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Developing and refining a FRAP-based transport assay in combination with RNAi to identify key molecular players regulating peripheral ER to nuclear envelope connections.

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Clemens Geppner BSc

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Shotaro Otsuka PhD

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Abstract

The endoplasmic reticulum (ER) is an extensive membrane network that spans large parts of the cellular complex and facilitates a variety of critical functions. It is in contact with most cellular compartments, but most critically, one portion of the ER, the nuclear envelope (NE) encapsules the entire nucleus and therefore is indirectly and directly involved in maintaining correct cellular function and health. The focus of this thesis lies specifically on the interface between the peripheral ER and the nuclear envelope (NE). We hypothesize that these ER-NE connections are at the center of many crucial transport processes and therefore potentially linked to various disease phenotypes.

It was established in previous experiments, that there are measurable differences in the way the endoplasmic reticulum (ER) and the nuclear envelope (NE) interface, when compared to ER-ER connections. While ER-ER connections, or three-way junctions, generally appear to be larger in terms of the luminal space, they also show more size variation when measured. ER-NE connections however appear to be much more tightly restricted and show little variability. When analyzing electron microscopy images of HeLa wild-type cells, it was shown that the ER-NE connections are constricted down to a size of only 20nm.

Seeing how tightly these connections seem to be regulated, it can be speculated that some sort of control mechanism is in place that maintains this ER-NE interface. However, how this connection is regulated or what molecular players are involved is not known. When looking at which proteins could be involved in the regulation of ER-NE connections, we have to differentiate between two areas of interest: Firstly, ER-NE connections can affect the luminal transport of molecules to and from the nucleus. Secondly, as the ER-NE connections also connect the outer membranes of the ER and NE, membrane bound proteins could traverse these connections, unless there is some kind of restriction happening.

In this work, we focus on the luminal transport and which molecular players are potentially involved in its regulation. To analyze this, we decided on a targeted approach that knocks-down candidate genes via RNAi followed by a fluorescence recovery after photobleaching (FRAP) based transport assay using a confocal spinning disc microscope for measuring recovery kinetics in live-cells. The resulting recovery curve was fitted to a one-phase association model from which a recovery rate was extracted to directly compare all generated data.

Initial transport screenings highlighted the limitations of the chosen approach by yielding inconsistent results with poor reproducibility. In an iterative manner, we improved the method little by little until we were able to generate reproducible results. This then allowed us to show how certain genes affect the overall transport rates throughout the entire ER network. At this stage however, no conclusive evidence, directly linking one of the investigated genes to altered ER-NE connections has been found.

Zusammenfassung

Das endoplasmatische Retikulum (ER) ist ein weitreichendes Netzwerk aus Membranen, welches viele wichtige Funktionen ermöglicht und mit den meisten Bereichen der Zelle in Kontakt steht. Besonders wichtig ist die Kernhülle, ein Teil des ER, der den Zellkern umschließt und daher an der Aufrechterhaltung der Zellfunktion und -gesundheit beteiligt ist. Der Hauptfokus dieser Arbeit liegt besonders auf der Verbindung des ER mit der Kernhülle (nuclear envelope, kurz: NE). Unsere Hypothese lautet hier, dass diese ER-NE Verbindungen unmittelbar für die korrekte Funktion zellulärer Transportprozesse verantwortlich sind und daher potenziell an der Ausbildung von Krankheitsbildern beteiligt.

Frühere Experimente haben gezeigt, dass es Unterschiede zwischen den Verbindungen des ER-ER und ER-NE gibt. ER-ER-Verbindungen scheinen im Allgemeinen größer zu sein und weisen mehr Variation in den gemessenen Durchmessern des Lumen Raums auf, während ER-NE Verbindungen enger sind und nur wenig Variabilität aufweisen. Ein Versuch an HeLa-Wildtyp-Zellen zeigte, dass die ER-NE-Verbindungen auf einen Durchmesser von nur etwa 20 nm verengt sind, was deutlich kleiner ist als die ER-ER-Verbindungen. Dies deutet auf eine strenge Regulierung der ER-NE Verbindungen hin.

Die enge Regulierung der ER-NE Verbindungen könnte auf ihre Funktion in der Zellkernintegrität und Genexpression zurückzuführen sein. Wie genau diese Verbindungen jedoch reguliert werden und welche Gene daran beteiligt sind ist noch unerforscht. Es gilt weiters zu unterscheiden, dass die ER-NE Verbindungen sowohl Einfluss auf den luminalen Transport als auch auf den Transport von membrangebundenen Proteinen nehmen können.

In dieser Arbeit beschränke ich mich auf den luminalen Transport und von welchen Genen dieser reguliert sein könnte. Hierfür wird ein gezielter Ansatz zum knock-down bestimmter Gene mittels RNAi gekoppelt mit einem FRAP (fluorescence recovery after photobleaching) -basierten Transport Assay verwendet. Die mikroskopische Bilderfassung erfolgt in einem „confocal spinning disc microscope“ welches die Messung der „fluorescence recovery“ an lebenden Zellen erlaubt. Mit Hilfe eines mathematischen Modells konnte die Geschwindigkeit der Transportprozesse somit ermittelt werden. Dies ermöglichte einen direkten Vergleich sämtlicher durchgeführter Experimente.

Die ersten Versuche zeigten jedoch deutlich die Limitierungen des gewählten Ansatzes auf, da die Ergebnisse stark schwankten und nicht reproduzierbar waren. Durch gezielte Eliminierung von externen Einflüssen, konnte die Methode Stück für Stück verbessert werden, wodurch es letztendlich möglich war, reproduzierbare Ergebnisse zu erzielen. Wir konnten somit zeigen, dass bestimmte Gene einen Einfluss auf die generelle Transportkinetik des ERs haben. Es war jedoch noch nicht möglich ein Gen zu identifizieren welches einen nachweisbaren Einfluss auf die Regulierung der ER-NE Verbindungen hatte.

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1. Introduction

1.1 The endoplasmic reticulum (ER) and the nuclear envelope (NE)

The endoplasmic reticulum (ER) is a large, continuous membrane-bound organelle consisting of several structurally and functionally distinct domains. Due to its expansive nature, the ER is in direct contact with most other cellular compartments and therefore acts as a central hub, both structurally and functionally, in maintaining proper cellular function.

The ER has first been observed in electron microscopic (EM) in 1945 (Porter et al., 1945) and has since then remained an important area of research. Figuratively and literally speaking, the ER sits at the core of cellular functionality and is involved in a plethora of biological processes. And while there was a slight stagnation in the volume of publications in the late 1970s, recent advances, especially in the fields of electron tomography and confocal fluorescence microscopy, have rejuvenated the subject and led to an ever-increasing scientific interest, particularly in exploring how dysregulation of ER associated processes might lead to disease development. Protein synthesis, folding and transport, lipid metabolism, and calcium storage are all implicated in disease phenotypes associated to pathogenic ER dysfunction (H. Chen, 2021; J. H. Lin et al., 2008).

Prominent examples of diseases caused by dysregulation of the ER include Alzheimer's disease, cystic fibrosis, and diabetes, among many others. Mutations in ER-shaping proteins, that lead to a disturbed ER morphology and consequently inhibit proper ER function, have been identified to be involved in the emergence of neurodegenerative disorders (H. Zhang & Hu, 2016).

The ER can be divided into nuclear and peripheral regions. The nuclear region, usually referred to as nuclear envelope (NE) is a highly regulated membrane barrier that separates the nucleus from cytoplasmic components in eukaryotic cells. While we distinguish between inner nuclear membrane (INM) and outer nuclear membrane (ONM), these two membranes are continuous and connected at the nuclear pore complexes (NPCs) (see Figure 1). Further, the outer nuclear membrane (ONM) is continuous with the membrane of the peripheral ER which has already been shown in early electron microscopic (EM) images (Watson, 1955). Additionally, the general structure of the NE has been conserved in all eukaryotes (Wilson & Dawson, 2011).

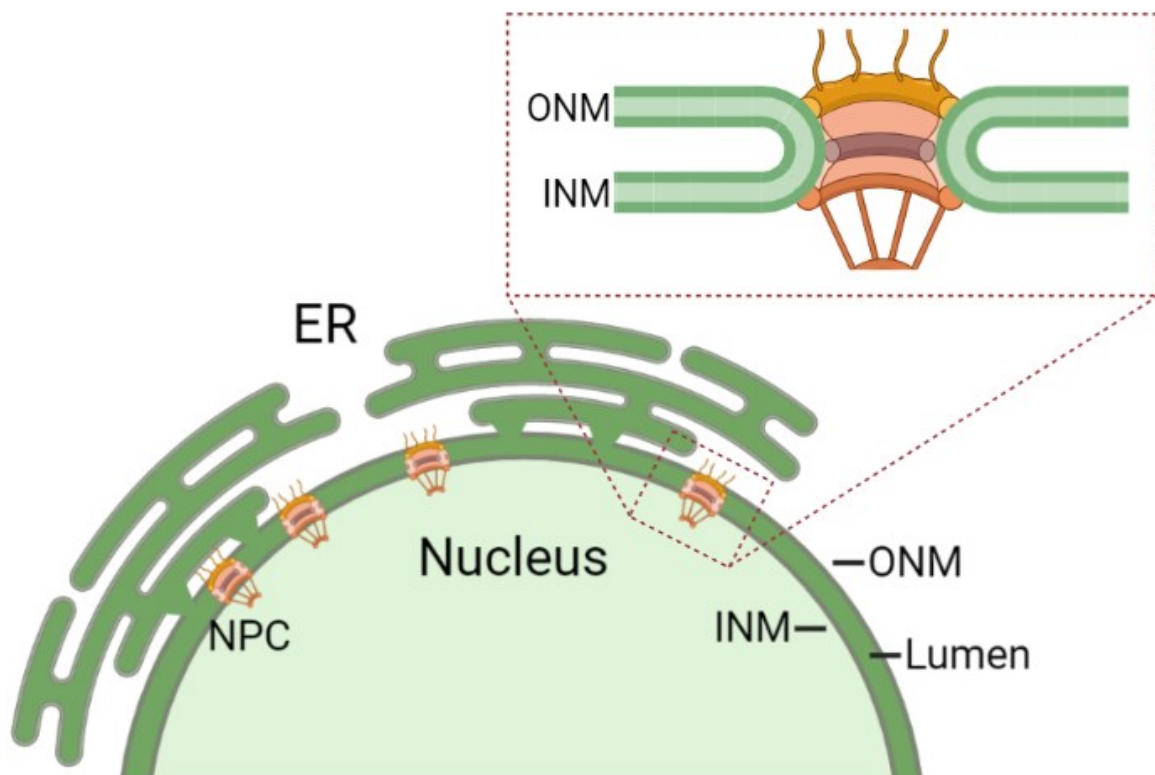


Figure 1. Schematic representation of the endoplasmic reticulum (ER). Peripheral ER connects to the nuclear envelope (NE) at ER-NE junctions. The nuclear envelope encloses the nucleus and is interrupted by nuclear pore complexes (NPC) – enlarged schematic depiction of one NPC displays the continuity of the inner nuclear membrane (INM) to the outer nuclear membrane (ONM). Created in BioRender.com.

The peripheral ER regions form a reticulated membrane network system of membrane tubules and flattened cisternae, called sheets. These two domains can be sub-categorized into smooth-ER (ribosome free) and rough-ER (ribosome bound). Structurally, ER-sheets display low curvature except at their edges (Shibata et al., 2009). In contrast, membrane tubules are characterized by high membrane curvature which, due to being energetically unfavorable, demands a high degree of stabilization facilitated by proteins of the reticulon and DP1/Yop1p families. Importantly ER tubules are highly dynamic structures undergoing constant remodeling and rearrangement. This structural plasticity allows the ER to form a loose meshwork, mostly interconnected at three-way junctions, spanning almost the entirety of the cell (Westrate et al., 2015). The luminal diameter of the tubular ER and the thickness of

the membrane sheets are very similar in animal cells at about 50 nm (Bernales et al., 2006; Shibata et al., 2010). Since the distance between the ER-membranes is roughly unchanged across the ER, membrane curvature presents itself as a key factor for characterization of structure and function of endoplasmic sub-compartments.

1.1.1 Endoplasmic reticulum function

The ER is the largest organelle in the cell and plays many roles including the protein synthesis, lipid metabolism, calcium storage and carbohydrate metabolism (Schwarz, 2015). The structure of the ER is primarily affected by reticulons (RTN), which promote formation of ER tubules by stabilizing high curvature (De Craene et al., 2006; Voeltz et al., 2006), atlastin (ATL), promoting ER-ER fusion (Orso et al., 2009) and lunapark (LNPK) proteins, stabilizing ER three-way junctions (S. Chen et al., 2015). Regulators of sheet-like structure like CLIMP-63 and TMEM170A have been shown to positively affect ER sheet formation when overexpressed (Shibata et al., 2010; Christodoulou et al., 2016).

And while the ER is one continuous organelle, this wide array of functions demands sub-compartmentalization into distinct domains. The primary distinction that can be made is between rough- and smooth-ER. The rough-ER (rER) is studded with membrane-bound ribosomes which perform translation of mRNA into protein. Therefore, the rER is involved in the biosynthesis, facilitation of correct folding and modification of secretory and integral membrane proteins (Shibata et al., 2006). Structurally the rER is characterized by a low membrane curvature and is of sheet-like nature. While there does not appear to be any active retention of ribosomes to the cisternal ER, a widely accepted hypothesis is that high membrane curvature is energetically unfavorable for membrane-bound ribosomes. In the absence of ribosomes, the smooth-ER (sER) is typically found in the tubular regions of the ER and is significantly involved in processes such as biosynthesis of phospholipids, cholesterol, and steroids, detoxification and intracellular Ca^{2+} -storage (Baumann & Walz, 2001). Lipid metabolism specifically happens in the endomembrane compartment that includes the ER and Golgi apparatus (Schwarz, 2015).

The cell types and especially their function in the context of an organism made up of different tissues play an important factor dictating relative amounts of rER and sER. Prominent examples for cells with predominantly cisternal ER are pancreatic secretory cells and B-cells, both of which notorious for synthesizing vast amounts of secretory proteins. Conversely, adrenal, liver and muscle cells are all characterized by their mainly tubular ER which can be ascribed to the higher demand for Ca^{2+} in the case of muscle cells, the secretory nature of adrenal cells and the need for detoxification in the liver respectively. (Baumann & Walz, 2001).

Although this distribution of rER and sER should be viewed as a guideline, rather than a clear-cut set of rules, it still serves the purpose of organizing our mental image of the endoplasmic reticulum and its functional sub-compartmentalization.

1.1.2. Nuclear envelope function

The innermost part of the ER, called nuclear envelope (NE) is in direct contact with the nucleus and performs the crucial role of separating nuclear components from the cytosol. Structurally, we differentiate between the inner nuclear membrane (INM), the outer nuclear membrane (ONM) and the nuclear pore complexes (NPCs) which connect these two membranes and provide a gateway between the nucleoplasm and the cytoplasm. To ensure proper function of the NE, both INM and ONM require a distinct protein composition and strict maintenance thereof. The ONM is continuous to the ER and shares certain functions and proteins, including ribosomes and ER specific proteins. Yet it still has a set of proteins unique to the ONM. Similarly, the INM, despite being connected to the ONM via the NPCs exhibits its own set of integral membrane proteins (Schirmer & Foisner, 2007). Nuclear envelope transmembrane proteins (NETs) span across the perinuclear lumen and facilitate a wide array of crucial functions on either side of the nuclear envelope. NETs serve as anchor points for cytoplasmic filaments connected to the ONM of the nucleus and connect to lamin type V intermediate filaments that form the majority of the nuclear lamina (Dechat et al., 2008; Zuleger et al., 2011). The INM has a highly specific set of proteins that allow its involvement in various cellular processes including the sensation and buffering of mechanical forces, the organization and regulation of the genome as well as its inheritance and lipid metabolism (Lee & Burke, 2018; Romanauska & Köhler, 2018; Ungricht & Kutay, 2017).

As briefly mentioned above, the nuclear lamina is at the center of many critical functions performed at the nuclear side of the inner nuclear membrane. Intermediate filaments (primarily type V intermediate filaments, A- and B-type lamins) line the surface of the inner nuclear membrane, thereby stabilizing it. Tightly associated with this lamin scaffold are lamin-associated polypeptides (LAPs). LAPs exhibit enzymatic activity but most often are part of large multi-protein complexes and are therefore involved in a wide range of functions, including but not limited to histone modification and transcriptional repression (Schirmer & Foisner, 2007).

Another key component of the INM are lamin-associated domains (LADs) which bind heterochromatin, organize the genome spatially and are involved in regulation of gene repression (van Steensel & Belmont, 2017). First discovered in electron microscopy, it was later shown that genomic loci which are close to the nuclear lamina (later defined as LADs) often appear to be relatively transcriptionally inactive (Fawcett, 1966; Lanctôt et al., 2007).

For correct cellular function the positioning of the nucleus is vital. One passive mode of manipulating the nuclear position within the cell would be achieved through changing local densities of actin or intermediate filament in the cytosol. Through size exclusion, the nucleus would be forced to remain in a loosely controlled space. A second mechanism involves the active movement of the nucleus through direct coupling of the cytoskeleton to the nuclear envelope. Merely attaching the cytoskeleton to the ONM however would not be sufficient to perform the necessary functions of this nuclear displacement mechanism but instead a connection that reaches the INM is needed (Reinsch & Gonczy, 1998).

A second system of vectorial movement of the nucleus involves so-called LINC complexes (Linker of Nucleuskeleton and Cytoskeleton). LINC complexes are universally found in all nucleated cells known to date (Gundersen & Worman, 2013) and can therefore be viewed as immensely important to correct cellular function. LINC complexes consist of two set proteins interacting across the luminal space of the nuclear envelope. At the inner nuclear membrane, SUN proteins are found which form trimers spanning the largest part of the perinuclear space. Close to the ONM, the SUN (SAD1 and UNC-84) trimer interacts with luminal domains of the KASH (Klarsicht, ANC-1, SYNE homology) proteins which in turn enable binding to the cytoskeleton on the cytoplasmic side of the ONM with help of Nesprin-2 (Cain et al., 2018).

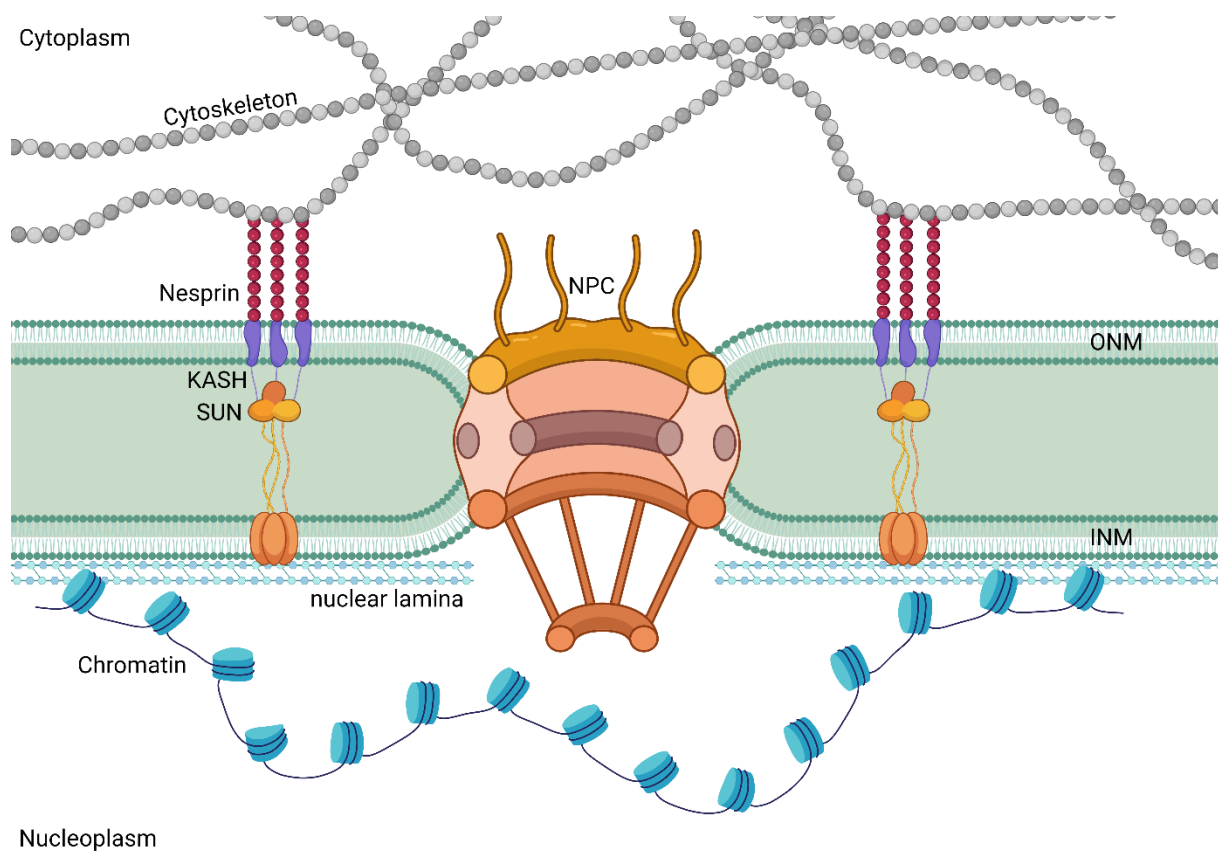


Figure 2. Simplified representation of how cytoplasm and nucleoplasm are separated by the nuclear envelope. The nuclear pore complex is represented as a cross-section and shows how inner- (INM) and outer nuclear membrane (ONM) are continuous. Various examples of proteins and how they interact with nearby systems are shown as well. Created in BioRender.com.

Only briefly mentioned thus far but no less important are the protein complexes that fuse the inner and outer nuclear membranes and form aqueous channels for transport of molecules between cytoplasm and nucleoplasm. These nuclear pore complexes are mainly composed of nucleoporins (NUPs) and exhibit an 8-fold rotational symmetry with a web-like, intrinsically disordered center channel for selective transport protecting the nuclear environment (Beck & Hurt, 2017; Wong, 2021). Transport through NPC channels can happen passively through diffusion. For larger molecules and protein complexes (>40 kDa) an active, targeted transport process is required (D. H. Lin & Hoelz, 2019). Generally, the type of signal sequences (NTRs, nuclear transport receptors) present determines the fate of proteins in terms of their

localization in respect to the nuclear envelope. Nuclear export signals (NESs) facilitate the export of molecules through Ran-GTPase-dependent nuclear transport factors (Exportins) (Matsuura, 2016). Antagonistically, nuclear localization signals are bound by Import-Karyopherin- β s (Importins) and transport their cargo into the nucleus (Soniati & Chook, 2015). Recently, various new hypotheses for the involvement of NPCs outside of cellular transport processes have emerged. This includes the suggested function as a mechanosensor (Matsuda & Mofrad, 2022) or the involvement of NPCs in gene regulatory processes through interaction with chromatin through manipulation of genome architecture, transcription complex assembly, chromatin remodeling and coordination of transcription and mRNA export (Pascual-Garcia & Capelson, 2021).

The tight regulation of nuclear transport processes has been shown to be critical in the maintenance of healthy cellular function and the dysregulation of transport processes across NPCs has been suggested to be causal in a diverse set of disease (Sakuma & D'Angelo, 2017). Various neurodegenerative disorders such as frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), and Parkinson's disease have been linked to NPC biology (K. Zhang et al., 2015; Gleixner et al., 2022).

Given the central role of the nucleus in a cellular context, tight regulation of processes in and around it, is to be expected. The diseases mentioned above are only a few NPC specific examples that showcase the necessity for proper regulation of nucleus associated processes.

1.2 The ER and NE membranes and the lumen are continuous.

The ER is universal and each cell has only a single copy forming an extensive 3D-network that encompasses the entire cell from INM all the way to peripheral regions (Levine & Rabouille, 2005).

The continuity of ER and NE membranes was first suggested in 1955 in early electron microscopy images (Watson, 1955). From these EM images, schematic drawings were created already showing how the inner and outer nuclear membranes were connected at nuclear pores. The nuclear pore complexes that form these channels were described shortly afterwards (Watson, 1959). The continuity of the ER and NE lumen was shown in further studies utilizing fluorescent dyes (Terasaki et al., 1994; Villa et al., 1991).

This continuity between the NE and the ER is the baseline for the hypothesis that this thesis explores. More specifically, the interfaces between the peripheral ER and the NE, where the ER membranes transition into the ONM, which shall be referred to as "ER-NE connections", are of major interest.

Electron microscopy has shown differences in the way that ER-ER connections (junctions) are regulated versus ER-NE connections. ER-ER connections, mostly three-way junctions depict much lower levels of structural and spatial regulation when compared to ER-NE connections which appear much more strictly regulated. This regulation was shown through quantitative analysis of ER-NE connections from EM images of HeLa cells and showed that ER-NE connections were very consistent in size at roughly

20 nm in diameter (see Figure 3)(Bragulat-Teixidor et al., 2023). The consistency in size clearly suggests a regulating mechanism that is yet to be discovered.

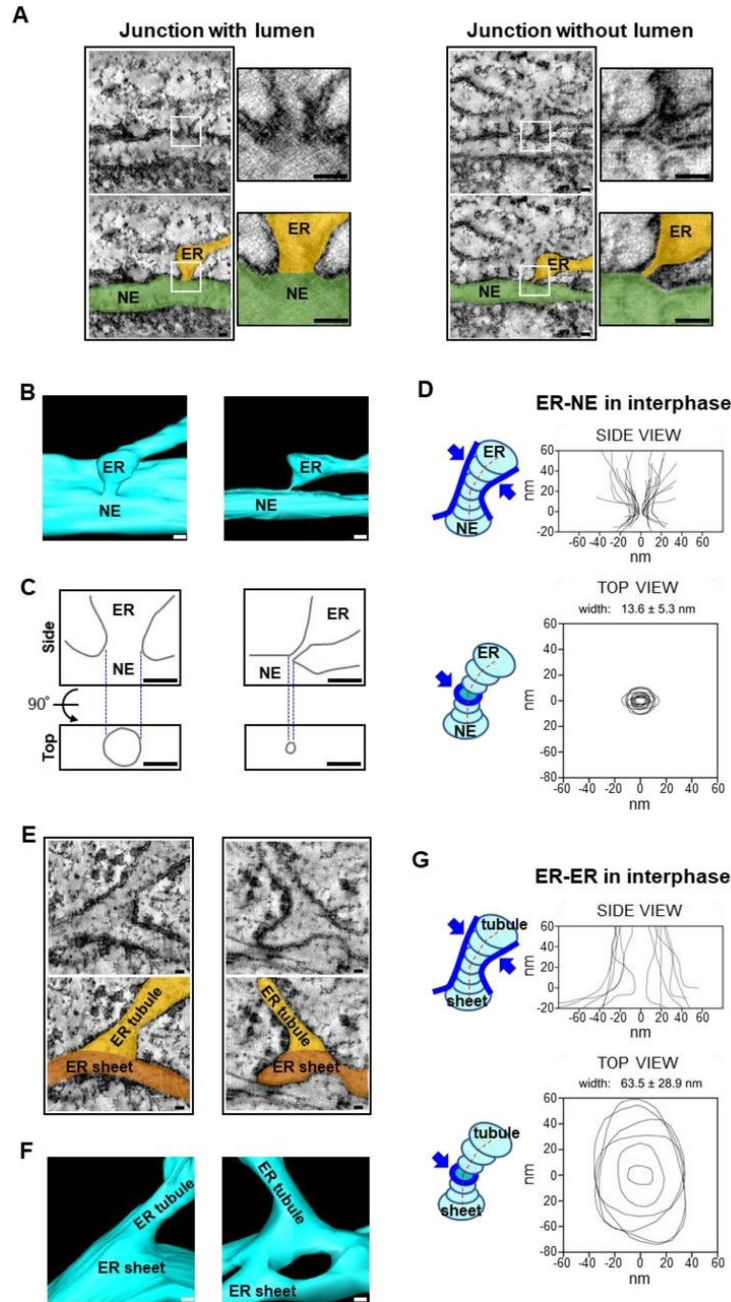


Figure 3. The membranous junctions that link the ER to the NE in interphase are narrow, constricted, and distinct from the junctions within the ER.

(A) Electron tomographic slices of ER-NE junctions with and without a lumen at their sagittal planes. For each junction, an enlargement of the region containing the junction is displayed. The top row shows raw EM data; the bottom row, the EM data on which the ER and the NE are colored in orange or green, respectively. (B) 3D meshes of the ER-NE junctions shown in A. (C) Membrane profiles of the side and top views of the junctions shown in A. (D) Plots overlaying membrane profiles of the side and top views of ER-NE junctions found in interphase cells. $n = 14$ junctions from 4 cells. (E) Electron tomographic slices of ER-ER junctions (in which an ER tubule is fused perpendicularly to an ER sheet) at their sagittal planes. The top row shows raw EM data; the bottom row, the EM data on which an ER tubule and an ER sheet are colored in orange or brown, respectively. (F) 3D meshes of the ER tubule-sheet junctions shown in (E). (G) Plots overlaying the side and top views of the ER tubule-sheet junctions found in interphase cells. $n = 6$ junctions from 4 cells. Scale bars for A-C, E, F: 20 nm. (Bragulat-Teixidor et al., 2023)

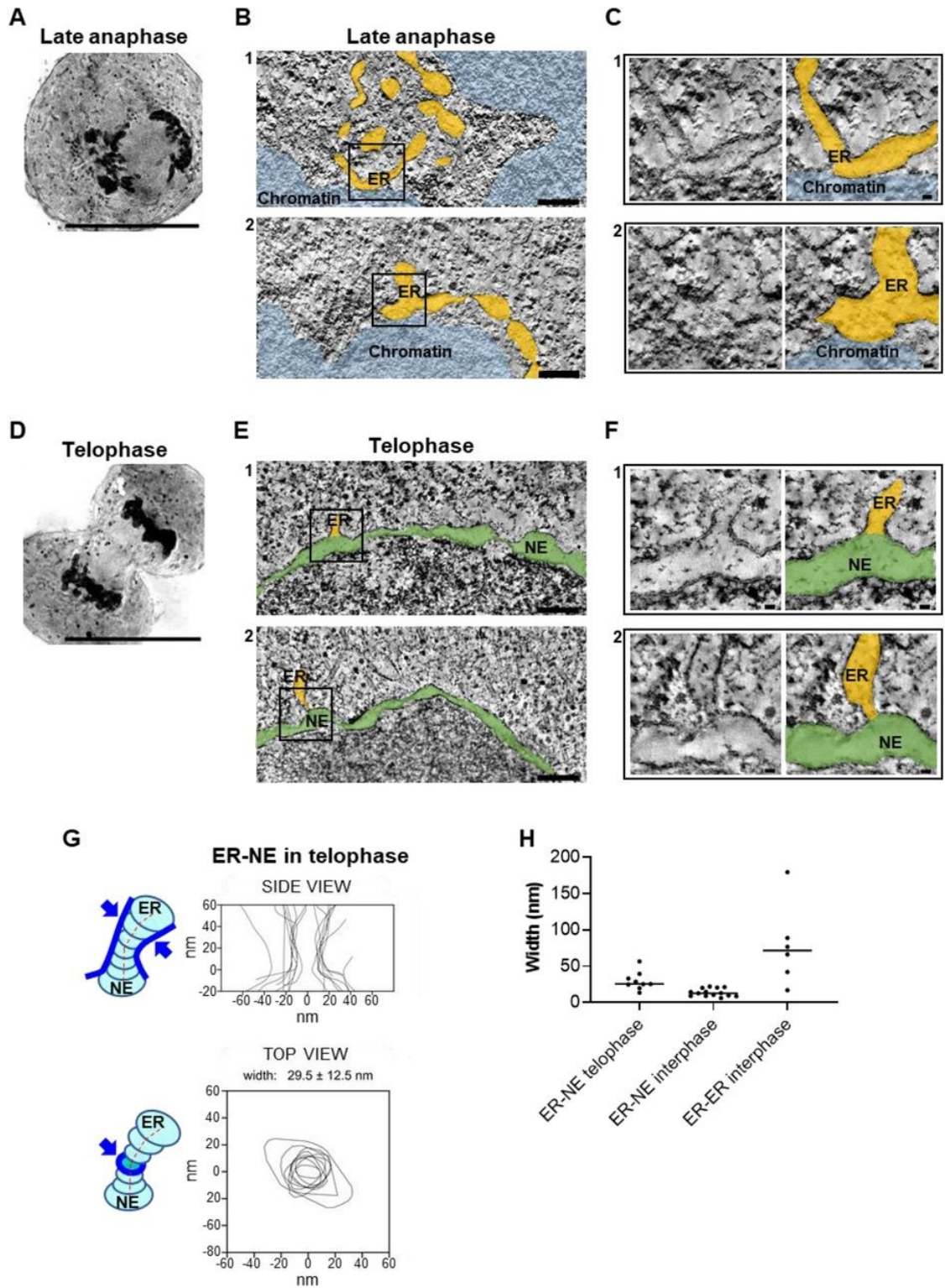


Figure 4. ER-NE junctions get progressively constricted during mitotic exit. (A) 2d EM micrograph of a cell in late anaphase. (B) tomographic slices of cells in late anaphase showing that the er (orange) starts to contact the chromatin (blue), but the ne is still indistinguishable. (C), enlargement of the regions indicated in b. For each junction, the left image shows raw EM data; the right one, the EM data on which the er and the chromatin are colored in orange or blue, respectively. (D) 2d EM micrograph of a cell in telophase. (E) tomographic slices showing that the ne is established in telophase and er-ne junctions are present. (F) enlargement of the regions indicated in e. For each junction, the left image shows raw EM data; the right one, the EM data on which the er and the ne are colored in orange or green, respectively. (G) plots overlaying the side and top views of the er-ne junctions found in telophase cells. N = 9 junctions from 2 cells. (H) the cross-section width of the constricted neck of er-ne junctions in telophase and interphase, as well as the width of er tubule-sheet junctions. N = 9, 14, and 6 for ER-NE telophase, ER-NE interphase, and ER-ER interphase. Scale bars for a, d: 20 μ m; scale bars for b, e: 200 nm; scale bars for c, f: 20 nm. (Bragulat-Teixidor et al., 2023)

1.2.1 Protein transport and molecular players involved

Protein transport from ER to the nucleus is an essential function that involves a multitude of proteins but is not fully understood yet. Two main pathways for protein translocation have been described but the amount and diversity of proteins present around the ER and the NE demands further investigation into additional mechanisms at play.

Generally, we differentiate between two main pathways of protein incorporation into the ER, co-translational and post-translational.

The majority of proteins entering the secretory pathway are first targeted to the Sec61 translocon via recognition of the hydrophobic nascent N-terminal signal or transmembrane domain by the signal recognition particle (SRP). The nascent peptide together with the associated ribosome are then bound to the ER membrane. (Keenan et al., 2001; Kurzchalia et al., 1986; Voorhees et al., 2014; Wiedmann et al., 1986).

Protein translocation can also happen independently of the SRP in a post-translational manner which is required for tail-anchored and small secretory proteins. The signal sequence is tail-anchored, it cannot be recognized co-translationally but only once the peptide has been fully synthesized and the C-terminus has fully emerged from the ribosomes. Posttranslational translocation is facilitated by chaperones targeted to the Sec61 complex (Ast et al., 2013) and requires two additional membrane proteins, Sec62 and Sec63 to function properly (Meyer et al., 2000, p. 62; Rothblatt et al., 1989).

ER membrane integration is only the first step in a protein's path to its target destination. Both INM and ONM proteins utilize unique pathways for correct transportation such as the well-established diffusion retention model as well as more active modes of transport and translocation (Ungricht et al., 2015). Theoretically, protein import can happen in an NPC-dependent or NPC-independent manner, however mechanistical details of these processes are still heavily discussed (Mudumbi et al., 2020).

1.3 Aim

The key aspect, discussed in this introduction, that lends this thesis its importance, is that throughout all structural and functional aspects of the nucleus, the nuclear envelope and the peripheral ER, a lot of high-resolution information and detailed mechanistical insight is still missing. The multitude of disease associated to mutations of nucleus or ER components further highlights how crucial correct maintenance of these complex systems really is. Specifically, around the regulation of the ONM protein composition huge knowledge gaps exist that might prove worthwhile investigating.

The focus of this thesis are the ER-NE connections and their possible regulation. We believe that gaining insight into structure, quantity and function of these connections would greatly improve our understanding of cellular function and could serve as the basis for many future studies. ER-NE junctions provide an important interface between these two functionally distinct sub compartments of the

endoplasmic reticulum. Supplying the nuclear envelope with phospholipids and NE-specific proteins, synthesized in the peripheral ER, relies on intraluminal transport through ER-NE junctions. Dysregulation of these connections might therefore lead to impaired maintenance of the INM, which is essential for nuclear organization and function or altered protein composition of the NE further restricting correct function. Additionally, the structure and quantity of ER-NE connections could affect nuclear shape, size, and positioning within the cell. Thereby influencing nuclear and genomic organization, gene expression patterns and therefore differentiation and development of the cell.

The approach that was chosen is based on work done by a previous student, Julia Scholz, and relies on live-cell imaging techniques to identify molecular players involved in regulation of the ER-NE connections. Since the list of potential candidates is extensive, a relatively high-throughput method is needed until prime candidates can be identified that justify high-effort experiments.

As mentioned above, this thesis is a continuation of previous work and aims at refining previously established methods to achieve highly reliable and reproducible results. In an iterative process, gene-knockdown through RNAi, transport measurements and quantification of imaging data were continually refined to find highly promising candidates.

1.4 Approach

As shown in Figure 3 the connections between the ER and the NE appear to be tightly regulated in terms of the luminal “channel size”. If regulatory mechanisms involved in maintaining these ER-NE connections can be altered in a way that changes the diameter of these channels, this should be measurable in a transport assay tracking diffusion rate between the peripheral ER lumen and the NE luminal space.

This approach involves knock-down of target genes followed by a reliable quantification of diffusion rates in living cells. This is achieved through gene knock-down by RNAi and a FRAP-based transport assay respectively.

1.4.1 FRAP – fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) is a widespread technique used for quantifying protein and lipid dynamics indirectly via coupled fluorophores. Fluorescently labelled reporters are precisely and selectively bleached by a high-powered excitation laser temporarily reducing signal intensities in the target area. If reporters are not stationary within the system, recovery of lost signal can be tracked as a function of time. These recovery kinetics are a strong indicator of the dynamic properties of the system.

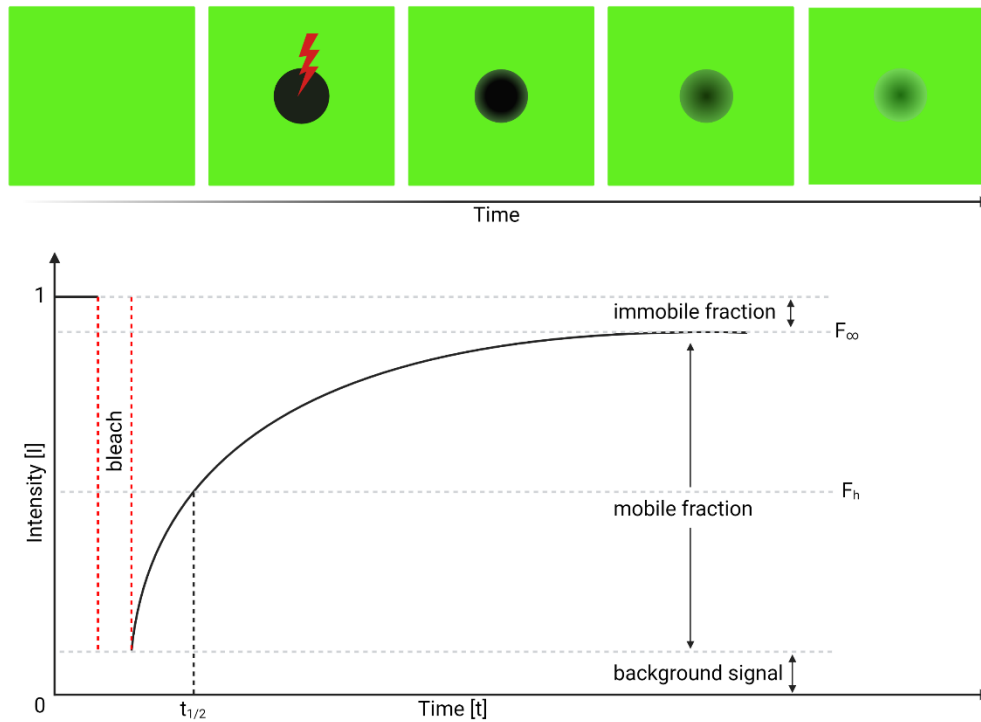


Figure 5. Fluorescence recovery after photobleaching (FRAP). This schematic explains the general working principle of FRAP and shows a typical signal intensity curve throughout the process. The upper graphic shows how the signal recovers within the bleached area across time and that this recovery might not necessarily be complete which is represented in the graph below by the immobile fraction. After bleaching, the signal intensity drops down all the way to the level of background signal and then recovers exponentially until a plateau (F_{∞}) is reached. $t_{1/2}$ is the timepoint at which the signal recovery has reached 50% relative to the plateau. Individual time points measured will be fitted with a one-phase association $f_{\infty}(t) = f_{\infty} \cdot (1 - e^{-(k \cdot t)})$. From this fitting, the k -value, describing the recovery kinetics, can be extracted. Created in BioRender.com.

When signal intensities are plotted as a function of time, a recovery curve can be generated. Individual time points can then be fitted with a one-phase association from which a singular value, the k -value, can be extracted which describes the recovery kinetics. When setting up a FRAP experiment, it is important to choose a suitable reporter that tracks dynamics in the desired region of the system. In our approach, ER luminal reporters were chosen, as they need to diffuse through the narrow ER-NE connections to travel between the peripheral ER and the NE. Further, luminal reporters can be adapted in size, to change the time needed to complete a full recovery. Smaller reporters display faster diffusion rates allowing for a faster and potentially higher-throughput assay needed for initial phases of identifying promising candidate genes. Larger reporters can then be used to increase the resolution of the transport assay as changes in the spatial regulation of ER-NE connections are exaggerated. The possible range of sizes suitable for reporters is dictated by the number of possible time points within a given time frame on the lower end and the ability to diffuse through the narrow ER-NE connection on the higher end of the reporter size spectrum. Additionally, to represent the recovery in a meaningful way, it is always necessary to reach the plateau phase of the recovery process within the possible time frame for transport measurements.

1.4.2 RNAi screen - targeted depletion of target proteins

To identify key players in the regulation of ER-NE connections we compare the transport kinetics within a cell, based on the transport assay mentioned above, of cells in a native, or wild type (WT) state and after depleting the cells of certain genes. The direct comparison of WT cells and cells with altered gene expression should then give insight into possible dysregulation happening in the absence of the depleted gene product.

While there are multiple approaches to achieving altered gene-expression in a targeted manner, our approach requires cells to be alive and relatively healthy. Preferably we want to measure the direct effect of the altered gene expression and minimize secondary affects this unavailability of certain proteins might induce. Since complete knock-outs fully deplete the cells of the desired gene, correct growth and function or even cell viability are not guaranteed, systems like Crispr-Cas9 are not suitable. In the example of a Crispr-Cas9 genomic deletion of target genes, effort and time requirements also restrict the throughput of our method which at this stage is a major factor.

To increase our experimental throughput, we therefore decided on an RNA interference (RNAi) based approach.

RNA mediated interference is a eukaryotic defense mechanism used to silence foreign double-stranded RNA molecules (Shrivastava & Srivastava, 2008). Silencing happens through degradation of RNA through ribonucleases (Agrawal et al., 2003). This conserved mechanism is already found in eukaryotes such as yeast but can be adapted for experimental use, in post-transcriptional manipulation of gene expression in mammals (Geley & Müller, 2004; Hannon, 2002).

When transfected into cells, these double-stranded “short interfering RNAs” (siRNAs) integrate into RNA-induced silencing complexes (RISCs), where one siRNA strand is cleaved. The now fully assembled siRNA-RISC complex can bind to a complementary mRNA sequence, leading to mRNA degradation (Leuschner et al., 2006; G. Tang, 2005). Absence of mRNA leads to inhibited translation and stops protein expression.

By changing the siRNA sequence, this method can be adapted rapidly to perform a broad RNAi screen for a variety of potential candidates which are assumed be play a potential role in regulation of ER-NE connections. Through literature research a list of potential candidates was established, and subsequently fitting siRNAs were used to individually knock-down candidate genes.

Table 1. Candidate genes

Candidate genes
ATL2 (Atlastin-2)
ATL3 (Atlastin-3)
SYNE1 (Nesprin-1)
SYNE3 (Nesprin-3)
SYNE4 (Nesprin-4)
REEP2 (Receptor expression-enhancing protein 2)
REEP4 (Receptor expression-enhancing protein 4)
ACTN (Alpha-actinin-1)
LMNA (Prelamin-A/C)
LNPK (Endoplasmic reticulum junction formation protein Lunapark)
CTDNEP (CTD nuclear envelope phosphatase 1)
SIGMAR1 (Sigma non-opioid intracellular receptor 1)
PCYT1A (Choline-phosphate cytidylyltransferase A)
SEC13 (Protein SEC13 homolog)
TMEM170 (Transmembrane protein 170A)

1.4.3 Candidate Genes

1.4.3.1 *Atlastins – ATL2 & ATL3*

The Atlastin (ATLs) family of proteins are a class of dynamin-like GTPases necessary for homotypic ER membrane fusion and ER tubular network biogenesis (Orso et al., 2009). Knock-out of ATLs results in elongated and unbranched, less mobile ER tubules (Hu et al., 2009). Atlastins are further involved in various ER-regulatory processes such as calcium level maintenance, lipid metabolism, membrane trafficking and microtubule dynamics (Lü et al., 2020; L. Niu et al., 2019; Park et al., 2010). Depletion of ATL also resulted in a reduced ER-export rate and negatively affected the correct exit of proteins into the secretory pathway (Behrendt et al., 2019; L. Niu et al., 2019). This ultimately also hinders the efficiency or proteins targeted to the INM reaching their destination (Pawar et al., 2017).

The large variety of functions performed by the Atlastin family of proteins still has not been investigated fully and additional functionality cannot be ruled out. ER-membrane fusion at the three-way junctions of the tubular ER-network could be regulated very similarly for ER-NE connections and Atlastins should therefore be considered a prime candidate for regulation of these connections.

1.4.3.2 *Nesprins - SYNE1, SYNE3 & SYNE4*

Nuclear envelope spectrin-repeat proteins (Nesprins), are protein family belonging to the spectrin-repeat super family and contain a highly conserved C-terminal Klarsicht ANC-1 Syne Homology (KASH-)

domain linking them to the nuclear envelope. Nesprins connect the NE to various cytoskeletal components like actin, intermediate filaments and microtubules (Roux et al., 2009; Wilhelmsen et al., 2005; Q. Zhang et al., 2002).

The proximal localization of Nesprins to ER-NE connections could mean that they are involved in regulatory processes. ER-NE connections could also be regulated through connections to the cytoskeleton, mediated by Nasrin proteins.

1.4.3.3 REEPs – REEP2 & REEP4

REEPs, short for receptor expression-enhancing proteins, were initially discovered for increasing expression of cell surface G-protein coupled receptor (GPCRs). REEPs are a protein sub-family of the Yip (YPT interacting proteins) superfamily with some members being associated to functions in ER-structure (Björk et al., 2013). A total of six REEP proteins have been identified and Yop1 was identified as a yeast homolog. Atlastin-1 GTPase interacts with Spastin and REEP1 forming protein complexes binding tubular ER. REEP proteins were shown to be required for ER network formation (Park et al., 2010). It has also been shown that yeast homologs are heavily involved in ER tubule formation (Shibata et al., 2008). ER-NE connections appear to exhibit a more tube-like morphology pointing towards regulation through mechanisms shared or similar to those found in ER-tubule formation. Dp1/Yop1p proteins would fit that description and therefore the mammalian counterpart being the REEP family are solid candidate genes to be evaluated.

1.4.3.4 ACTN

Alpha-actinin-1 is a member of the filamentous actin crosslinking superfamily and binds actinin through formation of anti-parallel dimers (Oikonomou et al., 2011; Sjöblom et al., 2008). Besides binding to actin filament, it plays a role in various regulatory and structural mechanisms involving the cytoskeleton. Alpha-actinin has been shown to bind other cytoskeletal structures and signaling molecules (Sjöblom et al., 2008). A 2004 review on alpha-actinin by Carol Otey collected a vast number of different binding partners of actinin and summarized them in an easy-to-read table. This table quickly gives insight into the huge variety of function that alpha-actinin is potentially involved in and strongly suggests that it is not simply an Actin-binding protein but a potentially much more important component of overall cell health (Otey et al., 1993; Otey & Carpen, 2004). ACTN deficient cells have been shown to lose anterior-posterior polarity as a consequence of altered actin organization. Further, loss of actinin function greatly reduced motility of cells (Hamill et al., 2015).

1.4.3.5 LMNA

Through alternative splicing the LMNA gene encodes Lamin A and Lamin C isoforms of the Lamin protein family (F. Lin & Worman, 1993). Lamins are the main components of the nuclear lamina and play a vital role in the structural maintenance of nuclear membrane integrity. The nuclear lamina provides an anchor point for chromatin and, among many others, the inner-nuclear components of the

LINC complex, Nesprins (Méjat & Misteli, 2010; Schirmer & Foisner, 2007; Wilson & Foisner, 2010). Mutations in LMNA are one of the primary causes for Dilated Cardiomyopathy one of many laminopathies caused by aberrations in LMNA function. Depletion of functional LMNA is assumed to cause instability of the nuclear lamina and all related functions to this critical component of the nuclear (Brayson & Shanahan, 2017). However, the exact mechanisms at play are yet to be discovered.

Lamins being so closely located to the ER-NE connections of interest to this thesis, as well as their central role in the cellular context make it an important candidate for further testing.

1.4.3.6 LNPK

As the full name of this protein, endoplasmic reticulum junction formation protein Lunapark suggests, the main function associated with Lunapark is the stabilization of nascent three way junctions (S. Chen et al., 2015). It is part of a process assigned to three protein families cooperating in the formation and stabilization of an interconnected tubular membrane network. Due to the close interplay of these three protein families, Lunapark is often mentioned alongside Atlantins and the Reticulon protein families (S. Wang et al., 2016). Lnp consists of two transmembrane Zinkfinger domains and is assumed to stabilize ER three-way junctions as its depletion leads to fewer junctions and a more densely reticulated ER network (S. Chen et al., 2012, 2015). The role of Lunapark is not fully agreed upon yet as there have been conflicting results when observed in mammalian or yeast cells. While it has been proposed that Lnp stabilizes three-way junction, as observed in mammalian cells, results based on yeast gathered by Shemesh et al., suggested a function in the removal of junctions (ring closure) (S. Chen et al., 2015; Shemesh et al., 2014).

If Lunapark is indeed involved in the stabilization of ER-junctions it might very well also play a key-role in the stabilization of ER-NE junction and is therefore one of the primary candidate proteins.

1.4.3.7 CTDNEP

The C-terminal domain nuclear envelope phosphatase promotes synthesis of membrane phospholipids through phosphatidic acid phosphatase lipin. CTDNEP regulates phospholipid flux and thereby prevents the accumulation of excess ER-sheets around the nucleus which would entail problems in nuclear envelope breakdown (Bahmanyar et al., 2014). CTDNEP is assumed to modulate the lipid composition of the NE and peripheral ER which could lead to altered membrane dynamics if not maintained correctly (Kim et al., 2007).

1.4.3.8 SIGMAR1

Sigma non-opioid intracellular receptor one, often also abbreviated as SGMR1 is a ubiquitously expressed signaling chaperone performing a plethora of different roles. It has been shown to locate to various membranes, like the plasma-membrane where it is involved in potassium ion channel inhibition (Aydar et al., 2002, 2004; T.-P. Su et al., 2009) and cholesterol rich parts of the ER membranes (ER lipid

droplets) and the NE (Dussossoy et al., 1999; Hayashi & Su, 2003). Sigmar is originally known for the broad spectrum of drugs it can bind (Martin et al., 1976) but subsequently was shown to play a role in cancer biology (Crawford & Bowen, 2002), regulation and modulation of neurotransmitter release and function involving memory processes and mood alterations (T. P. Su, 1993) as well as other types of disease like schizophrenia (Ishiguro et al., 1998). Silencing of the sigma-1 receptor induces cell death leading to an even broader spectrum of processes involving this non-opioid receptor (L. Wang & Duncan, 2006).

The multi-compatible nature of SIGMAR1 serves as the baseline for justification for further research for a potential involvement in the regulation of ER-NE connections.

1.4.3.9 PCYT1A

The PCYT1A gene encodes for choline-phosphate cytidylyltransferase A (or phosphocholine cytidylyltransferase) which regulates phosphatidylcholine biosynthesis at the inner nuclear membrane (Lykidis et al., 1999). Phosphatidylcholine (PC) is the most abundant phospholipid (PL) found in eukaryotic membranes. PCYT1A was shown to sense PL composition of the inner nuclear membrane and locates to it as a response to required de-novo synthesis of PLs in yeast and mammalian cells (Haider et al., 2018). In cells where extensive ER remodeling processes are required, such as differentiating cells, increased PCYT1A activity was measured (Fagone et al., 2007). In the case of B-lymphocytes differentiating into plasma cells, an increase in rough-ER is needed to facilitate the higher protein synthesis rates needed for immunoglobulin production. Similarly, increased phosphocholine cytidylyltransferase (CTP) can also be an indicator of a progressing cell cycle, especially during S-phase in preparation for mitosis (Banchio et al., 2003).

As discussed in greater detail, by Bahmanyar and Schlieker, the lipid compositions between the peripheral ER and the NE are asymmetrically regulated by a variety of proposed mechanisms and for different suggested reasons (Bahmanyar & Schlieker, 2020). However, this asymmetry might very well be important for the regulation of the ER-NE connections placed right at the interface of those two membrane systems and could involve the presence of phosphocholine cytidylyltransferase. We therefore consider PCYT1A as a candidate gene for our assay.

1.4.3.10 SEC13

Mammalian Sec13, also called protein Sec13 homolog, is the mammalian homolog to Sec13p in yeast. It functions as a component of the nuclear pore complex (NPC) through interaction with Nup96 and the COPII coat which mediates protein traffic from ER to Golgi. It is also directly involved in the biogenesis of the COPII complex (Barlowe et al., 1994; Enninga et al., 2003, p. 13; B. L. Tang et al., 1997). Sec13 loss of function was shown to cause ER-structure disintegration and subsequently lead to cell-cycle arrest and apoptosis of affected cells (X. Niu et al., 2012). The exact mechanisms that involve Sec13

have not been discovered yet, however the shuttling of Sec13 between the nucleus and the cytoplasm could indicate a regulative role between these compartments.

1.4.3.11 TMEM170

The last candidate gene on our list was only functionally investigated recently but is very promising as it is involved in various ER and NE regulatory processes. The transmembrane protein 170, encoded by TMEM170 is a regulator of ER and NE morphogenesis and pushes the ER network towards a more sheet-like morphology. TMEM170A localizes to ER and NE and specifically co-localizes with ER proteins calnexin and RTN4. It has a transmembrane domain anchoring it in the ER and NE membranes, with a lumenally oriented N-terminus and a cytoplasmic C-terminus. TMEM170 acts ER-sheet promoting and therefore as an antagonist to RTN4. Further, it promotes nuclear envelope formation and assists NPC assembly (Christodoulou et al., 2016, p. 17).

Due to its very recent functional characterization, TMEM170 has not been at the forefront of ER science and could be considered a “sleeper” candidate.

2. Material and Methods

2.1 Cell culture

2.1.1 Cell lines and culture

Human cervical adenocarcinoma cell line HeLa wild type. HeLa moxGFP-KDEL small ER-luminal GFP reporter (stable transfection, created by Tamara Völkerer from HeLa wild type). Human retinal epithelial-1 cells RPE-1 (courtesy of Gerlich Lab). RPE-1 moxGFP-KDEL small ER-luminal GFP reporter (stable transfection). RPE-1 NusA_moxGFP2-KDEL large ER-luminal GFP reporter.

All cell lines were kept under equal growth conditions at 37 °C + 5 % CO₂ and held in culture for no more than 15 passages. Growth medium consisted of DMEM (Sigma-Aldrich, D6046-500ML) with 10 % fetal calf serum (FCS)(Sigma-Aldrich, F7524-500ML) and 1 % penicillin/streptomycin (Pen/Strep)(Sigma-Aldrich, P4333-100ML).

2.1.2 Splitting cells

Cells were routinely split after two or three days or when they reached a confluency of 90 %. Fresh growth medium and Trypsin-EDTA (0.25 %, Sigma T4049-100ML) were preheated beforehand. The old growth medium was aspirated, the cells were washed with 1x PBS (Sigma-Aldrich, D8537-500ML) once and 2 mL of Trypsin-EDTA were added and spread across the dish evenly before aspirating most of the Trypsin again. The cells were then incubated for roughly 3 minutes. 9.5 mL of fresh medium were added and resuspended thoroughly to singularize cells and break up cell clusters. When splitting the cells at 20 % (2 mL cell suspension + 8 mL DMEM) the cells reach confluency after 2 days. For 3 days, the cells were split at 10-15 %.

2.1.3 Live cell imaging medium

To improve image quality and provide ideal detection of fluorophores in live cell imaging a clear imaging medium was used and applied to the cells one hour prior to imaging. For this, clear DMEM without Riboflavin and Phenol Red (Thermo Fisher Scientific, Gibco 041-96205M) containing 10 % FBS and 1 % Pen/Strep was used. Importantly, a wash step was performed after aspirating the standard growth medium. This was done with imaging medium and not with PBS because the latter caused a noticeable change in cell morphology that was apparent for at least two hours. SiR-Hoechst (tebu-bio, SC007; final concentration 50 nM, stock 100 µM in DMSO) was added to the imaging medium to visualize cell nuclei.

2.1.4 Freezing cells

Cells were trypsinized and resuspended in 10ml medium and then centrifuged for 3 minutes at 1000 rcf. The supernatant was aspirated, and the remaining cell-pellet was suspended in pre-chilled (4 °C) freezing medium (90 % FBS + 10 % DMSO) (Sigma, C6164-50ML). The suspension was transferred to cryovials, initially stored at -80 °C for one week and then at -150 °C for long term storage.

2.1.5 Thawing cells

To minimize the stress, cells undergo while thawing, a 10 cm culture dish containing 10ml of medium is pre-warmed and saturated with CO₂ for 30 minutes in the incubator. The cells in the cryovials are then rapidly thawed and transferred onto the prepared culture dish to avoid long exposure of the cells to high concentrations of DMSO at 37 °C. Even distribution of the cells throughout the culture dish was ensured before incubating at 37 °C/ 5 % CO₂ over-night. On the following day, medium was exchanged. From now on normal cell-maintenance protocols apply. To ensure constant conditions throughout all experiments performed, cells were passaged at least 4 times before being used.

2.2 Transfection and RNAi

2.2.1 RNAi

Creating a transport-assay based loss of gene function analysis, required a highly efficient and specific but minimally invasive method of silencing the expression of target genes.

To achieve this, RNA-Interference (RNAi) was used to inhibit the translation of target mRNAs through initiating their depletion. This function is performed by an RNA-induced silencing complex (RISC), which identifies the target mRNA via the antisense strand of the siRNA which it has incorporated. The target mRNA is cleaved at a specific site and targeted for degradation.

All siRNAs used were acquired from Thermo Fisher Scientific. Their *Silencer Select* series of siRNAs was chosen for their high potency and efficiency even at low concentrations (intended concentration of 10 nM) and low off-target interaction.

2.2.2 Cy3-labelling of siRNAs

For conjugating cy3 dyes to the target siRNAs used in following experiments, an siRNA labelling kit was used (Thermo Fisher Scientific, AM1632). The provided protocol was followed except the washing step which was left out to increase signal strength of the cy3 conjugate.

Labelled siRNAs were stored at -80 °C instead of -20 °C due to a potentially quicker degradation at higher temperatures. Additionally, labelled siRNA stocks were not used beyond ten freeze/thaw cycles to ensure proper function.

2.2.3 Co-transfection of plasmid DNA and target siRNA

The nature of the transport assay setup we used, required both siRNA gene knock-down and transient transfection of a reporter gene. Therefore, our initial approach consisted of a simultaneous co-transfection of both siRNA and plasmid DNA. The general experimental setup is visualized in a simplified manner in Figure 6.

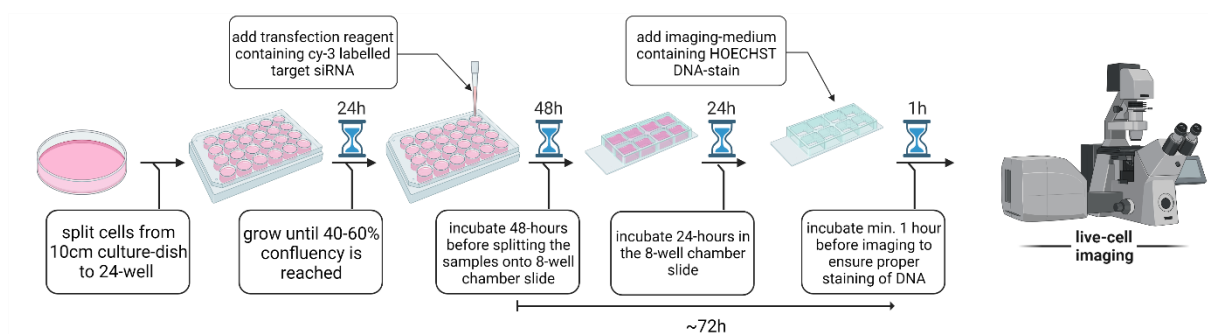


Figure 6. Flowchart describing the experimental setup for the FRAP transport assay coupled with siRNA knock-down of target genes.

Total incubation time before imaging, after siRNA knock-down has been induced, is 72 hours.

Cells were seeded onto 24-well culture dishes (Corning Costar, 3523), pre-filled with 550 μ L culture media such that after 24 hours of incubation, roughly 40-60 % confluency was reached. After one day, culture medium was replaced with Pen/Strep-free medium and incubated for 1h at 37 $^{\circ}$ C/ 5 % CO_2 before commencing the co-transfection.

Transfection was performed using 0.75 μ L of LipofectamineTM 3000 Transfection Reagent (Thermo Fisher Scientific, L3000001) plus 480 ng of plasmid DNA mixed with 25 μ L Opti-MEMTM I Reduced Serum Medium (Thermo Fisher Scientific, Gibco 31985-062). In a separate Eppendorf tube, target siRNA was added to 25 μ L of 25 μ L Opti-MEMTM I Reduced Serum Medium such that a final concentration of 10nM in 600 μ L was achieved. The contents of both tubes were vortexed and centrifuged separately, combined and then again vortexed briefly. After combining the reagent was incubated at RT for 10 minutes and then added to the desired well on the 24-well culture dish.

The cells were then incubated at 37 $^{\circ}$ C/ 5 % CO_2 for 72 h.

2.2.4 Separate RNAi and transfection of plasmid DNA

Slight adaptation of the protocol used in 2.2.3 Co-transfection of plasmid DNA and target siRNA. In an effort to maximize the signal quality of the moxGFP reporters that were used in the transport assay, the processes of RNAi (RNA-Interference) and plasmid transfection were split and performed on two days.

While the basic experimental layout remained the same, a few key adaptations were made. Primarily, instead of LipofectamineTM 3000 Transfection Reagent, LipofectamineTM RNAiMAX Transfection Reagent (Thermo Fisher Scientific, 13778030) was used.

0.5 μ L RNAiMAX were added to 25 μ L OptiMEM and combined with 25 μ L of OptiMem + siRNA (10 nM in final volume). The two reagent cocktails were vortexed and centrifuged before and after combining. After 10 minutes at RT, this reagent mix was added to the 24-well dish and incubated for 24h at 37 $^{\circ}$ C/ 5% CO_2 . The following day, the medium was replaced with fresh Pen/Strep-free medium to prepare for plasmid DNA transfection. This was performed as described in chapter 2.2.3 Co-transfection of plasmid DNA and target siRNA.

Then cells were incubated for another 48 h resulting in a total of 72 h of RNAi and 48 h after transfection of plasmid DNA before imaging.

2.3 Molecular cloning

While the initial transport assays were performed using transiently transfected cells that expressed our desired reporter gene, later methodical refinements included establishing stable cell lines expressing reporter genes.

We used standard molecular cloning techniques to stably integrate a reporter gene DNA into RPE-1 cells, starting from previously established plasmid DNAs and utilizing a lentiviral backbone construct.

2.3.1 Designing a cloning strategy

The moxGFP and NusA-moxGFP2 reporter genes were already established by previous members of our lab and tested thoroughly. Therefore, creating a stable cell line was a matter of transferring the gene of interest into a suitable vector for integrating it into our target cell-line. A lentiviral vector was chosen.

Suitable restriction enzyme sites were chosen with help of Benchling (Cloud-Based Informatics Platform for Life Sciences R&D). A 20-nucleotide long sequence containing the restriction enzyme sites and used as a baseline for our PCR primers. Forward primers were complementary to the anti-sense DNA strand. Reverse primers were to be complementary to the sense strand.

The general layout of the cloning process was as follows:

Initially, the genes of interest plus the restriction enzyme sites (RES) were amplified via PCR and the lentiviral backbone was digested at the corresponding RES. After purification the PCR amplified insert and the opened backbone were ligated and transformed into competent *E. coli*. These transformed *E. coli* were then plated on selective agar containing the appropriate antibiotics to select for colonies containing the resistance gene cassette. After incubation overnight at 37 °C, multiple resistant colonies were picked and cultured in suspension overnight. Most but not all the bacteria (a small amount was kept for future amplification) were then centrifuged to remove excess LB-medium was discarded. Plasmid DNA was extracted via mini-prep and the correct insertion was verified via restriction digest followed by sequencing. Once a clone displaying correct insertion was identified, the corresponding colony was cultured in 100 mL LB-Medium overnight to express large quantities of plasmid DNA. The plasmid DNA was then purified via Midi-Prep. Before continuing with the lentiviral transduction, the correct function of the created plasmids was verified by transiently transfecting the reporter genes into the final target cell line.

2.3.2 PCR amplification of insert

From the plasmids containing our desired reporter genes which were used for transient transfection in initial experiments, the inserts were amplified via PCR.

Forward and reverse primers were created using Benchling and consisted of a 20 nucleotide long complementary sequence, the sequence corresponding to the restriction enzyme sites used to cut open the backbone and three additional randomly chosen nucleotides. These additional nucleotides ensure correct cleavage by offering additional space for the restriction enzymes to align properly to the RES.

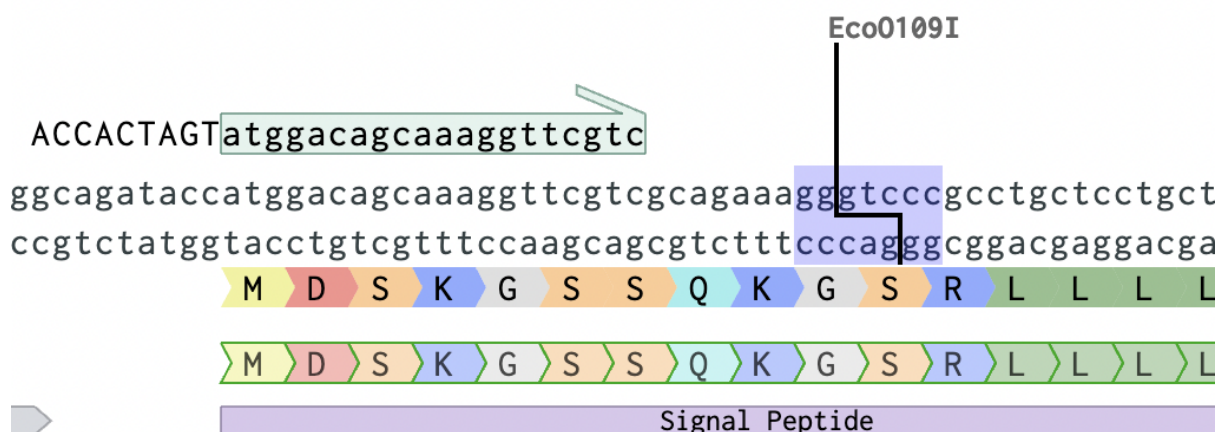


Figure 7. Screen capture of Benchling showing the forward primer:

Green arrow, 20 nucleotide long complementary sequence, 5' upstream of which the sequence of the SpeI restriction enzyme (5'...ACTAGT...3') as well as three random nucleotides (5'...ACC...3').

To ensure correct function and high specificity of the primers, potential off-target interactions were ruled out by seeing whether the last seven nucleotides of our primers would bind somewhere else within the plasmid. Additionally, the melting temperature was checked to be within a reasonable range to avoid secondary annealing. This was done with help of the NEB-Tm calculator for high fidelity Phusion Polymerases. Finally, self-dimerization/-annealing as well as primer pair dimerization tendencies were checked using the *Multiple Primer Analyzer* by *ThermoFischer Scientific*. These custom primers were then ordered from *Microsynth AG*.

Table 2. PCR amplification mix

Reagent	Amount
MiliQ dH ₂ O	fill to V _{tot} of 50 µL
5x Phusion HF Buffer (NEB, B0518S)	10 µL
MgCl ₂ (25mM)	1 µL
dNTPs (10mM)	1 µL
Forward Primer (10µM)	2.5 µL
rRverse Primer (10µM)	2.5 µL
Template DNA	5 ng
DMSO	1.5 µL
Phusion HF Polymerase (NEB, M0530S)	2 µL

All components were pre-chilled and kept on ice until PCR amplification. The high-fidelity Phusion Polymerase was added right before transferring the PCR tubes into the Eppendorf Mastercycler Nexus GX2 which was used. The PCR program can be seen in the table below. The PCR cyclers allow applying a temperature gradient to the heater block and to test and find the ideal conditions for annealing, a temperature gradient between 65 °C and 71 °C was set.

Table 3. PCR protocol

Step	Temperature	Duration
Initial denaturation	98 °C	30 s
Amplification (35 cycles)	98 °C	8 s
	65-71 °C	20 s
	72 °C	15 s/kb
Final extension	72 °C	8 min
Hold	4 °C	∞

The amplified DNA was then purified through an agarose gel. The agarose content was adapted to fit the expected size of the insert DNA. To 50 µL of sample, 10 µL 6x loading dye (NEB #B7025) were added prior to loading. To visualize DNA, the agarose gel contained SYBR Safe DNA Gel Stain (Thermo Fischer Scientific, Invitrogen, S33102).

Initially, the gel was run at 80 V until the sample transitioned into the gel fully before increasing the voltage to 120 V. Once the two fractions were nicely separated the process was stopped. The desired fraction was then cut from the surrounding gel and purified. Purification was performed according to manufacturer protocol using the innuPREP DOUBLEpure Kit (Analytik Jena, 845-KS-5050050). The purified DNA was eluted in 10 µL total volume.

2.3.3 Restriction digest of insert

After PCR, the blunt ends of the amplified sequences needed to be cut to enable proper ligation in later steps. This was achieved via restriction digest using the previously chosen enzymes. The reaction was set up as follows:

Table 4. Insert restriction digest mix

Reagent	Amount
MiliQ dH ₂ O	fill to V _{tot} of 25 µL
10x CutSmart Buffer (New England Biolabs, B7204S)	2.5 µL
DNA eluate	10 µL (300-500 ng)
Restriction enzymes	0.5 µL (each)

After combining, the reaction mix was incubated for 30 minutes at 37 °C.

The restriction digest was then purified via a quick column purification according to the protocol supplied with the QIAquick PCR Purification Kit (250x, QIAGEN, 28106). The purified DNA was finally eluted in 10 µL MiliQ dH₂O. DNA concentration was then determined using a DeNovix DS-11+ spectrophotometer/fluorometer.

2.3.4 Restriction digest of backbone

To prepare the vector plasmid for ligation with the insert DNA, the backbone must be cut open via the restriction enzyme sites include in the previously used PCR primers. The reaction mix for the restriction digest of the backbone was prepared as follows:

Table 5. Backbone restriction digest mix

Reagent	Amount
MiliQ dH ₂ O	fill to V _{tot} of 50 µL
10x CutSmart Buffer (New England Biolabs, B7204S)	5 µL
Vector DNA	1 µg
Restriction enzymes	1 µL (each)

After combining, the reaction mix was incubated for 30 minutes at 37 °C.

After the restriction digest, the two components, backbone and cut fragment, were separated on an appropriate agarose gel.

The agarose content was adapted to fit the expected size of the insert DNA. To 50 µL of sample, 10 µL 6x loading dye (NEB #B7025) were added prior to loading. To visualize DNA, the agarose gel contained SYBR Safe DNA Gel Stain (Thermo Fischer Scientific, Invitrogen, S33102).

Initially, the gel was run at 80 V until the sample transitioned into the gel fully before increasing the voltage to 120 V. Once the two fractions were nicely separated the process was stopped. The desired fraction was then cut from the surrounding gel and purified. Purification was performed according to

manufacturer protocol using the innuPREP DOUBLEpure Kit (Analytik Jena, 845-KS-5050050). The purified DNA was eluted in 10 μ L total volume. DNA concentration was then determined using a DeNovix DS-11+ spectrophotometer/fluorometer.

2.3.5 Ligation

The complementarity of the opened backbone and the insert DNA allows for efficient ligation of both sequences. The ratio of backbone to insert was calculated using a tool provided by New England Biolabs (*NEBioCalculator*). The ligation was catalyzed by a T4 DNA ligase in a reaction that was set up as follows:

Table 6. Ligation reaction mix

Reagent	Amount
MiliQ dH ₂ O	fill to V _{tot} of 10 μ L
Vector/Backbone DNA	25 ng
Insert DNA	ratio of x*:1 to vector (*depending on insert and vector length)
10x Ligation Buffer (New England Biolabs, B0202S)	1 μ L
T4 DNA ligase ((New England Biolabs, M0202S)	0.5 μ L

After combining, the reaction mix was incubated for 30 minutes at room temperature.

2.3.6 Transformation

The following steps were performed in a sterile environment maintained by a Bunsen burner. Chemically competent *E.coli* were thawed gently and kept on ice. To 100 μ L of thawed competent cells, 10 μ L of pre-chilled assembled product were added and mixed by flicking the Eppendorf tube. This mixture was kept on ice for 30 minutes before heat-shocking at 42 °C for 30 seconds. The tubes were then put back on ice for 2 minutes after which, 250 μ L of room-temperature LB were added. The tubes were then vigorously shaken for 45 minutes at 37 °C and 300 rpm. After recovery was finished, the cells were added to pre-warmed selective culture dishes containing the appropriate antibiotics. This simplifies and increases efficiency of selection for correctly transformed colonies. After 20 minutes incubation at room temperature the plates were then incubated at 37 °C overnight.

2.3.7 Verifying correct transformation

After overnight incubation, only clones that had the correct resistance due to successful transformation should have grown on the selective agar plates. Several colonies were picked and inoculated separately in 3.5 mL selective LB (+ antibiotic) and again grown at 37 °C and 180 rpm overnight.

The following day, a mini-prep was performed for further verification of correct ligation.

2.5 mL of overnight culture were centrifuged at 4 °C at 6700RCF for 3 minutes. The supernatant was discarded; the pellet was resuspended thoroughly in 150 µL cold Buffer P1 and transferred to a 1.5 mL Eppendorf tube. Then 150 µL of room temperature lysis Buffer P2 were added and mixed gently by inverting the tube multiple times. To stop the lysis and to neutralize the mix, 150 µL of cold Buffer P3 were added and again mixed by inverting the tube.

This mixture was then centrifuged at 4 °C, maximum speed in a tabletop centrifuge (Eppendorf Centrifuge 5427R). The supernatant was transferred to a fresh tube and the DNA precipitated with 240 µL of Isopropanol. The samples were mixed via vortexing and left at RT for 5 minutes before centrifuging at maximum speed in a 4 °C tabletop centrifuge. The supernatant was aspirated and the remaining pellet containing the precipitated DNA was washed with 250 µL room temperature Ethanol (96 %). This wash step was once again followed by centrifuging the sample for 5 minutes at RT in the tabletop centrifuge (maximum speed). The supernatant Ethanol was aspirated, and the remaining pellet left to air dry at RT for 15 minutes. Once dry, the precipitated DNA was dissolved in 100 µL MiliQ dH₂O. DNA concentration was then determined using a DeNovix DS-11+ spectrophotometer/fluorometer.

Table 7. Mini-prep buffers

Mini-prep buffers	Components
Buffer P1	50mM Tris-HCl, pH 8.0 10mM EDTA 100µg/ml RNase A
Buffer P2	200mM NaOH 1% SDS
Buffer P3	3M potassium acetate, pH 5.5

With the purified DNA a restriction digest was performed to check the correct ligation and whether the fragment sizes are roughly correct when separating them on an agarose gel.

The restriction digest was set up as follows:

Table 8. Verification of correct ligation restriction digest mix

Reagent	Amount
MiliQ dH ₂ O	Fill to V _{tot} of 20 µL
10x CutSmart Buffer (New England Biolabs, B7204S)	2 µL
DNA	500-1000 ng
Restriction enzymes	0.5 µL (each)

After combining, the reaction mix was incubated for 15 minutes at 37 °C before loading the samples on an appropriate agarose gel.

Once a correct ligation can be presumed from the resulting bands on the agarose gels, the respective clones were sent to Microsynth for a final verification via Sanger sequencing.

2.3.8 Midiprep

To get large quantities of our desired DNA, a 100 mL overnight culture of the correct clone (verified through Sanger sequencing) was created and purified via Midiprep. The Midiprep was performed close to manufacturer specifications supplied with the QIAfilter Plasmid Midi Kit (25) (Qiagen, 12243).

First, the overnight culture was harvested by separating it into two 50ml falcon tubes and centrifuging them at 6000 x g for 15 minutes at 4 °C (Eppendorf, Centrifuge 5810R). Then, the supernatant was discarded from both tubes and 2ml of cold Buffer P1 were used to resuspend the remaining pellets before combining the two resulting pellets into one 50 mL falcon tube. 4 mL of Buffer P2 were added to the resuspension and mix through inverting 4-6 times. This was left at room temperature at 5 minutes before adding 4ml cold Buffer P3 to the lysate. Once again mixed by inverting and left on ice for 15 minutes. After this incubation period, the lysate was centrifuged at 4 °C for .60 minutes at 3220 x g. Meanwhile, 4 mL of Buffer QBT were used to equilibrate a QIAGEN-tip. The supernatant was transferred onto the equilibrated QIAGEN-tip and left to enter the tip via gravity flow. This was followed by two washing cycles using 10 mL of Buffer QC each. The DNA was then eluted with 5 mL of warm (50 °C) Buffer QF into a fresh tube. To precipitate the DNA, 3.5 mL of room-temperature isopropanol (absolute) were added and mixed before centrifuging at 6000 x g for 30 minutes at 4 °C. The supernatant was decanted carefully as to not lose the pelleted DNA. Then 2 mL of 70 % ethanol were added to wash the DNA before centrifuging again at 6000 x g for 10 minutes at 4 °C. The supernatant was decanted carefully, and the remaining pellet left to air dry for 15 minutes at RT. The DNA pellet was dissolved in 100 µL MiliQ dH₂O and the DNA concentration determined using a DeNovix DS-11+ spectrophotometer/fluorometer.

Before continuing with the viral transduction, the created plasmids were tested for functionality by transiently transfecting the target cells via lipofection.

Cells were seeded onto 24-well culture dishes (Corning Costar, 3523), pre-filled with 550 µL culture media such that after 24 hours of incubation, roughly 40-60 % confluency was reached. After one day, culture medium was replaced with Pen/Strep-free medium and incubated for 1h at 37 °C/ 5 % CO₂ before commencing the co-transfection.

Transfection was performed using 0.75 µL of Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, L3000001) plus 480ng of plasmid DNA mixed with 25 µL Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific, Gibco 31985-062). Reagent was incubated at RT for 10 minutes and then added to the desired well on the 24-well culture dish.

The cells were then incubated at 37 °C/ 5 % CO₂ for 48 h before inspection at the live cell spinning-disc confocal microscope.

2.3.9 Viral transduction

After the correct function was confirmed, the next goal was to stably integrate the established DNA plasmids into the target cell line to improve our imaging workflow regarding its reproducibility.

Viral transduction was selected as the means to achieve this. In a series of steps, across five days, the target plasmids were transfected into lenti-virus producing HEK293T cells (through co-transfection of viral plasmids). After 48 h of incubation the lenti-virus containing supernatant culture medium was filtered and transferred onto RPE-1 cells where stable integration of plasmid DNA into the host genome is facilitated. All steps where lenti-viruses are involved are strictly performed using special lab equipment reserved for this purpose. This includes laminar-flow hoods, incubators and all tools required for lenti-viral cell culture.

On day one, HEK293T cells are seeded onto a 10cm culture dish, aiming for 50 % confluency on the following day (1:6 split).

On the second day, the HEK293T cells are transfected using poly-ethyleneimine (PEI). To a 50 % confluent 10 cm dish, 10 µg of plasmid DNA are added alongside the vector of interest, a viral packing plasmid psPAX and an envelope expressing plasmid pMD2.g in a 1/3 : 1/2 : 1/6 ratio (vector : psPAX : pMD2.g).

To 300 µL of OptiMem, 30 µL of PEI are added and gently mixed through resuspension. In a separate 1.5 mL tube, 10 µg of plasmid DNA are added too 300 µL of OptiMem. Both tubes are then combined, and the contents dispersed through resuspension. The combined tube is left to incubate at RT for 15 minutes. Culture medium is changed to antibiotic free medium before the previously prepared transfection reagent is added dropwise to the 10 cm culture dish. The cells are then left to incubate for 48 h at 37 °C/ 5 % CO₂ in an incubator reserved for lenti-viral use.

On day three, RPE-1 target cells are seeded onto 6-well plates with a target confluency of 50 % on the following day.

On day four of the transduction process, the viral transduction of the target is performed. To 0.5 mL of fresh growth medium, 5 µL of polybrene are added. The supernatant medium of the virus producing HEK293T cells is collected and transferred to a sterile syringe, combined with the polybrene-medium mix, and filtered through a 0.45 µm filter into a fresh 50ml falcon tube.

The sterile filtered supernatant is then transferred onto the RPE-1 target cells and incubated at 37 °C/ 5 % CO₂ for 24 h in an incubator reserved for lenti-viral use.

On day five, the supernatant medium is discarded, and the cells are thoroughly washed with PBS before splitting and seeding them in selective antibiotic medium. The transduction process is now complete

and only cells that stably integrated target plasmid can grow under antibiotic selective growth conditions. The stable cell line can now be considered free of lenti-viruses and used as such. The correct function of the integrated plasmid was finally observed in the spinning-disc live-cell microscope before the cells were expanded and aliquots frozen and stored as described in paragraph 2.1.4 Freezing cells.

2.4 Live spinning Disc

After inducing RNAi and transfecting cells, time lapse imaging of live cells was done to measure transport dynamics and kinetics of reporter protein throughout the ER. The imaging was performed on a live-cell spinning-disc confocal microscope. The preparation of sample cells is described in paragraph 2. Transfection and RNAi.

Technical specifications as kindly supplied by the Max Perutz Laboratories BioOptics Facility.

Visitron Live Spinning Disk/Nanodissection Unit

Stand: Zeiss Axio Observer Z1, inverse, fully motorized, with hardware autofocus (Zeiss Definite Focus 2).

Objectives:

- A-Plan 5x/0.12 (working distance 10.1 mm, for overview images)
- EC Plan-Neofluar 20x/0.50 (working distance 2.0 mm, for sample viewing only)
- Plan-Apochromat 63x/1.4 Oil DIC III
- EC Plan-Neofluar 63x/1.25 Oil (preferential use for 355 nm laser applications)
- EC Plan-Neofluar 100x/1.30 Oil Iris (preferential use for 355 nm laser applications)

Lasers:

- 405 nm laser diode (120 mW)
- 488 nm laser diode (100 mW)
- 561 nm DPSS laser (200 mW, AOTF-controlled)
- 640 nm laser diode (150 mW)
- 355 nm passively Q-switched pulsed laser (16 mW average power, 0.8 J/pulse, repetition rate 21 kHz, pulse width 400 ps)

Spinning Disk Unit: Yokogawa CSU-X1-A1 Nipkow spinning disc unit (pinhole diameter 50 μm , spacing 253 μm)

Settings:

Pixel size: 211.6 nm

Image size (total resolution): 512 x 512 px (108.36 nm x 108.36 nm)

Binning: 1

Transmission: 100 %

Channels:

- sdc-GFP+FRAP (488 nm): laser intensity 3 %, exposure 75, gain 250
- sdc-Cy3 (561 nm): laser intensity 10 %, exposure 150, gain 720
- sdc-Cy5+FRAP (640 nm): laser intensity 3 %, exposure 150, gain 500

FRAP488 laser for photobleaching: laser intensity 95 %

FRAP settings:

PreCount: 1

Timepoints: 61

Interval: 0.5 s or 1 s, sample dependent – specified in results description

Bleaching time: 10 ms/px

Bleaching cycles: 1

Image acquisition: sdc-GFP+FRAP and sdc-Cy5+FRAP channels were alternately imaged between after bleaching for the specified period. For ER-ER transport measurements, only sdc-GFP+FRAP channel was imaged.

2.4.1 FRAP (fluorescence recovery after photobleaching)

Depending on which area of a cell was bleached, the bleached area as well as the area within which fluorescence recovery was measured were set up differently and refined to yield higher quality and more comparable results. The basic setup involved a polygonal selection of an area that was to be bleached at the live cell spinning disc followed by the acquisition timelapse capturing the fluorescence recovery process. In post processing of the timelapse image file, a small subregion of the bleached area was defined of which the mean signal intensities were measured for each frame and tracked numerically for further evaluation.

For ER-ER measurements the initial FRAP setup had a large portion of peripheral ER bleached (Figure 8, indicated by the dotted red line) before measuring the fluorescence recovery within the yellow circle (10 px diameter). The position of the red polygon was chosen such that the nuclear envelope was not bleached.

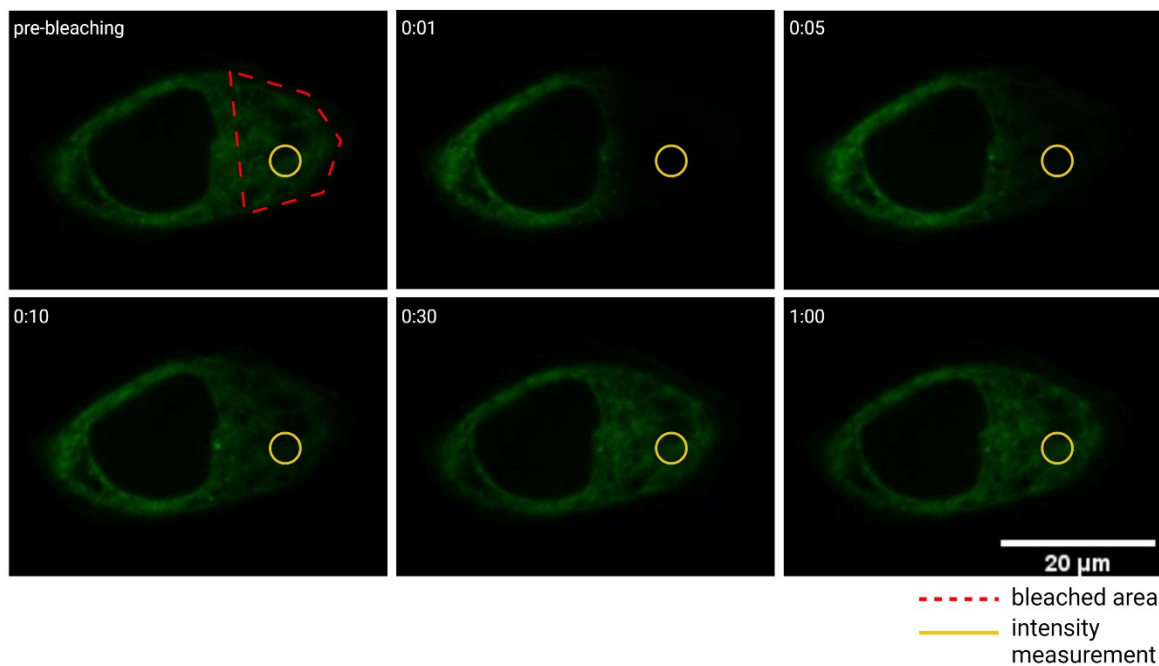


Figure 8. FRAP setup for ER-ER transport measurement.

The area inside the dotted red line is bleached with a 488nm Laser for 10 ms/pixel. Across 60 time points after the bleach the fluorescence signal recovery is measured in the area enclosed by the yellow circle (radius: 10 px) in 1s intervals. Time in min:sec.

To reduce the influence of unwanted effects on measured fluorescence intensities, the FRAP setup for ER-ER measurements was adapted after the first round of experiments was finished. The bleached area was changed from a variable polygonal selection covering large portions of peripheral to a smaller circular area of constant size (Figure 9). Additionally, the timelapse interval was reduced to 500 ms.

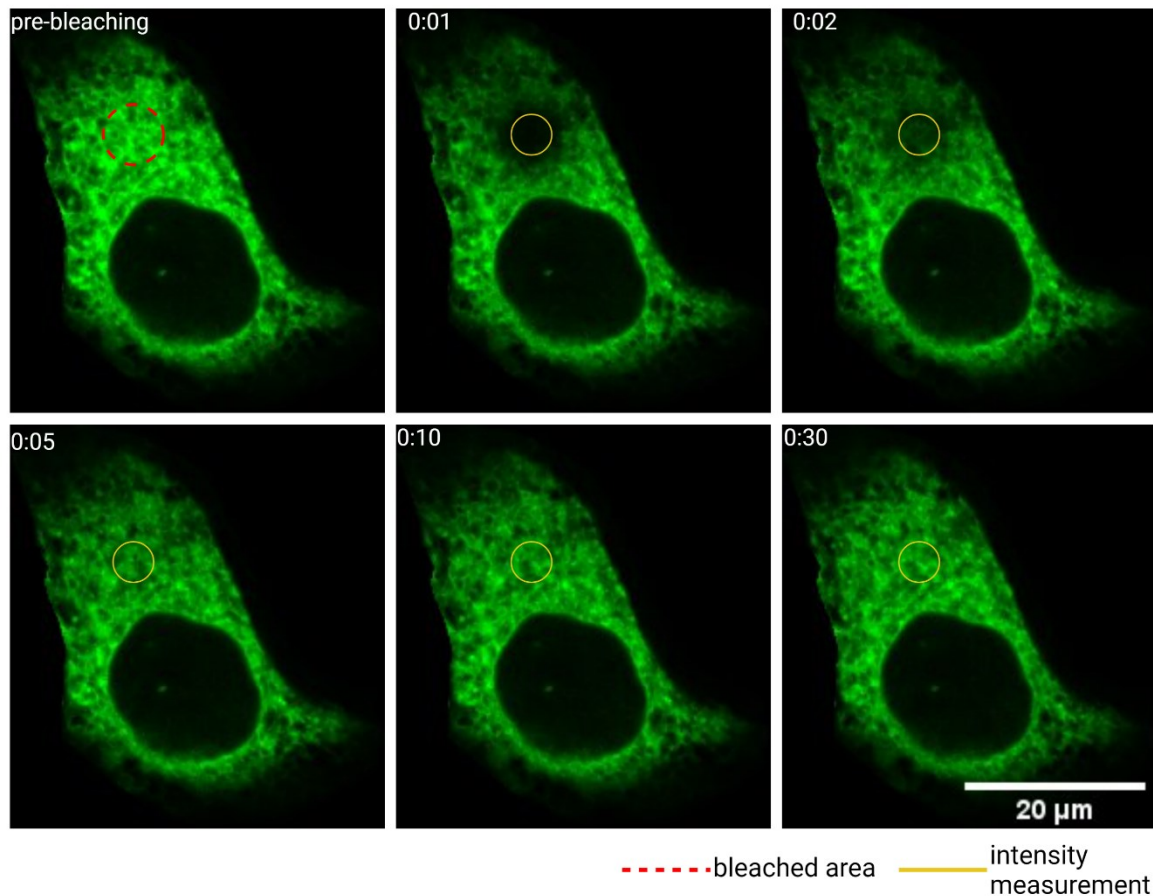


Figure 9. Adapted FRAP setup for ER-ER transport measurement.

The area inside the dotted red line is bleached with a 488nm Laser for 10ms/pixel. Across 60 time points after the bleach the fluorescence signal recovery is measured in the area enclosed by the yellow circle (radius: 10 px) in 500 ms intervals. The change to a smaller circular bleaching area was made to reduce inconsistencies in transport measurements presumably caused by reporter molecules coming from the NE and the overall distance reporter molecules might have to travel throughout the cell. Time in min:sec.

For ER-NE measurements the entire nucleus was selected for bleaching and a timelapse acquired. Imaging was done in sdc-GFP+FRAP and sdc-Cy5+FRAP channels for each timepoint to streamline the post-processing workflow. An ImageJ macro (courtesy of Julia Scholz), tracing the outer border of the nucleus was done from the images acquired in the sdc-Cy5+FRAP channel. From this outline, a 2-pixel wide mask of the NE could be created to measure the recovery of signal intensity in the sdc-GFP+FRAP channel (Figure 10). As this mask was generated for each image within the timelapse, it closely followed the shape of the NE throughout the timelapse which is often subject to morphology changes or other types of drift and movement. Recovery of signal intensity was then measured for each frame and expressed numerically for further evaluation.

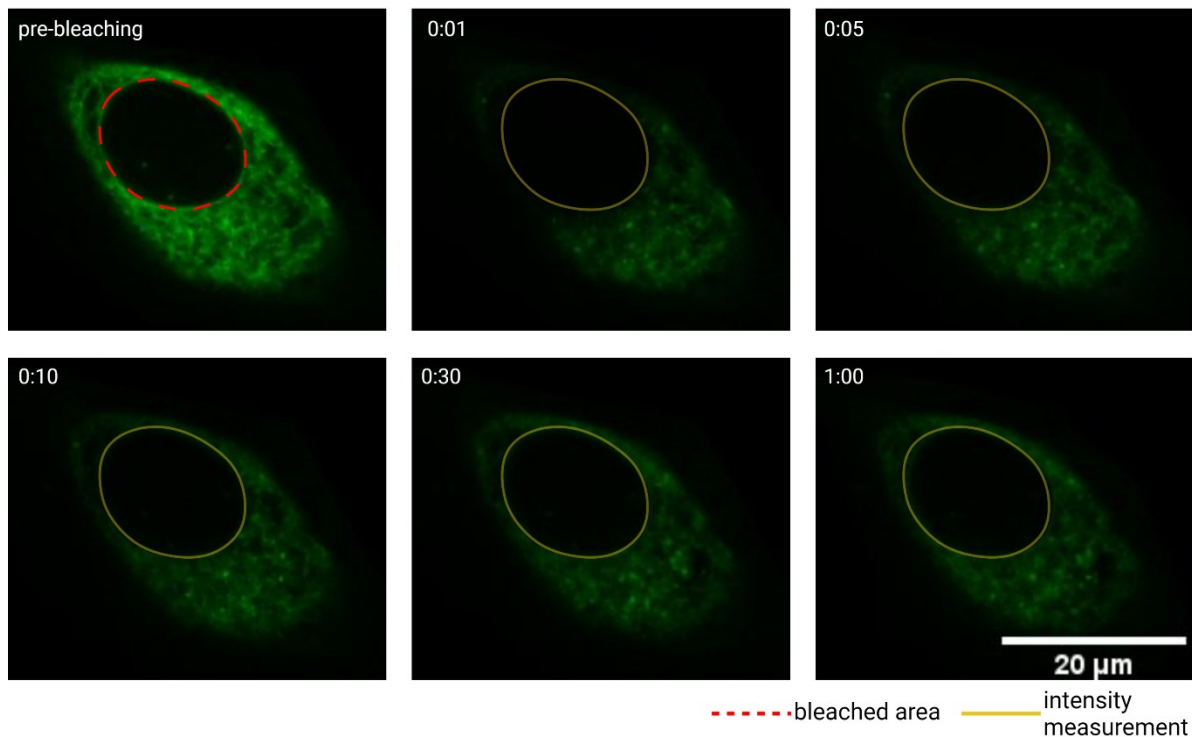


Figure 10. FRAP setup for ER-NE transport measurement.

The area inside the dotted red line is bleached with a 488 nm Laser for 10 ms/pixel. Across 60 time points after bleaching the fluorescence signal recovery is measured in a 2-pixel wide area represented by the yellow line in 1 s intervals. The yellow line is generated for each frame by masking the SiR-HOECHST signal of the nucleus and creating a 2-pixel wide outline at the. Time in min:sec.

2.5 Western Blot

Western Blots were performed to verify that RNAi sufficiently knocked-down the expression of a target gene.

2.5.1 Protein harvesting

Protein was always harvested from cells that were prepared for analysis at the live-cell spinning disc to allow for maximum comparability between results generated from the transport assay and the Western Blot. Cells were seeded onto 6-well culture dishes and harvested once a linear growth phase could be assumed.

The 6-well culture dishes were placed onto ice to stabilize the proteins before 400 μ L of lysis buffer (10 mM TRIS-base, 150 mM NaCl, 0.1 % SDS, 1 % Triton X-100/pH 7.5) and 8 μ L protease inhibitor (Sigma-Aldrich, P8340-1ML) were added. After regularly tapping the culture dish and redistributing the lysis mixture for a total of 30 minutes of incubation the lysate cell suspension was transferred to small flip-cap tubes and centrifuged for 20 minutes at 4 $^{\circ}$ C at 16.1 x 1000 RCF (Eppendorf centrifuge 5415 R). The supernatant was carefully removed from the tube and stored in a fresh tube at -80 $^{\circ}$ C. Before freezing, protein concentrations were determined via Bradford Assay (Bradford Reagent: Bio-Rad, #5000205).

2.5.2 SDS-PAGE

Based on protein concentration determined by Bradford Assay, equal amounts of protein were used (5-30 µg) and filled up to 10 µL with MiliQ H₂O. To this, 10 µL of 2x SDS-buffer (20 % glycerol, 0.01 % bromophenol blue, 10 % β-mercaptoethanol, 5 % SDS) were added and heated to 95 °C for 5 minutes for protein denaturation.

A 7.5 % separating + stacking SDS-PAGE gel was prepared fresh or stored at 4 °C wrapped in wet paper towels.

Table 9. SDS-PAGE

7.5% separating gel	Amount per gel
ddH ₂ O	2.5 mL
30 % Acrylamide/Bis	1.25 mL
1.5 M Tris (pH 8.8)	1.25 mL
20 % SDS	25 µL
10 % APS	25 µL
TEMED	2.5 µL
Total volume	5 mL
Stacking gel	Amount per gel
ddH ₂ O	2.1 mL
30 % Acrylamide/Bis	500 µL
1.5 M Tris (pH 6.8)	375 µL
20 % SDS	15 µL
10 % APS	15 µL
TEMED	1.5 µL
Total volume	3 mL

The gel was submerged in fresh 1x running buffer (35 mM SDS, 250 mM Tris, 1.92 M glycine) and samples were loaded alongside a size ladder (New England Biolabs, P7719S, Color pre-stained Protein Standard, Broad Range 10-250 kDa)

The gel was initially run at 60 V until a nice front formed at the edge of the separating gel. Voltage was then increased to 110 V.

The gel was then assembled in a full-wet blotting transfer setup and blotted in 1x transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) onto a nitrocellulose membrane (Amersham Protran, 10600004). Blotting was performed at 40 mA and 4 °C overnight.

2.5.3 Detection

Immediate verification of electrophoresis and blotting transfer success was verified using Ponceau S red stain solution (0.1 % Ponceau, 5 % acetic acid). After submerging the nitrocellulose membrane in Ponceau S solution for 2 minutes and further 2 minutes of washing in ddH₂O, protein bands are stained, and the quality of protein separation can be judged at a glance.

The nitrocellulose membranes were then submerged in blocking solution (5 % milk powder in PBS+0.1 % Tween) for 30 minutes on a rocking table in the cold room lab (4 °C). Primary antibody incubation was usually performed overnight. Primary antibodies were diluted in 10ml blocking solution and added to a 50 mL Falcon tube together with the nitrocellulose membrane. The tubes were then placed on a rolling table in the cold room overnight (4 °C). The following day, the membranes were washed three times using PBS+0.1 % Tween for 5 minutes respectively. Secondary antibodies were then also diluted in 10ml blocking solution and added to a 50 mL Falcon tube together with the membrane. The tube was then rolled for 30 to 45 minutes at 4 °C. The membrane was then washed three times using PBS+0.1 % Tween for 5 minutes each.

Table 10. Antibodies

Primary antibodies	Concentration
Mouse anti-GAPDH (Abnova, ABNOMAB9748)	1:10.000
Rabbit anti-ATL2 (Proteintech, 16688-1-AP)	1:1.000
Mouse anti-Alpha Tubulin (Abcam, ab23273)	1:5000
Secondary antibodies	Concentration
HRP Conjugate, IgG (H+L) anti-mouse (Promega, W4011)	1:2500
HRP Conjugate, IgG (H+L) anti-rabbit (Promega, W4021)	1:2500

Secondary antibodies were conjugated with HRP (horseradish peroxidase). For detection, highly sensitive detection reagent was used (ECLTM Select Western Blotting Detection Reagent, Sigma-Aldrich, RPM2234 S1/S2). The membrane was placed on a polypropylene film. Right before detection in the ChemiDoc Touch Imaging System, 600 µL of both detection reagent components were mixed and applied to the membrane. Exposure settings were usually set to auto and only rarely adapted for poor detection.

2.6 Data analysis and quantification

Image analysis was done in Fiji ImageJ2 which bundles a lot of useful plugins for scientific image processing and analysis. The built in macro feature, based in the IJ1 macro language was utilized to semi-automate the analysis process and increase throughput significantly.

Alongside the development on signal intensities throughout the bleaching process, background levels and unintended bleaching were accounted for through various parallel measurements that were taken. The resulting values were transformed and represented graphically using a combination of Microsoft Excel and GraphPad Prism version 8.3.1+ (Dotmatics, GraphPad Software, www.graphpad.com).

After subtracting the background signal intensities and correcting for unintended photobleaching throughout the time-lapse imaging process, the resulting signal recovery intensities were transferred to GraphPad Prism and for statistical analysis and curve fitting using a one-phase association.

- I. $f(t) = f_{(0)} + (f_{(\infty)} - f_{(0)}) \cdot (1 - e^{-k \cdot t})$
- II. $f(t) = f_{(\infty)} \cdot (1 - e^{-k \cdot t})$

In one-phase association (I.) $f_{(0)}$ (Y_0) describes the Y-axis value at X-axis value 0 (time = 0). $f_{(\infty)}$ describes the Y-axis value forming a plateau at an infinite time. K is the rate constant expressed as a reciprocal of X-axis units. Since time is in seconds (s), k is expressed as s^{-1} . Expression II. is a derivative of expression I. achieved through normalizing the background signal levels to 0 effectively cancelling out $f_{(0)}$.

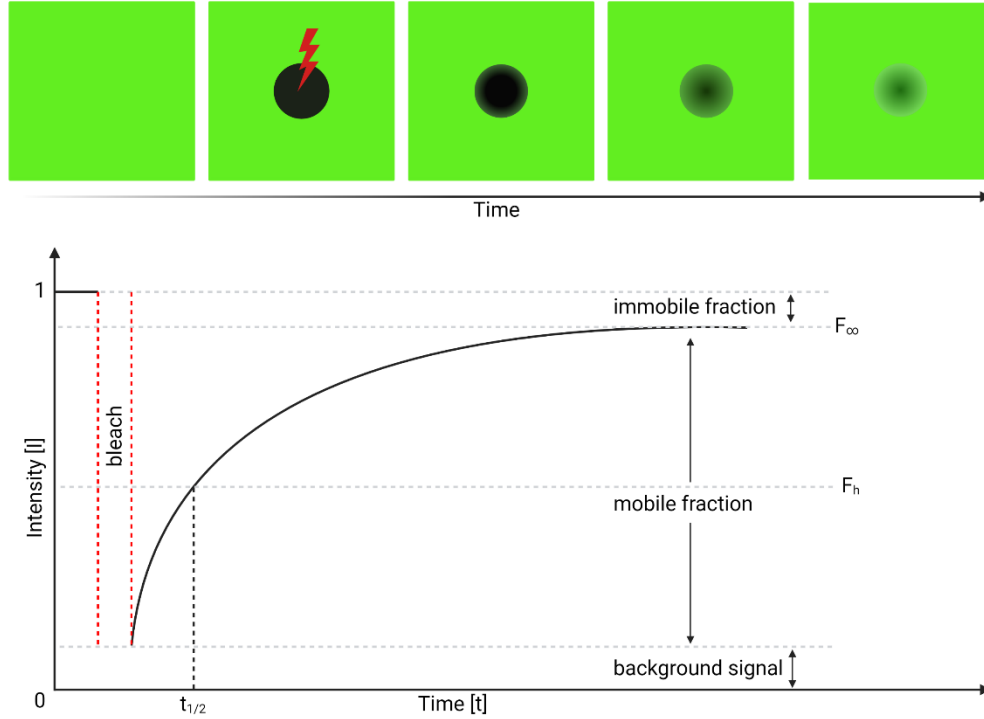


Figure 11. Fluorescence recovery after photobleaching (FRAP).

This schematic explains the general working principle of FRAP and shows a typical signal intensity curve throughout the process. The upper graphic shows how the signal recovers within the bleached area across time and that this recovery might not necessarily be complete which is represented in the graph below by the immobile fraction. After bleaching, the signal intensity drops down all the way to the level of background signal and then recovery exponentially until a plateau (F_{∞}) is reached. $t_{1/2}$ is the timepoint at which the signal recovery has reached 50% relative to the plateau. Individual time points measured will be fitted with a one-phase association $f(t) = f_{\infty} \cdot (1 - e^{-k \cdot t})$. From this fitting, the k -value, describing the recovery kinetics, can be extracted.

To qualify for transport measurement, sample cells needed to fulfill certain criteria. This aimed at maximizing repeatability and traceability of results. Only cells in interphase with an approximately round nucleus and typical ER structure were utilized. Further, only cells exhibiting strong Cy3 signals. This allows the assumption of presence of Cy3 labelled siRNA within the cell and subsequent target gene knockdown.

For each condition a minimum of 10 cells were chosen to allow to statistical analysis. Sample size (n) and p -value are indicated whenever relevant for each figure. T-tests were performed in GraphPad Prism from which statistical significance (p -values) could be calculated.

3. Results

3.1 Overview of all reporters

The analysis of various types of reporters that could be used in the establishment of a FRAP-based reporter gene assay was performed by Julia Scholz during her master's project upon which this thesis further expands (Figure 12). From this work we deduced that luminal reporters are best suited for our purposes (Figure 13). Specifically, the small luminal reporter SP-moxGFP-KDEL was a highly promising reporter candidate as it yielded excellent brightness while minimally affecting the ER/NE morphology. Additionally, the small size meant that it could diffuse through the ER lumen significantly faster which allows for faster screening and consequently higher data output. For all initial transport assays, SP-moxGFP-KDEL was used.

Reporter							
Name	SP-moxGFP-KDEL	SP-4XHA-NusA-moxGFP-KDEL	SP-4XHA-NusA-moxGFP2-KDEL	SP-4XHA-moxGFP-Sec61 β -KKLL	4XHA-moxGFP-Sec61 β	4XHA-Dronpa-Sec61 β	4XHA-NusA-Dronpa2-Sec61 β
Fluorophore	moxGFP	moxGFP	moxGFP	moxGFP	moxGFP	Dronpa	Dronpa
Expected localization	ER lumen	ER lumen	ER lumen	ER/ONM/INM	ER/ONM/INM	ER/ONM/INM	ER/ONM
Brightness	+++	++	++	+	+	++	++
ER/NE morphology	+++	+++	+++	++	+	+++	+++

Figure 12. Overview of all reporters used in the initial experiments to establish a FRAP-based transport assay. The brightness and the impact of the reporters on overall ER/NE morphology (compared to wildtype) were graded using “+” ranging from +++ being the highest relative score and + being the lowest. This evaluation was performed by Julia Scholz during her master's project and serves as a baseline for the more in-depth analysis performed in this thesis.

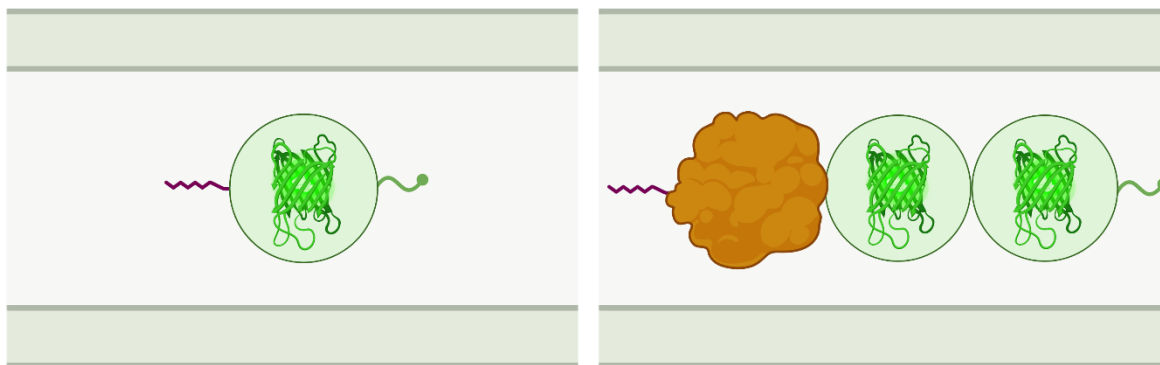


Figure 13. Schematic representations of the two main reporter genes selected for the following experiment. Small luminal reporter (SP-moxGFP-KDEL ~30kDa, top-left) and the large luminal reporter (SP-NusA-moxGFP-KDEL ~120kDa, top-right). From left to right: signal peptide (SP, purple line) – NusA protein (only in large reporter, orange) – moxGFP (fluorescent protein, green) – KDEL (ER retention signal, green line).

3.2 Transport Assay – developing the approach

To efficiently test various candidate genes regarding their effect on transport kinetics between the ER and the NE of cells, it is necessary to first establish a robust and repeatable approach that places high priority on minimizing tertiary effects that skew the resulting data. The initial testing was done in HeLa wildtype cells which were transiently co-transfected with the small luminal reporter SP-moxGFP-KDEL and cy3-stained scrambled siRNA. The small size of the reporter and the non-targeting scrambled siRNA offer a reference point that serve as a control for future gene-specific testing. Figure 14 depicts live-cell images captured 72 h after transfection of SP-moxGFP-KDEL and scrambled siRNA. Notably, picture A shows the ER network, how it extends to the periphery of the cell and how it surrounds the nucleus. The lumen of the NE can also be observed clearly. Picture B shows regularly shaped nuclei. Picture C shows the cy3-siRNA conjugates and their distribution throughout the cell. This also allows for an educated guess on the transfection efficiency and subsequent efficiency of the knock-down to be made, as the amount of siRNA that reached a target cell closely correlated with the knock-down efficiency (see Figure 15).

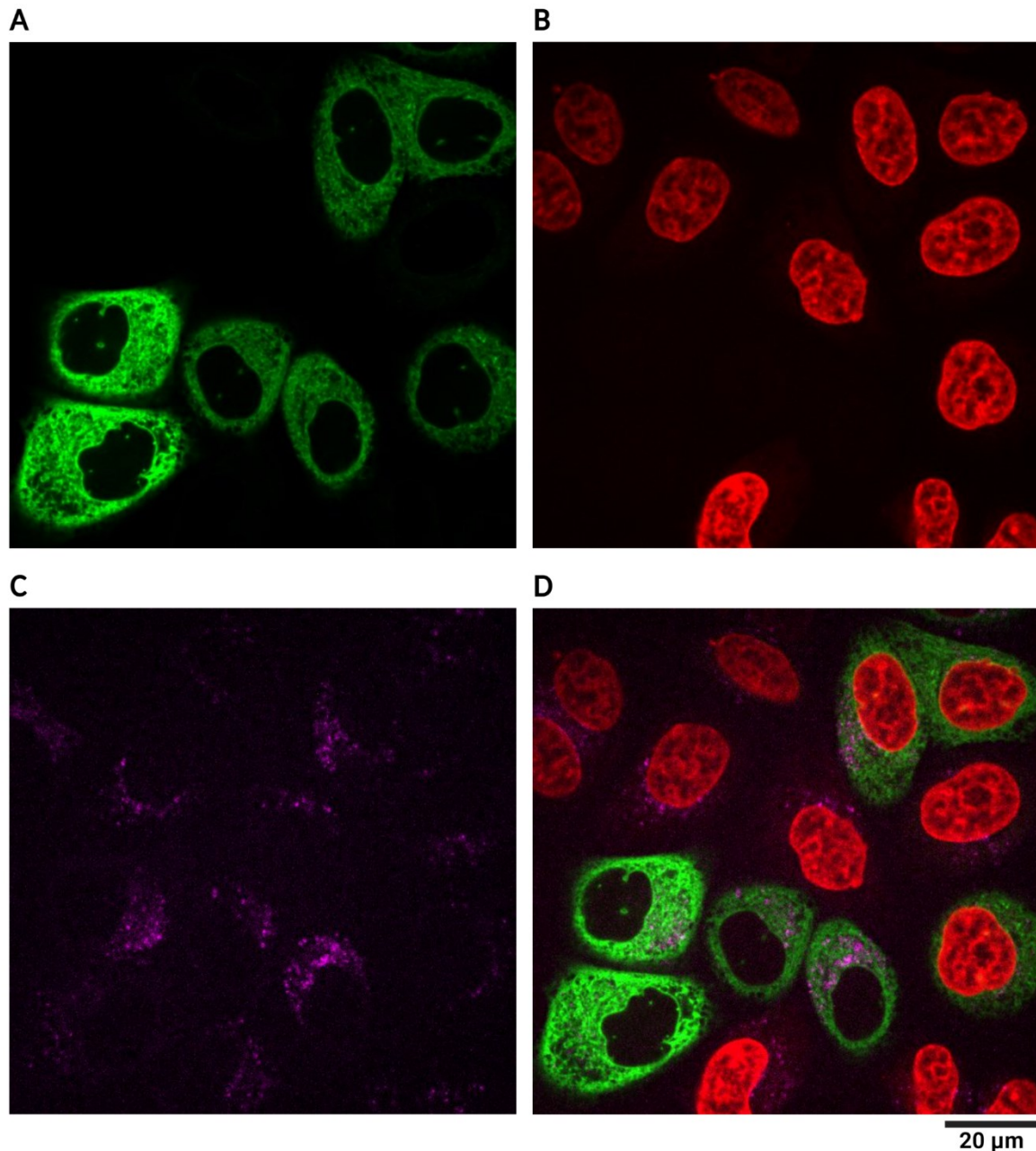


Figure 14. Live-cell spinning-disc confocal microscopy of HeLa wildtype cells transiently co-transfected with SP-moxGFP-KDEL ER luminal reporter and cy3-stained scrambled siRNA 72h prior to imaging. **(A)** green fluorescence signal at the ER and NE of SP-moxGFP-KDEL retained to the ER lumen. **(B)** red fluorescence signal of DNA stained with SiR HOECHST. **(C)** magenta fluorescence signal of Cy-3 labeled scrambled siRNA. **(D)** merged image of the previous three channels. Laser power and exposure times vary between channels. Brightness and Contrast have been adjusted for each channel individually.

3.2.1 Cy-3 signal intensity correlates with knock-down efficiency

To be viable for FRAP-measurement, cells need to be of regular size and morphology, and display sufficient reporter gene signal and siRNA levels. Cells with obvious malformations While irregularities in size, shape and morphology might be direct consequences of knocking-down genes which are potentially crucial for correct cellular function, it is not possible to determine what the source of cellular-stress is. Experience has shown that even co-transfection with reporter gene and scrambled siRNA resulted in cells of irregular morphology, we determined that the siRNA cy3-signal intensity is a more

reliable and objective means of judging the knock-down efficiency within a given cell. This approach is further supported by the results displayed in Figure 15 where it was shown that siRNA signal intensity largely correlates with the knock-down efficiency. Cy-3 labelled siRNA against GFP was used to show how siRNA amount affects median GFP signal intensity.

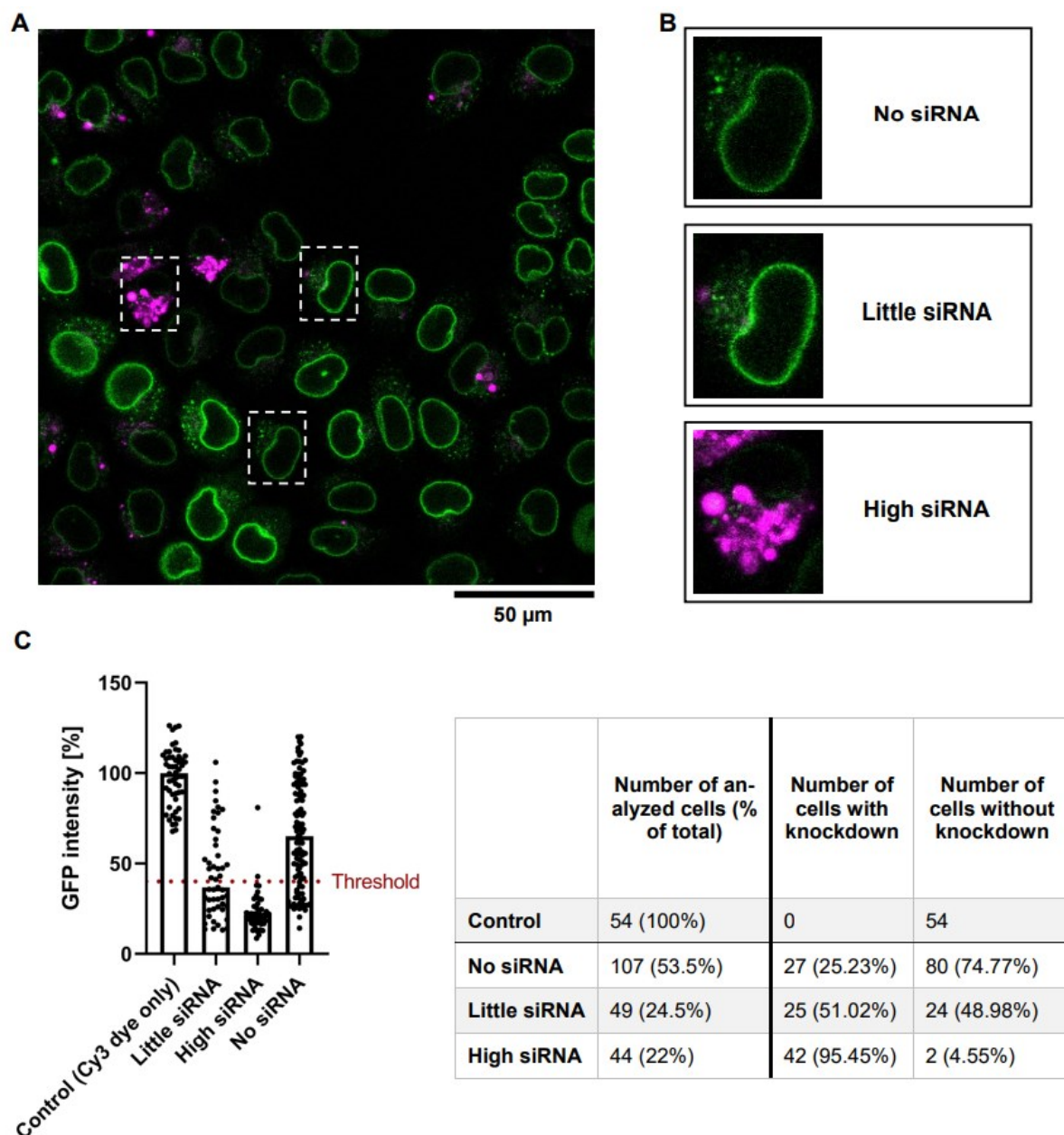


Figure 15. Signal intensity of cy-3 labeled siRNA correlates with knockdown efficiency.

(A) Classification of cy-3 labeled GFP-siRNA correlated to the knockdown of GFP. (B) Cells were categorized roughly into three distinct categories. No visible cy-3 signal (no siRNA), with low cy-3 signal intensity (little siRNA) and cells with high cy-3 signal (high siRNA). Cells where the nuclear envelope was not fully in frame were excluded from analysis. Representative cells for each of the three categories are shown. (C) Quantification of GFP signal around the nuclear envelope of categorized cells. The relative median GFP intensity is shown for each category normalized to the control (cy-3 dye only). A threshold of 40% was set to classify cells as successfully knocked down. n=200 siRNA-cy3 transfected cells, n=54 for control (cy-3 dye only) cells. The table presents the quantified data numerically for all categories. Cells are categorized into cells with or without knock-down according to the threshold of 40% set previously. (Figure 33 from Julia Scholz', MSc master thesis, classification and quantification performed by Tamara Völkerer (formerly Groffics)).

3.2.2 Quantifying targeted knock-down efficiency after 72 h

Further, western-blotting was used to determine the overall knock-down efficiency across all cells within a culture dish. After 72 h of total incubation time with target siRNA (see Figure 16, three different siRNAs against Atlastin-2), proteins were harvested from an entire culture dish and separated on an SDS-PAGE gel for western-blot analysis. The corresponding band for Atlastin-2 is indicated by the black arrow and corresponds to the predicted band at 66kDa. Above that, an unspecific band can be seen, that does not appear to be affected by Atlastin-2 knock-down and therefore can be assumed to be independent of ATL-2 levels. The three different siRNAs that were used for specifically knocking-down Atlastin-2 all show similar results.

Generally, it was shown that various siRNAs with different target sequences were able to knock-down the target gene very effectively. While live-cell microscopy showed that cy3 signal intensities can vary greatly across individual cells, the western-blot gives us reasonable confidence that the absolute amount of target protein is sufficiently reduced.

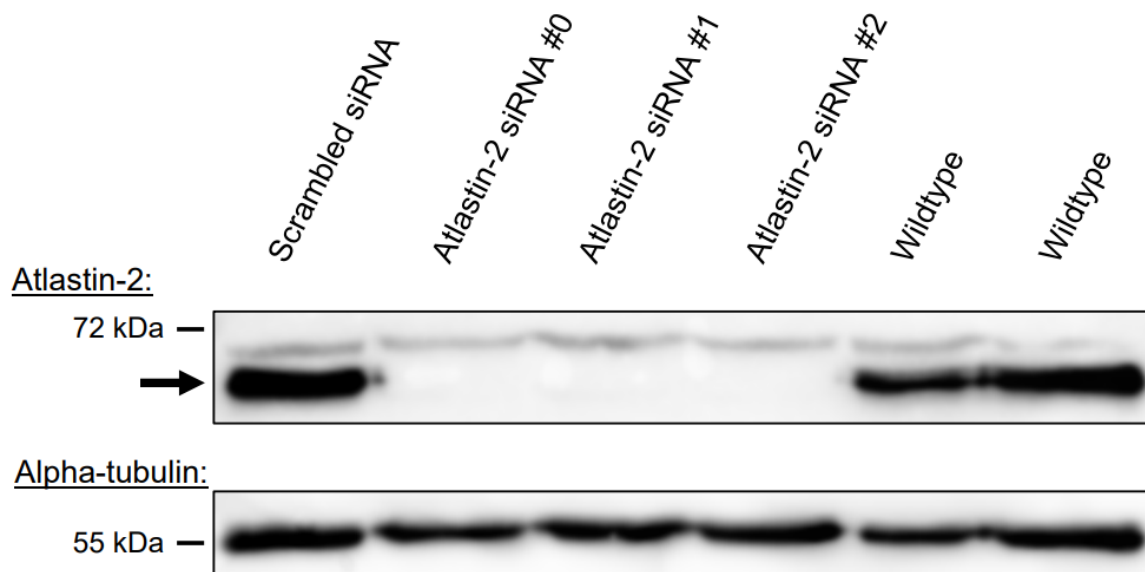


Figure 16. Verifying the efficiency of siRNA knockdown after 72 h.

Atlastin-2 was sufficiently knocked down. The western-blot (7.5 % SDS-PAGE) shows HeLa wildtype cells with Atlastin-2 knockdown with three different siRNAs. As controls, scrambled siRNA, and HeLa wildtype (no siRNA) cells were used. Alpha-tubulin acted as our loading control. Atlastin-2 specific bands are indicated with an arrow. Atlastin-2 antibody (rabbit, 1:1000), Alpha-tubulin antibody (mouse, 1:5000). Secondary antibodies (1:2500). (Data has been acquired by Julia Scholz, MSc, as part of her master-thesis. Fig.30)

3.3 RNAi screen of multiple candidate genes

3.3.1 initial FRAP transport assays

The first round of RNAi screening was performed on a large set of potential candidate genes and aimed at narrowing this wide list down and gaining some insight into the viability of our approach. The list of candidate genes is comprised of genes that could potentially be involved in the regulation of ER-NE connections due to their cellular location or their previously established function (see 1.4.3 Candidate Genes for a more detailed description of all candidate genes).

In this first set of tests we used HeLa wildtype cells and co-transfected the small luminal reporter SP-moxGFP-KDEL alongside the cy3-labelled siRNA against the respective target. The small luminal reporter was chosen as its relatively fast diffusion rate allows for faster screening. The results, displayed in Figure 17 and Figure 18, are corresponding sets of data acquired from the same cells only differing in whether the transport was measured between ER-ER or ER-NE. The resulting recovery curves of the transport assays were fitted to a one-phase association. The resulting k-values were then normalized to the k-values acquired by transport measurements of scrambled negative controls from the same day of testing. The k-value ratios of all measurements were then combined to get an easy overview. Each dot represents one cell. Significance levels were then determined via t-test analysis for each sample versus its corresponding scrambled dataset and are represented as asterisks.

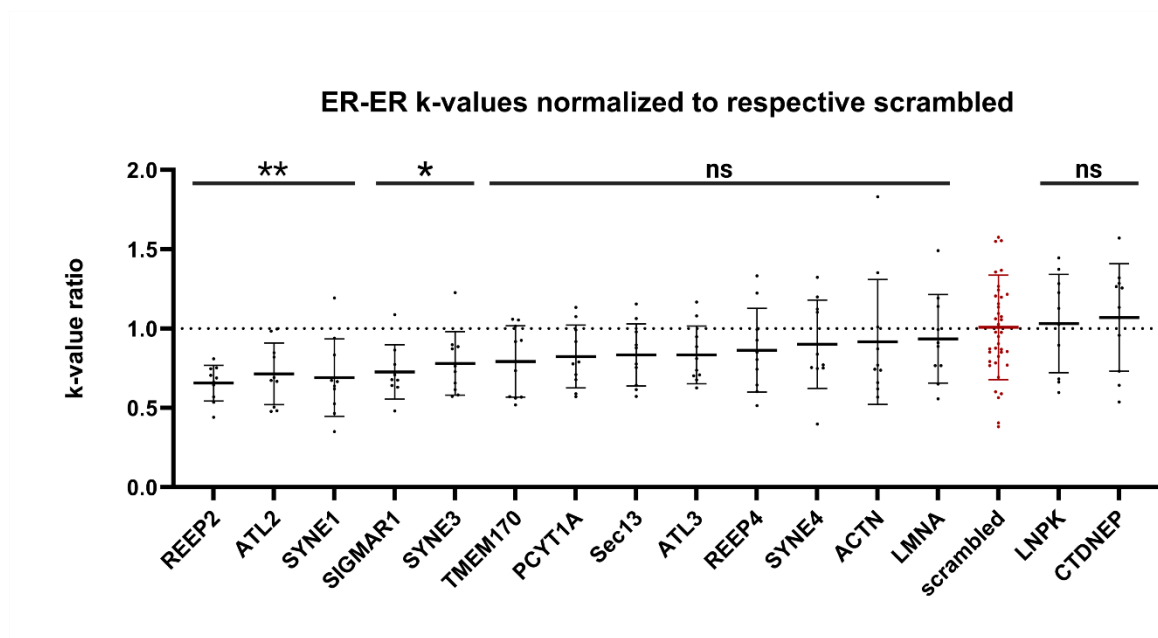


Figure 17. Initial FRAP ER-ER transport-assay of HeLa wildtype co-transfected with SP-moxGFP-KDEL and cy3 labelled target siRNA 72 h prior to imaging.

The FRAP was performed as described in Figure 3 and the recovery curve of each measurement was fitted to a one-phase association from which k-values can be extracted. All k-values are displayed as ratios normalized to the scrambled negative control k-values taken on the same day. All scrambled k-values have been combined and normalized to 1. Results from the individual gene knockdowns have been sorted in an ascending manner. Each dot represents one cell. Statistical significance was determined via a t-test for each sample versus the scrambled negative control. Significance levels are displayed as asterisks.

* = $p < 0.05$, ** = $p < 0.005$, ns means not significant.

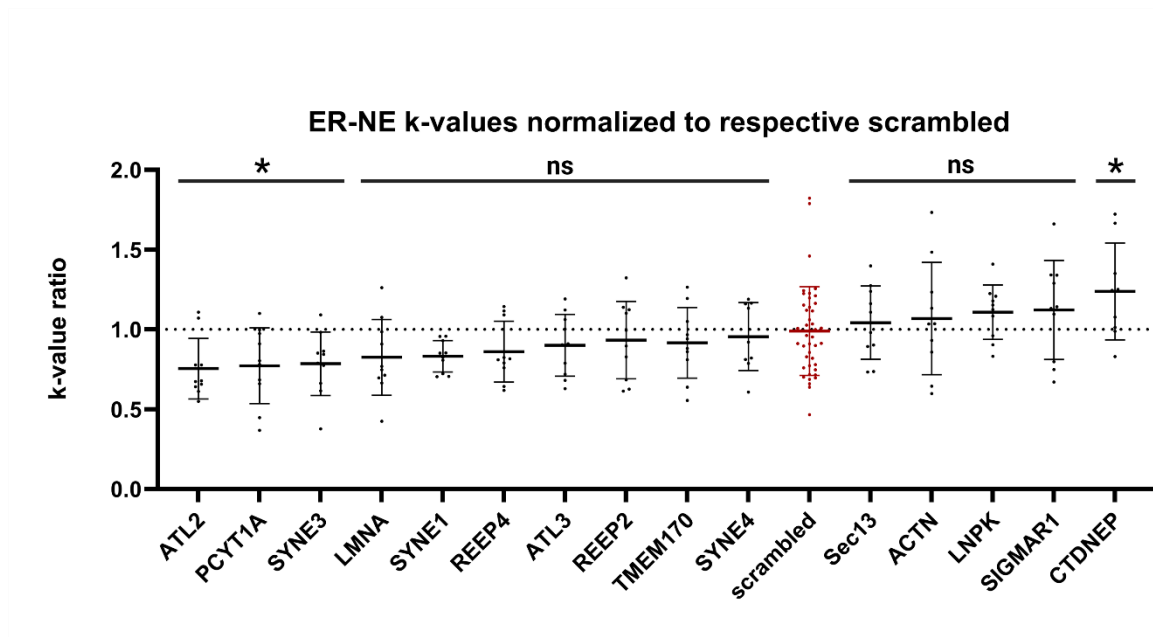


Figure 18. Initial FRAP ER-NE transport-assay of HeLa wildtype co-transfected with SP-moxGFP-KDEL and cy3 labelled target siRNA 72 h prior to imaging.

The recovery curve of each measurement was fitted to a one-phase association from which k-values can be extracted. All k-values are displayed as ratios normalized to the scrambled negative control k-values taken on the same day. All scrambled k-values have been combined and normalized to 1. Results from the individual gene knock-downs have been sorted in an ascending manner. Each dot represents one cell. Statistical significance was determined via a t-test for each sample versus the scrambled negative control. Significance levels are displayed as asterisks. * = $p < 0.05$, ns means not significant.

While across both ER-ER and ER-NE data sets a general tendency, of more gene knock-outs resulting in slower transport rates, can be observed, it has to be considered that only few result in statistically significant changes. Further, changes in ER-NE recovery rate can be an indirect consequence of altered ER-ER transport. Especially results that show similar changes in both measurements (e.g. ATL2 and CTDNEP) must be interpreted very carefully. Conversely, inverse effects as seen in SIGMAR1 knock-out measurements for example could be very promising in future experiments. At this stage however it is unwise to draw any conclusions as individual measurements deviate heavily and statistical significance is not given.

3.3.2 refining FRAP transport assays for promising candidate genes

After the overview gained by the initial set of FRAP transport assays, we narrowed the list of candidates down to focus our resources and generate more meaningful results. By looking at the known cellular functions of genes and their gene products in combination with the results gathered, we decided to continue testing with ATL2, CTDNEP, REEP4, SIGMAR1, SYNE1 and SYNE3. The experimental setup was left unchanged except for how ER-ER transport measurements were gathered, regarding the shape and size of the bleached area. The changes to the FRAP setup are explained in Figure 9 in more detail. The adaptations made ensured a constant size of the bleached area and aimed at making FRAP recovery measurements more robust to cell size and shape and less likely to be influenced by reporters diffusing from distant regions of the cell.

The results of this second iteration of screening are displayed in Figure 19 for ER-ER and Figure 20 for ER-NE transports. The datasets for each gene knockdown were always acquired in parallel and normalized to the scrambled negative control measurements performed on the same day. The normalized k-value ratios were then sorted in an ascending manner and combined on one figure. Statistical significance levels were determined via t-test. Significance levels are displayed above the corresponding dots.

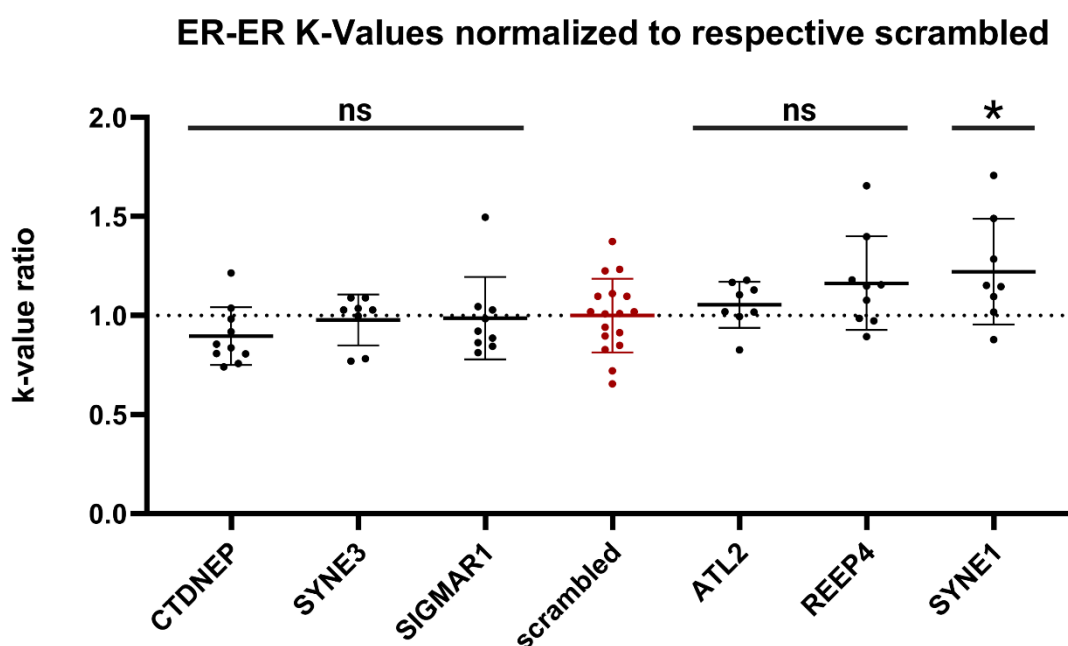


Figure 19. Second iteration of FRAP assays.

For the second test-run promising candidates from the first run were chosen and the FRAP method was slightly adapted to account for possible inconsistencies caused by the large areas that have been bleached initially. The new approach is shown in Figure 8. HeLa wildtype cells were co-transfected with SP-moxGFP-KDEL and cy3 labelled target siRNA 72 h prior to imaging. The recovery curve of each measurement was fitted to a one-phase association from which k-values can be extracted. All k-values are displayed as ratios normalized to the scrambled negative control k-values taken on the same day. All scrambled k-values have been combined and normalized to 1. Results from the individual gene knock-downs have been sorted in an ascending manner. Each dot represents one cell. Statistical significance was determined via a t-test for each sample versus the scrambled negative control. Significance levels are displayed as asterisks. * = $p < 0.05$, ns means not significant.

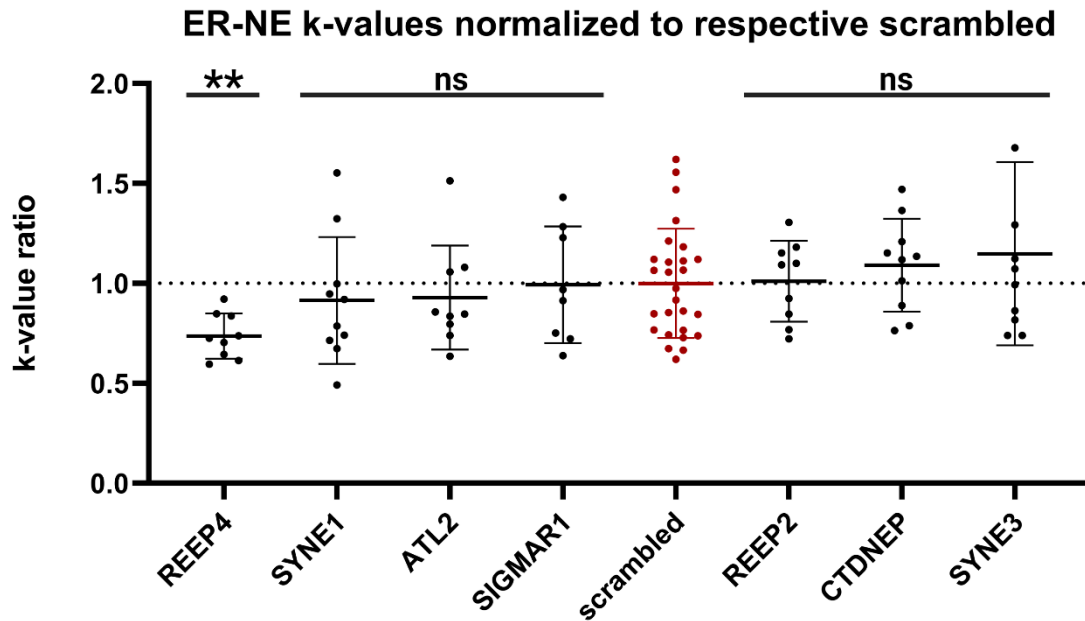


Figure 20. Second iteration FRAP ER-NE transport-assay of HeLa wildtype co-transfected with SP-moxGFP-KDEL and cy3 labelled target siRNA 72h prior to imaging.

The recovery curve of each measurement was fitted to a one-phase association from which k-values can be extracted. All k-values are displayed as ratios normalized to the scrambled negative control k-values taken on the same day. All scrambled k-values have been combined and normalized to 1. Results from the individual gene knock-downs have been sorted in an ascending manner. Each dot represents one cell. Statistical significance was determined via a t-test for each sample versus the scrambled negative control. Significance levels are displayed as asterisks. ** = $p < 0.005$, ns means not significant.

While the second iteration of RNAi screening offered additional insight into changes of transport rates as a consequence of gene knock-down, it also highlighted a few key shortcomings of the approach taken and methods applied. Not only are the results mostly statistically insignificant but they also appear to be rather inconsistent. This inconsistency is true at the single cell level but even more critically across entire data sets too.

Generating more meaningful data therefore required primarily two things: an increased sample size as well as a robust methodical approach that minimized external factors as much as possible.

3.3.3 Results from RNAi screens vary among different experiments and are not reproducible

After adapting our approach slightly in the second round of FRAP assays, we did a third round of testing to increase our total number of available data points and test how well data sets acquired on different days can be compared. Since this RNAi screen is directly based on previous work done by Julia Scholz during her master's thesis, we also included data acquired by her whenever available and compared the averages of the individual runs in Figure 21. It is immediately noticeable that almost all gene knock-downs yielded heavily varying results despite the fact, that all measurements were taken under circumstances that could be considered equal. The ER-NE ATL2 and SYNE measurements are rare exceptions to this phenomenon.

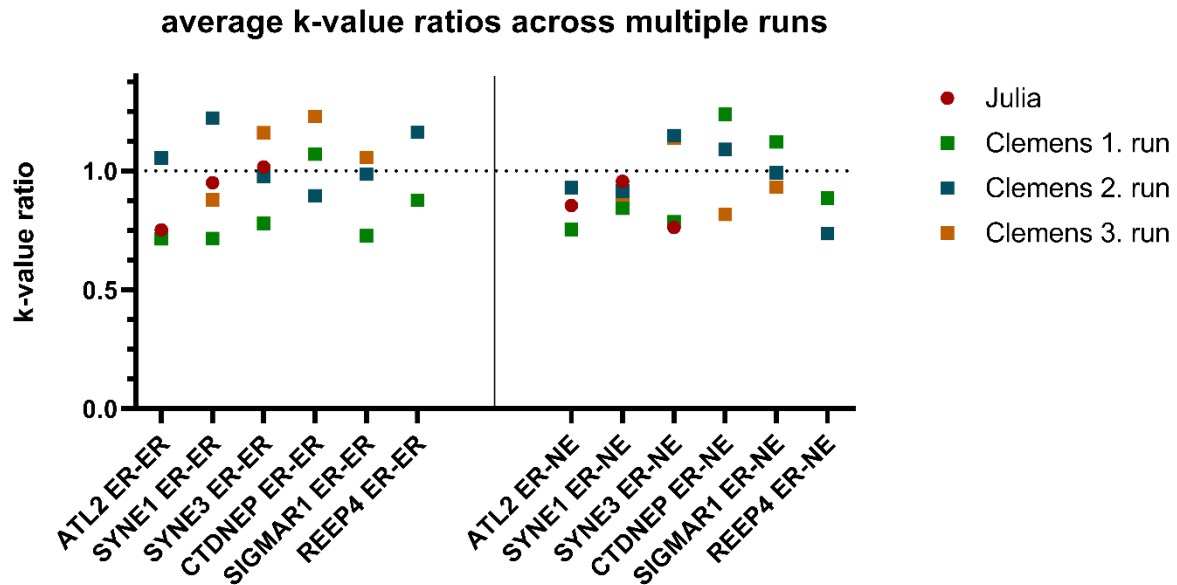


Figure 21. The comparison of average k-value ratios from multiple FRAP assays reveals an inconsistency of results for some samples while others appear to be rather consistent across multiple runs. Experiments from data set A (red) have been performed by Julia Scholz, MSc as part of her master's project.

The general experimental setup was designed with repeatability and maintenance of steady conditions in mind. The selection of cells for FRAP also followed objectively set rules to minimize the influence of potential personal bias. To identify potential factors that could affect transport measurements that are seemingly unrelated to the specific gene knock-down we therefore analyzed how cell shape, size and ER distribution around the nucleus influenced transport kinetics.

3.3.4 k-values are inversely proportional to cell size

Cell size or more specifically, how far the ER extends outward from the NE can have a significant effect on fluorescence signal recovery. With an increased distance between the outer edges of the ER and the NE, it takes longer for reporters to diffuse towards the bleached area of the cell which deflates k-values. This presumption is further supported by the data displayed in Figure 22. Plotting scrambled ER-NE k-values against their relative cell-size showed that k-values are indirectly proportional to the maximum ER distance from the nucleus.

The experiments were performed in HeLa wildtype cells, co-transfected with SP-moxGFP-KDEL and cy-3 labelled scrambled siRNA. After 72 h incubation cells were imaged and transport measurements performed. Using a fully automated script, the distance between the NE and the outermost point of ER was determined. The distance unit (px) is an arbitrary unit.

Figure 22 shows an example of how this ER to NE distance is measured and then plotted. When analyzing the trend these data points follow, it was determined that k-values relate to cell size with a Pearson correlation-coefficient of $R=-0.4743$ with a statistical significance of $p=0.000196$ (***). This represents a highly significant but moderate negative correlation of k-values to cell size.

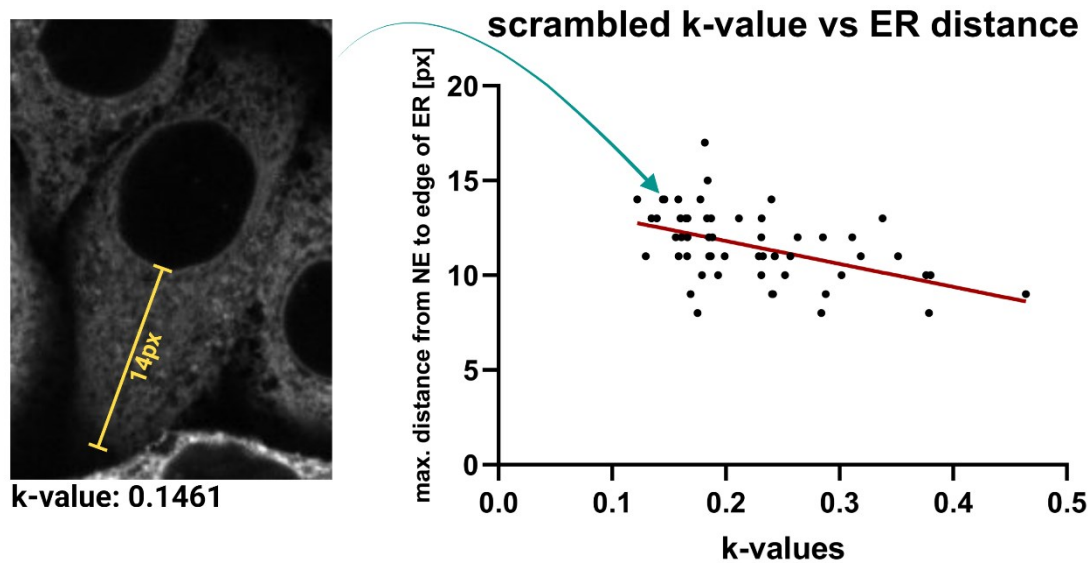


Figure 22. ER-NE k-values are indirectly proportional to cell size. The further a cell extends into at least one direction, the longer the signal recovery takes resulting in a smaller k-value. Scrambled k-values from multiple FRAP assays in HeLa wild-type cells co-transfected with SP-moxGFP-KDEL and cy-3 labelled scrambled siRNA (72 h prior to imaging) were plotted against the maximum distance from the edge of the ER to the NE in the corresponding cell. The cell shown in the left image represented in the graph by the dot indicated by the cyan arrow. The distance unit [px] is an arbitrary unit that is determined via a fully automated script that masks the nuclear envelope and as the ER and can measure the maximal distance between those. A Pearson correlation-coefficient of $R = -0.4743$ with a significance level of $p = 0.000196$ was determined.

With enough data, this analysis can be performed for each data point and used as a correction factor. However, if cell sizes follow a roughly even distribution, this effect should even out across the entirety of a data set rendering this additional step in the process more or less worthless.

3.3.5 ER-ER recovery is slower than ER-NE recovery

Transport assays performed during previous experiments already showed that ER-ER recovery was consistently slower than corresponding ER-NE recovery. It was speculated that both the differences in morphology as well as the slightly different experimental approach of measuring transport kinetics might be responsible for this discrepancy.

To investigate the differences in recovery between ER-ER and ER-NE, we first adapted the experimental approach for the FRAP assay. While the way we select the FRAP region for ER-NE remained unchanged throughout all experiments, selecting the FRAP regions for ER-ER measurements was adapted significantly. Initially, ER-ER bleaching was done by selecting large parts of the peripheral ER and bleaching it entirely before measuring the recovery within a small circular area. This approach did not lend itself to achieving steady and repeatable results as it was vulnerable to subjective bias and differed greatly from the way ER-NE measurements were done. As seen in Figure 8, selecting the FRAP region was done using a polygon line tool and followed no objective guidelines. This meant that the selected area is never the same size. Additionally, when measuring the recovery for ER-NE, the nuclear envelope is surrounded by peripheral ER and diffusion can happen omnidirectionally. The initial ER-ER FRAP region however covered an entire side of the peripheral ER, which restricts the potential for fluorescence

recovery to effectively one direction. Together with potentially further distances that reporters must cross to reach the bleached areas, a significant part of why there are differences between ER-ER and ER-NE recovery can be explained by this suboptimal approach.

To address the most plausible shortcomings of the initial FRAP region selection we established a new way of selecting this region that is more resistant to subjective bias and resembles the bleached area of the ER-NE measurements more closely. Figure 9 shows a representation of the newly adapted ER-ER bleaching method that uses a circular area of fixed size. Positioning this FRAP region is easier to do in a repeatable manner and ensures that the area where recovery is measured is surrounded by unbleached ER.

While these changes still make a direct comparison of ER-ER and ER-NE difficult, they definitely help in creating a better experimental approach that ultimately leads to generating more meaningful data. That said, the overall method is still inherently susceptible to external influences and controlling external factors is both complex and time consuming but ultimately worthwhile.

3.3.6 using stable cell lines to reduce influence of external factors

Transfection of plasmid DNA for fluorescent reporter gene expression yielded varying results in terms of fluorescence signal measured in sample cells. To combat these inconsistencies, cell lines with stably integrated reporter genes were used. HeLa cells, with a stably integrated small luminal reporter moxGFP-KDEL were used to test the general viability of this approach. The cell line was created using standard cloning techniques described in chapter 2.3 Molecular cloning of this thesis. To analyze the performance of these reporter expressing cell lines, we set up another round of transport assays where like previously, cy-3 siRNAs against ATL2 and CTDNEP were added 72 h before imaging.

The results of this experiment are summarized in Figure 23. Similarly, to some previous experiments, knock-down of ATL2 resulted in a significant reduction of signal recovery speeds in ER-ER measurements and a less significant tendency of reduced recovery rates in ER-NE measurements. And while the changes of k-values as a consequence of ATL2 knock-down are at not always statistically significant, the overall trends appear to be comparable across multiple datasets and suggest that using stable cell-lines is indeed a viable option for future testing.

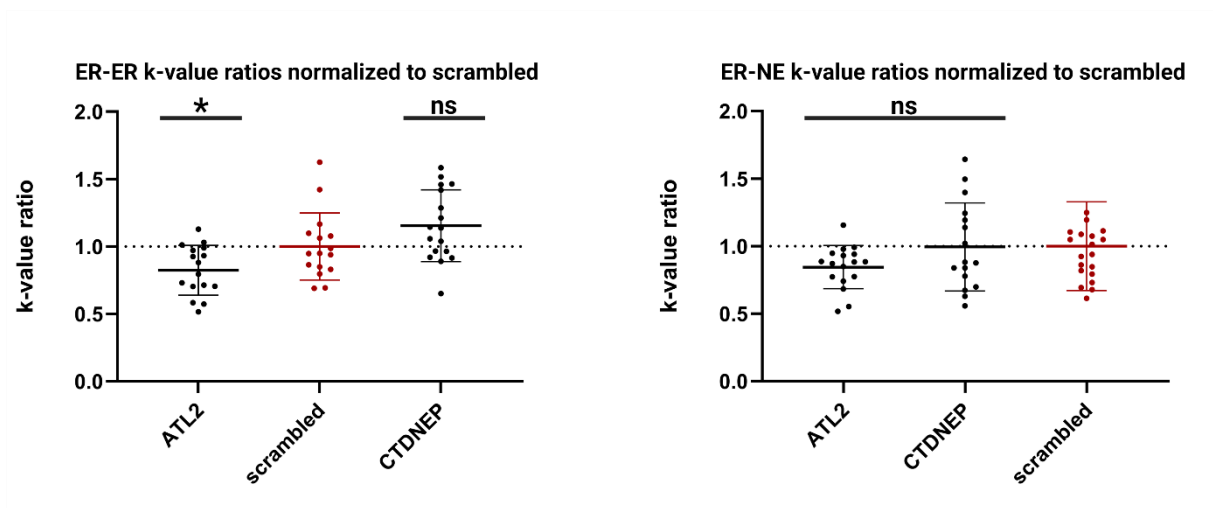


Figure 23. FRAP assays have been performed with a stable moxGFP-KDEL HeLa cell line to verify that the previously established method of siRNA knock-down followed by a FRAP assay after 72h of incubation also works in cell-lines that stably express reporter genes.

This cell-line expresses a SP-moxGFP-KDEL soluble reporter retained in the lumen of the ER and the NE. The recovery curve of each measurement was fitted to a one-phase association from which k-values can be extracted. All k-values are displayed as ratios normalized to the scrambled negative control k-values taken on the same day. All scrambled k-values have been combined and normalized to 1. Results from the individual gene knock-downs have been sorted in an ascending manner. Each dot represents one cell. Statistical significance was determined via a t-test for each sample versus the scrambled negative control. Significance levels are displayed as asterisks. * = $p < 0.05$, ns means not significant.

3.3.7 Expanding the experimental approach to another cell-line

For this experimental approach to be viable for gaining insight into how knock-down of target genes might affect transport kinetics through the endoplasmic reticulum, it has to be adaptable to various cell types. Ideally, an identical approach can be used in a variety of cell types from different tissues or species to compare effects of altered gene expression.

To test this, we used the previously established protocol of co-transfecting a reporter gene alongside a targeted cy3-stained siRNA for a total duration of 72 h and applied to RPE-1 cells (Human retinal epithelial-1 cells, see chapter 2.1.1 Cell lines and culture).

Figure 24 shows a direct comparison between the previously used HeLa cells versus the RPE-1 cells when observed through a live-cell spinning disc confocal microscope. Laser intensities used as well as exposure, brightness and contrast settings are the same across the two sets of images to allow for direct comparison. The images were chosen to be representative for each cell-line. The overall cell size and shape are rather similar, however, the distribution of the ER network around the nucleus appear to be more biased towards one side in RPE-1 cells when compared to HeLa cells. This trend was noticeable across the majority of cells observed.

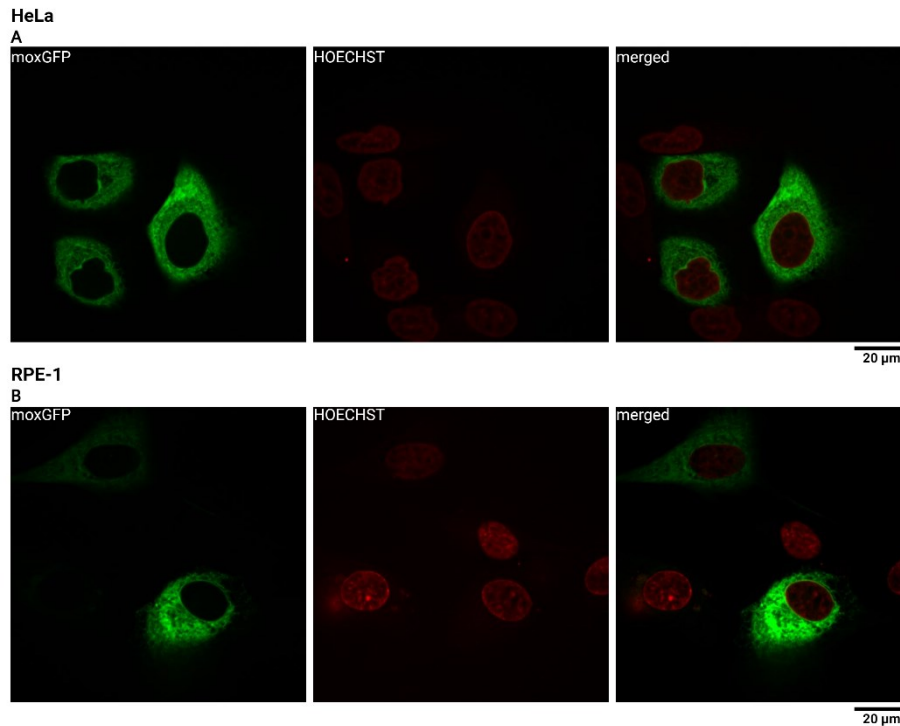


Figure 24. Live-cell spinning disc confocal microscopy comparison between HeLa (A) and RPE-1 (B) cell-lines transiently transfected with SP-moxGFP-KDEL (green) and DNA stained with SiR-HOECHST (red). Imaging was done 72 h after transfection and approximately 1 h after HOECHST staining. Laser intensities and exposure as well brightness and contrast are the same across the two sets of images.

In a proof-of-concept study, RPE-1 cells were transiently co-transfected with the small luminal reporter SP-moxGFP-KDEL and cy3-stained siRNAs against ATL2 and CTDNEP. After 72 h incubation, cells were used in a FRAP transport assay and proteins harvested for evaluating the knock-down efficiency via western blot.

The summary of this study is displayed in Figure 25 and shows that ATL-2 KD cells show a significant reduction of Atlastin-2 protein levels (B) when compared to Atlastin-2 levels in scrambled negative control cells. Sub-figures C and D show changes in transport rates for ER-ER and ER-NE measurements. The results of this transport assay nicely align with previously established data. Especially for ATL-2 knock-downs, a repeated tendency of significant reduction in ER-ER transport and a less pronounced reduction in ER-NE transport was observed across different experiments and cell lines.

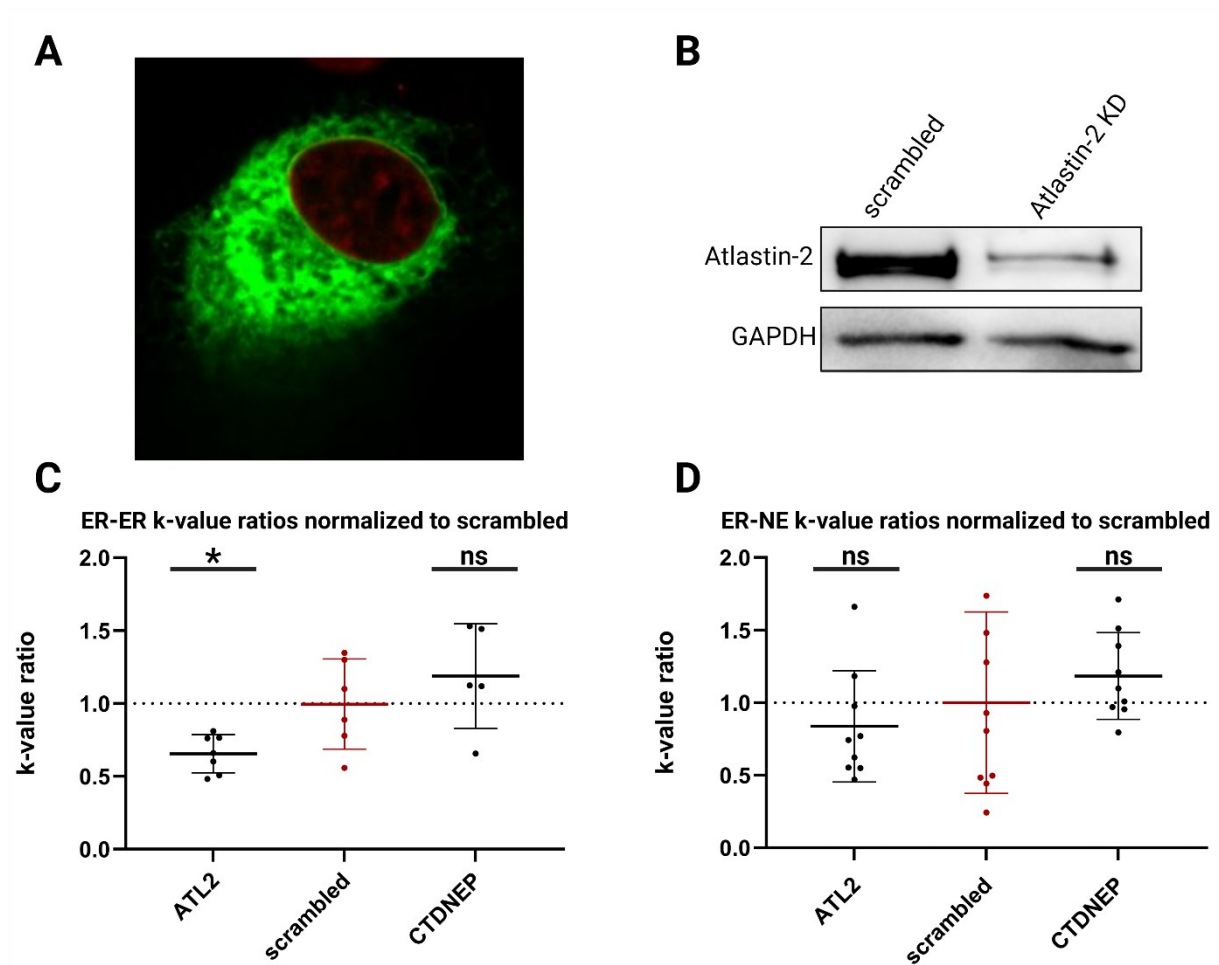


Figure 25. Proof-of-concept experiment verifying that the previously in HeLa cells established experimental setup for siRNA knock-down followed by a FRAP assay can be applied to RPE-1 cells.

(A) Shows a representative live-cell spinning disc confocal image of an RPE-1 cell taken 43x magnification. The cell has been transiently transfected with SP-moxGFP-KDEL, a soluble luminal reporter retained in the lumen of the ER and NE, 72h prior to imaging (green). The DNA was stained with SiR-HOECHST 1h prior to imaging (red). (B) Atlastin-2 (ATL-2) is sufficiently knocked-down after 72 h of incubation with an ATL-2 specific cy-3 labelled siRNA. The western-blot shows RPE-1 cells that were co-transfected with SP-moxGFP-KDEL and the target specific cy-3 labelled siRNA, either against ATL-2 or a scrambled negative. GAPDH served as a loading control. Antibody concentrations used: anti-GAPDH (mouse 1:10.000), anti-ATL2 (rabbit 1:1.000), secondary antibodies (1:2000). (C-D): FRAP assays of transiently transfected RPE-1 cell lines expressing SP-moxGFP-KDEL. Transfection with cy-3 labelled target siRNA was done 72h before imaging. Transfection of the reporter was done 48h prior to imaging. The recovery curve of each measurement was fitted to a one-phase association from which k-values can be extracted. All k-values are displayed as ratios normalized to the scrambled negative control k-values taken on the same day. All scrambled k-values have been combined and normalized to 1. Results from the individual gene knock-downs have been sorted in an ascending manner. Each dot represents one cell. Statistical significance was determined via a t-test for each sample versus the scrambled negative control. Significance levels are displayed as asterisks. * = $p < 0.05$, ns means not significant.

In contrast to ATL-2, CTDNEP knock-outs did not produce repeatable results and lacked statistical significance. While originally considered one of the more interesting candidate genes, it failed to deliver on that promise and a change in the experimental approach might be necessary to draw any meaningful conclusions.

3.3.8 Utilizing larger reporters to slow down transport measurements for more pronounced changes in transport kinetics

The iterative process of developing this FRAP based transport assay for analyzing the effects of targeted gene knock-downs has thus far shown that it is necessary to minimize the influence of external factors as much as possible and that the main difficulties encountered are the, at times, poor reproducibility of results and the poor statistical significance of which.

To combat issues that might stem from any variabilities caused by the experimental setup, we previously established that stable cell lines can be used to potentially minimize inconsistency caused by transiently transfecting reporter genes.

To address the poor statistical significance of results that have been generated to this point, we identified the two main components (aside from fluctuations in measured k-values) that negatively impact statistical significance of our results and what potential solutions might be:

Firstly, the low number of individual data points often is insufficient to generate significant changes in k-values. While the average change in k-value might be reasonably pronounced, a higher number of measurements would be needed to properly support these propositions suggested by the relative change in transport rate. This issue is difficult to address as transport measurements are time consuming and progressively harder to do, the longer a microscopy session lasts. The available time slots for using the required microscope are scarce. Additionally signal intensities progressively decrease due to the extended periods of high-intensity light exposure.

The second cause for low significance might be due to the nature of the small luminal reporter we used for transport measurements. While it was initially chosen for its fast diffusion rate through the ER, to allow for high throughput screening of multiple genes, this exact feature might also be the tradeoff when it comes to producing highly significant results. If the constraints, dictating the shape of ER-ER and ER-NE connections are altered, having a very small, highly mobile reporter might mask the resulting changes in morphology. Conversely, a large reporter might highlight these changes, which is why we decided on establishing an RPE-1 cell line which stably expresses the larger luminal reporter SP-NusA-moxGFP2-KDEL additionally to a cell line expressing the small luminal reporter SP-moxGFP-KDEL. In Figure 26 a schematic representation of the small luminal reporter (top-left) and the larger luminal reporter (top-right) as well as representative images capture in a live-cell spinning-disc confocal microscope are shown.

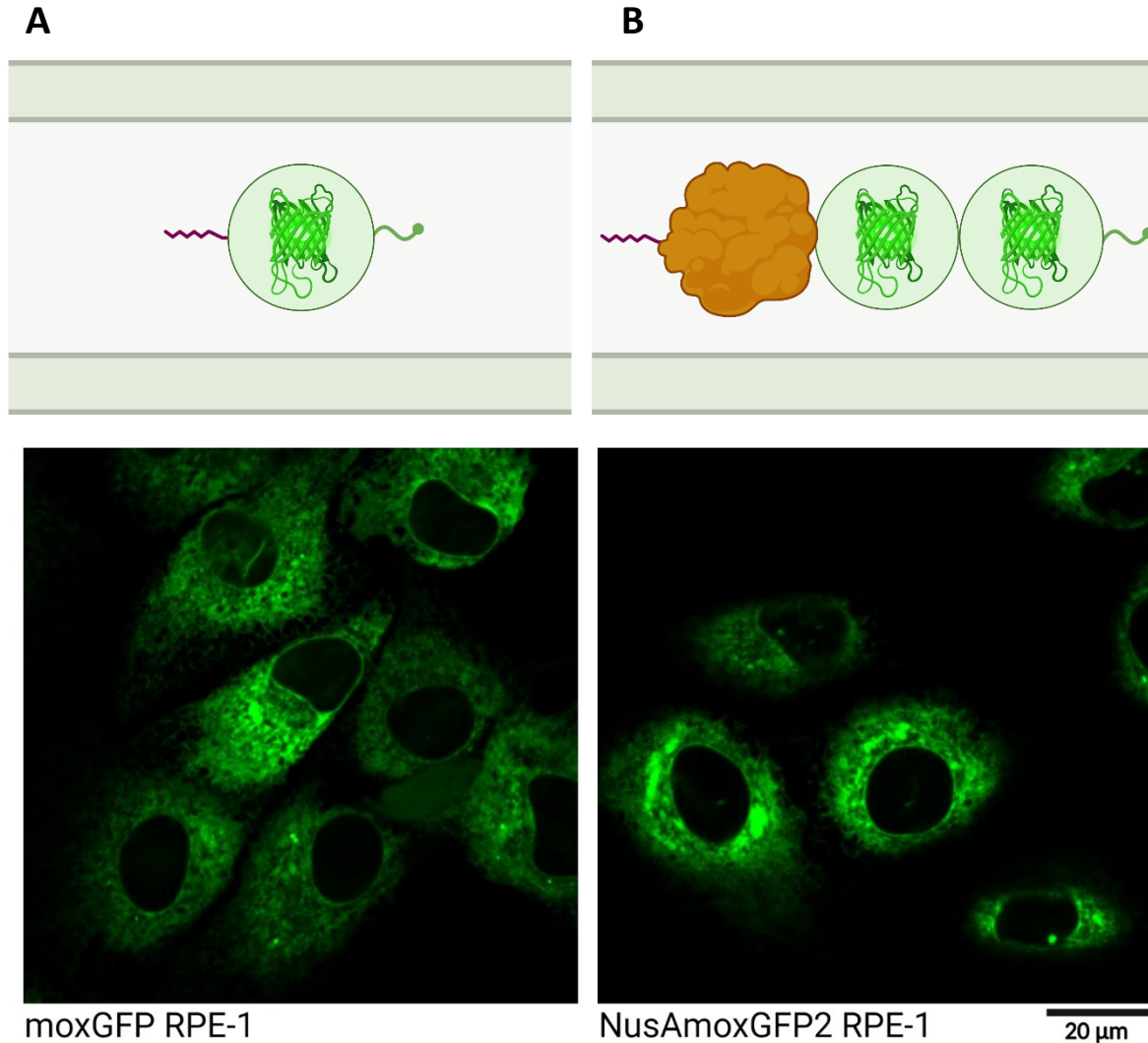


Figure 26. Overall cell morphology is not visibly different when comparing the two luminal reporters that were used. Schematic representations of the small luminal reporter (SP-moxGFP-KDEL ~30 kDa, top-left) and the large luminal reporter (SP-NusA-moxGFP2-KDEL ~120kDa, top-right). (A) stable RPE-1 cell line expressing the small luminal reporter SP-moxGFP-KDEL. (B) stable RPE-1 cell line expressing the large luminal reporter SP-NusA-moxGFP2-KDEL. Images were taken in equal conditions at the live cell spinning disc confocal microscope. Brightness and contrast were changed for both images individually. Generally, the large luminal reporter displays a significantly lower signal strength.

Having these stable RPE-1 cell-lines expressing two different luminal reporters at our disposal allows for a direct comparison between effect of gene-knock down when measured using reporters of different mobility. Additionally, the previously discussed advantage of using stable cell-lines minimizes potential external effects which leaves us with a great baseline for further RNAi screening.

3.3.9 FRAP transport assays using stable cell-lines expressing fast and slow ER luminal reporters

Based on previously generated data, we set up another round of RNAi screening using the newly established RPE-1 cell-lines expressing the small and large luminal reporters, SP-moxGFP-KDEL and SP-NusA-moxGFP2-KDEL. The difference in transport rate and the resulting recovery is shown in Figure 27. It is visible immediately that the small reporter takes only a fraction of the time to reach a plateau when compared to the larger reporter. The imaging interval was extended accordingly.

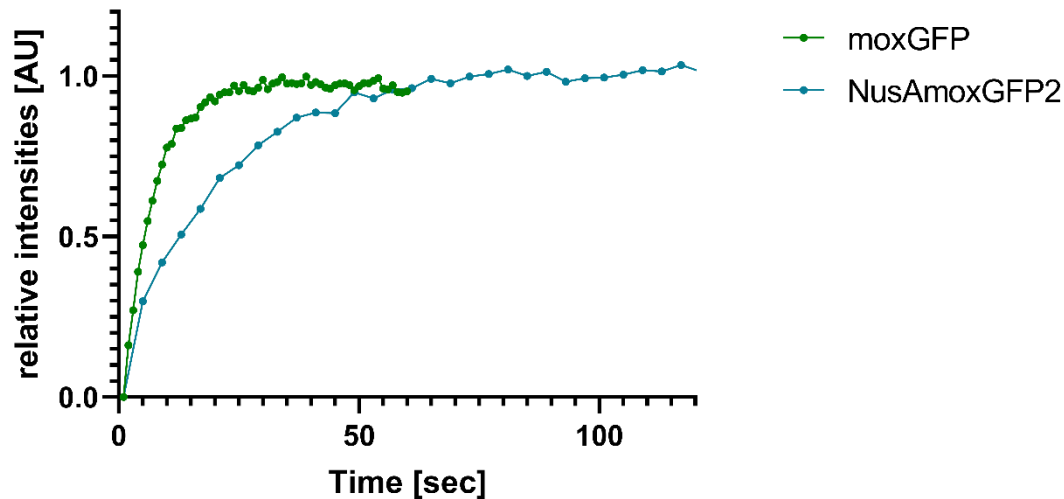


Figure 27. The small luminal reporter SP-moxGFP-KDEL displays significantly faster recovery kinetics over the larger one. When comparing the recovery curves one can clearly see that the green curve, representing the small luminal reporter, reaches its plateau within a fraction of the time it takes the large reporter to reach its plateau. Both curves were normalized to their plateau respectively. The interval of measurements for SP-moxGFP-KDEL was [1] second. The interval of measurements for SP-NusA-moxGFP2-KDEL was [4] seconds. Data was acquired from stable RPE-1 cell lines expressing the respective reporters which were additionally transfected with scrambled siRNA.

When looking at data generated from transport assays using these reporter gene expressing cell lines, we have shown that using larger reporters can indeed be a viable way of magnifying the changes in transport kinetic caused by gene knock-down, when measured using smaller reporters. In Figure 28 this is particularly well demonstrated by the results of ATL2 knock-downs. The reduction of k-value measured using the small luminal reporter seems to be even more pronounced when using NusAmoxGFP2. The results for SYNE3 and REEP4 are harder to interpret as significance levels are lacking. While SYNE3 could show a similar effect, where the change in transport kinetics is much more apparent in measurements performed using the larger reporter, we are careful not to overestimate meaningfulness of this observation. Nonetheless, the previously generated, repeatable results for ATL2 as well as the now established difference in measured k-values when comparing the reporters use give us reasonable confidence that this approach can be viable for certain genes.

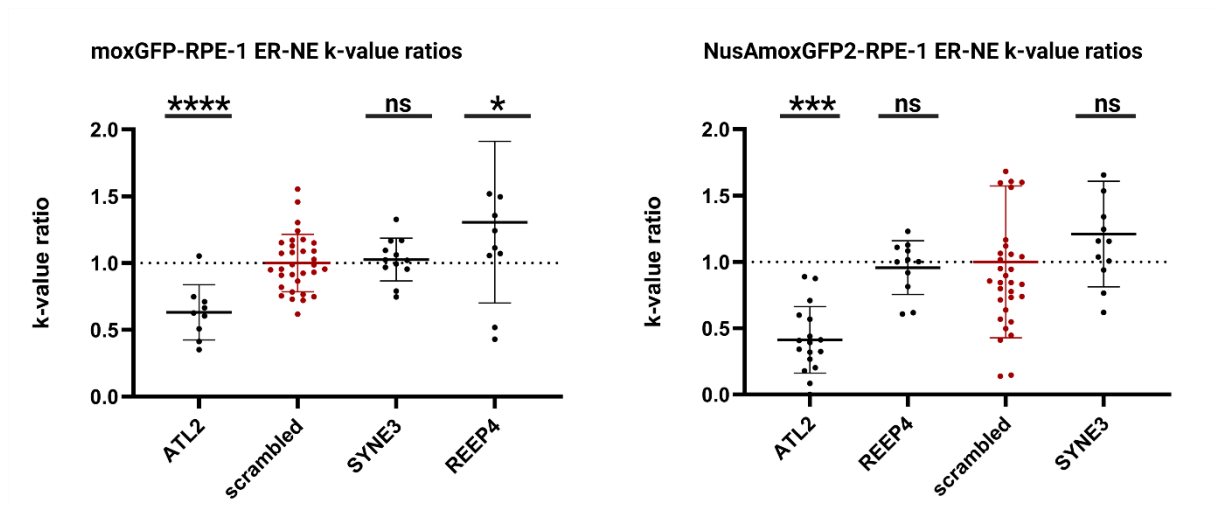


Figure 28. FRAP assay of stable RPE-1 cell lines expressing a small luminal ER reporter (SP-moxGFP-KDEL - left) and large-luminal ER reporter (SP-NusA-moxGFP2-KDEL - right) respectively.

Targeted siRNA knock-down was induced 72h prior to imaging. The large luminal reporter exhibits slower recovery speeds which can potentially magnify changes in the transport kinetics caused by the knock-down of target genes. This effect can be observed for ATL-2 and potentially also for SYNE-3 where the determined k-values show similar tendencies for both reporters but to a lesser extent in the cells expressing the small luminal reporter. All k-values are displayed as ratios normalized to the scrambled negative control k-values taken on the same day. All scrambled k-values have been combined and normalized to 1. Results from the individual gene knock-downs have been sorted in an ascending manner. Each dot represents one cell. Statistical significance was determined via a t-test for each sample versus the scrambled negative control. Significance levels are displayed as asterisks. * = $p < 0.05$, *** = $p < 0.0005$, **** = $p < 0.0001$ ns means not significant.

4. Discussion

The aim of this thesis was to build upon the foundation laid by previous members of the lab and refine the process of using RNAi to knock-down specific genes and subsequently quantify changes in transport rates across the ER and NE to a point where it can be used to create reliable and meaningful data. One of the biggest issues is the poor reproducibility of the transport assay, which prevented the reliable assessment of candidate genes. Here I will discuss the possible reasons for the poor reproducibility and propose potential improvements.

4.1 ER-NE recovery rates can be influenced by changes in peripheral ER morphology

The experimental setup was designed to allow relatively fast and efficient screening of various candidate genes to identify those playing a role in regulating ER to NE connections. Since not much is known about how these connections are maintained or whether they even are actively regulated in the first place, the experimental approach of investigating these questions is inherently riddled with shortcoming and trade-offs. And while we believe that the approach, we have chosen was the best option available to us at the time, we were aware that achieving highly precise data was always unlikely. As previously mentioned, we placed a lot of focus on minimizing inconsistencies and external factors. One of our key findings in this regard was that the recovery of signal in the nuclear envelope was directly dependent on the recovery rate within the peripheral ER as well as the shape of which. After complete photobleaching of the nuclear envelope, signal recovery is entirely dependent on influx of unbleached reporter proteins from the peripheral ER. This effectively dictates that changes in ER-ER recovery rate necessarily affect the recovery of the ER-NE signal. It is however not possible to determine to what extent these two recovery measurements are linked.

This is especially important when interpreting acquired FRAP assay data. If ER-ER and ER-NE recovery rates are directly proportional to one another, one must be particularly cautious about ascribing the observed effect of a gene knock-down on the recovery rate of ER-NE solely to changes caused to the morphology of said connections.

Further we discovered that the shape and size of the peripheral ER around the nucleus also have a measurable effect on ER-NE recovery rates. The corresponding data is depicted in Figure 22 and explains how signal recovery of the nuclear envelope gets increasingly slower as the ER extends further away from the nucleus. We can assume that this is simply caused by the increased distance reporters must travel in cells that have an extended ER network. Our method of determining the correlation between recovery rates (k-values) and the distance between the nucleus and furthest part of the ER is well suited to be used as a correction factor. If the determined correlation can be replicated across various data sets one could justify using it to correct previously acquired data for ER size and possibly extract more information from it.

4.2 Large reporter SP-NusA-moxGFP2-KDEL yields higher FRAP assay data quality

The main reporters used for FRAP transport assays were the small luminal reporter SP-moxGFP-KDEL and SP-NusA-moxGFP2-KDEL. At a size of roughly 30 kDa and 120 kDa respectively, reporter size is the main differentiator. Other than that, both carry an N-terminal signal peptide for translocation into the ER-lumen and a C-terminal KDEL ER-retention signal to prevent secretion.

The initial experimental setup was based around the idea that the small size of SP-moxGFP-KDEL was well suited for rapid screening of a large number of potential candidate genes that can be used as a baseline for following analyses. While this approach was sensible in theory we found that the resolution this yielded was most likely insufficient for drawing any meaningful conclusions for most candidate genes. This meant that among many external factors, the fast recovery displayed could be at the core of what is summarized graphically in Figure 21. Many transport measurements contradicted what has been found in previous runs but also lacked statistical significance.

Applying what we learned from these initial screenings, we could deduce that slowing the recovery down by means of using a larger reporter should highlight changes in transport kinetics as a consequence of changes in ER-NE morphology and deliver more meaningful results.

This turned out to be a promising path in the experiments performed as seen when looking at k-value ratios for ATL2 depicted in Figure 28. While both small and large reporters showed a similar tendency of ATL2 slowing down the ER-NE recovery, it is immediately noticeable that the large reporter appears to be significantly slower than the small reporter. The relative k-value normalized to scrambled is 0.631 for SP-moxGFP-KDEL and 0.412 for SP-NusA-moxGFP2-KDEL which equates a decreased recovery speed of roughly 35 %.

4.3 Stable cell lines reduce inconsistencies caused by cellular stress due to transient transfection.

Throughout the initial experiments, the reporter genes used were transiently transfected using lipofection 72-48 hours before imaging. Not only does this constitute a source of cellular stress but it also introduces inconsistencies due to reporters degrading at a non-steady rate. At the time of imaging signal levels across cells varied heavily which made selecting suitable cells difficult and subject to personal bias. Since the imaging parameters were kept unchanged for all samples, an ideal range for signal intensity was determined ahead of time ensuring minimal influence of bias. Nonetheless, it was noticeable how inconsistent the degradation of fluorescent protein was across individual imaging session.

To address this issue, we decided on establishing cell-lines that stably expressed the desired reporter genes by means of standard cloning techniques. Two different cell lines were used: HeLa wt and RPE-1, to create cell lines that stably express the small and large luminal reporters respectively.

These stable cell-lines provided steady signal intensities across most cells with hardly any outliers to be found. Proof-of-concept experiments (see Figure 23 and Figure 25) showed that previously established protocols could be adapted to suite the new cell-lines very well.

5.Outlook

Despite making significant progress in making the scientific approach of studying the ER-NE connections using a FRAP-based transport assay more viable, the overall outcome of the results generated is still to be treated with caution. Among other things, the repeatability of generated results has posed one of the greatest hurdles on our scientific journey of understanding how ER-NE might be regulated.

As previously mentioned, the most promising strategy of adapting the established method to generate more meaningful results appears to be the utilization of larger reporter genes for the transport assay. Slower moving reporters showed great promise in enhancing the detectability of changes in transport rates which can ultimately lead to highly significant results. Once highly promising candidate genes are identified, the next step would be to return to the very beginning of this project and look at whether and how knock-down of said gene affects the structure of ER-NE connections when observed through an electron microscope. Confirming a change in this specific structure or any other critical change in ER or NE morphology would constitute a breakthrough in this field and provide an excellent baseline for future research.

The Otsuka Research Group also worked on a non-targeted approach of identifying possible candidates using a biotin based proximity labelling technique, which will complement the targeted approach for identifying genes.

The correct formation of the ER and its proper connection to the NE ensures efficient cellular homeostasis which is crucial for distributing molecules throughout the cell and supplying the nucleus with all necessary proteins, lipids, and ions throughout its life cycle. Dysregulation of these processes however might very easily topple the balance of this process and potentially lead to a plethora of disease phenotypes. Not only do we expect issues to arise when transport is not facilitated quickly enough but given how the ER-NE connections appear to be narrower and more constricted than most ER junctions we can also speculate that transport can also be too fast. The importance of a viable nucleus for cell function can hardly be overstated and the number of diseases that could directly or indirectly be affected by altered regulation of ER-NE appears to be virtually limitless.

Understanding this connection might prove invaluable in ways that far exceed what is immediately obvious though. Many cellular processes are indirectly linked and therefore dependent on the ER. Examples for this include the involvement of the ER in protein quality control as well as signal transduction pathways critical for cell growth, differentiation, and stimulus-response. Any regulatory mechanisms at play might therefore be the basis for novel therapeutic strategies in medicine and drug development. Beyond disease, understanding ER-NE connection might further improve our understanding of aging and cell senescence as ER-maintaining systems such as the unfolded protein response (UPR) have been shown to be less efficient in ageing cells (Brown & Naidoo, 2012).

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