Accessory proteins of the zDHHC family of S-acylation enzymes

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Abstract

Almost two decades have passed since seminal work in *Saccharomyces cerevisiae* identified zinc finger DHHC domain containing (zDHHC) enzymes as S-acyltransferases. These enzymes are ubiquitous in the eukarya domain, with twenty-three distinct *ZDHHC* genes in the human genome. zDHHC enzymes mediate the bulk of S-acylation (also known as palmitoylation) reactions in cells, transferring acyl chains to cysteine thiolates, and in so-doing affecting the stability, localisation and function of several thousand proteins. Studies using purified components have shown that the minimal requirements for S-acylation are an appropriate zDHHC enzyme-substrate pair and fatty acyl-CoA. However, additional proteins including GCP16, Golga7b, huntingtin and selenoprotein K have been suggested to regulate the activity, stability and trafficking of certain zDHHC enzymes. In this Review, we discuss the role of these accessory proteins as essential components of the cellular S-acylation system.

Introduction

S-acylation is a common post-translational modification of cellular proteins involving the attachment of fatty acids onto cysteine residues (Chamberlain and Shipston, 2015). This modification occurs on several thousand proteins (Blanc et al., 2015; Blanc et al., 2019) that have accessible and reactive cysteine residues positioned at the cytosol-membrane interface (Rana et al., 2018; Rana et al., 2019). These proteins include ion channels, receptors, signalling proteins, molecular scaffolds and chaperones (Chamberlain and Shipston, 2015). The effects of S-acylation are substrate-specific but this modification typically affects the stability, localisation and interactions of modified proteins (Blaskovic et al., 2014; Chamberlain and Shipston, 2015; Essandoh et al., 2020; Gok and Fuller, 2020). S-acylated proteins have important physiological functions throughout the human body and many diseases are linked to perturbations in S-acylation (Chamberlain and Shipston, 2015).

S-acylation reactions are mediated by a family of zinc finger DHHC domain containing (zDHHC) proteins encoded in humans by 23 distinct genes (see Box 1) (Fukata et al., 2004; Greaves and Chamberlain, 2011; Mitchell et al., 2006). All zDHHC enzymes are predicted to be polytopic membrane proteins with four-six transmembrane (TMD) domains (Rana et al., 2018; Rana et al., 2019) (Fig. 1A). The catalytic DHHC (aspartate-histidine-histidine-cysteine) cysteine-rich domain (CRD) is present on a cytosolic loop (Rana et al., 2018; Rana et al., 2019) (Fig. 1A). Most zDHHC enzymes localise to the endoplasmic reticulum (ER) and Golgi, although a small number associate with the plasma membrane and endosomes (Greaves et al., 2011; Noritake et al., 2009; Ohno et al., 2006).

Elegant *in vitro* studies using purified yeast and mammalian zDHHC enzymes revealed that the S-acylation occurs *via* a ping-pong reaction mechanism, in which the cysteine of the DHHC motif reacts with a fatty acyl-CoA forming an “autoacylated” enzyme intermediate (Jennings and Linder, 2012; Mitchell et al., 2010) (Fig. 1B). The acyl chain can then either be hydrolysed through a reaction with water (releasing a fatty acid) or transferred to a cysteine thiolate of a substrate protein (Fig. 1B). This reaction process is likely to be similar for all zDHHC enzymes as substitution of the cysteine of the DHHC motif invariably leads to loss of enzyme activity (Fukata et al., 2004), although different zDHHC isoforms are likely to exhibit marked differences in relative reactivity (Lemonidis et al., 2014). Palmitoyl-CoA appears to be the preferred lipid substrate used in S-acylation reactions (Muszbek et al., 1999), reflected in the common usage of the term “palmitoylation” to describe protein S-acylation. However, different zDHHC enzymes display distinct selectivity profiles for fatty acyl-CoAs of different carbon chain length (Greaves et al., 2017; Rana et al., 2018).

In general, zDHHC enzymes are thought to be intrinsically active and to function as either monomers or higher oligomers (either homo-oligomers or hetero-oligomers with other zDHHC enzymes) (Fang et al., 2006; Lai and Linder, 2013). However, emerging evidence suggests that many zDHHC enzymes may be regulated by accessory proteins that control their activity, stability and/or localisation. Here, we discuss current knowledge about the role of Erf4, GCP16, Golga7b, huntingtin, and selenoprotein K in the regulation of zDHHC enzymes and cellular S-acylation.

Erf4 and GCP16: accessory proteins for Erf2 and zDHHC9, respectively

The zDHHC enzyme Erf2 and its accessory protein Erf4 were identified in a genetic screen for *S. cerevisiae* mutants that lead to a loss of function of an S-acylation-dependent Ras2 allele (Bartels et al., 1999) (Fig. 2). The specific Erf2 mutants that were characterised encoded truncated Erf2 proteins or led to amino acid substitutions in and around the DHHC-cysteine-rich domain (CRD) (Bartels et al., 1999), whereas missense mutations (leading to substitutions of S128P, V148K, and L204P) and a nonsense mutation (W180X) were identified in Erf4 (Bartels et al., 1999; Zhao et al., 2002). Erf2 and Erf4 form a protein complex that requires a hydrophobic region between amino acids 167–187 of Erf4 and associates with ER membranes (Bartels et al., 1999; Zhao et al., 2002). Analysis of the purified Erf2-Erf4 complex confirmed its S-acyltransferase activity as it enhanced the rate of S-acylation of Ras2 by 160-fold over basal incorporation rates (Lobo et al., 2002).

The levels of the Erf2 protein are reduced in cells lacking Erf4 (Lobo et al., 2002), and assays using cycloheximide inhibition of protein translation demonstrated a pronounced decrease in the half-life of Erf2 in Erf4-mutant cells, with an approximately 40-fold reduction in the steady-state levels of Erf2 (Mitchell et al., 2012). This rapid degradation of Erf2 in the absence of Erf4 was prevented when six lysine residues in the C-terminal 58 amino acids were substituted with arginine residues, suggesting that Erf4 protects Erf2 from ubiquitination-dependent degradation (Mitchell et al., 2012). In support of this idea, expression of His_6-ubiquitin and capture on Ni^{2+}-NTA agarose illustrated Erf2 polyubiquitination in Erf4-mutant strains, and the half-life of Erf2 in the absence of Erf4 was enhanced (and approached that seen in wild-type yeast) when components of the ubiquitination-mediated ER-associated degradation (ERAD) system were deleted (Mitchell et al., 2012). These results are therefore consistent with a model in which Erf4 protects Erf2 from ubiquitination and destruction via the ERAD pathway (Fig. 3).

Although Erf4 plays a key role in controlling Erf2 stability, this does not appear to be the only function of this accessory protein as a stabilised Erf2 mutant containing six lysine-to-arginine

substitutions was unable to suppress the growth defects seen in Erf4-mutant strains (Mitchell et al., 2012). Thus, Erf4 must exert additional effects on the S-acylation process. Indeed, when partially purified Erf2-Erf4 complexes were incubated with a fluorescent acyl-CoA substrate (Bodipy C12:0-CoA) and detected using a gel-based assay, the authors could show that the steady-state level of Erf2 autoacylation was substantially reduced in the absence of Erf4 (Mitchell et al., 2012). This could reflect either a slower rate of autoacylation, or a faster hydrolysis rate in the absence of Erf2 (Fig. 1B), and to assess these two possibilities, an additional autoacylation assay was employed that measures the release of CoASH, a by-product of the autoacylation reaction. This showed that after steady-state is reached, there was a more rapid palmitoyl-Erf2 hydrolysis rate in the absence of Erf4 (Mitchell et al., 2012), suggesting that the Erf4 accessory protein might shield the active site of Erf2 from water molecules (Mitchell et al., 2012) (Fig. 1B). The C-terminus of Erf2 might also contribute to the protection of the autoacylated intermediate as removal of the C-terminal 58 amino acids also enhanced the hydrolysis rate in both the presence and absence of Erf4 (Mitchell et al., 2012), suggesting that perhaps Erf4 acts by stabilising an interaction between the C-terminus and DHHC-CRD of Erf2. Interestingly, Erf4 was also shown to be important for successful transfer of the acyl group from Erf2 to a Ras2 substrate, implying that Erf4 also regulates the second stage of the S-acylation reaction either directly, or perhaps it has an additional role in the recognition of Ras2 (Mitchell et al., 2012).

The mammalian homologue of Erf2 was identified as zDHHC9 (which shares 31% amino acid identity with Erf2) (Fig. 2; see alignment in supplementary Fig. S1), whereas GCP16 (also known as Golgin subfamily A member 7, Golga7) was identified as the homologue of Erf4 (Swarthout et al., 2005) (Fig. 2; see alignment in supplementary Fig. S2). The functional relationship between zDHHC9 and Erf2 was confirmed by work showing that human zDHHC9 can at least partially rescue the phenotypes of Erf2 mutants of *S. cerevisiae* (Mitchell et al., 2014) and *Schizosaccharomyces pombe* (Young et al., 2014). However, rescue in *S. cerevisiae* also required GCP16, suggesting that this protein and Erf4 are not functionally interchangeable (Mitchell et al., 2014). zDHHC9 and GCP16 were shown to form a stable complex in both HEK293 and Sf9 insect cells (Swarthout et al., 2005), and indeed partial proteolysis of zDHHC9 occurred in the absence of GCP16, suggesting a similar stabilising effect of this protein on zDHHC9 as seen for Erf4-Erf2 (Fig. 3) (Swarthout et al., 2005). Furthermore, zDHHC9 was unable to S-acylate H-Ras in the absence of GCP16, suggesting that the function of the GCP16 is not simply to stabilise zDHHC9, but that it also contributes to the S-acylation reaction (Swarthout et al., 2005). Indeed, analysis of partially purified zDHHC9 showed that the enzyme is still able to undergo autoacylation in the

absence of GCP16 but had a faster rate of hydrolysis (Mitchell et al., 2014) (Fig. 1B), consistent with a role for GCP16 in stabilising the acylated zDHHC9 intermediate similar to that observed with Erf2-Erf4 (Mitchell et al., 2012). Although the zDHHC9-GCP16 complex appears functionally similar to Erf2-Erf4, the mammalian proteins predominantly co-localise at the Golgi, with some partial zDHHC9 fluorescence detected on ER membranes (Ohta et al., 2003; Swarthout et al., 2005). GCP16 is a peripheral membrane protein that is targeted to Golgi membranes through the S-acylation of two cysteines (cysteine-69 and cysteine-72; suppl. Fig. S2) (Ohta et al., 2003), and S-acylation of these residues is important for both the rescue activity in *S. cerevisiae* and interaction with zDHHC9 (Mitchell et al., 2014).

Thus, Erf4 and GCP16 appear to be essential accessory proteins that regulate the stability and S-acylation activity of Erf2 and zDHHC9, respectively. Although a short hydrophobic sequence in Erf4 has been implicated in interaction with Erf2 (Mitchell et al., 2012), our understanding of how Erf4/GCP16 function as accessory proteins is hindered by a general lack of knowledge about how the proteins interact with their respective partners. The ability of Erf4 to shield the active site of Erf2 might suggest that Erf4 and GCP16 interact with the DHHC-CRD of Erf2 and zDHHC9, respectively. Alternatively, as the C-terminus of Erf2 appears to be involved in regulating its autoacylation status (Mitchell et al., 2012), Erf4 and GCP16 might interact with the C-tail of Erf2 and zDHHC9 and perhaps reorientate this region of the zDHHC enzymes to protect their active site.

Regulation of zDHHC5 by GCP16 and Golga7b

Two recent studies have suggested that the regulatory role of GCP16 and its related isoform Golga7b (which share 61% amino acid identity; see suppl. Fig. S2) also extend to the plasma membrane-localised zDHHC5 enzyme (Fig. 2). In neuronal cells, zDHHC5 enters a dynamic trafficking pathway that is responsive to neuronal activity. The activity-dependent internalisation of zDHHC5 allows S-acylation of δ-catenin and subsequent trafficking of this cell adhesion regulator to dendritic spines where it facilitates synapse enlargement and recruitment of glutamate receptors (Brigidi et al., 2015; Brigidi et al., 2014).

A recent study investigated the role of Golga7b in zDHHC5 trafficking and localisation in non-neuronal cells (Woodley and Collins, 2019). This interaction is of interest as Golga7b and zDHHC5 have been previously shown to interact in a protein interactome study (Huttlin et al., 2015). This work confirmed that the proteins interact by co-immunoprecipitation and further showed that S-acylation of Golga7b is substantially reduced following siRNA-mediated depletion of *zDHHC5* (Woodley and Collins, 2019). S-Acylation by zDHHC5

appears to stabilise Golga7b, as an S-acylation-deficient Golga7b mutant could only be detected when proteasome activity was inhibited by MG132 (Woodley and Collins, 2019). The zDHHC5-Golga7b interaction requires three cysteine residues (cysteine-236, cysteine-237 and cysteine-245) in the C-terminal tail of zDHHC5, which were previously shown to be S-acylated (Collins et al., 2017; Woodley and Collins, 2019; Yang et al., 2010); this suggests that these cysteines modulate the structure or orientation of this region to facilitate Golga7b interaction.

Interestingly, siRNA-mediated depletion of *Golga7b* caused a reduction in the plasma-membrane levels of zDHHC5 in HeLa cells (Woodley and Collins, 2019), suggesting that Golga7b imparts a reciprocal regulation on zDHHC5 by stabilising the enzyme at the plasma membrane. This regulatory effect of Golga7b also requires its S-acylation by zDHHC5, as an S-acylation-deficient mutant of Golga7b is less able to enrich zDHHC5 at the cell surface (Woodley and Collins, 2019). Thus, a model emerges, whereby zDHHC5-mediated S-acylation of Golga7b allows Golga7b to regulate the cell-surface expression of zDHHC5. This raised the questions of how Golga7b affects zDHHC5 localisation? The plasma-membrane association of zDHHC5 in the presence of the S-acylation-deficient Golga7b mutant could be rescued either by pharmacological inhibition of clathrin-mediated endocytosis, or siRNA-mediated depletion of the μ subunit of the clathrin adaptor AP2 (Woodley and Collins, 2019). Furthermore, their assessment of endocytosis showed that expression of Golga7b reduced zDHHC5 internalisation, whereas the Golga7b S-acylation deficient mutant enhanced the endocytosis of zDHHC5 (Woodley and Collins, 2019). Based on these findings, the authors proposed that S-acylated Golga7b retains zDHHC5 at the plasma membrane by preventing its internalisation through clathrin-mediated endocytosis (Woodley and Collins, 2019) (Fig. 4A).

Counter-intuitively, although mutation of the S-acylated cysteines in zDHHC5 blocked its interaction with Golga7b, this zDHHC5 mutant was stabilised at the plasma membrane (Woodley and Collins, 2019). This was suggested to reflect the fact that endocytosis of zDHHC5 is dependent on its S-acylation status (Woodley and Collins, 2019). Taken together, these findings highlight a complex relationship between the interaction of zDHHC5 with Golga7b and S-acylation in regulating its endocytosis; although S-acylation is essential for internalisation of zDHHC5, only its S-acylated form interacts with Golga7b, which, in turn, stabilises the complex at the plasma membrane by blocking its internalisation. Thus, it is somewhat unclear under what conditions zDHHC5 is normally endocytosed.

Golga7b was also found to have a dramatic effect on the interactome of zDHHC5 as it promotes its association with a large number of proteins, notably components of

desmosomes, structures involved in cell adhesion (Woodley and Collins, 2019). Indeed, depletion of zDHHC5 or Golga7b caused a decrease in cell adhesion (Woodley and Collins, 2019). This study thus highlights a role for Golga7b in the regulation of zDHHC5 localisation that appears to be distinct from the direct effect of GCP16/Golga7 on the autoacylated intermediate of zDHHC9 (Mitchell et al., 2012). Importantly, the effect of siRNA-mediated depletion of Golga7b on the plasma-membrane localisation of zDHHC5 could not be rescued with the expression of GCP16 (Woodley and Collins, 2019), suggesting that there may be a specific role of the extended N- and/or C-termini that are present in Golga7b in its interaction with zDHHC5 (suppl. Fig. S2).

Another recent study also reported a functional interaction between Golga7 proteins and zDHHC5 by investigating the factors required for the cell lethal effect of the small molecule CIL56 (Ko et al., 2019). CIL56 induces an unconventional form of cell death that is associated with an expansion of the Golgi and accumulation of intracellular vesicles (Ko et al., 2019). These effects of CIL56 are thought to reflect the inhibition of anterograde Golgi transport by an unknown mechanism and a subsequent imbalance between anterograde and retrograde trafficking pathways (Ko et al., 2019). Indeed, CIL56 treatment leads to the accumulation of the epidermal growth factor receptor and transferrin receptor in intracellular vesicles (Ko et al., 2019).

A genome-wide shRNA screen was undertaken to explore the mechanism of action of CIL56. This screen revealed that the toxic effect of CIL56 was ablated to the greatest extent by the knockdown of either zDHHC5 or Golga7, in this case, the GCP16 isoform rather than Golga7b (Ko et al., 2019). The S-acyltransferase activity of zDHHC5 was important for CIL56-mediated toxicity as expression of wild-type, but not a catalytically-dead mutant of zDHHC5 restored CIL56 sensitivity in zDHHC5-depleted cells (Ko et al., 2019). The cell-death-promoting effect of CIL56 was also blocked by triascin C, an inhibitor of fatty acyl CoA biosynthesis, further supporting the involvement of S-acylation in the toxicity mechanism (Ko et al., 2019).

Furthermore, immunoprecipitation assays performed in this study showed that GCP16 interacts with both zDHHC9 and zDHHC5, and mutations that blocked the GCP16-zDHHC5 interaction (mutation of C-terminal S-acylation sites in zDHHC5 or the S-acylated cysteines in GCP16) prevented the restoration of CIL56 toxicity in zDHHC5- or GCP16-depleted cells, providing evidence that the function of zDHHC5 and GCP16 in CIL56-induced toxicity requires the formation of a complex between these proteins (Ko et al., 2019). However, previous work characterised GCP16 as a Golgi protein and, therefore, at a different subcellular location to zDHHC5 (Ohta et al., 2003; Swarthout et al., 2005). In contrast, Ko *et*

al found that Flag-tagged GCP16 was present at the plasma membrane in HT-1080 cells and that this localisation was independent of zDHHC5 (Ko et al., 2019). This suggests that there are either cell-type specific differences in GCP16 localisation, or that distinct pools of the protein are localised to different subcellular compartments. Indeed, the finding that GCP16 co-precipitated with both zDHHC5 and zDHHC9 (Ko et al., 2019) implies that GCP16 can form complexes with zDHHC enzymes at both the plasma membrane (zDHHC5) and the Golgi (zDHHC9) in the same cell type.

Although both recent studies support the formation of a functional zDHHC5-Golga7b/GCP16 complex there are clear differences between them. In particular, Ko *et al* reported that wild-type zDHHC5 (without GCP16 co-expression) was efficiently targeted to the plasma membrane, whereas a zDHHC5 mutant with disruption of the C-tail S-acylation sites accumulated in cytoplasmic puncta (Ko et al., 2019), which was opposite to the findings of Woodley and Collins who showed that zDHHC5 required Golga7b for efficient plasma membrane targeting and that zDHHC5 accumulated at the plasma membrane when the C-tail S-acylation sites were mutated (Woodley and Collins, 2019). A more recent study using total internal reflection fluorescence (TIRF) microscopy showed that C-tail S-acylation was associated with a stabilisation of the enzyme at the plasma membrane of neonatal rat ventricular cardiomyocytes, (Chen et al., 2020). Thus, in this cell type S-acylation is linked to increased localisation of zDHHC5 at the plasma membrane, consistent with the study by Ko et al. (Ko et al., 2019). How can the different reported effects of S-acylation on zDHHC5 localisation be reconciled? One possibility is that cell-type specific differential S-acylation of the three C-tail cysteines could lead to differences in zDHHC5 localisation. In addition, different cellular expression profiles of the large number of zDHHC5 interactors (Woodley and Collins, 2019) could also have an effect on S-acylation of zDHHC5 and its localisation. It will be interesting to explore these possibilities in future work.

Another interesting question is what is the mechanistic link between zDHHC5-GCP16 and sensitivity to CIL56? As discussed above, depletion of these proteins prevents the CIL56-mediated expansion of the Golgi and accumulation of intracellular vesicles (Ko et al., 2019); however this occurs despite a continued block in anterograde Golgi transport (Ko et al., 2019). As zDHHC5 has previously been linked to retrograde flux from the plasma membrane/endosomes (Lin et al., 2013; Sergeeva and van der Goot, 2019), it was instead proposed that depletion of zDHHC5 and GCP16 inhibits retrograde protein trafficking and thus limits protein and vesicle accumulation at the Golgi when anterograde transport is blocked by CIL56 (Fig. 4B).

Collectively, the above studies expand our knowledge of the regulatory effects the GCP16/Golga7b accessory proteins have on the zDHHC family. A critical next step will be to define the regions and/or domains of zDHHC9, Erf2 and zDHHC5 that interact with GCP16, Erf4 and Golga7b as this will allow to develop a more comprehensive understanding of the functions of these interactions. It is interesting to note that work to-date on Erf2/zDHHC9 has highlighted a role for Erf4/GCP16 in regulating the enzyme active site and catalytic process, whereas the regulatory mechanism of Golga7b/GCP16 on zDHHC5 is less well characterised. For instance, the question remains whether Golga7b/GCP16 is only involved in the trafficking and localisation of zDHHC5 and/or its endocytic functions, or whether these accessory proteins also directly impact on the S-acylation reaction, as is the case for GCP16 regulation of zDHHC9? It is also possible that future studies will uncover similar regulatory effects of the Erf4/Golga7b/GCP16 accessory proteins across different zDHHC enzyme isoforms. Indeed the study of Ko *et al* showed that expression of zDHHC8,which is closely related to zDHHC5, in zDHHC5-depleted cells could restore CIL56 sensitivity and that this enzyme also formed a complex with GCP16 (Ko et al., 2019).Given the lack of sequence identity outside of the DHHC domain in zDHHC5/8 and zDHHC9 (Greaves and Chamberlain, 2011), it could be that the DHHC domain plays a central role in the interaction of these enzymes with GCP16, and, that this accessory protein, in fact, interacts with many more members of the zDHHC family. Finally, it is important to note that S-acylation of GCP16/Golga7b by zDHHC5 could indirectly influence the activity of other zDHHC enzymes that are regulated by these accessory proteins, and thus depletion of zDHHC5 may have indirect effects on the cellular S-acylation machinery.

Regulation of zDHHC17 by huntingtin

Huntington's disease (HD) is an autosomal-dominant neurodegenerative disorder caused by expansion of a CAG repeat sequence in the *huntingtin* (*HTT*) gene, which encodes a poly-glutamine (polyQ) domain in the HTT protein (Tabrizi et al., 2020). A yeast two-hybrid screen for new interactors of huntingtin (HTT) identified a novel protein named huntingtin-interacting protein 14 (HIP14), and the authors showed that the HTT-HIP14 interaction was disrupted by pathogenic expansion of the polyQ domain (128Q compared with 15Q for the wild-type protein) (Singaraja et al., 2002). In the same study, a closely related protein HIP14L was also identified (Singaraja et al., 2002), and it was subsequently shown that HIP14 and HIP14L are members of the mammalian zDHHC family, corresponding to zDHHC17 and zDHHC13, respectively (Fukata et al., 2004; Huang et al., 2004) (Fig. 2).

The interaction of zDHHC17 or zDHHC13 with HTT is mediated by their ankyrin-repeat domains and leads to S-acylation of cysteine-214 in HTT (Huang et al., 2009; Huang et al., 2011; Yanai et al., 2006). Consistent with loss of interaction (Singaraja et al., 2002; Yanai et al., 2006), expansion of the polyQ region in HTT also disrupts its S-acylation (Huang et al., 2009; Huang et al., 2011). Interestingly, mutation of the S-acylation site or knockdown of zDHHC17 increased the formation of intracellular inclusions formed by mutant HTT (Yanai et al., 2006). As inclusion formation is a hallmark of Huntington's disease (Group, 1993), this finding suggests that defects in HTT S-acylation could be a contributing factor of the disease.

A surprising twist in the tale of the HTT-zDHHC17 interaction came from a study suggesting that HTT could function as a positive modulator of the S-acyltransferase activity of zDHHC17 (Huang et al., 2011). Specifically, the S-acylation status of zDHHC17 was decreased both in brain extracts from Huntington's disease gene homologue $(hdh)^{+/-}$ mice that have a 50% reduction in the levels of HTT protein, as well as in cortical neuronal cultures treated with HTT anti-sense oligonucleotides (Huang et al., 2011). The S-acylation status of zDHHC enzymes is often taken as a readout of activity as "autoacylation" is part of the catalytic process (Fig. 1). However, it is worth noting that several zDHHC enzymes (including zDHHC17) are also S-acylated outside of their DHHC domain (Collins et al., 2017), and the S-acylation status of zDHHC enzymes thus may not always accurately reflect their catalytic activity. Nevertheless, follow-up biochemical analyses supported the idea that HTT enhances zDHHC17 activity. Specifically, it was shown that cell extracts expressing wild-type (but not polyQ mutant) HTT enhance S-acylation of the model substrate protein GST-SNAP25 by GST-zDHHC17, which both were purified from a bacterial host (Huang et al., 2011). Furthermore, activity of zDHHC17 that has been immunoprecipitated against SNAP25 on $hdh^{+/-}$ mouse brain extracts was decreased compared to wild-type extracts (Huang et al., 2011). Finally, S-acylation of SNAP25 and the GluA1 subunit of AMPA receptors was decreased in brains of $hdh^{+/-}$ mice or following knockdown of HTT in cortical cultures (Huang et al., 2011).

Evidence linking zDHHC17 and HTT was further strengthened by the analysis of genetrap mice with ablated expression of zDHHC17. These mice displayed similar features to Huntington's disease, including a reduced number of medium spiny neurons in the striatum (Singaraja et al., 2011). Interestingly, however, the phenotype of these mice occurred in the absence of any change in HTT S-acylation, likely owing to a partial functional redundancy between zDHHC17 and zDHHC13 (Singaraja et al., 2011). Instead, the similarities in phenotypes between zDHHC17-mutant mice and HD mouse models are suggested to indicate that loss of zDHHC17 activity in the presence of polyQ mutant HTT contributes to

some features of Huntington's disease. In support of this idea, zDHHC17 immunoprecipitated from the YAC128 HD mouse model, which expresses human HTT with 128 glutamines in the polyQ region (Slow et al., 2003), displayed reduced S-acylation (used as a proxy for activity) and reduced activity towards SNAP25, consistent with the idea that wild-type (but not polyQ mutant) HTT is an accessory protein of zDHHC17 (Singaraja et al., 2011). It is also interesting to note that zDHHC13 also exhibits a reduced interaction with polyQ mutant HTT, indicating a role for this enzyme in HD pathogenesis as supported by a zDHHC13-mutant mouse line, which also displayed an HD-like phenotype (Sutton et al., 2013).

Overall, the decrease in zDHHC17 S-acylation status either in the presence of polyQ mutant HTT, or when levels of wild-type HTT are reduced, are consistent with a role for the HTT-zDHHC17 interaction in accelerating formation of or stabilising the autoacylated enzyme intermediate. However, the data supporting this idea is limited due to the absence of studies using purified proteins and lack of evidence that any S-acylated zDHHC17 species that are monitored reliably reflect the active form of the enzyme (Huang et al., 2011). An important breakthrough in this area should come from a detailed description of the HTT-zDHHC17 interaction interfaces. Interestingly, mutagenic analyses suggest that a major basis for the interaction of HTT to zDHHC17 is similar to that seen with its other substrates such as SNAP25, and involves the binding of a [VIAP][VIT]xxQP consensus motif in the substrate to a pocket in the ankyrin-repeat domain of zDHHC17, including key contacts with Asn-100 and Trp-130 (Lemonidis et al., 2015; Verardi et al., 2017). However, it is difficult to reconcile how competitive binding of HTT to the same site in the ankyrin-repeat domain as other substrate proteins could positively modulate zDHHC17 activity. In this context, a different study used immunoprecipitation analysis to suggest that there may be additional binding sites in HTT the full-length zDHHC17 can interact with (Sanders et al., 2014), and perhaps these other binding sites are the basis of the regulatory effect of HTT on the S-acyltransferase activity of zDHHC17. Alternatively, as this study used immunoprecipitation analysis, it is also possible that their results are based on indirect interactions between HTT and zDHHC17 that are facilitated by bridging proteins. In this respect it is interesting to note that a yeast two-hybrid study suggested that HTT and zDHHC17 share many common binding partners (Butland et al., 2014), and these might be potential candidates.

Regulation of zDHHC6 by Selenoprotein K

Selenoproteins are a group of cellular proteins that incorporate selenocysteine within their polypeptide chains, and they often have anti-oxidant and oxidoreductase activities due to the

reducing power of selenocysteine (Zhang et al., 2020). One of these proteins, Selenoprotein K (SelK) (see suppl. Fig. 3), has been shown to be important for store-operated Ca^{2+} entry in immune cells (Verma et al., 2011). Store-operated Ca^{2+} entry is activated in response to depletion of endoplasmic reticulum Ca^{2+} stores, which is triggered by inositol trisphosphate (IP_3) interaction with the IP_3 receptor (Prakriya and Lewis, 2015). Experiments using caged IP_3 in macrophages and T cells suggested that the IP_3 receptor function was perturbed in cells depleted of SelK (Fredericks et al., 2014). Indeed, IP_3 receptor protein levels (but not their mRNA) were reduced in certain tissues from SelK- knockout mice, including the spleen; here, reduced expression of the IP_3 receptor was found in T and B cells, as well as macrophages (Fredericks et al., 2014). A reduction in IP_3 receptor protein expression was also observed in Jurkat T cells that were grown in medium containing low selenium levels, which reduces SelK expression (Fredericks et al., 2014). Collectively, these observations suggest that there is a link between SelK and synthesis or stability of the IP_3 receptor. Previous work had shown that S-acylation of the fatty acid transporter CD36 is reduced in bone marrow-derived macrophages from SelK-knockout mice (Meiler et al., 2013), and this led to an interest in investigating whether the effects of SelK on the IP_3 receptor are linked to its S-acylation.

Indeed, acyl-biotin exchange revealed that the IP_3 receptor is modified by S-acylation in HEK293 cells, and mass spectrometry analysis identified cysteine-56 and cysteine-849 as the likely sites of modification (Fredericks et al., 2014). Because SelK is localised to the ER, its potential role in regulating ER-localised zDHHC enzymes was explored. SelK co-immunoprecipitates with zDHHC6, and this was dependent on the SH3-binding domain of SelK, which presumably mediates interaction with the SH3 domain of zDHHC6 (Fredericks et al., 2014). Indeed, limited depletion of zDHHC6 in Jurkat T cells led to a reduction in IP_3 receptor S-acylation and protein levels, and a corresponding decrease in IP_3-dependent Ca^{2+} flux (Fredericks et al., 2014). Although these analyses suggest that zDHHC6 can S-acylate the IP_3 receptor, no direct evidence was presented to show that S-acylation of the receptor is SelK-dependent.

To investigate the regulatory effects of SelK on zDHHC6 activity, in a follow-up study, the authors isolated ER microsomes from splenocytes of wild-type and SelK-knockout mice and used them as a source of enzyme for S-acylation of a fluorescent CD36 peptide (MGCDRNCK) (Fredericks et al., 2017). Thin-layer chromatography analysis of the reaction products revealed that the CD36 peptide was S-acylated to a lesser extent with microsomes from SelK-knockout mice than with wild-type microsomes (Fredericks et al., 2017). To further resolve the catalytic process, purified components were used, including a zDHHC6 construct consisting of the catalytic DHHC-CRD coupled to the C-terminal tail of the enzyme

containing the SH3 domain by a flexible glycine-serine linker (Fredericks et al., 2017). This zDHHC6 construct was used with full-length SelK that had either a selenocysteine at position 92 (Sec-92) (see suppl. Fig. S3), or a substitution with an alanine or cysteine residue (Fredericks et al., 2017). Interestingly, autoacylation of zDHHC6 by the fluorescent lipid substrate NBD-palmitoyl CoA was higher with Sec-92 SelK than with the other variants, implying that Sec-92 is important for the regulation of the autoacylation status of zDHHC6 (Fredericks et al., 2017). Unfortunately, owing to the nature of the assay used, a direct comparison of zDHHC6 autoacylation in the absence and presence of SelK was not possible (Fredericks et al., 2017). As Sec-92 SelK also increased the autoacylation of zDHHC6 at pH 6.8, which stimulates hydrolysis of the thioester, to a greater extent than the Ala- and Cys-SelK variants, the authors suggested that Sec-92 SelK may act by stabilising the autoacylated zDHHC6 intermediate (Fredericks et al., 2017) (Fig. 1B). There are caveats that should be noted about the use of the zDHHC6 recombinant protein described in this study. In particular, the appendage of the catalytic site to the C-terminus of the protein and removal of the transmembrane domains, which play a critical role in the S-acylation reaction by interacting with the acyl chain of acyl-CoA (Box1) (Rana et al., 2018), has the potential to disrupt the normal activity profile of the enzyme. It would be appropriate to also examine activity of this construct with a substitution of the active site cysteine to confirm S-acylation is occurring through the conventional mechanism.

To generate a better understanding of the wider role of SelK in regulating zDHHC6, it will be interesting to examine a range of zDHHC6 substrates and their S-acylation following SelK knockdown or selenium deprivation. In addition, S-acylation assays using purified components could be employed to more directly test the role of SelK in zDHHC6-mediated S-acylation, beyond the importance of the selenocysteine at position 92. In particular, it will be interesting to use full-length zDHHC6 in *in vitro* assays, as it is well-established that the TMDs of zDHHC enzymes play an essential role in the S-acylation reaction process in cells (Rana et al., 2018). Finally, it is interesting to note that although CD36 S-acylation was reported to be reduced in SelK-knockout cells (Meiler et al., 2013), more recent work has suggested that zDHHC4 and zDHHC5 (not zDHHC6) mediate the S-acylation of this protein in HEK293T cells and mouse adipocytes (Wang et al., 2019). In light of this, it will be interesting to determine if SelK also has any effects, either directly or indirectly, on other members of the zDHHC enzyme family.

Conclusions and perspectives

The role of Erf4 and GCP16 as regulators of Erf2 and zDHHC9, respectively, is supported by detailed kinetic analyses using purified proteins and by yeast genetic manipulation experiments. This work has provided clear evidence that the Erf4/GCP16 accessory proteins function by stabilising the autoacylated enzyme intermediate, with an additional role in acyl chain transfer to substrate proteins (Mitchell et al., 2012; Mitchell et al., 2014). The physiological importance of efficient autoacylation is highlighted by the finding that point mutations in zDHHC9 that cause intellectual disability either increase hydrolysis of the autoacylated intermediate or decrease the burst phase of autoacylation (Mitchell et al., 2014). A central question that emerges is whether Erf2 and zDHHC9 are unique in requiring an accessory protein to limit hydrolysis of the autoacylated intermediate and, if so, why? Whilst the analyses of HTT and SelK lack the detailed kinetic measurements performed on Erf4/GCP16, the results are consistent with an effect of these accessory proteins on the autoacylated state of zDHHC17 and zDHHC6, respectively (Fredericks et al., 2017; Huang et al., 2011), suggesting that, in fact, many zDHHC enzymes may require accessory proteins to facilitate efficient substrate S-acylation.

The finding that GCP16 also interacts with zDHHC5 and zDHHC8 (Ko et al., 2019) raises the possibility that GCP16 may act as an accessory protein for several zDHHC enzymes, perhaps by recognising conserved features of the DHHC-CRD. Although the effect of GCP16 on the catalytic activity of zDHHC5 was not examined, the related Golga7b protein was suggested to regulate the plasma membrane levels of zDHHC5 by preventing its endocytosis (Woodley and Collins, 2019). This observation, together with the finding that Erf4/GCP16 prevents degradation of Erf2/zDHHC9 (Mitchell et al., 2012; Swarthout et al., 2005) show that a single accessory protein can have multiple regulatory effects on zDHHC enzyme function.

A more refined understanding of the mechanistic actions of accessory proteins should emerge from detailed structural and biochemical analysis of their interactions with zDHHC enzymes, in particular, by mapping the relevant interacting domains and determining in which cases the actions of a single accessory protein span multiple zDHHC enzymes. Cell-based studies of zDHHC enzymes deficient in interacting with the accessory protein will then allow a detailed description of the importance of these protein complexes for the activity, stability, localisation and recognition of substrate proteins to emerge.

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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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BOX 1- The zDHHC Family of S-acylation Enzymes

The discovery that zinc finger DHHC domain containing (zDHHC) proteins are S-acyltransferase enzymes was a watershed moment for the S-acylation field. The first

breakthroughs came from analyses of the S-acylation of Ras2 and Yck2 in *Saccharomyces cerevisiae*, which identified effector of Ras function (Erf) 2 (Lobo et al., 2002) and Akr1 (ankyrin-repeat containing protein 1) (Roth et al., 2002), respectively, as the modifying enzymes. Erf2 and Akr1 share a common zinc finger-like cysteine-rich domain containing a DHHC tetrapeptide motif (Putilina et al., 1999) (DHYC in Akr1), which is critical for enzyme activity. These pioneering studies led the way to the identification of a family of twenty-three zDHHC isoforms in mammals (Fukata et al., 2004; Huang et al., 2004; Keller et al., 2004). Although some zDHHC enzymes appear to be quite promiscuous, other zDHHC enzymes display substrate selectivity, which is often driven by defined domains or amino acid motifs (Lemonidis et al., 2017; Roth et al., 2006).

The crystal structure of human zDHHC20 was recently reported (Rana et al., 2018; Rana et al., 2019). This structure showed that the four transmembrane domains of zDHHC20 arrange into a tepee-like cavity in the membrane, providing a space to accommodate the acyl chain of fatty acyl CoA (Rana et al., 2018). Furthermore, specific amino acids within the acyl chain binding cavity in the transmembrane domains were important in determining the length of the acyl chain that could be accommodated (Greaves et al., 2017; Rana et al., 2018). The catalytic DHHC motif was shown to be positioned at the membrane-cytosol interface, and the DHHC-CRD is stabilised by the binding of two zinc ions (Rana et al., 2018). Analysis of zebrafish zDHHC15 revealed a similar arrangement, suggesting this overall structure is likely representative of all zDHHC enzymes.

Figure Legends

Fig. 1. Autoacylation and deacylation of the catalytic cysteine in the DHHC motif of zDHHC enzymes. (A) Membrane topology of DHHC enzymes. The transmembrane domains (TMDs) are shown in *red*. Most zDHHC enzymes have four TMDs, whereas zDHHC13 and zDHHC17 have six TMDs (the additional two TMDs in these enzymes are indicated by a dashed outline and slightly lighter shade of red). The DHHC cysteine-rich domain (CRD) is shown in *yellow*. (B) Autoacylation of the DHHC cysteine occurs following reaction with a fatty acyl-CoA (the figure shows palmitoyl-CoA). For clarity only the DHHC domain is shown (*yellow*). The autoacylated state is unstable and can undergo hydrolysis to revert back to a deacylated state. Evidence suggests that Erf4 and GCP16 protect the

acylated state of Erf2 and zDHHC9, respectively. SelK may also stabilise the acylated state of zDHHC6. If a substrate protein is available (*orange*), the acyl chain can be transferred from the autoacylated DHHC cysteine to a suitable cysteine in the substrate, and the DHHC reverts to a deacylated state.

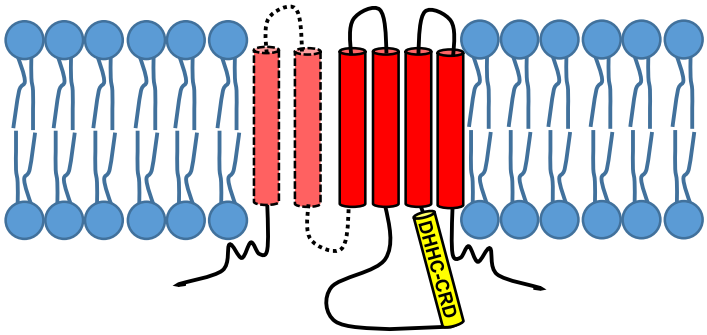
Fig. 2. zDHHC enzymes and their proposed regulators. Overview of the discussed zDHHC enzymes and the accessory proteins that are proposed to regulate their activity, localisation or stability in the cell. The figure indicates that zDHHC5 is regulated by GCP16 and Golga7b, zDHHC9 by GCP16, zDHHC17 by huntingtin (HTT), Erf2 by Erf4, and zDHHC6 by selenoprotein K (Selk). The S-acylation of GCP16, Golga7b and HTT is highlighted (*black squiggles*). The intracellular localisation of the proteins is also shown.

Fig. 3. Regulation of Erf2/zDHHC9 stability by Erf4/GCP16. In the absence of Erf4, Erf2 undergoes ubiquitination and is targeted for destruction *via* the ER-associated degradation (ERAD) pathway (the dashed outlines indicate that the degradation of the protein is increased). In the absence of GCP16, zDHHC9 is also destabilised, but its ubiquitination and/or enhanced degradation has not been demonstrated.

Fig. 4. Regulation of zDHHC5 localisation by Golga7b and the role of zDHHC5-GCP16 in CIL56-mediated cell toxicity. (A) Golga7b has been proposed to promote the plasma-membrane localisation of zDHHC5 by preventing its endocytosis. **(B)** *Top panel:* CIL56 promotes cell toxicity, which is associated with expansion of the Golgi and accumulation of intracellular vesicles; this has been proposed to occur due to an imbalance in anterograde and retrograde trafficking pathways that is caused by a block in anterograde Golgi transport. zDHHC5 and GCP16 have been proposed to promote retrograde transport. *Bottom panel:* Depletion of zDHHC5 or GCP16 relieves the cell toxic effect of CIL56, possibly by inhibiting retrograde transport, thus limiting vesicle accumulation, Golgi expansion and intracellular accumulation of (unknown) proteins that contribute to cell toxicity.

FIGURE 1

A


DHHC-CRD

B

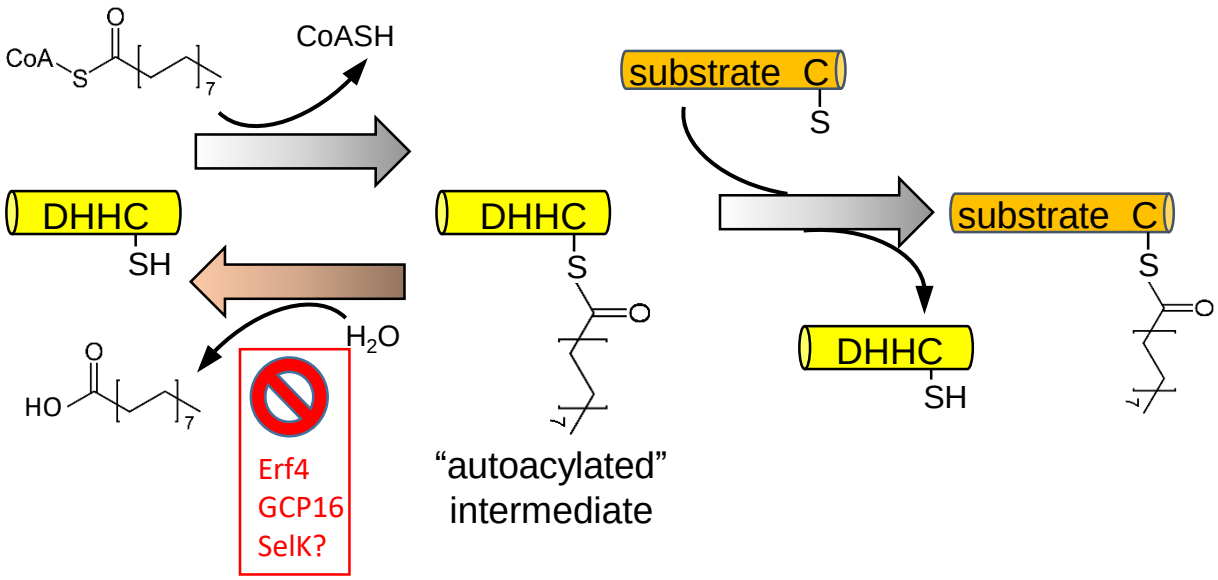

CoA
S
O
7
CoASH
DHHC
SH
DHHC
S
O
7
substrate C
S
substrate C
S
O
7
DHHC
SH
H2O
HO
O
7
Erf4
GCP16
SelK?
"autoacylated"
intermediate

FIGURE 2

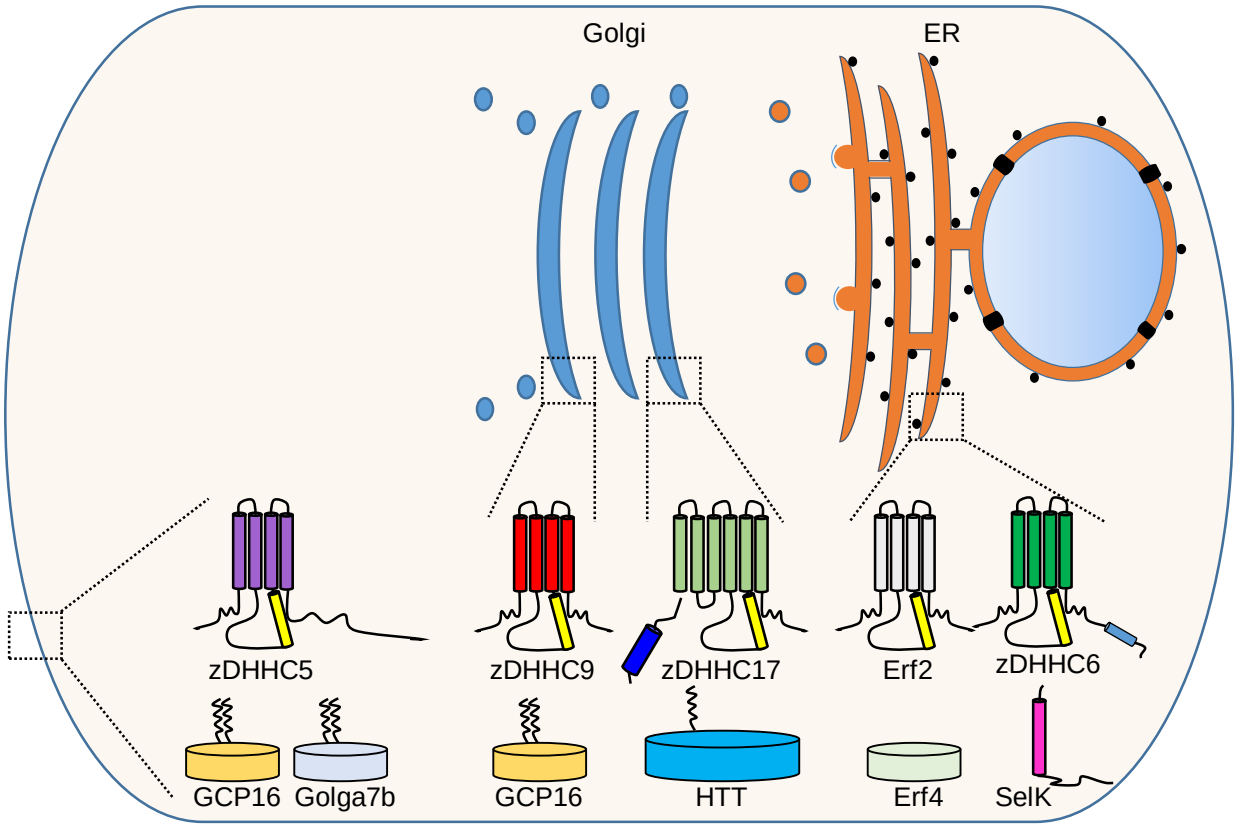

Golgi
ER
zDHHC5
zDHHC9
zDHHC17
Erf2
zDHHC6
GCP16
Golga7b
GCP16
HTT
Erf4
SelK

FIGURE 3

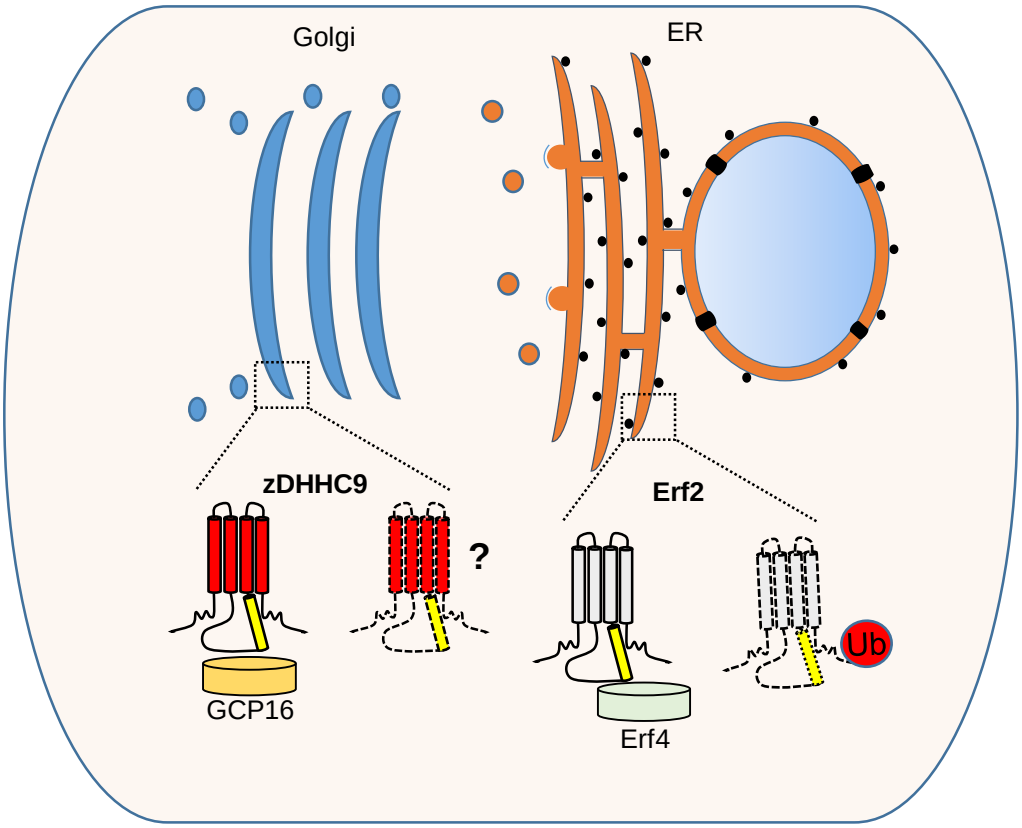
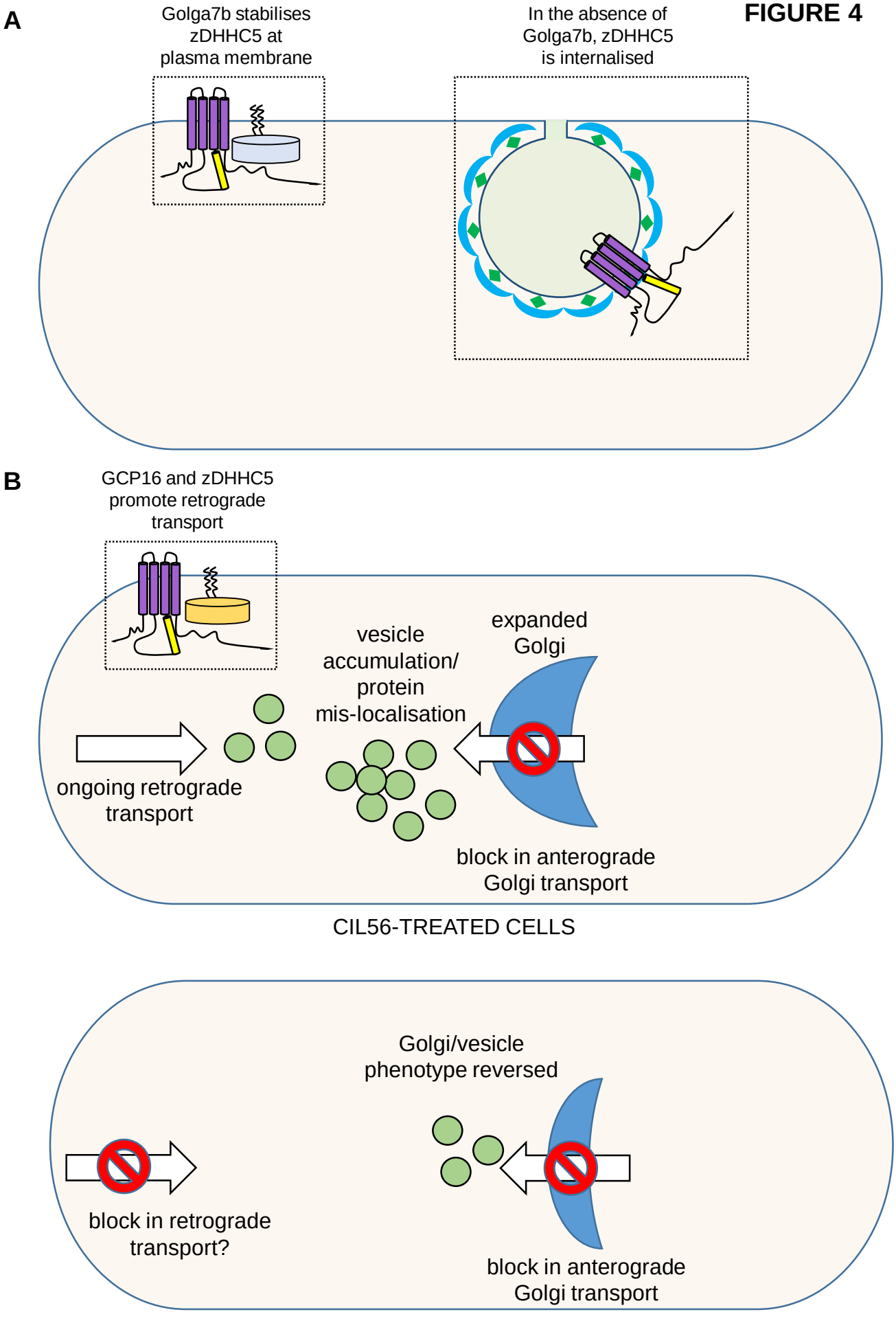


FIGURE 4


A
Golga7b stabilises zDHHC5 at plasma membrane
In the absence of Golga7b, zDHHC5 is internalised
B
GCP16 and zDHHC5 promote retrograde transport
vesicle accumulation/ protein mis-localisation
expanded Golgi
ongoing retrograde transport
block in anterograde Golgi transport
CIL56-TREATED CELLS
Golgi/vesicle phenotype reversed
block in retrograde transport?
block in anterograde Golgi transport
CIL56-TREATED CELLS WITH ZDHHC5/GCP16 DEPLETION