessarily be interlinked as they could be acting on different populations of lysosomes. Lysosomes became enlarged when any of these proteins were depleted suggesting that dynein activity is needed for lysosome function as enlarged lysosomes are indicative of a slowing or block in proper lysosomal degradation (Fukuda et al., 2006). The most striking phenotype seen following depletion was the LAMP1- and CD63- LEs and lysosomes that became greatly enlarged in Lis1 Kd cells (Figure 3.10 and 3.13). The extreme enlargement of these vesicles would suggest that Lis1, specifically, has a very important role in maintaining organelle function in the late in the endocytic pathway.

Although there are similarities in certain phenotypes seen when these dynein subunits and regulators are depleted, they seem to have differing roles in maintaining organelle morphology, size and distribution as summarised in figure 3.15. It is worth considering the effect of protein depletions on cell size might affect the interpretation of organelle positioning. Both LIC depletion and IC depletion led to cells that were much larger than control cells. Many of the Nde1/Ndel1 Kd cells fall into two shape categories, extremely large or long and thin. The long thin cells were not included when counting organelle positioning phenotypes as organelle distribution in these cells was not comparable to
control and it was difficult to judge whether organelles were truly peripheral if the cell was extremely narrow. Lis1 Kd cells were not strikingly different in size when compared to control cells, but did tend to be qualitatively slightly smaller.

The fact that each of these Kds result in a pronounced disruption to the position and size of endocytic organelles shows how important the correct regulation of dynein is for maintenance of the endocytic pathway. The variance not only between phenotypes in different Kds but also the varying importance of each protein for the size and position of different organelles gives an insight into how complex and finely tuned the regulation of dynein is within the endocytic pathway is.
Figure 3.15: Different endocytic phenotypes observed after protein depletions. 1, Early endosome rings; 2, Early endosome peripheral arrays; 3, Early endosome aggregation; 4, Early endosome general dispersion; 5, Enlarged lysosomes; 6, Peripheral arrays of lysosomes; 7, Peripheral clusters of lysosomes; 8, Greatly enlarged lysosomes; 9, Enlarged CD63-positive late endosomes and lysosomes.
Chapter 4

Results: Dynein recruitment to intracellular membranes
4. Results: Dynein recruitment to intracellular membranes

The results from the previous chapter show that depletion of dynein LIC or IC had differing effects on the position of endocytic membranes. It seemed possible that the peripheral redistribution seen in these protein depletions may be due to an inability to recruit dynein to the surface membrane of these organelles. To investigate whether dynein IC or LIC have roles in dynein-membrane association, these proteins were depleted and the recruitment of dynein to membranes in their absence was analyzed. Previous data published from our lab show that loss of Lis1 or both Nde1 and Ndel1 leads to loss of dynein from pelleted membranes (Lam et al., 2010), thus these protein depletions were not carried out as part of this biochemical analysis of dynein membrane recruitment.

4.1 The effect of IC and LIC depletion on dynein and dynactin recruitment to intracellular membranes

HeLaM cells were treated with control, LIC or IC siRNA then homogenised. The membrane and cytosol fractions were separated using high speed centrifugation and the protein contents of each fraction was analysed by Western blotting (Figure 4.1).

CRM1 is a protein found in cytosol (Paraskeva et al., 1999) and therefore acts as a cytosolic marker and loading control for the cytosolic fraction. The membrane fractions from these preparations are not contaminated with cytosol, as demonstrated by the lack CRM1 (Figure 4.1). The membrane fractions obtained would be expected to contain intercellular membranes derived from ER, Golgi, mitochondria and transport vesicles. Due to the limited volume of membrane fraction available it was not feasible to blot for proteins from all of these different organelles. TfR was chosen as a membrane marker probe due to the sensitivity of the antibody. Its absence from the cytosolic fractions indicates an efficient separation of membranes and cytosol (Figure 4.1). The IC and LIC1 levels are indicative of an efficient depletion of the respective proteins.

4.1.1 Dynein and dynactin recruitment to membranes in cells lacking IC

Depletion of IC leads to a substantial loss of dynein from the membrane fraction as seen by the reduction in of DHC and LIC1 (Figure 4.1). Note there is less TfR in the control membrane fraction compared with IC Kd and therefore this increases the relative loss of dynein seen in IC Kd membrane fraction (Figure 4.1).
A semi-quantitative method was applied to give an indication of how much dynein was present in the membrane and cytosolic fractions. The fractionation of membranes was repeated in triplicate and the band intensity of LIC1 and p150 was measured from Western blots. These were normalised using the loading control intensities and the normalised LIC1 and p150 levels were then expressed as a fraction of the control (Figure 4.2). For this method to be fully quantitative LIC1, p150, TfR and CRM1 would need to be purified and varying known concentrations of each protein would have to be blotted alongside each membrane and cytosol sample in order to create a concentration/intensity curve that would allow the protein concentration to be calculated. This was not possible at the time of experimentation as there was no available TfR and CRM1 DNA constructs in our lab that would enable purification of these proteins. As such, it was decided that this semi-quantitative approach would give an adequate indication of dynein and dynactin on the membrane. The isolation of membranes and Western blotting of DHC are technically challenging techniques and unfortunately the DHC blot in one of the experimental repeats was not clean enough for quantification. The LIC and IC bands were therefore chosen as a readout for dynein in the membrane fraction.

There was approximately a 10-fold reduction in normalised LIC1 band intensity from membrane fractions of IC Kd cells compare with control; p<0.0001 (Figure 4.2). Although it is not statistically significant there is an increase in the normalised LIC1 band intensity in the IC Kd cytosolic fractions compared with control. This indicates that dynein may be dissociating from membranes in the absence of IC and relocating the cytosol. Surprisingly, there was approximately a 3-fold reduction in normalised dynactin p150 band intensity in the IC Kd membrane fractions compared with control; p<0.01. There was an increase in the normalised dynactin band intensity of the IC Kd cytosolic fractions compared with control, although this is not statistically significant (Figure 4.2). It is therefore possible a loss IC can cause dynactin to dissociate from some cargo.

4.1.2 Dynein and dynactin recruitment to membranes in cells lacking LIC

When LICs were depleted there was a reduction in dynein, demonstrated by the reduction of IC and DHC, in both the cytosolic and membrane fraction. The reduction in the cytosolic dynein is more noticeable visually (Figure 4.1) but note that the TfR band in the control membrane fraction is smaller than that of the LIC Kd fraction. Quantification of IC band intensity, normalised to loading controls, showed there was approximately a 4-fold decrease in the cytosolic fraction and a 2-fold decrease in the membrane fraction, which
were statistically significant (Figure 4.3, p<0.001 and p<0.01 respectively). These data would suggest the dynein complex lacking LIC is less stable and could be being degraded. There is less decrease in the normalised dynein band intensity of the membrane fraction compared with the cytosol fraction. It is possible that due to a general reduction of dynein in the cell, a higher proportion of total dynein that is able to associate with cargo independently of LIC is doing so. Alternatively, dynein may be more stable when it associated with the membrane. In contrast to what was seen following IC Kd, there was no significant difference in the normalised dynactin band intensity of either the cytosol or the membrane fractions of LIC Kd cells compared with control (Figure 4.3).
Figure 4.1: Representative blot of fractionated cell homogenates depleted of LIC and IC. HeLaM cells were treated with siRNA for 65 hours, trypsinised and homogenised. Unbroken cells, plasma membranes and nuclei were removed using low speed centrifugation. Intracellular membranes were separated from the cytosol by centrifuging the post nuclear supernatant at 88,000g through a 0.5M sucrose layer onto a 2M sucrose cushion. The fractions were analysed using western blotting using the antibodies indicated.
Figure 4.2: The dynein and dynactin distribution in the cytosol and membrane fractions of IC Kd cells. Control and IC Kd cells were separated into cytosolic and membrane fractions by centrifugation. Dynein LIC1 and dynactin p150 band intensity was measured, normalised to TfR and CRM1 and expressed as a fraction of the control (n=3 independent experiments). There is a 10-fold reduction in dynein LIC1 and a 3-fold reduction in dynactin p150 relative intensity in the membrane fraction of IC Kd cells, **** = p<0.0001, ** = p<0.01, unpaired t-test. Error bars represent standard error of the mean.
Figure 4.3: The dynein and dynactin distribution in the cytosol and membrane fractions of LIC Kd cells. Control and IC Kd cells were separated into cytosolic and membrane fractions by centrifugation. Dynein IC (IC74 antibody) and dynactin p150 band intensity was measured, normalised to TfR and CRM1 and expressed as a fraction of the control (n=3 independent experiments). There is a 4-fold reduction in dynein IC relative intensity in the cytosol and a 2-fold reduction in the membrane fraction of IC Kd cells, *** = p<0.001, ** = p<0.01, unpaired t-test. Error bars represent standard error of the mean.
4.2 Isolation of specific endocytic membranes

The results from bulk membrane preparations (Figure 4.1) show that IC is involved in dynein recruitment to membranous cargo, while the role of LIC is less obvious. As depletion of LIC leads to specific EE, LE and lysosomal phenotypes (Figure 3.5, 3.9, 3.11), it is possible that LIC is important for recruiting dynein specifically to membranes in the endocytic pathway. To investigate this, an assay was developed in which endocytic compartments (EE or lysosomes) could be isolated.

The assay was adapted from previously published work (Loubery et al., 2008) and a full description of this assay can be found in section 2.11 and 2.12. Briefly, maghemite nanoparticles (NP) were synthesised and coated in dimercaptosuccinic acid, a carboxyl acid that can bind non-specifically to the PM. Cells were incubated with NP at 4°C for 60 minutes to allow PM saturation, followed by 10 minutes incubation at 37°C. Cells were washed and incubated with NP-free media at 37°C for a further 10 minutes (EE isolation) or 80 minutes (lysosome isolation). Cells were then homogenized, subjected to low speed centrifugation and a magnet was attached to the tube containing the resulting PNS in order to isolate the endocytic vesicles containing NP, termed the magnetic fraction (MF). The non-magnetic fraction was subjected to high-speed centrifugation in order to separate the non-magnetic membrane (NMM) from the cytosol. These factions were then analysed using Western blotting (Figure 4.4).

This approach led to a clean preparation of endocytic compartments. A 10 minute incubation of NP-free media produced a fraction enriched with TfR, indicating EE/RE. An 80 minute incubation led to a fraction with less TfR and more LAMP1, indicating the NP had reached lysosomes (Figure 4.4). The NMM from 80 minutes NP-free media chase had depleted LAMP1. In contrast TfR, Golgin84 and PDI levels were relatively similar between the two NMM fractions. This would indicate efficient lysosome isolation. The fractions were not contaminated with Golgi or ER membranes as demonstrated by the lack of PDI (an ER luminal protein) and Golgin84 (a protein that associates with Golgi apparatus membranes).

There was very little detectable dynein, dynactin, kinesin, Nde1 or Lis1 in the MF. It was thought that the PM, which is saturated with NP during the hour incubation at 4°C, could be vesiculating during homogenisation and contaminating the MF. As the dynein does not associate with the PM in interphase cells it was thought this might account for the high levels of TfR but low levels of motor protein (Figure 4.4). To reduce this, the incubation of
NP at 4°C was removed from the protocol in order to reduce the amount of NP binding the PM. Cells were therefore pulsed with NPs for 15 minutes at 37°C, then chased for 15 and 105 minutes to increase the amount of NP endocytosed from the PM.

To assess whether the NP are still present on the PM after these two isolation approaches, HeLaM cells were treated with NP as described above, but not homogenised. The cells were instead visualised using transmission electron microscopy (Figure 4.5), which was kindly performed by Dr. Ling Zhang. Both 15 and 105 minute chases were performed to look at NP localisation, but the NP were difficult to see in cells that had not been incubated at 4°C and had been chased for 105 minutes. For this reason 105 minute chase cells have not been included in Figure 4.5 because a direct comparison could not be made between the two conditions. However, cells that were incubated at 4°C for one hour and chased for 105 minutes still had NP associated with the PM (Appendix 2). An hour incubation with NP at 4°C led to more NP inside the cells but also far more on the PM. Interestingly, the vesicles that NP were found in under both conditions were electron dense (as demonstrated by their darker colour) which is indicative of rich protein content and usually associated with LEs and lysosomes. This may indicate the NP are either reaching LE after a 15 minute pulse and 15 minute chase or that they are localised to unusually protein-rich EEs.

Using the modified protocol, membranes were isolated and analysed biochemically. The modified approach led to less TfR in the MF relative to the NMM fraction indicating there may be less PM contamination (Figure 4.6). Surprisingly, the amount of TfR in the MF did not decrease after 105 minutes of chase, perhaps suggesting that endosomes containing NPs might not efficiently recycle their contents to the PM. The adjustments in the protocol led to an increase in detectable motors in the MF (Figure 4.6). Despite NMM being diluted in approximately 4-times more buffer than MF, there was far more detectable LIC1. In order to visualise LIC1 properly on the MF the intensity while scanning the blot had to be increased (Figure 4.6, bottom box) which led to overexposure of NMM (cropped from image). Even when LIC1 was poorly detected at low scan intensity (Figure 4.6 LIC upper box) the NMM bands were saturated. Lis1 was present in relatively large amounts when comparing the Lis1 detectable in MF and NMM (Figure 4.6). Kinesin heavy chain 1 (Kif5) was present in the MF, however this antibody is very sensitive and can detect extremely low levels of protein. The band was so intense in the cytosol and NMM it led to bleed-
through when scanning the blot in another wavelength (Figure 4.6 LAMP1 box, non-specific bands).
Figure 4.4: Isolation of magnetic fractions enriched with endocytic membranes. HeLaM cells were incubated with magnetic nano-particles for 60 minutes at 4°C, followed by 37°C for 10 minutes. The media was changed to nano-particle free complete media and cells were incubated for 10 or 80 minutes at 37°C. Cells were homogenised and nuclei and plasma membrane were removed by low speed centrifuging. The post-nuclear supernatant (PNS) was placed next to a magnet at 4°C overnight to isolate membranes containing magnetic nano-particles. The magnetic fraction was collected and the non-magnetic membranes were separated from the cytosol by centrifuging at 136,000g. Non-magnetic membranes that had been pelleted were resuspended in HB. The protein content of each fraction was analysed using western blotting, using the antibodies indicated.
Figure 4.5: Electron microscopy images comparing different treatments with magnetic Nano Particles (NP). A. HeLaM cells were pre-treated with NP at 4°C for 1 hour prior to 37°C incubation (left) or incubated at 37°C immediately after addition of NP (right). After 15 minutes 37°C, NP were removed and cells were incubated with complete media for a further 15 minutes at 37°C. Nuclei are indicated with an N. Extracellular Space is indicated with ES. Cells were fixed in glutaraldehyde and imaged using transmission electron microscopy. Cell fixation and imaging was kindly carried out by Dr. Ling Zhang. Scale bars represent 0.5μm. B: Enlargements of sections highlighted in panel A.
Figure 4.6: Isolation of magnetic fractions enriched with endocytic membranes and containing detectable motor proteins. HeLaM cells were incubated with magnetic nano-particles at 37°C for 15 minutes followed by nano-particle complete media for 15 or 105 minutes at 37°C. Cells were homogenised and nuclei and plasma membrane were removed by low speed centrifuging. The post-nuclear supernatant (PNS) was placed next to a magnet at 4°C overnight to isolate membranes containing magnetic nano-particles. The magnetic fraction was collected and the non-magnetic membranes were separated from the cytosol by centrifuging at 136,000g. Non-magnetic membranes that had been pelleted were resuspended in HB. The protein content of each fraction was analysed using western blotting, using the antibodies indicated.
The anti-LIC1 is the most sensitive antibody available in our laboratory for detecting dynein by Western blotting. Although the MF contained detectable LIC1, there was not enough dynein present for anti-IC or anti-DHC antibodies to detect dynein (data not shown) in the MF, thus this preparation method is not suitable for LIC depletion experiments as there would unfortunately be no way to assess the effect LIC Kd has on dynein recruitment to these endocytic membranes.

4.3 Dynein recruitment to RILP-positive membranes
As the magnetic isolation method was not suitable to determine how LIC depletion affects recruitment of dynein to endocytic organelles, another method for assessing its role was required. Due to the abundance of dynein in cells, both attached to membranous cargo and unbound in the cytosol, there are very few examples in which dynein can be visualised on cargo using fluorescence microscopy. An example is the large membrane structures that form when RILP is overexpressed. As LIC has been implicated in the recruitment of dynein to these structures (Tan et al., 2011), this seemed like a logical assay to use in order to further investigate potential roles LIC may play in dynein recruitment to endocytic membranes. Published data indicate there are two recruitment mechanisms for dynein association with RILP-positive membranes, LIC-dependent and dynactin-dependent (Johansson et al., 2007; Jordens et al., 2001; Tan et al., 2011). As such, it was of particular interest to see whether dynactin is recruited to RILP membranes in the absence of LIC. If there were two independent recruitment pathways it would be expected that dynactin would remain on the RILP-positive membrane structures after loss of LIC.

4.3.1 Dynein and dynactin recruitment to RILP-positive membranes after LIC depletion
As published work shows LIC1 may play a more prominent role in dynein recruitment to RILP (Tan et al., 2011), individual LIC1 and LIC2 depletions were carried out alongside depletion of both LIC1 and LIC2. HeLaM cells were treated with siRNA for 65 hours and transfected with GFP-RILP 16 hours prior to fixation. Dynein IC was visualised on the RILP-positive membrane structures (Figure 4.7A). Depletion of both LIC1 and LIC2 led to a loss of dynein from RILP-positive membrane structures but individual depletion of LIC1 or LIC2 did not. Dynein IC was present on the RILP structures in 84.4% of control cells, compared with 14.1% of cells lacking both LIC1 and LIC2 Kd. A one-way ANOVA with Dunnett’s post-hoc test shows that there were significantly fewer cells that have RILP-positive membrane structures with dynein present after depletion of both LIC1 and LIC2 (p<0.0001) but not
after single depletions (Figure 4.7B). Loss of both LIC1 and LIC2 led to a partial dispersion of RILP-positive membranes and this was not observed as frequently in control or individual LIC1 and LIC2 depleted cells, where the RILP-positive membranes were often found in one central cluster (Figure 4.7A). This correlates with dynein being visible on these RILP membranes and together these data would suggest either LIC1 or LIC2 is needed for dynein-RILP association, but there is a level of redundancy between the two homologues in dynein recruitment to RILP-positive membranes.

As loss of dynein from the membrane fraction appears to lead to a significant reduction in dynactin associated with membranes (Figure 4.1 and 4.2), it is possible that dynactin needs to be in complex with dynein in order to be recruited to membranous cargo. In order to test this, control or LIC1 and LIC2 in combination or individually were depleted and cells were transfected with GFP-RILP. Dynactin was visualised on the RILP-positive membrane structures (Figure 4.8A). Reflecting the loss of dynein seen after depletion of both LIC1 and LIC2 there was a loss of dynactin from RILP-positive membranes whereas individual depletions of LIC1 or LIC2 had no obvious effect on dynactin association with RILP. Dynactin was present on the RILP structures in 72.4% of control cells, compared with 11.4% of both LIC1 and LIC2 Kd cells (p<0.001) but there was no significant difference in RILP-dynactin association in cells that have been depleted of LIC1 or LIC2, individually (Figure 4.8B). These results indicate that dynein requires LIC to associate with RILP-positive membranes and in the absence of dynein on these membranes, dynactin is unable to associate. These data would suggest the recruitment of dynein to RILP-positive membranes does not occur via two independent LIC- and dynactin-regulated mechanisms and the two proteins are part of the same functional pathway in which LIC is upstream of dynactin.

As LIC-mediated recruitment of dynein to RILP has been proposed as an LIC1-specific mechanism (Tan et al., 2011) LIC1 protein levels were analysed via Western blotting. Cells from the same well as the coverslips used in the RILP overexpression fluorescence microscopy experiments (Figure 4.7 and 4.8) were lysed and analysed using Western blotting (Figure 4.9) and showed that LIC1 protein was being efficiently depleted. Although LIC2 proteins levels could not be assessed in this way (instead Golgi scattering was assessed), it is clear is that LIC1 was efficiently depleted in both LIC1 and LIC2 Kd cells and LIC1 Kd cells (Figure 4.9). Additionally, the LIC1 depletion seen here was much more efficient than that described in the published work proposing an LIC1-specific mechanism for dynein-RILP association (Tan et al., 2011). As dynein and dynactin are only lost from
RILP-positive membranes when both LIC1 and LIC2 are depleted, but remain when LIC1 is depleted and LIC2 is present in LIC1 Kd cells, this would strongly support a model in which there is redundancy between LIC1 and LIC2 in the recruitment of dynein to RILP and would suggest that this is not an LIC1-specific mechanism.
Figure 4.7: Dynein recruitment to RILP-positive membranes after LIC depletion. A: HeLaM cells were treated control siRNA or siRNA to LIC1 and LIC2 individually or in combination for 65 hours. 16 hours prior to fixation cells were transfected with GFP-RILP. Cells were fixed in methanol and stained with anti-IC (IC74) antibody (red). The blue staining in the merge is DAPI. Scale bars represent 20µm. B: The mean percentage of transfected cells with dynein present on RILP-positive membrane structures (minimum of 100 cells counted per siRNA treatment, n=3 independent experiments). Error bars represent standard deviation, **** = p<0.0001, one-way ANOVA with a Dunnett’s post-hoc test.
Figure 4.8: Dynactin recruitment to RILP-positive membranes after LIC depletion. A: HeLaM cells were treated as described in Figure 4.7, except cells were stained using anti-p50 antibody (red). Scale bars represent 20µm. B: The mean percentage of transfected cells with dynactin present on RILP-positive structures (minimum of 100 cells counted per siRNA treatment, n=3 independent experiments). Error bars represent standard deviation, *** = p<0.001, one-way ANOVA with a Dunnett’s post-hoc test.
Figure 4.9: The level of LIC1 protein in LIC depleted cells expressing GFP-RILP. HeLaM cells were grown in 12 well dishes containing coverslips and treated with control siRNA or siRNA to LIC1 and LIC2 individually or in combination for 65 hours. 16 hours prior to lysis cells were transfected with GFP-RILP. Coverslips were removed for cell fixation (Figure 3.7 and 3.8) and the remaining cells were washed in PBS and lysed by scraping in boiling sample buffer. The proteins were separated using SDS-PAGE and LIC1 levels were analysed using western blotting. Anti-tubulin (TAT1) was used as a loading control.
4.3.2 Dynein and dynactin recruitment to RILP-positive membranes after Lis1 and Nde1/Ndel1 depletion

Lis1, Nde1 and Ndel1 have been shown to play an important role in the recruitment of dynein to membranous cargo (Lam et al., 2010). Lis1 and Nde1/Ndel1 depletion also affected the size and distribution of lysosomes, which would be expected to be Rab-7-positive membranes (Figure 3.13 and 3.14). Additionally, isolation of membranes enriched with lysosomes showed that Nde1 is present on these membranes, and relative to NMM, there was a substantial amount of Lis1 associated with this fraction (Figure 4.6). Based on these observations Lis1, Nde1 and Ndel1 seemed to be likely candidates for dynein regulation in RILP-membrane recruitment. To investigate this Lis1, Nde1 and Ndel1 were depleted using siRNA, GFP-RILP was overexpressed and dynein was visualised on the RILP-positive membranes (Figure 4.10A). As Nde1 and Ndel1 have redundancy in dynein membrane recruitment (Lam et al., 2010), they were depleted together.

Lis1 depletion led to a loss of dynein from RILP-positive membrane structures as well as their dispersion. Surprisingly, though, Nde1/Ndel1 depletion did not have the same effect (Figure 4.10A). Dynein LIC1 was present on the RILP-positive membrane structures in 90.6% of control cells, compared with 17.5% of Lis1 Kd cells (p<0.0001) but there was no statistically significant decrease in dynein association with RILP in Nde1/Ndel1 Kd cells (Figure 4.10B). The RILP-positive membranes in Lis1 Kd cells were also more dispersed in keeping with loss of dynein from these membrane structures. These data suggest that dynein recruitment to RILP-positive membranes requires Lis1 but not Nde1 and Ndel1.

To investigate whether the dissociation of dynein from RILP in Lis1 Kd cells (Figure 4.10), effects dynactin recruitment to these structures, dynactin was visualised in control, Lis1 Kd and Nde1/Ndel1 Kd cells overexpressing GFP-RILP (Figure 4.11). Lis1 depletion led to a loss of dynactin from RILP-positive membranes whereas Nde1/Ndel1 Kd had no obvious effect on dynactin-RILP association. Dynactin was present on the RILP-positive membranes in 89.6% of control cells, compared with 6.1% of Lis1 Kd cells (p<0.0001) but loss of Nde1/Ndel1 has no statistically significant reduction in dynactin-RILP association (Figure 4.11B). The loss of dynactin from RILP structures after Lis1 Kd-mediated dynein displacement further supports the hypothesis that dynein association with RILP-positive membranes is a prerequisite for dynactin association.
4.3.3  Lis1, Nde1 and Ndel1 localisation to RILP-positive membranes

As Lis1 is required for the localisation of dynein and dynactin to RILP-positive membrane structures it seemed likely Lis1 would localise to these structures. Unfortunately there were no working antibodies available that could detect endogenous Lis1 by fluorescence microscopy. Instead, GFP-RILP was co-overexpressed with myc-Lis1 and the Lis1 was visualised using anti-Myc antibodies. Although Nde1 and Ndel1 do not appear to be needed for dynein recruitment to RILP-positive membranes, it was possible they may still be present on these membranes and so they were visualised in the same way.

HeLaM cells were co-transfected with GFP-RILP and either pcDNA3.1 containing c-Myc, Myc-Lis1, Myc-Nde1 or Myc-Ndel1, 16 hours prior to fixation. Due to the high levels of Lis1 and Nde1/Ndel1 found in the cytosol, it was necessary to pre-extract the cytosol prior to fixation of cells in order to visualise the Lis1, Nde1 and Ndel1 that might be present on RILP-positive membrane structures. Cells were treated with saponin to permeabilise the PM only and then fixed in 3% formaldehyde. Although this pre-extraction and fixation method in combination with anti-Myc antibody detection did not produce staining that is as clear as methanol fixation with endogenous dynein and dynactin staining, Myc-Lis1 could still be seen localised to RILP-positive membranes in 72.1% of cells (Figure 4.12A and B). Myc-Nde1 and Myc-Ndel1 were seen on RILP-positive membranes structures in very few cells (8.6% and 8.0%, respectively), which was similar to the number of control cells in which c-Myc alone localised to RILP (3.7%). The localisation of Myc-Lis1 and absence of Myc-Nde1 and Myc-Ndel1 on RILP-positive membranes supports a model in which Lis1 is involved in the recruitment of dynein to RILP-positive membranes in an Nde1/Ndel1-independent manner.

As depletion of Lis1 leads to a dissociation of dynein and dynactin from RILP it was possible that dynein might be being recruited to RILP via Lis1. If this were the case it would be expected that depletion of LIC, which also leads to a loss of dynein and dynactin from RILP-positive membrane, would not displace Myc-Lis1 from RILP. To test this, HeLaM cells were either treated with control siRNA or depleted of both LIC1 and LIC2 and cotransfected with GFP-RILP and Myc-Lis1. The cytosol was again pre-extracted via saponin treatment and cells were fixed and stained with anti-Myc (Figure 4.13A). Depletion of LIC led to the loss of Myc-Lis1 from RILP-positive membranes (Figure 4.13A and B; p<0.0001). This would indicate that although Lis1 is essential for proper dynein recruitment to RILP-positive membranes,
Lis1 does not interact with these membrane independently of dynein. It is likely that dynein may need to be in complex with Lis1 for either protein to be recruited to these membranes.
Figure 4.10: Dynein recruitment to RILP-positive membranes after Lis1 or Nde1/Nde1 depletion. A: HeLaM cells were treated with control, Lis1 or both Nde1 and Ndel1 siRNA for 65 hours. 12 hours prior to fixation cells were transfected with GFP-RILP. Cells were fixed in methanol and stained using anti-LIC1 antibody (red). The blue staining in the merge is DAPI. Scale bars represent 20μm. B: The mean percentage of transfected cells with dynein present on RILP-positive membrane structures (minimum of 100 cells counted per siRNA treatment, n=3 independent experiments). Error bars represent standard deviation of the mean, **** = p<0.0001, one-way ANOVA with Dunnett’s post hoc test.
Figure 4.11: Dynactin recruitment to RILP-positive membranes after Lis1 or Nde1/Nde1 depletion. A: HeLaM cells were treated as described in Figure 4.7, except cells were stained using anti-p50 antibody (red). Scale bars represent 20μm. B: The mean percentage of transfected cells with dynactin present on RILP-positive membrane structures (minimum of 100 cells counted per siRNA treatment, n=3 independent experiments). Error bars represent standard deviation of the mean, **** = p<0.0001, one-way ANOVA with Dunnet’s post hoc test.
Figure 4.12: Lis1, Nde1 and Ndel1 localisation to RILP-positive membranes. A: HeLaM cells were co-transfected with GFP-RILP and pcDNA3-c-Myc, Myc-Lis1, Myc-Nde1 or Myc-Ndel1 16 hours prior to fixation. The cells were permeabilised with saponin in order to extract the cytosol and then fixed in formaldehyde. Cells were stained with anti-Myc antibody. The blue staining in the merge is DAPI. Scale bars represent 20μm. B: The mean percentage of transfected cells with Myc present on RILP-positive structures (minimum of 100 cells counted per siRNA treatment, n=3 independent experiments). Error bars represent standard deviation of the mean, ** = p<0.01, one-way ANOVA with Dunnett’s post-hoc test.
Figure 4.13: The effect of LIC depletion of Lis1 localisation to RILP-positive membranes. A: HeLaM Cells were treated with control or both LIC1 and LIC2 siRNA for 65 hours. 16 hours prior to fixation cells were co-transfected with GFP-RILP and Myc-Lis1. The cells were fixed and stained as described in Figure 4.12. Scale bars represent 20μm. B: The mean percentage of transfected cells that have Myc-Lis1 present on RILP-positive membrane structures (minimum of 100 cells counted per siRNA treatment, n=3 independent experiments). Error bars represent standard deviation, **** = p<0.0001, unpaired t-test.
4.3.4 GFP-RILP co-immunoprecipitates with Lis1 and dynein

It is known that RILP can interact directly with dynactin (Johansson et al., 2007), but there is no published data regarding direct interactions of RILP and dynein or Lis1. To further investigate the involvement of Lis1 in dynein recruitment to RILP-positive membranes, co-IP experiments were carried out to see whether Lis1 interacts with the RILP-dynactin-dynein complex in vivo. HeLaM cells were transfected with GFP C1 parental vector, GFP-RILP or GFP-Lis1 for 16 hours. Cells were lysed and incubated with anti-GFP antibody, followed by protein G beads. The beads were washed and then boiled in Laemmli SB. The proteins from the IP beads and lysis inputs were analysed using Western blotting (Figure 4.14).

The co-IP experiments shown in figure 4.14 have been performed in triplicate and this representative blot was chosen as it shows GFP-RILP and GFP-Lis1 co-IP experiments on the same blot for direct comparison. The anti-GFP blot show that the IPs were efficient and that more GFP-RILP had been expressed in cells than GFP-Lis1. Although the lysates used in these IPs were adjusted in attempt to make the total protein concentrations equal, it would seem the GFP-RILP lysate still had less protein in, which was shown by the weaker TfR band (used as an input loading control and to show the IP samples were not contaminated with unrelated proteins).

As expected, GFP-RILP immunoprecipitated in complex with LIC and DHC. GFP-RILP also appeared to co-IP with Lis1. Although the band was weak, the lysate input also had very little Lis1 present and as such the GFP-RILP immunoprecipitated Lis1 fairly efficiently. Other IP experiments that had more Lis1 present in the input showed similar proportions of Lis1 being immunoprecipitated from the lysate (Appendix 3). GFP-RILP immunoprecipitated with ORP1L, but surprisingly the amount of p150 that co-immunoprecipitated with GFP-RILP was not much more than that of GFP-control. A non-specific band was present in the IP lane when blotting Nde1 (highlighted with an arrow). Despite this, there was no Nde1 present in the GFP-RILP IP lane, which would support the hypothesis that dynein interactions with RILP are independent of Nde1/Ndel1. Due to the limited volume of IP samples available for blotting, the RILP- or Lis1- Ndel1 interaction could not be assessed. The Nde1 antibody was chosen as it has cross-reactivity with Ndel1. However, it is far less sensitive in its detection of Ndel1 compared with Nde1 (Appendix 1), so it is still possible Ndel1 may be present in the IP.
GFP-Lis1 co-immunoprecipitated with DHC and LIC, as would be expected. GFP-Lis1 immunoprecipitated in complex with both Nde1 and p150, which is surprising as dynein IC has been reported to interact with either Nde1 or p150 (McKenney et al., 2011). There are two possibilities that might explain this. The GFP-Lis1 may be forming two complexes that are immunoprecipitating, one which contains GFP-Lis1-dynein-p150 and another that contains Lis1-Nde1-dynein. The other possibility is that GFP-Lis1 is immunoprecipitating dynein, Nde1 and dynactin simultaneously. If this were occurring then Lis1 may be interacting with dynactin via p50 (Tai et al., 2002) so that an interaction between p150 and dynein-IC is not required. GFP-Lis1 appeared to interact with endogenous Lis1 because the Lis1 IP lane contained GFP-Lis1 (~80kDa) and endogenous Lis1 (~40 kDa). This could be due to GFP-Lis1 dimerising with endogenous Lis1. There were two intermediate bands between GFP-Lis1 and endogenous Lis1 in the IP lane, which could indicate GFP-Lis1 is being cleaved. There was no available antibody that could detect endogenous RILP via Western blotting but GFP-Lis1 also co-immunoprecipitated with ORP1L. This may be an indication that Lis1 is in complex with RILP-dynein/dynactin complex. Taken together these co-IP experiments indicate RILP interacts with dynein in the presence of Lis1 and dynactin but not Nde1.
Figure 4.14: Immunoprecipitations of GFP-RILP and GFP-Lis1. HeLaM cells were transfected with GFP C1 parental vector, GFP-Lis1 or GFP-RILP, 16 hours prior to lysis and incubated with anti-GFP IgG followed by protein-G beads. Lysate input and washed beads were separated using SDS-PAGE and proteins were analysed by western blotting, using indicated antibodies. Input is 3% of IP.
4.4 Characterising RILP-positive membranes

4.4.1 RILP-positive membrane structures constitute a subpopulation of lysosomal membranes

As RILP is Rab7-interacting and known to associate with LE and lysosomal membranes (Jordens et al., 2001), cells that were expressing GFP-RILP were stained with LAMP1 to confirm the membrane clusters that were being induced were indeed lysosomal. The RILP-positive membranes present in transfected cells were extremely strongly labelled with LAMP1 and needed to be taken at low exposure to avoid image saturation, but this low exposure meant that when imaging LAMP1 it was difficult to see membranes not associated with this bright central cluster. Cells were therefore imaged at low exposure to focus on the RILP-membrane cluster (Figure 4.15 A bottom row) and a higher exposure to show non-central LAMP1-positive membranes (Figure 4.15 A top row). Many of these non-central LAMP1-positive vesicles do not have any associated GFP-RILP (Figure 4.15B). This indicates that the RILP membranes present at the centre of the cell are lysosomal, but not all lysosomal membranes are RILP-positive, therefore RILP-positive membranes constitute a subpopulation of lysosomes.

4.4.2 RILP-positive membranes can colocalise with the autophagy marker LC3

As the RILP-positive membranes constituted a subset of lysosomes, other markers associated with lysosomal membranes were used to characterise the membrane composition. One of these markers was LC3, which associates with autophagic compartments. 41.8% of cells counted contained RILP-positive membranes that were also LC3-positive (Figure 4.16A and B upper rows and C). In cells that were expressing more GFP-RILP and thus had brighter GFP-RILP membrane clusters, the RILP-positive membranes tended not to be LC3-positive. It was hypothesised that this may be due to earlier transcription of GFP-RILP post-transfection, leading to the RILP-membrane structures forming sooner and LC3 being digested by lysosomal proteases. To test this, cells were treated 0.1mM leupeptin and 1.5mM pepstatin (blocking lysosomal degradation) for 16 hours during transfection and this lead to an increase to 83.4% (p<0.001) of cells with LC3 and RILP colocalisation (Figure 4.16A lower row and C). This would indicate that RILP-positive membranes may be autophagic but after over-expression the large RILP-positive membrane vesicles that form are able to digest the autophagy protein LC3. As HeLaM cells are known to have background levels of autophagy under normal conditions it is possible that RILP overexpression is increasing the dynein recruited to Rab7-membranes, which
includes LE, lysosomes and autolysosomes. This may be causing an initial accumulation of LC3 in the RILP structures, which is subsequently degraded.

4.5 Conclusions
The results in this chapter indicate that IC may play an important role in dynein-cargo association on many internal membranes of the cell whereas LIC may play more cargo-specific roles, such as in the recruitment of dynein to RILP-positive membranes. Here it is shown the membranes that form after RILP overexpression constitute a subpopulation of lysosomal membranes and could be associated with autophagy. Recruitment of dynein and dynactin to these membranes is dependent on both LIC and Lis1 and independent of Nde1 and Ndel1 (Figure 4.17). A common feature observed in this results chapter is that the loss of dynein from organelle membranes leads to a loss or reduction in associated dynactin. It is possible that on some cargo, like RILP-positive membranes, dynactin is recruited to membranes via dynein, not vice versa.
Figure 4.15: LAMP1 distribution in GFP-RILP expressing cells. A: HeLa M cells were transfected with GFP-RILP 16 hours prior to fixation. Cells were fixed in methanol and stained with anti-LAMP1 antibody (red). Images of cells were taken at low exposure and high exposure to show central RILP- and LAMP1-positive structures and peripheral smaller non-central structures, respectively. The blue staining in the merge is DAPI. Scale bar represents 20μm. B: Enlarged 20μm x 20μm sections from panel A. Many of the visible lysosomes visible are not RILP-positive.
Figure 4.16: LC3 localisation to RILP-positive membranes. A: HeLaM cells were transfected with GFP-RILP and incubated with (+) or without (-) 1.5mM leupeptin and 0.1mM pepstatin, 16 hours prior to fixation. Cells were fixed methanol and stained using anti-LC3 antibody. The blue staining in the merge is DAPI. Scale bars represent 20μm. B: 25μm x 25μm enlargements from panel A, showing GFP-RILP structures to which LC3 localises. C: The percentage of transfected cells with LC3 present at RILP-positive membrane structures (minimum of 100 cells counted per siRNA treatment, n=3 independent experiments). Error bars represent the standard deviation of the mean, *** = p<0.001, unpaired t-test.
Figure 4.17: The interactions of dynein with RILP-positive membranes. A: Dynein and dynactin localise to RILP on Rab7 positive membranes (Johansson et al., 2007). It is possible, based on the results here that dynein may also interact with RILP via Lis1 and LIC. B: Loss of Lis1 leads to dissociation of dynein and dynactin from RILP-positive membranes. C: Loss of LIC leads to dissociation of dynein, dynactin and Lis1 from RILP-positive membranes.
Chapter 5

Results: Dynein regulation in autophagic flux and endocytic progression
5. Results: Dynein regulation in autophagic flux and endocytic progression

5.1 The role of dynein regulation in autophagy

Dynein has been shown to play an important role in the movement of autophagic vesicles (Kimura et al., 2008), so it seemed possible that depletion of the dynein subunits LIC and IC and also the dynein accessory proteins Lis1, Nde1 and Ndel1, might perturb the autophagy pathway. To investigate whether any of these proteins play specific roles in the regulation of the autophagy pathway they were depleted in HeLaM cells using siRNA and the number of LC3 puncta and their localisation was observed. It is worth noting LC3 puncta can be seen in control HeLaM cells so these observations reflect the amount of autophagy taking place, the flux through the pathway and transport of autophagic membranes rather than the induction of autophagy. In all LC3 imaging experiments described below, the LC3 in control and Kd cells were imaged at the same exposure, camera settings and were processed similarly to prepare figures.

5.1.1 LC3 puncta after loss of Lis1

As depletion of Lis1 leads to enlarged lysosomes, the composition of these swollen lysosomes was investigated to see whether they were autophagic. LAMP1 and LC3 were stained in control and Lis1 Kd cells (Figure 5.1). The loss of Lis1 led to an accumulation of LC3 that appeared to be internally localised in enlarged lysosomes. LC3 appeared to decorate the inner surface of extremely enlarged lysosomes (Figure 5.1B middle row), and puncta of concentrated LC3 labelling could be observed within these lysosome, which indicates these vesicles may have become swollen with contents and the LC3 is still bound to the internal face of the membrane. The LC3-positive lysosomal structures appear to be autolysosomes.
Figure 5.1: The effect of Lis1 depletion on LC3 and LAMP1 distribution. A: HeLaM cells were treated with control or Lis1 siRNA for 65 hours and fixed in methanol. Cells were stained with anti-LC3b and anti-LAMP1. The blue staining in the merge is DAPI. Scale bars represent 20μm. B: Enlargement of the 20μm x 20μm sections highlighted on panel A.
5.1.2 LC3 puncta after loss of dynein LIC and IC

As loss of IC and LIC leads to some lysosomes becoming enlarged it was thought that these too could be autolysosomes. LAMP1 and LC3 were stained in control, LIC Kd and IC Kd cells (Figure 5.2 and Figure 5.3). Although there did appear to be an increase in LC3 puncta in LIC Kd cells compared with control (Figure 5.2A), most did not appear to localise with LAMP1-positive lysosomes. IC Kd led to a visible increase in LC3 puncta, some of which colocalised with LAMP1-positive lysosomes (Figure 5.3B). Although there did appear to be slightly more LC3 puncta that colocalise with lysosomes than in LIC Kd cells, IC Kd did not lead to the same striking colocalisation observed in Lis1 Kd cells. These results indicate loss of LIC or IC leads to an increase in the number of autophagic vesicles but does not cause autolysosomes to form in the same was as Lis1 depletion.

5.1.3 LC3 puncta after loss of Nde1 and Ndel1

To investigate whether the enlarged lysosomes in some of the Nde1/Ndel1 Kd cells were autophagic, control and Nde1/Ndel1 Kd cells were stained with LAMP1 and LC3 (Figure 5.4). There may have been a slight increase in LC3 puncta when Nde1 and Ndel1 were depleted, but it was difficult to be sure through visual observation alone (Figure 5.4A). Some of the LC3 puncta that form did colocalise with LAMP1-positive lysosomes (Figure 5.4B), but certainly not in a manner that is as striking the autolysosomes that form in Lis1 Kd cells or even the less striking phenotype of IC Kd cells. It is possible that loss of both Nde1 and Ndel1 may be increasing the number of autophagic vesicles in cells but it is not at all clear using observations from microscopy alone.
Figure 5.2: The effect of LIC depletion on LC3 and LAMP1 distribution. A: HeLaM cells were treated with control or LIC siRNA for 65 hours and fixed and stained as described in Figure 5.1. Scale bars represent 20μm. B: Enlargement of the 20μm x 20μm sections highlighted on panel A.
Figure 5.3: The effect of IC depletion on LC3 and LAMP1 distribution. A: HeLaM cells were treated with control or IC siRNA for 65 hours and fixed and stained as described in Figure 5.1. Scale bars represent 20μm. B: Enlargement of the 20μm x 20μm sections highlighted on panel A.
Figure 5.4: The effect of Nde1/Ndel1 depletion on LC3 and LAMP1 distribution. A: HeLaM Cells were treated with control or Nde1 and Ndel1 siRNA for 65 hours and fixed and stained as described in Figure 5.1. Scale bars represent 20μm. B: Enlargement of the 20μm x 20μm sections highlighted on panel A.
5.1.4 Quantification of LC3

As there is so much variation in the number of puncta observed between different siRNA treatments Western blotting analysis of the LC3b-II intensity was used as an unbiased approach to quantifying the amount of LC3 that was membrane associated. There are two isoforms of LC3: LC3A and LC3B. Both can be present in two forms, LC3-I and LC3-II, the latter of which is lipidated with phosphatidyl ethanolamine and thus can associate with autophagic membranes (Klionsky et al., 2008). LC3b-I has an apparent molecular weight of 17kDa but the LC3b-II lipidation means it migrates faster on SDS-PAGE gels (around 15kDa) (Klionsky et al., 2008). HeLaM cells were treated with control, IC, LIC, Lis1 or both Nde1 and Ndel1 siRNAs for 65 hours. Cells were lysed and the LC3b-I and LC3b-II levels were visualised using Western blotting, with tubulin being used as a loading control (Figure 5.5A).

Control HeLaM cells had LC3b-II present (Figure 5.5A), which correlates with the observation of LC3 puncta via fluorescence microscopy. The intensity of LC3b-II was measured for each siRNA treatment and normalised to tubulin intensity. Figure 5.5B shows the mean intensities (n=3) expressed as fraction of the control. A one way ANOVA with a Dunnett’s post hoc test shows that loss of Lis1, but not IC, LIC or Nde1/Ndel1, leads to statistically significant increases in the amount of LC3b-II present in cells. This semi-quantitative measurement of LC3b-II show Lis1 Kd led to the largest increase of LC3b-II (4.4 fold). IC Kd and LIC Kd led to an increase (2.0 fold and 2.1 fold, respectively) of LC3b-II whereas in Nde1/Ndel1 Kd LC3b-II did not increase. This is similar to the observation of LC3 puncta, in that Lis1 Kd leads to the most striking phenotype and LIC and IC depletions show a visually obvious increase in LC3 puncta. This LC3b Western blotting analysis data in combination with observations of LC3 puncta would suggest there is no increase in membrane associated LC3 in Nde1/Ndel1 Kd cells.
Figure 5.5: The levels of LC3-II in cells depleted of Lis1, LIC, IC or Nde1/Nde1. A: siRNA treated HeLaM cells were lysed and analysed by western blotting, using LC3b and tubulin (TAT-1) antibody. A representative blot is shown. LC3-I is the upper band at 17kDa and LC3-II is the lower band at 15kDa. B: Mean LC3-II band intensity (n=3 independent experiments) normalised to tubulin band intensity, expressed as a fraction of the control. Error bars represent standard error of the mean, ** = p<0.01, one-way ANOVA with a Dunnett’s post hoc test.
Based on these results, it can be concluded that depletion of LIC and IC lead to an increase in the number autophagic vesicles. Lis1 Kd also leads to an accumulation of autophagic vesicles but the phenotype observed here seems to be distinct, with large autolysosomes forming and a statistically significant increase in LC3-II. It is possible that loss of IC and LIC is leading to reduced dynein function that prevents or slows proper flux through the autophagy pathways, whereas Lis1 depletion is having some other effect. Based on the observation that LC3 is not being degraded it is possible the depletion of Lis1 is leading to a block in lysosomal degradation. Additionally, the role that Lis1 is playing regulating flux through the autophagy pathway appears to be independent of Nde1 and Ndel1 as their depletion does not have the same, or indeed any, effect on LC3-II levels or number of puncta.

5.2 Further characterisation of Lis1 knock down induced LC3-positive membranes

As Lis1 depletion led to such a striking phenotype it was decided that the autolysosomes that form in Lis1 Kd cells would be studied further.

5.2.1 Transmission Electron Microscopy (TEM) imaging of Lis1 knock down cells

To further investigate the composition of the autolysosomal membranes formed after depletion of Lis1, Lis1 Kd cells were imaged using TEM (imaging carried out by Dr. Ling Zhang). Large aberrant membrane structures were observed in many Lis1 Kd cells that are similar in morphology and size to autolysosomes which lack the double membranes that characterise AP (Figure 5.6). These structures seemed to be filled with membranes and materials which would be indicative of a swollen lysosome, or autolysosome, that is filled with undegraded material as opposed to an empty swollen vacuole-like structure.
Figure 5.6: Electron microscopy images of Lis1 Kd cell showing structures that appear to be autolysosomes. A: HeLaM cells were treated with Lis1 siRNA for 65 hours, fixed in glutaraldehyde and imaged using transmission electron microscopy. Fixation and imaging was performed by Dr. Ling Zhang. B: Enlargements of from panel A, highlighting aberrant structures they may be autolysosomes.
5.2.2 Autolysosome formation after lysosomal protease inhibitor treatment

Due to the fact that LC3 is not being degraded in the LC3-positive lysosomes that form in Lis1 Kd cells (Figure 5.1) and the autolysosome-like structures observed by EM are filled with material (Figure 5.6), it seemed possible there might be an issue with lysosomal protein degradation in Lis1 Kd cells. HeLaM cells were therefore treated with 0.1mM leupeptin and 1.5mM pepstatin for 24 hours in order to inhibit proteases in the lysosome to see whether this had a similar effect on lysosomes and LC3. Control (untreated) and protease inhibitor-treated cells were then fixed and stained for LC3 and for LAMP1 (Figure 5.7).

Blocking lysosomal protein degradation led to a similar accumulation of autolysosomes to that seen in Lis1 Kd cells (Figure 5.7 A and B). Although the LC3-positive lysosomes did not seem as large as those observed in many Lis1 Kd cells, this may be due to the fact the cells are only treated with protease inhibitors for 24 hours. Lis1 Kd cells are treated with siRNA for 65 hours, meaning that if lysosomal degradation is being inhibited, it may be over a longer period of time resulting in larger LC3-positive lysosomes. The similarity in the LC3-positive lysosomes that form after lysosomal degradation has been blocked and when Lis1 has been depleted further supports a model in which Lis1 is needed to maintain lysosomal protein degradation.

5.2.3 Lis1 knock down rescue experiments

To assess whether the Lis1 Kd lysosome phenotype could be rescued by reintroducing Lis1, an siRNA-resistant (SR)-GFP-Lis1 was overexpressed in Lis1 Kd cells. HeLaM cells were treated with Lis1 siRNA and 18 hours prior to fixation they were transfected with the SR-GFP-Lis1. SR-GFP-Lis1 was able to rescue the Golgi apparatus positioning phenotype in Lis1 Kd cells (Figure 5.8C) in a similar manner to that which has previously been described (Lam et al., 2010), so it was clear that the SR-GFP-Lis1 was working appropriately. These data are preliminary and need to be repeated with a GFP only expression control, but overexpression of SR-GFP-Lis1 in Lis1 Kd cells led to a redistribution of the LC3 and LAMP1 to the centre of the cell. However, many of lysosomes were still LC3-positive (Figure 5.8B). These observations suggest that the reintroduction of Lis1 can restore dynein-mediated transport of lysosomes to the cell centre but the lysosomes may take longer than the few hours of Lis1 expression to become properly functional in protein degradation.
Figure 5.7: The effects of inhibiting lysosomal protein degradation. A: HeLaM cells were incubated with either complete media (Control) or complete media with the addition of 1.5mM pepstatin and 0.1mM leupeptin for 24 hours. Cells were fixed in methanol and stained with anti-LC3 and anti-LAMP1 antibody. The blue staining in the merge is DAPI. Scale bars represent 12μm. B: Enlargement of the 12μm x 12μm sections highlighted on panel A.
Figure 5.8: The effect of SR-GFP-Lis1 on LAMP1 and LC3 distribution in Lis1 depleted cells. A: HeLaM cells were treated with Lis1 siRNA for 65 hours and transfected with SR-GFP-Lis1 16 hours prior to fixation. Cells were fixed in methanol and stained using anti-LC3 and anti-LAMP1. Scale bar represents 20μm. Transfected cells are indicated with an asterisk (*). B: 20μm x 20μm sections from panel A, showing central LAMP1- and LC3- positive vesicles. C: Cells treated as described in A, but stained using anti-GM130. The blue staining in the merge is DAPI. Scale bar represents 20μm.
5.3 Investigating the role of Lis1 in the PAFAH complex during autophagy

As depletion of Lis1 causes a lysosome and LC3 phenotype that is distinct from IC Kd and LIC Kd as well as DHC Kd (data not shown), it seemed possible that Lis1 may be regulating lysosomal function via a dynein-independent mechanism. As Lis1 is part of the PAFHA1B complex, which has been implicated in membrane tubule formation (Bechler and Brown, 2013; Bechler et al., 2009), this complex seemed to be a likely candidate for dynein-independent regulation of lysosomal function. The α1 and α2 subunits, which form homo- and heterodimers and associate with Lis1 in the PAFAH complex, were therefore depleted using siRNA in order to observe the effects on lysosome size, distribution and composition. If the phenotype seen after loss of Lis1 involved the PAFAH1B complex it would be expected that LC3 puncta and lysosomes would increase in number and size after the loss of the α1 and α2 subunits.

5.3.1 Characterisation of α1 and α2 knock down

HeLaM cells were treated with control or both α1 and α2 siRNA for 65 hours prior to fixation and lysis. The cells were fixed and the Golgi apparatus and MTs were stained (Figure 5.9A). Cells depleted of both α1 and α2 had a much more compact Golgi apparatus compared with control. Published work shows that loss of α1 and α2 leads to Golgi membrane fragmentation but these fragments are still very central and clustered (Bechler and Brown, 2013), which is similar to the Golgi apparatus observed after Kd in this project. When Lis1 is overexpressed in cells the Golgi apparatus becomes very compact (Lam et al., 2010), so it is possible that loss of α1 and α2 frees more endogenous Lis1 which can then regulate and activate dynein function, thus leading to more compact and central Golgi apparatus. To test the Kd efficiency, the levels of α1 and α2 in whole cell lysates of α1/α2 Kd cells were analysed using Western blotting (Figure 5.9B). These blots showed that the α1 and α2 protein levels were substantially reduced. No α1 was detectable and α2 protein levels were greatly reduced. Efficiency of α1 and α2 depletion in subsequent experiments was judged by Western blotting or changes to Golgi apparatus morphology.
Figure 5.9: Characterisation of PAFAH alpha 1 and alpha 2 depletion. A: HeLaM cells were treated with control or Alpha 1 and Alpha 2 siRNA for 65 hours and fixed in methanol. The Golgi apparatus was stained using anti-GM130, and microtubules were stained using YL1/2. The blue staining in the merge is DAPI. Scale bar represents 20μm. B and C: Whole cell lysates were analysed using western blotting and anti-Alpha 1 (B) and anti-Alpha 2 (C) antibodies. Tubulin (TAT-1) was used as a loading control.
5.3.2 Loss of α1 and α2 does not induce autolysosome formation

To assess whether loss of α1 and α2 affects autophagy within cells, control and α1 and α2 Kd cells were fixed and stained with LAMP1 and LC3 (Figure 5.10A). The number of LC3 puncta did not obviously increase in α1/α2 Kd cells compared with control but both the LC3 and LAMP1 appeared to be more centrally clustered. This is similar to the clustered morphology of the Golgi apparatus after α1 and α2 depletion. Again, this may be due to more free Lis1 promoting dynein-driven transport toward the MTOC.

Western blotting was used to analyse the LC3-II levels after α1 and α2 Kd and tubulin was used as a loading control. There was no obvious increase in LC3-II band intensity in α1 and α2 Kd cell lysates compared with control (Figure 5.10B), although measurement of the LC3-II band intensity (normalised to tubulin intensity and expressed as a fraction of the control) showed a two-fold increase in LC3-II levels (Figure 5.10C). However, there was considerable variability between experiments and an unpaired t-test showed the difference was not statistically significant. These data indicate the PAFAH complex is not important for autophagy membrane regulation. It is, however, possible that the role of Lis1 in the PAFAH complex and regulating dynein function have a combined effect on autophagy. To test this dynein would need to be inhibited or depleted in combination with an α1/α2 Kd.
Figure 5.10: The effect of alpha 1 and alpha 2 depletion on LC3 and LAMP1 distribution.
A: HeLaM cells were treated with control or PAFAH Alpha 1 and Alpha 2 siRNA for 65 hours prior to fixation. Cells were fixed in methanol and stained using anti-LC3 and anti-LAMP1. The blue staining in the merge is DAPI. Scale bars represent 20μm. B: Control cells and cells depleted of PAFAH Alpha 1 and Alpha 2 were lysed and analysed by western blotting, using LC3b and tubulin (TAT-1) antibody. A representative blot is shown. LC3-I is the upper band at 17kDa and LC3-II is the lower band at 15kDa. C: Mean LC3-II band intensity (n=3 independent experiments) normalised to tubulin band intensity, expressed as a fraction of the control. Error bars represent standard error of the mean.
5.4 The role of Lis1 in endocytic trafficking

As loss of Lis1 seems to be preventing proper lysosomal degradation and potentially causing a block in autophagic flux, it seemed likely that its loss was affecting traffic through the endocytic pathway. If the loss of Lis1 was leading to a general deficiency in lysosomal degradation of protein it would be expected that Lis1 Kd would prevent the degradation of endocytosed material. To test this possibility, EGF pulse chase experiments were carried out. In all EGF imaging experiments described below the EGF was imaged at the same exposure, camera settings and images were processed similarly to prepare figures.

5.4.1 EGF reaches early endosomes in control and Lis1 knock down cells after 25 minutes

Control or Lis1 Kd cells were incubated with Alexa 488-conjugated EGF for 5 minutes, immediately followed by incubation with unlabelled EGF for 25 minutes. Cells were fixed and stained with anti-EEA1 to visualise EEs (Figure 5.11). As expected, the EEs in Lis1 Kd cells were dispersed compared with control. After a 25 minute chase with unlabelled EGF, the Alexa 488-EGF had reached the EE in both control and Lis1 Kd cells.

5.4.2 EGF is degraded after 180 minutes in control cells but remains undegraded in Lis1 knock down cells

Next, control and Lis1 Kd cells were pulsed with Alexa 488-EGF for 5 minutes and chased with unlabelled EGF for 180 minutes. Cells were fixed and labelled with LAMP1 (Figure 5.12A). In control cells almost all the EGF had been degraded and the small amount that was left seemed to localise mostly at the lysosomes (Figure 5.12 A and B). Lis1 Kd cells still had a great deal of EGF present and it was not localised to the lysosomes (Figure 5.12 A and B). This would suggest EGF in the Lis1 Kd cells is not reaching the lysosomes after 180 minutes and therefore not being degraded.
Figure 5.11: The effect of Lis1 depletion on EGF localisation after 5 minutes pulse and 25 minutes chase. HeLaM cells were treated with control or Lis1 siRNA for 65 hours. The cells were incubated with Alexa 488-conjugated EGF for 5 minutes, followed by unlabelled EGF for 25 minutes. Cells were fixed in formaldehyde and stained with anti-EEA1 antibody. The blue staining in the merge is DAPI. Scale bars represent 20µm.
Figure 5.12: After the effect of Lis1 depletion on EGF localisation after 5 minutes pulse and 180 minutes chase, relative to LAMP1-positive lysosomes. A: HeLaM cells were treated with control or Lis1 siRNA for 65 hours. The cells were incubated with fluorescently labelled Alexa488-conjugated EGF for 5 minutes, followed by unlabelled EGF for 180 minutes. Cells were fixed in formaldehyde and glutaraldehyde and stained for LAMP1. The blue staining in the merge is DAPI. Scale bars represent 20μm. B: 20μm x 20μm enlargements of highlighted sections in panel A.
To visualise where the EGF might be localised to in Lis1 Kd cells after 180 minutes, cells were pulsed for 5 minutes with Alexa 488-EGF and chased with unlabelled EGF for 180 minutes and the same cells were stained for EEA1 and CD63 (Figure 5.13). Some of the Alexa 488-EGF was still located in EEA1-positive EE that appeared to be somewhat aggregated and central (Figure 5.13). None of the Alexa 488-EGF was in the enlarged CD63-positive LE and lysosomes seen in Lis1 Kd cells (Figure 5.13). A large proportion of the Alexa 488-EGF appeared to localise to small peripheral puncta. This would suggest that EGF is not being trafficked from EE to MVB and LE correctly and is instead accumulating in vesicles that are relatively peripheral. It is possible that these vesicles do not undergo dynein-driven transport and that is why many are located away from the cell centre.

5.4.3 Dynein inhibition through p50 overexpression slows endocytosis but does not prevent early endosomes to MVB/LE trafficking

To assess whether this block in traffic between EE and MVB/LE is Lis1 Kd specific or due to a general misregulation of dynein, HeLa M cells were transfected with the dynactin subunit p50 in order to inhibit dynein function. Although this loss of dynein function did slow EGF movement through the endocytic pathway as previously reported (Driskell et al., 2007), Alexa 488-EGF still reached CD63-positive lysosomes and LE (Figure 5.14). After 180 minutes chase with unlabelled EGF, control cells degraded most of the visible Alexa 488-EGF whereas in p50 overexpressing cells the Alexa 488-EGF was localised almost exclusively to CD63-positive compartments. This indicates that, as we would expect, inhibiting dynein function slows the movement through the endocytic pathway, but it does not prevent EE to LE transport. The block in trafficking in Lis1 Kd cells is therefore likely to be a Lis1-specific phenotype rather than an effect caused by general dynein inhibition.
Figure 5.13: A: The effect of Lis1 depletion on EGF localisation after 5 minutes pulse and 180 minutes chase, relative to EEA1- and CD63-positive compartments. HeLaM cells were treated with control or Lis1 siRNA for 65 hours. The cells were incubated with Alexa 488-conjugated EGF for 5 minutes, followed by unlabelled EGF for 180 minutes. Cells were fixed in formaldehyde and stained for EEA1, CD63 and with DAPI. Scale bars represent 20μm. B: Enlarged 50μm x 50μm regions from Figure 5.13, with merge.
Figure 5.14: The effect of dynein function inhibition, by p50 overexpression, on EGF endocytosis. A: HeLaM cells were transfected with mCherry or mCherry-p50 16 hours prior to incubation with EGF. The cells were incubated with Alexa 488-conjugated EGF for 5 minutes, followed by unlabelled EGF for 180 minutes. Cells were fixed in formaldehyde and stained for CD63. The blue staining in the merge is DAPI. Scale bars represent 20μm.
5.5 Conclusions

The results from this chapter show that dynein activity is not only important for endocytic trafficking but also autophagic flux. Data from Lis1 Kd cells show that Lis1 is needed for proper protein degradation in lysosomes, as loss of Lis1 led to an accumulation of LC3 in autolysosomes and a reduced ability to degrade endocytosed EGF. The pulse chase data suggest this inability to degrade protein in the lysosome may be due to Lis1 depletion preventing trafficking from EE to LE. The role Lis1 plays in aiding lysosomal protein degradation is unclear, as perturbing dynein function does not have the same effect as a Lis1 Kd nor does disruption of the PAFAH complex. It is possible that Lis1 regulating both these protein complexes is needed for proper maintenance of lysosome activity. Irrespective of the mechanisms Lis1 is using to regulate lysosome function it is clearly essential for maintenance of proper protein degradation within these organelles and this implicates Lis1 in a novel role in regulating endocytic pathway progression.
Chapter 6
Discussion
6. Discussion

The results from this project highlight some of the similarities and differences between IC, LIC, Lis1 and Nde1/Ndel1 in their ability to influence membrane trafficking. When these proteins are depleted they do not just affect one process, but many. The multiple effects of these Kds may be due to the proteins playing different roles within individual processes but in some instances they also reflect how various processes within the cell are interlinked. In the next sections the results from this project will be considered in the context of dynein recruitment to membranes, endocytic pathway progression and autophagy regulation.

6.1 Dynein recruitment to membranes

The way in which dynein is recruited to organelle cargo is not completely understood at present. At best we can say the recruitment mechanisms are complex, involve many proteins and vary depending on the context of the cargo being transported. For a time it seemed possible that dynactin could act as a general membrane adaptor for dynein via the interaction of Arp1 and β-III spectrin (Holleran et al., 2001). Subsequent studies have provided substantial evidence to suggest that this may not be true as the loss of Arp1 or breaking apart the association between p150 and Arp1 does not reduce dynein associated with the membrane (Flores-Rodriguez et al., 2011; Haghnia et al., 2007). Furthermore, Nde1, Ndel1 and Lis1 have been shown to be very important for general membrane recruitment. Loss of Nde1 and Ndel1 together results in dynein being displaced from a general membrane fraction and loss of Lis1 also lead to a clear reduction (Lam et al., 2010). It is not understood how these proteins achieve this because to date they have not been shown to interact with membrane proteins or lipids. Ndel1 can undergo palmitoylation but it is not thought the single lipid that is added to the Ndel1 protein is sufficient to induce a membrane interaction (Shmueli et al., 2010). Dynein recruitment to membranes may be achieved by specific interactions between membrane proteins on organelles interacting with dynein subunits or accessory proteins. There are many examples of cargo specific proteins such as Rabs, Rab effectors, SNXs and other membrane associated proteins that interact with dynein, dynactin or other accessory proteins (Cai et al., 2010; Fejtova et al., 2009; Hong et al., 2009; Horgan et al., 2010a; Horgan et al., 2010b; Hunt et al., 2013; Johansson et al., 2007; Jordens et al., 2001; Matanis et al., 2002; Rayala et al., 2006; Tan et al., 2011; Traer et al., 2007; Wanschers et al., 2008; Yadav et al., 2012). All these interactions between membrane proteins and the dynein-dynactin complex may provide a means for dynein recruitment to specific membranes. If the interactions between
dynein/dynactin and cargo proteins can be either promoted or inhibited, this provides a mechanism for regulating membrane transport and trafficking.

6.1.1 Dynein LIC and IC in membrane recruitment
Looking at bulk membrane preparations, there is a reduction in dynein-membrane association after LIC Kd, but there is also a reduction of dynein in the cytosol (Figure 3.1 and 3.3). This might be indicative of dynein being less stable in the absence of LIC and does not give us a clear insight into what role LIC may play in dynein membrane recruitment. However, dynein LIC may play an important role in recruitment to specific organelles, such as RILP-positive LE and lysosomes (discussed in section 6.1.3).

The data from this project would suggest that in the absence of dynein IC a large proportion of dynein is being lost from cell organelles (Figure 3.1 and 3.2). It is possible that IC acts as a ubiquitous membrane adaptor for dynein recruitment to cargo. This may account for why apoptotic cleavage of IC (and p150) leads to dynein dissociation from membranes (Lane et al., 2001). If IC is acting as a general membrane adaptor then interactions of other subunits with membrane proteins, such the interaction seen between LIC and SNX or RILP, might play a regulatory role in conferring membrane association specificity (Hunt et al., 2013; Tan et al., 2011). However, in some cases dynein IC defines recruitment specificity, as seen with its interactions with Golgin160 and Snapin (Cai et al., 2010; Yadav et al., 2012). It is possible that dynein is associating with membranes via a mechanism that involves dynein IC interacting with Nde1/Ndel1, as loss of either protein leads to a displacement of dynein from the bulk membrane preparations (Figure 4.1 and 4.2) (Lam et al., 2010). It is also possible that dynein needs to be in complex with dynactin for membrane association since loss of IC abolishes the dynein-dynactin interaction, so both would dissociate from the membrane. The binding of dynein to dynactin or Nde1 is thought to be mutually exclusive (McKenney et al., 2011), so if both proteins play a role in dynein membrane association it is likely to be either through two different mechanisms or in a sequential fashion. However since dynein IC interacts with both proteins, its loss would affect any pathway involving either protein.

6.1.2 Dynactin in membrane recruitment
The results presented here suggest that dynactin requires dynein in order to interact with membranous cargo. The reduction in dynein-membrane association after IC depletion is accompanied by a significant reduction in dynactin (Figures 3.1 and 3.2). Additionally, loss
of dynein from RILP-positive membranes after LIC or Lis1 Kd leads to a loss of dynactin (Figures 4.7 and 4.10). It is therefore possible that dynactin is recruited to membranes via dynein, which associates with membranes independently of a dynactin. This model is supported by the observation that overexpressing p50 and breaking apart the dynactin complex does not displace dynein or p150 from membranes (Flores-Rodriguez et al., 2011). It is, however, possible that the p50-p150 subunits, which are likely to be associated with dynein, could be contributing to membrane recruitment independently of Arp1. It would be interesting to see how overexpression of the dominant-negative CC1 region of p150 that interacts with IC would affect the recruitment of dynein and dynactin to membranes. It would show whether dynein or dynactin can interact with membranes independently of each other and reveal if dynactin is recruited to the membrane through IC interaction.

6.1.3 Dynein recruitment to late endosomes and lysosomes via RILP

RILP is a Rab7 interacting protein that can interact with dynactin p150 and is known to recruit dynein to membranes in a process that involves LIC (Johansson et al., 2007; Jordens et al., 2001; Tan et al., 2011). Something that is worth taking into account when interpreting the RILP experiments from this project is the fact the membrane structures observed after overexpressing GFP-RILP are never observed in control cells and can be therefore thought of as somewhat aberrant. It would be interesting to assess how dynein interacts with endogenous RILP but the protein was difficult to detect in control cells. Although there are RILP antibodies that can detect overexpressed GFP-RILP by microscopy or Western blotting, no endogenous RILP could be detected in control cells using either method. This could be due to a lack of antibody sensitivity but may also suggest RILP is expressed at very low levels in the cell. Overexpressing GFP-RILP and introducing much more of the protein than is normally present in cells may affect the endocytic pathway in many ways independently of any dynein interaction. Indeed, it is well established that overexpressing Rab proteins of the endocytic pathway is not an ideal way to investigate their function as this leads to aberrant and enlarged organelles. As RILP is an effector of Rab7, it is possible that its overexpression has the same implications. We can therefore consider the GFP-RILP-positive membranes to be a poor experimental system for investigating the role of RILP as a Rab7 effector in membrane trafficking. Instead, overexpression of RILP provides a simple experimental approach for dissecting the proteins needed for dynein recruitment to RILP-positive LE and lysosomes. The functional implications of this in control cells are presently not well defined.
Published data has placed dynein recruitment to RILP-positive membranes downstream of dynactin due to an established interaction of p150 and RILP along with overexpression of a RILP construct lacking its p150-binding region (NΔRILP), which led to a loss of dynactin from NΔRILP-positive membranes (Johansson et al., 2007; Jordens et al., 2001). There is conflicting evidence from another study that suggests overexpressing dynactin p50 displaces p150 but not dynein from RILP-positive membranes (Tan et al., 2011). This is surprising because even if dynein is recruited to RILP independently of dynactin via LIC, p50 overexpression would not be expected to prevent dynactin association as it could still be recruited to these membranes via a p150-RILP or p150-dynein IC interactions.

The data in this thesis would point strongly towards a model in which LIC or Lis1 are necessary but not sufficient to recruit dynein and dynactin to RILP-positive membranes. Additionally, here it is shown that LIC1 and LIC2 share functional redundancy in this role, which is contrary to published data that suggest this mechanism is LIC1-specific (Tan et al., 2011). Interestingly, the role that Lis1 is playing in terms of dynein recruitment to RILP appears to be independent of Nde1 or Ndel1. Published data usually show that Nde1/Ndel1 and Lis1 work together in regulating dynein (Kardon and Vale, 2009), so it is intriguing to see a potential role for Lis1 that does not involve Nde1 or Ndel1. Additionally, Nde1 and Ndel1 are often observed to play a more important role than Lis1 in recruiting dynein to cargo (Lam et al., 2010; Vergnolle and Taylor, 2007), so a mechanism in which Lis1 is helping recruit dynein to a cargo independently of Nde1/Ndel1 is novel.

Looking at dynein/dynactin interaction with RILP only represents one mechanism for dynein recruitment to LE and lysosomes. It is apparent that there are other mechanisms by which dynein can associate with LE: Snapin, for example, is part of the LE-associated BLOC1 complex and can interact with dynein IC (Cai et al., 2010; Lu et al., 2009). The mechanism by which dynein is recruited to LE or lysosomes may be dependent on the proteins within these vesicles that are being transported, i.e. different membrane proteins may be responsible for dynein recruitment depending on the cargo within the vesicle.

The data presented in this thesis would suggest that dynactin requires dynein to associate with RILP-positive membranes but does not indicate whether dynein requires dynactin for association. Overexpression of p50 is unlikely to displace dynein as the p150 subunit will still be able to interact with dynein IC and RILP. Additionally, as Lis1 is playing a key role in dynein and dynactin recruitment to RILP-positive membranes, it is possible that Lis1 interacting with p50 could recruit dynactin and therefore p50-p150 without Arp1 would
still be able to interact with both dynein and Lis1. Finally, published data show that
depletion of β-spectrin has no effect on dynactin association with RILP-positive membranes
indicating dynactin recruitment is independent of Arp1 (Johansson et al., 2007), again
reinforcing the conclusion that breaking apart the complex is unlikely to affect recruitment
events. Taking this into account the best way to assess the role of dynactin recruitment to
RILP-positive membranes is by inhibiting dynein-dynactin interactions, which will be
discussed in section 6.4.

6.1.4 Conclusions surrounding dynein recruitment to membranes
The recruitment of dynein to membranes is fundamental for its function. The results from
this project suggest that dynein IC plays an important role in dynein recruitment to
membranes, although it is not clear by what mechanism. LIC and Lis1 are crucial for
recruitment of dynein to RILP—positive membranes, which highlights the complexity of
dynein regulation; different dynein subunits and accessory proteins play different roles in a
cargo-dependent manner. One of the most striking observations in these dynein-
recruitment data is that dynactin requires dynein to associate with membranes. The results
from the RILP-overexpression experiments suggest dynactin needs dynein to be
membrane-associated before dynactin is recruited, as loss of dynein from these
membranes leads to a loss of dynactin. These data open up the possibility that dynactin
associates with membranes via an interaction with dynein and thus is downstream of
dynein in membrane recruitment. This would fit with the proposal that dynactin’s primary
function is that of a dynein activator (see section 1.3).

6.2 Dynein and the endocytic pathway
Dynein is responsible for MT minus end-directed transport through the endocytic pathway
and dynein, dynactin, Lis1, Nde1 and Ndel1 are all involved in the correct positioning of EE,
LE and lysosomes \textit{in vivo} (Aniento et al., 1993; Burkhardt et al., 1997; Flores-Rodriguez et
al., 2011; Lam et al., 2010; Yeh et al., 2012; Zhang et al., 2011). Dynein is also important for
progression through the endocytic pathway and when its function is inhibited, both
endocytic recycling and delivery of material for degradation are significantly slowed
(Driskell et al., 2007).

There are two main elements in dynein function that can be regulated to influence the
transport of endocytic vesicles: the association with cargo and the activity of dynein that
produces movement. The association of dynein with endocytic vesicles can be achieved
through a variety of dynein subunit and accessory protein interactions with cargo. The
proteins dynein associates with are often involved in protein sorting and thus they may provide a link between sorting and transport. For example, several SNX proteins have been shown to interact with different dynein and dynactin subunits (Hong et al., 2009; Hunt et al., 2013; Traer et al., 2007). Rab proteins and their effectors can also interact with dynein and dynein accessory proteins (Horgan et al., 2010a; Horgan et al., 2010b; Jordens et al., 2001; Matanis et al., 2002; Tan et al., 2011; Wanschers et al., 2008). Another interesting group of proteins that may help recruit dynein to membranes in response to regulatory signalling pathways are JIPs, which act as scaffolds for JNK (Whitmarsh, 2006). Dynein has been implicated in JIP-mediated transport of endosomes, lysosomes and neuronal transport vesicles (Drerup and Nechiporuk, 2013; Fu and Holzbaur, 2013; Montagnac et al., 2009). The BLOC complex is required for correct trafficking of lysosomal resident proteins and Snapin, which is part of the complex, interacts with dynein IC (Cai et al., 2010). The ability of dynein and accessory proteins to interact with proteins that regulate trafficking in the endocytic pathway shows that the processes of protein sorting and vesicle transport are intrinsically linked.

Dynein activity may affect the way it moves membranes in the endocytic pathway. For instance, it has been proposed that Lis1 is only found on LE and lysosomes that are large in size and require the extra force production needed in order to move their relatively large mass (Yi et al., 2011). Lis1 has also been shown to be important in initiating transport of smaller vesicles, but is not present on their surface when they are moving (Egan et al., 2012; Lenz et al., 2006). Dynactin seems to be needed for transport initiation of endocytic vesicles at the +TIPs of MTs, whereas Lis1 is needed for initiation events along the whole length of MTs (Moughamian and Holzbaur, 2012; Moughamian et al., 2013). The transport initiation events that require Lis1 may be due to Lis1 inducing an idling complex of dynein, which enables dynein to be localised to cargo prior to MT minus end-directed transport (Yamada et al., 2008). Another possibility is that dynein requires the extra force that Lis1 and Nde1/Ndel1 can induce (McKenney et al., 2010), to initiate transport. Once moving, Lis1 might dissociate and as dynein interacts with either Nde1 or dynactin (McKenney et al., 2011), it may switch from Nde1/Ndel1 to dynactin for ongoing transport.

Another aspect of endocytic transport that dynein may influence is vesicle association with actin. When DHC is depleted, lysosomes accumulate in the cell periphery and this can be rescued by depolymerising actin filaments (Loubery et al., 2008). This led to the suggestion the effect DHC loss has on lysosomes is due to a misregulation of actin by dynein. Data
from this project alongside published work show loss of many different dynein subunits and accessory proteins disrupts lysosome distribution (Burkhardt et al., 1997; Lam et al., 2010; Tan et al., 2011; Yi et al., 2011). It seems unlikely that all these proteins are involved in regulating actin dynamics. Instead, the movement of endocytic vesicles may be achieved by cooperation between actin- and MT-driven processes. Htt has been shown to mediate a switch between lysosome association with MTs to actin filaments at the cell periphery and HAP40 may be involved in a similar process with EEs (Caviston et al., 2007; Pal et al., 2006).

The combined effects of membrane recruitment, force-production, transport initiation and actin association are likely to influence the positioning of endocytic vesicles. In this project the focus was how dynein subunits and accessory proteins may affect this positioning and how this might in turn change the size and trafficking capacity of these organelles.

6.2.1 The effects of dynein subunit or accessory protein loss on early endosome positioning

When dynein IC is depleted, EEs become aggregated (Figure 3.6), which is similar to the phenotypes observed when DHC is depleted (Caviston et al., 2010). This would suggest loss of IC is having a similar effect as loss of dynein all together. This may be due to the role dynein IC has in membrane association. In contrast, loss of Lis1 or Nde1/Ndel1 leads to EEs becoming dispersed throughout the cytoplasm (Figures 3.7 and 3.8) similar to previous reports (Lam et al., 2010). As Lis1 and Nde1/Ndel1 have been shown to be important for dynein membrane recruitment (Lam et al., 2010), inefficient dynein-membrane association may be causing this phenotype. Conversely, as Lis1 and Nde1/Ndel1 increase the force producing ability of dynein (McKenney et al., 2010), in their absence force-production might not be regulated properly and this could be leading to inefficient transport of EEs, which might contribute to their dispersed positioning. Additionally, as Lis1 plays a role in vesicle transport initiation (Egan et al., 2012; Lenz et al., 2006; Moughamian et al., 2013), it is possible that the Lis1 Kd or Nde1/Ndel1 Kd is reducing the number of transport events leading to less efficient transport. The similar EE distribution after Lis1 or Nde1/Ndel1 Kd would suggest they may be regulating dynein-driven positioning of these organelles together. However, Nde1/Ndel1 Kd leads to rings of EE forming at the cell periphery (Figure 3.8) which is not seen in Lis1 Kd cells, suggesting loss of Nde1/Ndel1 is having a specific effect on EE positioning that is independent of Lis1. The same peripheral EE ring formations, although smaller, can be observed in LIC Kd cells (Figure 3.5) so it may indicate LIC and Nde1/Ndel1 are involved in some EE positioning mechanism together.
Aside from the ring formations that can be seen in some Nde1/Ndel1 Kd cells, the depletions of IC, Lis1 or Nde1/Ndel1 do not cause an obvious peripheral accumulation of EEs despite the phenotypes of these Kds suggesting dynein is not moving EEs properly (figures 3.6-3.8). In contrast, LIC Kd does cause peripheral accumulation of lysosomes in some cells, indicating LIC has some roles in EE distribution that are independent of IC, Lis1 and Nde1/Ndel1 (discussed further in section 6.2.4). The lack of peripheral EEs in IC, Lis1 and Nde1/Ndel1 Kd cells could imply that kinesins may not be important for EE transport, since if they were, it would be expected when dynein is not moving EEs properly, EEs would localise to the cell periphery. This fits with published data that show kinesin-1 is associated with EEs and is needed for bidirectional movement, but that inhibition of kinesin-1 does not affect overall movement of EEs (Loubery et al., 2008). It is possible that kinesin-1 associated with EEs, mediates short bursts of bidirectional transport in order to overcome obstacles in the cell, but does not actively transport EEs long distances to MT plus ends.

### 6.2.2 The effects of dynein subunit or accessory protein loss on lysosome positioning

The lysosomes and LEs in Lis1 Kd cells are much larger than in any other protein depletions carried out in this project. A consideration when thinking about the transport of lysosomes in Lis1 Kd cells is that it has been reported that Lis1 is only needed for transport of larger LEs and lysosomes (Yi et al., 2011), but as most of the LEs and lysosomes in Lis1 Kd cells have become enlarged, it is probable that Lis1 would be needed for their transport. As such greatly enlarged lysosomes are not present in Nde1/Ndel1 Kd, IC Kd or LIC Kd cells it is possible that Lis1 has a role in influencing lysosome size that is independent of Nde1/Ndel1 and dynein. The role of Lis1 in the PAFAH complex seemed like a likely candidate for dynein-independent membrane trafficking regulation, but the depletion of the α1 and α2 subunits has no obvious effect on lysosome size (Figure 5.10). Whether Lis1 is influencing the size of lysosomes and LEs independently of dynein or not remains to be fully established and it is possible that α1 and α2 need to be depleted in conjunction with DHC to see the same effects as a Lis1 Kd.

If RILP-mediated recruitment of dynein is responsible for a proportion of dynein-driven transport within the cell, this may go some way to explaining why Nde1/Ndel1 Kd does not lead to the same peripheral clustering of lysosomes seen in some LIC and Lis1 Kd cells. The peripheral accumulation of lysosomes in IC Kd cells may be due to loss of general IC-mediated dynein membrane recruitment. However, only a subset of lysosomes reside in peripheral clusters so it is more likely to be due to a specific interaction of IC with a subset
of lysosomes, for example via the interaction with Snapin (Cai et al., 2010). Additionally, dynein IC can interact with Htt, and Htt has been shown to be important for lysosome transport (Caviston et al., 2010). If the lysosomes present at the cell periphery are due to an inability of dynein to interact with RILP, Htt or Snapin, this may account for why a proportion of lysosomes that are often enlarged are located towards the centre of the cell, as loss of these interactions may only prevent a subset of lysosomes being transported. In this model, loss of dynein function leads to a moderate enlargement of lysosomes, which is seen in all the Kd experiments carried out in this project (except Lis1 Kd where the enlargement is more pronounced), and specific subunit interactions with LE and lysosomal proteins mediate transport of subsets of lysosomes.

6.2.3 Lis1 and trafficking

Lis1 has a profound effect upon trafficking in the endocytic pathway. Lis1 depletion seems to prevent traffic between EE and LE as displayed by the failure of Alexa 488-conjugated EGF to move from EEs into LEs (Figure 5.12 -5.13). The presence of LC3 in the lumen of the enlarged lysosomes of Lis1 Kd cells (Figure 5.1) would indicate proteins in these organelles are not being degraded properly. This is further supported by the observation that blocking protein degradation in cells treated with lysosomal protease inhibitors results in LC3-positive lysosomes that are similar to those observed in Lis1 Kd cells (Figure 5.7). LC3 does not reach the lysosome/autolysosome via the endocytic pathway like most lysosomal proteins but arrives via the fusion of AP with lysosomes (Lamb et al., 2012), and therefore is unlikely to encounter an issue in being trafficked from EE to LE.

It is not clear whether the enlarged CD63-positive membranes in Lis1 Kd cells are LAMP1-positive (i.e. lysosomal) as CD63 can be found in both LE and lysosomes (Scita and Di Fiore, 2010; van Niel et al., 2011), and it was not possible to co-stain for the endogenous proteins to check. The presence of LC3 in autolysosomes that form in Lis1 Kd cells would suggest these membranes can fuse in a normal way as APs uses the same machinery as LEs to fuse with lysosome (Syntaxin 7, Syntaxin 8 and VAMP7) (Lamb et al., 2012). The retained ability for organelle fusion would mean it may be possible that the enlarged CD63 and LAMP1 membranes seen in Lis1 Kd cells are the same organelle. If LE and lysosomes were fusing to form these enlarged vesicles, it may be one of the contributing factors to their size.

The block in EE to LE traffic seen in Lis1 Kd cells may be preventing the delivery of newly synthesised lysosomal resident proteins to lysosomes via the maturation of EE into LE,
which can then fuse with lysosomes (Figure 6.1). Some of these resident lysosomal proteins might include hydrolases and if these are not being replenished in the lysosome, it could lead to lysosomes lacking full degradative function. If this were the case, enlarged lysosomes containing undegraded proteins would indeed be expected to form. A similar phenotype is observed in neuronal cells lacking Snapin, where GFP-LC3 accumulates and LAMP1 lysosomes become enlarged. It is thought that this is due to hydrolases not being transported to the lysosome via BLOC1 and dynein (Cai et al., 2010). The similarity in phenotypes suggests that Lis1 might play a role in regulating the Snapin-dynein IC interaction.

As SR-GFP Lis1, which is siRNA resistant, has been shown to the rescue Lis1 Kd phenotypes (Lam et al., 2010), it was though it might rescue the LC3-positive lysosome phenotype as well. However, SR-GFP-Lis1 did not reduce the amount of LC3 puncta, which could be because the time the Lis1 is expressing in cells (approximately 16 hour transfection period), may not be long enough to rectify trafficking of proteins from EE to LE, as this would involve the transcription and translation of the proteins that lysosomes require to be functional, and delivery to lysosomes in order for proper protein degradation to resume. Additionally, if the aberrant autolysosome compartments that form after Lis1 Kd are actually due to an autophagy-specific effect that is not directly linked to the endocytic pathway, it may take more time for the these compartments to be cleared from the cell.
Figure 6.1: Protein trafficking events in a control cell and a possible model for trafficking misregulation in Lis1 Kd cells. Here, proteins are not reaching the LE and are being transported to an unknown vesicle. This leads to non-functional lysosomes that fuse with autophagosomes to form enlarged organelles that accumulate undegraded protein.
6.2.4 Early endosome and lysosome distribution in LIC Kd cells with lamellipodia-like structures

The depletion of LIC causes EEs and lysosomes to distribute at the cell periphery in striking arrays or bands across the cell edge (Figures 3.5 and 3.11). These arrays form lamellipodia-like regions in the LIC Kd cells, which are structures rarely seen in control or IC, Lis1 or Nde1/Ndel1 Kd cells. It is possible the array formation of EE, LE and lysosomes is linked to the formation of these lamellipodia. LIC Kd cells that have not formed lamellipodia, often have lysosomes in peripheral clusters, similar to that seen in IC and DHC Kd cells (Figures 3.11 and 3.12) (Caviston et al., 2010). This suggests LIC is needed for lysosomal transport and in the absence of lamellipodia they accumulate in clusters, but when lamellipodia are present they get stuck in these structures and form arrays.

It is possible that LICs may be associating with EE and lysosomes through JIP proteins. The neuronal JIP3 is thought to mediate lysosomal transport in an LIC-specific manner and JIP4 has been implicated in transport of FIP3-Rab11 membranes, which are known to require LIC for proper transport and interact with LIC1 and LIC2 via FIP3 (Drerup and Nechiporuk, 2013; Horgan et al., 2010a; Horgan et al., 2010b; Montagnac et al., 2009). Unpublished data from our laboratory show that depletion or overexpression of JIP4 produces a peripheral array of lysosomes that is very similar to those seen in LIC Kd cells. It is therefore possible that LIC may play some role in JIP interactions that can promote endocytic vesicle transport.

The lamellipodia that form in some LIC Kd cells may be in part due to misregulation of cell polarisation. Dynein is needed for correct MTOC orientation to face a wound edge in scratch wound assays and this could be mediated via LIC2 as it interacts with Par3, which has been shown to regulate centrosome positioning (Palazzo et al., 2001; Schmoranzer et al., 2009). It is therefore possible that depleting cells of LIC1 and LIC2 leads to misregulation of cell polarisation in some way and this causes lamellipodia-like structures to form that are not normally observed in HeLaM cells. LIC may also play a role in Rac1 signalling in lamellipodia formation. Rac1 is needed to regulate actin and drive lamellipodia formation (Ridley et al., 1992). Data from our laboratory show that LIC1 can interact with Rac1 in JNK regulated fashion and this influences cell spreading and migration. Loss of LIC1 leads to increased cell spreading and lamellipodia formation (McNee et al., manuscript in preparation). This not only provides evidence for a novel mechanism in regulating lamellipodia formation in migrating cells but also fits in with the observations from this project. Furthermore, if LIC is interacting with JIPs on endocytic membranes this may link
JNK regulation of lamellipodia formation and endocytic transport. Additionally, it explains the lamellipodia structures observed in LIC Kd cells. In the absence of LIC, Rac may be overactive and cause lamellipodia to form, in which EEs and lysosomes that are not being transported by dynein are getting stuck (Figure 6.2). Indeed, it has been observed that lysosomes that are not associated with dynein get enmeshed in cortical actin (Caviston et al., 2010), so the actin filaments that are enriched in lamellipodia may be trapping the EEs and lysosomes in a similar manner. As it has previously been shown that depleting Htt can prevent this actin-association (Caviston et al., 2010), it would be interesting to see whether the peripheral arrays of EEs and lysosomes are lost if LIC and Htt are depleted simultaneously.

The spatial separation of CD63-positive LEs/lysosomes and EEs in LIC Kd cells that have formed lamellipodia is very striking. It would suggest the vesicles are being transported away from the cell periphery and thus not getting trapped in lamellipodia. IC and Nde1/Ndel1 Kd have very little effect on CD63 distribution in cells. Lis1 Kd leads to enlarged CD63-positive membranes and this is probably linked to trafficking issues (discussed in section 6.2.3), but the CD63-positive vesicles are not peripheral. Together these data would suggest that LIC, IC, Lis1 and Nde1/Ndel1 are not involved in regulating minus end-directed MT transport of CD63-positive vesicles. It is possible dynein may not be involved in this process at all, although overexpression of dynactin p50 does seem to cause CD63-positive compartments to become more dispersed (Figure 5.14). Dynein recruitment to these membranes may occur via other accessory protein or DHC directly. Alternatively, it could involve the subunits and accessory proteins studied here, but they could share redundancy so that loss of one protein alone does not affect CD63 distribution overall. One way to test whether dynein is involved in transport of these vesicles would be to deplete DHC and observe the positioning of CD63-positive vesicles. In summary, the movement of CD63-positive vesicles does not require LIC, IC, Lis1 and Nde1/Ndel1 and in LIC Kd cells this leads to spatial separation from EEs, which appear to be trapped in lamellipodia (Figure 6.2).

Dynein is regulated in many ways and thus the different dynein subunits and accessory proteins that are needed to modulate dynein function play different roles in the movement of diverse organelles. The results from this project highlight the similarities and differences in dynein subunit and accessory proteins in influencing endocytic compartment size (figure 6.3).
Figure 6.2 A possible model for LIC and JIP interactions in endocytic transport.
Figure 6.3: A summary of possible models that might account for distribution of size of endocytic vesicles in LIC, IC, and Lis1 and Nde1/Ndel1 Kd cells.
6.3 Dynein and Autophagy

Autophagy is the process by which cells can sequester a portion of the cytoplasm and target it for degradation in order to produce amino acids, sugars and lipids that can be reused by the cell. APs are transported by dynein and this has been linked to their maturation, which culminates in them fusing with lysosomes to become autolysosomes capable of protein degradation (Kimura et al., 2008; Lamb et al., 2012; Maday et al., 2012). APs need to interact with the endocytic pathway by fusing with the EEs and lysosomes and many of the proteins that regulate the endocytic pathway also regulate autophagy (Lamb et al., 2012). As HeLaM cells have a basal level of autophagy occurring, they provide an easy system for assessing autophagic vesicle size and position as autophagy does not need to be induced. The results in this thesis therefore indicate whether autophagy is being upregulated or whether there is a block in the autophagic pathway rather than giving any indication on autophagy induction. The data provide an initial insight into how specific dynein subunits or accessory proteins may be involved in this pathway.

6.3.1 LC3 puncta in LIC, IC, Lis1 and Nde1/Ndel1 depleted cells

IC and LIC depletion both lead to an increase in LC3 puncta (Figures 5.2 and 5.3). As dynein transport is linked to the progression through the autophagy (Maday et al., 2012), it is possible that dynein malfunction in LIC and IC Kd cells is slowing transport and leading to inefficient clearance of LC3. Conversely, the LC3 puncta increase might not be due directly to an issue in AP transport but rather via changes in the endocytic pathway. Preventing dynein-based transport has been shown to slow progression through endocytosis (Figure 5.14) (Driskell et al., 2007; Tan et al., 2011) and as endocytosis and autophagy are so closely linked this may also lead to a slowing in autophagy and thus to accumulation of LC3. Snapin (part of the BLOC1 complex) and its association with dynein IC are important for LE/lysosome transport in neurons, which mediate encounters with APs to promote fusion (Cai et al., 2010). Additionally, RILP is upregulated during autophagy (Bains et al., 2011) and overexpression of GFP-RILP leads to some RILP-positive membranes being LC3-positive (Figures 4.16). It is therefore possible that RILP might be important for LE/lysosome transport and fusion with APs. As loss of LIC may prevent dynein-RILP association (Figure 4.7) (Tan et al., 2011), it may prevent these LE and lysosomes being transported towards to AP and so reduce fusion events. Indeed, in either an IC or LIC Kd the loss of RILP- or Snapin-mediated dynein recruitment to LE/lysosomes may result in subsets of lysosomes being immobile and not coming into contact with APs. In this situation, the alternative
mechanism (RILP or Snapin recruitment) might be able to compensate partially for loss of the other, but may lead to less efficient lysosome-AP encounters and cause the accumulation of LC3 that is observed.

The Lis1 Kd phenotype seen is different to that of IC and LIC Kd and is much more striking. The autolysosomes that form would suggest that the AP and lysosomes are fusing but failing to degrade proteins (Figure 5.1). When these inactive lysosomes fuse with autophagic vesicles they do not degrade protein and LC3 accumulates, leading to an enlarged vesicle. Another factor to consider is that the inability of Lis1 Kd cells to traffic protein through the endocytic pathway (Figures 5.12-5.13) may also cause cells to become stressed and upregulate the amount of autophagy taking place in order to produce more nutrients. The potential upregulation of autophagy might contribute to the amount of LC3 present in cells (Figures 5.1 and 5.5).

Nde1/Ndel1 Kd does not have a profound effect on the abundance of LC3 puncta or LC3-II in the cell (Figures 5.4). One possibility is that Nde1/Ndel1 do not contribute to dynein-driven movement of APs. However, it does correlate with the effect depleting Nde1/Ndel1 has on lysosomes, which results in a less extreme phenotype than depleting IC, LIC or Lis1 Kd (Figures 3.11-3.14). This might suggest it is indeed the disruption to the endocytic pathway that is affecting the autophagy in cells. In IC and LIC Kd cells where lysosomes do not appear to be transported properly, the LC3 accumulates. This is probably due to inefficient encounters of AP with lysosomes. In Lis1 Kd cells where lysosomes seem to be functionally inactive, large autolysosomes form due to an inability to degrade LC3. In summary, the results from this project show how the regulation of dynein in the endocytic pathway might directly influence progression through autophagy.

### 6.3.2 A potential role for dynein in influencing autophagy via endocytic trafficking events

The Rab7 GEF HOPS promotes autophagy, endosome maturation and is important for LE tethering (Sun et al., 2010; van der Kant et al., 2013). RILP can interact with dynactin and HOPs simultaneously implicating Lis1 and LIC as potential regulators for HOPS since they are needed for the recruitment of dynein and dynactin to RILP (Figure 4.7-4.11) (van der Kant et al., 2013). Further evidence that dynein may be involved in HOPS regulation comes from observations in which depletion of the HOPS subunit hVps39 leads to EE clustering and lysosomal peripheral clusters (Flinn et al., 2010), which is similar to LIC and IC Kd phenotypes (Figures 3.6, 3.11 and 3.12). If dynein or Lis1 are involved in regulating HOPS
via RILP then this may have implications in autophagy as HOPS has been shown to promote autophagy progression (Sun et al., 2010). The HOPS-mediated Rab5/Rab7 switch has been shown to be needed for Rheb activation of mTORC1 (Flinn et al., 2010; Rink et al., 2005), which leads to inactivation of ULK-1 and prevents further autophagy initiation. If dynein and Lis1 contributed to a RILP-mediated regulation of the HOPS Rab5/Rab7 switch, loss of either protein from the complex may decrease mTORC1 activation and increase autophagy.

6.3.3 Dynein influencing autophagy outside of membrane trafficking
LC8 has been shown to regulate autophagy induction by sequestering Beclin via Ambra or Bim to MTs via dynein (Di Bartolomeo et al., 2010; Luo et al., 2012). Autophagy is already occurring in HeLaM cells but loss of IC may free LC8 and thus Ambra- and Bim-associated Beclin from MTs to upregulate autophagy. However, if LC8 has dissociated with dynein after IC depletion, LC8 could still be in complex with Ambra and Bim and this may be enough to inhibit Beclin activity.

6.3.4 Conclusions surrounding dynein and autophagy
The results from this project indicate LIC, IC and Lis1 influence autophagy progression. The data is relatively preliminary and there may be many reasons that loss of these proteins causes an increase in LC3 in cells. In light of the importance of dynein in progression through the endocytic pathway, the most likely explanation for dynein LIC and IC to be affecting autophagy seems to be via their role in promoting EE and lysosome fusion with AP through transport events. The extreme autolysosome phenotype seen in Lis1 Kd cells seems likely to be caused by lysosomes lacking the ability to degrade proteins, which caused a block in autophagy flux.

6.4 Future perspectives
The results from this project give an insight into how LIC, IC, Lis1 and Nde1/Ndel1 proteins influence transport and progression through the endocytic pathway and how this may affect autophagy. To further investigate the specific mechanisms that cause the phenotypes observed after protein depletion there are several approaches that could be taken. The next section will explore additional experiments that could be carried out to provide further information about how these proteins regulate movement within these pathways.
Lis1 kd has generated some intriguing results so far. One important question that remains to be answered is where EGF is being trafficked to in Lis1 Kd cells. An approach to investigate this would be to perform a screen to immunostain many different membranes after an EGF pulse chase experiment. Membrane proteins that would be of particular interest are those associated with EEs, SEs and LEs such as Rab5, Rab7, Hrs and APPL1/2. It is possible that EGF is being erroneously sent to REs as well so Rab11, Rab4 and TfR could be visualised to test this. In addition, it would be good to know whether the block in endocytic progression and extreme lysosome phenotype is a result of simultaneous misregulation of dynein and PAFAH or whether Lis1 has a role outside of these complexes. To test this, DHC and the α1 and α2 subunits of PAFAH could be depleted together. If the large autolysosomes that form after Lis1 Kd is a result of both these complexes being misregulated, it would be expected that the same large autolysosomes would form after their co-depletion. If the effects of Lis1 depletion are not due to a combined misregulation of PAFAH and dynein then it is possible that Lis1 is interacting with other proteins involved in the endocytic pathway. To investigate this GFP-Lis1 could be overexpressed in cells and immunoprecipitated. The IP could be analysed using mass spectrometry to see whether any proteins that are part of the endocytic pathway are interacting with Lis1.

One possibility discussed is that Lis1 Kd cells may be unable to send hydrolases and other lysosomal proteins to the lysosome and that this is resulting in a non-functional organelle. To test this, fluorescently tagged lysosome resident proteins (e.g. GFP-CD63) could be overexpressed in Lis1 Kd cells and their localisation could be assessed using immunostaining. If the incorrect trafficking between EE and LE in Lis1 Kd cells is linked to the large non-functional lysosomes it would be expected that the CD63 might localise to either EE or the compartment EGF is being sent to in these cells. To further define the large autolysosomes that form after a Lis1 Kd, correlative light microscopy-EM could be performed to test whether the aberrant structures observed in figure 5.6 are indeed LC3- and LAMP1-positive membranes.

The over-expression of GFP-RILP has proved to be a useful assay for studying dynein recruitment. However, what is needed now is better insight into how endogenous RILP behaves and whether the interaction between RILP, dynactin and HOPS (van der Kant et al., 2013), affects dynein-driven membrane traffic. One way to investigate this would be to express an N-terminal region of RILP that binds p150 and HOPS but not Rab7. This would likely block the interaction between endogenous RILP and dynactin/HOPS and prevent...
them associating with Rab7. The effect this has on LE and lysosomes would give a better indication of endogenous RILP function.

In terms of dynein and dynactin recruitment to RILP, this project has established that dynactin requires dynein, but not whether dynein requires dynactin. A simple way to test this would be to inhibit dynein IC interaction with p150 by overexpressing the IC binding regions of p150, CC1. If dynein requires dynactin for RILP association via a RILP-p150-IC interaction then dynein would be displaced from the membrane in the presence of CC1 (Figure 6.4B). If dynein is lost, then the results from this project would suggest that endogenous dynactin should be absent too. If dynein and Lis1 are present but endogenous dynactin is not, then it would be likely that dynein and Lis1 are interacting with RILP directly and recruiting dynactin via dynein IC (Figure 6.4C). Finally, if all 3 proteins were present it would be possible that dynactin is being recruited to RILP via the interaction of p50 and Lis1 (Figure 6.4D).

The co-IP experiments carried out give a good indication of the protein complexes present in vivo but with such a complex set of interactions it does not give any information about direct protein-protein binding. To find out whether LIC or Lis1 can interact directly with RILP, in vitro protein binding assays would need to be carried out. During this project RILP was sub-cloned into a GST vector (pGEX4T1). The purification of GST-RILP was attempted using E. coli under various conditions but the GST-RILP was insoluble. In future, differing approaches could be attempted, such as sub-cloning RILP into a different purification vector or trying to purify fragments of RILP that might be more soluble. If RILP or RILP fragments were purified, in vitro protein interactions could be tested. Additionally, during this project GFP-RILP co-IPs were carried out after Lis1 or LIC depletion, to see whether dynein no longer co-immunoprecipitated. Unfortunately, even when there was no detectable Lis1 or LIC protein in the input, a small amount of protein was found in the IP lanes of Western blots, making the experiment difficult to interpret. As GFP-RILP is so well expressed in transfected cells, the large amount of RILP may be interacting with the tiny amounts of protein remaining. To better control this, purified RILP or RILP fragments could be used to perform pull downs in which the amount of RILP protein introduced into the experiment is more tightly controlled.
Figure 6.4: Dynein interactions with RILP-positive membranes and possible outcomes of CC1 overexpression.
This project has shown intriguing differences in the behaviour of LEs and lysosome subpopulations. CD63 is found on LE and LAMP1-positive lysosomes. As both CD63- and LAMP1-positive vesicles become enlarged after Lis1 Kd it would useful to know whether they are the same compartment or two separate subsets of vesicles that have become enlarged, as this would tell us whether the compartments are fusing. Additionally, in LIC Kd cells LAMP1-lysosomes seem to form bands at the cell periphery but CD63-positive LE and lysosomes are often spatially separated from EE. It would therefore be interesting to see whether CD63-positive vesicles are spatially separated from LAMP1-lysosomes in these cells or whether the staining overlaps. There are no working antibodies available in the laboratory that work together to test this, so to get around this, fluorescently tagged CD63 could be expressed in cells and LAMP1 staining could be carried out to visualise these protein markers together. The distribution of EE and lysosomes in peripheral bands in LIC Kd cells seemed to be linked to the formation of lamellipodia. To test whether EE and lysosomes are getting stuck in actin networks in the lamellipodia as hypothesised, LIC Kd cells could be treated with Latrunculin B or cytochalasin D to depolymerise actin. If actin is holding the vesicles in these peripheral array arrangements, it would expected that after drug treatment this would no longer be observed. Htt has a role in vesicle-actin and -MT association (Caviston et al., 2010), so to test whether Htt is influencing these EE and lysosome peripheral arrays, LIC and Htt could be depleted simultaneously to see whether the peripheral bands of vesicles still form. If JIP proteins are involved in LIC-mediated EE and lysosome positioning, the JIP4 Kd and overexpression experiment mentioned earlier could be repeated to confirm the same lysosome phenotype is observed and to investigate whether the same effects on EE and CD63 are seen. Additionally, other JIPs could be depleted or overexpressed in the same way.

The results surrounding LC3 accumulation in Lis1, LIC and IC depleted cells indicate these proteins may influence autophagy. The data is somewhat preliminary though and it remains to be established how the proteins are affecting autophagy. Based on the results of this project it seems most likely that loss of these proteins is affecting flux through the pathway. To investigate whether these Kds are indeed preventing flux through the pathway or are actually promoting autophagy, a cell line in which autophagy needs to be induced would have to be used. If these proteins are influencing autophagy flux, LC3 puncta would not be expected to accumulate after protein depletions unless autophagy was induced by serum starvation.
The experiments in this project have involved biochemical analysis or observations based on fixed cells. As dynein is a motor protein that moves cargo the next logical step to investigate some of the phenotypes seen here would be to look at cargo moving in live cells. Our laboratory has previously tracked Rab5 expressed at low levels (Flores-Rodriguez et al., 2011). Using the same technique here to observe transport events and the speed of endocytic vesicle transport after these depletions would provide important functional data. It would of interest to find out how the EE move in IC Kd cells. They are aggregated but still quite central so using the tracking software we would be able to see whether they are being actively transported in either direction in the absence of IC. It would be useful to perform live cell imaging of CD63 and Rab5 simultaneously in LIC Kd cells as tracking the movement of EE and the CD63-positive LEs might tell us more about the spatial divide seen in fixed cells.

Since there is considerable evidence that endosome motility contributes to the function of the endocytic pathway, an outstanding issue is to what extent the functional changes brought about by Lis1Kd are due to alterations in endosome motility. To study this, live cell imaging could be used to analyse the movement of Rab5 EEs. In addition, as there is an problem with EGF trafficking between EEs and LEs following Lis1 depletion, it would be informative to determine whether the Rab5 to Rab7 transition (Rink et al., 2005) is taking place properly. Additionally, in Lis1 Kd cells the EGF is being trafficked to unknown vesicles at the cell periphery. To investigate this further, live cell imaging of cells from EGF pulse chase experiments would be informative. If they were carried out in cells expressing moderate levels of Rab5 they would tell us when EGF is leaving the EE and moving to the cell periphery.
6.5 Final conclusions

Dynein regulation is very complex. The results from this project give an insight into how dynein subunits and accessory proteins can contribute to dynein function within membrane recruitment, the endocytic pathway and autophagy. Dynein IC appears to be important for membrane recruitment generally, while LIC seems to be important for specific recruitment events. The results show dynactin requires dynein for membrane association in many contexts and opens up the possibility dynactin is recruited to membranes downstream of dynein. In terms of dynein interactions with RILP-positive membranes this project has demonstrated that the LIC1 and LIC2 share redundancy in this role and surprisingly Lis1, independently of Nde1 and Ndel1, is required. The data presented here also shows how dynein-driven transport of LE and lysosomes may contribute to autophagy flux. A novel role for Lis1 has been found in protein trafficking between EE and LE that has implications for protein degradation and autophagy. The way dynein recruitment to membranes and regulation of movement through the endocytic pathway feed into autophagy are particularly important in terms of understanding disease mechanisms. Impaired dynein function in neurodegenerative diseases has been linked to cells that cannot properly complete autophagy, which means aggregated proteins are not cleared from cells and this can contribute to toxicity and disease progression (Hara et al., 2006; Ravikumar et al., 2005). The results from this thesis give an insight into how some of the proteins involved in dynein regulation may have roles in these processes and thus contribute to proper protein degradation and therefore may have implications for protein aggregate clearance in cells.
7 References


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Appendix

Appendix 1: Western blots showing that the anti-Nde1 antibody recognises both Nde1 and Ndel1. Prior to lysis HeLaM cells were treated with control, both Nde1 and Ndel1, Nde1 only or Ndel1 only siRNA for 72 hours. Tubulin was probed as a loading control with TAT1.
Appendix 2: Magnetic Nano-Particle (NP) localisation after 1 hour at 4°C and chased for 105 minutes with NP-free media. After 1 hour at 4°C with NP, cells were incubated with the NP for a further 15 minutes at 37°C and chased with complete media lacking NP for 105 minutes. NP are highlighted with arrows. Extracellular space indicated with ES. Scale bar represents 0.5μm.
Appendix 3: Co-Immuno-Precipitations of GFP-RILP. HeLaM cells were transfected with GFP C1 parental vector or GFP-RILP, for 16 hours prior to lysis and incubated with anti-GFP IgG followed by protein-G beads. Lysate input and washed beads were separated using SDS-PAGE and proteins were analysed by western blotting, using indicated antibodies. Input is 3% of IP.