Aus dem Munich Cluster for Systems Neurology (SyNergy)
am Institut für Schlaganfall und Demenzforschung
Institut der Ludwig-Maximilians-Universität München
Vorstand: Prof. Dr. Martin Dichgans


# *Dissecting cellular consequences of neuropathy-associated TECPR2*

Dissertation
zum Erwerb des Doktorgrades der Naturwissenschaften
an der Medizinischen Fakultät der
Ludwig-Maximilians-Universität zu München

vorgelegt von
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aus
Dillingen/Saar

2023

Gedruckt mit Genehmigung der Medizinischen Fakultät

der Ludwig-Maximilians-Universität München

| | |
|---|---|
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| Tag der mündlichen Prüfung: | 18. Januar 2024 |

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# Table of Abbreviations

| | |
|---|---|
| AHA | Azidohomoalanine |
| ALG-2 | Apoptosis-linked gene 2 |
| AMBRA1 | BECN1-regulated autophagy protein 1 |
| AMPK | AMP-activated protein kinase |
| AP | Adaptor protein complex |
| AREX | Auto-regulation of ER export |
| ARL8B | ADP-ribosylation factor-like protein 8B |
| Arp2/3 | Actin related protein 2/3 complex |
| ASO | Antisense oligonucleotide |
| ATG | Autophagy-related |
| BECN1 | Beclin-1 |
| BET1 | bet1 Golgi vesicular membrane trafficking protein |
| BLOC-1 | Biogenesis of lysosome-related organelles complex 1 |
| BPAN | Beta-propeller protein-associated neurodegeneration |
| BRUCE | BIR repeat-containing ubiquitin-conjugating enzyme |
| CHEVI | Class C homologues in endosome-vesicle interaction |
| CKII | Casein kinase II |
| CMT | Charcot-Marie-Tooth disease |
| COPI | Coat protein complex I |
| COPII | Coat protein complex II |
| CORVET | Class C core vacuole/endosome tethering complex |
| CSNK1D | Casein kinase I isoform delta |
| cTAGE5 | Cutaneous T-cell lymphoma-associated antigen 5 |
| C-terminal | Carboxy-terminal |
| DFCP1 | Double FYVE-containing protein 1 |
| EARP | Endosome-associated recycling protein |
| EEA1 | Early endosome antigen 1 |
| EPG5 | Ectopic P-granules autophagy protein 5 |
| ER | Endoplasmic reticulum |
| ERES | ER exit sites |
| ERGIC | ER-Golgi intermediate compartment |
| ESCRT | Endosomal sorting complex required for transport |
| FAM21 | WASH Complex Subunit 2C |
| FBXW5 | F-box/WD repeat-containing protein 5 |
| FIP200 / RB1CC1 | RB1-inducible coiled-coil protein 1 |
| FYCO1 | FYVE and coiled-coil domain-containing 1 |
| GABARAP | Gamma-aminobutyric acid receptor-associated protein |
| GAP | GTPase-activating protein |
| GARP | Golgi-associated retrograde protein |
| GOLGA2 | Golgi matrix protein GM130 |
| Golgi | Golgi apparatus |
| GOS28 | Golgi SNARE of 28 kDa |
| GOSR2 | Golgi SNAP receptor complex member 2 |
| GPI | Glycosylphosphatidylinositol |

| | |
|---|---|
| GTPase | Guanosine triphosphatase |
| HERC2 | E3 ubiquitin-protein ligase HERC2 |
| HOPS | Homotypic fusion and vacuole protein sorting |
| Hrr25 | Casein kinase 1 Hrr25 |
| HSAN | Hereditary sensory and autonomic neuropathy |
| HSP | Hereditary spastic paraplegia |
| LAMP2A | Lysosome-associated membrane protein 2A |
| LC3 | Microtubule-associated protein 1 light chain 3 protein |
| LIR | LC3-interacting region |
| LTK | Leukocyte tyrosine kinase |
| LysoIP | Lysosome immunoprecipitation |
| mTORC1 | Mechanistic target of rapamycin complex 1 |
| NHS | N-hydroxysuccinimide |
| NRBF2 | Nuclear receptor-binding factor 2 |
| N-terminal | Amino-terminal |
| p125A | Phospholipase A1-like factor |
| p62 / SQSTM1 | Sequestosome 1 |
| PDCD6 | Programmed cell death protein 6 |
| PDCD6IP | Programmed cell death 6-interacting protein |
| PE | Phosphatidyl-ethanolamine |
| PI(3,5)P2 | phosphatidylinositol 3,5-bisphosphate |
| PI3KC3 | Class III phosphatidylinositol 3-kinase complex |
| PI3P | Phosphatidylinositol 3-phosphate |
| PI4P | Phosphatidylinositol 4-phosphate |
| PLEKHM1 | Pleckstrin homology domain-containing family M member 1 |
| RAB1 | Ras-related protein Rab-1 |
| RAB11 | Ras-related protein Rab-11 |
| RAB5 | Ras-related protein Rab-5 |
| RAB7 | Ras-related protein Rab-7 |
| RME-8 | Receptor mediated endocytosis 8 |
| Sar1 / SAR1 | Secretion associated ras-related GTPase 1 |
| Sec / SEC | Protein transport protein |
| SNAP29 | Synaptosomal-associated protein 29 |
| SNAREs | Soluble N-ethylmaleimide-sensitive-factor attachment receptors |
| SNX | Sorting nexin |
| SPECS | Secretome protein enrichment with click sugars |
| SPTLC1 | Serine palmitoyltransferase, long chain base subunit 1 |
| STX17 | Syntaxin 17 |
| STX5 | Syntaxin-5 |
| SURF4 | Surfeit locus protein 4 |
| SUSPECS | Surface-spanning protein enrichment with click sugars |
| TANGO1 | Transport and Golgi organization protein 1 |
| TECPR | Tectonin beta-propeller repeat |
| TECPR1 | tectonin beta-propeller repeat containing protein 1 |
| TECPR2 | Tectonin beta-propeller repeat containing protein 2 |
| TFG | TRK-fused gene protein |
| TGFα | Transforming growth factor alpha |
| TGN | trans-Golgi network |

| | |
|---|---|
| TMED9 | Transmembrane emp24 domain-containing protein 9 |
| TRAPPI | Transport protein particle I |
| ULK | Unc-51 like autophagy activating kinase |
| UPR | Unfolded protein response |
| USO1 | General vesicular transport factor p115 |
| UVRAG | UV radiation resistance-associated gene protein |
| VAMP8 | Vesicle-associated membrane protein 8 |
| VAP | VAMP-associated protein |
| VPS | Vacuolar protein sorting |
| WASH | Wiskott-Aldrich syndrome protein and scar homolog complex |
| WD | Tryptophan-aspartic acid repeat |
| WIPI2 | WD repeat domain phosphoinositide-interacting protein 2 |
| YKT6 | Synaptobrevin homolog YKT6 |

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**Nalbach, K.**, Schifferer, M., Bhattacharya, D., Ho-Xuan, H., Tseng, W., Williams, L. A., Stolz, A., Lichtenthaler, S.F., Elazar, Z., Behrends, C.

Nat Commun 14, 870 (2023)

**II. Lysosomal targeting of autophagosomes by the TECPR domain of TECPR2.**

Fraiberg, M., Tamim-Yecheskel, B. C., Kokabi, K., Subic, N., Heimer, G., Eck, F., **Nalbach, K.**, Behrends, C., Ben-Zeev, B., Shatz, O., & Elazar, Z.

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**III. Developing antisense oligonucleotides for a TECPR2 mutation-induced, ultra-rare neurological disorder using patient-derived cellular models.**

Williams, L. A., Gerber, D. J., Elder, A., Tseng, W. C., Baru, V., Delaney-Busch, N., Ambrosi, C., Mahimkar, G., Joshi, V., Shah, H., Harikrishnan, K., Upadhyay, H., Rajendran, S. H., Dhandapani, A., Meier, J., Ryan, S. J., Lewarch, C., Black, L., Douville, J., Cinquino S., Legakis H., **Nalbach K.**, Behrends C., Sato A., Galluzzi L., Yu T. W., Brown D., Agrawal S., Margulies D., Kopin A., Dempsey, G. T.

Mol Ther Nucleic Acids, 29, 189-203 (2022)

# 1 Zusammenfassung

Proteinhomöostase ist entscheidend für die zelluläre Integrität. Sie wird durch streng regulierte Prozesse aufrechterhalten, welche Synthese, Transport und Abbau von Proteinen integrieren. Die überwiegende Mehrheit aller Säugetierproteine, welche zum Endomembransystem oder zur Zelloberfläche transportiert werden, verlässt das ER an spezialisierten, COPII-beschichteten Austrittsstellen und wandert anschließend in Transporteinheiten zum ER-Golgi-Zwischenkompartiment. Im Golgi werden diese Proteine verarbeitet und in verschiedene intrazelluläre Transportwege sortiert. Dazu gehören der Transport zur Plasmamembran, Sekretion in den extrazellulären Raum, Transport durch das endosomale System oder Rückleitung zum ER in einem COPI-abhängigen Prozess. Endosomen umfassen verschiedene Unterkompartimente um mehrere Funktionen wie Recycling, retrograden Transport und lysosomalen Abbau zu trennen. Zusätzlich zum endo-lysosomalen System mündet auch die Makroautophagie, ein entscheidender Prozess zum Abbau und Recycling verschiedener Zellkomponenten, in lysosomaler Degradation. In Säugetierzellen beginnt die Makroautophagie an spezifischen ER-Mikrodomänen mit der Bildung einer Phagophore. Mehrere Proteinkomplexe vermitteln sowohl die Erweiterung der Phagophorenmembran, als auch das selektive Anreichern verschiedener Komponenten in von einer Doppelmembran umschlossenen Autophagosomen, welche schlussendlich mit hydrolytisch aktiven Lysosomen fusionieren. Da beide Prozesse am ER in räumlicher Nähe beginnen, überrascht es nicht, dass sich COPII-vermittelte Sekretion und Makroautophagie gegenseitig beeinflussen und sich darüber hinaus mehrere regulatorische Faktoren teilen.

TECPR2 ist ein Protein, das kürzlich mit beiden Prozessen in Verbindung gebracht wurde. Darüber hinaus sind TECPR2 Mutationen ursächlich für HSAN9, einer tödlichen, extrem seltenen Neuropathie, die sich durch fortschreitende Lähmung, autonome, respiratorische und sensorische Dysfunktion, Entwicklungsverzögerung sowie geistige Behinderung auszeichnet. Ursprünglich als Bindungspartner autophagosomaler ATG8-Proteine identifiziert, erwies sich TECPR2 auch als Interaktor der endo-lysosomalen Proteinkomplexe HOPS und BLOC-1 sowie als Regulator der Makroautophagie.

Darüber hinaus stabilisiert TECPR2 ER-Austrittsstellen, reguliert den funktionalen ER-Export einiger Reporterproteine und moduliert somit frühe sekretorische Prozesse. Tatsächlich interagiert TECPR2 mit SEC24D, einer Komponente der COPII-Hülle und reguliert diese Transportstrukturen zusammen mit dem ATG8-Protein LC3C. Bemerkenswerterweise wirkt sich TECPR2 auch auf die frühe Phagophore aus, was eine potenzielle Rolle an der Schnittstelle beider Prozesse nahelegt. Die mechanistische Beteiligung TECPR2s, insbesondere in der Verknüpfung von Autophagie und COPII-vermittelter Sekretion sowie deren Auswirkungen sind jedoch weitestgehend unklar. Ziel meiner Arbeit war es die zellulären Folgen krankheitsassoziierter TECPR2-Varianten im

Detail aufzuklären und somit dazu beizutragen, den bisher unverstandenen HSAN9 Pathomechanismus zu entschlüsseln.

In meinem Promotionsprojekt konzentrierte ich mich auf die Beteiligung TECPR2s an COPII-vermittelten sekretorischen Prozessen. Ein besonderer Fokus lag hierbei auf den Effekten verkürzter, pathogener TECPR2 Varianten, welche in Zellmodellen untersucht wurden. Um Defekte entlang des Sekretionswegs zu erforschen, verfolgte ich mehrere ergänzende proteomische Ansätze, die verschiedene Schritte des Sekretionsprozesses untersuchten. Durch den Einsatz von APEX2-vermittelter Nachbarschafts-Proteomik konnte ich zeigen, dass verkürztes TECPR2 zu desintegrierten ER-Exportstellen führt, möglicherweise durch Beeinflussung der Anordnung von COPII-Proteinen. Interessanterweise wurden Golgi-assoziierte Komponenten in der Nähe von COPII-Bestandteilen angereichert, was auf eine mögliche Umverteilung von (individuellen) Hüllproteinen hindeutet. Als nächstes untersuchte ich, wie sich pathogenes TECPR2 auf die Fracht der COPII-behüllten Transportstrukturen auswirkt. Durch die Etablierung eines neuen proteomischen Ansatzes, der die Bestimmung des Inhalts von Transportstrukturen mit synchronisiertem, RUSH-vermitteltem ER-Austritt kombiniert, stellte ich wesentliche Veränderungen im endogenen ER-Export diverser Proteine fest. Zusätzlich untersuchte ich die Zusammensetzung nachgeschalteter Zielkompartimente wie der Plasmamembran, dem extrazellulären Raum sowie von Lysosomen und konnte ausgeprägte TECPR2-vermittelte Veränderungen in allen drei Kompartimenten detektieren. Bemerkenswerterweise war der Transport mehrerer Proteine mit distinkten neuronalen Funktionen betroffen, was eine erste potentielle Verbindung zwischen TECPR2-abhängiger sekretorischer Fracht und klinischen HSAN9 Merkmalen herstellt. Um die Verbindung TECPR2s mit Transportprozessen weiter zu charakterisieren, führte ich vergleichende Interaktionsanalysen mit wild-typischem und verkürztem TECPR2 durch. Damit konnte ich nachweisen, dass wildtypisches TECPR2 mit mehreren membranassoziierten Komponenten an der ER-Golgi-Grenzfläche interagiert, wodurch seine Rolle über die COPII-Hülle hinaus erweitert wird. Außerdem konnte ich zeigen, dass TECPR2 mit der cytosolischen Oberfläche von Membranen interagiert. Zusammengefasst bringt diese Forschung das Verständnis der sekretorischen Rolle TECPR2s erheblich voran und bietet eine reichhaltige Ressource für nachfolgende mechanistische Studien (Veröffentlichung I).

Durch die Ausweitung meiner Bindestudien trug ich zu zwei weiteren TECPR2-bezogenen Publikationen bei: In Zusammenarbeit mit Zvulun Elazar charakterisierte ich die Funktion von TECPR2 in Patientenzellen mit Blick auf Autophagie. Insbesondere untersuchten wir die Fusion von Autophagosomen mit Lysosomen und deckten einen neuen VAMP8-vermittelten Mechanismus auf. Durch endogene TECPR2-Pulldowns konnte ich erstmals die endogene Bindung von TECPR2 mit nicht-überexprimierten Komponenten des HOPS-Komplexes zeigen (Veröffentlichung II).

Derzeit ist keine TECPR2-spezifische Behandlung verfügbar. Ausgehend von der Tatsache, dass eine leserasterverschiebende Mutation in Exon 8 maßgeblich zur HSAN9 Pathogenese beiträgt, untersuchte ich zusammen mit Graham Dempsey das therapeutische Potenzial eines durch Antisense-Oligonukleotide induzierten Exon-Überspringens, wodurch die pathogene Veränderung umgangen wird und Teile des Proteins wieder translatiert werden. In diesem kollaborativen Projekt entdeckten wir, dass TECPR2 Proteinlevel sowie seine subzelluläre Lokalisierung in Fibroblasten von Patienten und in iPSC-differenzierten Neuronen wiederhergestellt werden können. Durch proteomische Bindestudien konnte ich zeigen, dass TECPR2, dem das übersprungene Exon 8 fehlt, einen signifikanten Anteil der Wildtyp-Interaktionen bewahrt. Wichtig ist, dass das verwendete Oligonukleotid *in vivo* gut vertragen wurde, effektiv in das zentrale Nervensystem eines nichtmenschlichen Primaten eindrang und somit einen vielversprechenden neuen Therapieansatz darstellt (Veröffentlichung III).

# 2 Summary

Protein homeostasis is pivotal for cellular integrity and is therefore maintained by tightly regulated processes that integrate synthesis, trafficking and degradation. In order to be directed towards the endomembrane system or cell surface, newly synthetized proteins canonically enter the ER, pass through quality control checkpoints and are directed to their respective destination. The vast majority of mammalian proteins leave the ER at specialized ER exit sites via COPII-coated structures and subsequently traffic towards an ER-Golgi intermediate compartment. During this classical anterograde transport, the double-layered COPII coat facilitates multiple functions, including cargo selection. Upon entering the Golgi, cargo is further processed and targeted towards different intracellular trafficking routes. These encompass classical transport to the plasma membrane and exocytosis into the extracellular space, trafficking through the endo-lysosomal system or redirection towards the ER in a COPI-dependent process. Endosomes typically comprise different sub-compartments, separating multiple endomembrane trafficking pathways such as recycling, retrograde transport or targeting towards hydrolytically active lysosomes. In addition to their role in the endo-lysosomal system, lysosomes are also involved in macroautophagy, a crucial process responsible for the degradation and recycling of various cellular components, including protein aggregates and damaged organelles. In mammalian cells, macroautophagy starts at specific ER microdomains with the formation of a phagophore. Multiple components facilitate both, phagophore expansion and selection of cargo designated for turn-over. Upon closure, mature double-membraned autophagosomes fuse with lysosomes, resulting in cargo degradation. Since both processes start at specific ER sites, it is not surprising that COPII-mediated secretion and macroautophagy influence each other and share several regulatory factors.

TECPR2 is a neuropathy-associated protein that was recently implicated in both processes. Several mutations in the gene encoding TECPR2 are described in HSAN9, a fatal and extremely rare disease clinically characterized by progressive spasticity, developmental delay, autonomic, respiratory and sensory dysfunction as well as intellectual disability. Originally identified as an interactor of human ATG8 proteins, TECPR2 was also found to be a positive regulator of autophagy-mediated degradation. TECPR2 moreover interacts with the lysosome- and endosome-associated complexes HOPS and BLOC-1, indicating a role in autophagosome-lysosome fusion. TECPR2 is also required to maintain ER exit sites and proper ER export of a specific reporter protein, thus modulating early secretory processes. Indeed, TECPR2 interacts with SEC24D, a component of the mammalian COPII coat, possibly in concert with LC3C, a member of the ATG8 protein family. Notably, TECPR2 also affects the early phagophore, supporting a potential function at the interface of both processes. However, TECPR2's mechanistic involvement, particularly in linking macroautophagy, COPII-mediated secretion and downstream effects, remains elusive. Hence, I aimed to elucidate and dissect in detail the cellular consequences of disease-associated TECPR2 variants and shed light on the function of TECPR2 in both, autophagy and secretion.

Firstly, I focused on TECPR2's involvement in COPII-mediated secretory processes using disease-mimicking TECPR2 variants. In order to profile defects along the secretory pathway, I used several unbiased proteomic approaches targeting different steps of secretory processes. By employing APEX2-mediated proximity proteomics, I was able to show that truncated TECPR2 results in disintegrated ER export sites, possibly by affecting the assembly of COPII components. Interestingly, Golgi-facing components were enriched in the vicinity of COPII constituents, suggesting a potential redistribution of (individual) coat proteins. Secondly, I assessed how TECPR2 deficiency affects the cargo of these COPII-coated structures. By establishing a novel proteomic approach combining proximity cargo profiling and synchronized trafficking with the RUSH-system, I detected substantial changes in ER export of endogenously trafficking proteins. Next, I investigated defects in downstream target compartments, namely the plasma membrane, the extracellular space and the lysosomal composition. In doing so, I identified pronounced TECPR2-dependent changes in all three compartments. Strikingly, trafficking of several proteins linked to neuronal function was affected, providing a first link between TECPR2-based secretory cargo disruption and clinical HSAN9 features. To further characterize TECPR2's involvement in trafficking processes, I performed comparative interactomics of wild-type vs. truncated TECPR2. Remarkably, full-length but not disease-linked TECPR2 interacts with several membrane-associated components at the ER-Golgi interface, expanding its role beyond the core COPII coat. I was also able to show that TECPR2 associates with membranes, presumably at their cytosolic interface. Taken together, this publication significantly advances our understanding of TECPR2's role in the secretory pathway and provides a rich resource for subsequent mechanistic studies (Publication I).

By extending my interactomic approaches, I contributed to two other TECPR2-related studies: In collaboration with the group of Zvulun Elazar, I characterized the function of TECPR2 in autophagy. Together, we uncovered that TECPR2 plays a role in VAMP8-mediated autophagosome-lysosome fusion. Through endogenous pulldowns, I was able to demonstrate TECPR2's interaction with endogenous HOPS complex components (Publication II).

Currently, there is no TECPR2-specific treatment available. Based on the fact that a deleterious frameshift mutation in exon 8 contributes significantly to HSAN9, I collaborated with Graham Dempsey to investigate the therapeutic potential of antisense oligonucleotide-induced exon skipping, bypassing pathogenic modifications. In this collaborative effort, we discovered that TECPR2 protein levels as well as its subcellular localization can be restored in patient fibroblasts and iPSC-derived neurons. Through proteomic interactome studies, I demonstrated that exon 8-missing TECPR2 partially retains a significant fraction of wild-type interactions. Importantly, the oligonucleotide used was well tolerated *in vivo* and effectively penetrated the non-human primate CNS, hence providing a promising novel therapeutic strategy (Publication III).

# 3 Introduction

A crucial feature of any biological entity, from single cells to complex organisms, is the ability to maintain a constant state, often referred to as cellular homeostasis. Through complex and tightly regulated processes, living organisms are able not only to coordinate anabolic and catabolic processes but also to react to cellular stress and to adapt to external stimuli. When cellular homeostasis is disrupted, survival, growth and signaling processes can be severely compromised.

Proteins, which are a diverse class of genetically-encoded macromolecules, play critical roles in maintaining physiological functions. Depending on their cellular localization and regulatory post-translational modifications, proteins can facilitate numerous functions. Newly synthetized proteins traffic to specific cell areas or are secreted into the extracellular space. Simultaneously, several quality control and degradative processes constantly monitor the cellular proteome, maintaining regular protein turnover and specific removal of abnormal protein species. Eukaryotes have developed the so-called endomembrane system, a remarkable compartmentalized structure ensuring proper quality control, regulation and transport of proteins and other biomolecules.

## 3.1 Protein sorting, trafficking and secretion

### 3.1.1 Endomembrane vesicle system

The endomembrane system is a group of diverse and distinct membranous compartments and vesicles that are unique to eukaryotic cells. By separating cytosolic and membrane-bound compartments, cells can not only generate distinct micro-environments for specific biological processes, but also spatially organize anabolic, secretory and catabolic processes [1]. These compartments are tightly regulated and typically comprise the nuclear envelope, the endoplasmic reticulum (ER), the Golgi apparatus (Golgi), the endo-lysosomal system, the plasma membrane as well as several vesicles and vacuolar structures (Figure 1).

Despite their discrete organization, all compartments interact extensively with each other and exchange proteins, lipids as well as other biomolecules through bidirectional transport systems decorated with specialized coat proteins (Figure 1). Upon entering the ER, proteins destined for conventional secretion are processed and transported towards the Golgi, where they are further modified and directed either towards the cell surface or endosomes (anterograde transport). In parallel, proteins can also traffic back towards the Golgi and ER (retrograde transport) or enter the endocytic pathway, a process by which cell surface proteins are internalized and subsequently sorted in endosomal compartments. Early trafficking processes at the ER-Golgi interface are regulated by the evolutionary conserved coat protein complex I (COPI) [2,3] as well as complex II (COPII). Later steps include clathrin-coated vesicles, five clathrin-associated or -independent

adaptor protein complexes 1-5 (AP1-5) and numerous effectors [4]. Non-conventional trafficking routes such as direct Golgi-to-plasma membrane secretion and direct ER-organelle contact sites further extend signaling and trafficking complexity [5]. Some proteins are secreted in COPII-independent processes, summarized as unconventional protein secretion. These processes can be divided into two groups: direct translocation of cytosolic proteins across the plasma membrane and secretion via membranous compartments of varying origin [6,7] (Figure 1).

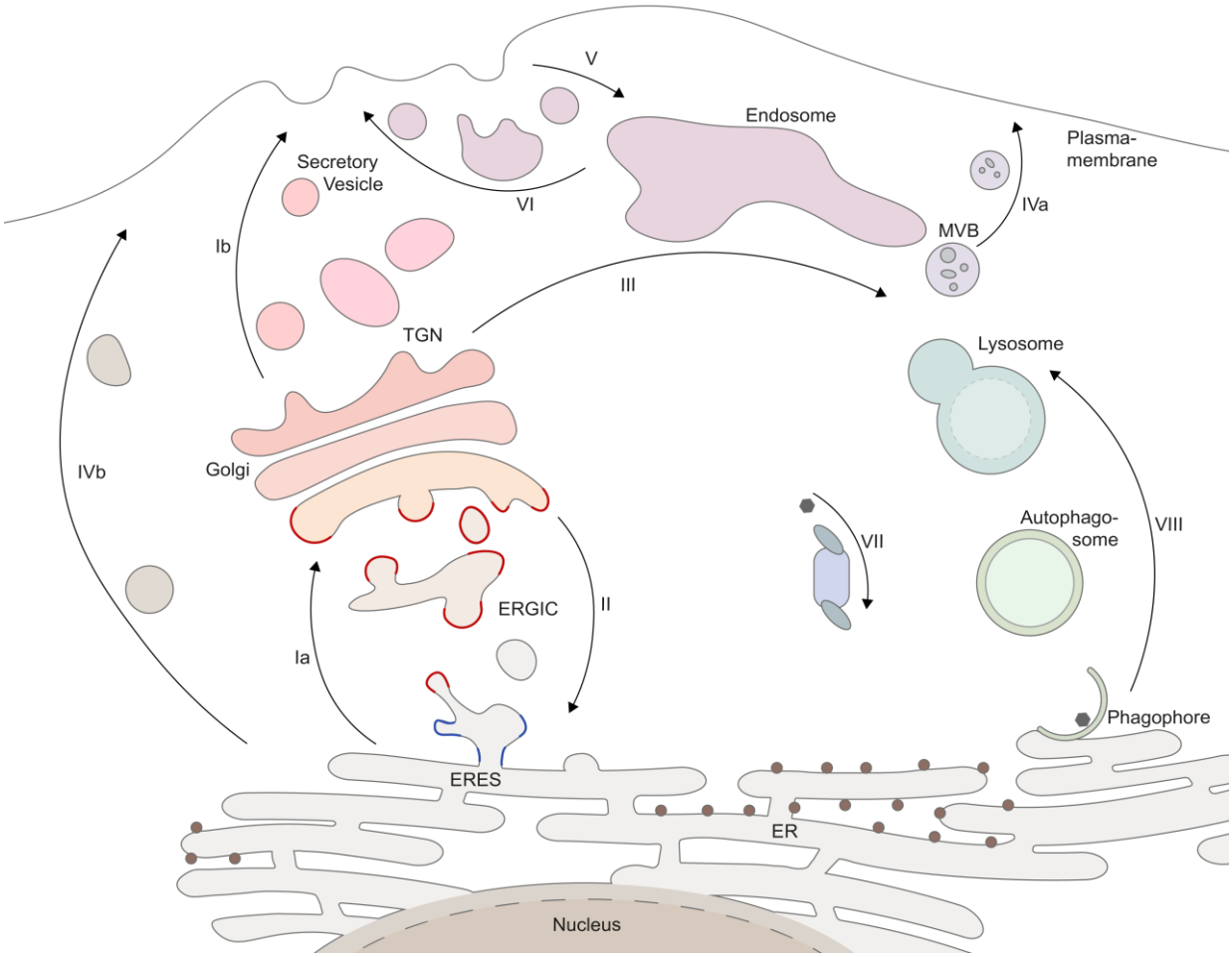


**Figure 1 - Trafficking routes in the endomembrane system.** Proteins destined for conventional secretion exit the ER at ERES and are transported towards the ERGIC / Golgi (Ia) in COPII-coated carriers, ultimately reaching the cell surface (Ib). Retrograde transport from the Golgi to the ER is facilitated by COPI vesicles (II). Upon sorting in the TGN, cargo can also enter the endo-lysosomal system and be transported towards the lysosome (III) or unconventionally secreted through MVB-fusion with the plasma membrane (IVa). Other unconventional secretory pathways can bypass the Golgi (IVb). External and cell surface-resident cargo can be internalized in a process called endocytosis (V). This endocytic cargo is sorted in endosomes and directed either back towards the plasma membrane (VI), the lysosome or the TGN. To maintain cellular homeostasis and degrade misfolded proteins, cargo can be directed towards the proteasome (VII) or engulfed in autophagosomes (VIII). ER, endoplasmic reticulum; ERES, ER exit sites, ERGIC, ER-Golgi intermediate compartment; TGN, trans-Golgi network; MVB, multivesicular bodies; blue markings, COPII; red markings, COPI.

Before leaving the ER, trafficking proteins must pass a complex system of chaperones and protein folding-associated proteins, ensuring that only properly folded and modified proteins enter the secretory pathway. In order to deal with aberrant proteins, cells have developed several degradation machineries, such as the cytosolic proteasome or lysosome-associated autophagy. Abnormal internalized proteins can either be routed towards lysosomal degradation or redirected to the cell surface, regulated by a process termed endosomal sorting.

### 3.1.2 COPII-mediated ER-to-Golgi trafficking

Over 40 % of the human proteome traffics within the endomembrane system, either as transmembrane or soluble (luminal) proteins. For this purpose, they conventionally enter the ER, often upon recognition of a specific signal peptide. The majority of proteins destined to traffic to the Golgi and beyond enter a specific secretory route referred to as early secretory processes. Here, the coat complex COPII assembles at specialized ER micro-domains called ER exit sites (ERES) and facilitates anterograde ER-to-Golgi trafficking [8]. This highly conserved membrane coat was first described in yeast studies as a coat for vesicular structures by the Schekman lab directly bridging ER and Golgi [9-12]. However, the mammalian ER-Golgi interface is organized in a more intricate way. Here, the COPII coat enables transport from the ER towards an ER-Golgi intermediate compartment (ERGIC) in a complex system of tubular-vesicular structures. After cargo transfer to the ERGIC, COPI-coated transport carriers facilitate downstream targeting towards the Golgi [13-15]. Different modes of action and connectivity have been proposed, including passive bulk flow and active, receptor-specific ER exit.

#### Formation of COPII carriers

The COPII coat assembles at specific, ribosome-free ER areas in a hierarchical manner [16,17]. In yeast, it comprises five core proteins called secretion associated ras-related GTPase 1 (Sar1p), protein transport protein (SEC) 13 (Sec13p), Sec23p, Sec24p and Sec31p, accompanied by several accessory proteins including COPII coat assembly protein SEC16 (Sec16p) and the guanine nucleotide-exchange factor SEC12 (Sec12p). Given the intricate ER-Golgi interface in mammals, a complex set of human paralogs has been identified for most COPII components and regulatory proteins, such as SAR1A-B, SEC23A-B, SEC24A-D, SEC31A-B and SEC16A-B [18].

Formation of COPII-coated transport carriers is initiated by the cytosolic guanosine triphosphatase (GTPase) SAR1. Upon activation by ER-anchored SEC12 [19-21], GTP bound SAR1 associates to the ER membrane, followed by a conformational change and insertion of a helix into the ER membrane [22-24]. During this process, the ER membrane slightly bends, which in turn increases the membrane affinity of SAR1 [25]. Activated SAR1

additionally recruits the scaffold protein SEC16 to forming ERES, which in turn assists in the assembly of the COPII coat by establishing a specialized ER microdomain [26-29] (Figure 2).

Active, GTP-bound SAR1 recruits the inner COPII coat consisting of SEC23-SEC24 heterodimers to ERES via direct interaction with SEC23 [30]. SEC23 and SEC24 share a similar structure and organize in a bow tie-like unit [30]. SEC23 functions as a GTPase-activating protein (GAP), promoting hydrolysis of SAR1-bound GTP, while SEC24 mainly functions in cargo selection and concentration at ERES [20,31-34] (Figure 2).

Assembly of the inner coat recruits the outer coat composed of SEC13-SEC31 hetero-tetramers via direct interaction of SEC31 with SAR1-SEC23 complexes [11,31,35,36]. Structurally, SEC13 and SEC31 share beta-propeller like structures and organize in an elongated rod [37,38]. Upon recruitment, SEC13-SEC31 units assemble into an octahedral cage that in turn reorganizes the inner coat [39,40,18]. Complete coating stimulates the GAP activity of SEC23, leading to hydrolysis of SAR1-bound GTP and subsequent release of SAR1 [12,41,42]. In doing so, SAR1 is proposed to regulate the formation of a tight constricting neck at the rim of ERES [43] (Figure 2).

Although initially described to coat distinct vesicular trafficking carriers, the localization of the COPII coat has recently been restricted to the neck between the ER and forming tubular-vesicular ERES clusters, possibly functioning as a gate-keeper regulating ER exit. ERES are furthermore enriched in phosphatidylinositol 4-phosphate (PI4P), lysophosphatidyl-inositol and cholesterol, potentially supporting ERES formation through decreased membrane rigidity [44-47].

## Cargo sorting

Cargo recruitment and sorting can follow two different modes of action. On the one hand, cargo can passively exit the ER in a so called bulk flow [48]. To prevent exit of unfolded or aberrant proteins, upstream quality control machineries in the ER lumen might restrict trafficking by occupying said proteins in a COPII-independent manner. On the other hand, the COPII coat can actively and specifically mediate cargo selection, providing an additional level of ER exit quality control and the ability to adapt ER export to different stimuli [49,50]. Cargo selectivity can be driven by direct interactions of cargo sorting motifs with COPII components or indirectly via membrane-associated cargo receptors. Interestingly, ERES are mainly devoid of classical ER constituents, promoting an additional layer of COPII cargo selectivity [12].

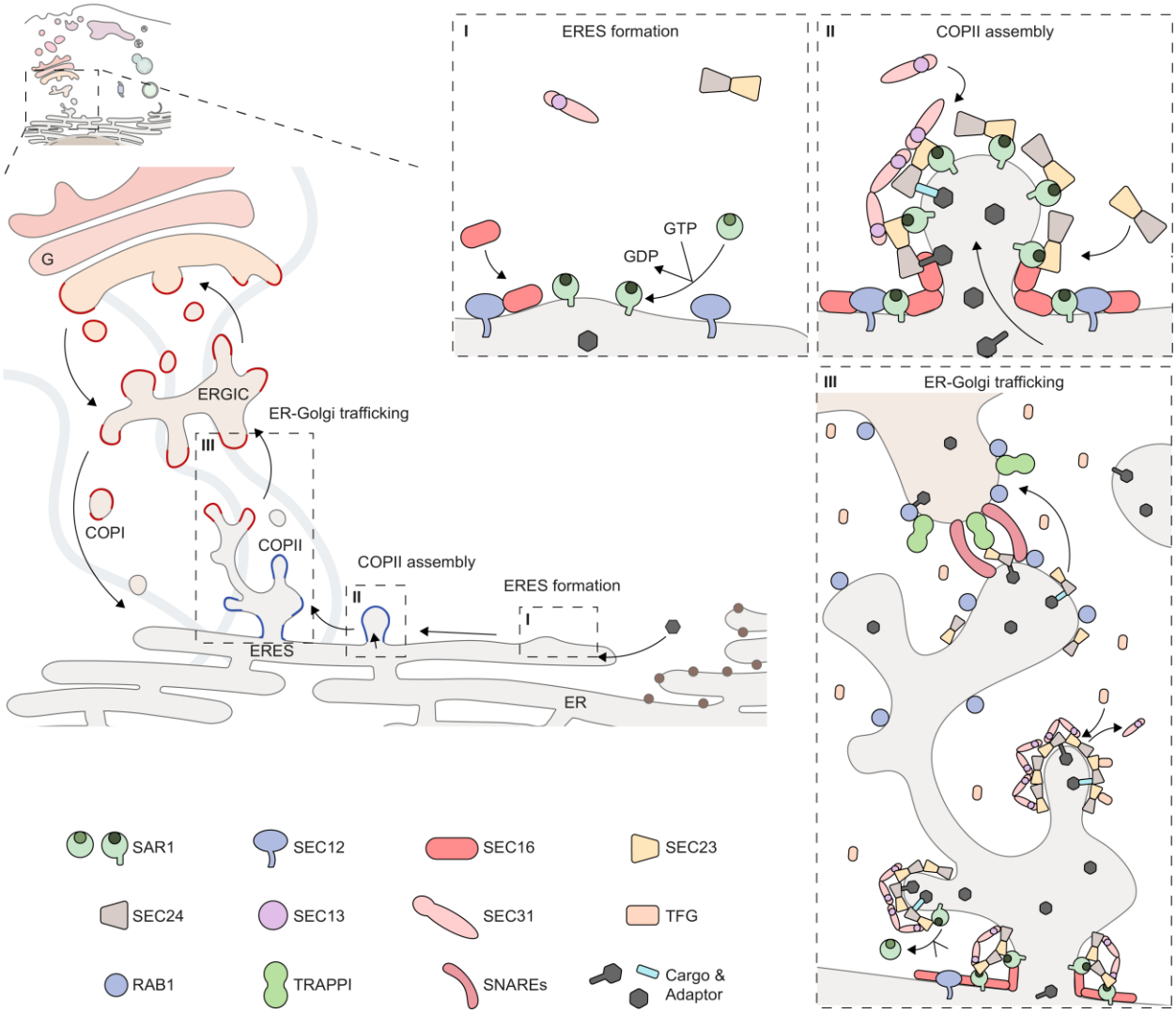


**Figure 2 - Formation of ERES and COPII-mediated ER-to-Golgi trafficking.** Functional ER-to-Golgi transport via classical COPII-mediated secretion is established by stepwise assembly and disassembly of coat components and regulatory factors. (I) This process starts with the activation of SAR1 by SEC12. Active SAR1 assembles at specialized ER microdomains enriched in SEC16. (II) Both, inner (SEC23-SEC24) and outer (SEC13-31) COPII coats assemble in a coordinated manner. SEC23 mediates the bridging of SAR1 and SEC31, while SEC24 facilitates cargo sorting. (III) ERES transform into tubular-vesicular networks and enable ER-Golgi traffic. During extension towards the ERGIC, the COPII coat disassembles and carriers are partially coated with COPI. Contact with the ERGIC is established by many factors including RAB1, TRAPPI and several SNAREs. ER, endoplasmic reticulum; ERES, ER exit sites, ERGIC, ER-Golgi intermediate compartment; G, Golgi apparatus; blue markings, COPII; red markings, COPI.

The only COPII component that directly interacts with cargo is SEC24, which harbors several distinct binding regions for specific interactions [51]. The four different human SEC24 paralogs might facilitate complementary functions, combining enhanced cargo specificity and high cargo variability with increased redundancy for certain cargos. Indeed, the SEC24 family can be clustered into two groups that show higher similarity and group-

specific binding motifs, SEC24A and SEC24B or SEC24C and SEC24D, respectively [33,52,53]. Interestingly, SEC24C and SEC24D have been found to be essential factors in neuronal development and trafficking of neurotransmitter transporters [54-56].

COPII components, predominantly SEC24 paralogs, furthermore associate with transmembrane and luminal ER cargo receptors that bind secretory cargo, thus physically bridge cargo and COPII subunits. Cargo receptors either traffic alongside their cargo towards the ERGIC/cis-Golgi compartments or just facilitate cargo loading into COPII carriers and reside within ERES. For instance, ERGIC-53, an ERGIC marker, is a transmembrane adaptor for soluble proteins that cycles between the ER and ERGIC [57,58]. Since ERGIC-53 contains a lectin domain, it mediates efficient SEC24-dependent secretion of glycoproteins [59,60]. Interestingly, some ERGIC-53-dependent sorting signals are functional only after receptor dimerization, providing an additional layer of selectivity [61]. The transmembrane and ERGIC-localized protein surfeit locus protein 4 (SURF4), exhibits a similar mechanism but acts as an adaptor protein for a wide range of soluble secretory cargos, including lipoproteins and lysosomal proteins such as progranulin [62-66]. Strikingly, a recent report proposed a SURF4-specific ERGIC route, further extending potential cargo selection and trafficking mechanisms [67]. Other adaptors include members of the p24 family for luminal glycosylphosphatidylinositol (GPI)-anchored membrane proteins [68,69] and chronicon receptor proteins for transmembrane G protein-coupled receptors as well as transforming growth factor α (TGFα) [70,71].

Since tubular-vesicular COPII transport carriers resemble cauliflower-like structures of up to 400 nm in diameter, with individual tubules and beads seldomly exceeding 60 nm [47], bigger cargo such as procollagen fibrils rely on additional steps modifying the COPII machinery [72,73]. In order to adapt to large cargos, the classical COPII coat is reorganized by the collagen-adaptor proteins transport and Golgi organization protein 1 (TANGO1) and cutaneous T-cell lymphoma-associated antigen 5 (cTAGE5) [74,75,38,76-78,46]. This reorganization also affects accessory COPII proteins such as SEC16 and SEC12 [79,80]. Moreover, large cargos might be transported in non-conventional transport carriers directly bridging ER and Golgi compartments [81].

## Targeting of COPII carriers to cis-Golgi structures

In contrast to distinct and mobile COPII-coated vesicles in yeast, the mammalian COPII envelope rapidly disassembles after scission of transport carriers [14,41], resulting in concurrent COPII-coated and -free structures [82]. While COPII components are almost always present at ERES and remain rather immobile, cargo can traffic in COPII-free transport carriers [83]. The majority of these carriers is juxtaposed to the ERGIC, not the cis-Golgi, a compartment where COPI-coated vesicles are proposed to mediate trafficking. [83,84].

In recent years, evidence for non-vesicular COPII-coated carriers has been accumulating [85,86,47]. Currently, ERES are understood as intertwined tubular-vesicular structures. In this model, preliminary ERES accumulate COPII components and swell into complex tubular structures devoid of SAR1, which are still connected with the ER over a tight constricting neck [47]. In accordance with the observation of COPII-free transport carriers, only specific ERES areas directly adjacent to the ER are COPII-coated. These ERES give rise to beaded, COPI-coated tubules which facilitate trafficking towards the ERGIC [47]. Interestingly, these carriers are also positive for ras-related protein Rab-1 (RAB1A/B), a protein that is not involved in cargo loading but in vesicle targeting at the ER-Golgi interface [87,88,86] (Figure 2).

Although not involved in ER export and ERES formation [89], microtubules are tightly associated with expanding tubular-vesicular ERES structures [47]. Microtubules are part of the cellular cytoskeleton and consist of polymeric tubulin subunits. At the ER-interface, they are involved in the correct positioning of the ERGIC as well as in bidirectional transport of cargo carriers by their associated motor proteins [89-92].

Targeting of cargo carriers towards the ERGIC is supported by the TRK-fused gene protein (TFG). Upon recruitment to ERES through direct interaction with SEC23, TFG supports COPII disassembly by outcompeting the outer-coat and is organized in a gradient-like manner at the ER-Golgi interface. Thereby, TFG retains transport carriers and directs them to the ERGIC, possibly in an apoptosis-linked gene 2 (ALG-2) - dependent manner [84,93,94] (Figure 2).

Exposure of the inner COPII coat at the ERGIC interface facilitates binding to ERGIC-resident tethering and fusion complexes such as the transport protein particle I (TRAPPI) complex. This hetero-heptameric complex is proposed to capture ERES-derived carriers through the interaction of its subunit TRAPP3 with SEC23 [95,96], thus bringing together ERES-derived and ERGIC compartments. Furthermore, phosphorylation of the inner COPII coat by the cis-Golgi-localized casein kinase I isoform delta (CSNK1D) displaces the TRAPPI complex and prepares transport carriers for fusion [97] (Figure 2).

Once in close proximity, interaction between soluble N-ethylmaleimide-sensitive-factor attachment receptors (SNAREs) on both membranes facilitates fusion. SNAREs involved in mammalian cells include SEC22, syntaxin-5 (STX5), Golgi vesicular membrane-trafficking protein p18 (BET1), Golgi SNAP receptor complex member 2 (GOSR2), synaptobrevin homolog (YKT6) and Golgi SNARE of 28 kDa (GOS28) [98-101]. An additional important cis-Golgi tethering complex consists of STX5, Golgi matrix protein GM130 (GOLGA2), general vesicular transport factor p115 (USO1) and RAB1 [102-105] (Figure 2).

Since COPII uncoating is primarily driven by GTP hydrolysis of SAR1, regulators of this process might also contribute to localized destabilization of the COPII coat and subsequently promote fusion events. Indeed, one important stabilizer of active SAR1, the COPII accessory protein SEC16, is only present at the ER and adjacent constricting

necks of ERES [106,43]. Hence, more distal areas of the COPII coat might not be supported by SEC16's stabilizing function.

**Regulation of COPII-dependent trafficking**

In order to maintain and modify early secretory processes, tight regulation of said trafficking is necessary. One way to stabilize the COPII coat is through proteins that bridge the inner and outer envelope. Indeed, several accessory proteins including phospholipase A1-like factor (p125A), programmed cell death protein 6 (PDCD6) and ALG-2 directly interact with SEC23 and SEC31 [107-110]. Apart from direct COPII components, almost 150 proteins were identified to modify the ER-Golgi interface through diverse and complex signaling pathways [111].

Furthermore, all human COPII components can be regulated by different post translational and transcriptional modifications, ranging from microRNAs and ubiquitination to phosphorylation and glycosylation [112]. Individual modifications include O-linked β-N-acetylglucosamine glycosylation of SEC23A, SEC24C, SEC31A and TFG, supporting COPII mediated export [113-115], or cullin-mediated monoubiquitinylation of SEC31A, which drives ERES enlargement [116-118]. Additional modifications include several phosphorylation events such as the phosphorylation of SEC31A by the casein kinase II (CKII) or of SEC12 by the leukocyte tyrosine kinase (LTK), potentially destabilizing the COPII coat [119-121].

Furthermore, complex cellular processes, such as ER stress and its response, can regulate the formation of ERES by impairing the assembly of the inner coat [122]. ERES also rapidly adapt to alterations in cargo load, for instance by enhancing ER export in a SEC16-dependent manner, or by responding to mechanical strains [123,124]. Recently, a novel regulatory mechanism called auto-regulation of ER export (AREX) has been described. Here, SEC24 detects increased cargo load and then activates signaling cascades that inhibit protein synthesis and simultaneously stimulate ER export [125].

### 3.1.3 Endosomal protein sorting and trafficking

Upon reaching the Golgi via COPII-dependent trafficking, cargo is transported through its cisterna towards the trans face and undergoes further processing [126]. While Golgi-resident proteins are either prevented from leaving the Golgi cisterna or are actively transported back towards the cis face, secretory cargo leaves the Golgi in vesicular and tubular structures called the trans-Golgi network (TGN) [127-129]. Within this heterogeneous array of distinct transport carriers, cargo is either transported towards the cell surface or to other intracellular compartments such as the endosome, often in a microtubule-associated manner [130-133].

The highly dynamic plasma membrane provides an interface between the cell and the extracellular space. Here, TGN-derived secretory vesicles fuse at specific sites and either insert their membrane-associated cargo into the plasma membrane or secrete soluble proteins into the extracellular space in a process called exocytosis [134]. *In vivo*, the extracellular space is organized in a matrix-like structure that facilitates cell-cell and cell-matrix interactions. By providing growth factors, signaling molecules and other cell survival factors, this matrix is essential for cell viability and proper organization of multicellular structures. Apart from its main component, the family of collagens, the mammalian extracellular matrix comprises many other proteins such as fibronectin, laminins, collagens and glycosaminoglycans [135,136]. Specialized areas, so-called focal-adhesions, translate extracellular stimuli into intracellular signaling events. They often assemble as large multiprotein structures connecting transmembrane, signal-transducing integrins with the cytoskeleton [137-139].

**Endosomal compartmentalization**

An alternative trafficking route directs TGN cargo towards endosomes. These highly dynamic and heterogenous compartments sort and deliver many trafficking cargos towards particular acceptor compartments. In order to enable specialized functions, endosomes undergo a process summarized as endosomal maturation. Here, endosomes change their lipid and protein composition as well as their luminal pH, leading to several distinct subtypes (Figure 3).

Early endosomes, canonically decorated with phosphatidylinositol 3-phosphate (PI3P), ras-related protein Rab-5 (RAB5) and early endosome antigen 1 (EEA1) [140,141], constitute the main sorting unit within the endosomal system.

Most proteins destined for the cell surface pass through the recycling endosome, a tubular structure enriched in ras-related protein Rab-11 (RAB11) [142]. Due to its high variability and similarity with early endosomes, both compartments are often considered a continuum. Several specific RAB microdomains ensure rapid association with early endosomes and efficient cargo sorting [143,144].

Cargos targeted towards the lysosome enter late endosomes. This subtype is characterized by a low pH, the presence of phosphatidylinositol 3,5-bisphosphate (PI(3,5)P2) and ras-related protein Rab-7 (RAB7) [145-147]. Similar to lysosomes, late endosomes can facilitate mechanistic target of rapamycin complex 1 (mTORC1) signaling [148].

Endosomes constantly receive freshly internalized cargo by a process called endocytosis. Here, cargos retrieved from the plasma membrane traffic in small, often clathrin-coated vesicles towards the early endosome [149,150]. Endocytic cargo is subsequently targeted for recycling to the plasma membrane through recycling endosomes, for lysosomal

degradation via the late endosome or for retrograde transport towards the TGN [151-153] (Figure 3).

**Endosomal sorting and tethering complexes**

Although cargo sorting mainly occurs in early endosomes, retrieval of recycling cargo from late endosomes is also possible [154,155]. Functional cargo sorting relies on specialized multi-protein complexes as well as proper association with the cytoskeleton [156,157].

A key regulator in endosomal sorting is the retromer complex. This multimeric complex associates with tubular endosomes and regulates cargo trafficking towards the TGN or the cell surface [158]. The mammalian retromer complex consists of a core heterotrimer of vacuolar protein sorting 26 (VPS26), VPS29 and VPS35 and a membrane-bound sorting nexin (SNX) dimer comprising SNX1, SNX2, SNX5 or SNX6 [159-162]. This classical retromer complex binds to endosomal membranes in the presence of RAB7 [163,164]. Additional non-canonical retromer complexes harboring SNX3 or SNX27 are involved in the trafficking of specialized cargos [165,166,158]. Interestingly, SNX27 facilitates recycling to the plasma membrane and is not involved in TGN trafficking. Additionally, SNX27 can bind to the classical retromer complex, thus expanding its cargo recognition machinery [167]. Although cargo sorting and signal sequence recognition is mainly enabled by the VPS trimer [168,169,161], the variance in SNXs could facilitate an additional layer of trafficking regulation [170,171] (Figure 3).

The retromer recruits the Wiskott-Aldrich syndrome protein and scar homolog (WASH) complex, a large multi-protein complex, to the endosome [172]. This recruitment is mediated by the interaction of the retromer subunit VPS35 with the WASH component WASH complex subunit 2C (FAM21) [173,174]. Another protein bridging both complexes through direct interactions is receptor mediated endocytosis 8 (RME-8) [175,176]. Since RME-8 modulates endosomal trafficking and morphology, it is proposed to coordinate retromer and WASH function [176]. Upon recruitment, the WASH complex nucleates the actin related protein 2/3 complex (Arp2/3), resulting in actin filamentation on endosomes and subsequent endosomal tubulation [177]. Thereby, the WASH complex is essential for the endosomal tubulation during retromer-connected cargo sorting [178]. In concert with VAMP-associated proteins (VAPs), usually localized at the ER-Golgi interface, the WASH complex can generate ER-endosome contact sites. These contact sites modulate ER tubulation and Arp2/3 activity [179,180]. Other sorting machineries involved in retrograde transport include clathrin-coated carriers as well as clathrin-independent transport via AP-5, RAB9 or the retromer-related retriever complex [181-185,155] (Figure 3).

During their maturation, endosomes can accumulate intraluminal vesicles and transform into multivesicular bodies (MVBs). In doing so, they internalize cytoplasmic and transmembrane cargo including cell surface receptors. After quality control and sorting, the cargo is targeted towards either lysosomal degradation or secretion via exosomes [186-188]. Along with other regulatory components such as tetraspanins and clathrins,

endosomal sorting complexes required for transport (ESCRTs) are critical for cargo recruitment and vesicle fission [189]. The evolutionary conserved ESCRT machinery is divided into several complexes that mediate distinct functions: ESCRT-0, ESCRT-I and ESCRT-II together with programmed cell death 6-interacting protein (PDCD6IP) mainly recruit ubiquitinated cargo, facilitate ESCRT assembly and induce membrane curvature [190-195]. In concert with VPS4, ESCRT-III then drives vesicle fission [196,197] (Figure 3).

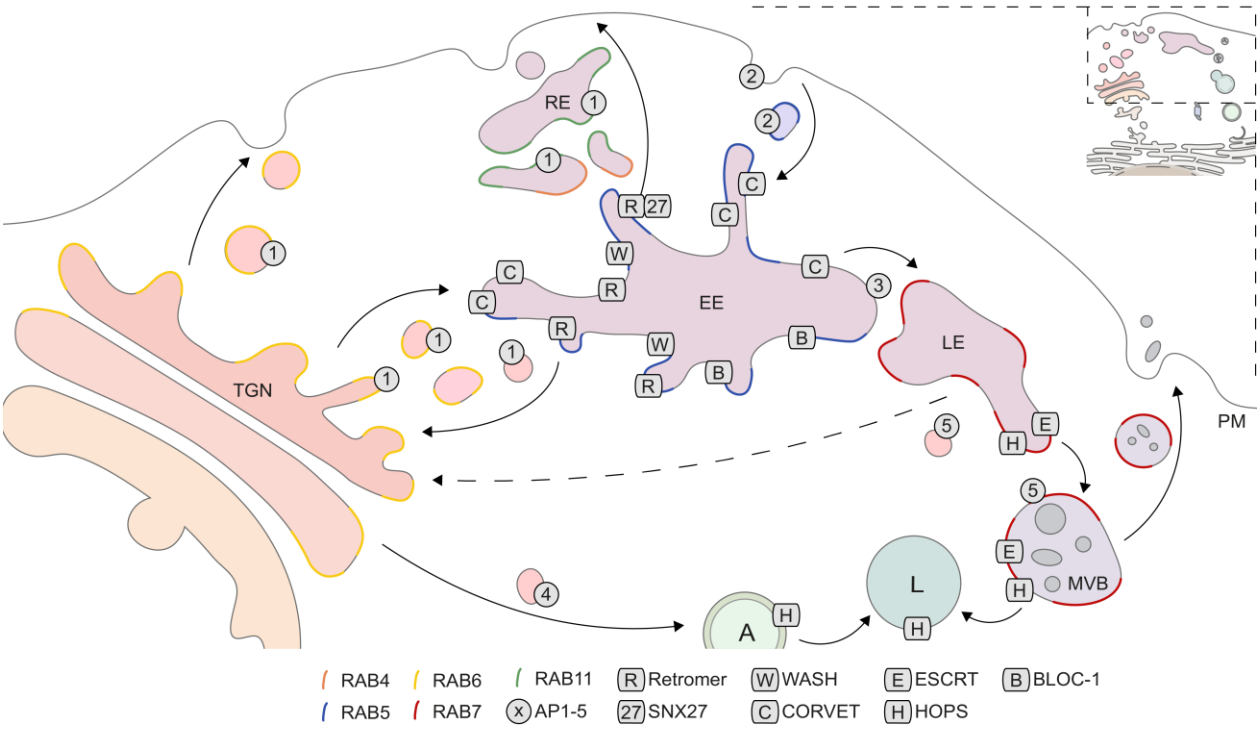


**Figure 3 - Endosomes are a crucial cellular sorting entity.** Proteins exiting the Golgi are either directed to the cell surface as secretory cargo, packaged to support autophagy or transported to the endosomal system. Based on their particular function and composition, endosomes can be divided into highly dynamic compartments. Mediated by specialized sorting machineries such as the Retromer, WASH and BLOC-1 complexes, early endosomes direct incoming cargo towards the TGN, late or recycling endosomes. While recycling endosomes facilitate transport towards the cell surface, late endosomes convert into MVBs in an ESCRT-dependent manner. Components such as the HOPS complex can direct late endosomes and MVBs to lysosomal degradation. All sub-compartments are decorated with specific components such as different Rab and adaptor proteins. TGN, trans-Golgi network; EE, early endosomes; RE, recycling endosomes; LE, late endosomes; MVB, multivesicular bodies; PM, plasma membrane; L, lysosome; A, autophagosome.

By bridging and establishing contact between different membranes, specific tethering complexes are required for endosomal fusion with other membranous compartments. One of them is the homotypic fusion and vacuole protein sorting (HOPS) complex. This hetero-hexameric complex comprises a core of three VPS proteins (VPS11, VPS16 and VPS18), accompanied by the three accessory proteins VPS33A, VPS39 and VPS41 [198,199]. While the inner core facilitates complex formation and recruitment of the auxiliary proteins, VPS39 and VPS41 bind to endosome-localized RAB7, and VPS33A

mediates SNARE function [200-204]. In binding both RAB7 and SNAREs, HOPS is not only involved in tethering but also SNARE-dependent fusion of lysosomes with late endosomes and autophagy-related vesicles [205-207] (Figure 3).

Similar to HOPS, the tethering complex class C core vacuole/endosome tethering (CORVET) complex shares the same core composition (VPS11, VPS16 and VPS18), but associates with accessory proteins VPS3, VPS8 and VPS33A [208]. Since VPS3 and VPS8 interact with RAB5, CORVET acts upstream of HOPS in early endosomes and mediates fusion with new endocytic vesicles [209-212]. Other complexes involved in endosomal membrane tethering comprise the class C homologues in endosome-vesicle interaction (CHEVI) complex and the endosome-associated recycling protein (EARP) complex, both of which act on recycling endosomes [213,214], as well as the Golgi-associated retrograde protein (GARP) complex, involved in retrograde-trafficking towards the Golgi and organization of the TGN-interface [215-217].

Another component of the endo-lysosomal sorting and tethering machinery is the octameric biogenesis of lysosome-related organelles complex 1 (BLOC-1) [218]. Originally described in lysosome-related organelles of specialized cells, BLOC-1 further localizes to early endosomes and associates with AP-3. Here, BLOC-1 is involved in PI4P- and Arp2/3-dependent endosomal tubule remodeling and regulates cargo sorting towards the lysosome [219-223]. Interestingly, BLOC-1 might also be involved in the trafficking of synaptic vesicles and neuronal receptors, thus perform distinct functions in a neuronal context [224,225] (Figure 3).

## 3.2 Quality control and degradation

Several cellular disposal mechanisms are involved in maintaining functional homeostasis and engage a wide range of disruptions, spanning aberrant proteins and dysfunctional organelles to invading pathogens. Besides the cytosolic ubiquitin-proteasomal system, which is primarily involved in the degradation of regulatory, short-lived and misfolded proteins, a variety of cellular components can also be degraded by a lysosome-dependent process called autophagy.

### 3.2.1 Autophagy as a major degradative process

Autophagy, originally described as a recycling mechanism in starving cells, directs a wide range of cargos, including organelles and protein aggregates to lysosomal degradation. Regarding their mode of action, autophagic processes can be differentiated into several distinct subtypes. During microautophagy, cargo is directly engulfed by late endosomes and lysosomes via membrane invagination [226]. Soluble proteins can also be recognized by chaperons and are subsequently translocated by the glycoprotein lysosome-associated membrane protein 2A (LAMP2A) in a process called chaperone-mediated autophagy [227]. In contrast, during macroautophagy (herein referred to as autophagy), cargo is first engulfed by a specialized, double-membrane structure called autophagosome and then directed to lysosomal degradation [228]. This highly conserved process can facilitate nonselective degradation of cytosolic components or selectively capture particular cargo in a mechanism called selective autophagy. Here, several cargo receptors typically containing an LC3-interacting region (LIR) bind and deliver autophagic cargo to autophagosomes. Based on their differing architecture, cargo receptors can mediate the sequestration of a wide range of cargos, including protein aggregates by sequestosome 1 (p62/SQSTM1) and different organelles such as mitochondria, lysosomes or the ER [229-232].

Due to its involvement in early secretory processes, autophagosome biogenesis and quality-control of newly synthetized proteins, tight regulation of ER function is pivotal for cellular homeostasis. ER malfunction activates the unfolded protein response (UPR), a complex system inducing the expression of chaperones and glycosylases, expansion of the ER and inhibition of ER export [233,234,122]. Misfolded proteins are subsequently ubiquitinated, transported into the cytosol and targeted for proteasomal degradation, a process referred to as ER-associated degradation [235]. ER stress also induces autophagy, and severely disrupted ER fractions are degraded in a selective process called ER-phagy [236-238]. Several ER-phagy specific receptors harboring a cytosolic LIR motif have been identified, including ATL3, RNT3, SEC62, CCPG1, CALCOCO1 and FAM143B [239-245]. Interestingly, COPII components of the inner coat (e.g. SEC24C) can also function as receptors for ER-phagy [246].

### Initiation of autophagy and phagophore nucleation

Under physiological conditions, cells usually exert autophagy at very low levels (basal autophagy). Specific stimuli trigger the autophagic machinery which prompts a massive increase in the autophagic flux and overall degradative capacity (induced autophagy). Induction triggers range from starvation, protein aggregation and infections to oxidative stress and damaged organelles.

Starvation leads to the activation of the multimeric Unc-51 like autophagy activating kinase (ULK) complex, comprising the serine/-threonine kinases ULK1/2, the autophagy-related (ATG) proteins ATG13 and ATG101 as well as RB1-inducible coiled-coil protein 1 (RB1CC1, also known as FIP200) [247,248]. This upstream autophagy complex is regulated by two nutrient and energy-sensing kinases: mTORC1 associates with the ULK1 complex under basal conditions, which results in inactive ULK and inhibits autophagy. This association is decreased upon starvation, leading to an activated ULK complex [249-252]. Concurrently, AMP-activated protein kinase (AMPK) is activated upon ATP depletion, which negatively regulates mTORC1 and directly activates the ULK complex [253,254]. Activated ULK localizes to specialized ER areas and initiates downstream events that drive autophagosome biogenesis (Figure 4). During this process, ATG101, ATG13 and FIP200 stabilize the ULK complex, mediate membrane association and promote the catalytic activity of the ULK1/2 subunit [248,255,256]. ULK1 and FIP200 further associate with VAPs at ER-organelle contact sites, enhancing recruitment and stabilization of the ULK complex [257].

Upon phosphorylation by active ULK, class III phosphatidylinositol 3-kinase complexes (PI3KC3) are recruited to the ER membrane where they generate PI3P [258] (Figure 4). PI3KC3 complexes comprise a core of VPS34, VPS15, beclin-1 (BECN1) and exclusively integrate either ATG14 or UV radiation resistance-associated gene protein (UVRAG) [259-262]. ATG14-containing PI3KC3 is active at the ER surface, generates a PI3P-rich ER microdomain and can be regulated by additional factors such as nuclear receptor-binding factor 2 (NRBF2) and activating molecule in BECN1-regulated autophagy protein 1 (AMBRA1) [263-268]. In turn, ER-localized PI3P stabilizes the membrane association and activity of the ULK complex, providing a positive feedback loop [256]. This gives rise the so-called omegasome, an ER-extension pivotal for the generation of the early phagophore [269,270]. Contrary, the UVRAG-containing PI3KC3 complex regulates later steps of autophagy and endosomal trafficking [271-274].

The formation of the phagophore is supported by distal membrane-bound compartments, including ATG9A-positive vesicles. To supply the growing phagophore, vesicles containing this multi-pass protein relocate from the TGN and endosomes to the omegasome upon autophagy induction [275-277]. This autophagy-supporting ATG9A redistribution partially depends on ULK kinase activity and association of ATG9A with AP complexes [278,279]. Notably, ATG9A vesicles do not fuse with the phagophore [280]. During this transient interaction, ATG9A is functioning in concert with ATG2, a protein essential

for autophagosome formation [281]. ATG2 acts at ER-phagophore contacts sides [282]. Here, it transfers lipids to the growing phagophore, a mechanism also proposed for its coordinated function with ATG9A [283-285,282,286] (Figure 4).

Phagophore nucleation is furthermore supported by additional PI3P-effectors such as the PI3P-binding proteins double FYVE-containing protein 1 (DFCP1) and WD repeat domain phosphoinositide-interacting protein 2 (WIPI2) [269,287]. Together, they link the generated PI3P signal to downstream autophagic machineries [288,289] (Figure 4).

**Phagophore elongation and maturation**

In order to mature into a functional autophagosome, the phagophore requires massive expansion. This elongation is accomplished through the recruitment and incorporation of mammalian ATG8 proteins in a ubiquitin-like conjugation, a cascade consisting of activating E1, conjugating E2 and ligating E3 enzymes [290,291]. To achieve this processing, cytosolic precursor ATG8 proteins are carboxy-terminally cleaved by ATG4 and bound by ATG7 (E1) [292]. Activated ATG8s are then transferred to ATG3, functioning as E2 [293]. Together with FIP200 and phosphoinositide-binding motifs, WIPI2 drives membrane association of the ATG12/ATG5/ATG16L1 complex (E3) [288,294,295]. This complex captures cytosolic ATG8-ATG3 conjugates and links processed ATG8s to phagophore-resident phosphatidyl-ethanolamine (PE) [291,296] (Figure 4).

The six mammalian ATG8 proteins cluster into the microtubule-associated protein 1 light chain 3 proteins A, B and C (LC3A, LC3B and LC3C) and the gamma-aminobutyric acid receptor-associated proteins (GABARAP, GABARAPL1 and GABARAPL2) [297]. While LC3s are involved in phagophore elongation, GABARAPs are necessary for several steps in autophagosome formation and lysosomal targeting, suggesting that GABARAPs are essential for the overall autophagic flux [298-300]. LC3 proteins are preferentially involved autophagosome trafficking and selective cargo sequestration [301].

ATG8 proteins provide a platform for binding and selective enrichment of LIR-containing proteins [302,303]. LIR motifs canonically contain two hydrophobic flanking amino acids, separated by two random amino acids. Structurally, the two hydrophobic flanks bind to hydrophobic pockets on ATG8 proteins [303]. Functioning as cargo receptors, LIR-containing proteins direct cargo (often ubiquitinated) to the forming autophagosome by bridging ATG8 and cargo proteins.

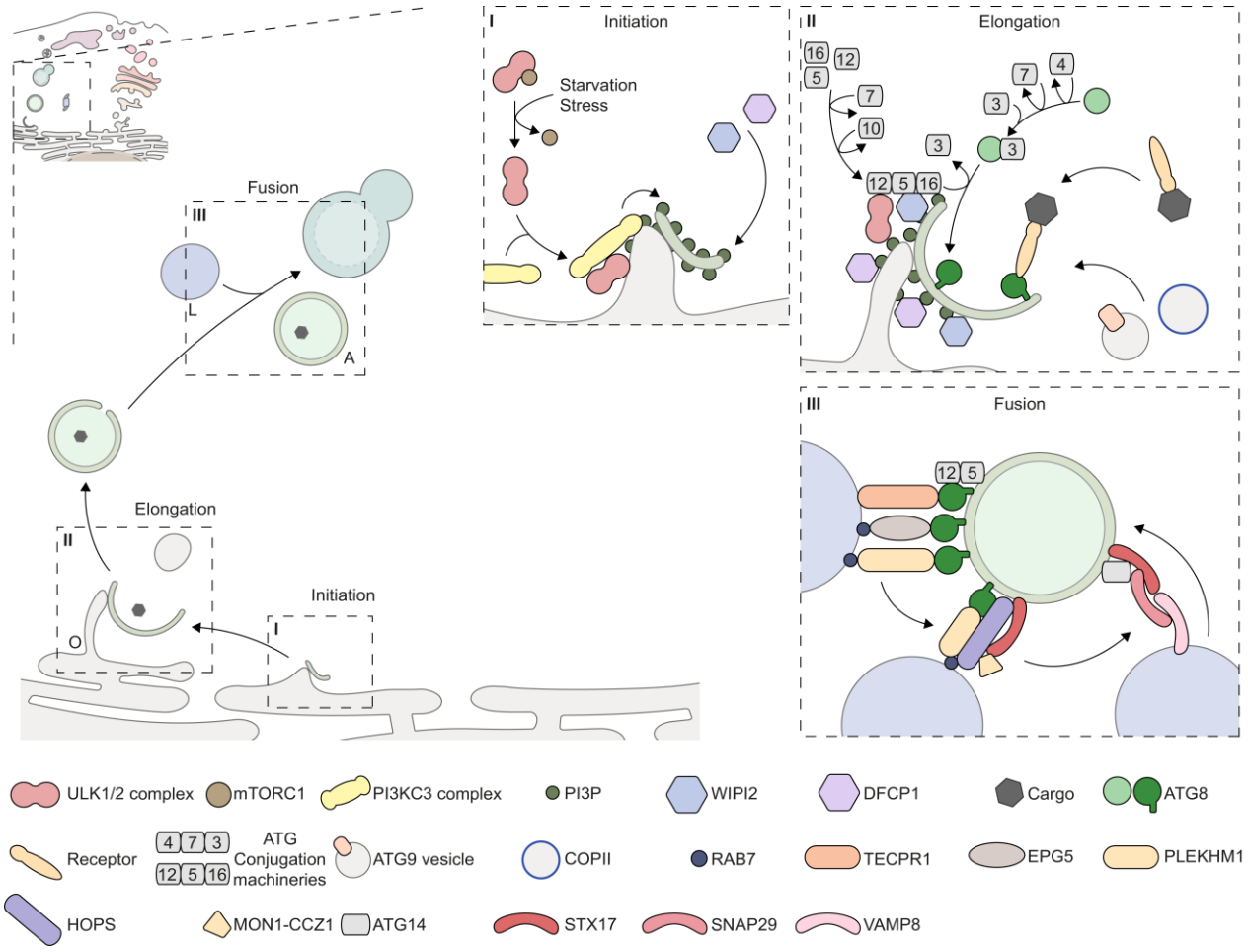


**Figure 4 - Relevant steps during macroautophagy.** During macroautophagy, a diverse range of cargos is engulfed by a double-membraned autophagosome which fuses with hydrolytic active lysosomes. (I) Stimulating factors such as starvation and cellular stress mediate ULK1/2 complex activation, which leads to the formation of specialized ER microdomains called omegasomes. Here, PI3KC3 complexes assemble and generate PI3P enriched membrane patches providing a platform for phagophore assembly, particularly by binding PI3P effectors such as WIPI2 and DFCP1. (II) Phagophore formation and expansion is supported by multiple sources providing membrane components and autophagic factors, including ATG9-containing vesicles and COPII-coated structures. Elongation is performed by two ubiquitin-like conjugation machineries, decorating the emerging autophagosome with ATG8 proteins that also facilitate selective capturing of cargo receptors. (III) Sealed autophagosomes are targeted towards lysosomes by several tethering proteins and complexes including TECPR1, EPG5, PLEKHM1 and HOPS, bridging autophagosomal ATG8s and lysosomal RAB7. Small distance between both compartments supports the assembly of several SNARE complexes, such as the STX17-SNAP29-VAMP8 assembly, which subsequently mediate autophagosome-lysosome fusion. O, omegasome; A, autophagosome; L, lysosome.

## Formation of degradative active autolysosomes

Ahead of lysosomal fusion, autophagosomes usually close both membrane layers. Closure is mediated by the ESCRT machinery, with ESCRT-I/-II recruiting ESCRT-III, which then seals the autophagosome [304-307]. After fusion, hydrolytic lysosomal enzymes such as cathepsins degrade autophagic contents and the inner membrane of

autophagosomes. The outer membrane fuses with that of the lysosome and is quickly covered by the protective lysosomal glycocalyx [308,309].

Several factors facilitate docking and fusion of autophagosomes with lysosomes or late endosomal compartments, likely mediated by a LIR-dependent association with autophagosomal GABARAPs [299,310,311]. In agreement with general membrane fusion events, a SNARE complex comprising autophagosome-decorating syntaxin (STX) 17 (STX17) and synaptosomal-associated protein 29 (SNAP29) as well as the lysosomal vesicle-associated membrane protein 8 (VAMP8) are required for autolysosome formation [312-314] (Figure 4). A second SNARE complex involved in autophagosome-lysosome fusion consists of YKT6, lysosomal STX7 and SNAP29 [315,316].

SNARE-mediated membrane fusion is supported by tethering complexes, GTPases and scaffolding proteins, bringing lysosomes and autophagosomes together in close proximity. By stabilizing STX17-SNAP29 on autophagosomes, ATG14 primes their interaction with VAMP8 [317], thus promoting membrane tethering. RAB7, a central GTPase in the formation of autolysosomes, is essential for the recruitment of several factors involved in membrane trafficking [318,319]. These include lysosome-associated ectopic P-granules autophagy protein 5 homolog (EPG5), which recruits autophagosomes via interaction with ATG8 proteins and SNAREs [320]. In addition to its endosomal localization, RAB7 can also be recruited to late autophagosomes by the vacuolar fusion protein homologs MON1 and CCZ1, in concert with autophagosomal GABARAPs [321,300,322,323] (Figure 4). Other RAB7 effectors include FYVE and coiled-coil domain-containing 1 (FYCO1), possibly involved in LC3 and PI3P-dependent autophagosome movement [324,325], GABARAP interactor BIR repeat-containing ubiquitin-conjugating enzyme (BRUCE) [326], and the pleckstrin homology domain-containing family M member 1 (PLEKHM1). Localized on late endosomes and lysosomes, PLEKHM1 promotes autophagosomal-lysosomal fusion [327,299] (Figure 4).

In a coordinated fashion with ADP-ribosylation factor-like protein 8B (ARL8B), STX17 and UVRAG-containing PI3KC3, PLEKHM1 recruits the HOPS complex, a tether that additionally promotes SNARE assembly, autophagosome maturation and ultimately lysosomal fusion [328-333] (Figure 4).

### 3.2.2 Crosstalk between secretion and autophagy

Due to the ER's pivotal function in COPII-mediated ER export and concurrent involvement in the biogenesis of autophagosomes, it seems obvious that some ER components and machineries could be shared between both processes. In mammalian cells, autophagosomes form at specialized ER microdomains called omegasomes. Here, phagophores are directly connected to the ER, enabling fast transfer of membrane components and ER-assisted *de-novo* phospholipid synthesis [269,334-336]. Apart from the

ER itself, other membrane-bound compartments including the ERGIC and ER-mitochondria contact sites are implicated in phagophore expansion [337-339].

Strikingly, ERES were found to be in close contact to phagophores and are crucial for overall autophagosome biogenesis [340-343]. Indeed, autophagosomes can form at ERES, as demonstrated by the assembly and interaction of autophagic core components with ERES, and partially colocalize with the specific ERES marker SEC16 [344]. Loss of Sar1p, Sec12p or Sec16p inhibits autophagosome formation in yeast, indicating a role in early autophagic processes [345,344]. Moreover, upon autophagy induction, COPII-coated carriers localize from the ER towards the ERGIC which triggers budding of ERGIC-derived COPII-decorated vesicles. These carriers fuse with the expanding phagophore and provide an additional COPII-mediated membrane source that supports autophagosome formation [337,346,347,342]. Starvation also leads to enlarged ERES positive for SEC12, facilitated by the ULK complex subunit FIP200 and the transmembrane emp24 domain-containing protein 9 (TMED9) decorating the ERGIC [342,348]. Importantly, TMED9-mediated remodeling is not required for ER-Golgi trafficking and is independent of the canonical tether TFG [348].

In concert with the TRAPPIII complex, the GTPase RAB1 is recruited to sites of autophagosome biogenesis and mediates ATG9 trafficking [349-352]. TRAPPIII, usually assigned to autophagy, also localizes to the ER-Golgi interface and directly interacts with the SEC13-SEC31 tetramer as well as yeast Sec23p. In doing so, TRAPPIII likely directs COPII components to the phagophore and also affects ER-Golgi trafficking [340,353].

In addition to ERES components functioning in phagophore formation, key components of autophagy also regulate COPII assembly. For instance, the kinase ULK1 can phosphorylate SEC23A, which disrupts the assembly of the outer coat and inhibits ER-Golgi trafficking [354]. During starvation, ULK1 also phosphorylates SEC23B, which disrupts its F-box/WD repeat-containing protein 5 (FBXW5)-mediated proteasomal degradation. Stabilized SEC23B preferentially binds SEC24A/B and relocates to the ERGIC [355]. Both SEC23 modifications are thought to redirect COPII-coated structures to sites of autophagosome biogenesis. The importance of remodeled ERES is furthermore supported by the starvation-induced recruitment of TFG to the expanding phagophore, mediating both ULK1 and LC3C [356]. ERGIC reorganization is also involved in xenophagy, where STING translocation from the ER towards the Golgi promotes COPII- and WIPI2-dependent phagophore elongation [357]. ULK1/2-mediated phosphorylation of mammalian SEC16A promotes COPII-assembly and ER-Golgi trafficking independent of other autophagic components [358]. Since protein secretion and SEC16 levels are reduced during starvation [359-361], SEC16 might couple cellular energy levels and secretory processes. In affecting different ERES components, ULK1-dependent phosphorylation might be involved in this integration. Consistently, COPII trafficking of metabolic liver components dynamically responds to nutrient availability [362].

Other posttranslational COPII modifications, including phosphorylation of yeast Sec24p by the casein kinase 1 (Hrr25) or ubiquitination of COPII-carriers transporting large collagen cargo, also disrupt conventional COPII transport and direct said carriers towards autophagy [363,364]. Besides immediate ERES-dependent effects, disturbed COPII trafficking might further affect autophagy indirectly, for instance through altered trafficking of endosomal and lysosomal components [365].

### 3.2.3 Disturbed homeostasis in disease

Proper protein homeostasis, established through tight regulation of protein synthesis, trafficking and degradation, is crucial for cell survival and development. This is of paramount importance in highly specialized, post-mitotic and polarized cell types such as neurons. Indeed, disturbed autophagy and protein secretion is implicated in many neurodevelopmental and -degenerative diseases, prominently affecting neurons of the cortico-spinal tract, cerebral motor cortex and cerebellum. In addition to well-known disorders such as Alzheimer's disease, Parkinson's disease and Amyotrophic lateral sclerosis, many more, often less well understood and extremely rare movement disorders and neuropathies with affected protein homeostasis have been described [366].

Among them is a class of hereditary peripheral neuropathies with movement disorder phenotypes. Charcot-Marie-Tooth disease (CMT), a group of heterogenous genetic disorders, is the most prevalent subtype of inherited motor diseases associated with sensory neuropathies. Key clinical features include progressive distal amyotrophy, chronic muscle weakness, sensory deficits, dystonia, tremor and areflexia due to demyelination of both motor and sensory neurons [367-369]. Historically, CMT was divided into four subtypes, differentiated by their mode of inheritance and pathological features, with CMT1A being the most common subtype affecting more than 70 % of CMT patients. Overall, more than 80 genes have been linked to CMT [370]. Among them, several proteins involved in protein trafficking, transport as well as the endo-lysosomal system (*LITAF*, *GJB1*, *RAB7A*, *SH3TC2*) have been reported [371-375].

Another rare group of movement disorders is the heterogenous class of hereditary spastic paraplegia (HSP). Despite its clinically diverse presentation, all subtypes share lower extremity weakness and slow progressive spasticity as their predominant feature, affecting mainly neurons in the corticospinal motor tract. Depending on the presence of additional neurological signs, HSPs are classified as uncomplicated or complicated subtypes [376,377]. Patients affected by uncomplicated HSP clinically present with ataxic gait and general walking difficulties as well as partial lower-body paresthesia. Complicated HSP is characterized by the additional presence of peripheral neuropathy, cerebellar ataxia, seizures, cognitive impairment, dementia and overall poor prognosis. More than 80 HSP-linked genotypes are described, with autosomal dominant subtypes accounting for three quarters of all cases [376,378].

Regarding the pathophysiology of axonal degeneration, various proteins involved in processes linked to proper protein homeostasis are affected. Mutations in the gene encoding the protein spastin account for almost 50 percent of inherited HSPs. Importantly, this protein is involved in several trafficking processes from the ER to the endosomal system. Together with two other proteins involved in HSP, atlastin-1 and REEP1, it functions in shaping ER architecture, specifically ER tubules, as well as the downstream Golgi morphology [379-383]. Furthermore, mutations in spastin were linked to disruptions in endosomal fission as well as abnormal lysosomal morphology [384,385]. Other ER-, endosomal-, and trafficking-associated proteins implicated in HSP include reticulon-2 [386], spartin [387,388], AP4 subtypes [389,390], TFG [391], SPG8 [392] and VPS37A[393]. In addition, mutations in the autophagy- and lysosome-associated protein ZFYVE26 and its interactor spatacsin have been linked to HSP [394-396].

A third class of hereditary neuropathies with predominant sensory and autonomic abnormalities and optional motor deficits is classified as hereditary sensory and autonomic neuropathy (HSAN). HSAN is further subdivided into eight heterogenous HSAN subtypes based on mode of inheritance, genetic causes and primary clinical features [397]. Depending on the subgroup, clinical features range from sensory loss (pain, temperature), motor dysfunction and hyporeflexia to cardiovascular and gastrointestinal dysfunction, often accompanied by recurrent pneumonia and apnea [398,397]. Patho-physiologically, sensory fibers are more severely affected than motor neurons, including axonal loss and demyelination [399]. HSAN1, further divided into several subtypes, is the most prevalent class of HSAN. Prototypical HSAN1 is caused by autosomal dominantly-inherited mutations in the gene serine palmitoyltransferase, long chain base subunit 1 (*SPTLC1)* [400], additional subtypes by mutations in *SPTLC2* [401], atlastin-1 [402] and atlastin-3 [403], the latter two involved in modulating ER morphology. Proteins functioning in membrane organization, protein trafficking in the endolysosomal-system and autophagy are also implicated in the other HSAN subgroups, such as FAM134B in HSAN2 [404], high affinity nerve growth factor receptor in HSAN4 [405] and dystonin in HSAN6 [406].

Apart from the neuropathies described above, dysfunction in autophagy, protein trafficking and organelle organization is involved in many other neurological diseases, such as autosomal-recessive ataxia syndromes via mutated SNX14 and ATG5 [407-409] as well as beta-propeller protein-associated neurodegeneration (BPAN) caused by mutations in *WDR45* coding for WIPI-4 [410]. Other neurological diseases are directly linked to disturbed ER homeostasis and protein secretion, for instance by mutations in *SEC31A* [411] and *TFG* [412].

Due to their partially overlapping clinical features, many hereditary peripheral neuropathies present on a symptomatic spectrum with no clear distinction between different subtypes.

## 3.3 TECPR2-related neuropathy

### 3.3.1 HSAN9

In 2012, the group of Doron Lancet identified a novel mutation linked to hereditary peripheral neuropathy within the gene encoding for tectonin beta-propeller repeat containing protein 2 (TECPR2) [413]. Due to prominent lower limb spasticity and intellectual disability, the TECPR2-related syndrome was first classified as SPG49, a subtype of complicated HSP. With the identification of additional disease-linked TECPR2 mutations and the clinical examination of more patients, sensory and autonomic deficits became more evident, leading to a reclassification as a new subtype of HSAN, HSAN9.

So far, less than 40 patients have been documented with pathogenic mutations within *TECPR2*. Despite presenting with heterogenous phenotypes, all individuals diagnosed with HSAN9 share global developmental defects, intellectual disability, neurological and behavioral irregularities as well as autonomic and sensory disturbances [413-421].

Developmental delay almost always includes moderate to severe intellectual disability and speech impairment, with some individuals remaining nonverbal. In spite of delayed motor learning, most patients temporarily achieve the ability to walk independently. More than half of monitored patients present a wide range of behavioral abnormalities, varying from hyperactivity and erratic, sometimes aggressive, behavior to apathy [418,421].

Key neurological abnormalities, present in almost all patients, include muscular hypotonia, lower extremity spasticity and dyskinesia, leading to gait ataxia and dysarthria (if acquired). Affected individuals usually lose the ability to walk independently during adolescence. Sensory neuropathy frequently presents with hypo- to areflexia, especially in the lower limbs, accompanied by decreased pain sensitivity [418,421].

Autonomic dysfunctions often involve apnea and central hypoventilation, leading to hypercapnia and asphyxia, dysphagia, recurrent aspiration and gastroesophageal reflux disease. Recurrent, often chronic, respiratory infections and pneumonia contribute considerably to mortality [421].

Common additional findings encompass dysmorphic abnormalities such as microcephaly and distinctive facial features as well as defects in vision, temperature and blood pressure stability. Neuroimaging often reveals a thin corpus callosum, mild ventriculomegaly, atrophy of the cerebrum and cerebellum as well as delayed myelination [421].

Overall, HSAN9 presents itself as a complex neurodevelopmental disease with progressive neurodegeneration and worsening of symptoms - all individuals described so far died by the age of 20. To date, no specific treatment for HSAN9 exists. Clinical management relies mainly on symptomatic treatment, supportive care and tight surveillance of critical body functions [418].

### 3.3.2 TECPR2

#### Structural features and domains

Human TECPR2 is encoded by a gene called *TECPR2*, located on chromosome 14q32.31. There are two described isoforms, produced by alternative splicing. The longer, canonical isoform 1 encodes 20 exons which are translated into a 1411 amino acid long protein that is described in this chapter. The shorter isoform 2 lacks the last three exons and encodes a 1267 amino acid long protein.

TECPR2 can be divided into highly structured amino- and carboxy-terminal parts (N-terminal / C-terminal) as well as a disordered central region ranging from amino acid position 379 to 779 [418] (Figure 5). Both structured ends contain several repeat domains forming beta-propeller secondary structures: tryptophan-aspartic acid repeat (WD repeat) domains in the N-terminal part and tectonin beta-propeller repeat (TECPR) domains in the C-terminal part with a LIR motif at the very end (Figure 5). TECPR2 lacks a signal peptide and is localized in the nucleus and the cytoplasm.

WD repeats are one of the most abundant protein interaction domains present in a wide range of proteins. They canonically comprise about 40 amino acid long sequences of four to 16 repeat units separated by variable amino acids. WD repeat domains fold into seven-fold symmetric beta-propeller structures with a key central pore devoid of any catalytic activity. Because they provide protein interaction platforms, WD repeat containing proteins are involved in many cellular processes, including protein complex assembly, signal transduction and intracellular transport [422-425]. TECPR2 contains 7 WD repeats that are distributed between amino acids 23 and 343 and form a seven-bladed beta-propeller fold [413,418] (Figure 5).

Due to its C-terminal TECPR domains located between amino acids 900 and 1400, TECPR2 additionally belongs to the tectonin beta-propeller repeat containing protein family. This group of repeats was initially identified in the tectonin protein family found in *Physarum polycephalum* (slime mold) and is proposed to function as lectins involved in signaling processes [426]. This notion is supported by its presence in the *limulus* (horseshoe crab) lectin L-6 [427]. To date, TECPR domains are annotated in several thousand proteins, including two additional human proteins, tectonin beta-propeller repeat containing protein 1 (TECPR1) and the E3 ubiquitin-protein ligase HERC2 (HERC2) [InterPro consortium, EMBL]. Despite the formation of beta-propeller structures, implying a function in protein interaction, the molecular function of TECPR repeats remains poorly understood. In TECPR2, the six TECPR repeats form a double propeller structure reminiscent of that of WD repeats.

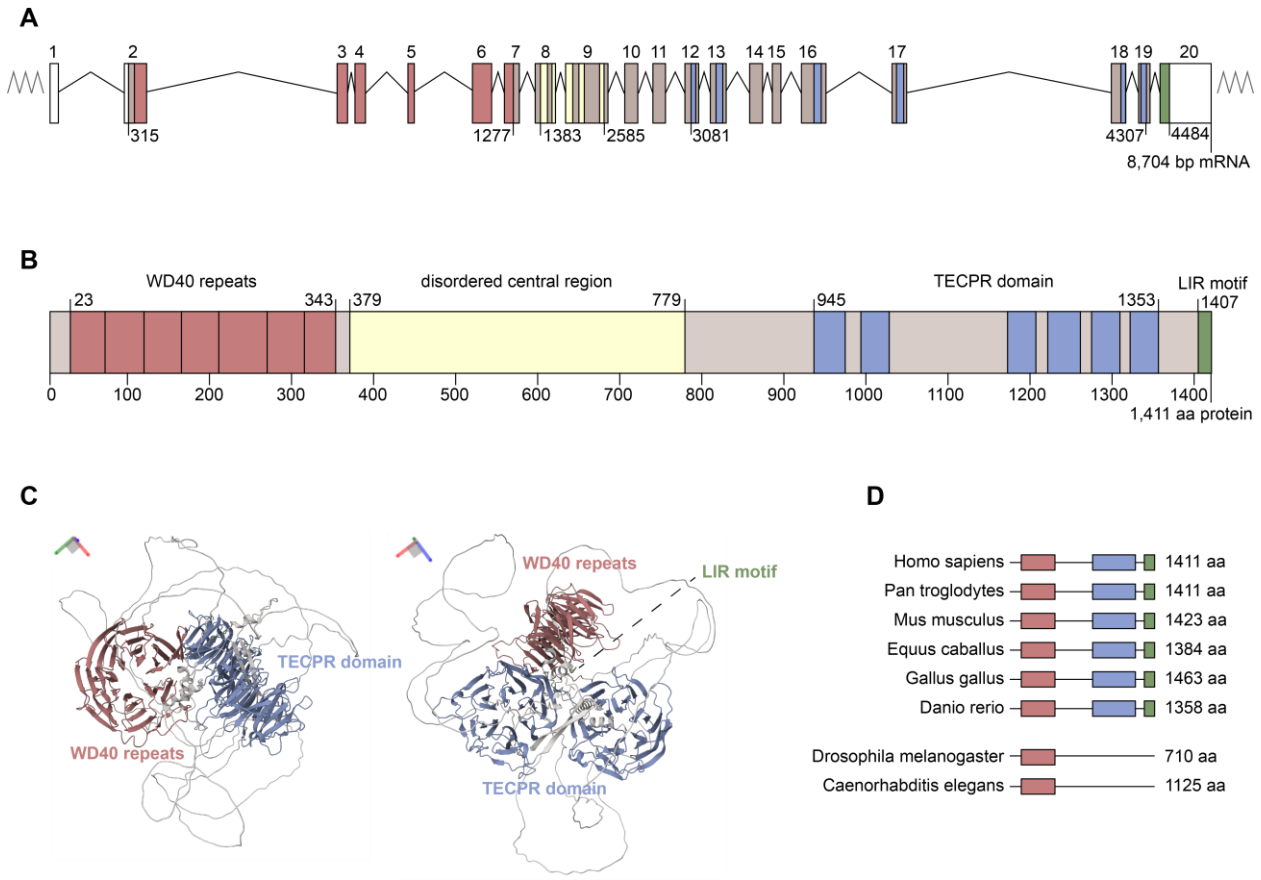


**Figure 5 - TECPR2 contains multiple interaction motifs.** (A) Structure of the *TECPR2* gene, divided into 20 exons and encoding a 4235 long coding sequence. Numbers on top indicate exons, numbers below sequence areas coding specific structural features. Boxes represent exons, lines introns. bp, base pair; mRNA, messenger ribonucleic acid. (B) Structure of the canonical 1411 amino acid long TECPR2. Colored boxes highlight WD40 repeats, the central and disordered region, TECPR domains and the carboxy-terminal LIR motif. Numbers on top indicate the localization of each domain cluster. aa, amino acid. (C) Structural model of TECPR2, predicted by AlphaFold (DeepMind) as of April 2023. Left and right model show different viewing angles. WD40 repeats, TECPR domains and the LIR domain are highlighted. (D) TECPR2 shows high homology within vertebrates. Boxes represent WD40 repeats, TECPR domains and the LIR motif. aa, amino acid.

Since both conserved domain families share similar secondary structures and are implicated in protein-protein interactions, it is proposed that TECPR2 functions as a multi-scaffolding or linker protein, bringing together and stabilizing different protein (complexes) of diverse sub-cellular backgrounds.

In comprising both WD repeat and TECPR domains, TECPR2's architecture is specific to vertebrates and was likely acquired during evolution, as non-vertebrate orthologs show only one of these two domains [413]. In vertebrates, however, TECPR2's architecture is highly conserved [341] (Figure 5). TECPR2 shows some sequence similarity to human TECPR1, which is involved in xenophagy and autophagosomal assembly [428] as well as

to the human member of the BLOC-2 complex HPS5, implicated in the lysosome-associated Hermansky-Pudlak syndrome [429].

According to expression data, TECPR2 mRNA is expressed in almost all tissues. Interestingly, it is most abundant in the human brain, particularly in basal ganglia, the cerebral cortex and the corpus callosum/white matter [Human protein atlas]. Single-cell transcriptomics indicate high expression in oligodendrocytes, excitatory neurons and astrocytes [Human protein atlas]. A recent bioinformatic analysis linked transcriptional regulators that bind to *TECPR2* upstream elements to brain and neuronal development [430]. Expression of *TECPR2* increases prenatally throughout the brain and resembles the expression pattern of common neurodevelopmental genes [431,430]. Furthermore, TECPR2 is co-expressed with other neurodevelopmental genes involved in neuronal signaling and synapse functioning, but not with ribosomal proteins implicated in translation, and might have co-evolved with other genes involved in brain development [430]. Combined with the finding that *TECPR2* is slightly downregulated in the prefrontal cortex of individuals affected by Alzheimer's or Huntington's disease, *TECPR2* shares genetic traits implying involvement in neuronal processes [430].

**Disease-linked TECPR2 mutations**

Indeed, 17 clinically significant miss- and nonsense TECPR2 variants have been observed in HSAN9 patients over the past decade (Figure 6). In total, about 100 potentially pathogenic variants are reported in the ClinVar archive, most of which have not yet been described in HSAN9-affected individuals. Disease-linked mutations cluster mainly in TECPR2s conserved C- and N-terminal parts, implying disruption of secondary structures.

In 2012, Doron Lancet's group reported the first disease-associated TECPR2 founder variant in five individuals of Jewish-Bukharian background. All patients shared the homozygous nonsense mutation c.3416delT in exon 16, resulting in a premature stop codon and loss of four TECPR domains (p.Leu1139Argfs*75) [413]. Since patients sharing this autosomal-recessive variant presented mainly with progressive spasticity and weakness of the lower limbs as well as cognitive decline and intellectual disability, the respective disease was first classified as SPG49 [413].

In 2016, the second pathogenic TECPR2 founder variant was identified in a Jewish-Ashkenazi background. This nonsense c.1319delT mutation within exon 8 also leads to a premature stop codon with increased truncation and loss of two-thirds of the protein, including all TECPR domains (p.Leu440Argfs*19) [416]. This mutation was either present in a homozygous state or in combination with the previously identified c.3416delT or the novel c.C566T variants [416]. The latter missense mutation in exon 5 results in a substitution

of a highly conserved and structurally important amino acid within the third WD repeat (p.Thr189Ile) [416].

Both founder mutations have also been reported in several other affected individuals, either in a homozygous or compound heterozygous state with each other, or as additional mutations [414,418]. Several additional homozygous or compound heterozygous frameshift and missense variants cluster in either conserved region [417,418,420] and were predicted to be deleterious. Moreover, some described missense variants, such as c.2050C>G (p.Leu684Val) and c.2708C>T (p.Thr903Met) exert unclear pathogenicity [415]. Due to a less severe phenotype and the additional presence of a mutation in the protein paraplegin, linked to a subtype of HSP, both of these TECPR2 mutations might be benign or contribute only in combination with other mutations [415,432]. Recently, two missense mutations of unclear pathogenicity were discovered in a Chinese patient, expanding HSAN9 presence to new ethnic backgrounds [433].

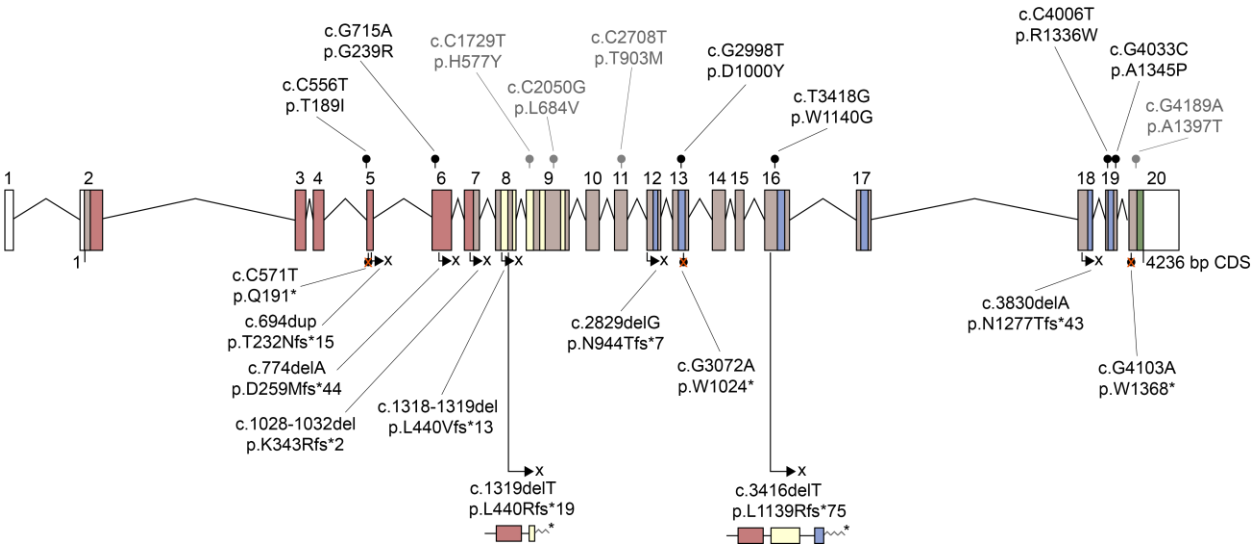


**Figure 6 - Pathogenic mutations within TECPR2.** Multiple mutations within *TECPR2* have been linked to neuropathy, based on their evidence divided into likely (black) or potentially (grey) pathogenic. Missense point mutations are displayed on top, frameshift and nonsense mutations below the gene structure. Point mutations are indicated by a dot, frameshift mutations by an arrow and immediate translation stop by a cross. Each mutation is specified by the nucleotide modification (c.) and amino acid substitution (p.), the two founder mutations additionally with a small model depicting the remaining protein domain with stars indicating a stop. Amino acids are noted by their single letter code. Boxes and colors highlight WD40 domains, the unstructured central region, the TECPR domains and the LIR motif. bp, base pair; CDS, coding sequence.

Since patients carrying missense variants can be affected just as severely as individuals with nonsense mutations, a similar pathomechanism, possibly involving the disruption of conserved N- or C-termini, seems plausible. Indeed, some truncated TECPR2 variants have been shown to be proteasomally degraded in cultured cells, including patient-derived fibroblasts [413,418]. Given the lack of antibodies targeting the N-terminal region of

TECPR2 and conflicting reports on mRNA stability of pathogenic variants, further studies on expression and protein stability are necessary [413,418,434].

Due to the prominent involvement of frameshift or missense variants and the potentially non-redundant function of TECPR2 [430], mutant TECPR2 may not be able to exert its regular functions such as stabilizing protein interactions. Indeed, a recent *tecpr2* knockout mouse model exhibits behavioral and neurodegenerative dysfunctions, reinforcing the idea of a loss-of-function mechanism contributing to HSAN9 pathology [434].

### Autophagy-related dysfunctions

A proteomic screen investigating the human autophagy network identified TECPR2 as an interactor of human Atg8 homologs, thus providing the first evidence for its involvement in autophagy [302]. Further research focused on this process and positioned TECPR2 as a positive modulator of autophagy (Figure 7). Skin fibroblasts from patients carrying the c.3416delT (p.Leu1139Argfs*75) mutation as well as TECPR2-depleted HeLa cells showed decreased levels of lipidated LC3B under basal, as well as induced and blocked autophagy [413,341]. Interestingly, p62 showed accumulation under basal conditions in immunofluorescence analysis [413,341], suggesting reduced autophagy-mediated degradation. In basal and induced autophagy, TECPR2 depletion also reduces both, the abundance of WIPI2 and the formation of WIPI2 positive puncta [341] (Figure 7). According to these findings, TECPR2 depletion is thought to affect the autophagic flux, possibly by disturbing phagophore formation, and to attenuate lysosomal delivery, resulting in overall impaired autophagy [302,413].

Based on structural analyses and interactomics, TECPR2 was found to interact with LC3B, LC3C and GABARAP proteins in a LIR-dependent manner [302,341] (Figure 7). Loss of the TECPR2-LC3C interaction reduces WIPI2 levels under basal and induced conditions, implying a concerted effect on autophagosome formation sites. Interestingly, no consistent colocalization of TECPR2 or LC3C with WIPI2 or DFCP1 could be observed, ruling out that TECPR2 is a permanent component of the forming autophagosome [341].

Attenuation of autophagy was also observed in the brainstem and spinal cord of *tecpr2* knock-out mice, mimicking HSAN9 pathology [434]. A recent comparative analysis of interaction partners of TECPR2 reported a strong enrichment of autophagy-associated proteins, further strengthening TECPR2's potentially diverse involvement in functional autophagy [430]. Interestingly, its closest human sibling, TECPR1, has also been identified as a positive regulator of autophagy. TECPR1 was shown to bind to ATG5 and LC3C, and to support lysosomal targeting of autophagosomes, especially in the context of xenophagy and aggrephagy [428,435,436]. However, apart from its general effect on

autophagy, the mechanistic details of TECPR2's involvement in autophagy remain elusive.

**Disruption of conventional secretion**

Based on the intertwined relationship between autophagy components and the COPII-machinery as well as the prominent involvement of ER- and Golgi-associated proteins in neurological diseases, the role of TECPR2 in ERES maintenance has also been subject of research. Indeed, in 2015, the group of Christian Behrends provided the first evidence for the involvement of TECPR2 in mediating conventional COPII-mediated secretory processes and suggested potential links to autophagy [341] (Figure 7).

Several distinct cellular trafficking components were found in a TECPR2 interactomics screen. Apart from further downstream components such as constituents of the BLOC-1 (DTNBP1, BLOC1S1, BLOC1S3) and HOPS complex (VPS41 and VPS11), TECPR2 interaction was also validated for the COPII coat protein SEC24D (Figure 7). Moreover, TECPR2 forms cytoplasmatic puncta that independently colocalize with SEC24D or to a lesser extend with VPS41 and DTNBP1 [341]. Together, these observations expand the interaction landscape of TECPR2 beyond the initially described interaction with members of the ATG8 family and further strengthen TECPR2's potential scaffolding function, possibly by bringing together different distinct cellular compartments.

Interestingly, association with SEC24D and components of BLOC-1 and HOPS partially requires the LIR-mediated binding of TECPR2 to LC3C, expanding the role of ATG8 proteins beyond autophagy. Interaction with LC3C stabilizes their respective complexes and protects individual constituents from proteasomal degradation, as shown for the formation of SEC23A-SEC24D heterodimers. TECPR2 itself is not subjected to autophagosomal degradation [341].

Since depletion of LC3C or TECPR2 not only reduces WIPI2-positive puncta but also ERES levels, a shared effect on both, sites for ER export and autophagosome formation, seems plausible [341] (Figure 7). TECPR2 deficiency furthermore delayed ER export of several reporter proteins, both in HeLa cells and patient-derived fibroblasts carrying the c.3416delT (p.Leu1139Argfs*75) variant [341].

Consistently, TECPR2 knockdown leads to expanded ER sheets and condensed Golgi, thus also affects the ER-Golgi interface [341]. Interestingly, the expression pattern of TECPR2 is negatively correlated with that of ribosomal proteins [430], indicating a potential role in the synchronization of protein synthesis, trafficking and degradation.

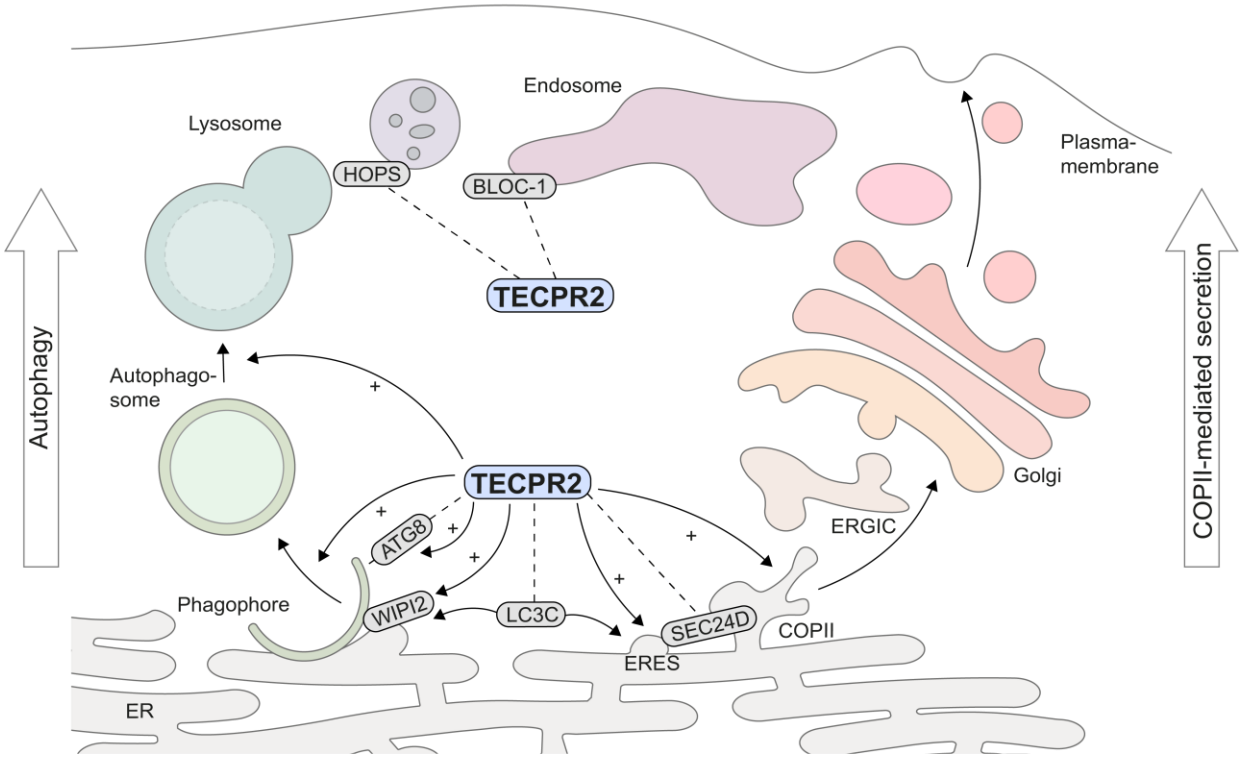


**Figure 7 - TECPR2 regulates autophagy and COPII-mediated secretion.** TECPR2 is a positive modulator of autophagy, interacts with ATG8 proteins and affects the formation of WIPI2 puncta, possibly in a LC3C-dependent manner. TECPR2 furthermore regulates ER-to-Golgi trafficking. Besides effects on ERES levels and core COPII components, TECPR2 also interacts with downstream trafficking components HOPS and BLOC-1. ER, endoplasmic reticulum; ERES, ER exit sites; ERGIC, ER-Golgi intermediate compartment; dotted lines indicate interactions.

Despite these recent findings, the involvement of TECPR2 in maintaining functional ERES and early secretory processes as well as general trafficking to downstream compartments remains poorly understood. It also remains unclear to what extent TECPR2-related ERES defects precede the autophagic phenotype, possibly due to disturbed supply of cellular constituents. Therefore, further studies dissecting the mechanistic role of TECPR2 are needed, as well as unbiased approaches investigating broad effects on general secretory cargo and trafficking-acceptor compartments.

## 3.4 Approaches for studying protein trafficking and secretion

In the past decades, several biochemical and genetic methods have been developed to study protein trafficking and secretion. These range from bioinformatic prediction of signal peptides and cellular localization to *in vitro* assays investigating specific overexpressed proteins by immunoblotting and -fluorescence analysis. Although these approaches allow for an in-depth characterization of a specific target protein, they are insufficient to analyze global effects on the cellular proteome. For this reason, mass spectrometry-based assays became increasingly popular in recent years, as they allow to assess alterations of thousands of proteins in an unbiased manner. Thereto, complex protein samples are often specifically processed and digested. The resulting peptide mixture is usually separated by liquid chromatography, ionized and injected into the mass spectrometer, where the peptides are further separated and often fractionated into smaller analytes. Based on these fragments, the protein composition of complex samples can be identified and even used to quantify proteomic changes. Moreover, mass spectrometry can be coupled to a variety of upstream analyses such as protein and sub-cellular enrichments, making it an exquisite method for studying complex biological systems that may require the combination of different biological approaches.

### 3.4.1 Proximity profiling of ER-export components

Since a huge proportion of trafficking and secreted proteins start their journey at ERES, the composition and modulation of these sites is crucial for proper ER export, including transient regulations. As classical interaction studies often lack these temporary elements, proximity profiling is an excellent tool for a more comprehensive mapping of ERES components. In this way, all proteins that are adjacent to a certain bait protein can be labelled and then analyzed by mass spectrometry. Although this technique always relies on tagging a bait protein with a specific enzyme, several different approaches using distinct enzymes have been developed. On the one hand, mutants of the *Escherichia coli*-derived biotin ligase BirA (often referred to as BioID, TurboID and miniTurboID) are used to covalently biotinylate proximal lysine residues in neighboring proteins with a labelling radius of about 10 nm [437-439]. On the other hand, the soybean ascorbate peroxidase APEX and its improved variant APEX2 is used to covalently attach a biotin phenol radical to electron-rich residues (e.g. in tyrosines) in neighboring proteins with a labelling radius of approximately 20 nm [440,441]. Both approaches result in biotinylated proteins that can be enriched and coupled to mass spectrometry. Since APEX-based techniques require an initial $H_2O_2$ pulsing, this profiling method cannot be applied in *in vivo* animal experiments. However, since biotinylation is achieved by short lived (<1 ms) radicals and performed with very short labeling times (<1 min), APEX2-based profiling is able to assess dynamic, short-lived proximity associations and is compatible with electron microscopy [442]. BioID-based approaches require longer biotinylation times (>10 min) and are therefore more suited for the analysis of static associations, but can be performed *in vivo*.

### 3.4.2 RUSH-System for monitoring trafficking proteins

Besides the identification of trafficking and secretion regulators, the content of secretory compartments, the actual secretory cargo, is of particular interest as disturbances of vertical transport likely account for many disease-associated phenotypes.

Studying the cargo of COPII-coated structures and general ER export, a major hurdle is their non-synchronized trafficking as these compartments constantly emerge at ERES. In the last decades, several approaches have been developed to overcome this issue, ranging from temperature-dependent blockage of ERES export due to slower kinetics [443,444] to temperature-induced (and reversible) aggregation of trafficking receptors or specifically tagged proteins, ultimately blocking ER export [445-447]. However, these techniques suffer from major drawbacks, as they are performed under non-physiological conditions, often rely on gradient centrifugation and only follow individual reporter proteins. In 2012, Franck Perez's group presented a novel approach for studying ER export that overcomes conditions using temperature-blockage termed retention using selective hooks (RUSH) system. The RUSH system elegantly combines the expression of two fusion-proteins, an ER-resident hook tagged with a streptavidin and an endogenously trafficking protein linked to a streptavidin-binding protein and a reporter such as GFP [448]. Under basal conditions, the reporter protein is retained in the ER due to its streptavidin-mediated interaction with the hook. Upon addition of biotin, the reporter construct is released and traffics in a synchronized manner to its acceptor compartment, e.g. the Golgi or PM. Since its introduction, the RUSH system has been used with a variety of reporter proteins to study the effect of genetic modifications and drug treatments on ERES architecture, export dynamics and involved regulators [86,449]. Despite these advances, the RUSH system is still limited to monitoring a specific trafficking reporter.

Due to their ability to detect and quantify many proteins, MS-based techniques might be a way forward to an unbiased and simultaneous profiling of different COPII cargos. Indeed, a recent publication investigated the core proteome of *in vitro* reconstituted COPII coated structures, providing novel insights into COPII machinery and biogenesis [450]. However, as this approach relies on *in vitro* reconstitution with recombinant coat proteins, enrichment via gradient centrifugation and limited presence of physiological accessory proteins, its predictive value on general ERES and COPII cargo remains insufficient. To overcome this issue, coupling of MS-based cargo profiling with RUSH-dependent synchronized trafficking might combine both, physiological formation of ERES and an unbiased detection of endogenous cargo proteins. In this light, APEX2-mediated proximity profiling of established RUSH-reporter proteins could offer an excellent approach, as biotin phenol radicals are membrane-constrained and can therefore be used to specifically analyze the content of ERES and COPII-coated structures. Interestingly, our lab recently developed a similar approach to systematically profile the content of autophagosomes by coupling APEX2 proximity proteomics with selective protein digestion, serving as a potential blueprint for an ERES-adapted profiling [451,232].

### 3.4.3 Proteomic analysis of subcellular acceptor compartments

Depending on their function and life cycle, trafficking proteins can be targeted to a variety of subcellular compartments. In classical secretory processes, functional proteins often migrate to the plasma membrane or are secreted into the extracellular space. Apart from that, proteins destined for recycling or degradation can traffic through the endo-lysosomal system towards degradative-active lysosomes. As these compartments are highly specialized, sophisticated methods are necessary to reach adequate compartment separation and specificity for their proteomic profiling.

Given that a high percentage of secreted proteins are glycosylated, approaches analyzing the surface proteome and secretome often exploit this feature for enrichment. Based on mode of action, classical techniques include glycol-capture by post-harvest modification of glycans [452,453] or biotinylation of protein amino-termini and lysine residues with N-hydroxysuccinimide (NHS)-biotin [454]. Since these methods do not differentiate between cell- or media-derived proteins, unspecific identification of background proteins is a common issue in these approaches, regularly resulting in complete incompatibility with secretome analysis.

One way to distinguish between cell-derived and unspecific proteins is by utilizing azidohomoalanine (AHA) labeling, in which an azide-containing analog of methionine is incorporated in newly synthetized proteins and thus can be used for specific enrichment [455]. A similar approach compatible with serum-complete media, which has reduced toxicity compared to AHA labeling, has been developed by the group of Stefan Lichtenthaler. This technique relies on the metabolic labeling of newly synthetized glycoproteins by incorporation of a modified N-azidoacetylmannosamine into the glycan-tree and its subsequent biotinylation via copper-free click chemistry using specific biotin-derivates. Several approaches, including streptavidin-based enrichment of biotinylated proteins, are then used to purify samples for proteomics analysis. This principle has been applied to analyze both the surface membranome as well as the secretome and is referred to as either surface-spanning protein enrichment with click sugars (SUSPECS) [456,457] or secretome protein enrichment with click sugars (SPECS) [458,459].

Regarding intracellular and membrane-bound acceptor compartments such as lysosomes, disturbed trafficking towards these targets can be analyzed by proteomic profiling of respective compartments. This often includes differential centrifugation, which works well with large amounts of input material but also leads to loss of a significant amount of sample during processing. Hence, methods relying on other purification principles such as immune-based approaches may be advantageous over classical differential centrifugation techniques, especially for low-abundant compartments. Indeed, the metabolic composition of lysosomes was recently analyzed using lysosome immunoprecipitation (LysoIP) [460] after enrichment of intact lysosomes via a tagged lysosomal protein.

## 3.5 Aim of the study

Mutations in the gene encoding TECPR2 are associated with HSAN9, a fatal neurological disease. TECPR2 is involved in two essential processes maintaining cellular homeostasis: Classical COPII-mediated secretion and autophagy. Due to its protein structure, TECPR2 presumably acts as a critical scaffolding factor that stabilizes protein complexes and integrates various pathways. However, the mechanistic involvement of TECPR2 in either of these processes remains unclear.

The aim of my main thesis project is to elucidate the role of TECPR2 in early secretory processes and the downstream cellular defects that arise from TECPR2 deficiency. Based on the hypothesis that pathogenic mutations affect secretory processes not only at the level of ERES but also the composition of downstream acceptor compartments, I will exploit several unbiased proteomic approaches targeting different steps of secretory processes and intracellular compartments. Initially, I seek to identify changes in the composition, formation and vicinity of ERES by utilizing APEX2-driven proximity proteomics of core COPII components in wild-type and disease-mimicking cell lines. To profile the content of ERES and COPII-coated carriers, I strive to establish a novel APEX2-RUSH approach in different TECPR2 backgrounds. Further on, I will analyze the composition of downstream target compartments such as the plasma membrane, extracellular space and lysosomes by metabolically labeling secreted glycoproteins and enriching intact lysosomes. To elucidate TECPR2's mechanistic involvement, I furthermore seek to characterize how TECPR2 associates with the ER-Golgi interface by combining interactomics, differential centrifugation and compartment enrichment approaches (Publication I).

In a collaborative effort with Zvulun Elazar's group, focusing on TECPR2's involvement in autophagy, we seek to characterize the role of TECPR2 in patient-derived fibroblasts. In order to identify the domains involved in the regulation of autophagy, different TECPR2 variants will be analyzed using biochemical and cell-biological approaches. To test TECPR2's endogenous interactions, I strive to establish and perform endogenous TECPR2 immunoprecipitations and verify interactors so far only investigated in over-expression studies (Publication II).

Since there is no cure for HSAN9, we will collaborate with Graham Dempsey's team to evaluate the therapeutic potential of an antisense oligonucleotide (ASO)-based approach aimed at rescuing TECPR2 function in patients. After screening for the most suitable ASO, we will assess the lead candidate's ability to restore TECPR2 protein levels, its localization and function in different model systems. Both, efficacy and tolerability, will be validated in a non-human primate. To investigate a potential ASO-driven rescue of TECPR2 interactions, I strive to investigate the interaction landscape of different exon 8-related TECPR2 variants by performing pulldown proteomics (Publication III).

# 4 Publications and contributions

## 4.1 Publication I

**Spatial proteomics reveals secretory pathway disturbances caused by neuropathy-associated TECPR2.**

**Nalbach, K.**, Schifferer, M., Bhattacharya, D., Ho-Xuan, H., Tseng, W., Williams, L. A., Stolz, A., Lichtenthaler, S.F., Elazar, Z., Behrends, C.

Nat Commun 14, 870 (2023)

DOI: 10.1038/s41467-023-36553-6

Due to lack of research addressing the role of TECPR2 in early secretory processes and protein trafficking, corresponding cellular consequences for overall secretion and trafficking upon TECPR2 deficiency remain elusive.

By performing complementary proteomics, this work dissected TECPR2-dependent defects in the secretory pathway. In this way, we provided a resource for alterations occurring at different steps of secretory processes and trafficking compartments.

As the first author of this publication, I conducted and analyzed all experiments except electron-microscopy experiments (Figure 6, a) and ER-phagy assays (Figure S7, h). I generated all cell lines in TECPR2 wild-type and deficient backgrounds. I furthermore designed all figures, wrote and edited the manuscript and was instrumental in the conceptual design of the study.

## 4.2 Publication II

**Lysosomal targeting of autophagosomes by the TECPR domain of TECPR2.**

Fraiberg, M., Tamim-Yecheskel, B. C., Kokabi, K., Subic, N., Heimer, G., Eck, F., **Nalbach, K.**, Behrends, C., Ben-Zeev, B., Shatz, O., & Elazar, Z.

Autophagy, 17(10), 3096-3108 (2021)

DOI: 10.1080/15548627.2020.1852727

Although the role of TECR2 in autophagy has been studied more comprehensively than its involvement in secretory processes, underlying mechanisms remain unclear. In particular, it is not known how TECPR2 affects later stages of the autophagic flux, including targeting of autophagosomes to lysosomes.

In this study, we investigated the link between autophagy and TECPR2 in patient-derived material and revealed a new link between TECPR2 and lysosomal-targeting of autophagosomes via the SNARE VAMP8, possibly in conjunction with the HOPS complex and ATG8 proteins.

I contributed to this publication by establishing and performing endogenous immunoprecipitations of endogenous TECPR2 with subsequent identification of components of the HOPS complex via immunoblotting (Figure S3, B).

## 4.3 Publication III

**Developing antisense oligonucleotides for a TECPR2 mutation-induced, ultra-rare neurological disorder using patient-derived cellular models.**

Williams, L. A., Gerber, D. J., Elder, A., Tseng, W. C., Baru, V., Delaney-Busch, N., Ambrosi, C., Mahimkar, G., Joshi, V., Shah, H., Harikrishnan, K., Upadhyay, H., Rajendran, S. H., Dhandapani, A., Meier, J., Ryan, S. J., Lewarch, C., Black, L., Douville, J., Cinquino S., Legakis H., **Nalbach K.**, Behrends C., Sato A., Galluzzi L., Yu T. W., Brown D., Agrawal S., Margulies D., Kopin A., Dempsey, G. T.

Mol Ther Nucleic Acids, 29, 189-203 (2022)

DOI: 10.1016/j.omtn.2022.06.015

To date, TECPR2-linked HSAN9 remains an incurable, complex and fatal disease that severely affects patients' daily routines. Due to its broad involvement in autophagy and secretory processes as well as its rare frequency, the identification of therapeutically effective drugs remains challenging.

This research article reports the first strategy to combat HSAN9 by targeting pathogenic *TECPR2* mutations via an ASO exon-skipping strategy. The lead ASO candidate was able to rescue TECPR2 protein levels as well as its subcellular localization and functional interactions in multiple disease-models, while exhibiting acceptable *in vivo* tolerance.

I contributed to this publication by performing all mass spectrometry-based interactome experiments, including generation and validation of cell lines re-expressing wild-type and disease-mimicking TECPR2 variants (Figure S9).

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# Acknowledgements

At the end of my thesis, I would like to thank everyone who accompanied and supported me during this time:

First and foremost, I would like to express my gratitude to my doctoral advisor, Christian, for enabling me to perform my Ph.D. thesis in his lab and for his support, guidance and expert opinion, even after regular working hours and on weekends. Due to his support, I was able to work on a variety of fascinating projects, had the experimental freedom to explore different topics and gained new insights. My main TECPR2 project was an incredible opportunity to experience both the frustration and fulfillment that comes along with venturing down less-traveled paths.

I would also like to extend my thanks to the examination and doctoral boards for taking their time and reviewing this thesis as well as to my colleagues and friends for proofreading it. I am grateful for all my cooperation partners, whether related to TECPR2 or other projects.

The company of the whole Behrendslab was crucial for realizing this work. I would like to thank all members for the wonderful time, endless fun and enjoyable six years of research. Your friendship, support, team spirit and fellowship were invaluable. You were the best colleagues I could have dreamt of and made me look forward to coming to work every day, whether by car or as part of the Radl-Gang. This also accounts for the whole building, particularly the members of the Eddie, Haass and Steiner labs. Thank you for providing not only an exceptional scientific environment and support but also for your friendship, shared meals and outdoor adventures. I am looking forward to further joint adventures. Additionally, I would like to thank all the supportive staff that is crucial for performing exquisite science, including animal care takers and administrative personnel.

I would like to thank all of my friends, both in Munich and elsewhere – Munich expats, friends from my student days or old school friends - for their unwavering support and distractions that carried me through the highs and lows. I am grateful for each and every one of you.

Lastly, I would like to thank my family, especially my parents. Without your support and encouragement throughout my academic studies and doctoral work, this thesis would not have happened. Danke für alles!

*“Still round the corner there may wait/ A new road or a secret gate.*
*And though we pass them by today/ Tomorrow we may come this way*.”

- J.R.R. Tolkien. The Lord of the Rings: The Return of the King