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The big and intricate dreams of little organelles:

Embracing complexity in the study of membrane traffic

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Running title: Complexity of organelles and membrane traffic

Table of contents synopsis: Organelle dynamics and membrane traffic are integrated within complex, multiscale, and non-linear regulatory networks that impact virtually every aspect of cell physiology. In this review, we discuss systematic approaches that have revealed the complexity of these phenomena, and discuss molecular versatility and organelle heterogeneity, as well as organelle adaptation under specific cellular conditions. Organelle dynamics and membrane traffic are functionally heterogeneous and adaptable processes that coordinate with higher-order system behaviour to optimize cell function under a range of contexts.

Keywords: heterogeneity, adaptation, systems biology, signaling, transcription, computational modeling, lysosomes, clathrin-mediated endocytosis, lipids, phosphoinositides

Abstract

Compartmentalization of eukaryotic cells into dynamic organelles that exchange material through regulated membrane traffic governs virtually every aspect of cellular physiology including signal transduction, metabolism and transcription. Much has been revealed about the molecular mechanisms that control organelle dynamics and membrane traffic and how these processes are regulated by metabolic, physical, and chemical cues. From this emerges the understanding of the integration of specific organellar phenomena within complex, multiscale, and non-linear regulatory networks. In this review, we discuss systematic approaches that revealed remarkable insight into the complexity of these phenomena, including the use of proximity-based labelling proteomics, high-throughput imaging, transcriptomics, and computational modeling. We discuss how these methods offer insights to further understand molecular versatility and organelle heterogeneity, phenomena that allow a single organelle population to serve a range of physiological functions. We also detail how transcriptional circuits drive organelle adaptation, such that organelles may shift their function to better serve distinct differentiation and stress conditions. Thus, organelle dynamics and membrane traffic are functionally heterogeneous and adaptable processes that coordinate with higher-order system behaviour to optimize cell function under a range of contexts. Obtaining a comprehensive understanding of organellar phenomena will increasingly require combined use of reductionist and system-based approaches.

Abbreviations

APEX, ascorbate peroxidase; BioID, Proximity-dependent biotin identification; CCP, clathrin-coated pit; CIE, clathrin-independent endocytosis; CLEAR coordinated lysosomal expression and regulation; CME, clathrin-mediated endocytosis; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ER, endoplasmic reticulum; GFP, green fluorescent protein; MITF, Melanogenesis Associated Transcription Factor); mTORC1, mechanistic target of rapamycin C1; ODE, ordinary differential equations; PTF1, pancreas transcription factor 1; siRNA, small interfering ribonucleic acid; TFEB, Transcription factor EB; TfR, Transferrin receptor; TFE3, Transcription factor E3; TFEC, Transcription factor EC; XBP1, X-box binding protein 1.

Introduction

The organization of eukaryotic cells into membrane-bound organelle compartments and the dynamic traffic of material between these locales have an underlying role in controlling a wide range of cellular functions. These functions include nutrient uptake, waste extrusion, ion transport, intercellular signaling, cell adhesion and migration, molecular biosynthesis, and regulated catabolism [1–5]. Collectively these cellular functions, regulated by organelle dynamics and membrane traffic, integrate to control many broad aspects of human physiology, including infection and immunity, tissue development and homeostasis, and systemic metabolism (**Figure 1**). Many studies have used reductionist approaches to resolve the mechanisms and regulation of membrane traffic phenomena and organelle dynamics, as well as the contribution of these processes to various cellular functions. These approaches have been very informative and have revealed molecular mechanisms that control cargo sorting, membrane fission, fusion, remodeling, targeting, and the interactions of organelles with other cellular components such as the cytoskeleton. In addition, the regulation of specific membrane traffic phenomena within several physiological contexts has been extensively studied (**Figure 1**), such as regulated endocytosis and exocytosis of vesicles harboring the facilitative glucose transporter GLUT4 by insulin signaling [4].

The types of approaches that are used broadly in cell and molecular biology research have relied on several key underlying assumptions [6], which include (i) the universality of specific aspects of organelle regulation and function, both at the intra- and inter-cellular level, (ii) that the integration of understanding of individual molecular mechanisms allows understanding of a broader system, the so-called “clockwork” approach, and that (iii) individual molecular components have relatively narrow scope of functions and are part of linear regulatory systems, such that manipulations of molecules reveals largely direct consequences of experimental manipulations. Moreover, since the advent of stable culture of transformed and immortalized cells in the 1950 and 60s, many studies have investigated the mechanisms, regulation, and function of organelle dynamics under idealized conditions. This approach simplified the variability observed in the study of primary cells isolated from animals and allowed study of cell biological phenomena that are relatively consistent.

Indeed, much has been learned about the mechanisms, regulation, and function of membrane traffic and organelle dynamics from reductionist approaches that are collectively aimed at enhancing understanding of molecular mechanisms to ever-greater detail. However, recent advances and discoveries have brought the complexity of membrane traffic and organelle dynamics to the forefront. These include the emerging understanding of the molecular complexity and versatility of organelles and the heterogeneity of membrane compartments and traffic, suggesting that universal properties of organelle regulation and function must be complemented with understanding of their context-dependent regulation and function. Moreover, the emergence of the important role of organelles as part of complex, non-linear cellular regulatory networks that sense extrinsic and intrinsic cues in order to maintain cellular homeostasis indicates that organelles are subjected to adaptation and are also extensively integrated within broader biological phenomena.

From this emerges the importance of complementing informative reductionist approaches with those that embrace complexity in biological systems. Such approaches that embrace complexity include the use of systematic approaches to study the entire spectrum of molecules or phenomena that exhibit heterogeneity instead of relying on ensemble averaging, examination of the context-specific properties of organelles, and the use of computational models to integrate specific phenomena within complex regulatory networks. Many of these approaches will be familiar to systems biologists, and these strategies have the potential to reveal important new information about the key roles of membrane traffic and organelle dynamics in cellular and systemic physiology. In this review, we discuss research efforts that approach understanding the complexity within membrane traffic and organelle dynamics, and the functional consequences from a variety of perspectives. We first discuss studies that have undertaken systematic characterization of molecules that regulate organelles or are found within specific organelles (e.g. functional screens, and "omics"-type approaches). Next, we examine the evidence for versatility of molecules and assemblies, and the resulting heterogeneity of membrane traffic and organelles. Further, we discuss the adaptability of membrane traffic and organelles, such as that resulting from sensing of extracellular cues and integration with pathways that control gene expression.

Systematic molecular and functional characterization

The modern era of cell biology has been driven in part by several waves of technological advances, such as the development of electron microscopy to study subcellular organization in the 1950s and the re-emergence of confocal microscopy in the 1990s. Indeed the seminal work of Palade, Claude and de Duve that culminated in the 1974 Nobel Prize was based in part on electron microscopy and subcellular fractionation approaches to provide an integrated understanding of organelles and a map of the traffic routes between them [7,8]. Furthermore, the work of Schekman and colleagues in the 1970s and beyond (also leading to a shared Nobel Prize in 2013) was critical to establishing the molecular basis of membrane traffic and organelles through the use of genetic screens to identify genes important for specific membrane traffic pathways (e.g. [9]). As such, the study of the molecular basis of organelle dynamics and membrane traffic has been influenced from an early stage by systematic functional screening.

Quantitative information about the composition of organelles and vesicles and a comprehensive understanding of regulatory networks are often important precursors to understanding biological heterogeneity and adaptation. Over the decades, there have been numerous studies and approaches aimed at obtaining the complete molecular composition of specific organelles and vesicle populations, as well as obtaining a systematic understanding of the genes and proteins that control and regulate membrane traffic and organelle dynamics. These approaches include biochemical isolation and purification of specific organelles coupled to different proteomic analyses and high-content imaging-based approaches, which we discuss below. Collectively, these have contributed to a corpus of protein (and genetic) interaction data that is available in BioC-Biological General Repository for Interaction Datasets (BioGRID) [10] as well as detailed interactome maps [11,12] and a subcellular proteome map of 30 subcellular structures [13].

Organelle proteomics. Various methods have been used to qualitatively or quantitatively measure the proteome of specific organelles [14,15]. These methods initially sought to use classical subcellular fractionation experiments coupled to mass spectrometry. Subsequently, refinements of these methods by analyzing the protein enrichment profile within multiple fractions that corresponded to various

organelles revealed organellar proteomes with higher fidelity and specificity [16,17]. Others have used stable isotope labeling with amino acids in cell culture (SILAC) to differentially label subcellular fractions with distinct heavy isotopes to allow quantitative comparison of protein enrichment within cytosolic, nuclear and nucleolar fractions [18]. Subcellular fractionation-coupled mass spectrometry was complemented by studies involving high-content imaging of a yeast strain library with each gene fused to GFP, allowing assignment of the localization of proteins to 22 different locales [17,19]. More recently, methods have been developed to utilize proximity biotinylation. BirA, a biotin ligase, biotinylates a specific acceptor peptide sequence that is fused to a protein of interest. This has been used to tag and isolate ribosomes at the surface of mitochondria, followed by identification of specific transcripts undergoing translation in close proximity to the mitochondria by deep sequencing of ribosome-protected fragments [20].

Two recent technologies in spatially resolved proteomics, both based on proximity biotinylation, offer exceptional opportunities for extending the systematic characterization of molecular components of organelles to incorporate context-specific and heterogeneous dimensions. BioID, a method developed by Kyle Roux, utilizes a promiscuous BirA [21]. While wild-type BirA biotinylates (via a biotinoyl-5'-AMP intermediate) an acceptor peptide in a site- and sequence-specific manner and has been used previously for studying protein-protein interactions [22,23], the R118G BirA mutant promiscuously biotinylates primary amines in a proximity-dependent fashion [24]. Similarly, APEX, developed by Alice Ting, uses an engineered soybean ascorbate peroxidase (APEX) that biotinylates proteins in its immediate vicinity in the presence of biotin-phenol and hydrogen peroxide [25,26]. In both approaches, samples are lysed after biotinylation and biotinylated proteins can be enriched using streptavidin pull-down. These proteins can be analyzed by quantitative mass spectrometry to provide a comprehensive catalogue of proximal interacting proteins to the protein of interest.

One major advantage of both APEX and BioID for studying membrane trafficking is that biotinylation of proteins 'records' both transient and stable interactions during the labeling period and obviates the concern with standard affinity purification where protein interactions need to be kept intact through the purification steps. A major difference between APEX and BioID rests on the period of time required to biotinylated proteins, which ranges in hours for BioID and one minute for APEX. Thus, the applicability of either technique depends on biological processes that are considered rapid or slow. BioID has successfully been applied to ephrin type-A receptor 2 (EphA2) [27], cell junction proteins occludin and claudin [28], and fibroblast growth factor receptor FGFR4 [29]. These studies have all identified interacting proteins during membrane traffic phenomena. Exciting works are underway in the Gingras lab to identify proteins to subcellular organelles using BioID [30]. As a more recent technology, APEX has been applied to map the proteome of the mitochondrial intermembrane space [31] and of primary cilia [32]. More recently, APEX has been used to study G protein-coupled receptor interactome with spatial and temporal resolution as the receptor undergoes clathrin-mediated endocytosis (CME) and traffics through endosomes, which has led to the identification of previously unknown network components [33]. Both BioID and APEX have generated much excitement in the cell biology community and are primed for applying to problems in membrane trafficking.

High content imaging-based approaches. High content imaging and screening has traditionally been used for drug discovery research, but in the last decade it has been increasingly used to investigate membrane traffic and organelle function. Earlier work used a microscope-based assay to screen for and identify new proteins involved in secretory membrane traffic [34]. Using immunostaining and GFP-tagged open reading frames in a transport assay, 20 new proteins were found to affect either secretory transport or Golgi morphology. More recent studies coupled high content imaging of membrane traffic through the secretory system with genome-wide RNAi screening to identify >2000 genes that may regulate secretion [35]. Together with findings from analyses of protein localization conducted by high content imaging of GFP-tagged proteins [19,34,35], and computational analysis of known protein and gene interaction networks, these approaches have revealed new aspects of regulation within and among cellular systems. For example, this work revealed an enhanced loading of the secretory membrane traffic system by epidermal growth factor (EGF) receptor signaling [35].

The continuous improvement of imaging technology and the development of automated extraction of information from images and subsequent analysis provide an unbiased approach to decipher patterns of cellular activities that arise from intrinsic and extrinsic factors, and how these regulate the

dynamics and function of organelles beyond the secretory system. For instance, the combination of high content imaging with graphical and probabilistic models was used to uncover how virus infection, endocytosis and membrane lipid composition relate to specific cellular states that are defined by the population context of a cell (i.e. local cell density, cell size, and colony edge) [36]. To extend this type of systems biology approach to identify regulatory interactions within the endocytic membrane system, 13 imaging assays using endocytic pathway-targeting fluorescent cargos and molecular markers for endocytic organelles were used in conjunction with siRNA knockdown of over 1000 genes to obtain image data of 50 million single cells and over 30 billion measurements [37]. After extensive statistical modeling and the calculation of hierarchical interaction scores, the authors were able to infer functional interactions between different genes and create a map of regulatory functional interactions in the endocytic membrane system. Comparable approaches have been used to study other membrane traffic processes, for example the systematic examination of the genes and proteins that regulate the initial formation of clathrin-coated vesicles [38] and autophagy [39], each revealing novel regulatory mechanisms within and among cellular system and processes. This type of systems biology approach has also led to mapping of the *dynamic* localization of the yeast proteome to defined subcellular locales by using high content imaging of >2800 GFP-tagged ORFs, and systematic examination of localization changes of these proteins upon genetic or environmental perturbations [40]. Future systematic study of membrane traffic and organelle function will undoubtedly be aided by the emergence of screening gene function with whole-genome CRISPR-Cas9 libraries [41].

These approaches have collectively and systematically characterized the proteins within specific membrane compartments, as well as provided insights into the plethora of genes and proteins that function and regulate organelles and their trafficking routes. Other proteome-scale assays to systematically understand the interactions between proteins and small molecules also provide important information for drug discovery for membrane receptors, such as by the use of a receptor-based system which detects specific pairs of protein-protein or protein-drug interactions[42]. Collectively, these provide a wealth of information to form hypotheses for further reductionist approaches, which will without a doubt reveal further insight into the mechanism and regulation of specific processes. Importantly, these systematic analyses also highlight the non-linearity of regulation and inter-connectedness of organelle dynamics in cellular physiology, thus allowing a better understanding of the complexity of membrane traffic and organelle dynamics. These studies also form the basis for resolving the mechanisms that underlie organellar heterogeneity, versatility and adaptability, and how these phenomena govern various cell functions. We examine these concepts and processes next.

Versatility and heterogeneity

Many of the molecules and molecular assemblies that control membrane traffic and organelle dynamics are capable of existing in multiple distinct states, which we describe herein as *versatility* (**Figure 2A-B)**. In other words, versatility can describe the ability of a molecule to form distinct assemblies or complexes. In turn, this can lead to *heterogeneity* of molecular assemblies or the processes that they control, such as organelle dynamics and membrane transport (**Figure 2B**). Significant molecular heterogeneity can arise from diversity within classes of molecules important for membrane traffic, such as lipids and glycans, as well as both intra- and inter-cellular heterogeneity of organelles. By extension, molecular versatility and heterogeneity generate cellular heterogeneity with regards to membrane traffic and organelle dynamics, even when considering populations of cell cultures that are largely genetically homogeneous. These collectively lead to cellular heterogeneity, the relevance of which is now becoming appreciated. Importantly, the molecular, organellar and cellular heterogeneity observed have substantial deterministic components that are derived from the historical and physical contexts of a cell [43]. Thus, there is a lot to be learned about complex regulatory networks by studying individual molecular phenomena and organelles, as opposed to methods that involve ensemble averaging. When coupled to computational modeling approaches, these approaches provide powerful insight into fundamental regulatory mechanisms about membrane traffic and ultimately cell physiology.

Molecular heterogeneity and context. The advent of mass spectrometry technologies has revealed the molecular diversity within classes of specific molecules, which has been particularly evident for the study of lipids relevant to membrane traffic and organelle dynamics. Phosphoinositides (PIPs),

formed by regulated phosphorylation of the inositol headgroup of phosphatidylinositol, have been well established as key regulators of specific stages of membrane traffic [44,45]. For example, the regulation of synthesis and breakdown of phosphatidylinositol-4,5-bisphosphate at the plasma membrane controls the assembly, scission and uncoating of clathrin-coated pits (CCPs) during endocytosis [46–48], and control of phosphatidylinositol-3-phosphate at the early endosome controls membrane tethering and fusion, and a number of other functions [49–51]. Lipidomic studies have revealed the molecular complexity of phosphatidylinositol and PIPs, demonstrating that these classes of lipids defined by headgroup are actually comprised of a wide range of individual molecular species that exhibit differences in their acyl chain composition [52]. These acyl profile differences among lipids occur as a result of highly regulated processes, supported by the observation that different classes of phospholipids exhibit unique preferences of acyl species. For instance, phosphatidylinositol, but not phosphatidylcholine or phosphatidylethanomine, is enriched in 18:0/20:4 acyl species [52–55].

Hence, to understand the complex regulation of membrane traffic and organelles by lipids and lipid dynamics, there is a need to quantify lipids as individual molecular species and not just classes of molecules [56], as well as to understand the functional consequences of this molecular complexity. While lipidomic approaches have proven very effective at quantitative measurements of individual lipid acyl species, assigning function to such individual species is one of the challenges at the frontier of lipid research. Some insight into the function of specific lipid acyl species has been obtained from manipulation of specific acyltransferases and lipid-metabolizing enzymes, such as lysocardiolipin acyltransferase (LYCAT) [54,55], lysophosphatidylinositol-acyltransferase-1 (LPIAT1) [57,58], and diacylglycerol kinase ε (DGKε) [59]. These studies established that specific acyl species have unique functions, demonstrating that the molecular heterogeneity of PIPs impacts organelle dynamics and cell physiology.

Lipid metabolic pathways are intrinsically complex and interconnected, and thus lipid composition and properties of lipids such as acyl profile are acutely sensitive to diet [60], signaling pathways such as those controlled by p53 [61], and stress signals [62]. As such, understanding the functional outcomes of lipid diversity will require complementing reductionist approaches with systematic and computational modeling approaches that can resolve the emergent behaviour of membranes and lipid composition. In this vein, an important role for adaptation of lipid composition to cellular environment was uncovered by a combination of transcriptional profiling, modeling single-cell behaviour and lipidomic analysis [63]. This approach revealed that cellular crowding, sensed by focal adhesion kinase (FAK), impacted the expression of a wide variety of genes including the phospholipid and cholesterol transporter ABCA1, which in turn impacted cellular lipid composition. Hence, within a population of cells, those within a crowded local environment express high levels of ABCA1 and have lower content of sterol esters and a higher content of polyunsaturated lipids, which broadly impacts the acyl profile of multiple phospholipids [63]. Importantly, the unique lipid composition of cells in low versus high crowding resulted in unique collective behaviour of membranes in each state, as observed by measurement of lipid ordering and activation of PI3K-Akt signaling associated with membrane fluidity [63]. This study highlights the power of systems biology approaches for uncovering relationships between diverse lipid profiles, the collective behaviour of membranes, and both individual and collective cell behaviour. While we have highlighted phospholipid and especially phosphoinositide heterogeneity here, many other classes of molecules such as glycans [64] exhibit analogous heterogeneity, regulated by various parameters such as metabolism [65]. Hence, obtaining a complete understanding of how the biochemical diversity present within classes of molecules is regulated to control cell physiology will require embracing this complexity, uncovering the contexts that control molecular heterogeneity, and the use of systems biology approaches such as computational modeling.

Heterogeneity of molecular assemblies and organelles in membrane traffic. There are hundreds if not thousands of different types of integral membrane proteins at the cell surface and within the endomembrane system. The majority of studies have largely focused on measuring the traffic of a few key receptors and transporters such as transferrin receptor (TfR), epidermal growth factor receptor (EGFR) and low-density lipoprotein receptor (LDLR), using these as models of membrane traffic between compartments. However, it has become apparent that the membrane traffic of various proteins is distinct, not only with respect to the specific compartments through which each transits, but also with respect to the use of common but versatile molecular machineries for membrane fusion and fission events.

For example, while TfR, EGFR and LDLR each uses CME for internalization from the cell surface, there are marked differences in the mechanisms and regulation by which this occurs for each receptor. CME occurs by the regulated assembly of clathrin, AP2 and myriad other proteins from the cytosol into 50-100 nm CCPs at the plasma membrane, resulting in membrane invagination, cargo receptor recruitment, and in some cases, scission into clathrin-coated vesicles [66,67]. There are hundreds of CCPs at the surface of any given cell and importantly, distinct cargo such as TfR, EGFR and LDLR are found in separate CCPs [68,69]. Further comparative analysis of the CME of distinct receptors has found differences in requirements for lipids [70], auxiliary proteins [68,71–74], and regulation by intracellular calcium [75]. Collectively these studies have revealed that understanding the systematic regulation of CME may be best accomplished by complementing assays that monitor the traffic of individual cargo receptors with systematic study of CCPs.

To this end, many studies have combined time-lapse fluorescence microscopy of clathrin and other proteins with systematic computational detection and analysis of CCPs to study the mechanisms and regulation CME. This has revealed broad heterogeneity of CCPs, including in size, distribution within the cell surface, lifetime and protein composition [48,70,76–86]. In addition, specific CCP properties have been linked to cargo receptor content [78,85]. This type of data has allowed the construction of computational models to describe CCP assembly and scission [87], from which now emerges the synergism between predictions made by these computational models and experimental testing that are at the core of systems biology.

While we have focused here on the heterogeneity of CCP assembly that regulates proteins at the cell surface, the concept of intrinsically versatile molecular assemblies that heterogeneously gate membrane traffic events has also been suggested for the retromer complex [88]. This indicates that the concept of intrinsically versatile molecular assemblies may apply more broadly to gate and facilitate many diverse membrane traffic events that control organelle dynamics. In addition to the heterogeneity of molecular assemblies that gate membrane traffic events, there is also the emerging concept of heterogeneity of organelles themselves [89]. In particular, the heterogeneity of lysosomes with respect to protein composition was first noted several decades ago [90]. In fact, a single cell can contain well over one hundred lysosomes that differ in shape, location, acidification, degradation capacity, and motility [90–96].

Cellular heterogeneity related to membrane traffic. Understanding how the versatility of molecular assemblies and diversity within a class of organelles may contribute to or be caused by cell-to-cell heterogeneity is fundamentally important yet poorly understood. Heterogeneity can arise from deterministic or stochastic inputs, which can then lead to cell-to-cell heterogeneity of organelles or membrane traffic over multiple temporal and spatial scales that reflect the historical and physical contexts (deterministic causes) or not (stochastic causes) [43]. Notably, while some phenomena can have heterogeneous properties initially thought to arise from stochastic causes, subsequent new information can reveal these to actually be largely deterministic in nature [43].

Organelles and their related molecular assemblies are regulated by both intrinsic and extrinsic cues, and at multiple spatial and temporal scales. Organelle heterogeneity can result in part from highly localized cues, such as CCPs containing specific receptors and the position of lysosomes within cells. Importantly, approximately half of the 590 human kinases (including those with protein, lipid, and carbohydrate substrates) regulate either CME or clathrin-independent endocytosis (CIE), as measured by the membrane traffic of vesicular stomatitis virus (VSV) and/or Simian virus 40 (SV40) [97]. This indicates that transport vesicles and organelles are intimately integrated with many signal transduction pathways, including those that respond to extrinsic cues (e.g. growth factor) or intrinsic cues (e.g. metabolism) [4]. Cellular environment, in the form of adhesion context and cell shape and size, also contributes to deterministic factors that underlie organelle heterogeneity, as revealed by the mostly homogenous positioning of organelles within cells grown on constrained micropatterns that homogenize cell size and shape [98].

Thus, given the diversity of inputs that control endomembrane traffic and more broadly other organelles as well, there are many regulated sources of cellular heterogeneity [43], many of which have to do with cell population context that controls lipid composition [63], endocytosis rate [36], and transcriptional activity [99]. This cellular heterogeneity reflects complex regulatory networks that can either cause or be caused by systematic differences in organelles or organelle function between cells, even in a genetically uniform cell population.

Importantly, organelles may do more than simply reflect or add to cellular heterogeneity based on the versatility and heterogeneity of the organelles themselves, as they may instead also limit cell-to-cell heterogeneity caused by biochemical noise. Conditions found within cells, such as the low abundance of substrates and products for many reactions lead to substantial impact on reaction outcomes by stochastic fluctuations and noise [100]. Given the interconnectivity of biochemical reactions, it is possible that random fluctuations amplify over time to produce large fluctuations along biochemical or signaling pathways, thus greatly enhancing cellular heterogeneity [101], as suggested from study of gene expression networks [102]. As such, compartmentalization of signals provided by membrane microenvironments or within specific organelles may serve as a passive filter to reduce biochemical noise. Specifically, this compartmentalization separates signals that are also subject to stochastic fluctuations generated in one compartment from the location on which the signals eventually act, thus allowing only the regulated signals but not noise to propagate [103,104]. Hence, organelles may not only respond to cellular cues that eventually lead to cell-to-cell heterogeneity derived from regulated cues, but may also serve to limit stochastic contributions to cellular heterogeneity by passive noise filtering [104].

Thus, organelle heterogeneity arises in part from the regulated versatility of molecules and molecular assemblies that control membrane traffic. This allows the generation of specialized membrane traffic structures as illustrated by CCPs, or organelles within the same class with distinct properties and functions, as illustrated by lysosomes. This versatility and intracellular heterogeneity of organelles illustrate the central role of organelles as part of tunable, regulatory networks that have broad impact on cellular physiology.

Adaptability of membrane traffic and organelles

Related to the concept of organelle heterogeneity is that of the adaptability of organelles and membrane traffic phenomena. Here we define adaptation as long-term changes in organelles or membrane traffic events, often involving transcriptional regulation, much of which remains underexplored (**Figure 2C**). Specifically, there is a paucity of knowledge about how cells "measure and adapt" the size, number and activity of organelles such as lysosomes, the endoplasmic reticulum, peroxisomes, and mitochondria to match their differentiation status, cell cycle stage, metabolic activity, or extrinsic cues. Indeed, Mills and Taghert proposed the existence of a special class of transcription factors called "scaling factors" that can gradually increase or decrease the activity of an organelle accordingly to the needs of a cell [105]. Here, we focus on two transcription-driven programs of organelle adaptation involving transcriptional regulatory networks: (i) lysosome biogenesis by Transcription factor EB (TFEB) and related transcription factors and (ii) scaling of the secretory pathway in acinar cells. These examples illustrate how the combination of systematic study of complex networks complementing reductionist approaches can synergize to improve our understanding of organelle biogenesis and adaptation.

Lysosome biogenesis by TFEB and related transcription factors. Lysosomes are a heterogeneous network of acidic organelles that enact degradation of membrane and luminal content by interfacing with various pathways including biosynthesis, endocytosis, autophagy and phagocytosis [106–110]. Lysosomes are not terminal organelles, as they were so often portrayed – they serve as platforms to sense and govern various cellular functions including infection and nutrient availability [108,109,111–115]. From this an important question arises: how do cells decide on the number, size and activity of lysosomes they require?

A significant step towards understanding how cells adapt lysosome activity was taken by the discovery that TFEB controls the expression of a network of over 400 genes, many of which encode proteins that serve in lysosomes and autophagy [116,117]. This network became known as the Coordinated Lysosomal Expression and Regulation (CLEAR) gene network and was characterized by the presence of the CLEAR element, a sequence proximal to the promoter of these genes to which TFEB directly binds [116]. TFEB and the related MITF, TFE3 and TFEC transcription factors, all of which exhibit various splice variants and that can heterodimerize with each other, are thus part of a complex regulatory network that facilitates lysosome adaptation and scaling in response to a number of intrinsic and extrinsic cues [118–120].

Activation of TFEB and stimulation of the CLEAR network is best understood in the context of starvation and conditions that stimulate autophagy. Under amino acid-rich conditions, the kinase mTOR is recruited to the cytosolic face of lysosomes as part of

the mTOR Complex 1 (mTORC1). Through a multifaceted pathway that senses amino acid concentrations, mTORC1 is stimulated on lysosomes [121–124]. Subsequently, mTORC1 phosphorylates and maintains TFEB in the cytosol [115,125,126]. In contrast, during starvation, mTORC1 is inactivated and the phosphatase calcineurin is activated [111,115,125,126]. The combined inactivation of mTORC1 and calcineurin stimulation dephosphorylates TFEB, eliciting its nuclear entry and enhanced expression of the CLEAR network. A similar pathway may control TFE3, which also responds to starvation and mTORC1 activity [119,127]. In this way, TFEB and TFE3 sense the intrinsic and extrinsic cues of metabolism to coordinate two catabolic pathways – autophagy and lysosome function – to help liberate nutrients and energy during amino acid depletion.

Recently, the range of regulatory inputs controlling TFEB, TFE3 and MITF have expanded significantly to broaden the complexity of the circuitry surrounding lysosomal adaptation. First, and perhaps coupled to autophagy, TFEB induces lipid breakdown by stimulating lysosome and autophagy gene expression and re-wiring mitochondria and metabolic pathways by stimulating PGC-1α, a transcription factor that controls mitochondrial function [128,129]. Second, TFEB and TFE3 are activated by various stresses such as protein aggregation, mitochondria damage, ER stress, and lysosome damage [119,130–133]. Moreover, the role of TFEB in immunity may represent an ancestral function since bHLH-30, a *C. elegans* ortholog, is important for *C. elegans* to mount an immune response and suppress bacterial growth [134]. Similarly, in mammalian macrophages, bacteria and bacterial products like lipopolysaccharides activate TFEB and TFE3 leading to upregulation of immuno-modulating cytokines and chemokines *in vitro* and *in vivo* [134,135]. Lastly, phagocytosis by macrophages activates TFEB to stimulate lysosomal activity and improve bactericidal activity against subsequent rounds of phagocytosed bacteria, indicating that lysosomal adaptation within the innate immune system controls pathogenic clearance [136]. Strikingly, and speaking to the intense interest to better understand TFEB and related transcription factors, several additional modulators were recently discovered including the kinases glycogen synthase kinase 3β (GSK3β), protein kinase Cβ (PKCβ), and the microRNAs miR33 and miR33* [137–139]. Collectively these studies demonstrate that cells integrate a diverse range of cellular signals to modulate TFEB, TFE3 and MITF, thus adapting lysosomal content, size, number and function to specific conditions.

Secretory pathway scaling in acinar cells. The pancreas and salivary glands host specialized secretory tissues composed of acinar cells that undertake massive bursts of regulated secretion of enzymes during ingestion of food. To handle this secretory demand, during their differentiation, acinar cells expand the rough ER, enlarge the Golgi apparatus, and scale up the level of proteins that are involved in biosynthesis, protein folding, biogenesis of secretory granules (zymogen granules) and exocytosis [140,141]. This developmental and functional programming of acinar cells necessitates at least three transcription factors in mammalian cells: XBP1, PTF1 and Mist1 [142–144]. All three appear to form a transcriptomic network that coordinates and synergizes to adapt acinar cells to their secretory life style, since deletion of any of these factors impairs the development of the pancreas and differentiation of acinar cells.

XBP1 is a transcription factor involved in the unfolded protein response in the endoplasmic reticulum; however, XBP1 plays a specialized role in acinar cells of pancreas and salivary glands by upregulating the biosynthetic machinery [142,145–147]. Indeed, $XBP1^{-/-}$ knockout mice with a XBP1 "knock-in" in liver to rescue embryonic lethality showed a profound defect in ER and zymogen granule biogenesis [147]. In comparison, PTF1 is a multi-subunit transcription factor that is also essential for pancreatic development [143,148]. Interestingly, XBP1 and PTF1 seem to induce expression of Mist1, a transcription factor that further defines the final differentiation and function of acinar cells and zymogenic cells [144,145,149–152]. Indeed, $Mist1^{-/-}$ presumptive acinar cells display defective, mis-localized secretory granules [144,152], whereas Mist1 overexpression suffices to induce acinar functions in cells [153]. In yet another example of networking and/or co-dependence, not only does PTF1 drive expression of Mist1, they are found together associated with promoter regions of over 1000 acinar-expression genes where they additively and/or synergistically drive their expression [143,148]. Examples of these genes include IP_3R3 [144], Ca^{2+} channels [154], signaling proteins [155], and the GTPases Rab26 and Rab3A, which mediate granule secretion [156,157].

Overall, we detailed these two examples of organelle adaptation to illustrate how transcriptional networks exist to build and tune the endomembrane systems to various differentiation states and/or environmental conditions. The deep understanding of these pathways emerged by using transcriptomic tools such as RNA-seq, microarrays and/or deep

sequencing of promoter regions isolated during chromatin-immunoprecipitation of the indicated transcription factors. However, there are many questions that remain. For example, the regulation of TFEB is non-linear and much more complex than previously thought. TFEB regulation likely exploits differential pre- and post-transcriptional and post-translational mechanisms to generate specificity. In addition, it is not known whether Mist1-dependent adaptation of the secretory pathway is limited to terminal differentiation processes or if it might play a role in adapting cells to a temporary high-capacity secretory state due to a transient or reversible stress. The complexity of these regulatory networks governing organelle dynamics and membrane traffic requires computational modeling approaches to complement ongoing reductionist studies. Some examples of such approaches have revealed the control of lysosomal networks by glycosaminoglycan and glycosphingolipid pathways [158]. The development of computational models of the complex, non-linear regulatory pathways that gate adaptability of organelles will accelerate understanding and discovery of the mechanisms and impact of these phenomena.

Bringing it all together: modeling and integration into systems behaviours

Despite the progress that biologists have made using reductionism to explain cellular and molecular processes, the reductionist approach cannot account for the emergent properties and complexity of biological systems [159]. Computational modeling has become an essential tool and is an indispensible part of systems biology. Importantly, computational methods provide a means to integrate experimental data to build predictive models of complex biological processes [160]. Cell signaling, which intimately ties to membrane trafficking pathways, is a common system for building network models of molecular interactions.

Structural network methods have been used in genomic or proteomic studies to provide correlations between molecules in large networks. While the functional patterns can be inferred by statistical methods, it generally provides a static view of molecular interaction with limited predictive power. On the other hand, differential equation methods based on ordinary differential equations (ODEs) can be highly predictive. However, the predictive power relies on knowledge of kinetic parameters that are often unknown. For a characterized system, ODE models can be quite effective, as in the example of an experimentally parameterized two nonlinear ODE model to describe cell cycle oscillation in *Xenopus laevis* oocytes [161]. An intermediate compromise between structural analysis and ODE models is the logic-based network model pioneered by Kauffman [162]. Logic-based models approximate biochemical regulation and provide qualitative approximation of chemical reaction kinetics [163]. Boolean models, which are logic-based models with two binary states (*ON* and *OFF*), can be used to construct a signaling network that infers indirect molecular relationships from experimental data [164]. Rule-based modeling has gained a lot of attention recently due to the accessibility to biologists as rule-based models have simple syntax. The models can be used to generate computational models to provide quantitative or qualitative predictions on the system's emergent behaviours. For example, this approach has been used to uncover unexpected roles of a specific phosphatase in the regulation of early T-cell receptor signaling [165].

As membrane traffic and cell signaling occur at specific locations in a cell, spatio-temporal models that are formulated as reaction-diffusion systems can simulate collective behaviour of cellular processes. Several spatio-temporal models exist, including compartment-based models, agent-based models, and lattice-based models. Compartment-based models can capture the dynamic rearrangement of compartments and the molecular transport between them. Agent-based models consider a collection of decision-making entities, known as agents, which make decisions based on a set of rules and the environment that surround the agents. Agent-based modeling has been applied to study autophagy regulation [166] and the NF-κB signaling pathway [167]. The above short survey describes some of the common computational modeling approaches used in systems biology. In the sections below, we will specifically focus on modeling CCP assembly during CME and some conceptual framework to link membrane trafficking to other cellular processes.

Computational modeling. Mathematical and computational approaches can complement experimental studies in membrane trafficking to allow for physical understanding of the process and to explore parameters and their ranges that may not be easily accessible by experimental means. This is particularly attractive for modeling single molecular

assemblies, such as vesicle shape changes during endocytosis as there is a rich and deep understanding of the energetic cost for bending membrane as an elastic sheet using the classical Helfrich theory [168]. Taking the case of CME, computational modeling of the dynamics and energetics of membrane curvature is now an integrated approach for the study of systems biology of CME [169]. The experimental finding that membrane and cell tension regulate CCP dynamics [77,170,171] prompted several modeling works to investigate the role of tension in regulating CCP morphology and size. The requirement of actin dynamics to form a closed clathrin-coated bud shape in high-tension conditions provided support for two modeling studies that indicate that protein-induced snap-through instability can offset tension and drive CCP growth [172,173]. Further modeling revealed that energetic cost is sensitive to the geometry of membrane shape during vesicle formation [174] and confirmed experimental work that showed a reduction in CCP size at high tension [171]. Future modeling efforts should increasingly focus on multiscale approaches to integrate molecular dynamics simulation with continuum Monte Carlo simulation to study protein-membrane interactions in membrane trafficking processes [175].

Integrating membrane trafficking into systems behaviours. The emerging view that endocytosis and membrane trafficking are closely integrated with other cellular behaviours can be rationalized by their critical roles in regulating signal transduction [66,176]. It is also well appreciated from earlier work that endocytosis and exocytosis can be regulated by physical properties like membrane tension [177]. Thus, from both biochemical and physical angles, membrane trafficking can regulate membrane and protein compositions in a spatiotemporal manner that has a direct impact on cellular systems behaviours. Here we will highlight two major cellular processes, cell migration and cell division, that present dramatic cell morphological changes and interface with membrane trafficking.

Directional cell migration is a coordinated process of polarized membrane protrusion, attachment, contraction at the rear end, and detachment. It was recognized some time ago that cytoskeleton and membrane flow cooperate during cell migration [178]. Membrane or cell tension has been shown to regulate exocytosis and endocytosis [170,171,179,180], and membrane tension is also known to regulate cell migration [181]. Thus, it is entirely possible that endocytosis is an upstream process that regulates signaling pathways leading to actin cytoskeleton rearrangement. Interestingly, CCPs are spatially organized in a migrating cell along the posterior and anterior axis a well as between ventral and dorsal surfaces [182,183]. Furthermore, CCP dynamics slowed down during morphological changes in a *Drosophila* embryo, illustrating an effect of mechanical cues on endocytosis during development [184]. In addition to the endocytic regulation of cell surface receptors that are involved in cell migration, in recent years, other membrane trafficking machineries have been connected to cell migration. The endosomal sorting complex required for transport (ESCRT) was found to mediate the rapid closure of small wounds made at the plasma membrane [185]. Interestingly, ESCRT-III machinery is also involved in repairing nuclear envelope rupture during 3D cell migration under confined geometry [186,187]. This new finding is particularly exciting and highlights the intersection of membrane trafficking machineries with cell migration.

Cell division is a complex and heavily regulated process as the division of a parent cell to two daughter cells requires precise separation of chromosomes. Cell rounding by actomyosin contraction is a prerequisite to cell division and the accompanying increase in cortical tension has called to the question whether or not endocytosis is coordinated during cell division. Two opposing findings that endocytosis is continuous throughout the cell cycle [188] and endocytosis is strongly inhibited in mitosis [189] have led to disagreement in answering the aforementioned question. This apparent discrepancy was resolved when it was found that differences in how dividing cells are prepared and how temperature shift is performed could explain the different conclusions [190]. Furthermore, it was shown that actin engagement can restart CME during mitosis [191], which fits with the finding that actin dynamics can counteract membrane tension during CME [170]. Additionally, endocytic accessory proteins are part of a network that interfaces with actin polymerization and exocytosis [192], both of which are important in controlling cell shape during mitosis. Regardless of the exact nature of the relationship between endocytosis and mitosis, CME proteins and other membrane trafficking machineries such as those in recycling have been shown to play a role during cell division [188,193,194].

Conclusions

In this review, we discuss examples of membrane traffic phenomena about which our understanding has been greatly enhanced by complementing reductionist approaches aimed at understanding in-depth molecular mechanisms with approaches used by systems biologists to understand complex systems. Some of these approaches have coupled the use of data obtained from systematic study of molecular heterogeneity or regulatory networks to models of organelle dynamics and membrane traffic phenomena under various conditions, thus revealing new information about the interdependence of these processes within the context of cellular and systemic physiology.

There remains much to be explored and understood with respect to the cell physiological significance of molecular, organellar and cellular heterogeneity, as well as about the mechanisms that regulate organellar and cellular adaptation. A comprehensive understanding of the complexity of regulation and function of organelle dynamics will require study of the impact of various metabolic or physical cellular contexts, as well as the integrated impact of intrinsic or extrinsic signals. By undertaking these types of approaches, we can better understand how little organelles can have big impact on cell physiology as a result of being key components within intricate cellular regulatory networks.

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Figure Legends

Figure 1. Membrane traffic phenomena integrate a variety of signals and in turn exert control over cell physiology. Shown is a diagram depicting a variety of signals that each control specific membrane traffic processes. In the central panel, two model dynamic organelles are shown undergoing vesicle-dependent membrane traffic and selective binding of specific protein signals. In turn, each membrane traffic phenomenon can control a variety of specific signals, by regulating signal transduction, the access of proteins to substrates or products (e.g. in the extracellular milieu) or localization of transcription factors. Collectively, this allows specific stages of membrane traffic to function as key regulatory nodes at the intersection of complex cellular regulatory systems.

Figure 2. Organelles exhibit versatility, heterogeneity, and adaptation. Shown are model histograms depicting the frequency of organelles exhibiting a specific value for a particular property (e.g. size, location, composition, etc.), and outcomes associated with organelle(s) of that property. These models depict examples of (***A***) a relatively homogenous population of a specific organelle, (***B***) a specific class of organelle that is controlled by versatile molecules, giving rise to organelle heterogeneity, as shown by the example of three subpopulations of that organelle type, each with specific distinct properties and each with a specific outcome on cell physiology, and (***C***) a population of a specific organelle that undergoes adaptation to a new state in response to a signal or cue, thus leading to a new set of properties and alternative outcome on cell physiology.

Figure 1

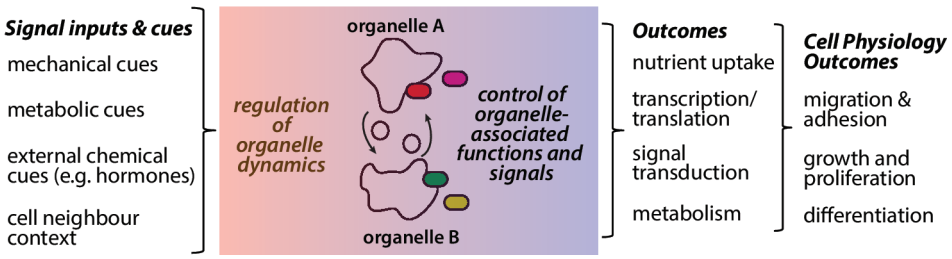


figure 1 - inputs and outputs final.eps

Figure 2

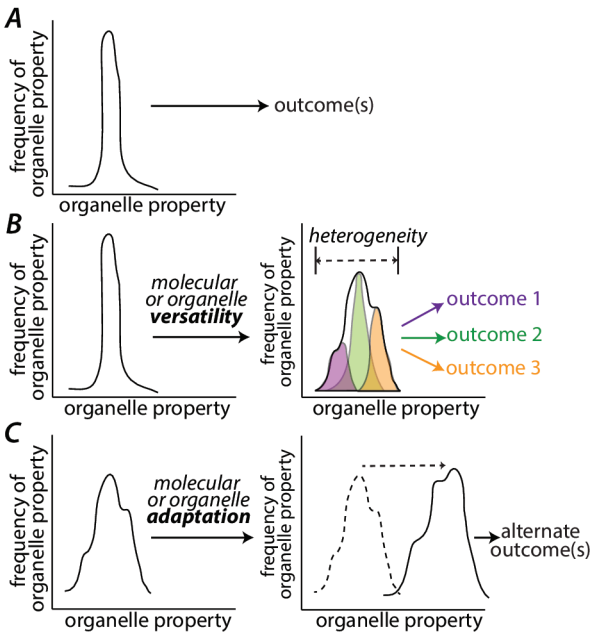
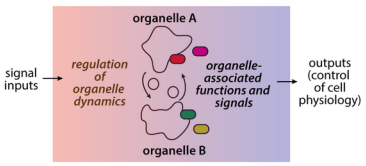


figure 2 - versatility heterogeneity and adaptability final.eps

Summary Figure for Table of Contents



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