# The classical secretion pathway during recombinant protein production in CHO cells: an omics perspective

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## Resumen

El desarrollo y la producción de proteínas recombinantes terapéuticas son primordiales para el tratamiento de diversas enfermedades. A pesar de que muchas de estas proteínas son producidas en células de ovario de hámster chino (CHO), aún se buscan estrategias para mejorar sus productividades. Las tecnologías ómicas han permitido identificar de forma profunda varios procesos que impactan en la expresión de estas proteínas. Sin embargo, dado que estos estudios se han realizado usando células completas, los resultados se encuentran sesgados hacia aquellas proteínas más abundantes. Por consiguiente, nuestro grupo de investigación ha empleado la proteómica subcelular como una alternativa viable para identificar proteínas de menor abundancia que participan en procesos asociados a la productividad celular, con énfasis en la vía clásica de secreción, pues esta ha sido descrita como un cuello de botella durante la síntesis y secreción de proteínas. Como resultado, el fraccionamiento subcelular permitió la identificación de 386 nuevos blancos, de los cuales alrededor del 30% son proteínas relacionadas con la vía clásica de secreción, revelando mecanismos moleculares poco explorados que podrán ser un punto de partida para la ingeniería de células CHO. En esta revisión discutimos sobre la intensificación de la búsqueda de datos asociados a la producción de proteínas recombinantes en células CHO, con particularidad en la vía de secreción clásica. Nuestro grupo se enfocó en esta vía mediante el uso de la proteómica subcelular, lo que nos permitió comprender la variación de mecanismos asociados al aumento de la productividad específica.

**Palabras clave:** Células CHO, proteínas recombinantes, fraccionamiento subcelular, ómicas, proteómica.

## Abstract

The development and production of therapeutic recombinant proteins is essential for the treatment of several diseases. Despite the fact that many of these proteins are produced in Chinese hamster ovary (CHO) cells, strategies are still being sought to improve their productivity. Omics technologies have made it possible to deeply identify several processes that impact on the expression of these proteins. However, since these studies have been carried out using whole cells, the results are biased towards those higher abundance proteins. Consequently, our research group has used subcellular proteomics as a viable alternative to identify lower abundance proteins that participate in processes associated with cellular productivity, with emphasis on the classical secretion pathway, since this has been described as a bottleneck during the synthesis and secretion of proteins. As a result, subcellular fractionation allowed the identification of 386 new targets, of which around 30% are proteins related to the classical secretion pathway, revealing little-explored molecular mechanisms that could be a

starting point for CHO cell engineering. In this review we discuss the intensification of the search for data associated with recombinant protein production in CHO cells, particularly in the classical secretion pathway. Our group focused on this pathway through the use of subcellular proteomics, which allowed us to understand the variation in mechanisms associated with increased specific productivity.

**Key words:** CHO cells, recombinant proteins, subcellular fractionation, omics, proteomics.

## Introducción

Recombinant proteins (RP) have become a crucial tool during last decades in academic research, biopharmaceutical industry and healthcare systems (Puetz & Wurm, 2019). Approved biopharmaceuticals have been increasingly marketed in US and EU, reaching a total of 155 products during the 2014-2018 period. Most of these biologics are monoclonal antibodies (mAbs), and the rest corresponds to hormones, blood related proteins, enzymes and vaccines (Walsh, 2018).

Biopharmaceuticals can be obtained from different hosts like bacteria, yeasts, insect and mammalian cells, where *Escherichia coli*, Chinese hamster Ovary (CHO) cells, *Saccharomyces cerevisiae* and *Pichia pastoris* are the preferred options (Tripathi & Shrivastava, 2019; Walsh, 2018). These RP are subjected to a wide range of post-translational modifications (PTM), according to protein properties and the selected host (Bandyopadhyay, 2008; Hajba et al., 2018; Qiu et al., 2019). As these PTM can modify the structural conformation and biological behavior of therapeutic proteins, mammalian cells are frequently chosen to perform modifications on biopharmaceuticals similar to those occurring in humans (Walsh, 2018).

Some advantages of mammalian expression systems are the secretion of RP to the extracellular medium (G. Zhang et al., 2017), performing complex PTM, successful expression of multi-subunit protein complexes (Baser & van den Heuvel, 2016) and high bioprocesses yields (Huang et al., 2010). Of these platforms, CHO, mouse myeloma (NS0), baby hamster kidney (BHK), human embryo kidney (HEK) and human retinal cells have gained regulatory approval (Dumont et al., 2016).

Despite their success, biologics prices limit the global access to this market. These high costs come partly by regulatory approval for intended use, and long-term treatment and/or high doses needed to achieve clinical efficacy (Deleuran et al., 2020; Puetz & Wurm, 2019). In this scenario, novel strategies to increase product titer are strongly encouraged, aiding to alleviate this hard panorama.

Many approaches have been developed to increase product yield, like optimizing protein and gene design (Cho et al., 2019), genetic vectors (Lin et al., 2017), delivery methods (Steger et al., 2015), selection procedures (Chin et al., 2015), cell line engineering (Shen et al., 2020) and bioprocess performance (Stepper et al., 2020). Among these, cell engineering has been noticed as a very promising approach, even though it has not been explored enough yet (Fischer & Otte, 2019). However, a prior understanding of cellular mechanisms behind protein production by omics is necessary for a systematic knowledge. Omics applied to clonal populations producing the same protein but differing in their specific productivity ($q_p$) have exposed several cellular processes contributing to protein production (Carlage et al., 2009; Hausmann et al., 2018; Kang et al., 2014; Ley et al., 2015; Orellana et al., 2015; Sommeregger et al., 2016). Nevertheless, a lower coverage of the classical secretory pathway (CSP) in comparison to other cell compartments has been achieved by these previous omics. This pathway plays a crucial role in several cellular processes such as lipid metabolism (Funato et al., 2020), autophagy (Morel, 2020) and apoptosis (Kara & Oztas, 2020), and of course, synthesis, modification and transport of secreted proteins (Barlowe & Miller, 2013).

Therefore, in this review we integrate a summary of previous transcriptomic and proteomic studies among CHO cell clones that differ in their $q_p$ and their association with the CSP. Then, we describe our subcellular proteomics results (Pérez-Rodriguez et al.,

2021), which highlights molecular mechanisms associated with changes in $q_p$ that operate in the CSP. Furthermore, we compare our recent findings with the literature. Accordingly, our data revealed that combining subcellular fractionation and compartmentalized proteomics allows a deep understanding of the secretory pathway and its relationship with the productivity level in CHO cell clones. The proposed targets are tools for future development that will have a positive impact on the performance of bioprocesses.

***Differential transcriptomic and proteomic studies of CHO cells differing in $q_p$***

Omics have shed light on some molecular processes sustaining a higher protein producer phenotype in mammalian cells. By studying CHO cells through transcriptomics and proteomics, it has been predicted that a higher protein synthesis supported by an active cell energy production (Ho, 2013; Nissom et al., 2006; Yusufi et al., 2017), DNA protection mechanisms (Kang et al., 2014; Yusufi et al., 2017), cell defenses against Reactive Oxygen Species (ROS) (Orellana et al., 2015; Yusufi et al., 2017), and modification of carbohydrates, lipids and aminoacids metabolism (Doolan et al., 2008; Ho, 2013; Kang et al., 2014; Ley et al., 2015; Meleady et al., 2008; Nissom et al., 2006; Orellana et al., 2015; Sommeregger et al., 2016; Yusufi et al., 2017), as well as an increased protein secretion to the extracellular medium (Orellana et al., 2015; Sommeregger et al., 2016), regularly take place in higher producer cells. Also, the cytoskeleton is rearranged most of the time during protein production, where differential abundance of many cytoskeletal proteins could lead to a deep restructuring of the filaments and microtubules (Carlage et al., 2009; Clarke et al., 2011; Hausmann et al., 2018; Kang et al., 2014; Meleady et al., 2008; Orellana et al., 2015; Sommeregger et al., 2016; Yusufi et al., 2017).

Occasionally, proteins participating in cell proliferation have lowered their abundance, which could redirect cell resources towards protein production (Ho, 2013; Meleady et al., 2008; Nissom et al., 2006; Orellana et al., 2015). However, since $q_p$ and growth rate do not correlate in all cases (Chusainow et al., 2009; Ley et al., 2015), these proliferation-related targets should be evaluated carefully before any modification. Apoptosis inhibition, which prolongs cell viability and increases the RP titer, is another Gene Ontology (GO) category enriched in higher producer cells (Doolan et al., 2008; Meleady et al., 2008; Sommeregger et al., 2016). Regarding secreted host cell proteins, the most common GO annotations among those differentially accumulated are cell signaling, cytoskeleton, intracellular transport, metabolism and apoptosis (Zhu et al., 2016), in agreement with the functions of secreted proteins in CHO cells (Kumar et al., 2015).

The impact of different signaling events on cell productivity in recombinant CHO cells has not been explored enough yet. In this sense, the regulation of calcium-dependent (Doolan et al., 2008; Kang et al., 2014; Sommeregger et al., 2016) and annexin-dependent responses (Nissom et al., 2006), and signaling from MAP kinases (Clarke et al., 2011; Doolan et al., 2008; Kang et al., 2014), Ras (Clarke et al., 2011), insulin (Edros et al., 2014), Toll-like receptors (Doolan et al., 2008), G proteins, GTPases of the Rho family, phosphatases and nuclear receptors (Sommeregger et al., 2016), have emerged as possible relevant pathways operating in these cells. A more in-depth study of these pathways in a RP production context is necessary to elucidate their role in the regulation of cellular productivity.

An augmented access of transcription machinery to DNA (chromatin remodeling), transcription and translation, coupled to an active protein catabolism (Bedoya-López et al., 2016; Carlage et al., 2009; Clarke et al., 2011; Hausmann et al., 2018; Kang et al., 2014; Meleady et al., 2008; Nissom et al., 2006; Sommeregger et al., 2016; Yusufi et al., 2017) have been other GO categories tightly linked to protein production. To distribute and secrete the protein overload, the CSP have shown a rearrangement of its intracellular traffic, where the increment of adapter subunits (AP2, AP3), proteins related to the formation and recruitment of vesicles (PDCD6), membrane fusion (tethering factors, NSF, SNAREs) and vesicle structure (COPA, COPG2, COPB1), molecular motors (kinesin, myosin) and small GTPases (Rabs, Sar1a,

Arfs), could promote the formation, transport, recognition and fusion of vesicles (Clarke et al., 2011; Doolan et al., 2008; Ho, 2013; Kang et al., 2014; Orellana et al., 2015; Sommeregger et al., 2016). As to chaperones, different sets have been uncovered depending on the RP expressed, which could be explained by the fact that each protein would require particular chaperones to avoid an incorrect folding within the endoplasmic reticulum (ER) and the further activation of stress responses (Carlage et al., 2009; Clarke et al., 2011; Doolan et al., 2008; Hausmann et al., 2018; Ho, 2013; Meleady et al., 2008; Nissom et al., 2006). Interestingly, although Unfolded Protein Response (UPR) and stress responses were significantly affected canonical pathways in rhBMP2 producer cells co-expressing PACE enzyme, by a transcriptomic study (Doolan et al., 2008), they were not differentially represented at the proteomic level (Meleady et al., 2008), probably indicating a limited capacity of whole cell proteomics to detect differences in low abundant proteins.

***Discovering novel targets and cellular processes associated to changes in $q_p$ in mAb producing CHO cells by a subcellular proteomics approach***

One big disadvantage of this afore mentioned proteomic reports is their use of whole cell homogenates. That kind of analysis is not representative of the entire proteome, because the relative abundance between cellular proteins may differ by up to 7-8 orders of magnitude, where ubiquitous and structural proteins can mask the detection of less abundant ones (Stasyk & Huber, 2004), such as those belonging to the CSP. However, subcellular fractionation prior to proteomics can bypass this limitation and eliminate this bias. Coupling of fractionation and proteomics represents an area called subcellular proteomics, which allows the identification of a greater number of proteins and the study of previously overlooked cellular processes (Michelsen & von Hagen, 2009; Paulo et al., 2013).

Given the benefits of subcellular proteomics and with the goal of a deeper insight into the CSP in mind, we carried out a proteomic comparison of ten subcellular compartments between two recombinant CHO cell lines. CRL-12444 (lower producer) and CRL-12445 (higher producer) anti hIL-8 mAb secreting cell lines, showing a 27-fold difference in their $q_p$, were chosen as cell models for subcellular proteomics. To resembling industrial cell culture conditions, cells were expanded in chemically defined medium in agitated Erlenmeyer flasks, at constant temperature (37°C) and atmosphere (5% $CO_2$), and sampled during exponential growth phase (Pérez-Rodriguez et al., 2021).

Prior proteomics, different cell fractionation protocols (Pérez-Rodriguez, de Jesús Ramírez-Lira, et al., 2020) and protein precipitation techniques (Pérez-Rodriguez, Ramírez, et al., 2020) were evaluated in order to isolate cell fractions enriched in organelles belonging to the CSP, namely ER, ER-Golgi intermediate compartment (ERGIC), Golgi Apparatus and vesicles, and to quantitatively precipitate their proteins before proteomic analysis. The fractionation protocol and composition of resulting compartments are delineated in Figure 1. After mechanical disruption, differential and isopycnic centrifugation, and acetone precipitation, samples were trypsin-digested and their peptides identified and quantified by label-free LC-MS/MS. As a result, 4952 total protein groups were identified and reliably quantified, from which 493 were differentially accumulated between both cell lines. In agreement with antecedent proteomics, metabolism of carbohydrates, lipids and aminoacids, cell signaling, proliferation, DNA repair, transcription, translation, synthesis and folding of proteins, intracellular transport, cytoskeleton organization and cellular mechanisms against ROS, were GO categories enriched among the differentially accumulated proteins (DAP) (Pérez-Rodriguez et al., 2021). Other categories recognized in our study with a lower representation in previous reports were nucleotide metabolism, autophagy, RNA splicing, protein degradation, cell adhesion, extracellular matrix (ECM) organization and ER stress-dependent responses. Among all DAP, 46 ones (21 upregulated and 25 downregulated) were consistently highlighted by both employed algorithms, SAM and ROTS, indicating that these targets should be explored first in future engineering strategies for recombinant CHO cells.

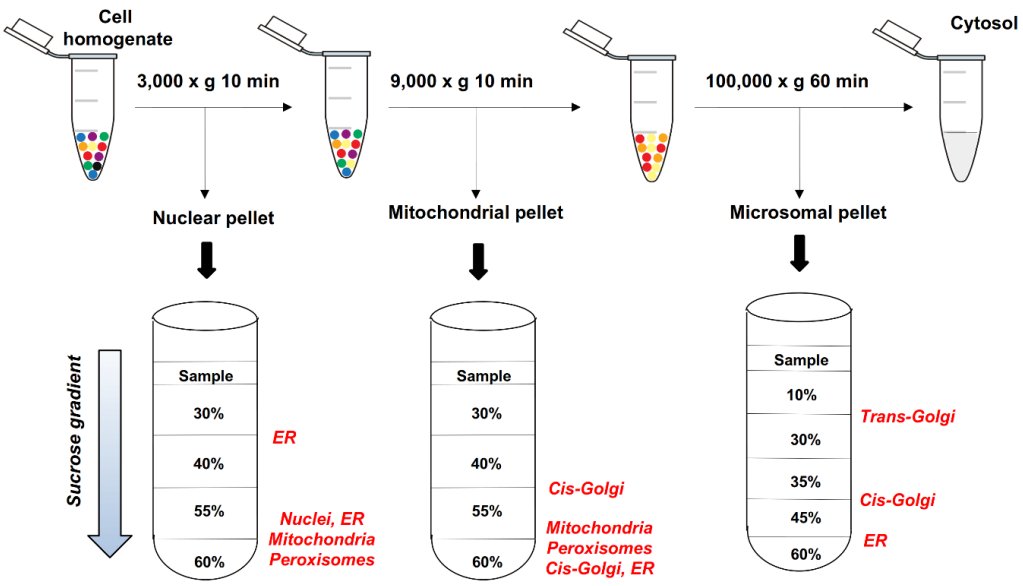


*Figure 1. Subcellular fractionation protocol of CHO cells by combining mechanical disruption, and differential and isopycnic centrifugation.*

By obtaining enriched fractions of subcellular organelles before proteomics characterization, we identified that near the third part of all proteins correlating with $q_p$ changes were from the CSP, mainly from the ER (74% upregulated and 67% downregulated) and Golgi Apparatus (59% upregulated and 54% downregulated) (Figure 2A). Additionally, about half of all DAP came from sucrose fractions enriched in CSP compartments, especially *trans*-Golgi and *cis*-Golgi (Figure 2B), which indicates an overrepresentation of this pathway in relevant $q_p$ associated processes.

With the aim of uncover those molecular mechanisms taking place in the CSP that are related to $q_p$ differences, 21 upregulated and 13 downregulated proteins, with known functions and identified by both comparison algorithms (SAM and ROTS), were classified into different biological categories. As a result, the synthesis and translocation of proteins to the ER, ER stress and UPR, homeostasis of ER and Golgi Apparatus, proteasomal degradation, antegrade and retrograde transport, production of ECM components and other secreted proteins, glycosylation and autophagy, seemed to play an important role during mAb production in CHO cells.

Protein translation and their import into the ER were enhanced in the high producing clone, probably associated with the increase in formation, stability and function of SRP through SRP72 (Becker et al., 2017), and with the SRP receptor (Mandon et al., 2003) and Sec62 translocon, which mediated protein internalization. Interestingly, most targets causing ER stress or involved in UPR (CLCC1, DNAJC3, EMC7, OS9, MINPP1, TMED4, UFC1, PRKCD, PITPNM1, SURF4) were decreased in the higher producer cells (Figure 3). The loss of CLCC1 (Jia et al., 2015) and SURF4 (Fujii et al., 2012) could trigger a stress signal and initiate UPR by blocking the entering of ions into the ER, which affect its homeostasis. Others have demonstrated to be required for a successful UPR by aiding in the folding, management and disposal of proteins in the overcrowded ER, and its homeostasis reestablishment, through their chaperone and ERAD-related (DNAJC3, ECM7, OS9)

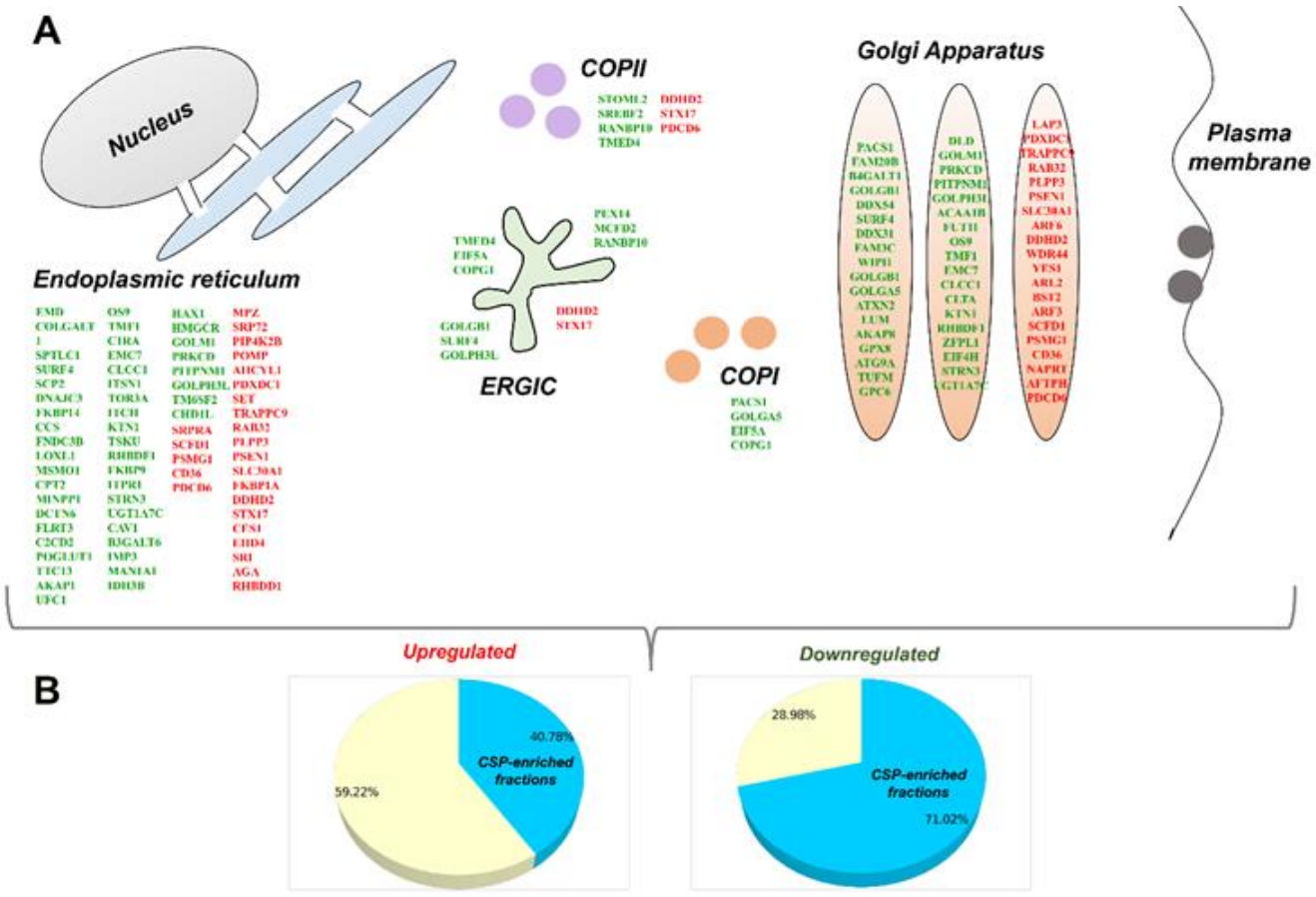


*Figure 2. Differentially accumulated proteins (DAP) belonging to the classical secretory pathway (CSP). (A) Upregulated (red) and downregulated (green) proteins mapped to organelles from the secretion pathway. (B) Percentage of DAP coming from sucrose fractions enriched in secretory organelles.*

(Christianson et al., 2012; A.-H. Lee et al., 2003; Melville et al., 1999; Satoh et al., 2015; van der Goot et al., 2018), PTM (UFC1) (Komatsu et al., 2004; Y. Zhang et al., 2012), kinase (PRKCD) (Lai et al., 2017), apoptosis (MINPP1, TMED4) (Hwang et al., 2008; Kilaparty et al., 2016) and signaling (PITPNM1) (Lev et al., 1999) activities. Downregulation of these proteins suggests that the higher producer cells could manage a high RP production without develop an ER stress nor UPR, in line with earlier omics research (Carlage et al., 2009; Meleady et al., 2008; Nissom et al., 2006).

Other proteins necessary for a proper functioning and homeostasis of the CSP were also differentially accumulated between both cell populations. The morphology, compaction level, relative position and functioning of ER, ERGIC, Golgi Apparatus and COPI vesicles, were probably disturbed by TMF1 (Yamane et al., 2007), GOLPH3L (Ng et al., 2013), GOLGA5 (Sohda et al., 2010), DDHD2 (Inoue et al., 2012), SCFD1 (Laufman et al., 2009) and STX17 (Muppirala et al., 2011), probably as a side effect of their functions in vesicle transport, while the disposal of incorrectly folded transmembrane proteins was enhanced

by RHBDD1 (Paschkowsky et al., 2016), together with a higher accumulation of proteasome chaperones (PSMG1 and POMP) (Fricke et al., 2007). ER calcium concentration appeared to be crucial, and it was maintained through stabilization of RyR and IP3R receptors by FKBP1A (Vervliet et al., 2015) (Figure 3).

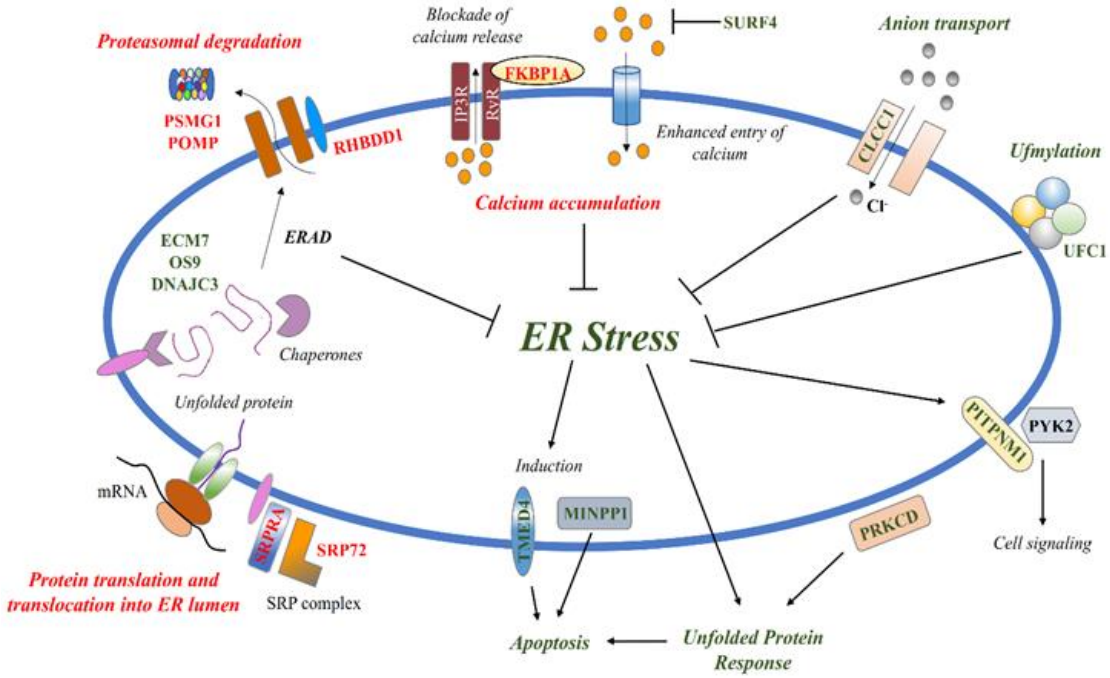


*Figure 3. Classification of differentially accumulated proteins (DAP) into molecular processes in the endoplasmic reticulum (ER), and their inter-relationship. Red: upregulated, green: downregulated.*

Vesicle trafficking was profoundly modified in the higher producer cells. Protein traffic in the early (ER, ERGIC, Golgi Apparatus) and late (*trans*-Golgi, plasma membrane (PM)) secretory, and endocytic pathways was favored, whereas the intra-Golgi transport was remodeled. Anterograde transport from ER to Golgi Apparatus seemed to be very active by a higher abundance of PDCD6, TRAPPC9, SCFD1 and ARF3 (Figure 4A), while retrograde traffic was supported by SCFD1, STX17 and ARF3 (Figure 4B). The calcium-binding protein PDCD6 and the small GTPase ARF3 aided in vesicle formation by facilitating the outer coat assembly, cargo loading of COPII (Kanadome et al., 2017; la Cour et al., 2013) and coatomer recruiting (Kuai et al., 2000; Volpicelli-Daley et al., 2005). Tethering, disassembling and fusion of vesicles was improved by TRAPPC9 (Zong et al., 2012), the SM protein SCFD1 (Demircioglu et al., 2014; Kosodo et al., 2003; Li et al., 2005) and the SNARE STX17 (Gordon et al., 2010). Vesicle traffic seems to be tight regulated given that protein secretion was not increased for all cargoes, such as those mediated by ZFPL1 (Chiu et al., 2008).

While some cargoes could be benefited from SCFD1 during intra-Golgi transport, others may be affected by downregulation of the tethering factors GOLGB1, GOLGA5 and TMF1, and the structural COPI component COPG1 (Figure 4C). Golgi resident proteins (Sohda et al., 2010), glycosylation enzymes (Stevenson et al., 2017), ECM components (Katayama et al., 2018) and PM proteins (Alvarez et al., 2001) are some of these cargoes, which suggests that a lower production of certain host biomolecules

saves limited cellular resources that could be used instead for RP expression (Kol et al., 2020).

In the endocytic and late secretory pathways, despite PACS1 downregulation, the significant augment of RAB32 and ARF3 could positively impact on endosome - *trans*-Golgi network (TGN) - PM traffic. RAB32 mediates in this context the biogenesis and trafficking of vesicles by interacting with adaptor proteins, LRRK2 and retromer complex (Waschbüsch et al., 2014, 2019) (Figure 4D). The activation of these transports has not been reported before and could be coupled with other cell activities like growth, signaling, survival and autophagy (di Fiore & von Zastrow, 2014).

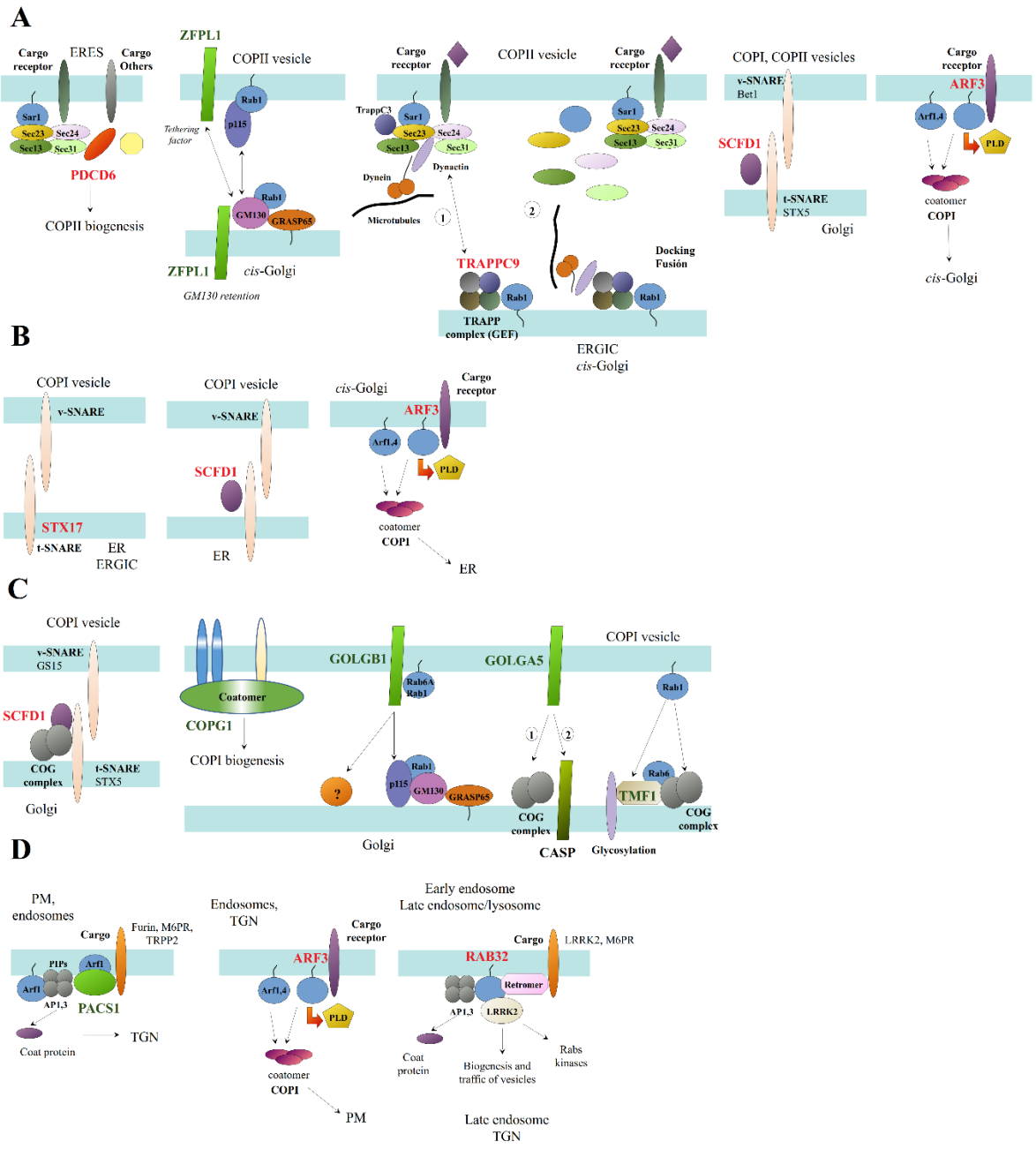


*Figure 4. Vesicle trafficking processes disturbed along the secretory pathway by differentially accumulated proteins. Red: upregulated, green: downregulated.*

Autophagy is a survival mechanism of eukaryotic cells to protect themselves from stressful conditions, by obtaining energy and biomolecule precursors, and the removal of damaged organelles (Kim et al., 2013). Given that only a handful of papers have studied its effect on recombinant CHO cells (Baek et al., 2016; Jardon et al., 2012; J. S. Lee et al., 2013; Nasseri et al., 2014; X. Zhang et al., 2018), the autophagy-related DAP found in our subcellular proteomic study are of great relevance (Figure 5). Autophagy could become activated in our mAb producing cells by loss of PRKCD (D. Zhang et al., 2017) and ZFPL1 (Xie et al., 2017), and the increase of RAB32, SCFD1 and STX17. While PRKCD and ZFPL1 trigger some stimuli, the other targets promote autophagosome formation (Hirota & Tanaka, 2009), lysosome maturation (Renna et al., 2011) and autophagosome-lysosome fusion (Vats & Manjithaya, 2019).

***Most $q_p$ associated targets uncovered by subcellular proteomics have not been reported before in whole cell proteomics***

The DAP identified in our subcellular proteomic study were compared with those identified in earlier whole cell proteomics (Carlage et al., 2009; Hausmann et al., 2018; Ho, 2013; Kang et al., 2014; Nissom et al., 2006; Orellana et al., 2015; Sommeregger et al., 2016), to assess the overlapping degree between both strategies. As shown in Table 1, only 33% of upregulated and 17% of downregulated ones matched with at least one report, indicating that 67% and 83% of upregulated and downregulated proteins from our strategy, respectively, constitute new targets to be modified. Only a portion of the proteins shared with earlier reports followed the same direction (up or down) in all cases, suggesting that targets from CHO cells could be RP, subline, clone, culture conditions or $q_p$-dependent.

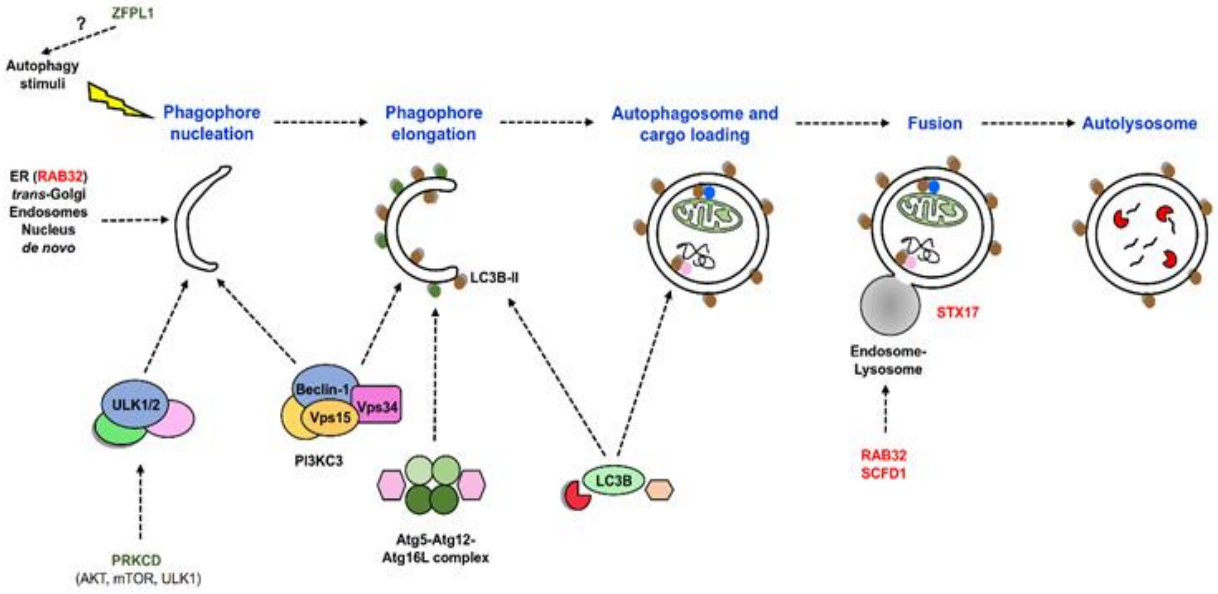


*Figure 5. Differentially accumulated proteins functioning on autophagy. ER: endoplasmic reticulum. Red: upregulated, green: downregulated.*

*Table 1. Comparison of differentially accumulated proteins (DAP) between subcellular proteomics and whole cell proteomics. CSP: classical secretion pathway.*

| DAP | Matched proteins | | New targets |
|---|---|---|---|
| | Same direction | Different directions | |
| ***All upregulated*** | 34 (20%) | 21 (23%) | 111 (67%) |
| ***All downregulated*** | 18 (6%) | 35 (11%) | 275 (83%) |
| ***Upregulated from CSP*** | 11 (32%) | | 23 (68%) |
| ***Downregulated from CSP*** | 10 (13%) | | 68 (87%) |

A closer inspection to the secretory pathway revealed that 68% upregulated and 87% downregulated proteins from this pathway did not match with previous studies, exposing new molecules and mechanisms that can be harnessed to augment protein yields.

## Conclusions

Omics technologies have been used to elucidate the molecular mechanisms involved in RP expression. Previous omics have tagged several proteins related to metabolism, proliferation, apoptosis, cell organization and cell signaling, as interesting targets from whole cell analysis. However, this approach has been biased towards higher abundance proteins, masking the importance of other less explored elements such as those from the secretory pathway. To examine this essential pathway, we applied subcellular proteomics to mAb producing CHO cell clones differing 26 times in their $q_p$. About 78% of all DAP between both cell populations were not found in earlier proteomics, being classified as new targets for CHO cell engineering. Interestingly, about one third of all DAP belonged to the CSP, revealing a tight relationship between processes taking place in these organelles and $q_p$. Among these previously overlooked mechanisms, stress responses, protein metabolism, vesicle traffic, ECM organization and autophagy can be cited.

These new targets and mechanisms together with the prior knowledge should be validated in many different industrial cell clones and culture conditions in order to obtain meaningful conclusions from all available data. Our work has unveiled subcellular proteomics as a viable alternative to whole cell proteomics for a closer look into the cellular mechanisms linked to protein production, and to develop new sublines with higher productivity.

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