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CCPG1 is a noncanonical autophagy cargo receptor essential for reticulophagy and pancreatic ER proteostasis

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ABSTRACT

Reticulophagy is the conserved macroautophagic/autophagic degradation of the endoplasmic reticulum (ER) in response to ER stress or general nutrient deprivation. Sequestration of the ER by phagophores plays an important role in regulating ER size and homeostasis. In their recent work, Smith et al. have discovered that the ER-localized protein CCPG1 is a novel mammalian reticulophagy receptor that interacts with core autophagy machinery components—LC3, GABARAP and RB1CC1—and regulates reticulophagy.

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Macroautophagy (hereafter, autophagy) is a conserved pathway for the degradation of cytoplasmic components, including long-lived biomolecules, protein aggregates and dysfunctional/superfluous organelles [1]. The autophagic degradation of organelles may occur randomly as a component of nonselective autophagy—a response to nutrient deprivation—or it may be highly regulated. Regulated removal of organelles occurs by a process known as selective autophagy that utilizes specific receptors for cargo recognition [2]. The selective autophagic degradation of the ER, known as reticulophagy, has recently been found to be important for maintaining ER size and regulating proteostasis within the ER. Recovery from ER stress, for example, is particularly dependent on reticulophagy [3]

The ER is the first step in the secretory pathway, and plays an instrumental role in the synthesis, folding and post-translational modification of proteins that are sorted through this pathway. This organelle is also a major site for lipid and sterol synthesis and calcium storage. Relative to its importance in cellular physiology, ER function is under stringent surveillance. Multiple ER quality control pathways exist including the unfolded protein response (UPR) and ER-associated degradation (ERAD). The ERAD pathway is dedicated to the recognition of terminally misfolded proteins and their delivery to the retrotranslocon to allow removal from the ER, followed by their proteasome-dependent degradation in the cytosol. The UPR, which is a more comprehensive response, occurs upon a severe disruption of ER function such as one leading to the accumulation of toxic aggregates of unfolded proteins. The UPR promotes the transcriptional upregulation of ER chaperones, downregulates general translation, and promotes expansion of the ER, thereby allowing more resources, time and space for proper folding of proteins. The UPR also engages the ERAD pathway for terminal degradation [4,5].

Approximately a decade ago, a link between ER stress and autophagy induction was reported [6], closely followed by the observation that the dilation of the ER, a consequence of ER stress, is subsequently rescued by autophagic sequestration of this organelle [3]. However, the mechanism of ER recognition by the autophagy machinery remained unclear, with one report suggesting that reticulophagy might operate independently of canonical autophagy-related proteins [7]. Recent research has identified ER-resident proteins that act as reticulophagy receptors—RETREG1/FAM134B [8] in mammalian cells, and Atg39 and Atg40 in yeast [9]. These proteins function like known autophagy receptors in that they interact with Atg8-family proteins (Atg8 in yeast, and LC3 and GABARAP in mammals), which drives sequestration within phagophores, the precursor to autophagosomes. Now, Smith et al. report the discovery of a hitherto unidentified reticulophagy receptor, the ER membrane protein CCPG1 (cell cycle progression 1) [10].

The authors identified CCPG1 from an unbiased affinity-purification mass spectrometry screen for binding partners of GABARAP [11]. CCPG1, a vertebrate-specific protein with unknown physiological function, was named such because of its ability to rescue cell-cycle arrest when expressed in yeast cells [12]. CCPG1 has an N-terminal cytosolic region and a C-terminal lumenal region on either side of the central transmembrane domain.

The authors first tested the interaction between CCPG1 and Atg8-family proteins—LC3B, LC3C and GABARAP—by investigating the ability of these proteins to affinity precipitate CCPG1 from HEK293 cell lysates. Indeed, GST fusions of not only GABARAP but also LC3B and LC3C pull down MYC-tagged CCPG1. However, GABARAP mutated at the LC3-interacting region (LIR) docking site (which interacts with the LIR motif of LC3/GABARAP-interacting proteins) fails to affinity precipitate CCPG1, indicating that interaction with the LIR docking site is important. The authors then show that the interaction between CCPG1 and GABARAP is direct (and not mediated by a LIR-

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containing intermediary) by successfully affinity purifying GABARAP with an N-terminal fragment of CCPG1 in vitro. This interaction is consistent with the ER-membrane orientation of CCPG1, where the N terminus is cytosolic and is thus able to interact with cytosolic proteins. In a subsequent step, the authors map the N-terminal domain (NTD) and identify a putative LIR motif (WTVI; amino acids 14–17) in CCPG1, which fits the LIR consensus sequence (W/F/Y-X-X-L/I/V) found in other Atg8-family interacting proteins. Interestingly, the authors find that this sequence in CCPG1 is conserved among vertebrates, indicating a critical role for this sequence in the function of the protein. Expectedly, mutating this putative LIR sequence leads to the loss of interaction between CCPG1 and LC3 or GABARAP, thereby confirming the sequence as a bona fide LIR motif.

To uncover other binding partners of CCPG1, the authors performed co-immunoprecipitation-based tandem mass spectrometry (MS/MS) analysis of HA-CCPG1 immunoprecipitates in A549 cells. Among many identified interaction partners were 2 components of the ULK1 complex, ATG101 and RB1CC1. In contrast to RB1CC1, ATG101, ULK1 and ATG13, which co-immunoprecipitated with CCPG1, ATG5 (an autophagy-related protein that is not a part of the ULK1 complex) did not interact with CCPG1. However, endogenous CCPG1 showed a robust interaction only with RB1CC1 in both the presence and absence of an autophagic stimulus. Smith et al. then established the CCPG1 NTD as the RB1CC1-binding region, because the CCPG1 NTD expressed alone affinity precipitates RB1CC1 from HEK293 lysates, whereas this is not seen with CCPG1 lacking the NTD. Furthermore, they establish the direct nature of this interaction in vitro.

RB1CC1 binding by CCPG1 is intriguing because there has been no previous instance of a LIR-motif containing Atg8-family binding protein that also binds RB1CC1. To map out the specific residues of the CCPG1 NTD responsible for binding RB1CC1 the authors carried out an indirect immunodetection based dot-blot assay using an immobilized peptide array spanning the N terminus of CCPG1 probed with recombinant RB1CC1. Three peptide regions showed interaction with CCPG1, of which one region encompassing amino acids 99–112 was confirmed to be the RB1CC1-interacting region. Indeed, a truncated NTD of CCPG1 containing the first 145 amino acids was sufficient for interaction with RB1CC1. A sequence within this short region (amino acids 99–112) was found to be evolutionarily conserved among vertebrates, indicating that it might be a module for RB1CC1 binding. A similar sequence was identified near the N terminus. While both sequences were shown to contribute to RB1CC1 binding, the region within amino acids 99–112 was found to be predominantly required for the interaction.

Smith et al. followed up the biochemical analyses with confocal imaging, and demonstrated that CCPG1 localizes to the ER and forms discrete foci when the cell is starved, a phenomenon that is abolished by mutations in the LIR motif or in the RB1CC1-interacting region. This finding indicates that CCPG1 might be an ER-localized autophagy receptor. Consistent with this hypothesis, CCPG1 colocalizes with different autophagy markers—ZFYVE1/DFCP1 and WIPI2—along with LC3B and RB1CC1 upon starvation. Using 3D-structured illumination microscopy, the authors convincingly demonstrate that in HeLa cells CCPG1 foci colocalize with LC3B puncta at the reticular network of the ER. They also find a subset of CCPG1 foci that colocalize with STX17 and LAMP2 but do not colocalize with ZFYVE1 or WIPI2, indicating that these CCPG1 foci represent mature autophagosomes and/or autolysosomes. As with other autophagy receptors, CCPG1 levels are reduced upon starvation, owing to its lysosomal delivery and degradation. The inhibition of lysosomal degradation by treatment with bafilomycin A_1 consequently prevents this reduction. Similarly, CCPG1 mutated at the LIR motif or the RB1CC1-interacting region does not show a reduction in protein level upon starvation and fails to colocalize with LC3B. Finally, to show that CCPG1 degradation is directly autophagy dependent, the authors knocked out or knocked down the genes encoding ATG5 or RB1CC1, and both perturbations block starvation-dependent CCPG1 degradation in A549 cells.

HeLa cells lacking CCPG1 or harboring CCPG1 mutated at either the LIR motif or RB1CC1-interacting region show an expansion of the ER, indicating a role for CCPG1 in regulating ER size under physiological conditions. Furthermore, CCPG1 expression is induced upon ER stress, which suggests that it may also play a role in regulating ER size in response to such stress. Consistent with this notion, *ccpg1* knockout cells treated with the ER stressor DTT show an expansion of the ER compared to wild-type cells. The *ccpg1* knockout phenotype is recapitulated by an *atg5* knockout indicating that autophagy is responsible for the reduction in ER size post ER stress. The authors then link CCPG1 activity with proteostasis within the ER. The authors generate a *Ccpg1* hypomorphic mouse model and examine the pancreas, a predominantly secretory organ that requires finely-tuned ER function. They observe that the reduction in CCPG1 expression correlates with abnormalities in pancreatic morphology. The authors then show via mass spectrometry (LC-MS/MS) and immunoblotting that protein insolubility within the ER lumen is dramatically increased in *Ccpg1* hypomorphic mice. These findings indicate that pancreatic abnormalities may be the direct result of increased protein insolubility, with most of the insoluble proteins being ER lumenal chaperones or secretory components of the ER in enzyme-secreting acinar cells.

Finally, the authors investigate the morphology of the pancreatic acinar cells in *Ccpg1* hypomorphic mice using a combination of immunohistochemical staining and electron microscopy and show that polarization of the acinar cells is lost in these mice. The authors find that in the acinar cells of the mutant mice, the ER is not restricted to the basolateral regions of the acinar cells (as is the case with wild-type mice) but expands into the apical region, which promotes the loss of polarization as well as the formation of rough ER inclusions. Consistent with this, acinar cells in wild-type mice exhibit dense protein granules in the apical region, whereas *Ccpg1* mutant mice exhibit a pan-cellular distribution of proteinaceous foci indicating a loss in polarization. Pancreata from the mutant mice show higher levels of expression of ER-stress related transcripts such as those encoding HSPA5/BiP and DDIT3/Chop as well as increased inflammation and cellular death. MKI67 staining, a proliferation marker, revealed increased proliferation in the mutant mice, which the authors suggest represents a physiological response to replace dead cells. The authors rule out the contribution of major developmental abnormalities by confirming the absence of differences in

mRNA expression of key differentiation markers including *Gata4* as well as in circulating levels of pancreatic amylase between wild-type and mutant cells. Overall, this observation indicates that the exocrine pancreas in *Ccpg1* hypomorphic mice is exclusively functionally impaired.

Smith et al. identify CCPG1 as a novel, noncanonical mammalian autophagy receptor and establish its interaction not only with LC3 and GABARAP but also with RB1CC1, a component of the ULK1 complex. They also demonstrate that CCPG1 plays a role in maintaining ER homeostasis during both physiological and stress conditions. With recent research highlighting the importance of autophagy in regulating ER homeostasis in the context of innate immunity [13], this work takes us one step closer to understanding reticulophagy. Further investigation will shed more light on CCPG1 function, including the importance of RB1CC1 binding during autophagy, as well as the role of CCPG1 in promoting selective sequestration of the ER into phagophores.

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