Import to the inner nuclear membrane
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2016

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 03-06-2019
Chapter 5

On the relationship between membrane protein import, nuclear envelope deformation and cell viability.

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Author contribution:
RAH and LMV designed the study. Experiments were performed and analyzed by RAH with help of MvR. RAH and LMV wrote the chapter.
Introduction

The overexpression of Heh2 or Heh2-derived reporter proteins that are targeted to the inner nuclear membrane affects viability and causes nuclear envelope (NE) deformation. Specifically, cell viability and NE integrity are only compromised when the NLS and an ID linker are encoded, the sorting signals responsible for targeting to the INM. Also, the expression of reporter proteins that are impaired in transport to the INM such as those with shorter or less flexible linkers (Popken et al, 2015), and those larger extraluminal domains (Laba et al, 2015) are less toxic to the cells. In both cases, the proteins that accumulate more at the nuclear envelope relative to the endoplasmic reticulum, indicative of INM localization, are more toxic. The fact that the effects on viability are so specific to INM localized proteins, rather than being a general overexpression effect, gives confidence that the cellular response may reveal some of Heh2’s functions. In the present chapter we thus study the effect of overexpression of Heh2-derived INM proteins on NE deformation and cell viability.

Results

The growth defect related to the expression of Heh2 and Heh2-derived proteins is dependent on a functional NLS signal

First, we establish that the viability of cells expressing Heh2 or the Heh2-derived reporter proteins depends on the potency of the NLS encoded on the membrane protein (Figure 5.1). The h2NLS is weakened in the reporters by mutation of the NLS residues that bind at the P2 and/or P2’ positions of the major and minor bindings sites of Kap60 (Lokareddy et al, 2015). Particularly the P2’ and P2P2’ mutants are mislocalized. Expression of these mutants is also least toxic to the cell, both in the context of full length Heh2 and the reporter protein. This correlation is not observed for the soluble reporter. Here, expression of the reporter proteins does not affect the viability, regardless of the nuclear accumulation level. Altogether, we consistently observe a correlation between viability of the cells and the presence of overexpressed membrane proteins that localize and concentrate at the INM.
Figure 5.1. Cell viability of strains overexpressing Heh2-derived reporter proteins. (A) Cell viability of cells expressing indicated Heh2-derived reporter proteins composed of GFP fused to Heh2(93-378) encoding the h2NLS, the ID linker and the first transmembrane segment as indicated in the cartoons. GFP in green, LEM (Lap2, emerin, Man-1)-family domain in pink, bipartite h2NLS in red, long intrinsically disordered linker in black curved line, lumenal domain in black line, TM domain in black cylinder and MCHD (Man1-C-terminal Homology domain) of Heh2 in blue. P2 and P2’ indicate an a mutation of arginine or lysine to Alanine at residues K126 and R103, respectively. 5 µL cells suspension of 10 fold serial dilutions were spotted on a SD-Ura plate+2% raffinose supplemented with and without 0.1% galactose and grown for 2 days at 30°C. (B) Same as A but with full length Heh2. (C) same as A but with a soluble reporter. (D) Confocal microscope images of cells expressing the GFP-tagged Heh2-derived reporter proteins. Scale bar is 5 µm.

The effects on viability are likely expression level dependent and hence we test several combinations of strains and promoters. In Figure 5.2A we established that the highest lethality is seen with W303 and when expressing from the GAL1 promoter while no growth defect is observed with the cupper promoter in DF5 or W303. We excluded a direct effect of the presence of cupper on viability, the cupper somehow favorably compensating for the negative effects of the expression of INM proteins (Figure 5.2B). The expression levels of the reporter proteins in the different expression systems are shown in (Figure 5.3). They do not explain the dramatic differences in viability, as for
example the expression levels of in W303 with the ADH1 or GAL1 promoters is similar while their viability is very different. We conclude that the differences in expression level cannot explain the dramatic differences in viability.

![Image of Figure 5.2]

Figure 5.2. Viability of DF5 and W303 strains expressing Heh2-derived reporter proteins from different promoters. (A) Viability of DF5 and W303 strains expressing GFP-h2NLS-L-TM from the constitutive ADH1 promoter and the inducible GAL1 and CUP1-1 promoters. Induction was with 0.1% galactose or 100 µM CuSO4 in the plates. (B) Effect of the presence Cu2+ and galactose on cell viability for cell that expressed GFP-h2NLS-L-TM under GAL1 and CUP1-1 promoters.

The growth defect related to expression of Heh2-derived proteins is not alleviated by overexpression of KAP60

As we observed that the lethality is directly related to the NLS sequence (Figure 5.1), and knowing that the h2NLS sequence has a high affinity for Kap60 (Lokareddy et al, 2015), we next ask if the viability effects are mediated through Kap60. A possible scenario is for example that the overexpression of the reporter proteins causes depletion of cytosolic Kap60, due to a lack of recycling of Kap60 to the cytosol, and this may impact import of critical components. To address this we perform a
complementation assay where we ask if exogenous overexpression of Kap60 may relieve the growth defect.

We co-expressed GFP-h2NLS-L-TM from the GAL1 promotor and Kap60-HA from a doxycycline-repressible Tet-off promotor in W303 and observed no rescue of the growth defect on plates (Figure 5.4). As a control, the induction of Kap60-HA alone has no effect on growth. (Figure 5.4). We then tested co-expression of Kap60 from the GAL1 promotor because this may result in higher expression levels of Kap60 as compared to the Tet-off promotor. Also, here we observed no rescue (compare Figure 5.4 to figure 5.2A).
Using growth assays in liquid cultures we could control the timing of the induction of the membrane protein and Kap60. Here we found that when Kap60 is overexpressed (Tet off, no doxycycline) before induction of GFP-h2NLS-L-TM, the growth is more affected as compared to when they were are expressed simultaneously with the GAL1 promoter (compare dash (-) and open circle lines in figure 5.4A). This suggests the timing of the expression of Kap60 and the membrane protein may be an important parameter for cell viability, although effects of the different expression kinetics and levels from the Tet-off and GAL1 promoters can also play a role. Regardless these subtle effects we conclude that depletion of Kap60 is unlikely to be a major cause of the growth defect.

Figure 5.4 Overexpression of Kap60 fails to rescue the growth of cells expressing GFP-h2NLS-L-TM under GAL1 promoter in W303 background. (A) Growth curve of co-expression GFP-h2NLS-L-TM and Kap60 under GAL1 and Kap60-HA under Tet-off promoter. Culture were grown for 16h at 30°C in a microtiter plate. OD600 was taken every 30 minutes. (B) Growth assayed on plates by splotting serial dilutions of a culture of cells co-expressing GFP-h2NLS-L-TM and Kap60 or Kap60-HA from the GAL1 or Tet-off promoter.
The growth defect from expression Heh2-derived proteins may be related to deformation of the NE

As a next entry into the problem of what is negatively affecting viability upon overexpression of Heh2-derived proteins we look at the NE morphology. We noted that upon expression of GFP-h2NLS-L-TM, the NEs appear deformed in fluorescence microscopy images. These deformations are not observed when overexpressing the same proteins lacking the NLS or ID linker. They are also not observed when expressing soluble variants that lack the transmembrane segment. There is thus a relationship between overexpression of a membrane protein that localizes to the inner membrane having an NLS-L sorting signal and nuclear envelope deformation. The nuclear envelope deformation may have more indirect effects on cell viability, for example by a disturbed chromatin organization at the NE periphery.

We first confirm on a population level that longer expression of GFP-h2NLS-L-TM results in an increase in the mean fluorescence intensity in the nucleus measured in a population of cells and simultaneously one observes more deformed nuclei (Figure 5.5C). The cells with deformed nuclei also have on average a higher fluorescence intensity than those cells that have a round NE. Consistently, expression of the NLS mutant, P2', impaired in INM import (Lokardedy et al, 2015), showed fewer deformed nuclei, even after prolonger expression of the protein. Altogether the data indicate that the NE is deformed when high concentrations of the Heh2-derived proteins appear at the INM.

To assess the impact of NE deformation on the cell cycle on a single cell levels, we follow the cells in a microfluidic device (Lee et al, 2012) after induction of the expression of GFP-h2NLS-L-TM. Images from such an experiment are shown in Figure 5.5B. We observe that the expression of GFP-h2NLS-L-TM protein interferes with progression through the cell cycle as follows: After 1 hour of induction, the cells annotated with A and 1 still have round nuclei. When entering G1/M, the nucleus of Cell 1 starts to deform (t=9) and it remains deformed. It then takes 120 minutes to complete its first cells division after induction (t=5-11). Also the nucleus of cell A deforms and cell A also takes 120 minutes to divide (t=9-15). When one hour later Cell 1 enters a second round of cell division it fails to pass the nuclei to its second daughter. Meanwhile the size of daughter cell increases (t=15-35). Cell A does go through a second cell cycle (starting t=19) although also much later than for wild type cells. It took 180 minutes to
complete that second cell cycle, which is approximately 1 h longer than a normal cell division.

Figure 5. 5 Expression level of GFP-h2NLS-L-TM but not GFP-h2NLS P2'-L-TM induced nuclear shape changes. (A) Confocal images of cells with normal nuclei (round shape; expressing GFP-h2NLS-L-TM P2') and deformed nuclei (expressing GFP-h2NLS-L-TM). B) Time lapse images from Delta Vision microscope at 20 minute time intervals of cells expressing GFP-h2NLS-L-TM after induction with 0.1% galactose at t=0. Parental cells are indicated A and 1. (C) Quantification of the mean intensity of GFP-h2NLS-L-TM and GFP-h2NLS P2'-L-TM in cells with round or reformed nuclei at indicated time points after start of induction. The red bar indicates the percentage of round and deformed nuclei at each time point; the blue bar indicates the mean intensity (AU).
Altogether these single cell observations confirm what was shown with growth assay, that the expression of the GFP-h2NLS-L-TM causes problems in cell division. What we learn additionally is that the nuclear deformation is visible after approximately 1 hour of induction, and more important, nuclear division and migration is impaired when overexpressing GFP-h2NLS-L-TM.

Conclusions

In the present chapter we studied the effect of overexpression of Heh2-derived INM proteins on NE deformation and cell viability. The experiments using point mutants of the NLS of Heh2 confirm that the overexpression of Heh2 or Heh2-derived reporter proteins affects only the viability of the cells in cases where the proteins accumulate at the INM. The reduced viability is not a general overexpression artifact as a clear-cut correlation between expression level and viability is not apparent. Moreover, the effects on cell viability are specific for membrane proteins that encode the wild type NLS. The growth defect is characterized by an arrest in the cell cycle at a stage before nuclear division.

Knowing still relatively little about the function of Heh2, we here tested two possible reasons for the reduced viability. First, we rule out that the overexpression of Heh2-derived reporters results in competition for available Kap60 in the cell such that nuclear import of essential nuclear proteins is reduced. Second, we establish that there is at least a correlative relationship between NE deformation and cell cycle arrest. At this point an explanation where the disorganized NE directly interferes with nuclear division and migration is less likely, as we identified mutants that can rescue the growth defect while still maintaining the NE deformation (Anton Steen, unpublished data). A possible explanation is that the presence of high levels of GFP-Heh2 or GFP-h2NLS-L-TM interferes with the role of Heh2 in in NPC quality control (Webster et al, 2014), which may signal a cell cycle arrest. Several alternative explanations may apply and future studies will be required to solve this.

Material and methods

Plasmids construction
For the construction of pADH1-GFP-h2NLS-L-TM (pRAH36), first the additional XbaI site at the end of GFP sequence was removed by introduction of a silent mutation at pRAH15 resulting pRAH42 (not listed). Then the GAL1 promoter sequence from pRAH42 was replaced it with the ADH1 promoter sequence fragment from pYMN-6 (Janke et al, 2004) using XbaI and SacI. GFP-Heh2 FL mutants (P2 and P2') were generated by site directed mutagenesis using methods explained in chapter 3. All other plasmids were published in the Meinema et al, 2011, Lokkaredy et al 2015 and explained at the previous chapter (chapter 4). The plasmids for overexpressing Kap60 and Kap60-HA under GAL1 and Tet-off promoters were gifts from David Goldfarb (University of Rochester).

Table 2. List of plasmid and yeast strains

<table>
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<tr>
<th>Descriptive name</th>
<th>Plasmids name</th>
<th>Description</th>
<th>Sources</th>
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<tr>
<td>GFP H2NLS-L-TM P2' (R103A)</td>
<td>pRAH16</td>
<td>pRAH15 containing mutation in arginine pos. 103 to alanin</td>
<td>Lokkareddy et al, 2015</td>
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<td>GFP H2NLS-L-TM P2 (K126A)</td>
<td>pRAH17</td>
<td>pRAH15 containing mutation in lysine pos. 126 to alanin</td>
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<td>GAL1 promoter sequence is replaced by ADH1 promoter</td>
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<td>pCUP1-1 GFP-h2NLS-L-TM</td>
<td>pRAH37</td>
<td>GAL1 promoter sequence is replaced by ADH1 promoter</td>
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<td>Gift from David Goldfarb</td>
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<td>HA-tagged Kap60, expressed under inducible Tet-off promoter and URA3 as a marker.</td>
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<td>W303, matα tor1-1 fpr1::NAT PMA1-2xFKBP12::TRP1</td>
<td>Haruki, 2008</td>
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Microscope

All the cell cultures used were grown at mid-exponential growth in SD-min without Ura/His/His,Ura supplemented by 2% raffinose. For overexpression of the reporter, cells were induced by addition of 0.1% Galactose or CuSO4 150 µM (final concentration) for 90’ before imaging, unless indicated otherwise (Fig 5.5). Images were taking taken from two different microscopes, the Confocal Microscope and Delta Vision. Data acquisition for each LSM 710 confocal microscope used identically setting as explained in chapter 3 and chapter 4. Data analyses at figure 5.5 were done (Carl Zeiss MicroImaging) using ZEN2010B package by measuring total GFP intensity of individual cell showing the normal and deformed nuclei. an objective C-Achromat 403/1.2NA, a solid-state laser (488 nm) for excitation, and a pixel dwell times of 101–177 ms. For wide-field deconvolution microscope (DeltaVision; Applied Precision/GE Healthcare) taking 60 x 0.2 mm sections, equipped with a 1003, 1.40 NA objective lens and solid-state illumination; deconvolution was performed using Softworx, ten iterations, and medium noise filtering. The images were acquired using a CCD camera (CoolSNAP HQ2; Photometrics). Analysis of data presented in figure 5.5 was done with the ZEN2010B package by measuring total GFP intensity of individual cells with normal or deformed nuclei.

Western blot

Cells were grown identical as for microscope imaging and harvest at OD600 1. Cytocoll crude extract were prepared according protocol of Barends et al, 2000. 7.5 µL sample were loaded into 10% SDS-PAGE gel on electrophoresis chamber followed by BIORAD western blot procedure. Anti-GFP antibody 1:5000 dilution (GE healthcare) and anti-Rabbit 1:10000 dilution as secondary antibody was used to detect the GFP-tagged reporter. Blot were developed using CSPD (tropix Inc.) imaged by ChemiDoc™ MP system Biorad. Before detecting Pgk1 as internal control, the membrane were stripped-off according to instruction from Abcam. Anti-Pgk1 (1:10000 dilution) was used as first antibody and anti-mouse as secondary antibody (1:10000) to detect the Pgk1.

Spot test assay
Cell viability experiment were done by growing pre-culture for 2 day in appropriate media (SD minimum without Histidin or Ura or compination of both). All cells cultures were measured and adjusted at OD600=1 as the highest concentration before spotted on agar plates. Each 5 µL cultures were spotted in 10x dilution series. The plates were stored at 30°C for 2 days to let the cells grow.

**Microtiter experiment**

Microtiter experiment were generated by growing pre-culture of cells in the medium SD minimum without Histidine for those that only expressing the GFP-h2NLS-L-TM reporter and in SD minimum without Histidine and Uracil for co-expression of Kap60 and GFP-h2NLS-L-TM reporter. Each of the wells were filled with 200 µL culture final volume containing inoculume with final OD_{600} ±0.1. Microtiter plate were shaked and temperature were maintained at 30°C for 16h (overnight) in Multiskan GO instument (Thermo Scientific).

**Microfluidics**

The microfluidic experiments were imaged on a Delta Vision microscope (Applied Precision/GE Healthcare) using an 100×, 1.40 NA objective lens and solid state illumination. The images were acquired using a charge-coupled device (CCD) camera (CoolSNAP HQ2; Photometrics). The medium and microfluidic chamber were pre-incubated for 2-3h to reach a temperature of 30°C. A drop of inoculume at mid-exponentially growth phase was inoculated to the microfluidic chamber with a flow of 2.07 mL/minutes of medium SD-Ura supplemented with 0.1% Galactose for 3 days. GFP was illuminated at 50% intensity of FITC 488 nm for 0.1 seconds and DIC images at 32% polarized light for 0.05 seconds. Images were taken every 20 minutes.

**Acknowledgments**

We would like to thanks to Georges Janssens for his help in setting up the microfluidic chip, Anton Steen for his valuable discussion and Bert Poolman for critical review of this chapter.
References


