Cell Systems
Review

Systems-wide Studies Uncover Commander, a Multiprotein Complex Essential to Human Development

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Recent mass spectrometry maps of the human interactome independently support the existence of a large multiprotein complex, dubbed "Commander." Broadly conserved across animals and ubiquitously expressed in nearly every human cell type examined thus far, Commander likely plays a fundamental cellular function, akin to other ubiquitous machines involved in expression, proteostasis, and trafficking. Experiments on individual subunits support roles in endosomal protein sorting, including the trafficking of Notch proteins, copper transporters, and lipoprotein receptors. Commander is critical for vertebrate embryogenesis, and defects in the complex and its interaction partners disrupt craniofacial, brain, and heart development. Here, we review the synergy between large-scale proteomic efforts and focused studies in the discovery of Commander, describe its composition, structure, and function, and discuss how it illustrates the power of systems biology. Based on 3D modeling and biochemical data, we draw strong parallels between Commander and the retromer cargo-recognition complex, laying a foundation for future research into Commander's role in human developmental disorders.

Large-scale exploratory studies of the mammalian interactome are providing unparalleled information about the architecture of biological pathways and protein complexes (Wan et al., 2015; Dey et al., 2015; Hein et al., 2015; Huttlin et al., 2015; Li et al., 2014). When considered together, these networks identify overlapping parts of an entirely new and highly conserved metazoan multiprotein complex that we have dubbed "Commander" (Figure 1; Box 1), named because it contains up to ten copper metabolism Murr1 domains (COMMDs). Current data lead us to estimate that the Commander complex comprises up to 14 different proteins that equal approximately 600 kDa in mass. Commander genes are highly conserved in metazoa and near-ubiquitously expressed across tissue types (Burstein et al., 2005; FANTOM Consortium and the RIKEN PMI and CLST (DGT) et al., 2014; Wang et al., 2015). Although the molecular function of Commander genes is largely unknown, they are linked to endosomal protein sorting (Bartuzi et al., 2016; Li et al., 2015; Phillips-Krawczak et al., 2015), as well as proinflammatory signaling (Maine et al., 2007; Starokadomskyy et al., 2013) and hypoxia adaptation (van de Sluis et al., 2010; van de Sluis et al., 2007). Defects in Commander and its interaction partners are also implicated in Ritscher-Schinzel (RS)/3C (cranio-cerebro-cardiac) syndrome (Elliott et al., 2013; Kolanczyk et al., 2015; Voineagu et al., 2012), a rare developmental disorder characterized by craniofacial abnormalities, cerebellar brain anomalies, congenital heart defects, and intellectual disability (Ritscher et al., 1987).

This review presents the combined evidence from systems-wide and small-scale proteomics experiments that Commander functions as a physically associated and fully integrated complex. More generally, the discovery and characterization of Commander serves as an example of the power of systems biology to uncover new biological machines and phenomena (Box 1). We describe the conservation and abundance of Commander, and preliminary functional analyses of complex subunits at both the molecular and organism level. Finally, we speculate on the similarities between the 3D structure, architecture, function, and molecular mechanism of Commander to retromer and NDC80, and the potential role of the complex in developmental disease.

Discovery of the 14-Subunit Commander Complex by Large-Scale Proteomics and Phylogenetic Profiling Efforts

Several recently published human interaction networks describe interrelated pieces of the Commander complex (Wan et al., 2015; Dey et al., 2015; Hein et al., 2015; Huttlin et al., 2015; Li et al., 2014). The independent approaches used by these studies, which apply a variety of experimental and bioinformatic strategies to build global molecular interaction maps, make this evidence especially compelling. Taken together, these provide an initial set of candidate proteins that comprise Commander (Figure 2).

A putative form of Commander was identified by our lab in a set of extensive experiments to examine the composition of soluble multiprotein complexes among diverse metazoan models (Wan et al., 2015; Drew et al., 2017). These involved biochemical fractionation of native macromolecular assemblies from different species followed by quantitative mass spectrometry (MS) to determine protein complex membership and produce a map of protein complexes common to animals. The results suggested that Commander, one of the largest novel complexes to be uncovered in this study, comprises up to 15 different structurally uncharacterized subunits (Figure 2). Ten of these,

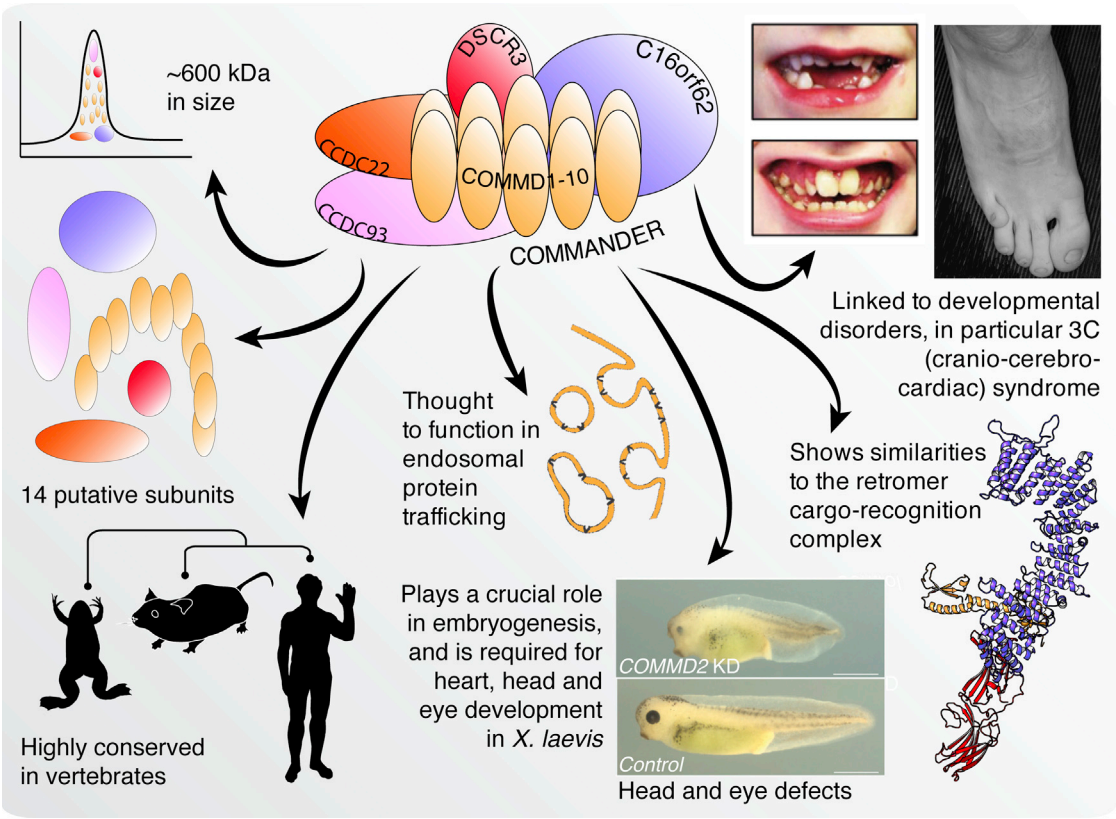


Figure 1. Overview of the Commander Complex
Commander is thought to contain up to 14 subunits equaling approximately 600 kDa in mass (Wan et al., 2015; Drew et al., 2017), is highly conserved in vertebrates, and functions in endosomal protein trafficking (Bartuzi et al., 2016; Li et al., 2015; Phillips-Krawczak et al., 2015). It also plays a crucial role in embryogenesis (Wan et al., 2015), and is linked to developmental disorders (Kolanczyk et al., 2015; Ritscher et al., 1987; Starokadomskyy et al., 2013). We suggest that Commander may function as a retromer-like sorter of cargo proteins within the endosomal system.

COMMD1-10, are structural and functional homologs from the COMMD family. They are defined by a unique and conserved C-terminal COMM domain that varies from 68 to 77 residues and mediates the formation of multimeric COMMD complexes (Burstein et al., 2005). The COMMDs also contain a more variable N-terminal α-helical domain of 18–151 residues (Sommerhalter et al., 2007). Other Commander components identified in this study include two coiled-coil domain proteins CCDC22 and CCDC93, and the protein C16orf62, all of unknown function, the endophillin-B1 SH3GLB1, and the Down-syndrome-related protein DSCR3.

Using perhaps the largest set of affinity purification-MS (AP-MS) experiments to date, Huttlin et al. (2015) profiled the BioPlex (biophysical interactions of ORFEOME-derived complexes) network. This involved 2,594 AP-MS experiments performed in human cell culture to characterize 23,744 interactions and connect 7,668 proteins. Although the BioPlex network of the human interactome is calculated by a different method to the metazoan complex map from co-fractionation-MS experiments, a complex almost equivalent to Commander was identified as a community of 18 proteins (Figure 2).

In a third independent large-scale proteomics study, Hein et al. (2015) applied a technique called quantitative bacterial artificial chromosome GFP interactomics to generate a library of 1,125 HeLa cell lines expressing GFP-tagged bait proteins that express under near-endogenous levels. By using these for GFP immunoprecipitation (IP)-MS experiments, the authors were able to detect specific interactions, estimate interaction stoichiometries, and measure cellular abundances within the human interactome, recovering a total of 28,504 interactions between 5,462 proteins. Several GFP-labeled bait proteins (CCDC22, CCDC93, and the Ran-binding protein RANBP1) pulled down prey proteins consistent with the Commander subunits predicted by the other two experimental interactome studies (Figure 2). At the time, this group was part of the largest assembly found in the study with no current annotation in the CORUM database (Ruepp et al., 2010).

Commander components have also been independently predicted by several large-scale bioinformatics studies (Dey et al., 2015; Li et al., 2014). Dey et al. (2015) applied phylogenetic profiling to determine functional links between human genes. This approach assumes that genes that function together are gained and lost together in evolution (Gabaldon and Koonin, 2013; Pellegrini et al., 1999). In this study, an extended phylogenetic analysis was used to compare the evolutionary history of orthologous groups of genes, including the many gene duplications present in the human genome. The authors report a set of co-evolving human orthogroup phylogenetic modules that

Box 1. Lessons from Commander

The Commander complex serves as a general example of the power of systems biology to uncover new biological phenomena by first visualizing the "big picture," then focusing on novel elements revealed from this vantage. Such holistic, top-down approaches highlight entire systems, and connect nicely with mechanistic and clinical studies on genes and processes of interest. System-wide studies also provide alternative contextualization for previously undertaken gene-specific studies. The latter provide mechanistic and disease-related links, leading to rapid generation and refinement of hypotheses about the system as a whole.

There are often clear trade-offs between large-scale and focused approaches. For example, the risk of losing information by simply not probing for the correct interaction is vastly reduced when many experiments are integrated in a quantitative discovery framework, with global control over false-positives, but the finer details of cellular machinery may be missed.

In the case of Commander, there was a mutually beneficial convergence of these discovery-based, proteome-wide, and specific smaller-scale approaches. Here, proteome-wide interactome studies, combining results from nearly 10,000 individual MS experiments, served to create an overview map of protein complexes in human and animal cells. The "strength in numbers" of the large-scale proteomics uncovered a large, poorly characterized cellular machine; comparison of the full complex with prior small-scale studies mutually supported and reinforced both datasets, and the resulting syntheses across these studies should now help guide targeted biochemical and structural studies to determine complex stoichiometry, architecture, and function.

represent predictions of functional units, one of which contains putative Commander subunits COMMD1, COMMD2, COMMD4, CCDC22, C16orf62, and DSCR3 (Figure 2). These predictions match those from an alternative phylogenetic profiling approach to identify new components of biological pathways called "clustering by inferred models of evolution" (CLIME) (Li et al., 2014). CLIME uses an input gene set of a known pathway to predict new genes that share the same evolutionary distribution, and places Commander subunits COMMD3, COMMD4, CCCDC22, CCDC93, and C16orf62 in the same evolutionary module (Figure 2). It is interesting to note that, although these bioinformatic analyses identify fewer potential Commander subunits compared with the experimental approaches, the particularly strong co-evolution of certain components suggests they represent a stable subcomplex.

We suggest that the strongest candidates as core components of Commander are the 14 protein subunits identified by at least three or more of the independent studies profiling the human interactome (Figure 2). These are COMMD1-10, CCDC22, CCDC93, C16orf62, and DSCR3 (Table 1). Structural modeling suggests that the COMM domain adopts a fold most similar to a pleckstrin homology domain, with an antiparallel β sheet followed by a C-terminal α helix (Table 1). CCDC22 and CCDC93 are predicted to be coiled-coil helical proteins that share common ancestry with the microtubule-associated proteins NDC80 and NUF2, and several proteins that form part of the intraflagellar transport (IFT) system key to the assembly and maintenance of microtubule-based cilia and flagella in eukaryotes (Rosenbaum and Witman, 2002; Schou et al., 2014). This suggests CCDC22 and CCDC93 might also form microtubule-bound complexes. C16orf62 is predicted to contain α-helical repeats, and DSCR3 is predicted to adopt an arrestin fold (Table 1). Both C16orf62 and DSCR3 are predicted to share strong structural homology with subunits in the retromer cargo-recognition complex, and we address this in more detail in a later section.

The results of these high-throughput screens are supported by more focused biochemical and cell biology experiments, many of which represent detailed work undertaken independently by the Burstein group (Bartuzi et al., 2016; Li et al., 2015; Mao et al., 2011; Phillips-Krawczak et al., 2015; Starokadomskyy et al., 2013; van de Sluis et al., 2010). A number of smaller-scale co-immunoprecipitation (coIP) assays have been reported that are consistent with the presence of stable Commander subcomplexes (Figures 3A–3F). All ten COMMDs can apparently interact with each other through COMMD1 (Burstein et al., 2005) (Figure 3A), individually bind to CCDC22 (Starokadomskyy et al., 2013) (Figure 3B), and uniformly precipitate CCDC93 (Phillips-Krawczak et al., 2015) (Figure 3C). Most strikingly, a three-subunit complex dubbed "CCC" of COMMD-CCDC22-CCDC93 has been well documented (Phillips-Krawczak et al., 2015; Li et al., 2015; Bartuzi et al., 2016). The CCC complex further interacts with C16orf62 and functions in endosomal trafficking (Phillips-Krawczak et al., 2015) (Figure 3D). CCC was first characterized containing COMMD1 (Phillips-Krawczak et al., 2015), but other COMMD proteins have been observed in combination with CCDC22 and CCDC93 in later studies (Figure 3E) (Bartuzi et al., 2016; Li et al., 2015). This makes Commander/CCC potentially very similar in composition; in this review, for simplicity's sake (and acknowledging that such definitions may evolve over time), we use the nomenclature Commander to refer to the full, putatively 14-member, complex (Figure 2), which contains the CCC subcomplex and its COMMD variants, as well as DSCR3 and C16orf62. We have independently verified many of the novel protein-protein interactions in the putative Commander complex by AP-MS experiments in human cell lines (Figure 3F) (Wan et al., 2015).

Proteomic data that characterize native human protein complexes based on size corroborate the hypothesis that the putative Commander components form a stable complex (Figure 3G) (Wan et al., 2015; Kirkwood et al., 2013). In these experiments, native size-exclusion chromatography is combined with MS analysis to characterize soluble protein complexes isolated from human osteosarcoma cells (Kirkwood et al., 2013). These data show the 14 most likely Commander components elute together with a mass of ~600 kDa (Figure 3G). Interestingly, a stable oligomeric complex of approximately 600 kDa containing endogenous COMMD1 was reported in a membrane fraction separated from human HepG2 (liver cancer) cells and analyzed by blue native PAGE (Burkhead et al., 2009), further suggesting that this corresponds to the molecular weight of endogenous human Commander. This mass of 600 kDa is slightly higher than the combined molecular weight of 497 kDa

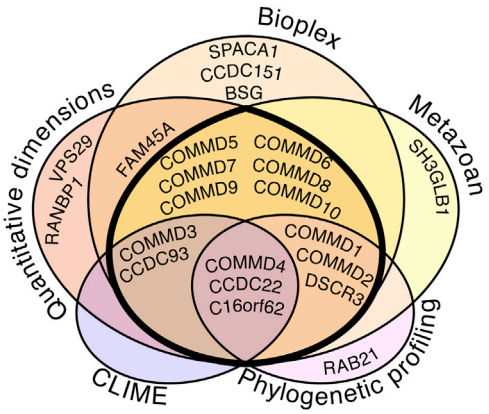


Figure 2. Putative Commander Subunits
Recent human interaction networks describe overlapping pieces of the Commander complex. Three are experimentally derived and include a metazoan complex map (Wan et al., 2015; Drew et al., 2017), the "BioPlex" network (Huttlin et al., 2015), and an interactome in "quantitative dimensions" (Hein et al., 2015). Two are computational networks calculated using phylogenetic profiling (Dey et al., 2015) or "clustering by inferred models of evolution" (CLIME) (Li et al., 2014). The 14 protein subunits identified by at least three studies are considered in this proposal as the strongest candidates to be core components of Commander (thick black line).

for the 14 putative Commander components (Table 1), and additional experiments are needed to determine if these subunits have a 1:1 stoichiometry within the complex, or if additional proteins identified in the human interaction networks are involved (Figure 2). Indeed, several of the COMMDs have been reported to oligomerize (Burstein et al., 2005; Burkhead et al., 2009), making it possible that Commander functions as a set of similarly sized heterogeneous complexes of several core subunits with stoichiometries of more than 1. In agreement with this, studies by the Burstein lab indicate that the CCC complex performs different functions with other combinations of COMMDs aside from COMMD1 (Bartuzi et al., 2016; Li et al., 2015; Phillips-Krawczak et al., 2015).

Commander's Broad Evolutionary Conservation and Ubiquitous Gene Expression Suggest a Fundamental Cellular Role in Animals

Current sequence annotations indicate that Commander genes are of old eukaryotic origin and derive from unicellular eukaryotes (Figure 4A). However, almost all have been lost in major eukaryotic clades, notably protists, fungi, and flowering plants. The subunits are highly conserved across metazoa, but with exceptions, including the loss of all COMMDs except COMMD4 in worm (*Caenorhabditis elegans*), and COMMD1, 6, 7, and 9 in fly (*Drosophila melanogaster*) (Figure 4A). These smaller Commander subunit groupings may represent less intricate versions of the human complex, and may give clues to its architecture and assembly. Notably, *Dictyostelium discoideum* (slime mold) contains orthologs of the entire putative complex (Figure 4A), which along with a near-complete complex in the protozoan parasite *Trichomonas vaginalis*, suggests that Commander is an ancient (>1 billion years old) complex that has been lost or significantly diverged in fungi and many protists and plants.

Old complexes tend to be broadly expressed and abundant, contain proteins with fewer domains, and are enriched for human disease interactions (Wan et al., 2015; Greco and Cristea, 2016; Marsh and Teichmann, 2015). In contrast, metazoan-only (i.e., new, and approximately 500 million years old) complexes are more specifically expressed, contain multidomain proteins, are enriched for cancer-related proteins, and tend to be involved in cell adhesion, organization, and differentiation (Wan et al., 2015). Commander genes are indeed strongly and ubiquitously expressed throughout human tissues at both the mRNA (FANTOM Consortium and the RIKEN PMI and CLST (DGT) et al., 2014) and protein (Wang et al., 2015) level (Figure 4B). This trend, and the deep evolutionary conservation of Commander, argue that it is likely a complex of central importance involved in fundamental cellular function.

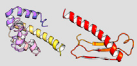
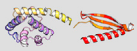
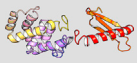
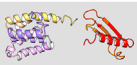
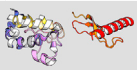
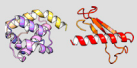
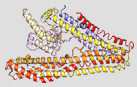
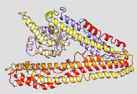
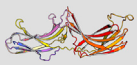
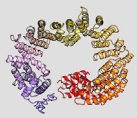
The reported subcellular localization of Commander varies. Subunits have been observed using immunofluorescence in human cells in more than one compartment, including the nucleus, cytoplasm, and vesicles (Figure 4C). This could mean that Commander proteins exist as full complexes, subcomplexes, and monomeric species in different cellular compartments. In addition, the COMMDs have two predicted nuclear export signals in the COMMD domain, suggesting that their transport from the nucleus to the cytosol is highly regulated (Muller et al., 2009). The observation of most Commander proteins in vesicles, endosomes, and/or membranes is consistent with a reported role of many Commander subunits in cellular trafficking, as we next discuss.

The Apparent Pleiotropic Functions of Commander Proteins

We can reconsider previously reported functional data on individual Commander proteins in the context of them operating as a large, multiprotein complex. COMMD1 is the most studied of the Commander proteins, and is linked to a diverse range of cellular processes including proinflammatory signaling (Burstein et al., 2005; Maine and Burstein, 2007; Starokadomskyy et al., 2013), hypoxia adaptation (van de Sluis et al., 2010; van de Sluis et al., 2007), copper metabolism (Phillips-Krawczak et al., 2015; van De Sluis et al., 2002), ubiquitination (Drevillon et al., 2011; Ganesh et al., 2003; Maine et al., 2007), and electrolyte transport (Biasio et al., 2004). COMMD1 has been identified as a therapeutic target in anti-inflammatory pathways (Bartuzi et al., 2013; de Becdelievre et al., 2013; de Bie et al., 2006; Ganesh et al., 2003; Maine et al., 2007), in hypoxia-related tumor invasion (van de Sluis et al., 2010), and in cystic fibrosis (de Becdelievre et al., 2013; Drevillon et al., 2011). Interestingly, with the exception of inflammation and hypoxic response, many reported functions of Commander proteins can be linked to the trafficking of transmembrane proteins. In particular, almost all of the predicted Commander subunits are directly implicated in protein sorting processes within the endosomal system (Bartuzi et al., 2016; Burkhead et al., 2009; Drevillon et al., 2011; Harbour et al., 2012; Li et al., 2015; Phillips-Krawczak et al., 2015), suggesting a key role for the whole complex in intracellular trafficking. We discuss a potential role for Commander in ubiquitination-related inflammatory pathways, hypoxia, and endosomal trafficking in more detail below.

The COMMDs regulate the activity of nuclear factor κB (NF-κB), a proinflammatory multiprotein transcriptional complex that controls almost 400 genes involved in immune responses,

Table 1. Putative Commander Protein Subunits

Subunit	Number of Residues	Molecular Weight (kDa)	Theoretical pI[a]	Predicted Structure[b]
COMMD1	190	21.2	5.9	
COMMD2	199	22.7	6.2	
COMMD3	195	22.2	5.6	
COMMD4	199	21.8	6.9	
COMMD5	224	24.7	6.5	
COMMD6	85	9.6	5.7	
COMMD7	200	22.5	5.7	
COMMD8	183	21.1	5.3	
COMMD9	198	21.8	5.6	
COMMD10	202	23.0	6.1	
CCDC22	627	70.8	6.3	
CCDC93	631	73.2	8.2	
DSCR3	297	33.0	7.6	
C16orf62	963	109.6	6.8	

The subunit name, residue number, molecular weight, predicted isoelectric point (pI), and predicted structure are shown.

[a]Theoretical pIs were calculated from the amino acid sequence using the ProtParam tool within ExPaSy (Wilkins et al., 1999).

[b]Protein structures shown are models generated by I-TASSER (Iterative Threading ASSEmbly Refinement) (Yang et al., 2015) or Robetta (Rosetta Comparative Modeling and Ab Initio Modeling) (Raman et al., 2009), which combine template-based and ab initio modeling. Where both models were calculated, we found they were in good agreement. Structure prediction for the COMMDs was performed on the individual N- and C-terminal domains to maximize accuracy. We did not model the relative orientation of the domains and they are depicted for maximum clarity only. PDB structures are shown for the N-terminal domains of COMMD1 (PDB: 2H2M) and COMMD9 (PDB: 4OE9), which represent the only experimentally available structural information for Commander subunits. The COMMD domains are predicted to adopt a fold similar to a pleckstrin homology domain, in agreement with a previous ab initio model calculated for COMMD1 (Burkhead et al., 2009).

immune system development, and cell survival and proliferation (Bartuzi et al., 2013; Maine and Burstein, 2007). Specifically, NF-κB is downregulated by the COMMDs, a necessary response after an inflammatory episode to prevent chronic inflammation. While all COMMD proteins are reported to interact distinctly with NF-κB (Burstein et al., 2005), a detailed mechanism of NF-κB regulation has only been proposed for COMMD1. This involves NF-κB ubiquitination and proteasomal degradation in the nucleus through an interaction of COMMD1 with a multimeric E3 ubiquitin ligase complex (Maine et al., 2007). It has been suggested that the mechanisms by which each COMMD regulates NF-κB are distinct (Bartuzi et al., 2013). We suggest that a role for Commander in this ubiquitination-controlled anti-inflammatory response should also be considered. Consistent with this, Commander subunit CCDC22 is required for the ubiquitination of IκB, an inhibitor of the NF-κB pathway (Starokadomskyy et al., 2013).

Gene inactivation studies in mice have shown that COMMD1 mediates hypoxia-inducible factor 1 (HIF-1) (van de Sluis et al., 2007), a heterodimeric transcription factor that regulates oxygen homeostasis (Gordan and Simon, 2007). HIF-1 controls energy metabolism, angiogenesis, erythropoiesis, and critical events during embryogenesis (Gordan and Simon, 2007). It plays a physiological role in the cellular adaptation to hypoxia, which occurs when available oxygen falls below 5%, and a pathophysiological role in cancer, where local hypoxia in rapidly growing tumors is thought to lead to HIF activation. COMMD1 inhibits HIF-mediated gene expression in cancer cells by physically associating with the amino terminus of HIF-1α, a subunit of the HIF heterodimer, preventing its dimerization and subsequent DNA binding and transcriptional activation (van de Sluis et al., 2010; van de Sluis et al., 2007). This suggests suppression of COMMD1 as a therapeutic avenue in cancer. Interestingly, studies have shown that there is extensive crosstalk between HIF and NF-κB pathways (Rius et al., 2008; van Uden et al., 2011), and although the involvement of other COMMDs in these processes is unstudied, taken as a whole, these data support a common role for Commander linking hypoxic response to innate immunity and inflammation.

Most strikingly, elegant studies on a substantial number of Commander components provide strong evidence that the complex plays a key role in endosomal protein sorting (Bartuzi et al., 2016; Drevillon et al., 2011; Harbour et al., 2012; Li et al., 2015; Phillips-Krawczak et al., 2015). This is consistent with the subcellular localization of many Commander proteins in vesicles and/or endosomes (Figure 4C). The endosomal system comprises a number of distinct and dynamic vesicular compartments (endosomes) that sort and deliver membrane protein cargo between the plasma membrane and the Golgi apparatus (Seaman, 2008; Soldati and Schliwa, 2006). The movement of endosomes within the cell is regulated by their interactions with cytoskeleton polymers such as microtubules and actin filaments (Soldati and Schliwa, 2006). Protein complexes that function to recognize and deliver cargo are coupled to cytoskeleton systems to drive membrane fission and vesicle movement. Endosomal protein sorting also requires localized actin polymerization, possibly to generate a local force to facilitate the production and/or scission of endosomal tubules (Seaman et al., 2013). Commander subunits have been linked to key components in the endosomal

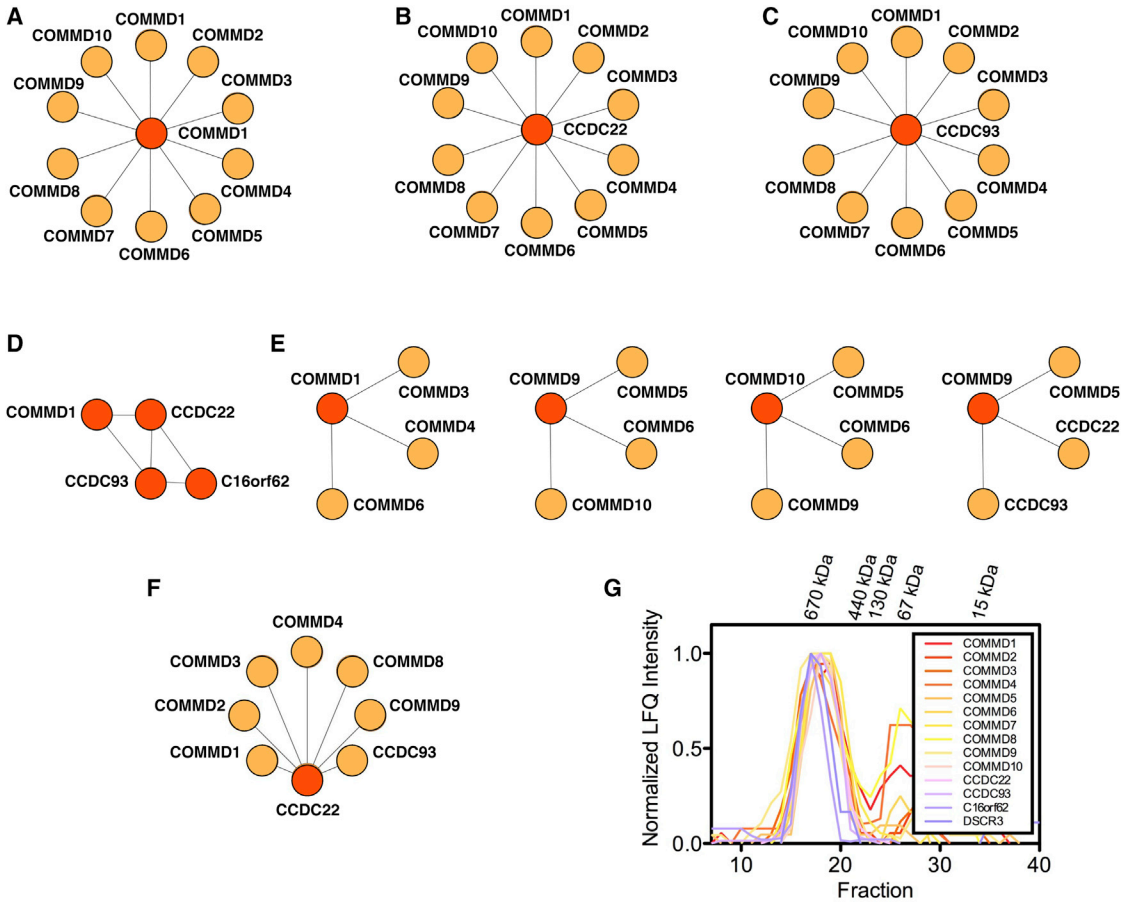


Figure 3. Evidence for Commander from Small-Scale Proteomic Studies
(A–F) Examples of published bait (red) and prey (orange) experiments with Commander subunits. (A) COMMD1 interacts with itself and all other COMMD proteins (Burstein et al., 2005). (B) CCDC22 binds COMMD1-10 (Starokadomskyy et al., 2013). (C) CCDC93 interacts with COMMD1-10 (Phillips-Krawczak et al., 2015). (D) A CCC complex of COMMD1-CCDC22-CCDC93 bound to C16orf62 has been extensively biochemically characterized, and is involved in endosomal protein trafficking (Bartuzi et al., 2016; Li et al., 2015; Phillips-Krawczak et al., 2015). (E) Other combinations of COMMDs have been seen to preferentially interact, for example COMMD3, -4, and -6 were identified in an early COMMD1 tandem affinity purification (TAP) screen (Burstein et al., 2005; Starokadomskyy et al., 2013). (F) We have verified several of the novel protein-protein interactions in the Commander complex by AP-MS (Wan et al., 2015).
(G) Size-exclusion chromatography of Commander measured by MS (Kirkwood et al., 2013). Elution profiles of all 14 putative Commander subunits indicate that they co-elute as a single entity of approximately 600 kDa in mass.

protein-sorting machinery by coIP, co-localization, phenotypic, and phylogenetic profiling studies (Bartuzi et al., 2016; Dey et al., 2015; Harbour et al., 2012; Kolanczyk et al., 2015; Li et al., 2015; Phillips-Krawczak et al., 2015). These include the Wiskott-Aldrich syndrome proteins and SCAR homolog (WASH) complex, which is the major endosomal actin-polymerization promoting complex (Derivery et al., 2009; Gomez and Billadeau, 2009; Jia et al., 2010; Seaman et al., 2013), and the retromer complex, the primary role of which is to select cargo proteins for retrograde endosomal transport to the Golgi (Hierro et al., 2007; Seaman et al., 2013).

WASH and retromer work together to facilitate the correct trafficking of many membrane cargo proteins from either the endosome-to-Golgi or endosome-to-cell surface (Seaman et al., 2013). Specifically, WASH is recruited to endosomes by the retromer to promote the formation of filamentous (F)-actin patches localized on endosomes, generating filaments that segregate membrane proteins (Harbour et al., 2012; Helfer et al., 2013; Jia et al., 2012) (Figure 5A). These branched actin patches define discrete domains into which specific proteins are sorted for transport to their destinations (Seaman et al., 2013). Thus, WASH function is linked to the cargo protein-sorting and membrane-tubulation activity of retromer. In the next sections we outline evidence to suggest that Commander is a retromer-like sorter of cargo proteins within the endosomal system. Analogous to retromer, we hypothesize that Commander likely recruits WASH to facilitate cargo trafficking (Figure 5A). The predicted fundamental role of the Commander complex as a key piece in the endosomal trafficking machinery helps to explain the apparent pleiotropic function of its subunits, its deep conservation (Figure 4A), and its broad expression (Figure 4B).

Commander's Role in Trafficking and Transport

Commander subunits COMMD1, CCDC22, CCDC93, and C16orf62 have recently been shown to function together to recognize and regulate endosomal cargo proteins such as copper

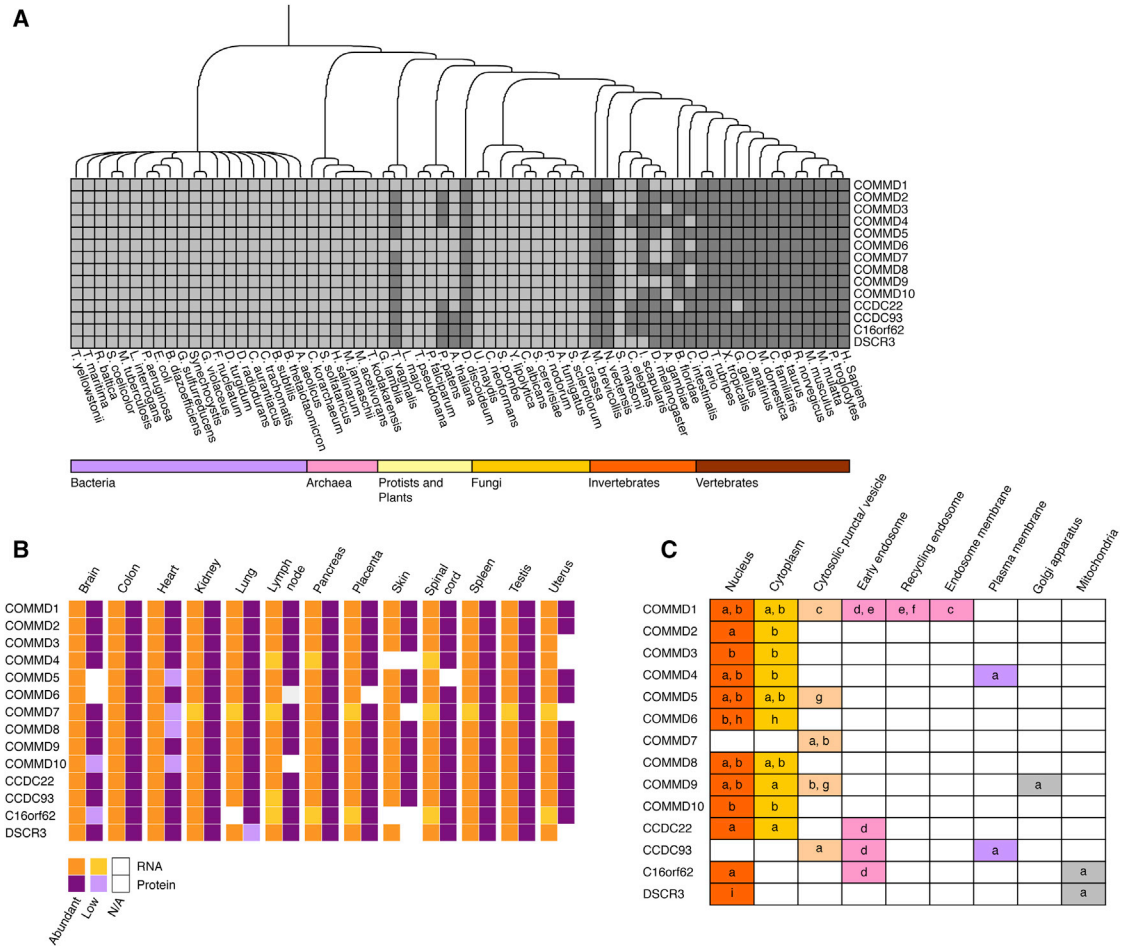


Figure 4. Commander Is Evolutionarily Conserved and Broadly Expressed across Human Tissues

(A) Phylogenetic profiles for the presence and absence of each of the Commander genes were calculated for each of 66 species' proteomes (Altenhoff et al., 2016). Dark gray, present; light gray, absent.

(B) RNA and protein expression profiles for Commander genes in human cells. Tissue types are shown for which transcripts (source: FANTOM5 project) and protein (source: PaxDB) data are readily available. For RNA expression profiles, abundant = 2–150 and low = <2 FPKM. For protein expression, abundant = 2–250 ppm and low = <2 ppm. ppm, parts per million. FPKM, fragments per kilobase of transcript per million mapped reads.

(C) Subcellular localization of predicted Commander proteins as reported in (Lindskog, 2015) (a); (Mao et al., 2011) (b); (Burkhead et al., 2009) (c); (Phillips-Krawczak et al., 2015) (d); (Drevillon et al., 2011) (e); (Chang et al., 2011) (f); (Li et al., 2015) (g); (de Bie et al., 2006) (h); and (Hu et al., 2006) (i).

transporters (Phillips-Krawczak et al., 2015), Notch receptors (Li et al., 2015), and lipoprotein receptors (Bartuzi et al., 2016). Three of these subunits form the CCC complex identified by the Burstein lab (Phillips-Krawczak et al., 2015) (Figure 3D), a likely subcomplex of Commander. An earlier study on the role of COMMD1-mediated ubiquitination in the regulation of CFTR trafficking in cystic fibrosis also proposed that COMMD1 has a specific function within the endocytic machinery (Drevillon et al., 2011).

Trafficking of copper transporters by the CCC complex involves early endosomal co-localization with WASH, retromer, and the copper transport protein ATP7A (Phillips-Krawczak et al., 2015). WASH is a pentameric complex of WASHC1, WASHC2 (FAM21), WASHC3 (CCDC53), WASHC4 (SWIP) and WASHC5 (strumpellin) (Jia et al., 2010), while mammalian retromer comprises a cargo-recognition complex and a sorting nexin (SNX) complex, each with distinct roles in endosome-to-Golgi protein retrieval (Seaman, 2008). The retromer cargo-recognition complex of three vacuolar protein sorting (VPS) proteins, VPS26, VPS29, and VPS35, is defined as such because VPS35 directly binds to the sorting motifs in cargo proteins (Nothwehr et al., 2000). SNXs are a family of proteins defined by the presence of a phospholipid-binding Phox (PX) domain (Teasdale and Collins, 2012). The SNX complex plays a structural role in deforming the endosomal membrane to generate cargo-loaded tubules for transport (Cullen and Korswagen, 2012). Several types of SNX proteins interact with retromer: SNX3, a sorting nexin consisting only of the PX domain (Harrison et al., 2014); the SNX-BAR SNX1/2-SNX5/6 heterodimers, which have an additional bar domain (Rojas et al., 2007; Wassmer et al., 2007); and the SNX-FERM protein SNX27, which has an additional PDZ and FERM domain (Steinberg et al., 2013).

Retromer recruits WASH to endosomes by an interaction between WASH subunit WASHC2 (FAM21) and retromer subunit VPS35 (Derivery et al., 2009; Gomez and Billadeau, 2009;

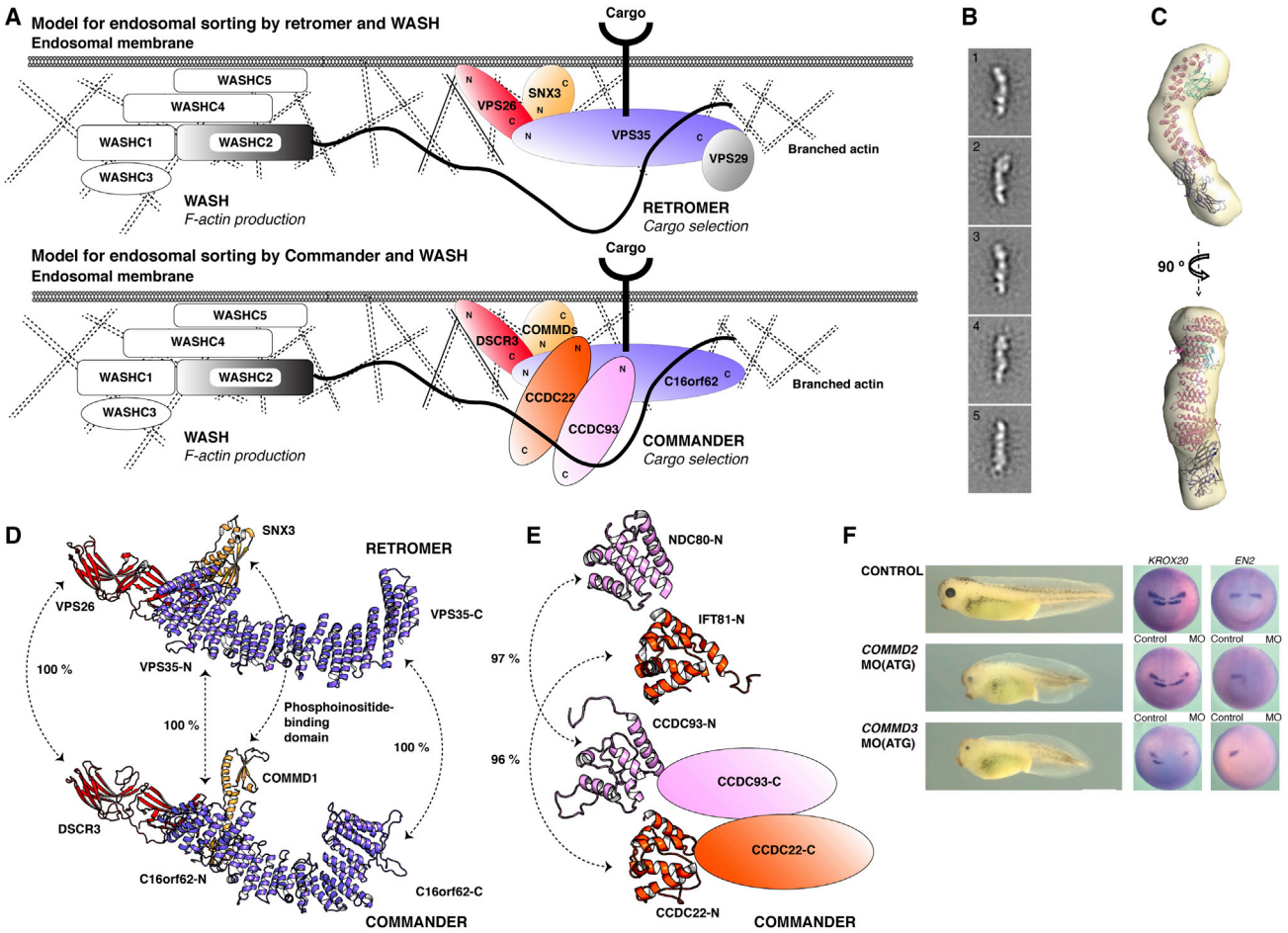


Figure 5. Structural Modeling and Functional Studies Suggest Roles for Commander

(A) Models for WASH-dependent endosomal protein trafficking by retromer (top) (based on Seaman et al., 2013) and Commander (bottom).

(B and C) Structural data for the retromer cargo recognition complex. (B) Negative stain electron microscopy images of the human retromer cargo recognition complex (VPS26-VPS29-VPS35) of five representative structural classes. Images are reproduced from Hierro et al. (2007). (C) Small-angle X-ray scattering (SAXS) model of the retromer VPS26 (purple)-VPS29 (green)-VPS35 (pink) complex, reproduced from Lucas et al. (2016).

(D) Structural comparison of the retromer cargo recognition complex and core Commander subunits. Top: a structural model of a partial retromer cargo recognition complex (VPS26, red; SNX3, orange; and VPS35, purple) based on crystal structures (2R17 and 5F0J), electron microscopy, and SAXS (Hierro et al., 2007; Lucas et al., 2016). Bottom: a proposed structural model for core components of the Commander complex (DSCR3, red; COMMD1, orange; and C16orf62, purple) based on their similarities to retromer subunits. For homologous domains, the probability score calculated by HHPred (Soding, 2005) is shown. This represents the likelihood that they are true homologs when using Pfam as a database of template hidden Markov models (Finn et al., 2014). A score of greater than 95% indicates that the homology is nearly certain. Crystal structures are available for the N and C termini of human VPS35 (VPS35-N, residues 9–462, PDB: 5F0J; VPS35-C, residues 483–780, PDB: 2R17) and human VPS26 (PDB: 2FAU). These were used as templates to generate models of C16orf62 (C16orf62-N, residues 182–620), C16orf62-C (residues 629–926); and DSCR3, respectively, using MODELLER (Sali and Blundell, 1993). Based on the predicted presence of a phosphoinositide-binding domain in the COMMDs, we suggest that they occupy a homologous binding pocket to SNX3.

(E) A structural comparison of the N-terminal domains of IFT81 (residues 1–109; PDB: 4LVP) and human NDC80 (residues 11–114; PDB: 2IGP) to the N-terminal domains of Commander CCDC22 (residues 1–109) and CCDC93 (residues 12–119), respectively. Homology scores were calculated by HHPred, and template structures were used to calculate models of CCDC22 and CCDC93 as described in (D). It is possible that the calponin homology (CH) domains in CCDC22 and CCDC93 function in microtubule binding. Interestingly, the C-terminal domains of both CCDC22 and CCDC93 show strong homology to tropomyosin (99% and 95%, respectively; PDB: 1C1G).

(F) Phenotypic traits of defective Commander. Morpholino knockdown of COMMD2 or COMMD3 in *X. laevis* embryos causes defective head and eye development (left), and COMMD2/3 knockdown animals show altered neural patterning (right), including a posterior shift or loss of expression of mid-brain marker EN2 and of KROX20 (EGR1), the latter specifically in rhombomeres R3/R5. Images reproduced from Wan et al. (2015).

Harbour et al., 2012; Helfer et al., 2013; Seaman et al., 2013) (Figure 5A). It is thought that this interaction enables WASH to generate F-actin-driven force to increase the efficiency of tubule scission by the retromer-SNX complex (Gomez and Billadeau, 2009). Key to the predicted function of the Commander complex, the Burstein lab have shown by silencing, localization, and coIP experiments, that WASHC2 (FAM21) is also required for the recruitment of the CCC complex to the endosome (Phillips-Krawczak et al., 2015). It follows that Commander, like retromer, may function in concert with the WASH complex (Figure 5A), as we discuss further below. Consistent with this, CLIME predicts that Commander subunits COMMD3, COMMD4, CCDC22, CCDC93, and C16orf62, co-evolved with the WASH complex, indicating a combined functional role (Li et al., 2014).

Proteomic, Biochemical, and 3D Structural Evidence Suggesting Commander Functions as a Retromer-like Sorter of Endosomal Proteins

Analysis of the CCC complex by the Burstein group using deletion constructs and IPs suggest an architecture where the C termini regions of CCDC22 and CCDC93 interact with the middle of the unstructured tail region of WASHC2 (Phillips-Krawczak et al., 2015) (Figure 5A). In addition, The COMMDs appear to interact using their C-terminal COMM domain with CCDC22 (Staroka-domskyy et al., 2013), an interaction that is independent of CCDC93 and C16orf62 (Phillips-Krawczak et al., 2015). IPs have also shown that the interaction of CCDC22 and CCDC93 with WASHC2 does not happen at the same time as the interaction of WASHC2 with the retromer subunit VPS35 (Harbour et al., 2012). We have consolidated structural, proteomic, and functional evidence to suggest that Commander in its entirety forms a retromer-like complex that binds to WASHC2 to traffic endosomal proteins (Figures 5A–5D).

A structural model for the architecture of the retromer cargo-recognition complex has been proposed based on data from crystal structures, electron microscopy, and SAXS (Hierro et al., 2007; Lucas et al., 2016) (Figures 5B–5D). Our preliminary structural analysis shows that COMMD1, DSCR3, and C16orfC2 have strikingly similar properties to a partial retromer cargo-recognition complex of SNX3, VPS26, and VPS35 (Hierro et al., 2007; Lucas et al., 2016) (Figure 5D). Commander protein DSCR3 and retromer VPS26 are direct homologs (Aubry et al., 2009; Shi et al., 2006), and likely act at the plasma membrane to sort specific cargos into endocytic vesicles (Zhang et al., 1997). The crystal structure of human VPS26 has been solved and adopts an arrestin fold (PDB: 2FAU) (Shi et al., 2006). We used the HHpred server for template-based structure prediction, which uses hidden Markov models to determine remote homologs (Soding, 2005), to show that VPS26 (327 residues) is the top match for DSCR3 (297 residues) with a probability score of 100% that the two are homologs. Likewise, retromer VPS35 (796 residues), whose amino (PDB: 5F0J) and carboxy (PDB: 2R17) termini adopt an α-helical solenoid (Hierro et al., 2007; Lucas et al., 2016), is the most likely structural homolog (probability = 100%) for the similarly sized Commander C16orf62 (963 residues).

Although not predicted to be direct structural homologs, the SNXs and the COMMDs share some striking similarities. Based on these, we hypothesize they have parallel functional roles, and occupy equivalent binding sites in retromer and Commander, respectively (Figures 5A and 5D). The predicted pleckstrin homology (PH)-fold of the COMM domains (Burkhead et al., 2009) (Table 1) is functionally analogous to the Phox homology (PX) domain found in the SNXs (Cullen and Korswagen, 2012). Both PH and PX domains are phosphoinositide-binding domains that target proteins to phospholipid-enriched membranes (Lemmon, 2008). The COMM domains in Commander may therefore have a membrane recruitment role similar to the retromer-SNX complex. In good agreement with this hypothesis, COMMD1 binds with high specificity to the lipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2; PIP_2), an interaction that is mediated by the COMM domain (Burkhead et al., 2009). It has been suggested that different combinations of retromer-SNX interactions give rise to different cargo selection specificities via higher order assemblies with different morphologies (Lucas et al., 2016). A similar scenario can be imagined for different combinations of COMMDs in Commander.

Finally, functional studies on the role of the CCC complex in Notch signaling report similar phenotypes in cells deficient in Commander components CCDC93 and C16orf62 and the retromer subunit VPS35, linking both Commander and retromer to Notch trafficking. Taken together, these structural, proteomic, and functional similarities between Commander and retromer lead us to hypothesize that core Commander subunits COMMD-DSCR3-C16orf62 function in a complex of similar architecture to the retromer cargo-recognition complex of SNX3-VPS26-VPS35 (Figure 5D). This model should be helpful in determining the specific events in the endosomal sorting process that are regulated by Commander, and the functional contributions of specific subunits.

It is interesting to note that, in a recent bioinformatics study, Commander subunits CCDC22 and CCDC93 were classified into a previously unidentified protein family of 11 proteins defined by a novel N-terminal calponin homology domain (Schou et al., 2014). Proteins within this family, which also include several IFT-B proteins, are predicted to share NDC80 and NuF2 from yeast as common ancestors, and have been implicated in actin and microtubule binding (Korenbaum and Rivero, 2002; Schou et al., 2014). We built homology models of the N-terminal domains of Commander CCDC22 and CCDC93 using the structures of IFT81 (PDB: 4LVP) and NDC80 (PDB: 2IGP) as templates, respectively, which have close to 100% probability of homology (Figure 5E). We suggest that CCDC22 and CCDC93 might function as direct points of contact between Commander and actin, perhaps by forming an actin-binding module similar to the NDC80-NuF2 (Ciferri et al., 2008) and IFT81-IFT74 (Bhogaraju et al., 2013) complexes (Figure 5E). This model can be used to direct further experiments into the architecture and function of Commander.

Strong Links between Commander and Its Interactome in Developmental Diseases

Reverse genetic experiments (Wan et al., 2015) (Figure 5F), combined with studies on individual Commander subunits (Kolanczyk et al., 2015; van de Sluis et al., 2007; Voineagu et al., 2012), provide useful insights into role of the complex in human development and disease. A clear importance of Commander proteins in vertebrate embryonic development has been demonstrated. In mouse, individual homozygous knockouts (KOs) of COMMD1, COMMD6, COMMD9, and COMMD10 are reported to cause embryonic-lethal phenotypes, and in some cases complex cardiovascular abnormalities (Bartuzi et al., 2013; Brown and Moore, 2012; Eppig et al., 2015; Li et al., 2015; van de Sluis et al., 2007). Interestingly, notable differences in the phenotype for embryonic lethality in KO COMMD1 and COMMD9 mice suggests non-redundant developmental functions for the two proteins, consistent with the idea of heterogeneous Commander assemblies (Li et al., 2015; van de Sluis et al., 2007). We have characterized several Commander genes in frog, which indicate that the complex has an explicit role in tadpole eye and head development (Figure 5F) (Wan et al., 2015). In particular, morpholino-oligonucleotide-mediated gene knockdowns (KDs) of either COMMD2 or COMMD3 in frog embryos give phenotypes with defective head and eye development along with altered

neural patterning. This includes defects in the patterning of the hindbrain, the precursor to the cerebellum (Figure 5F). Taken together, these findings in mouse and frog point toward a crucial role for Commander in vertebrate embryogenesis and provide the basis for more detailed genetic studies.

Consistent with these studies, defects in one Commander gene are implicated in human RS/3C syndrome, a rare disorder characterized by intellectual disability, cerebellar brain malformations, congenital heart defects, and craniofacial abnormalities (Ritscher et al., 1987). Specifically, missense alleles T17A and Y557C in CCDC22 were identified as causes of RS/3C syndrome in human patients (Kolanczyk et al., 2015; Voineagu et al., 2012), who display profound developmental phenotypes similar to our *Xenopus* Commander KD animals. Combined with the deep conservation of the entire Commander complex, this implies all Commander genes as strong candidates in the etiology of RS/3C syndrome. WASHC5 of the WASH complex, also known as strumpellin, has also been directly linked to RS/3C syndrome by the disease allele that leads to a substitution of the last 99 amino acids of the protein (Elliott et al., 2013). This suggests that the endosomal sorting processes governed by the interplay of the Commander and WASH (Figure 5A) are key to developmental processes, and disruptions in this machinery may cause disorders such as RS/3C syndrome. This is consistent with the documented crucial role of the CCC complex in the vesicular sorting and delivery of Notch proteins to the cell surface (Li et al., 2015), which are transmembrane receptors involved in many stages of development (High and Epstein, 2008). The links between COMMD1 and anti-inflammatory pathways (Bartuzi et al., 2013; de Becdelievre et al., 2013; de Bie et al., 2006; Ganesh et al., 2003; Maine et al., 2007), hypoxia-related tumor invasion (van de Sluis et al., 2010), and endosomal trafficking of the CFTR gene (de Becdelievre et al., 2013; Drevillon et al., 2011) warrant further research into the role of Commander and its protein-sorting function in diseases such as cancer and cystic fibrosis.

Future Outlook

We have presented an overview of recent proteome-wide human interactome experiments that, together with gene-focused studies, have uncovered a large new multiprotein complex called Commander. Commander is a deeply conserved, macromolecular machine of unknown architecture, and contains genes that are vital for vertebrate embryogenesis and implicated in developmental disease. The preliminary evidence we have summarized here suggests that Commander functions as an ancient complex of central importance in intracellular protein trafficking. By combining insights from both large- and small-scale studies, we have proposed a model of the Commander complex to help guide future studies into its structure and function. We expect future research to provide crucial mechanistic information about the role of the complex in developmental defects and rare disorders, and also chronic diseases such as cancer and cystic fibrosis.

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