Poor old pores
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Chapter 3

Flexible and extended linker domains support efficient targeting of Heh2 to the inner nuclear membrane

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Abstract

The nuclear pore complex (NPC) is embedded in the nuclear envelope (NE) and forms the main gateway to the nuclear interior and to the inner nuclear membrane (INM). The majority of INM-localized proteins is small enough to pass the NPC by diffusion and is then retained in the nucleus. For two INM proteins in yeast, and likely one in human, a selective transport mechanism exists that requires metabolic energy input, nuclear transport receptors and an unprecedented sorting signal. The sorting signals consist of a nuclear localization signal (NLS), separated from the transmembrane domain by a long intrinsically disordered (ID) linker. Recent structural data of the yeast NPC reveals that the approximate distance between the pore membrane and the inner surface of the NPC central channel is about 25 nm. We studied the ID linker of yeast Heh2 and asked whether INM targeting requires a linker that is flexible, and that can span such a distance. We used computational models to predict the dynamic conformations of ID linkers and analyzed the INM targeting efficiency of proteins with artificial linker regions with altered Stokes radii and decreased flexibilities. We find that flexibility, Stokes radius and the frequency at which the linkers are at an extended end-to-end distance larger than 25 nm are good predictors for the localization of the proteins at the NE. The data is consistent with a transport mechanism where INM targeting of Heh2 is dependent on an ID linker that facilitates the crossing of the NPC scaffold.

Introduction

The nuclear pore complex (NPC) is embedded in the nuclear envelope (NE) and forms the main gateway to the nucleus. NPCs are roughly built of a scaffold structure stabilizing a cylindrical opening in the nuclear envelope, and attached to this scaffold is a set of proteins that are intrinsically disordered (ID) and extend into the channel of the NPC. The full structure of the *Saccharomyces cerevisiae* NPC was recently solved at a resolution of 28 Å (Kim et al., 2018). Now for the first time, we have full insight into the dimensions of the different substructures of this large, 52 Mda structure. The structure reveals that the distance between the pore membrane and the inner surface of the NPC central channel is approximately 25 nm (Figure 1a) (Kim et al., 2018).

NPCs mediate rapid and energy dependent transport between the nucleus and the cytoplasm. This is best understood for soluble proteins (Aitchison and Rout, 2012) and less so for integral membrane proteins of the nuclear envelope. Several mechanisms have been proposed for the sorting of membrane proteins to the inner membrane of the nuclear envelope (reviewed in Antonin et al., 2011; Blenski et al., 2019; Boni et al., 2015; Burns and Wente, 2012; Goodchild...
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e et al., 2015; Katta et al., 2014; Laba et al., 2014; Lusk et al., 2007; Meinema et al., 2012; Pawar et al., 2017; Zuleger et al., 2012). The biogenesis and sorting of membrane proteins starts in the cytosol with co-translational or post-translational insertion of proteins into the endoplasmic reticulum (ER) membrane. The membrane proteins then potentially roam the entire NE-ER network, as the membranes of the peripheral ER and the outer and inner nuclear membrane (ONM and INM) of the NE form a continuous system. To reach the INM, membrane proteins pass the NPCs where the INM and ONM are fused. The route through the \textit{S. cerevisiae} NPC is spacious enough for passage of membrane proteins with extraluminal domain sizes of 90 kDa and thus the majority of monomeric proteins may enter the INM through the NPC even in the absence of specific sorting signals (Popken et al., 2015).

Many proteins indeed reach the INM by diffusion through the NPC and their accumulation at the INM is through retention mechanisms (Holmer and Worman, 2001). However, there are two \textit{S. cerevisiae} membrane proteins, Src1/Heh1 and Heh2, and likely a third human membrane protein, whose localization in the nucleus are depending on an active transport mechanism involving transport factors, FG-Nups and the RanGDP/RanGTP gradient (King et al., 2006; Kralt et al., 2015a; Meinema et al., 2011). The sorting signal of Src1/Heh1 and Heh2 consists of a very potent nuclear localization signal (NLS) (King et al., 2006; Lokareddy et al., 2015; Meinema et al., 2011, 2013), and additionally a long, intrinsically disordered (ID) linker (Meinema et al., 2011). Already in the ER, the NLS ensures recruitment of the transport factor Kap95 via the adapter protein Kap60 (Meinema et al., 2013). The model proposed is that the complex then shuttles through the NPC by binding to the FG-Nups in the NPC; a transport mode for which energy input is provided from the gradient Ran proteins across the NE (King et al., 2006; Meinema et al., 2011).

The ID linkers in Heh1 and Heh2 are approximately 200 amino acids long (Meinema et al., 2011). Shortening the linker decreases the sorting of the proteins to the INM, while randomization of the amino acid sequence, which keeps the linkers ID nature, maintains full functionality (Meinema et al., 2011). Thus, the length but not the sequence of the linker is important for efficient import to the INM. Unique to ID structures is that they are highly dynamic, readily changing between very extended and more collapsed conformations, while the energy input that is required to stabilize a specific conformation is small (Fuxreiter, 2019; Galea et al., 2008; van der Lee et al., 2014; Necci et al., 2016; Uversky, 2017). We proposed two prospective roles for the linker in INM
targeting (Meinema et al., 2011, 2012, 2013). In the first, the linker dodges into the scaffold of the NPC, allowing the transmembrane domain to remain in the membrane, while the NLS reaches out into the central channel of the NPC. If the linker indeed functions to enter the NPC, we expect that the fraction of time spent in an extended conformation, as well as flexibility, may be important parameters. Additionally or alternatively, we proposed that the linker may increase the efficiency of recruitment of Kap60 and Kap95 by positioning the NLS into the cytosol (Meinema et al., 2011, 2012, 2013). For this, the linker is effectively a spacer and we expect that also less flexible structures, such as alpha-helical segments, may suffice.

In this study we used Heh2 linker mutants and reporter proteins with various artificial linker domains, to study the role of length, charge content and flexibility of the linker in INM targeting. We altered the linker properties by replacing the full, or part of the linker sequence with polyproline sequences, or α-helices. Shorter stretches of polyproline peptides and α-helices have been used as molecular rulers in FRET, because they form rigid helices (Arai et al., 2001; Schuler et al., 2005). Multiple consecutive prolines form structured helical regions, of which the left-handed polyproline II helix (PPII) with residues in the trans conformation is energetically most favourable (Moradi et al., 2009). However, in longer polyproline peptides (24 residues) some residues are in the cis conformation (Hanson et al., 2012). Thus, most probably long stretches of prolines are not a rigid all-trans PPII helix, but even so, such proline-rich sequences are much less flexible than the native ID linker domain. α-Helices are right handed helices formed by hydrogen bonding between the N-H group and the backbone C=O group amino acid located three or four residues apart in the protein sequence. Alanine shows particularly high tendencies to form α-helical structure. α-Helical structures can be further stabilized by the formation of salt bridges, such as between glutamate and lysine/arginine residues; this increases the rigidity of the helix (Marqusee and Baldwin, 1987; Olson et al., 2001). Single charged α-helical segments of up to 60 amino acids have been suggested to act as relatively rigid spacers between protein domains (Süveges et al., 2009), while shorter artificial α-helices in the form of up to five EAAAK repeats have been reported to form stable alpha helices that show an approximately 80 % helicity (Arai et al., 2001).

In this study we aimed to describe structural features of the ID linker that are important for targeting of Heh2 to the INM. We studied the nuclear import of membrane proteins that have the ID linker replaced by α-helical segments of
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defined lengths, and we modulate the conformational freedom of the ID linker by introducing polyproline stretches into the ID sequence. A coarse-grained computational model (Ghavami et al., 2013b) was used to gain insight in the structure and dynamics of different linker structures. We find that while also helical and polyproline linkers support transport of the membrane proteins, a flexible and extended linker structure supports the most efficient translocation to the INM. We discuss our findings in the light of a potential transport path through the NPC, keeping in mind that the linkers have to extend 25 nm from the membrane to reach across the scaffold of the NPC.

Results

The ID linkers that best support import match the dimensions of the NPC scaffold

We set out to answer how the length and dynamics of ID linkers relate to the structure of the yeast NPC as reported in (Kim et al., 2018) (Figure 1A). To span the distance from the membrane across the NPC the linker would need to reach 25 nm. Previously a series of reporter proteins with truncated and randomized linkers was made (Meinema et al., 2011). The reporter proteins consist of amino acids 93 to 378 from Heh2, corresponding to the NLS, the linker domain and the first transmembrane segment of Heh2. Mutant versions of this reporter encoded ID linkers with randomized sequences (LR1 and LR2) and three to four truncated versions of the native and randomized linkers. The amino acid composition in the randomized sequences (LR1 and LR2) and the native linker (L) were the same, but the sequences were randomized. The localization of this set of reporters was studied and quantified as the fluorescence intensity at the NE relative to the ER (the NE/ER ratio) (Meinema et al., 2011). Now, we used a coarse grained 1-bead-per-amino-acid molecular dynamics model, developed to predict the behavior of intrinsically disordered proteins (IDPs) (Ghavami et al., 2013a, 2014), to simulate the dynamic structure of each of the linker domains. The Stokes radius is a measure of how extended the linker is. In Figure 1b we plotted the localization of the different proteins at the NE as a function of the Stokes radius of their linker domain. Indeed, the calculated stokes radius and the measured NE/ER ratio show a striking correlation (Figure 1b).

The simulations were also analyzed in terms of the end-to-end distances that the linkers visit in time. We see, that the ID linkers are highly dynamic, they frequently change conformations (Figure 1c) and cover a large range of end-to-end distances (Figure 1c and Figure 1d). The ID linkers only very infrequently reach end-to-end distances larger than 25 nm (Figure 1d and Figure 1e).
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Notably, ID linkers that never reach this end-to-end distance do not support accumulation of the membrane proteins at the NE. We found that the NE/ER ratio of the different ID linkers was closely correlated to the percentage of time that the linkers spend in this most extended conformations of end-to-end distances larger than 25 nm (Figure 1f), while no such correlation was found with the average end-to-end distance of the ID linkers.

The correlation between NE/ER ratio and end-to-end distance follows a negative exponential decay function, meaning the function plateaus at an NE/ER just below 50, when the linker spends more than 10% of its time at an end-to-end distance larger than 25 nm. We observe that NE/ER ratios above the threshold cannot be quantified, because the ER is not visible in those samples. However, we also consider that the NE targeting efficiency of the reporter proteins will be limited at some point, when other factors than the linker domain become rate-limiting for the INM targeting (i.e. the availability of NTRs to bind to the NLS). We conclude that INM targeting is effective with a linker domain that can extend to end-to-end distances larger than 25 nm, which co-insides with the approximate distance between the pore membrane and the central channel.
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Figure 1 The time that ID linkers spend in an extended conformation at an end-to-end distance larger than 25 nm correlates with the accumulation of reporter proteins at the INM.

a) Based on the structure of the NPC (Kim et al., 2018), an end-to-end distance of 25 nm is needed to span the distance between the pore membrane and the central channel.
b) Correlation between NE/ER ratio of membrane proteins and Stokes radius of linker. NE/ER ratio of GFP-h2NLS-linker-TM reporters with different linkers from (Meinema et al., 2011) plotted against Stokes radius predicted with the coarse-grained MD model (Ghavami et al., 2013a, 2014). Truncations of native linker (L, white circles), randomized linker 1 (LR1, dark grey triangles) and randomized linker 2 (LR2, light grey squares). Error bars are SEM for NE/ER ratio and SD for Stokes radius.
c) Example of end-to-end distance simulation of the Heh2 native ID linker. The red line marks an end-to-end distance of 25 nm.
d) Plot showing the frequency at which the linkers extend to the different end-to-end distances.
e) Like d), but showing only the frequency, at which ID linkers reach end-to-end distances larger than 25 nm.
f) The percentage of time that ID linkers spend in their most extended conformations, larger than 25 nm, correlates with NE accumulation.
Reporter proteins with inflexible linker regions show decreased accumulation at the NE

The correlations found in Figure 1 are consistent with a role for the ID linker in bridging a certain distance from the membrane and it fits with the distance required to bridge the scaffold of the NPC. If the linker is indeed dodging into the NPC we expect that flexibility of the linker region might also be relevant to facilitate INM targeting. To alter the flexibility of the linker region, we designed eight different artificial linker domains: four linker domains with the same length, but increasing proline content and four linker domains with \( \alpha \)-helical linkers of defined length. We increased the number of proline residues in the linker region by replacing regions of the ID linker with stretches of 5 to 10 prolines, which decreases the charge content and causes a more collapsed structure because of reduced repulsive energy. The resulting linkers contained a total of 57 (LP50), 75 (LP70), 100 (LP100) and 149 prolines (LP149), the last having a continuous stretch of 149 proline residues. The alpha helical linkers consisted of 5 (HL5), 10 (HL10), 20 (HL20) and 30 (HL30) EAAAK repeats, respectively (Figure 2a). \( \alpha \)-Helices in the form of EAAAK repeats (Arai et al., 2001) form a stable \( \alpha \)-helix that give the linkers an extended, yet less flexible structure, while keeping the charge content within the linker region high. Each \( \alpha \)-helical linker has two proline residues at the C-terminus, to set the \( \alpha \)-helix apart from the transmembrane domain. The reporter proteins consisted of GFP, NLS, an (artificial) linker and the transmembrane domain of Sec61 (Figure 2a).

First, we confirmed that all reporter proteins were expressed, although at reduced levels with increasing polyproline content (Figure 2b) and that they are correctly inserted into the membrane (Figure 2c). We quantified the efficiency of NE targeting as the ratio of fluorescence intensity in the NE over intensity in the peripheral ER (NE/ER ratio). As expected based on their length in amino acids, HL5 and HL10 linkers were too short to support the accumulation of the reporter proteins at the NE. The longer \( \alpha \)-helical linkers (HL20 and HL30) showed similar accumulation at the NE (median NE/ER of 8.5 and 9.2, respectively) (Figure 2c and Figure 2d). We conclude, that proteins with helical linkers also enrich at the NE albeit with lower levels compared to the native linker. The NE/ER ratio decreased with increasing proline content, with an exception for the reporter with the all proline linker LP149, which showed an intermediate accumulation (NE/ER = 19.4 compared to 29 for the native Heh2-linker L)(Figure 2c and Figure 2d). In the cells expressing the reporter with the LP100 linker a small soluble fraction can be observed in the nucleus (Figure 2c). This may be a degradation product of the reporter, containing at least the NLS and GFP, but it does not interfere with the analysis since the ratio of fluorescence intensity in different membrane compartments is used. Thus,
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Reporter proteins with inflexible linker regions show decreased accumulation at the NE. The correlations found in Figure 1 are consistent with a role for the ID linker in bridging a certain distance from the membrane and it fits with the distance required to bridge the scaffold of the NPC. If the linker is indeed dodging into the NPC we expect that flexibility of the linker region might also be relevant to facilitate INM targeting. To alter the flexibility of the linker region, we designed eight different artificial linker domains: four linker domains with the same length, but increasing proline content and four linker domains with \( \alpha \)-helical linkers of defined length. We increased the number of proline residues in the linker region by replacing regions of the ID linker with stretches of 5 to 10 prolines, which decreases the charge content and causes a more collapsed structure because of reduced repulsive energy. The resulting linkers contained a total of 57 (LP50), 75 (LP70), 100 (LP100) and 149 prolines (LP149), the last having a continuous stretch of 149 proline residues. The \( \alpha \)-helical linkers consisted of 5 (HL5), 10 (HL10), 20 (HL20) and 30 (HL30) EAAAK repeats, respectively (Figure 2a).

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**Figure 2** Reporters with long linkers express and accumulate at the NE.

a) Graphical representation of Heh2 and reporter proteins, where G stands for GFP and S stand for the transmembrane domain of Sec61. Schematic overview of the composition of different
artificial linker domains used in this study. Red bars are proline residues, blue bars are charged residues, other amino acids are represented by black bars.

b) Upper panel: Western blot from lysates of cells expressing reporter proteins, showing full-length expression of all reporter proteins, except the LP149. Lower panel: In gel fluorescence of indicated reporter proteins, confirming full-length expression of reporter proteins (indicated by the arrowheads). Although, the LP149 was not detected on the Western blot, a full-length green fluorescent signal was clearly detected on the SDS-PAA gel. On a Western blot, the reporter proteins run with for GFP-fusion proteins characteristic double bands, one representing the fusion protein with unfolded GFP, while the GFP in the second band is still folded (Geertsma et al., 2008), consequently only one of the two bands is fluorescent in gel.

c) Deconvolved images of cells expressing reporter proteins with indicated linker. Scale bar 5 µm.

d) Quantification of NE/ER ratio. Box plot shows the median ratios of the fluorescence at the NE over that at the peripheral ER (NE/ER). The box shows 25th and 75th percentile of the data. The whiskers extend to the data points, which are closest to 1.5 times above or below the inter quartile range; data points above or below this region are considered outliers and plotted individually (not all outliers are within the plotted area). Individual measurements are shown as grey dots, n=30.

Membrane proteins with polyproline linkers are targeted to the INM

Having established that the reporter proteins with helical or polyproline linkers are expressed and localize to the NE, we addressed whether the proteins are actually localized at the INM and not just at the ONM. A first indication was obtained using the inducible Kap95-anchor away system. When reporters were expressed in the presence of rapamycin, Kap95-FRB traps at Pma1-FKBP in the plasma membrane, hereby disabling active import of Kap60/Kap95-dependent cargo through the NPC (Haruki et al., 2008; Meinema et al., 2011). The expression under these conditions caused those reporters that were previously shown to be NE accumulated reporters, to mislocalize to the peripheral ER resulting in NE/ER ratios around 1.5 (Figure 3a and Figure 3b), showing that the accumulation at the NE is Kap95-dependent. This is consistent with the hypothesis that the enrichment at the NE is the result of nuclear import and that the proteins are localized in the INM.

A second indication that the proteins are INM localized comes from assessing the viability of cells expressing the different polyproline reporter proteins (Figure 3c). Previously we have seen that overexpression of reporter proteins that accumulate at the INM causes a reduction in cell viability, while overexpression of reporters without the NLS or linker does not (Laba et al., 2015). We do not understand what causes the toxicity of excess INM-localized membrane proteins, but we use it to predict if the proteins localize at the INM. Indeed, the reporters with polyproline linkers also show lethality upon overexpression, and there is a clear correlation between the viability of the strains and the NE/ER ratios: the proteins with highest accumulation at the NE show the lowest viability (compare e.g. LP50 and LP100 in Figure 2c and
Figure 2d). Thus, the viability analysis predicts that the reporters proteins with polyproline linkers are INM resident.

Lastly, for a subset of the reporters we used quantitative immuno electron microscopy (iEM) to determine their localization in relation to the INM (Figure 3d). Primary anti-GFP antibodies were visualized with colloidal gold. To allow quantitative and unbiased analysis, hundreds of yeast cells were imaged using a semi-automated large-scale EM at high resolution (also known as nanotomy; (van Rijnsoever et al., 2008)). The gold particles were assigned, in a blind-folded fashion, as nuclear (N) or cytoplasmic NOW when within 75 nm of the NE inside or outside the nucleus, respectively, or as ambiguous when they were in the lumen or at membrane (indicated by the arrowheads in Figure 3d). The LP149 reporters, with and without NLS, were compared with the corresponding reporters with the native linker L. The large FKBP-GFP-2xMBP-Sec61 (F-G-M2-S) was used as an control for a protein that is excluded from the INM (Popken et al., 2015). Because expression of strongly NE-accumulated reporters led to deformed nuclei, which made identification of the NE and assignment of the gold particles as INM or ONM difficult, expression levels were decreased by fusing a FKBP domain to the N-terminus of the INM reporters (F-G-NLS-L-S and F-G-NLS-LP149-S). For each reporter at least 200 gold particles were counted, and percentages of N, C or ambiguous are shown in Figure 3e. The distribution of the LP149 reporters are similar to their native linker counterparts, which shows that the different linker does not greatly affect the localization of the reporters, and that the reporter with LP149 and NLS is indeed accumulated in the INM.
Figure 3 Reporters with polyproline linkers accumulate at INM.

a) Deconvolved images of cells expressing reporter proteins with indicated linker in the presence of rapamycin, which blocks active import. Scale bar 5 µm.

b) Quantification of localization of reporter proteins, in the presence of rapamycin, n=30.

c) Spot assay with cells expressing reporter proteins with indicated linker. Top panel: control plate, bottom panel: plate containing 0.5% D-galactose which induces expression.

d) Two selected examples showing reporter proteins as indicated with mainly INM or mainly ONM localization. Nucleus is indicated. Arrowheads show assignment of gold particles. Green rightward: nuclear; red leftward: cytoplasmic; white upward: ambiguous; blue downward: not assigned (too far from membrane). Scale bar is 0.25 µm.

e) ImmunoEM analysis to determine INM localization of h2NLS-LP149-Sec61. Percentage of gold particles counted at nuclear side (black), cytoplasmic side (grey) of the membrane, or ambiguous (lumen or on membrane, white) are shown, with number of gold particles counted.
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listed. F-G-M2-S was included as a reference sample as this protein is excluded from the INM (Popken et al., 2015). Analysis was done blinded.

Disentangling import and efflux efficiency

To interpret the NE/ER ratios or the iEM data in terms of nuclear import efficiencies, it is critical to consider all parameters that determine the steady state localization of the reporters. Most relevant are synthesis rates, retention mechanisms and the rate of efflux and import through the NPC. We first compared the rates of efflux between the membrane proteins with different linker regions. The Kap95-AA system was used to study the efflux of reporters from the INM (Meinema et al., 2011). As expected, when rapamycin and glucose were added to cells expressing G-NLS-L-S, a gradual decrease in NE/ER ratio was observed over time (Figure 4a) as proteins diffuse from the INM to the ONM and ER. The simultaneous addition of glucose halted expression, reducing the impact of newly synthesized proteins interfering with the measurements. In contrast, the NE/ER ratio for the reporter with LP149 did not decrease after blocking its import and stopping synthesis but stayed accumulated at the NE (Figure 4a). This shows that the reporter with an LP149 linker was either retained in the INM at a nuclear component, or it can not passively go through the NPC to the ONM and ER.

The lack of diffusion to the ER upon depletion of Kap95 prompted us to validate if the protein with LP149 is mobile at the INM. Fluorescence recovery after photobleaching (FRAP) measurements showed the mobility of G-NLS-LP149-S to be comparable with G-NLS-L-S (Figure 4b), indicating that the lack of re-localization to the ER is not due to retention but rather to an inability to efflux through the NPC. Considering that accumulation is the result of import and efflux (assuming synthesis and degradation rates to be much slower than import), a low efflux rate would result in a higher NE/ER ratio if import rates are the same. The lack of leak for the reporter with LP149 thus shows that also the import is less efficient with this linker when compared to the reporters with similar NE/ER ratios. We conclude that the LP149-reporter is actively imported to the INM at reduced rates compared to other linkers, and its efflux through the NPC is severely hindered by the all-proline linker.
Stokes radii and flexibility of polyproline linkers

Next, we addressed which properties of the polyproline linkers, stokes radius or flexibility, are responsible for the decreased import to the INM. The polyproline linkers are less flexible than ID linkers, but also the charge content is decreased, which might cause a more collapsed structure. The simulation predicts that the Stokes radius decreases with more prolines present, up to LP100 (Figure 5a). Snapshots show that the short proline stretches, that can form PPII helices, fold back on top of each other. Combined with the lower charge content this makes a more collapsed structure more favorable. Simulations for the LP149 revealed 2 distinct states a single folded conformation and a double folded conformation (Figure 5b). The double folded conformation has lower Stokes radius than the single folded conformation and has higher stability than the single folded conformation (Figure S1, Table S1). While the single folded conformation never reaches the 25 nm end-to-end distance, the double folded conformation does (Figure S2).

Complementing the simulations, we experimentally determined the Stokes radius of the LP50-linker. LP50 was expressed in Lactococcus lactis as a fusion protein with C-terminal TEV-cleavage site, GFP and His-tag. After purification the GFP and His-tag were cleaved off and the Stokes radius was determined by size-exclusion chromatography (Figure 5c). The experimental values of 47 Å are in good correspondence with the Stokes radius obtained in the simulations (45 Å ± 4). The NE/ER ratio measured for reporters with polyproline linkers, the native Heh2-linker and two truncated ID linkers (Meinema et al., 2011), was plotted against their simulated Stokes radius (Figure 5d). The plot shows a clear
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correlation between the hydrodynamic radius of the linker with the accumulation at the NE for the random linkers and the polyproline linkers, which indicates that a more extended structure is favorable for efficient import and not the assumed rigidity of the linker.

Figure 5 Stokes radii of polyproline linkers and the correlation with import.

a) Snapshots from modelling of indicated linker domains (see movies 1-4 in Supplements).
b) Snapshots from modelling showing the two distinct conformations assumed by LP149 during the simulations differing in the folding of protein. On the left panel we show the double folded structure corresponding to lower Stokes radius and larger end-to-end distance. The right panel depicts a single folded conformation corresponding to a larger Stokes radius and lower end-to-end distance.
c) Stokes radius determination of LP50. Top graph shows elution profile from gel filtration of LP50 after GFP was removed by TEV-cleavage. Fractions indicated were analyzed together with the loaded sample (S) on SDS-PAGE (inset). Arrowhead indicates LP50, asterisk labels uncleaved LP50-GFP. Bottom graph shows fractional volume of reference proteins (squares) and LP50 (cross) plotted against Stokes radius.
d) NE/ER ratio of reporters (G-NLS-linker-S) with different linkers plotted against the Stokes radius from the computational model (shown in the table, along with the indicated standard deviation). White square: L, black circles: proline linkers, grey triangles: LR1 truncations LR1(138), LR1(78). The Stokes radius for the LP149, represents the double folded conformation. Error bars are SEM for NE/ER ratio and SD for Stokes radius.
The NLS-linker sorting signal is required for INM localization of native Heh2

We next asked whether the artificial linker domains tested in the context of reporter proteins would have a similar effect on the localization of natively expressed full length Heh2. Since previous research suggested that the LEM domain could be a nuclear retention signal (Grund et al., 2008; Taddei and Gasser, 2012), we first investigated whether the NLS-linker sorting signal was actually sufficient for Heh2 accumulation at the INM. The GFP-Heh2 fusions were expressed under the native heh2 promoter from the endogenous genomic location. The deletion of the ID linker domains, as well as the deletion of the NLS resulted in loss of INM accumulation of Heh2, which is visible as reduced fluorescent intensity at the NE and the appearance of a visible ER signal at the cellular periphery (Figure 6a and Figure 6b). Deletion of the LEM domain of Heh2 did not interfere with the NE localization of Heh2 (compare GFP-Heh2 with ΔLEM) and the LEM domain was insufficient to promote INM accumulation of Heh2ΔNLSΔL. The signals from the natively expressed GFP-Heh2 and GFP-Heh2 mutants are low, especially at the ER, and do not allow for a quantitative analysis of the fluorescence levels at the NE and ER, like we could do for the reporters. Nevertheless, the qualitative data clearly show that the NLS-linker sorting signal is required and sufficient for INM localization of natively expressed Heh2.

Next, we exchanged the native Heh2 linker for the polyproline and α-helical artificial linkers and integrated the different Heh2 linker mutants into the genome in the heh2 locus, where they are expressed from the native heh2 promoter. In line with previous data on the importance of the length of the linker region, we confirm that the α-helical linkers of 5 and 10 EAAAK repeats (HL5 and HL10) are too short to support NE accumulation; these mutants behave like a Heh2ΔL. All other linker mutants show high accumulation of Heh2 at the NE, indistinguishable from the native ID linker (Figure 6c). We conclude that sufficiently long linker regions, including those with polyproline stretches or helical linkers, support localization of Heh2 at the NE.
Chapter 3

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We next asked whether the artificial linker domains tested in the context of reporter proteins would have a similar effect on the localization of natively expressed full length Heh2. Since previous research suggested that the LEM domain could be a nuclear retention signal (Grund et al., 2008; Taddei and Gasser, 2012), we first investigated whether the NLS-linker sorting signal was actually sufficient for Heh2 accumulation at the INM. The GFP-Heh2 fusions were expressed under the native heh2 promoter from the endogenous genomic location. The deletion of the ID linker domains, as well as the deletion of the NLS resulted in loss of INM accumulation of Heh2, which is visible as reduced fluorescent intensity at the NE and the appearance of a visible ER signal at the cellular periphery (Figure 6a and Figure 6b). Deletion of the LEM domain of Heh2 did not interfere with the NE localization of Heh2 (compare GFP-Heh2 with ∆LEM) and the LEM domain was insufficient to promote INM accumulation of Heh2ΔNLSΔL. The signals from the natively expressed GFP-Heh2 and GFP-Heh2 mutants are low, especially at the ER, and do not allow for a quantitative analysis of the fluorescence levels at the NE and ER, like we could do for the reporters. Nevertheless, the qualitative data clearly show that the NLS-linker sorting signal is required and sufficient for INM localization of natively expressed Heh2.

Next, we exchanged the native Heh2 linker for the polyproline and ω-helical artificial linkers and integrated the different Heh2 linker mutants into the genome in the heh2 locus, where they are expressed from the native heh2 promoter. In line with previous data on the importance of the length of the linker region, we confirm that the ω-helical linkers of 5 and 10 EAAAK repeats (HL5 and HL10) are too short to support NE accumulation; these mutants behave like a Heh2ΔL. All other linker mutants show high accumulation of Heh2 at the NE, indistinguishable from the native ID linker (Figure 6c). We conclude that sufficiently long linker regions, including those with polyproline stretches or helical linkers, support localization of Heh2 at the NE.

Discussion

Heh1 and Heh2 are targeted to the INM by a mechanism that requires metabolic energy and specific transport factors (King et al., 2006). The targeting sequence of these proteins is comprised of an NLS and an ID linker domain (Meinema et al., 2011). We set out to study the ID linker as an essential part of the targeting sequence of INM proteins and asked if effective sorting to the NE inner membrane requires a linker that can span the 25 nm distance from the pore membrane to the central channel of the NPC (Kim et al., 2018). We simulated the highly dynamic end-to-end distances of the linker domain (Figure 1). Our simulated data show that the ID linkers spend a small fraction of time at this very extended conformation. Strikingly, the fraction of time that they extend at an end-to-end distance larger than 25 nm is a good predictor for targeting efficiency of the respected reporter proteins. The data thus support the hypothesis that the linker is needed to bridge the distance between the membrane and the central channel of the NPC (Meinema et al., 2011).

We found that the membrane proteins with mutant linker regions of different flexibility vary in their import and efflux dynamics. With the shorter ω-helical linker regions (HL5, HL10 and HL20), we confirm that a minimal length of the linker sequence is required to support INM targeting (Meinema et al., 2011).
Also the polyproline linkers support the proposed mechanism. From the most extreme linkers, HL30 and LP149, we learn that in addition to an extended structure also other properties are important. Based on the absence of leak for the LP149, the accumulation of this linker should be higher than what would be expected based on its Stokes radius. Also, the helical linkers never support transport as effectively as the ID linkers (not even the long HL30). The explanation may be that the lack of flexibility, as well as the diameter (thickness) of the helix limits INM targeting of membrane proteins. Our data are consistent with a narrow or highly curved passage way for the linker through the NPC scaffold.

It remains elusive, why Heh1 and Heh2 are transported by this mechanism rather than by a diffusion retention mechanism. Both proteins are part of an ESCRT-III/Vps4 dependent mechanism that senses misassembled NPCs and enables them to be cleared via the proteasome (Webster et al., 2014). Heh1 specifically is needed to initiate the recognition of misassembled NPCs. Heh2 is required for the clearance of misassembled NPCs, as is indicated by the fact that the deletion of Heh2 causes the accumulation of misassembled NPCs (Webster et al., 2014, 2016). One aspect of the mechanism to detect misassembled NPCs is related to the compartmentalisation of Chm7 (Thaller et al., 2019a). Future studies should resolve if the highly efficient INM targeting of Heh1 and Heh2 is a mechanism for sensing the structural integrity of the NPC. The selection of mutants created here will provide a tool for future studies aiming to resolve how the special active transport mechanism may be important in the context of NPC quality control.

Materials and methods

Strains, plasmids & growth conditions

All strains used in this study are listed in Table 1. All experiments with reporter proteins were done in the KAP95-AA strain (Haruki et al., 2008; Meinema et al., 2011), with the exception of the iEM control sample F-G-2M-S which was expressed in strain Htb2-FRB (Popken et al., 2015). Plasmids (Table 2) were generated by standard molecular biology techniques and validated by sequencing; details are available upon request. Amino acid sequences of the linker used in this study are listed in Table 3. Cells were grown at 30°C in selective drop-out medium, supplemented with 2% D-raffinose. Reporter proteins were expressed under control of the GAL1 promoter by 2 h induction with 0.5% D-galactose. Rapamycin (LC laboratories, Woburn MA) was used at
Flexible and extended linker domains support efficient INM targeting of Heh2

a final concentration of 5 µg/mL where appropriate. Incubation with 1% D-glucose was used to stop expression. Spot assays were done using exponentially growing cells, diluted to 1x10^6 – 1x10^3 with drops of 4 µL on SD-His plates supplemented with 2% D-raffinose and 0.5% D-galactose or only 2% D-raffinose as a control.

Table 1 Strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>KAP95-AA (W303, MATa tor1-1 fpr1::NAT PMA1-2×FKBP12::TRP1 Kap95-FRB::KanMX)</td>
<td>heh2Δ</td>
<td>(Haruki et al., 2008; Meinema et al., 2011)</td>
</tr>
<tr>
<td>Htb2-FRB (W303 MATa; tor1-1; fpr1::NAT; Htb2-FRB::kanMX6)</td>
<td>heh2Δ</td>
<td>(Popken et al., 2015)</td>
</tr>
<tr>
<td>heh2Δ (MATa ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 YDR458CA::kanMX4)</td>
<td>heh2Δ</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>AS1(BY4742 heh2::GFP-HEH2-NAT)</td>
<td>GFP-Heh2</td>
<td>(Kralt et al., 2015b)</td>
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<tr>
<td>AS2(BY4742 heh2::GFP-HEH2(Δh2NLS)-NAT)</td>
<td>GFP-Heh2ΔNLS</td>
<td>(Kralt et al., 2015b)</td>
</tr>
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<td>(BY4742 heh2::GFP-HEH2(Δ146-299)-NAT)</td>
<td>GFP-Heh2ΔL</td>
<td>This study</td>
</tr>
<tr>
<td>(BY4742 heh2::GFP-HEH2(Δ1-92)-NAT)</td>
<td>GFP-Heh2ΔLEM</td>
<td>This study</td>
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<tr>
<td>(BY4742 heh2::GFP-HEH2(Δ93-299)-NAT)</td>
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</tr>
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<td>GFP-Heh2-HL10</td>
<td>This study</td>
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**Table 2** Plasmids

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Descriptive name</th>
<th>Source</th>
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<tbody>
<tr>
<td>pACM023</td>
<td>GFP-h2NLS-L-TM</td>
<td>pUG34, GAL1 promoter (instead of Met25), GFP fused to h2NLS-L-TM (Meinema et al., 2011)</td>
</tr>
<tr>
<td>pSI6</td>
<td>GFP-h2NLS-L-Sec61 (G-NLS-L-S)</td>
<td>pACM023, where the TM is replaced for Sec61 (Residues 25-471)</td>
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<tr>
<td>pSI7</td>
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<td>pSI6, where L is replaced for LP50</td>
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<tr>
<td>pSI8</td>
<td>GFP-h2NLS-LP70-Sec61 (G-NLS-LP70-S)</td>
<td>pSI6, where L is replaced for LP70</td>
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<tr>
<td>pSI9</td>
<td>GFP-h2NLS-LP100-Sec61 (G-NLS-LP100-S)</td>
<td>pSI6, where L is replaced for LP100</td>
</tr>
<tr>
<td>pSI11</td>
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<td>pSI20</td>
<td>GFP-ΔNLS-L-Sec61 (G-ΔNLS-L-S)</td>
<td>pSI6, where h2NLS is removed</td>
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<td>pAS004</td>
<td>FKBP-GFP-h2NLS-L-Sec61 (F-G-NLS-L-S)</td>
<td>pSI6 with N-terminal 2×FKBP12</td>
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<td>pPP037</td>
<td>FKBP-GFP-h2NLS-LP149-Sec61 (F-G-NLS-LP149-S)</td>
<td>pSI11 with N-terminal 2×FKBP12</td>
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</table>
Flexible and extended linker domains support efficient INM targeting of Heh2

Table 2 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Descriptive name</th>
<th>Source</th>
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<tr>
<td>pACM023</td>
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<td>pUG34, GAL1 promoter (instead of Met25), GFP fused to h2NLS-L-TM (G-NLS-L-TM)</td>
</tr>
<tr>
<td>pSI6</td>
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<tr>
<td>pSI7</td>
<td>GFP-h2NLS-LP50-LSec61</td>
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<td>GFP-h2NLS-LP50-SSec61</td>
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<tr>
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<td>pPP011</td>
<td>FKBP-GFP-2xMBP-Sec61 (F-G-M2-S)</td>
<td>(Popken et al., 2015)</td>
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<td>pPP037</td>
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<td>pSI11 with N-terminal 2×FKBP12</td>
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<td>pSI15</td>
<td>h2NLS-LP50-GFP</td>
<td>This study</td>
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<tr>
<td>pACM044</td>
<td>GFP-h2NLS-LR1(138)-Sec61</td>
<td>Adapted from pACM023, where the L is replaced by a randomized and truncated version of 138 residues and TM is replaced for full-length Sec61 (Residues 25-471)</td>
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<td>Table 3 Amino acid sequence of linkers (including h2NLS) used in this study. NLS sequence is underlined. First TM of Sec61 starts after this sequence.</td>
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<table>
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<td>L</td>
<td>VKDENVETNKRKREQISTDNEAKMQIQEEKSPKKRRKRRSS KANKPESPPQSSDKGKATSADLTSELETVEELHKDSSDDK PRVKEKPKELPNLVNEFLAQLNKELASAATENYDHSIKS TDLSSIRIEEPIVGSTGAETRNESEVMENINLEVQPEVKEA KEELTKISETFDECEDTSRLSSHKKNIRSPKGRTRHFIGARK VPYNQK</td>
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<td>LP70</td>
<td>VKDENVETNKRKREQISTDNEAKMQIQEEKSPKKRRKRRSS KANKPESPPQSVDASEESSPPPLQFPSPSKNNPPPEREPED PTELPPPPPVPVSYPPEDPDPPPEALPPPIEPRIEPPPPNPWPPP PR VDPPPPVPNRLGITSAPPPPPRELDAEPETLPPPPETNDNES LSKPPPPPSNTHEPEKDPPPPKMVPKLVGARKVPYNQK</td>
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<td>LP100</td>
<td>VKDENVETNKRKREQISTDNEAKMQIQEEKSPKKRRKRRSS KANKPESPPQSVDASEESSPPPLQQPSPSKNNPPPEREPED PTELPPPPPVPVSYPPEDPDPPPEALPPPIEPRIEPPPPNPWPPP PR VDPPPPVPNRLGITSAPPPPPRELDPPLPPPPETNDNES LSKPPPPPSNTHEPEKDPPPPKMVPKLVGARKVPYNQK</td>
</tr>
<tr>
<td>LP149</td>
<td>VKDENVETNKRKREQISTDNEAKMQIQEEKSPKKRRKRRSS</td>
</tr>
</tbody>
</table>
Western blot and in gel fluorescence

Native whole cell protein extracts were used for western blots and in gel fluorescence. Approximately 2.5 OD units from an exponentially growing culture were harvested by centrifugation. The cells were resuspended in 450 µL 50 mM Tris-Cl pH 7.4, 0.33 M sucrose, 1 mM EDTA, 1 mM DTT, 1:100 solP. Up to 500 µl of resuspended cells were added to the fast prep cups together with ~450 µl glass beads (BioSpec, 0.5 mm diameter). Cells were lysed using the FastPrep-24 classic grinder (mpbio) on a standard yeast setting. The cell lysate was separated from the glass beads by pinching the fast prep tubes with hot needles (top and bottom) and draining the liquid into a fresh Eppendorf cup by centrifugation. 55 µl 10x SDS sample buffer was added to the samples, which were left at RT for a few minutes, before they were centrifuged at max speed for 30s to get rid of aggregates. Samples were separated on SDS-PAGE. In gel
fluorescence was detected, using the Fluorescein filter of a ChemiDocXRS (Bio-Rad).

For western blots, the SDS-PAGE was transferred to a PVDF membrane (semi dry transfer). An anti-GFP antibody (Merck, Darmstadt, Germany) at a dilution of 1:2,000 (v/v) and a secondary with anti-rabbit-alkaline phosphatase conjugate at 1:10,000 (v/v) (Sigma-Aldrich, St. Louis, USA), was used for detection of the reporter proteins.

Fluorescence Microscopy
Imaging was done on a DeltaVision Deconvolution Microscope (Applied Precision) at 30°C, using InsightSSITM Solid State Illumination of 488 nm and an Olympus UPLS Apo 100x oil objective with 1.4NA. Detection was done with a CoolSNAP HQ2 camera. SoftWoRx software was used, and image-stacks were deconvolved using standard settings. Data was analyzed with open source software Fiji (Schindelin et al., 2012). The data plotted in figure 1 and originating from Meinema et al., were measured on a confocal microscope.

FRAP was done on a LSM780 NLO confocal microscope (Carl Zeiss MicroImaging, Jena, Germany) at 30°C, using a “PlanNeofluar” 63x/1.3NA CorrDIC water or glycerine immersion objective, 34channelQuasar detector and ZEN acquisition software. Measurements were done essentially as described in (Meinema et al., 2013). Data were analyzed using the ZEN2010B software package (Carl Zeiss) and fitted to diffusion equation for membrane proteins (Ellenberg et al., 1997).

Purification and Stokes radius determination of LP50
*L. lactis* NZ9000 (Kuiipers et al., 1998) was used as expression host for LP50. Liquid cultures were grown standing at 30°C in M17 medium (Oxoid, Hampshire UK) supplemented with 0.5% glucose and 5 µg/mL Chloramphenicol. Expression of LP50 was induced at OD 0.5 with 0.01% nisin supernatant for 4 h. Purification and Stokes radius determination of LP50 was done as was described for h2NLS-L in (Meinema et al., 2011).

Molecular dynamics simulations
Simulations are performed using a one-bead-per-amino-acid coarse-grained molecular dynamics model (Ghavami et al., 2013a, 2014). The model accounts for hydrophobic and electrostatic interactions and can differentiate between all 20 naturally existing amino acids. The force field parameters of proline are specifically calibrated against the experimentally measured end to end distance of polyproline segments (Schuler et al., 2005). The proteins are set at the fully extended conformation at the beginning of the simulations and are allowed to...
freely move and rotate through the medium during simulation (see movies 1-4 in Supplements). The Stokes radii are calculated according to the method explained in (Ghavami et al., 2013a), in which each protein is simulated for $10^7$ steps and the average Stokes radius for the generated conformations (excluding the first $10^6$ steps) is calculated using the HYDRO computer program (Carrasco and García de la Torre, 1999). The standard deviation of the Stokes radii (Figure 5) is calculated based on the generated conformations after the first $10^6$ steps. LP149 showed 2 different stable conformations (see Figure S1). Thus, in order to acquire relevant statistics, we performed MD simulations for 6 different starting configurations where each simulation was run for $5 \times 10^7$ steps long. The Stokes radius and the end-to-end distance data for these 6 realizations are shown in Figure S2 and S3 and the time-averaged Stokes radius and end-to-end distance data is discussed in Table S1 and Table S2.

**Immuno-Electron Microscopy**

Sample preparation essentially the same as published before (Yewdell et al., 2011). Cells for immuno-EM (iEM) were fixed in 2% paraformaldehyde (Merck, Germany) and 0.2% glutaraldehyde (Polysciences, Germany) in PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM MgCl2, 10 mM EGTA, pH 6.9), washed with PHEM buffer and incubated for 1h with 1% periodic acid in PHEM buffer. After washing the cell pellet was infiltrated with 12% gelatin (Sigma, Germany) in PHEM buffer. The sample was cut in blocks and incubated in 2.3 M sucrose in PHEM buffer overnight. The blocks were mounted on aluminum pins and put in liquid nitrogen. 70 nm thin sections were cut with a cryo-ultramicrotome (Leica UC7, Austria) and picked up with 2.3 M sucrose (J.T. Baker, the Netherlands) in 2% methylcellulose (Sigma, USA) solution. The sections were placed on copper grids (Veco, the Netherlands) coated with formvar (Sigma, USA) support film sputtercoated (Leica, Austria) with carbon. Grids were placed on a solid gelatin plate at RT (Sigma, Germany; 2% in 0.1 M phosphate buffer), which was placed in an 37°C incubator (30 min). Grids were washed with PBS/glycine (Sigma, Japan) and subsequently blocked with PBS/1 % BSA (Sanquin, Netherlands). Next, GFP was probed (Abcam, rabbit anti GFP; 1:200; 2 hours), washed with PBS/0.1 % BSA and incubated with protein A gold (purchased from G. Posthuma, UMC-Utrecht; 1:50 for 30 minutes). After washing with PBS, grids were post-fixed with 1 % glutaraldehyde in PBS. After washing with MilliQ water the samples were contrasted with uranylacetate-acetate (4% uranylacetate in water, 3.8 % oxalic acid, pH 7) for 5 minutes and methylcellulose/uranylacetate (9 ml 2 % methylcellulose, 1 ml 4 % uranylacetate) for 5 minutes on ice.
Flexible and extended linker domains support efficient INM targeting of Heh2

Data was recorded using the Supra55 scanning electron microscope at 29 kV (SEM; Zeiss, Oberkochen), at 2.5 nm pixel size using the transmission detector essentially the same as described before (Sokol et al., 2015). Large-area scans were generated using the external scan generator ATLAS (Fibics, Canada) and tiles were stitched using Vviewer (Fibics) and exported both as a single TIF and a high-resolution html file, which contains the raw data and is open-access available via www.nanotomy.org. (Currently access is through: www.nanotomy.org/OA/Popken2015/index.html. Will be changed when data is published to www.nanotomy.org.)

Acknowledgements
This work was supported by the University of Groningen, the European Research Institute for the biology of Ageing, the Zernike Institute for Advanced Materials, the Ubbo Emmius Fund and the Netherlands Organization for Scientific Research (ALWOP.2015.053). Part of the work has been performed at the UMCG Imaging and Microscopy Center (UMIC), which is sponsored by NOW-grants 175-010-2009-023, ZonMW 91111.006 and STW 12718.
Supplementary Information:
The Stokes radii and end-to-end distance analysis for the LP149 revealed 2 distinct states (i) single folded conformation and (ii) double folded conformation. The single folded conformation has higher Stokes radius and lower end-to-end distance. On the other hand, the double folded conformation results in lower Stokes radius and larger end-to-end distance. Furthermore, Figure S2 and Figure S3 clearly show that the double folded conformation has higher stability as it is observed in all 6 realizations, whereas the single folded conformation is a local energy minimum where the protein resides for some time before assuming the more stable double folded conformation.

Figure S1: The Stokes radius of LP149 plotted as a function of time for 6 different initial configurations. The Stokes radius data for the three simulations on the top panel illustrates 2 stable states i.e., (i) larger Stokes radius (~ 46-47 Angstrom) corresponding to single folded conformation and (ii) lower Stokes radius (~ 41 Angstrom) corresponding to a double folded conformation. The data in the bottom panel show one stable state corresponding to a double folded conformation with Stokes radius ~ 41 Angstrom.
Supplementary Information:
The Stokes radii and end-to-end distance analysis for the LP149 revealed 2 distinct states (i) single folded conformation and (ii) double folded conformation. The single folded conformation has higher Stokes radius and lower end-to-end distance. On the other hand, the double folded conformation results in lower Stokes radius and larger end-to-end distance. Furthermore, Figure S2 and Figure S3 clearly show that the double folded conformation has higher stability as it is observed in all 6 realizations, whereas the single folded conformation is a local energy minimum where the protein resides for some time before assuming the more stable double folded conformation.

Figure S1: The Stokes radius of LP149 plotted as a function of time for 6 different initial configurations. The data for the three simulations on the top panel illustrates 2 stable states i.e., (i) larger Stokes radius (~ 46-47 Angstrom) corresponding to single folded conformation and (ii) lower Stokes radius (~ 41 Angstrom) corresponding to a double folded conformation. The data in the bottom panel show one stable state corresponding to the double folded conformation with end-to-end distance ~17 nm.

Figure S2: The end-to-end distance of LP149 plotted as a function of time for 6 different initial configurations. The data for the three simulations on the top panel illustrates 2 stable states i.e., (i) lower end-to-end distance (~ 8 nm) corresponding to single folded conformation and (ii) larger end-to-end distance (~ 17 nm) corresponding to the double folded conformation. The data in the bottom panel show one stable state corresponding to the double folded conformation with end-to-end distance ~17 nm.

Table S1: The time averaged Stokes radius (in Angstrom) of LP149 for 6 different initial configurations. The column “Total” corresponds to the Stokes radius averaged over entire simulation time of 1 μs. The Stokes radius listed under column “Single folded” and “Double folded” corresponds to the time averaged Stokes radius in the corresponding time domain where these conformations are observed.

<table>
<thead>
<tr>
<th>Realizations</th>
<th>Total</th>
<th>Single folded</th>
<th>Double folded</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>41.81</td>
<td>46.20</td>
<td>40.72</td>
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<tr>
<td>2</td>
<td>45.07</td>
<td>46.89</td>
<td>40.79</td>
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<tr>
<td>3</td>
<td>42.44</td>
<td>46.30</td>
<td>40.74</td>
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<td>4</td>
<td>40.88</td>
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<td>40.88</td>
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<tr>
<td>5</td>
<td>40.99</td>
<td>NA</td>
<td>40.99</td>
</tr>
<tr>
<td>6</td>
<td>40.94</td>
<td>NA</td>
<td>40.94</td>
</tr>
<tr>
<td>Average</td>
<td>42.02</td>
<td>46.46</td>
<td>40.84</td>
</tr>
<tr>
<td>Error (Std)</td>
<td>1.62</td>
<td>0.37</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Flexible and extended linker domains support efficient INM targeting of Heh2.
Chapter 3