The FTS-Hook-FHIP (FHF) complex interacts with AP-4 to mediate perinuclear distribution of AP-4 and its cargo ATG9A

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ABSTRACT The heterotetrameric adaptor protein complex 4 (AP-4) is a component of a protein coat associated with the trans-Golgi network (TGN). Mutations in AP-4 subunits cause a complicated form of autosomal-recessive hereditary spastic paraplegia termed AP-4-deficiency syndrome. Recent studies showed that AP-4 mediates export of the transmembrane autophagy protein ATG9A from the TGN to preautophagosomal structures. To identify additional proteins that cooperate with AP-4 in ATG9A trafficking, we performed affinity purification-mass spectrometry followed by validation of the hits by biochemical and functional analyses. This approach resulted in the identification of the fused toes homolog-Hook-FHIP (FHF) complex as a novel AP-4 accessory factor. We found that the AP-4–FHF interaction is mediated by direct binding of the AP-4 μ4 subunit to coiled-coil domains in the Hook1 and Hook2 subunits of FHF. Knockdown of FHF subunits resulted in dispersal of AP-4 and ATG9A from the perinuclear region of the cell, consistent with the previously demonstrated role of the FHF complex in coupling organelles to the microtubule (MT) retrograde motor dynein–dynactin. These findings thus uncover an additional mechanism for the distribution of ATG9A within cells and provide further evidence for a role of protein coats in coupling transport vesicles to MT motors.

INTRODUCTION

The heterotetrameric adaptor protein (AP) complexes AP-1, AP-2, AP-3, AP-4, and AP-5 are components of protein coats involved in the sorting of transmembrane protein cargo in post-Golgi compartments of the endomembrane system of eukaryotic cells (reviewed by Robinson 2015; Guardia et al., 2018). These complexes are composed of two large subunits (γ/α/β/ε/ζ and β1/β2/β3/β4/β5), a medium-sized subunit (μ1/μ2/μ3/μ4/μ5), and a small subunit (α1/α2/α3/α4/α5) (Robinson, 2004; Guardia et al., 2018). The composition of AP complexes is further diversified by the existence of paralogous isoforms of the γ, μ1, σ1, α3, β3, μ3, and ε3 subunits (Mattera et al., 2011). Each AP complex consists of a core formed by the medium and small subunits together with the N-terminal “trunk” domains of the large subunits; extending from this core are the two C-terminal “hinge” and “ear” domains of the large subunits (see Figure 1A for a scheme of AP-4). Each AP complex has a different localization within the cell: AP-1 is found at the trans-Golgi network (TGN) and endosomes, AP-2 at the plasma membrane, AP-3 on a tubular endosomal compartment, AP-4 at the TGN, and AP-5 at late endosomes (Robinson, 2004; Guardia et al., 2018). The complexes are recruited to membranes by interactions with small GTPases of the ARF family and/or membrane phosphoinositides, and associate with scaffolding and accessory proteins, resulting in the assembly of protein coats (Robinson, 2004; Guardia et al., 2018). APs then...
function to recognize signals in the cytosolic tails of transmembrane protein cargos leading to the incorporation of the cargos into transport carriers (Traub and Bonifacino, 2013).

The AP-4 complex (Figure 1A) was independently identified two decades ago by our laboratory (Dell'Angelica et al., 1999) and that of Margaret S. Robinson (Hirst et al., 1999). AP-4 was found to be present in a wide variety of eukaryotes, although not in the genetic model organisms Saccharomyces cerevisiae, Drosophila melanogaster, and Caenorhabditis elegans, probably due to gene loss during evolution (Hirst et al., 2014). Studies using mammalian cells showed that AP-4 associates with the TGN by virtue of interactions with ARF GTPases, and that it does not interact with the scaffolding protein clathrin (Dell'Angelica et al. 1999; Hirst et al. 1999; Boehm et al., 2001). At present it is unclear whether or not AP-4 interacts with a nonclathrin scaffold. Subsequent studies revealed that the μ4 subunit of AP-4 preferentially recognizes sorting signals fitting the consensus motif YXXΩE (where X is any amino acid and Ω is a bulky hydrophobic amino acid) (Burgos et al., 2010; Mattera et al., 2017), as well as noncanonical sequences (Yap et al., 2003). AP-4 has been the focus of renewed attention in recent years due to the finding that mutations in each of its subunits are the cause of a subset of autosomal-recessive hereditary spastic paraplegia (abbreviated HSP or SPG) types characterized by progressive spasticity and intellectual disability. This subset, referred to as “AP-4-deficiency syndrome,” comprises SPG47 (AP4B1, μ4), SPG50 (AP4M1, μ4), SPG51 (AP4E1, ε), and SPG52 (AP4S1, σ4) (mutated gene and protein subunit are indicated in parentheses; Verkerk et al., 2009; Abou Jamra et al., 2011; Moreno-De-Luca et al., 2011; for a review, see Tesson et al., 2015). The link of AP-4 to a human disease spurred further efforts to identify additional interactors and elucidate the molecular basis for the disease. Affinity purification and mass spectrometry (MS) analyses led to the identification of the accessory proteins tepsin (Borner et al., 2012; Mattera et al., 2015; Frazier et al., 2016), RUSC1 and RUSC2 (Davies et al., 2018), and the transmembrane protein cargos ATG9A (Mattera et al., 2015, 2017; Davies et al., 2018; Ivanovic et al., 2020), SERINC1, and SERINC3 (Davies et al., 2018) as relevant AP-4 interactors. Mutations in AP-4 were shown to result in defective transport of ATG9A, SERINC1, and SERINC3 from the TGN to the cell periphery (Mattera et al., 2017; Davies et al., 2018, De Pace et al., 2018; Ivanovic et al., 2020), indicating that AP-4 plays a role in protein export from the TGN.

The finding that ATG9A is an AP-4 cargo was particularly relevant because ATG9A is a critical component of the autophagy machinery, which contributes to the formation and expansion of preautophagosomal structures. ATG9A mutations in mice are associated with axonal dystrophy and a thin corpus callosum (Yamaguchi et al., 2018), as is also the case for mice with mutations in AP-4 subunits (De Pace et al., 2018; Ivanovic et al., 2020) and human patients with AP-4-deficiency syndrome (Verkerk et al., 2009; Moreno-De-Luca et al., 2011; Mattera et al., 2017, Lee et al., 2017; Davies et al., 2012; Mattera et al., 2015; Davies et al., 2018, De Pace et al., 2018; Ivanovic et al., 2020), indicating that AP-4 plays a role in protein export from the TGN.

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Bauer et al., 2012; Tüysüz et al., 2014; Abdollahpour et al., 2015; Hardies et al., 2015; Ebrahimi-Fakhari et al., 2018). In accordance with the role of AP-4 in TGN export of ATG9A, AP-4-deficient mice and humans were found to exhibit defects in autophagy, suggesting that impaired autophagy contributes to the pathogenesis of this disorder. Despite this progress in the understanding of AP-4 function, many aspects of its mechanism of action remain poorly understood.

To obtain additional insights into the physiological role of AP-4, we extended our analysis to other proteins that copurify with it in a tandem affinity purification (TAP). Our results revealed that AP-4 specifically interacts with a multimeric complex termed FHF, which comprises the proteins “fused toes homolog” (FTS), Hook, and “FTS and Hook-interacting protein” (FHIP) (Krämer and Phistry, 1999; Walenta et al., 2001; Xu et al., 2008). In mammals, FTS is encoded by a single gene, whereas Hook exists as three paralogues named Hook1, Hook2, and Hook3, and FHIP as two paralogues named FHIP and FHIP-L. Components of this complex were previously shown to function as adaptors of organelles to dynein–dynactin and kinesin microtubule (MT) motors (Krämer and Phistry, 1999; Maldonado-Báez et al., 2013; Bielska et al., 2014; McKenney et al., 2014; Zhang et al., 2014; Xiang et al., 2015; Guo et al., 2016; Olenick et al., 2016, 2019; Schroeder and Vale, 2016; Lee et al., 2018; Urnăcic et al., 2018; Dwiwedi et al., 2019). In line with this function, we found that knockdown (KD) of FHF subunits causes dispersal of AP-4 and ATG9A from the perinuclear area of the cell, consistent with a defect in dynein–dynactin-dependent centripetal transport of AP-4 vesicles. Our findings thus extend the AP-4 interactome to a complex involved in the coupling of organelles to a retrograde transport motor.

RESULTS
Identification of the FHF complex as an AP-4 interactor

We previously reported the identification of proteins copurifying with the ε subunit of AP-4 (Figure 1A) in TAP-MS experiments (Mattera et al., 2015; Dataset S1 in Mattera et al., 2017). Whereas in earlier studies we focused on tepsin (Mattera et al., 2015) and ATG9A (Mattera et al., 2017; De Pace et al., 2018), in this study we investigated four other copurifying proteins: Hook1 (product of the HOOK1 gene), FHIP (FTS- and Hook-interacting protein) (product of the FAM160A2 gene), the FHIP parologue referred to in this study as FHIP-L (for FHIP-L paralogue) (product of the FAM160A1 gene), and FTS (fused toes homolog) (product of the AKTIP gene) (Figure 1B). All of these proteins were identified with a relatively high peptide number, and had low scores (0/411 to 4/411) in the Contaminant Repository for Affinity Purification database (CRAPome, www.crapome.org; Mellacheruvu et al., 2013), suggesting that they were likely specific interactors. A similar TAP-MS analysis of proteins copurifying with the AP-4 accessory protein tepsin also yielded Hook1 as a high-ranking hit (Supplemental Table S1; see Supplementary Dataset S1 for a complete list of results). Hook1, FHIP, and FTS were previously shown to interact with each other as part of a complex named FHF, which may also include the Hook1 paralogues Hook2 and Hook3 (Xu et al., 2008) (Figure 1, C and D). The status of FHIP-L vis-à-vis the FHF complex was not previously established.

To confirm the AP-4–FHF interaction, we transiently transfected HEK293T cells with plasmids encoding two-Strap/one-FLAG (TSF)-tagged forms of AP-4 ε and the myrlysin subunit of the BORC complex (Pu et al., 2015) as a negative control. Pull down of cell lysates using StreptTactin beads followed by immunoblotting (IB) demonstrated the association of endogenous Hook1, Hook2, Hook3, and FHIP with AP-4 ε but not the myrlysin control (Figure 2A). In addition, we performed immunoprecipitation (IP) from lysates of H4 human neuroglioma cells stably transfected with TSF-tagged AP-4 ε using anti-AP-4 β4 or anti-GFP as a negative control. IB analysis revealed the co-IP of endogenous Hook1 and FHIP with antibody to endogenous AP-4 β4 but not to GFP (Figure 2B). These experiments thus confirmed the interaction of AP-4 with subunits of the FHF complex.

AP-4–FHF interaction is mediated by AP-4 μ4 and Hook proteins

We next used the yeast two-hybrid (Y2H) system to determine whether there is direct binding between AP-4 and FHF subunits. Interactions were detected by the ability of yeast expressing different combinations of AP-4 and FHF subunits fused to the Gal4-activation domain (AD) and Gal4-binding domain (BD) to grow on plates lacking histidine (~His) with varying concentrations of 3-amino-1,2,4 triazole (AT) to reduce background growth. The first set of assays (not including the FHIP-L paralogue) revealed interactions of the AP-4 μ4 subunit with Hook1 and Hook2, but not Hook3, FHIP, and FTS (Figure 3A). Control experiments showed interactions of tepsin with AP-4 ε and AP-4 β4, as previously reported (Borner et al., 2012; Mattera et al., 2015; Frazier et al., 2016), and revealed an additional interaction of tepsin with AP-4 μ4 (Figure 3A).

Further assays shown in Figure 3B demonstrated: 1) the ability of all Hook proteins to homodimerize, as previously shown for Drosophila Hook and mammalian Hook proteins (Krämer and Phistry 1996; Xu et al., 2008; Lee et al., 2018); b) the formation of Hook1-Hook3 heterodimers (Xu et al., 2008); c) the binding of FHIP to Hook proteins (see also Supplemental Figure S1A); and d) the interaction of FTS with all the other subunits of the FHF complex.

To date, FHIP-L has not been shown to be a component of the FHF complex. Y2H analysis using a BD-FHIP-L construct showed nonspecific interactions even at high (30 mM) concentrations of AT (Supplemental Figure S1B). To circumvent this problem, we performed additional experiments with a reverse configuration using AD-FHIP-L and BD-AP-4 μ4 constructs (Figure 3C). Using this configuration, we observed that FHIP-L binds to FTS and is therefore likely to be part of the FHF complex. Additionally, we found that, like FHIP (Figure 3A), FHIP-L does not interact with AP-4 μ4 (Figure 3C). The BD-AP-4 μ4 construct used in this configuration allowed us to confirm that AP-4 μ4 directly binds to Hook1 and Hook2 (Figure 3C), as previously seen with the AD-AP-4 μ4 construct (Figure 3A).

We also tested for interaction of Hook1 and Hook2 with μ subunits of other AP complexexes. The results showed that Hook1 exclusively interacts with AP-4 μ4, while Hook2 exhibits a broader pattern of interaction with AP-1 μ1A and AP-2 μ2, in addition to AP-4 μ4 (Figure 3D). Additionally, we found that the interaction of AP-4 μ4 with Hook1 and Hook2 is mediated by the C-terminal domain of AP-4 μ4, which is also involved in the recognition of YXXOε signals (Figure 3E) (Burgos et al., 2010).

Taken together, these experiments demonstrated that AP-4–FHF interactions are mediated by the corresponding μ4 and Hook1/ Hook2 subunits.

Mapping of Hook1 domains involved in interactions with AP-4 μ4 and other FHF subunits

We also used the Y2H system to map the Hook protein domains involved in interactions with AP-4 μ4 using Hook1 as a model protein (Figure 4A). Hook1 comprises a Hook domain (HD), four Hook domains, and a retrograde transport motor.
part of the region in Hook1 and Hook3 that is also important for
as well as with Hook1 and Hook3. Interestingly, these coiled-coils are
necessary and sufficient for interaction of Hook1 with AP-4 μ4 as
merization (Figure 4B). Thus, the first two coiled-coils in Hook1 are
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tion mutants (Figure 4A) showed that constructs having both the
Hook1 CC1 and CC2 domains (constructs spanning residues
predicted coiled-coil regions (CC1–CC4; CC4 is abbreviated as
“4” in the figure), a Spindly like motif (SM), and an FTS-BD (Walenta
et al., 2001; Xu et al., 2008, Schroeder and Vale 2016; Gama
et al., 2017, Lee et al., 2018) (Figure 4A). Analysis of various Hook1 deletion
mutants (Figure 4A) showed that constructs having both the
Hook1 CC1 and CC2 domains (constructs spanning residues 1–444, 1–658, 169–444, and 169–658) interacted with AP-4 μ4 (Figure 4B). In contrast, the individual HD (1–168), CC1 (169–239),
CC2 (240–444), or FTS-BD (658–728), or the CC3–CC4 combination
(483–658) (Figure 4A) did not interact with AP-4 μ4 in –His +2 mM AT plates, although weak interactions with constructs 1–239 and 658–728 could be detected in –His plates without added AT (Figure 4B). Of note, the combination of the Hook1 CC1 and CC2 domains in the 1–444 and 169–444 constructs was also able to mediate Hook1 homodimerization and Hook1-Hook3 heterodi
merization (Figure 4B). Thus, the first two coiled-coils in Hook1 are
necessary and sufficient for interaction of Hook1 with AP-4 μ4 as
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![Figure 2: Pull down and co-IP experiments confirm the interaction of the FHF complex with AP-4.](image)

Cellular distribution of Hook proteins and AP-4

The direct interaction between AP-4 and Hook1/Hook2 prompted us to analyze whether these proteins localized to the same subcel
lar compartments. To address this question, we used rabbit anti
 sera to Hook1 and Hook2 developed in the Krämer laboratory
(Walenta et al., 2001). Although these antibodies, as well as the rab
bit anti-Hook3 developed in the same lab, specifically recognized
their antigens in IBs (Figure 5A), the anti-Hook2 antibody was the
most specific for immunofluorescence (IF) microscopy. In agreement
with previous reports (Baron Gaillard et al., 2011; Dwivedi et al.,
2019a), IF microscopy with anti-Hook2 showed both perinuclear
tGN and peripheral punctate staining (Figure 5C). This pattern was
deemed specific, as it was markedly reduced in Hook2-KD cells
(Figure 5D). Similar to the observations with anti-Hook2, the anti
AP-4 ε IF showed both perinuclear and peripheral staining in most
cells (Figure 5C), which was reduced in the AP-4 ε KD cells (Figure
5E), as previously reported (Mattera et al., 2017). Comparison of
AP-4 ε and Hook2 immunostaining showed partial colocalization in
the perinuclear region (Figure 5C). The perinuclear staining of
FIGURE 3: Y2H assays demonstrate direct interaction of AP-4 μ4 with Hook1 and Hook2, and reveal subunit interactions in the FHF complex. (A) Y2H analysis of interactions between subunits of the AP-4 and FHF complexes. Constructs were subcloned in Gal4 AD and Gal4 BD vectors, as indicated. Following double transformation of the AH109 yeast strain, the cotransformants were plated in medium containing histidine (+His) as control of growth/loading and in medium without histidine (–His) to assay for activation of the HIS3 reporter gene on interaction of the constructs. The –His plates were supplemented with the indicated concentrations of AT, a competitive inhibitor of the His3 protein, to decrease background growth due to nonspecific interactions. Cotransformation of AD constructs with BD-p53 and of BD constructs with AD-SV40 large T antigen (T-Ag) provided negative controls, while double transformation with AD-T-Ag and BD-p53 was used as a positive control in the assays. The ε, β4, μ4, and σ4 constructs represent the different subunits of the AP-4 heterotetramer (Figure 1A). The results in the –His + 4 mM AT plate demonstrate the direct interaction of AP-4 μ4 with Hook1 and Hook2. In these experiments, we also used as control the AP-4 accessory protein tepsin which was previously shown to interact with both the ε and β4 subunits of AP-4 (Borner et al., 2012; Mattera et al., 2015; Frazier et al., 2016). We observed that the interaction of tepsin with AP-4 is actually trivalent, also involving direct binding to μ4 in addition to the ε and β4 subunits of this adaptor complex. (B) Y2H analysis of the assembly of the FHF complex. The results in the –His + 4 mM AT plate show: 1) the homodimerization of all Hook proteins and the formation of Hook1-Hook3 heterodimers, 2) the interaction of FHIP with Hook proteins (interactions with different Hook proteins were identified in –His plates + 4 mM AT depending on whether the AD-FHIP or BD-FHIP construct was analyzed; see also Supplemental Figure S1A for similar assays in the absence of AT), and 3) the interaction of FTS with all other subunits of the FHF complex. (C) Analysis of FHIP-L interactions using AD-FHIP-L and BD-AP-4 μ4 constructs. Results show the interaction of the AD-FHIP-L construct with BD-FTS and also confirm the interaction of Hook1 and Hook2 with AP-4 μ4, as seen with constructs in the opposite configuration (A). (D) Interaction of Hook1 and Hook2 with μ subunits of different AP complexes. Hook1 exhibits selective interaction with AP-4 μ4, while Hook2 exhibits a broader pattern of interaction. The TGN38 cytosolic tail was used as a positive control for interaction with μ subunits of AP-1, AP-2, and AP-3 complexes (see –His plate). The interactions of Hook2 in –His plates are not shown because of nonspecific reporter gene activation observed with the BD-Hook2 construct in the absence of AT (see Supplemental Figure S1A). (E) Interaction of Hook1 and Hook2 with the C-terminal fragment of AP-4 μ4 (residues 91–453 in the human protein) which is also involved in recognition of tyrosine-based sorting signals. The images separated by thin lines in B, D, and E correspond to different areas of the same assay plates.
Hook2 was not affected by AP-4 ε silencing and vice versa (Figure 5, D and E), indicating that, although the AP-4 and FHF complexes interact and partially colocalize, they do not depend on each other for their localization. Given that AP-4 is expressed at very low levels, we also examined its colocalization with Hook2 in cells transfected with expression vectors encoding all four subunits of AP-4. In transfected cells, we observed brighter AP-4 ε immunostaining in the TGN area compared with control cells, and this correlated with brighter immunostaining for Hook2 at the TGN (Figure 5F). The partial colocalization of Hook2 with AP-4 in the perinuclear area of the TGN is consistent with the idea that AP-4 ε and Hook2 may interact through their respective CC1 and CC2 domains.
Our observation of direct binding and partial colocalization of the AP-4 and FHF prompted us to analyze a possible functional relationship of these complexes. In view of the binding of Hook proteins to the dynein LIC and, possibly, to dynactin subunits, and of their effects on dynein–dynactin processivity (Schoeder and Vale, 2016; Lee et al., 2018; Qi et al., 2018; Urnavicius et al., 2018; Dwivedi et al., 2019), we investigated the effect of siRNA-mediated down-regulation of FHF subunits on the localization of AP-4 and its cargo ATG9A (Mattera et al., 2017; Davies et al., 2018; De Pace et al., 2019; Ivankovic et al., 2020). Due to a possible redundancy in the function of Hook proteins, we tested the effects of the combined KD of Hook1, Hook2, and Hook3 subunits. We also tested the individual KD of FHIP and FHIP-L, IB analysis showed that cells subjected to two rounds of treatment with siRNAs exhibited efficient silencing of their corresponding targets (Figure 5A). Silencing of FHIP-L could not be evaluated by IB due to the unavailability of specific antibodies to this protein and was therefore confirmed by quantitative real-time RT-PCR (Figure 5B). In these experiments, we included two additional conditions as controls. First, we examined changes in the distribution of AP-4 and ATG9A on overexpression of the GFP-labeled CC1 domain of the p150<sup>Glued</sup> dynactin subunit (GFP-p150-CC1), which is known to disassemble the endogenous dynein–dynactin complex and to prevent dynein–dynactin-dependent retrograde transport (Quintyne et al., 1999). Second, we examined the effect of AP-4 μ4 KD on the distribution of ATG9A, which requires AP-4 for export from the TGN (Mattera et al., 2017; Davies et al., 2018; De Pace et al., 2018; Ivankovic et al., 2020). We observed that overexpression of GFP-p150-CC1 resulted in marked dispersal of both AP-4 (Figure 6, A and B) and ATG9A (Figure 7, A and B) from the perinuclear area in virtually all cells (see quantification in Figure 8A), whereas AP-4 μ4 KD caused increased accumulation of endogenous and recombinant AP-4 ε, respectively). Images shown are multiple intensity projections prepared from Z-stacks. Scale bars; 5 μm for enlarged images (right column) and 10 μm for all other images.

**FIGURE 5:** Distribution of AP-4 and Hook2 in HeLa cells. (A) IB analysis of HeLa cells treated with siRNA pools as described in Materials and Methods. Control cells were treated with a nontargeting siRNA. Cells were lysed and cleared extracts were subjected to SDS–PAGE and IB with the indicated antibodies. The position of molecular mass markers (in kDa) is indicated at left of blots. Results are representative of at least three independent experiments carried out with different batches of silenced cells prepared on separate days (usually weeks apart). (B) The efficiency of FHIP-L silencing was analyzed by quantitative real-time RT-PCR because of the unavailability of a specific anti-FHIP-L antibody. Results shown represent absolute cDNA levels arising from reverse transcription of 15 ng of total RNA (mean ± SD of technical triplicates; *P < 10<sup>-6</sup>, unpaired one-tailed Student’s t test). The mRNA expression in FHIP-L-silenced samples relative to HeLa cells treated with nontargeted siRNA (Control) and normalized using β-actin as reference gene was 0.199. (C–E) Control, Hook2-, and AP-4 ε-siRNA-treated HeLa cells were immunostained for endogenous AP-4 ε, Hook2, and TGN46 and imaged by confocal fluorescence microscopy. (F) HeLa cells transfected with plasmids directing expression of all four AP-4 subunits (Rec. AP-4) were fixed, immunostained, and imaged as described for C–E. Single fluorescence microscope. (F) HeLa cells transfected with plasmids directing expression of all four AP-4 subunits (Rec. AP-4) were fixed, immunostained, and imaged as described for C–E. Single channel images in C–F are shown in inverted grayscale with DAPI staining of nuclei in magenta, while merged images depict staining of AP-4 ε, Hook2, and TGN46 in green, red, and blue, respectively, with nuclear staining in gray. Images in the last column are enlargements of the boxed areas in the merge panels. Although the antibodies to the different Hook proteins specifically recognized their antigens in IBs (A), the anti-Hook2 antibody was the most specific for IF microscopy analysis. The anti-Hook1 IF staining exhibited a perinuclear component in some cells together with small puncta scattered throughout the cytoplasm (possibly endosomes), along with an additional staining around the nuclear membrane that was also present in Hook1 KD cells (not shown). In contrast, immunostaining of Hook2 KD and AP-4 ε KD cells (D and E, respectively) demonstrated the specificity of anti-Hook2 and anti-AP-4 ε antibodies. Both AP-4 ε and Hook2 exhibited perinuclear and peripheral immunostaining (see C and F for staining of cells is consistent with the interaction of AP-4 with FHF occurring at this location. **KD of FHF complex subunits causes redistribution of AP-4 and ATG9A toward the cell periphery**

Due to a possible reductancy in the function of Hook proteins, we tested the effects of the combined KD of Hook1, Hook2, and Hook3 subunits. We also tested the individual KD of FHIP and FHIP-L, IB analysis showed that cells subjected to two rounds of treatment with siRNAs exhibited efficient silencing of their corresponding targets (Figure 5A). Silencing of FHIP-L could not be evaluated by IB due to the unavailability of specific antibodies to this protein and was therefore confirmed by quantitative real-time RT-PCR (Figure 5B). In these experiments, we included two additional conditions as controls. First, we examined changes in the distribution of AP-4 and ATG9A on overexpression of the GFP-labeled CC1 domain of the p150<sup>Glued</sup> dynactin subunit (GFP-p150-CC1), which is known to disassemble the endogenous dynein–dynactin complex and to prevent dynein–dynactin-dependent retrograde transport (Quintyne et al., 1999). Second, we examined the effect of AP-4 μ4 KD on the distribution of ATG9A, which requires AP-4 for export from the TGN (Mattera et al., 2017; Davies et al., 2018; De Pace et al., 2018; Ivankovic et al., 2020). We observed that overexpression of GFP-p150-CC1 resulted in marked dispersal of both AP-4 (Figure 6, A and B) and ATG9A (Figure 7, A and B) from the perinuclear area in virtually all cells (see quantification in Figure 8A), whereas AP-4 μ4 KD caused increased accumulation of endogenous and recombinant AP-4 ε, respectively). Images shown are multiple intensity projections prepared from Z-stacks. Scale bars; 5 μm for enlarged images (right column) and 10 μm for all other images.
ATG9A at the TGN (Figure 7, A and C). These control experiments demonstrated our ability to observe changes in AP-4 and ATG9A distribution on specific perturbations. Importantly, although less pronounced than the effect of GFP-p150-CC1 overexpression, combined silencing of Hook1, Hook2, and Hook3 (Hook1+2+3), as well as individual silencing of FHIP or FHIP-L, caused dispersal of both AP-4 (Figure 6, D–F) and ATG9A from the TGN toward the peripheral cytoplasm (Figure 7, D–F) in 50–87% of the cells (Figure 8A).

Because overexpression of GFP-p150-CC1 or siRNA of FHF subunits also caused some dispersal of the TGN marker TGN46 (Figures 6 and 7), we quantified changes in the Spearman's $r$ correlation coefficients for the colocalization of AP-4 or ATG9A with TGN46 (Figure 8, B and C). This quantification showed significant decreases in AP-4–TGN46 and ATG9A–TGN46 colocalization in GFP-p150-CC1-expressing cells (Figure 8, B and C) and a significant increase in ATG9A–TGN46 colocalization in AP-4 $\varepsilon$ KD cells (Figure 8C). We also observed that KD of Hook1+2+3, FHIP, or FHIP-L resulted in significantly lower Spearman's correlation coefficients for the colocalization of AP-4 and ATG9A with TGN46 (Figure 8, B and C). Interference with dynein–dynactin or FHF subunits thus caused greater dispersal of AP-4 and ATG9A than of TGN46.

Taken together, these observations indicated that silencing of FHF subunits caused dispersal of AP-4 and ATG9A toward the cell periphery, most likely by impairing the coupling of vesicles containing these proteins to dynein–dynactin, with consequent inhibition of retrograde transport toward the central region of the cell.

**Effect of KD of FHF complex subunits on autophagosomes**

Because ATG9A is involved in the early stages of autophagosome formation and maturation, we examined whether the dispersal of ATG9A in FHF-depleted cells affected autophagy. To this end, we performed IB analysis to monitor the conversion of LC3B-I to LC3B-II, which is used...
as a reporter for autophagy (Kabeya et al., 2004), in control cells versus cells subjected to either combined Hook1+2+3 silencing or individual silencing of FHIP or FHIP-L. These experiments showed no differences in the levels of the two LC3B species under both basal and starvation conditions, and also no difference in autophagic flux following inhibition of lysosomal degradation with bafilomycin A1 (Figure 9A). Likewise, IF microscopy showed no effect on the overall staining intensity of LC3B in control versus FHIP or FHIP-L KD cells (Figure 9, B–D). The only appreciable differences were alterations in the size and shape of LC3B-positive structures in a small percentage of FHF-deficient cells. In particular, we observed that ~3–7% of FHIP and FHIP-L KD cells exhibited LC3B-positive structures that were larger (0.9–1.6 μm diameter) than those in control cells (0.4–0.7 μm diameter) (Figure 9, B–D). In addition, we observed the appearance of tubular or “thread-like” LC3B-positive structures in ~4% of FHIP KD cells that were virtually absent in control cells (arrows in Figure 9C; see figure legend for quantification and statistical analysis). Thus, the dispersal of ATG9A in FHF-depleted cells was mostly inconsequential for autophagic flux, and only a small fraction of cells exhibited changes in autophagosome morphology.

DISCUSSION
Although the identification of ATG9A as an AP-4 cargo represented a significant advance in the understanding of AP-4 function (Mattera et al., 2015, 2017; Davies et al., 2018; De Pace et al., 2018; Ivankovic et al., 2020), a more complete picture of the AP-4 interactome is needed to fully explain the functional consequences of AP-4 deficiency. Our results contribute to this picture by revealing an interaction of AP-4 with the dynein–dynactin adaptor complex FHF and a role for the FHF complex in promoting the distribution of AP-4 and ATG9A to the perinuclear area of the cell.

Insights into the structure and function of the FHF complex
Xu et al. (2008) first demonstrated the existence of a complex termed FHF,
composed of the previously identified FTS (Lesche et al., 1997) and Hook proteins (Krämer and Phistry, 1999; Walenta et al., 2001), and an additional component named FHIP. Early studies showed that Hook proteins mediate attachment of organelles to MTs (Walenta et al., 2001) and participate in endosomal sorting of clathrin-independent cargo (Maldonado-Baéz, 2013). Subsequent studies revealed that Hook proteins and other subunits of the FHF complex function as “activating adaptors” linking the MT motors dynein–dynactin (Bielska et al., 2014; Yao et al., 2014; Zhang et al., 2016) and kinesin-3 (Bielska et al., 2014) to early endosomes in filamentous fungi. Further studies showed that mammalian Hook1 and Hook3 induce longer and faster runs of dynein–dynactin on MTs (Olenick et al., 2016; Schroeder and Vale 2016; Urnavicius et al., 2018) and suggested a similar role for Hook2 (Dwivedi et al., 2019a). These functional studies were extended to neurons with the demonstration that FHF complex subunits are required for dynein–dynactin-dependent retrograde axonal transport of Rab5- and transferrin-receptor-containing carriers (Guo et al., 2016) and BDNF-signaling endosomes (Olenick et al., 2019).

Recent structural studies shed light on the mechanistic bases for these functions of the FHF complex. The α-helical HD of Hook proteins was shown to bind to the C-terminal effector-BD of the dynein LIC (Schroeder and Vale, 2016; Lee et al., 2018; Dwivedi et al., 2019a). This binding as well as additional interactions with the Hook1 and Hook3 CC domains were found to be necessary for the productivity of dynein–dynactin (Olenick et al., 2016; Schroeder and Vale, 2016). Recent studies also indicated that Hook3 can function as a scaffold for bidirectional cargo transport due to its direct binding to both dynein–dynactin and the kinesin-3 protein KIF1C (Kendrick et al., 2019; Siddiqui et al., 2019).

Whereas the FHF complex was initially thought to comprise Hook1-3, FTS and FHIP (Xu et al., 2008), our TAP-MS and Y2H analyses, along with recent BioID-MS analysis of the dynein interactome (Redwine et al., 2017), indicate that the product of the FAM160A1 gene (herein referred to as FHIP-L) may also be part of this complex. Of note, a more distantly related gene product, FAM160B1, was also identified in the BioID-MS analysis of the dynein interactome (Redwine et al., 2017), suggesting that this protein could be a third alternative subunit of the FHF complex.

Our study also provides new insights into the assembly of the FHF complex. One aspect clarified by our Y2H analysis is the dimerization of Hook proteins. We observed that: 1) all Hook proteins homodimerize, six of them forming dimers of six different combinations, 2) Hook1 can also interact with Hook3, 3) Hook1 and Hook3 CC domains are necessary for the dimerization of Hook1 and Hook3, and 4) Hook proteins are involved in the disassembly of the FHF complex. These findings provide a mechanistic basis for the function of Hook proteins in the FHF complex.
2) Hook1 and Hook3 heterodimerize, 3) Hook2 does not heterodimerize, and 4) the combination of the Hook1 CC1 and CC2 domains is necessary and sufficient for the formation of Hook1 homodimers and Hook1-Hook3 heterodimers. These results extend previous observations on the role of CC domains in the assembly of Hook protein complexes (Krämer and Phistry, 1996; Xu et al., 2008; Lee et al., 2018). A second aspect highlighted by our experiments is that FHIP can bind directly to Hook proteins and is not just assembled into the FHF complex through its interaction with FTS (Xu et al., 2008). We also demonstrated that the C-terminal region of Hook1 (residues 658–728) binds to both FHIP and FTS and that different residues in this region appear to be involved in recognition of FHIP and FTS.

FIGURE 9: Conversion of LC3B-I to LC3B-II and morphology of autophagosomes in HeLa cells with KD of FHF subunits. HeLa cells were subjected to two rounds of treatment with the corresponding siRNA pools as described in Materials and Methods. (A) IB analysis of LC3B-I and LC3B-II in control and siRNA-treated cells seeded on 12-well plates and subjected to amino acid and serum starvation for 45 min at 37°C (image at left) or treatment with 100 nM bafilomycin A1 for 4 h at 37°C (image at right). IB for α-tubulin is shown as loading control. (B–D) Cells seeded on glass coverslips were fixed with methanol at –20°C and immunostained for endogenous LC3B, AP-4 ε, and TGN46 followed by confocal microscopy imaging. Anti-LC3B, anti-AP-4 ε, and anti-TGN46 immunostaining is shown in green, red, and blue, respectively. The panels at right depict merge images with DAPI staining of nuclei in gray (cells are outlined by dashed white lines). Arrows in C and D (left column) point at tubular or “thread-like” autophagosomes or large autophagosomes detected in a fraction of FHIP KD and FHIP-L KD cells, respectively (an enlargement of the area inside the white box in C is shown at the top right of the merge image). Scale bars: 5 μm for the enlargement in C (right column) and 10 μm for all other images. Cells were manually scored for the presence of LC3B-positive structures on threads or of large LC3B-positive structures (approximately 0.9–1.6 μm diameter range). We observed that 4.3 ± 0.7% of FHIP KD cells exhibited autophagosomes on threads compared with 0.3 ± 0.2% in control cells and 0 ± 0% in FHIP-L KD cells (mean ± SEM, P < 10−2 for FHIP-L KD compared with control cells, one-way ANOVA followed by two-tailed Dunnett’s test). We also observed that 3.1 ± 0.8% and 7.1 ± 0.8% of FHIP KD and FHIP-L KD cells, respectively, exhibited large LC3B-positive structures compared with 0 ± 0% in control cells (mean ± SEM; P < 5 × 10−2 for FHIP KD and P < 10−2 for FHIP-L KD compared with control cells, one-way ANOVA followed by two-tailed Dunnett’s test). Values shown are from triplicate measurements including cells from two independent silencing experiments. Total number of cells analyzed in the three scorings was 1217 (control), 1373 (FHIP KD), and 481 (FHIP-L KD).
Our observations on the predominant formation of Hook proteins homodimers and Hook1-Hook3 heterodimers, as opposed to heterodimers including Hook2, may have functional significance. Different Hook protein dimers could function at different cellular compartments as exemplified by the noninterchangeable roles of Hook paralogues during the initial stages of ciliogenesis (Baron Gaillard et al., 2011) and mitotic progression and cytokinesis (Dwivedi et al., 2019a). Alternatively, the differences in dimerization of Hook proteins could influence their function as dynein–dynactin adaptors. Each Hook protein dimer was initially assumed to bind two dynein LCs from a dynein dimer associated to a dynactin complex. However, recent cryo-electron microscopy and cryo-electron tomography structures, along with functional studies, revealed that Hook3 dimers favor the formation of complexes containing two dimeric dynein motors per dynactin, increasing the force and speed of the molecular motor (Grotjahn et al., 2018; Unravicius et al., 2018; reviewed in Reck-Peterson et al., 2018; Olenick and Holzbaur, 2019; and Dwivedi et al., 2019b). It will now be of interest to compare the effects of Hook1, Hook2, and Hook3 homodimers, and of Hook1-Hook3 heterodimers, on the stoichiometry of dynein recruitment and processivity of dynein–dynactin complexes.

FHF as an AP-4 accessory protein complex

Our TAP-MS data along with the pull down and co-IP studies demonstrated an interaction between the AP-4 and the FHF complexes. Other recent proteomic analyses, including BioID-MS of the Hook1 interactome (Redwine et al., 2017) and co-IP with GFP-tagged tepsin (Davies et al., 2018), also pointed to a link between AP-4 and FHF. The Y2H analyses in the present study demonstrated that the AP-4–FHF interaction reflects direct binding between the AP-4 μ4 subunit and the Hook1 and Hook2 subunits of the FHF complex. These analyses further showed that these interactions are mediated by the C-terminal domain of AP-4 μ4 and the CC1 and CC2 domains of Hook1. The formation of Hook1-Hook3 heterodimers discussed in the above paragraph is the likely explanation for the pull down of all three Hook proteins by AP-4 (Figure 2A) despite AP-4 μ4 only binding to Hook1 and Hook2 (Figure 3, A and C).

The C-terminus of AP-4 μ4 was previously shown to mediate recognition of tyrosine-based YXXØE-type signals in the cytosolic tail of transmembrane cargo proteins (Burgos et al., 2010), including ATG9A (Mattera et al., 2017). However, the C-terminal region of AP μ subunits and the μ homology domains (μHD) of related proteins such as the stonins and the muniscins Fcho1/Fcho2 also recognize sequences or domains other than tyrosine-based signals in either transmembrane or cytosolic proteins. For example, basic residue motifs mediate binding of the cytosolic tails of synaptotagmin 1 (Haucke et al., 2000), AMPA-type glutamate receptors (Kastning et al., 2007), and GABA receptors subunits (Kittler et al., 2005) to AP-2 μ2 and synaptotagmin 1 to stonin 2 (Martina et al., 2001; Maritzen et al., 2010). A basic motif within the synaptotagmin 1 C2B domain also mediates binding to AP-2 μ2 dependent on multimerization of the C2B domain (Grass et al., 2004). Other examples are the binding of AP-2 μ2 to stonin2 WxxF motifs (Walther et al., 2004) and the dishevelled2 DEP domain (Yu et al., 2010) and of Fcho1/Fcho2 to DPF motifs in Eps15 and Eps15R (Ma et al., 2016). Our results thus extend the function of μHDs to the recognition of CC domains in proteins lacking transmembrane domains. Further studies will be required to address the exact mode of recognition of the CC1 and CC2 domains in Hook1/Hook2 by AP-4 μ4, whether this recognition depends on the dimerization of the CC region and whether the AP-4 binding site for the YXXØE motif overlaps with that for Hook1/Hook2. In any event, our findings identify an AP-4 accessory factor that interacts with the μ4 subunit rather than the hinge-ear domains of the ε and β4 subunits, as is the case for tepsin (Borner et al., 2012; Mattera et al., 2015; Frazier et al., 2016) and for most accessory factors that bind to hinge-ear domains of other AP complexes.

At this time, we do not know whether binding of AP-4 μ4 to Hook1 and Hook2 may affect the function of these proteins as dynein–dynactin activating adaptors. The structural information available suggests that the Hook CC1-3 domains are important for high velocity and long run dynein–dynactin motility (Olenick et al., 2016; Schroeder and Vale, 2016). It is then tempting to speculate that AP-4 μ4 binding to the CC1 and CC2 domains of Hook1 and Hook2 homodimers or Hook1-Hook3 heterodimers may modulate the effect of these adaptors on the processivity of the dynein–dynactin complex. This possibility is reminiscent of the transition from autoinhibited to active state in another family of dynein adaptors, BicauD, which is regulated by cargo binding to its CC regions (Liu et al., 2013; Terawaki et al., 2015; Huynh and Vale, 2017; McClintock et al., 2018; Slawek et al., 2018; reviewed by Olenick and Holzbaur, 2019). Future studies should address whether AP-4 binding provides another regulatory layer modulating the ability of Hook proteins to activate the dynein–dynactin complex.

Role of the AP-4–FHF interaction in the cellular distribution of AP-4 and ATG9A

Combined silencing of all three Hook proteins or of FHIP or FHIP-L resulted in a more peripheral distribution of AP-4 and its cargo ATG9A, a phenotype similar to that caused by overexpression of the p150glued dynactin subunit CC1 domain. The dispersal of AP-4 and ATG9A was more pronounced than that of TGN46, indicating that it did not just correspond to the population of AP-4 and ATG9A at the TGN. This dispersal was thus likely caused by uncoupling of AP-4 and ATG9A-containing vesicles from dynein–dynactin, with consequent inhibition of their retrograde transport toward the cell center (Figure 10). We think that this role of FHF counteracts that of another AP-4 interactors, the RUN-domain-containing accessory protein, RUSC2, which promotes anterograde transport of ATG9A vesicles toward the cell periphery, most likely by coupling the vesicles to kinesin motor proteins (Davies et al., 2018) (Figure 10). As is the case for other intracellular organelles, the ability of AP-4/ATG9A vesicles to move in both anterograde and retrograde directions may be essential for the distribution of their functions through the entire cytoplasm.

Delivery of ATG9A to preautophagosomal structures is essential for the formation and expansion of autophagosomes (Orsi et al., 2012; Zavodsky et al., 2013), and its inhibition in AP-4-knockout cells results in impaired conversion of LC3B-I to LC3B-II (Mattera et al., 2017; Davies et al., 2018; De Pace et al., 2018; Ivankovic et al., 2020). KD of FHF subunits, however, did not affect LC3B-I to LC3B-II conversion or overall levels of LC3B in cells. The only noticeable effects were changes in the morphology
Interactions of FHF and AP-4 complexes

The human AP-4 ε and human tepsin constructs tagged at their N-terminus with a TSF epitope (pcDNA 3.1-TSF-AP-4-ε and pcDNA 3.1-TSF-tepsin) were described previously (Mattera et al., 2015). The pcDNA 3.1-TSF-myrsin (BORC subunit LOH12CR1) construct was described by Pu et al. (2015). The human Hook1, Hook2, and Hook3 constructs in pCMV-GST (pDEST27) and MSCV-N-HA/FLAG-human FTS (Xu et al., 2008) were a gift from J. Wade Harper (Harvard Medical School). The human Hook1, Hook2, Hook3, and FTS cDNAs were PCR-amplified and subcloned into the pcGBT9 and pGADT7 vectors (Clontech). The pcGBT9-FHIP (isoform 2 with 972 residues) was previously described (Guo et al., 2016). This FHIP construct was also subcloned in pGADT7. The cDNA encoding full-length human FHIP-L (FAM160A1 gene product) was PCR-amplified from a human brain library (Clontech) and subsequently subcloned into pcGBT9 and pGADT7 vectors. The human AP-4 ε, β4, and α4 constructs subcloned into pGADT7 (Boehm et al., 2001) and mouse AP-1 μ1A, human AP-1 μ1B, mouse AP-2 μ2, rat AP-3 μ3A, rat AP-3 μ3B, and human AP-4 μ4 constructs subcloned into pACT2 (Clontech) (Guo et al., 2013) were previously described. A cDNA fragment encoding human AP-4 μ4 followed by a GGSGGGSSG spacer and an HA tag was excised from the pC-neo-μ4-GGSGGGSSG-(HA3) construct (Mattera et al., 2014) and ligated into pcGBT9. A stop codon was subsequently introduced at the end of the μ4 coding sequence in order to generate pGBT9-human AP-4 μ4. The pcGBT9-human tepsin and pGBT9-rat TGN38 tail construct (residues 324–353) were previously described (Mattera et al., 2015). Sequences encoding human AP-4 ε fused to GST, TSF-tagged human AP-4 β4 fused to maltose-binding protein, and human AP-4 μ4 and human AP-4 α4 were cloned into pCAG-based vectors. Mutations were generated by site-directed mutagenesis and confirmed by DNA sequencing.

Antibodies

The following antibodies were used in this study: rabbit anti-Hook-1 (1:2,000 for IB; 1:1,000 for IF), rabbit anti-Hook2 (1:500 for IB, 1:750 for IF), and rabbit anti-Hook3 (1:2,000 for IB; 1:1,500 for IF) were gifts from Helmut Krämer (University of Texas Southwestern Medical Center); rabbit anti-FHIP (Abcam cat. ab184160, 1:1,000 for IB); mouse anti-AP-4 ε (BD Biosciences cat. 612028, 1:400 for IB, 1:75 for IF); mouse anti-AP-4 μ4 fused to GST, TSF-tagged human AP-4 β4 fused to maltose-binding protein, and human AP-4 μ4 and human AP-4 α4 were cloned into pCAG-based vectors. Mutations were generated by site-directed mutagenesis and confirmed by DNA sequencing.

MATERIALS AND METHODS

Recombinant DNA constructs

FIGURE 10: Proposed model for the MT-dependent transport of ATG9A-containing vesicles. AP-4- and ATG9A-containing vesicles are shown to undergo retrograde transport from the periphery to the center of the cell by virtue of coupling to dynein–dynactin via the FHF complex (this study) and anterograde transport from the center to the periphery of the cell by coupling to kinesin via the RUN and SH3 domain-containing protein 2 (RUSC2) (Davies et al., 2018). This proposed role of the FHF complex is based on the interaction of AP-4 with subunits of the FHF complex (Figures 1–4), the colocalization of AP-4 with FHF subunits (Figure 5), and the effects of silencing of FHF subunits on localization of AP-4 and ATG9A reported in this study (Figures 6–8). The FHF complex is shown to interact with AP-4 (this study), with cytoplasmic dynein (through binding of the HD of Hook proteins to the C-terminus of dynein LIC subunits) and to dynactin (resulting in stabilization of dynein–dynactin complex by coiled-coil domains of Hook proteins) (for review, see Dwivedi et al., 2019b). Two dimeric dynein complexes bound to dynactin-FHF are shown based on recent cryo-EM structural studies of Hook3-dynein–dynactin complexes (Grotjahn et al., 2018; Urnavicius et al., 2018). RUSC2 and kinesin are shown to remain associated with ATG9A vesicles after dissociation of AP-4, as reported by Davies et al. (2018). Coupling of AP-4- and ATG9A-containing vesicles to dynein–dynactin and kinesin allows distribution of these vesicles to different regions of the cell. FHF: fused toes protein homolog (FTS)/Hook/FTS– and Hook-interacting protein (FHIP) complex; MTOC: MT organizing center.

of LC3B-containing autophagosomes in a small fraction of FHIP- and FHIP-L-KD cells. These findings suggest that dispersal of ATG9A to the cell periphery, as seen in FHF-deficient cells, is less consequential for autophagy than its accumulation at the TGN, as observed in AP-4-deficient cells. The apparent lack of an effect of ATG9A dispersal on autophagy could be due to the cell type used in our study; perhaps effects could be seen in other cell types such as neurons. For example, the effects of AP-4 deficiency and retention of ATG9A in the TGN were more manifest in neurons, as evidenced by the impaired clearance of pathologic protein aggregates in the axon of AP-4-KO mice (De Pace et al., 2018; Ivankovic et al., 2020). Underscoring the importance of the FHF complex in the brain, reduced levels of Hook1 and Hook3 were found in brain tissue of patients with Alzheimer’s disease, and silencing of Hook3 was shown to enhance β-amyloid production (Herrmann et al., 2015). Hook2 was also found to regulate the formation of aggresomes (Szelenyi et al., 2007), junctional structures that accumulate misfolded proteins targeted for autophagy (Garcia-Mata et al., 2002). This could be due to a role of Hook proteins in coupling of autophagosomes to dynein–dynactin (Ravikumar et al., 2005; Kimura et al., 2008; Maday et al., 2012; Reck-Peterson et al., 2018). In light of our findings, however, some of the effects of Hook perturbations on autophagy could be due to its role in coupling ATG9A vesicles to dynein–dynactin.
human AP-4 ε or TSF-tagged human tepsin was performed as described previously (Mattera et al., 2015).

**Cell culture and transfection protocols**

H4 human neuroglioma cells, HeLa, and HEK293T cells were obtained from ATCC. H4 cells stably transfected with TSF-tagged human AP-4 ε or TSF-tagged human tepsin, HEK293T, and HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 4.5 g/l glucose and supplemented with 10% fetal bovine serum, 2 mM l-glutamine, and 100 U/ml penicillin–streptomycin (all reagents from ThermoFisher) at 37°C in a 5% CO2 atmosphere. Cells were intermittently grown in culture medium supplemented with MycoZap Prophylactic (Lonza) to prevent mycoplasma contamination. Cells plated on 100-mm dishes were transfected with 7 μg per of plasmid DNA using 27 μl of X-tremeGENE 9 reagent (Roche) in 500 μl of Opti-MEM I (ThermoFisher). Cells seeded on 6-well plates were cotransfected with a total of 1.4 μg of total plasmid DNA per well (0.35 μg of each of the four constructs encoding AP-4 subunits) using 5.4 μl of X-tremeGENE 9 reagent in 100 μl of Opti-MEM I.

**Pull down and co-IP experiments**

Experiments were performed in either transiently transfected HEK293T cells or H4 human neuroglioma cells stably transfected with TSF-tagged human AP-4 ε. Cells plated on 100-mm dishes were lysed in 0.8 ml of 50 mM Tris-HCl, pH 7.4, 0.8% (vol/vol) Triton X-100, and 75 mM NaCl supplemented with protease inhibitors (EDTA-free Complete, Roche). Following a 30-min incubation with rotation at 4°C, extracts were centrifuged for 15 min at 21,000 × g and 4°C and the supernatants were subjected to IP, pull down, or IP. Pull down of TSF-tagged constructs from lysates with Strep-Tactin beads (IBA) was performed as described in the preceding paragraph.

**Quantitative real-time RT-PCR**

Total RNA from HeLa cells treated with nontargeting siRNA (control) or ON-TARGETplus SMART pool siRNA targeting FHIP-L was prepared using the RNeasy Mini kit (Qiagen) following the manufacturer’s instructions. Samples containing 250 ng of total RNA were reverse transcribed using the Super Script VILO cDNA synthesis kit (Invitrogen). Levels of reverse-transcribed mRNA encoding FHILP-L were quantified using TaqMan gene expression assays (ThermoFisher) with FAM-probe/primer HS04935393_m1 for FAM160A1 (encoding FHILP-L) along with VIC-probe/primer HS01060665-g1 for ACTB (β-actin) as reference gene. PCR amplification was performed on triplicate cDNA samples originated from 15 ng of total RNA using TaqMan Fast Advanced Master Mix (ThermoFisher) and monitored in an AriaMX Real-Time PCR system (Agilent Technologies). Absolute expression levels were calculated from calibration curves obtained with serial dilutions of pGBT9-FHIP-L (10–3 to 10–7 μg/ml range). Relative expression was calculated using the 2–ΔΔCT method (Livak and Schmittgen, 2001), where ΔCT is the difference in threshold cycles (CT) between the target (FHILP-L) and the reference gene transcripts (β–actin) and ΔΔCT is the difference in ΔCT between the silenced (FHILP-L KD) and the control sample.

**IF microscopy**

Cells were fixed for 10 min in –20°C methanol and incubated with the indicated dilutions of primary (45–60 min at room temperature) and secondary antibodies (30–45 min at room temperature) in 0.1% bovine serum albumin, 0.1% saponin, and 0.02% sodium azide in PBS, DAPI (ThermoFisher) at 1:2,000 dilution and GFP-Booster Atto488 (Chromotech) at 1:400 dilution were added during incubation with secondary antibodies to stain nuclei and GFP constructs in transfected cells, respectively. Relative dispersal of AP-4 ε and ATG9A signals following treatment with siRNA pools or transfection with GFP-p150–CC1 was assessed by manual scoring of cells using a Zeiss Axio Imager.A1 fluorescence microscope (Plan Apochromat 63x/1.4 Oil DIC M27 objective) and by calculation of Spearman’s rank correlation values (next paragraph). Confocal microscopy images were obtained using Zeiss LSM 780 (63x/1.4 NA Plan Apochromat 63x objective) or Zeiss 880 (63x/1.4 NA Plan Apochromat 63x objective) laser scanning confocal microscopes.
Image analysis

Co-localization analysis was carried out using the Pearson–Spearman correlation plugin for ImageJ (French et al., 2008). Scatter plots of co-localization report the Spearman’s rank correlation value r, representing the relationship of the signal intensity from green and red channels of analyzed images. This value can range from −1 to +1, where 0 indicates no relationship, and −1 and +1 indicate a perfectly negative or positive correlation, respectively. The closer the r value is to +1, the more likely is the co-localization of signals. The plugin allows masking of areas to be included in the analysis. In a given image, TGN46-positive Golgi structures were masked prior to analysis using the selection brush tool as described (French et al., 2008).

A threshold level of 10 was set, under which pixel values were considered noise and not included in the statistical analysis. Statistical significance was analyzed by one-way ANOVA followed by two-tailed Dunnnett’s test using GraphPad Prism (GraphPad Software).

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