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Regulación de la función celular de la GTPasa Gpn3 a través de la modulación de su localización subcelular en células humanas

TESIS QUE PRESENTA

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PARA OBTENER EL GRADO DE DOCTORA EN CIENCIAS BIOMÉDICAS BÁSICAS

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“Un buen padre vale por cien maestros”

Jean Jacques Rosseau

A mi padre, madre y mejor amigo:
Ing. José Apolinar Peña Sánchez †

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Regulación de la función celular de la GTPasa Gpn3 a través de la modulación de su localización subcelular en células humanas.

Por Sonia Griselda Peña Gómez.

Nucleocytoplasmic shuttling of the GPN-loop GTPase Gpn3 is regulated by serum and cell confluence in MCF-12A mammary cells

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Abstract

The best-known function of the essential GPN-loop GTPase Gpn3 is to contribute to RNA polymerase II assembly, a prerequisite for its nuclear targeting. Although this process occurs in the cytoplasm, we have previously shown that Gpn3 enters the cell nucleus before being polyubiquitinated. Here, we show that inhibiting Crm1-mediated nuclear export with leptomycin B or the proteasome with MG132 caused the nuclear accumulation of endogenous and recombinant Gpn3RFlag in MCF-12A cells. When added simultaneously, leptomycin B and MG132 had an additive effect. Analysis of Gpn3 primary sequence revealed the presence of at least five nuclear export sequence (NES) motifs, with four having a higher exposure to the solvent in the GTP-bound than GDP-bound state in a structural Gpn3 model. Inactivation of any of these NESes led to some degree of Gpn3 nuclear accumulation, although mutating NES1 or NES3 had the more robust effect. MCF-12A cells expressing exclusively NES-deficient versions of Gpn3RFlag proliferated slower than cells expressing Gpn3RFlag wt, indicating that nuclear export is important for Gpn3 cellular function. Next, we searched for physiological conditions that may regulate Gpn3 nucleocytoplasmic shuttling. Interestingly, whereas Gpn3RFlag was mostly nuclear in sparsely growing MCF-12A cells, it was exclusively cytoplasmic in confluent cells. Furthermore, Gpn3RFlag was cytoplasmic, mostly perinuclear, in sparse but starved MCF-12A cells, and serum-stimulation caused a rapid, although transient, Gpn3RFlag nuclear accumulation. We conclude that Gpn3 nucleocytoplasmic shuttling is regulated by cell density and growth factors, and propose that Gpn3 has an unknown nuclear function positively linked to cell growth and/or proliferation.

Keywords: GPN-loop GTPase Gpn3, nucleocytoplasmic shuttling, perinuclear localization, nuclear export sequences, cell confluence, serum-stimulated nuclear entry.

1. Introduction

The GPN-loop GTPase subfamily is constituted by three members Gpn1, Gpn2 and Gpn3. These proteins are identified for having an invariable glycine-proline-asparagine motif, and the insertions I and II [1], [2]. All three GTPases display a high degree of sequence similarity and are essential proteins, indicating that they perform nonredundant critical functions in the cell [3]–[5]. It is known that Gpn3 physically interacts with Gpn1 and Gpn2, and with some subunits of RNA polymerase II (RNAPII) [3], [6], [7]. Gpn3 is required, together with the other two GPN GTPases, for the biogenesis and nuclear localization of RNAPII, both in yeast and human cells [4], [8]–[10].

Transport of proteins and RNAs from the nucleus to the cytoplasm is critical for many physiological processes in eukaryotes. Active nucleocytoplasmic transport through nuclear pore complexes (NPCs) requires the participation of the major export receptor Crm1 and the small GTPase Ran [11]–[15]. The exportin Crm1 recognizes a nuclear export signal (NES) in cargo molecules. The classical NES-consensus sequence is leucine rich, but in the four or five conserved positions are also allowed other hydrophobic amino acids, such as Leu, Ile, Val, Met and Phe [15]–[17]. Crm1 is selectively inhibited by leptomycin B (LMB) and others Crm1-blocking compounds by modification of its Cys528 [18]–[22].

Previous studies have shown that Gpn1 suffers a nucleocytoplasmic shuttling mediated by Crm1 [23], which recognizes a highly exposed nuclear export sequence identified by our group [24]. As Gpn1, Gpn3 is exclusively cytosolic although is we have previously shown that its polyubiquitination occurs in the cell nucleus [25]. However, it is still unknown if there is any physiological condition that regulates Gpn3 cellular function by controlling Gpn3 nuclear transport. In this study, we demonstrate that mutations in nuclear export signal motifs present in Gpn3 resulted in the nuclear accumulation of endogenous and recombinant protein Gpn3 in MCF-12A cells. In a structural model of Gpn3 we showed that these NESes are exposed to the solvent, and therefore for Crm1 binding, and that this exposure increased in the GTP-bound state compared to the GDP-bound conformation of Gpn3. We identify here two

physiological conditions that regulate Gpn3 nucleocytoplasmic shuttling in MCF-12A cells: cell confluence and the growth factors present in the growth media for these cells. In confluent MCF-12A cells Gpn3 is exclusively cytosolic, whereas it is mainly nuclear in sparsely growing cells, although in this case some Gpn3 is also detected in the cytoplasm. Furthermore, in subconfluent MCF-12A cells nutrient starvation causes Gpn3 to exit the cell nucleus, but stimulation with complete growth media or just starvation media supplemented with serum caused a rapid nuclear translocation of Gpn3. Interestingly, nuclear accumulation after stimulation of MCF-12A cells with complete media or just serum was only transient, and Gpn3 was back in the cytoplasm after approximately 4 h. These findings indicate that Gpn3 has additional functions to its known participation in RNAPII assembly in the cytoplasm. We showed that Gpn3 nucleocytoplasmic shuttling is regulated by cell confluence and growth factors in a manner pointing a Gpn3 having a still to identify nuclear function which is likely linked to cell growth and/or proliferation in a positive manner.

2. Materials and Methods

2.1 Reagents and antibodies

All chemicals and reagents were obtained from Sigma (St. Louis, MO, USA) and all cell culture reagents were from Invitrogen (Waltham, MA, USA). The rabbit polyclonal antibody against Gpn3 was generated as previously described [26]. Anti-mouse and anti-rabbit immunoglobulins G conjugated to horseradish peroxidase (HRP), and the mouse anti-tubulin antibody were obtained from Sigma. Alexa Fluor 488-conjugated anti-rabbit secondary antibody was acquired from Molecular Probes (Eugene, OR, USA).

2.2 Retroviral vectors to express Gpn3RFlag wt or NES deficient versions

Full-length cDNA for human Gpn3 was inserted into the pEYFP-N1 vector as previously described [25]. This cDNA had an in-frame oligonucleotide coding for the Flag peptide at the C-terminus of Gpn3. Another modification were three nucleotide

changes that did not modify the amino acid sequence of the protein but made the mRNA resistant to the shRNA g193, which we employ to suppress endogenous Gpn3 [25]. Gpn3 NES mutants were generated by site directed PCR mutagenesis using Gpn3RFlag as template. Mutagenic oligonucleotides were as follows: NES1 (V37A, L39A): 5'-ccctcaaccggtctgtccaagttgcaaacgcggatccagcagcagaacac-3' and 5'-gtgttctgctgctggatccgcgtttgcaacttggacagaccggttgaggg-3'; NES2 (I58A, V60A): 5'-gatggctgacatccgggaactggccgaggcggatgatgtaatggaggatg-3' and 5'-catcctccattacatcatccgcctcggccagttcccggatgtcagccatc-3'; NES3 (L165A,I167A): 5'-ccctgagtgccatgatctctgcagaagctccgcaagtcaacatcatgac-3' and 5'-gtcatgatgttgacttgcggagcttctgcagagatcatggcactcaggg-3'; NES4 (M176A, L178A): 5'-gtcaacatcatgacaaaagcggatgcgctgagtaaaaaagcaaaaaagg-3' and 5'-ccttttttgctttttactcagcgcatccgcttttgtcatgatgttgac-3'; NES 5 (F250A, I252A): 5´ cattgcattgcagcatattgatgctgccgctcaatatggagaagacc 3´ and 5´ ggtcttctccatattgagcggcagcatcaatatgctgcaatgcaatg 3´. Gpn3RFlag wt and NES mutants were subcloned into the Hind III and Not I restriction sites of the pLNCX2 retroviral vector. Briefly, retroviruses were produced in HEK 297T/17 cells by transfecting them with the corresponding plasmid together with gag-pol and vsv vectors by the calcium phosphate method. Retroviral particles were collected 48 h after transfection and diluted 1:1 in fresh culture medium containing 8 µg/ml polybrene to infect MCF-12A cells. 24 h after the beginning of infection, 0.5 mg/ml geneticin was added to eliminate noninfected cells.

2.3 Cell culture conditions

HEK 293/T17 and MCF-12A cells were obtained from ATCC (Manassas, VA, USA). HEK 293T/17 cells were grown and maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. MCF-12A cells were cultured in DMEM-F12 with 5% horse serum, 20 ng/ml EGF, 100 ng/ml cholera toxin, 10 µg/ml insulin and 0.5 µg/ml hydrocortisone. All culture medium contained 100 U/ml penicillin and 100 µg/ml streptomycin and were kept in a humidified air-CO_2 atmosphere at 37°C.

2.4 Suppression of Gpn3 expression

To suppress endogenous Gpn3 expression, we employed a specific short-hairpin RNA, shRNA g193, to suppress Gpn3 as previously described [27]. Retroviruses were generated as mentioned above and used to infect MCF-12A cells stably expressing Gpn3RFlag wt or one NES mutant. Noninfected cells were eliminated by adding 2 µg/ml puromycin to the culture media for 48 h.

2.5 Cell density

MCF-12A cells expressing recombinant Gpn3RFlag wt were seeded at low ($9x10^4$) or high ($2x10^5$) density per well into 12-well plates. The cells were grown for 24 or 48 h after plating, and they were examined for immunofluorescence as mentioned below.

2.6 Immunofluorescence assay

MCF-12A cells stably expressing recombinant Gpn3RFlag wt or one NES mutant version were washed twice with PBS and fixed with 3.7% formaldehyde in PBS (pH 7.4) for 15 min at room temperature. Cells were exposed to leptomycin B (20 nM) or/and the proteasome inhibitor MG132 (10 µM), and after 4 h cells were fixed as mentioned before. After rinsing the cells with PBS, they were permeabilized and blocked in one step by incubating them in PBS containing 0.2% Triton X-100 and 10% fetal bovine serum for 15 min at room temperature. Cells were subsequently incubated overnight with an anti-Gpn3 antibody at 4°C at a 1:1000 dilution. Next day, cells were rinsed with PBS, followed by a 1 hour-incubation with Alexa Fluor 488-conjugated anti-rabbit secondary antibody. Finally, the cells were counterstained with DAPI to visualize nuclei. Images were acquired at 60x with an Olympus inverted microscope equipped with an evolution MP camera. Data were analyzed using the ImageJ software (NIH, Bethesda, MD, USA) and the nucleus/cytoplasm fluorescence ratio (F n/c) was calculated in at least 300 cells, 100 from each experiment. To assess the difference between control and treated cells a Kruskal-Wallis and *post-hoc* test analysis for non-parametric data was performed (GraphPad Prism 9.0.0).

2.7 *Western blot assay*

Total protein cell extracts were obtained and separated on SDS 10% polyacrylamide gels [28] and then electrophoretically transferred onto polyvinyl difluoride membrane (0.2 μm, Millipore, Burlington, MA, USA), at 15 V for 30 min. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) for 1 h at 25°C, then incubated overnight at 4°C with an anti-Gpn3 or anti-tubulin antibody in a dilution 1:10,000 or 1.5,000, respectively. To detect the antigen-bound antibody, the membranes were treated with secondary antibody conjugated with horseradish peroxidase and immunoreactivity was detected using the enhanced chemiluminescence system.

2.8 Cell proliferation assay

Proliferation of MCF-12A cells stably expressing recombinant Gpn3RFlag wt or one NES mutant version was determined using the Cell Titer-Glow Luminescence Assay kit (Promega, Madison, WI, USA), by assessing relative adenosine triphosphate (ATP) levels. $1x10^3$ cells were plated per well into 96-well plates. Relative ATP levels were determined at different time points after platting, according to the manufacturer´s instructions, using the PerkinElmer VICTOR 4X plate reader.

2.9 Starvation conditions

Cells were plated at $9x10^4$ cells/35-mm-diameter dish and grown for 24 h. Cells were cultured for 4 h in a starvation medium as previously described [29]. Later, the media was removed and complete or starvation medium was added, and cells were fixed at the indicated times (between 0 to 4 h) to determine Gpn3RFlag subcellular distribution.

2.10 Molecular modeling

Modeling of the three-dimensional structure of Gpn3 was previously described [30]. Briefly, the crystallographic structure of Npa3 protein [2] was taken as a template to model Gpn3 protein (1-260 aa) in the modeller suite. The C-terminal region of Gpn3 (261 -284 aa) was modeled in the PEPFOLD3 server [31]. Docking of the C-terminal

region to the Gpn3 model was performed in the ClusPro 2.0 server [32]. Structure and geometry optimization of the model was accomplished with the program charmm_c40b2 [33]. Residue solvent accessibility was calculated using the programs Surface Racer and DSSP, by rolling a probe atom of 1.4 Å [34], [35]. Values reported in Table 1 are means per residue in each NES sequence.

3. Results.

3.1 Human Gpn3 shuttles between the cytoplasm and the cell nucleus in MCF-12A mammary cells.

To study the modulation of Gpn3 function through the regulation of its subcellular localization we first generated a derivative of MCF-12A cells expressing Gpn3RFlag, a wild type (wt), C-terminally Flag tagged, recombinant version of Gpn3 than in addition contains three silent nucleotide changes at the level of mRNA that make it resistant to shRNA g193, a highly effective shRNA to suppress expression of endogenous Gpn3 in this cell type, without altering Gpn3 primary sequence [27]. To this end, we infected MCF-12A cells with retroviral particles expressing Gpn3RFlag together with a geneticin-resistance gene, which allowed us to select a pure population of cells expressing Gpn3RFlag by adding geneticin to the culture media for 4 days to eliminate non infected cells (Figure 4C). Subsequently, we suppressed endogenous Gpn3 expression by a second round of retroviral infection, this time to express the shRNA g193, followed by elimination of non-infected cells by exposure to puromycin for 48h (Figure 4D). Thus, we successfully replaced endogenous Gpn3 by Gpn3RFlag. In contrast to transient expression of Gpn3-GFP (results not shown), this version of Gpn3 was detected mostly in the cytoplasm of MCF-12A cells (Figure 1A). Gpn3 was almost completely absent from the nuclei, counterstained with DAPI. To determine if Gpn3 shuttles between the cytoplasm and the cell nucleus, we exposed MCF-12A cells expressing Gpn3RFlag to 20 nM leptomycin B (LMB), an inhibitor of the exportin Crm1, for four hours. After this period, Gpn3 was clearly detected in the cell nucleus (Figure 1A). This result indicates that in MCF-12A cells

Gpn3 is constitutively mobilized between the cytoplasm and the cell nucleus, and that this protein is effectively exported from the nucleus to the cytoplasm by the most common exportin, Crm1. Exposure of MCF-12A cells to 30 μM MG132, an inhibitor of the proteasome, also resulted in a nuclear accumulation of Gpn3RFlag. Interestingly, the effect of LMB and MG132 retaining Gpn3RFlag in the cell nucleus was clearly additive (Figure 1A). To evaluate these results in a quantitative manner we determined the nuclear/cytoplasmic fluorescence ratio of the Gpn3RFlag signal yielded by the rabbit polyclonal antibody for Gpn3 (Figure 1B). Importantly, we showed that endogenous Gpn3 displayed the same nucleocytoplasmic shuttling as Gpn3RFlag, and that this protein accumulated in the cell nucleus in the presence of LMB or MG132, with the effect of these compounds also being additive (Figure C and D), as observed with Gpn3RFlag. Gpn3 also accumulated in the cell nucleus with fairly the same time course as Gpn3RFlag in MCF-12A cells exposed simultaneously to LMB and MG132 (Figure 1E).

3.2 Human Gpn3 contains at least five nuclear export signal motifs that can potentially be recognized by Crm1.

The exportin Crm1, the target of leptomycin B, contains five hydrophobic pockets to accommodate 4 or 5 hydrophobic amino acids (ϕ0-ϕ1-4) in the cargo proteins that are exported from the nucleus to the cytoplasm [15]. An analysis of the Gpn3 primary sequence in ELM (The Linear Eukaryotic Motifs predictor) revealed the presence of one short motif that matches the Crm1 binding consensus sequence (amino acids 27-39). Visual inspection of the protein allowed us to identify other four motifs that could potentially be recognized by Crm1 (Figure 2). Thus, human Gpn3 possess at least five nuclear export sequences for potential Crm1 binding.

3.3 Molecular modeling of Gpn3 indicates that NES1 to NES3 and NES5, but not NES4 display a higher solvent exposure in the GTP-bound, active state than in the GDP-bound inactive state.

The position of the short sequences that adjust to a NES consensus sequence in the protein may determine if they are available for Crm1 binding or not, as they may

be locate on the protein surface or internally. Thus, we generated a molecular model of human Gpn3 based on the available crystallographic structure of Npa3 [2], the yeast ortholog of human Gpn1, and determined the position of the five NESes in the Gpn3 structure. Npa3 acquires a closed or open conformation when GDP or GTP are bound [2], thus we modeled human Gpn3 in both nucleotide-bound states (Figure 3A and D), and identified the position of each NES on the protein surface (Figure 3B-C and E-F). We then calculated the degree of exposure for each of the five NESes to the solvent, and therefore to Crm1 for potential binding in both, the closed and open Gpn3 conformations (Table 1). All five NESes displayed some degree of solvent exposure in both states, which was quantitatively determined with two different programs, DSSP and Surface racer, giving both comparable values. In the GDP-bound, closed state of Gpn3 NES4 and NES5 displayed lower solvent exposure values than NES1-NES3 (Table 1). Interestingly, NES1-NES3 and NES5, but not NES4, showed a higher exposure to the solvent in the GTP-bound open state, the expected active form of this GTPase, compared to the GDP-bound, closed and inactive Gpn3 state.

3.4 Gpn3 contains several functional Crm1 binding nuclear export sequences. As all five potential NESes displayed some degree of exposure to the solvent in either the GDP- or GTP-bound form, we decided to evaluate the functional importance of all five potential NESes in Gpn3 nuclear export. To determine the involvement of these short sequences in Gpn3RFlag nuclear export, we mutated the invariable hydrophobic amino acids 3 and 4 to alanine in each of the five potential NESes. Gpn3RFlag wt and the NES1-NES5 mutated versions were stably expressed in MCF-12A cells employing a retroviral vector carrying a geneticin-resistance gene. Finally, endogenous Gpn3 expression was suppressed by shRNA g193 expressed from a second retroviral vector carrying a puromycin-resistance gene (see Materials and Methods). In summary, we replaced endogenous Gpn3 by Gpn3RFlag wt or one of five NES mutant derivative version. Both steps, the expression of Gpn3RFlag (Figure 4C), as well as the suppression of endogenous Gpn3 (Figure 4D), were confirmed by Western Blot analysis. The presence of the

Flag peptide at the C-terminus of Gpn3 allowed us to distinguish Gpn3RFlag from the endogenous Gpn3, as the former migrated with a slightly higher molecular weight than the endogenous protein (Figure 4C), due to the Flag peptide added to the C-terminus of Gpn3. NES2 and NES4 Gpn3RFlag mutants reached protein levels comparable to the wild type Gpn3RFlag protein, but NES1 and NES3 Gpn3RFlag mutants protein levels were detectably lower than Gpn3RFlag wt, and NES5 Gpn3RFlag mutant was barely detectable (Figure 4C). We then evaluated the subcellular distribution of all Gpn3RFlag versions by immunofluorescence (Figure 4A) employing the same polyclonal antibody specific for Gpn3 used before. We observed that all Gpn3RFlag NES1-5 mutants accumulated to some extent in the nucleus of MCF-12A cells, but the accumulation was more notable for NES1 and NES3 Gpn3RFlag mutants (Figure 4A). Of note, the low signal observed in both assays, Western blot and immunofluorescence, in cells expressing the Gpn3RFlag NES5 mutant in the absence of endogenous Gpn3 is evidence that the rabbit polyclonal antibody for Gpn3 we employ in our experiments is in fact detecting Gpn3 and no other protein in the immunofluorescence experiments. Interestingly, both NES1 (Figure 5A) and NES3 (Figure 5B) are evolutionarily conserved, as shown in the sequence alignment of these motifs in Gpn3 from several species. Thus, we conclude that Gpn3 possess at least two evolutionarily conserved, functionally important, NESes that are recognized by the exportin Crm1 to mobilize the protein out of the cell nucleus.

3.5 Nucleocytoplasmic shuttling is essential for Gpn3 cellular function.

To investigate the importance of Gpn3 nucleocytoplasmic shuttling in Gpn3 cellular function we took advantage of the fact that Gpn3 is an essential protein. Thus, we evaluated cell proliferation as a proxy for Gpn3 functional integrity. We assessed cell proliferation rate in the MCF-12A derivatives expressing Gpn3RFlag wt or a mutated version in NES1 or NES3 in the absence of the endogenous Gpn3 protein (see Materials and Methods). Cells were seeded in 96-well plates and ATP levels were determined by using the luciferin/luciferase system at days 1, 4 and 6 after seeding. Culture media was changed at days 2 and 4. ATP levels are directly proportional to

the number of viable cells at both, low and high numbers of cells, making this method highly precise to assess cell proliferation. Proliferation of MCF-12A cells expressing only Gpn3RFlag wt was undistinguishable from that of unmodified MCF-12A control cells (Figure 6), indicating that the experimental manipulations associated with the molecular replacement of endogenous Gpn3 by Gpn3RFlag did not interfere negatively with Gpn3 cellular function, and therefore, with cell proliferation. MCF-12A cells expressing exclusively the Gpn3RFlag NES3 mutant also proliferated normally. However, cell proliferation rate was markedly reduced in MCF-12A cells expressing only the Gpn3RFlag NES1 mutant (Figure 6). These results support the proposal that an intact transport between the cytoplasm and the cell nucleus is essential for a normal cellular function of human Gpn3.

3.6 Gpn3 nucleocytoplasmic shuttling is sensitive to cell density.

Next, we asked if Gpn3 nucleocytoplasmic shuttling was modulated by a physiologically relevant stimulus or condition. In our experiments to determine the subcellular distribution of Gpn3RFlag we noticed that in low cell density areas of the culture wells Gpn3RFlag was uniformly distributed between the nucleus and cytoplasm or almost completely nuclear, whereas it was mainly cytoplasmic in densely populated areas. Thus, we hypothesized that cell density might be a physiological condition that regulates Gpn3 nucleocytoplasmic shuttling. To test this proposal, we seeded MCF-12A cells at low or high density and evaluated Gpn3RFlag subcellular distribution two days later, when the cells seeded at high density were already confluent. Interestingly, Gpn3RFlag concentrated mainly in the cell nucleus in low density growing MCF-12A cells (Figure 7), but it was totally excluded from the nucleus in confluent MCF-12A cells (Figure 7). Thus, we identified cell density as a key physiological regulator of Gpn3 nucleocytoplasmic shuttling, a result that points to a nuclear function of Gpn3 likely related positively to cell growth and/or cell proliferation, as both aspects are highly active in low density growing cells but are markedly decreased in confluent cells by contact inhibition.

3.7 Gpn3 accumulates rapidly in the cell nucleus after stimulation with complete growth culture media in starved MCF-12A cells.

We next investigated more directly if Gpn3 nucleocytoplasmic shuttling may be influenced by growth factors normally present in the culture media of MCF-12A cells, as these factors stimulate cell growth and proliferation. To this end we first starved MCF-12A cells by incubating them in their regular culture media lacking horse serum, EGFG and insulin, three key components, followed by determining the effect of stimulating these starved cells with complete, nutrients-rich complete media, on the subcellular distribution of Gpn3RFlag. MCF-12A cells expressing Gpn3RFlag seeded in 12-well plates were incubated for 4h in starvation media, followed by stimulation with either starvation or complete media for different periods of time, between 0.5 and 4 h. After this time cells were fixed and the subcellular distribution of Gpn3RFlag was determined by immunofluorescence using a rabbit polyclonal antibody specific for Gpn3. In starved MCF-12A cells Gpn3RFlag was completely cytoplasmic, displaying a perinuclear distribution (Figure 8A and 9A, left panel). Exposing these cells to fresh starvation media did not alter Gpn3RFlag localization in the cell at any time point examined. However, stimulation of starved MCF-12A cells with complete media containing the three components missing in the starvation media, i.e., 5% horse serum, EGF and insulin, resulted in a rapid accumulation of Gpn3RFlag in the cell nucleus (Figure 8A) in close to 50% of cells (Figure 8A, right panel). After stimulating starved MCF-12A cells with complete media Gpn3RFlag was observed to concentrate in the cell nucleus after 30 min, the earliest time point examined, and this distribution remained unaltered for 1-2 h. However, the percentage of cells displaying Gpn3RFlag mainly in the cell nucleus decreased to approximately 23% after 4 h of stimulation with the complete media. These results clearly showed that exposing MCF-12A cells to the growth factors present in their culture media cause an accumulation of Gpn3 in the cell nucleus, supporting the proposal that Gpn3 may have a still to define nuclear function. The apparently transient nature of nuclear accumulation observed in these conditions point to Gpn3 nuclear export as an important regulatory branch of this putative Gpn3 nuclear function.

3.8 Gpn3 translocates to the cell nucleus of starved MCF-12A cells in response to stimulation with 5% horse serum, an essential component of their growth media.

In the past section nuclear accumulation of Gpn3RFlag was observed after stimulating starved MCF-12A cells with complete media containing horse serum, EGF and insulin. To determine which of the three components was responsible for the marked change in Gpn3RFlag subcellular distribution, we stimulated starved MCF-12A cells with each one of these components separately. Insulin stimulation did not induce the nuclear translocation of Gpn3RFlag and EGF had a small effect (results not shown). However, Gpn3RFlag concentrated in the cell nucleus of starved MCF-12A cells after only 30 min of being stimulated with 5% horse serum in the absence of EGF and insulin (Figure 9A, right panel). However, the percentage of cells exhibiting Gpn3RFlag nuclear accumulation was slightly lower (Figure 9B) in cells stimulated with horse serum compared to the one observed after stimulating starved MCF-12A cells with complete media (Figure 8B). These results point to the existence of some synergy between the effect of horse serum and EGF to induce the nuclear accumulation of Gpn3. However, the most important component in the complete media responsible for inducing the nuclear accumulation of Gpn3 was, with no doubt, the horse serum, a regular component of the growth media used to culture MCF-12A cells.

4. Discussion.

Despite being an essential protein, very scarce information is available on the cellular and molecular functions of the GPN-loop GTPase Gpn3. Although Gpn3´s involvement in RNA polymerase II assembly must occur in the cytoplasm, Gpn3 has also been described in the nuclear fraction [10] and we have previously shown that Gpn3 is polyubiquitinated in the cell nucleus [25]. However, neither the mechanisms of nucleocytoplasmic shuttling nor the physiological conditions that regulate this process have been investigated. In this study we showed that Gpn3 is indeed

constitutively mobilized between the cytoplasm and the cell nucleus in MCF-12A cells, as the inhibition of Crm1-mediated nuclear export with leptomycin B resulted in a nuclear accumulation of Gpn3. Our mutagenesis studies confirmed the presence of functionally important NESes in Gpn3 that were initially identified by a bioinformatic analysis, as inactivation of these sequences lead to the nuclear accumulation of Gpn3. Two of the three NESes we studied in detailed are the more likely to be used in vivo, NES1 and NES3, as they display a high value of exposure to the solvent (and therefore to Crm1), its inactivation interfered negatively with MCF-12A proliferation, and are evolutionarily conserved. We also identified two physiological conditions that regulate nucleocytoplasmic shuttling of Gpn3 in MCF-12A mammary cells: 1) Gpn3 accumulates in the cell nuclei after stimulation of MCF-12A cells with horse serum, a growth media component rich in growth factors and whose presence is absolutely required for proliferation in these cells, and 2) cell density, with Gpn3 being mostly nuclear in sparse cells, but only cytoplasmic in high-density, confluent cells. The presence of Gpn3 in the nuclei of MCF-12A after growth factor stimulation and in the more metabolically active sparse but not in confluent cells suggests that Gpn3 has a still to identify nuclear function, likely with a positive effect on cell growth and/or cell proliferation.

We previously identified one functional nuclear export sequence in the GPN-loop GTPase Gpn1 [24], a result that stimulated us to investigate if its paralogue Gpn3 also underwent this type of regulation in the cell. However, our initial efforts in this direction were hampered by the permanent nuclear accumulation of Gpn3-GFP observed after transiently transfecting HEK293T cells, probably due to the artificially high protein levels reached in these conditions. Interestingly, even in these conditions Gpn3-GFP was retained in the cytoplasm by co-expressing Gpn1, another GPN-loop GTPase that associates tightly with Gpn3 [7]. Interestingly, Gpn3 was almost completely cytoplasmic when expressed from a retroviral vector, showing that this was a more appropriate system to study the modulation of Gpn3 function by controlling its subcellular distribution [25], [26]. This is most likely due to the lower protein levels attained when expressing a protein from a retroviral vector compared to the levels reached after transfection. We took advantage of this

expression system to study the regulation of Gpn3 cellular function by modulating its nucleocytoplasmic shuttling. Clearly, the Gpn3 protein levels are strictly controlled in MCF-12A cells, as the expression of recombinant Gpn3RFlag with a retrovirus caused a notable decrease in the protein levels of endogenous Gpn3 (Figure 4C). Additionally, it was also clear that recombinant Gpn3RFlag protein levels increased after suppression of endogenous Gpn3 expression by the shRNA g193 (Figure 4D). It seems clear that the cell possesses mechanisms to maintain fairly constant the total Gpn3 protein levels. Inhibition of protein nuclear export with leptomycin B or proteasome inhibition with MG132 induced the nuclear accumulation of Gpn3 in HEK293T cells. In the present study we employed a retroviral vector to stably express Gpn3 wt or the NES-deficient versions in MCF12A mammary cells, followed by suppressing endogenous Gpn3. Employing this expression system, we found that Gpn3 localizes in the cytoplasm, at least under some specific conditions. Exposure of MCF-12A cells to both leptomycin B and MG-132 led to a notable nuclear accumulation of Gpn3, and their effect was additive. Although the cause of MG132-induced Gpn3 nuclear accumulation in MCF-12A cells is not clear, in HEK293T cells the polyubiquitinated fraction of Gpn3 was enriched in the nuclear fraction [25], an indication that Gpn3 is targeted for proteasomal degradation in the cell nucleus.

Analysis of the Gpn3 primary sequence in ELM (The Eukaryotic Linear Motifs predictor) identified only one putative nuclear export sequence, corresponding to our NES1, comprising amino acids 27-39. This sequence contains the five hydrophobic residues present in a functional NES that bind the same number of hydrophobic pockets in the exportin Crm1. Indeed, this prediction was accurate, as mutating the invariable hydrophobic amino acids 3 and 4 in NES1 to alanine resulted in a clear nuclear accumulation of Gpn3 to an extent practically undistinguishable from the caused by leptomycin B. NES1 in Gpn3 belongs to the class 1a, according to the classification of Lee *et al*., 2019 [15]. Inactivation of any of the other putative four NES sequences with the ϕ0-ϕ1-4 characteristic, resulted in some degree of Gpn3 nuclear accumulation, but this effect was stronger after inactivating NES1 or NES3. Interestingly, these NESes were the ones showing a higher value of exposure to the solvent calculated by two different programs. These results confirm that the

functionality of a predicted NES is not only determined by the motif sequence, but also for the position of that motif in the protein structure, as buried NES motifs will not be available for Crm1 binding. Although the three-dimensional structure of human Gpn3 has not been experimentally determined, the molecular model we generated for this protein based on Npa3 structure [2] was precise and sensitive enough to detect differences in solvent exposure among the five different predicted NESes. NES3 belongs to class 1b, but NES5 did not conform to any type of the classification proposed by Lee *et al*., 2019 [15]. Despite complying with most of the requisites with regard to possessing five hydrophobic amino acids and most spacing between those residues, NES5 does not comply with required spacing between residues ϕ3-ϕ4, as there is only one residue present, instead of the two or three observed in all experimentally tested NESes [15]. Additionally, mutating NES5 in Gpn3 was associated with a marked decrease in protein levels compared to Gpn3 wt and the other NES mutants (Figure 4C and D), probably due to a destabilizing effect of the mutation on Gpn3 stability.

We believe that one or more of the functional NESes we identified here in Gpn3 are indeed employed in a physiological context to export Gpn3 from the cell nucleus. At least the nuclear accumulation of Gpn3 induced by stimulating starved MCF-12A cells with complete media or horse serum is only transient, and the subcellular distribution of Gpn3 is practically undistinguishably before and 4 h after the stimulation (2 h in the case of serum). Although formally, we cannot rule out the possibility that the decrease in the nuclear fluorescence signal was due to degradation of Gpn3. However, the slower proliferation rate observed for MCF12A expressing only NES-deficient versions of Gpn3 supports the proposal that nuclear export is critical for Gpn3 cellular function. Although the function of Gpn3 in the nucleus remains to be determined, the fact that the growth factors present in the serum induce the nuclear accumulation of Gpn3 make us to propose that nuclear Gpn3 positively correlates with cell growth and/or proliferation. The second physiological condition we identified here as a critical regulator of the subcellular distribution of Gpn3 was cell confluence. Here too, nuclear Gpn3 seems to be positively linked to cell growth and/or proliferation, as Gpn3 was mostly nuclear in

sparse cells, but completely cytoplasmic in confluent cells. As MCF-12A cells are not tumorigenic, their proliferation is stopped by contact inhibition, a condition reached by cell confluence. Gpn3 is clearly excluded from the cell nucleus in confluent cells. It is not clear why Gpn3 requires more than one functional NES to be totally exported from the nucleus. One possibility is that distinct pools of Gpn3 molecules associate with additional proteins, each masking one or more NESes. In this case that fraction of Gpn3 molecules will be exported from the nucleus by using a different NES. Thus, the presence of more than one functional NES in Gpn3 may assure its exit from the cell nucleus, even when associated with other proteins, i.e., Gpn1, that may mask a specific NES. An extended presence of Gpn3 in the nucleus may be deleterious to the cell and detected as abnormal, leading to Gpn3 ubiquitination and degradation [25]. Interestingly, protein levels of the two Gpn3 NES mutants NES1 and NES3 are clearly lower than Gpn3 wt, and one possibility is that this is the result of degradation of the protein due to an abnormally extended stay in the cell nucleus. Although the identification of functional nuclear export sequences in Gpn3 shed some light on the mechanism of nuclear export, our attempts to understand nuclear entry were not successful, as mutating short regions enriched in positive amino acids present in Gpn3, the characteristic of nuclear import sequences recognized by most importins, did not prevent nuclear entry as expected (results not shown).

Altogether, our results identified several functional nuclear export signals in Gpn3, and identified two physiological conditions that regulate the nuclear accumulation of this GTPase, i.e., serum stimulation and cell confluence. Gpn1, another GPN-loop GTPase has also been found in the nuclear fraction [10], to copurify with elongating, and therefore chromatin-associated, nuclear RNA polymerase II [36] and to shuttle between the cytoplasm and the cell nucleus [8], [23] in part by using a nuclear export sequence that is highly conserved during evolution [24]. Interestingly, Gpn1 and Gpn3 seem to be mobilized between the cytoplasm and the cell nucleus even when they are physically associated to form a single complex [25]. Altogether, these results strongly support the proposal that both Gpn1 and Gpn3 GTPases are involved in cellular functions beyond RNA polymerase II assembly in the cytoplasm. The presence of both Gpn1 and Gpn3 in the cell nucleus point to a still to define

nuclear function for these proteins. Although nuclear export of Gpn1 has been reported to be important for RPAP2/Rtr1 nuclear export in both human [37] and *Saccharomyces cerevisiae* yeast cells [38] by a piggy-back mechanism, future work will be needed to uncover more nuclear functions of these essential GTPases. Based on our results, we anticipate that Gpn3 will perform a nuclear function that positively supports cell growth and/or cell proliferation. This nuclear function of Gpn3 induced by growth factor stimulation may be proportional to Gpn3 protein levels, therefore related to our previous observation that a higher expression of the GPN3 gene decreases the period of life in breast cancer patients [39].

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Figure legends.

Figure 1. Human Gpn3 accumulates in the cell nucleus after inhibition of Crm1-mediated nuclear export with leptomycin B (LMB) or/and proteasome inhibition by MG132 in MCF-12A cells. A) MCF-12A cells expressing recombinant Gpn3RFlag in the absence of endogenous Gpn3 (see Materials and Methods) were exposed to 20 nM LMB, 10 μM MG132 or both compounds for 4h. Cells were then fixed and the subcellular distribution of Gpn3RFlag was determined with a rabbit polyclonal antibody specific for Gpn3. B) The extent of Gpn3R nuclear accumulation was expressed as the nuclear/cytoplasm fluorescence ratio in at least 300 cells, 100 from each experiment, using Image J. Individual data points are shown, together with the median and interquartile range. A Kruskal-Wallis and post-hoc test analyses were performed to determine the statistical significance. ****P<0.0001. C) Unmodified MCF-12A cells were exposed to 20 nM LMB, 10 μM MG132 or both compounds for 4 h, and the subcellular distribution of endogenous Gpn3 was visualized with a rabbit polyclonal antibody under a fluorescence microscope. D) The extent of Gpn3R or Gpn3 nuclear accumulation was expressed as the nuclear/cytoplasm fluorescence ratio in at least 300 cells, 100 from each experiment, using Image J. Individual data points are shown, together with the median and interquartile range. A Kruskal-Wallis and post-hoc test analyses were performed to determine the statistical significance. ****P<0.0001. E) Time course of Gpn3RFlag and endogenous Gpn3 nuclear accumulation in MCF-12A exposed simultaneously to 20 nM LMB and 10 μM MG132. Cells were fixed after the indicated time periods and processed as in A and C.

Figure 2. Human Gpn3 contains at least five putative nuclear export sequences (NES) that can potentially be recognized by the leptomycin-sensitive exportin Crm1 to mobilize proteins from the cell nucleus to the cytoplasm. The short motifs in human Gpn3 that fit the Crm1 binding consensus sequence were identified using The Eukaryotic Linear Motifs predictor and visually. NES1 was the only predicted NES in ELM. NES2 through NES5 were identified by visual inspection of

human Gpn3 primary sequence. The Crm1 binding consensus sequence is shown at the top with the four or five invariable hydrophobic (ϕ) amino acids marked in red.

Figure 3. Localization of the putative nuclear export sequences in a homology-based structural model of human Gpn3. A) Ribbon representation of the molecular structure of full-length human Gpn3 in the GTP-bound, open conformation. B) Lateral view of the open conformation model of Gpn3 protein in surface representation showing the solvent accessibility of NESes 1, 2, 4 and 5. C) 180° rotation of the Gpn3 model in B showing solvent accessibility of NESes 1, 2, 3 and 5. D) Ribbon representation of the molecular structure of full-length human Gpn3 in the GDP-bound, closed conformation. E) Lateral view of the closed conformation model of Gpn3 protein in surface representation showing the solvent accessibility of NESes 1, 2, 4 and 5. F) 180° rotation of Gpn3 model in E showing solvent accessibility of NESes 1, 2, 3, 4 and 5. All five NESes in Gpn3 were color-coded for illustrative purposes only. The quantitation of surface exposure for each NES, in both the open and closed conformations, was calculated with DSSP and Surface racer and the results are shown in Table 1.

Figure 4. Functionality of predicted nuclear export sequences (NES) in human Gpn3. A) Endogenous Gpn3 was replaced by recombinant, shRNA-resistant with a C-terminal Flag peptide, Gpn3RFlag wt or a mutant version with the invariable hydrophobic amino acids 3 and 4 in each NES mutated to alanine in MCF-12A cells (see panels C and D). Gpn3RFlag was visualized with a rabbit polyclonal Gpn3 antibody under a fluorescence microscope. Nuclei were counterstained with DAPI, and microphotographs were taken at 60x. B) The extent of Gpn3RFlag nuclear accumulation was expressed as the nuclear/cytoplasm fluorescence ratio in at least 300 cells, 100 from each experiment, using Image J. Individual data points are shown, together with the median and interquartile range. A Kruskal-Wallis and post-hoc test analyses were performed to determine the statistical significance. ****P<0.0001. C) Western Blot of MCF-12A cells after infection with a retroviral vector expressing the indicated NES mutant version of Gpn3RFlag, and elimination

of non-infected cells with geneticin. Gpn3RFlag migrates slightly slower than endogenous Gpn3 due to the presence of the Flag peptide. Tubulin was used as a control of total protein loading. D) Western blot of the same MCF-12C cell derivatives described in C, but after infection with a retroviral vector expressing the shRNA g193 to suppress selectively endogenous Gpn3 expression but nor recombinant Gpn3RFlag, and selection of infected cells with puromycin. Tubulin was used as a control of total protein loading.

Figure 5. Nuclear export sequences NES1 and NES3 in Gpn3 are evolutionarily conserved. The amino acid sequence corresponding to NES1 (A) or NES3 (B) in Gpn3 from indicated species were aligned. The nuclear export signal consensus sequence containing the five hydrophobic amino acids ϕ0-ϕ4 present in most NESes is shown at the top of both panels. The corresponding hydrophobic amino acids present in Gpn3 were also marked in red in every Gpn3 sequence. The amino acid sequences were obtained from the Homologene section in the NCBI server.

Figure 6. MCF-12A cells expressing NES-deficient forms of Gpn3 exhibit defects in proliferation rate. MCF-12A cells expressing Gpn3RFlag wt or NES1 or NES3 derivative Gpn3RFlag mutants, all immediately after suppression of endogenous Gpn3 expression with the shRNAg193 (see Materials and Methods), were seeded in 96-well plates and cell viability was evaluated as a proxy for cell proliferation. ATP levels were determined at the indicated times by a commercially available luminescence kit assay. The same number of cells were seeded in all cases, and ATP levels assessed at day 1 were taken as the internal reference for subsequent times in each MCF-12A cell derivative. Two-way ANOVA followed by Dunnett´s multiple comparisons test was performed. Shown are median and interquartile range of 3 experiments. ***P<0.0001, ns, non-significant,

Figure 7. Gpn3 is mostly nuclear at low density but only cytoplasmic at high density MCF-12A cells. MCF-12A cells were seeded at low or high density and Gpn3RFlag subcellular distribution was assessed by immunofluorescence

employing a rabbit monoclonal antibody specific for Gpn3. Nuclei were counterstained with DAPI. Microphotographs were taken at 60x under a fluorescence microscope. Three independent experiments were performed. The results of one representative experiment are showed.

Figure 8. Stimulation of starved MCF-12A cells with complete, nutrients-rich media induce a rapid, but transient, nuclear accumulation of Gpn3. A) Starved MCF-12A cells expressing Gpn3RFlag in the absence of endogenous Gpn3 were stimulated with either the same starvation media lacking serum, EGF and insulin (left panel, Starvation media), or complete media (right panel, Nutrient-rich media containing all three components). Cells were fixed at the indicated time points after the stimulation. The subcellular distribution of Gpn3RFlag was determined by immunofluorescence using a rabbit polyclonal antibody specific for Gpn3. Nuclei were counterstained with DAPI. Microphotographs were taken at 60x under a fluorescence microscope. B) Quantitation of results shown in A. At least 150 cells, 50 from each experiment, were classified visually as having Gpn3RFlag in either the cytoplasm (C) or nucleus (N), and results are expressed as the percentage in each category after stimulating the cells with either starvation media (blue bars) or nutrient-rich media (orange bars) at the indicated time points. Shown are median and interquartile range of 3 experiments. Statistical analysis was performed with a mixed-effects model and Tukey´s multiple comparisons test. **P=0.0038; ****P<0.0001.

Figure 9. Serum stimulation of starved MCF-12A cells induce a rapid accumulation of Gpn3 in the cell nucleus. A) Starved MCF-12A cells expressing Gpn3RFlag in the absence of endogenous Gpn3 were stimulated with either the same starvation media (left panel, Starvation media) or with starvation media containing 5% horse serum, the concentration in media to grow these cells, (right panel, HS), but neither EGF nor insulin. Cells were fixed at the indicated time points and Gpn3RFlag subcellular distribution was determined and documented as in Figure 8. B) Quantitation of results shown in A. At least 150 cells, 50 from each

experiment, were visually classified as having Gpn3RFlag in either the cytoplasm (C) or nucleus (N), and results are expressed as the percentage in each category after stimulating the cells with either starvation media (blue bars) or starvation media supplemented with 5% serum (green bars). Shown are median and interquartile range of 3 experiments. Statistical analysis was performed with a mixed-effects model and Tukey´s multiple comparisons test. ****P<0.0001; ns, non- significant.

References

[1] S. Gras *et al.*, "Structural insights into a new homodimeric self-activated GTPase family," *EMBO Rep*, vol. 8, no. 6, pp. 569–575, Jun. 2007, doi: 10.1038/sj.embor.7400958.

[2] J. Niesser, F. R. Wagner, D. Kostrewa, W. Mühlbacher, and P. Cramer, "Structure of GPN-Loop GTPase Npa3 and Implications for RNA Polymerase II Assembly," *Mol Cell Biol*, vol. 36, no. 5, pp. 820–831, Mar. 2016, doi: 10.1128/mcb.01009-15.

[3] B. Alonso *et al.*, "Eukaryotic GPN-loop GTPases paralogs use a dimeric assembly reminiscent of archeal GPN," *Cell Cycle*, vol. 12, no. 3, pp. 463–472, Feb. 2013, doi: 10.4161/cc.23367.

[4] S. W. Minaker, M. C. Filiatrault, S. Ben-Aroya, P. Hieter, and P. C. Stirling, "Biogenesis of RNA polymerases II and III requires the conserved GPN small GTPases in Saccharomyces cerevisiae," *Genetics*, vol. 193, no. 3, pp. 853–864, 2013, doi: 10.1534/genetics.112.148726.

[5] G. Giaever *et al.*, "Functional profiling of the Saccharomyces cerevisiae genome," 2002. [Online]. Available: http://www.kegg.com

[6] B. Alonso, G. Chaussinand, J. Armengaud, and C. Godon, "A role for GPN-loop GTPase yGPN1 in sister chromatid cohesion," *Cell Cycle*, vol. 10, no. 11, pp. 1828–1837, Jun. 2011, doi: 10.4161/cc.10.11.15763.

[7] L. E. Méndez-Hernández *et al.*, "Gpn1 and Gpn3 associate tightly and their protein levels are mutually dependent in mammalian cells," *FEBS Lett*, vol. 588, no. 21, pp. 3823–3829, Nov. 2014, doi: 10.1016/j.febslet.2014.08.038.

[8] D. Forget *et al.*, "The protein interaction network of the human transcription machinery reveals a role for the conserved GTPase RPAP4/GPN1 and microtubule assembly in nuclear import and biogenesis of RNA polymerase II," *Molecular and Cellular Proteomics*, vol. 9, no. 12, pp. 2827–2839, 2010, doi: 10.1074/mcp.M110.003616.

[9] M. R. Calera *et al.*, "Parcs/Gpn3 is required for the nuclear accumulation of RNA polymerase II," *Biochim Biophys Acta Mol Cell Res*, vol. 1813, no. 10, pp. 1708–1716, Oct. 2011, doi: 10.1016/j.bbamcr.2011.07.005.

[10] C. Carré and R. Shiekhattar, "Human GTPases Associate with RNA Polymerase II To Mediate Its Nuclear Import," *Mol Cell Biol*, vol. 31, no. 19, pp. 3953–3962, Oct. 2011, doi: 10.1128/mcb.05442-11.

[11] M. Fornerod and M. Ohno, “CRM1 Is an Export Receptor for Leucine-Rich Nuclear Export Signals,” 1997.
[12] M. Fukuda *et al.*, “CRM1 is responsible for intracellular transport mediated by the nuclear export signal,” *Nature*, vol. 390, no. 6657, pp. 308–311, Nov. 1997, doi: 10.1038/36894.
[13] B. Ossareh-Nazari, F. Bachelerie, and C. Dargemont, “Evidence for a Role of CRM1 in Signal-Mediated Nuclear Protein Export,” *Science (1979)*, vol. 278, no. 5335, pp. 141–144, Oct. 1997, doi: 10.1126/science.278.5335.141.
[14] K. Stade, C. S. Ford, and C. Guthrie, “Exportin 1 (Crm1p) Is an Essential Nuclear Export Factor,” 1997.
[15] Y. Lee, J. Pei, J. M. Baumhardt, Y. M. Chook, and N. V. Grishin, “Structural prerequisites for CRM1-dependent nuclear export signaling peptides: accessibility, adapting conformation, and the stability at the binding site,” *Sci Rep*, vol. 9, no. 1, Dec. 2019, doi: 10.1038/s41598-019-43004-0.
[16] L. Gerace, “Nuclear Export Signals and the Fast Track to the Cytoplasm,” 1995.
[17] X. Dong *et al.*, “Structural basis for leucine-rich nuclear export signal recognition by CRM1,” *Nature*, vol. 458, no. 7242, pp. 1136–1141, Apr. 2009, doi: 10.1038/nature07975.
[18] N. Kudo *et al.*, “Leptomycin B inactivates CRM1exportin 1 by covalent modification at a cysteine residue in the central conserved region,” 1999. [Online]. Available: www.pnas.org.
[19] M. Köster *et al.*, “Ratjadones inhibit nuclear export by blocking CRM1/exportin 1,” *Exp Cell Res*, vol. 286, no. 2, pp. 321–331, Jun. 2003, doi: 10.1016/S0014-4827(03)00100-9.
[20] T. Meissner, E. Krause, and U. Vinkemeier, “Ratjadone and leptomycin B block CRM1-dependent nuclear export by identical mechanisms,” *FEBS Lett*, vol. 576, no. 1–2, pp. 27–30, Oct. 2004, doi: 10.1016/j.febslet.2004.08.056.
[21] S. Bonazzi *et al.*, “Anguinomycins and derivatives: Total syntheses, modeling, and biological evaluation of the inhibition of nucleocytoplasmic transport,” *J Am Chem Soc*, vol. 132, no. 4, pp. 1432–1442, Feb. 2010, doi: 10.1021/ja9097093.
[22] A. Dickmanns, T. Monecke, and R. Ficner, “Structural basis of targeting the exportin CRM1 in cancer,” *Cells*, vol. 4, no. 3. MDPI, pp. 538–568, Sep. 21, 2015. doi: 10.3390/cells4030538.
[23] F. Lembo *et al.*, “ MBD in , a Novel MBD2-Interacting Protein, Relieves MBD2 Repression Potential and Reactivates Transcription from Methylated Promoters ,” *Mol Cell Biol*, vol. 23, no. 5, pp. 1656–1665, Mar. 2003, doi: 10.1128/mcb.23.5.1656-1665.2003.
[24] H. Reyes-Pardo *et al.*, “A nuclear export sequence in GPN-loop GTPase 1, an essential protein for nuclear targeting of RNA polymerase II, is necessary and sufficient for nuclear export,” *Biochim Biophys Acta Mol Cell Res*, vol. 1823, no. 10, pp. 1756–1766, Oct. 2012, doi: 10.1016/j.bbamcr.2012.07.001.
[25] L. E. Méndez-Hernández, A. Y. Robledo-Rivera, M. Macías-Silva, M. R. Calera, and R. Sánchez-Olea, “Gpn3 is polyubiquitinated on lysine 216 and degraded by the proteasome in the cell nucleus in a Gpn1-inhibitable manner,” *FEBS Lett*, vol. 591, no. 22, pp. 3757–3770, Nov. 2017, doi: 10.1002/1873-3468.12883.

[26] A. A. Barbosa-Camacho *et al.*, "The Gpn3 Q279* cancer-associated mutant inhibits Gpn1 nuclear export and is deficient in RNA polymerase II nuclear targeting," *FEBS Lett*, vol. 591, no. 21, pp. 3555–3566, Nov. 2017, doi: 10.1002/1873-3468.12856.
[27] R. Sanchez-Olea *et al.*, "Parcs is a dual regulator of cell proliferation and Apaf-1 function," *Journal of Biological Chemistry*, vol. 283, no. 36, pp. 24400–24405, Sep. 2008, doi: 10.1074/jbc.M804664200.
[28] U. K. LAEMMLI, "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4," *Nature*, vol. 227, no. 5259, pp. 680–685, Aug. 1970, doi: 10.1038/227680a0.
[29] J. Debnath, S. K. Muthuswamy, and J. S. Brugge, "Morphogenesis and oncogenesis of MCF-10A mammary epithelial acini grown in three-dimensional basement membrane cultures," *Methods*, vol. 30, no. 3. Academic Press Inc., pp. 256–268, Jul. 01, 2003. doi: 10.1016/S1046-2023(03)00032-X.
[30] G. R. Cristóbal-Mondragón *et al.*, "FRET-based analysis and molecular modeling of the human GPN-loop GTPases 1 and 3 heterodimer unveils a dominant-negative protein complex," *FEBS Journal*, vol. 286, no. 23, pp. 4797–4818, Dec. 2019, doi: 10.1111/febs.14996.
[31] A. Lamiable, P. Thevenet, J. Rey, M. Vavrusa, P. Derreumaux, and P. Tuffery, "PEP-FOLD3: faster denovo structure prediction for linear peptides in solution and in complex," *Nucleic Acids Res*, vol. 44, no. 1, pp. W449–W454, Jul. 2016, doi: 10.1093/nar/gkw329.
[32] D. Kozakov *et al.*, "The ClusPro web server for protein-protein docking," *Nat Protoc*, vol. 12, no. 2, pp. 255–278, Feb. 2017, doi: 10.1038/nprot.2016.169.
[33] J. Huang *et al.*, "CHARMM36m: An improved force field for folded and intrinsically disordered proteins," *Nat Methods*, vol. 14, no. 1, pp. 71–73, Dec. 2016, doi: 10.1038/nmeth.4067.
[34] W. Kabsch and C. Sander, "Dictionary of Protein Secondary Structure: Pattern Recognition of Hydrogen-Bonded and Geometrical Features."
[35] O. V. Tsodikov, M. Thomas Record, and Y. V. Sergeev, "Novel computer program for fast exact calculation of accessible and molecular surface areas and average surface curvature," *J Comput Chem*, vol. 23, no. 6, pp. 600–609, Apr. 2002, doi: 10.1002/jcc.10061.
[36] L. Staresincic, J. Walker, A. B. Dirac-Svejstrup, R. Mitter, and J. Q. Svejstrup, "GTP-dependent binding and nuclear transport of RNA polymerase II by Npa3 protein," *Journal of Biological Chemistry*, vol. 286, no. 41, pp. 35553–35561, Oct. 2011, doi: 10.1074/jbc.M111.286161.
[37] D. Forget, A. A. Lacombe, P. Cloutier, M. Lavallée-Adam, M. Blanchette, and B. Coulombe, "Nuclear import of RNA polymerase II is coupled with nucleocytoplasmic shuttling of the RNA polymerase II-associated protein 2," *Nucleic Acids Res*, vol. 41, no. 14, pp. 6881–6891, Aug. 2013, doi: 10.1093/nar/gkt455.
[38] G. Guerrero-Serrano *et al.*, "Npa3/ScGpn1 carboxy-terminal tail is dispensable for cell viability and RNA polymerase II nuclear targeting but critical for microtubule stability and function," *Biochim Biophys Acta Mol Cell Res*, vol. 1864, no. 3, pp. 451–462, Mar. 2017, doi: 10.1016/j.bbamcr.2016.12.010.
[39] B. Lara-Chacón *et al.*, "Gpn3 Is Essential for Cell Proliferation of Breast Cancer Cells Independent of Their Malignancy Degree," *Technol Cancer Res Treat*, vol. 18, pp. 1–11, 2019, doi: 10.1177/1533033819870823.

Figures

Figure 1

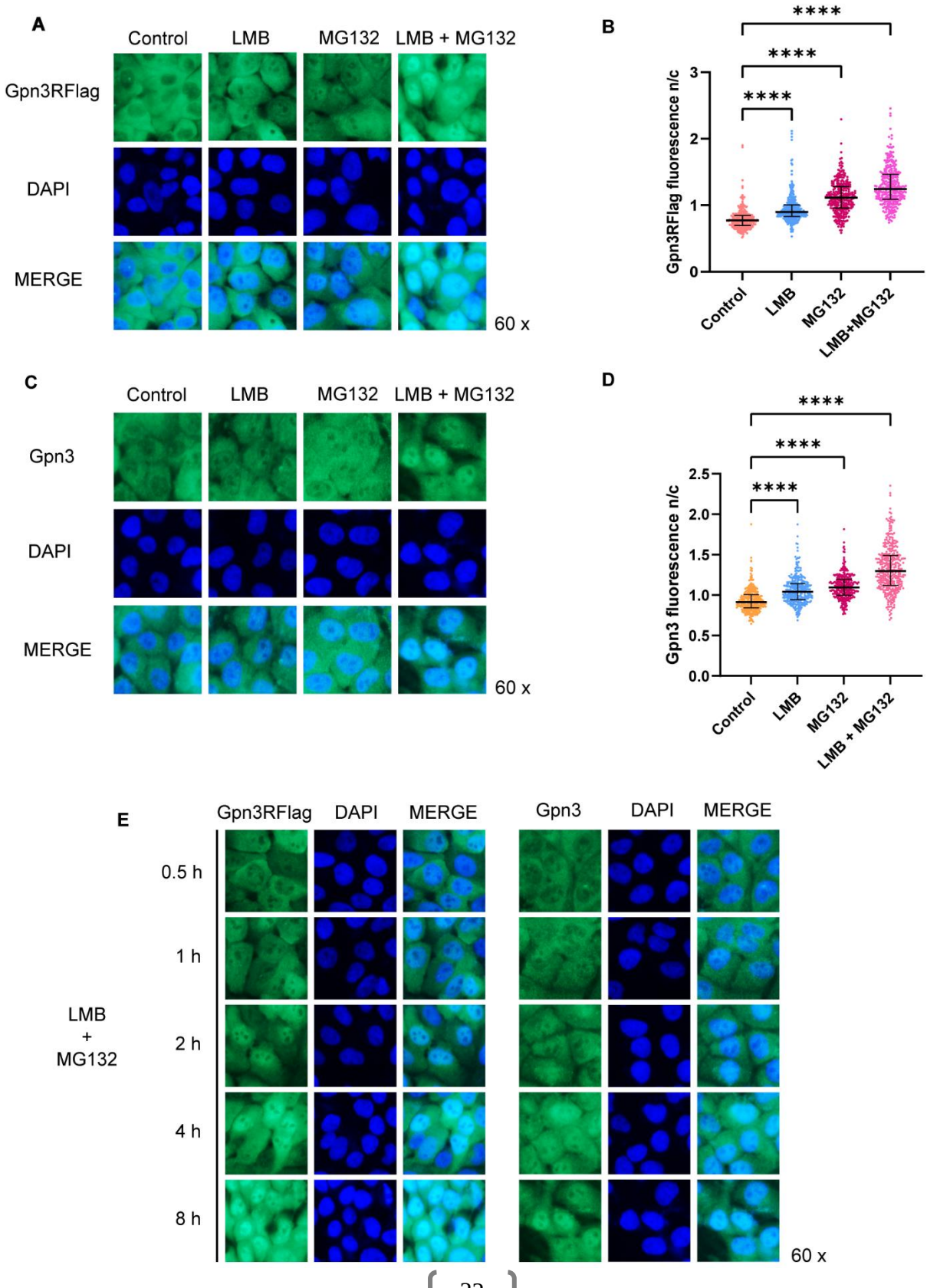


Figure 2

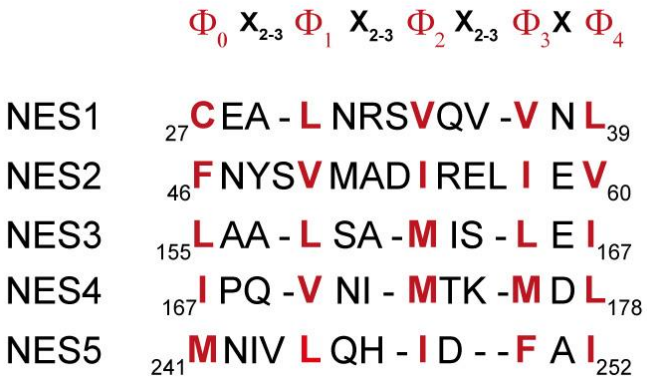

Φ0 X2-3 Φ1 X2-3 Φ2 X2-3 Φ3X Φ4
NES1 27CEA-L NRSVQV-V NL39
NES2 46FNYSVMADIRELI EV60
NES3 155LAA-L SA-MIS-L EI167
NES4 167I PQ-V NI-MTK-MD L178
NES5 241MNIV L QH-I D--F A I252

Figure 3

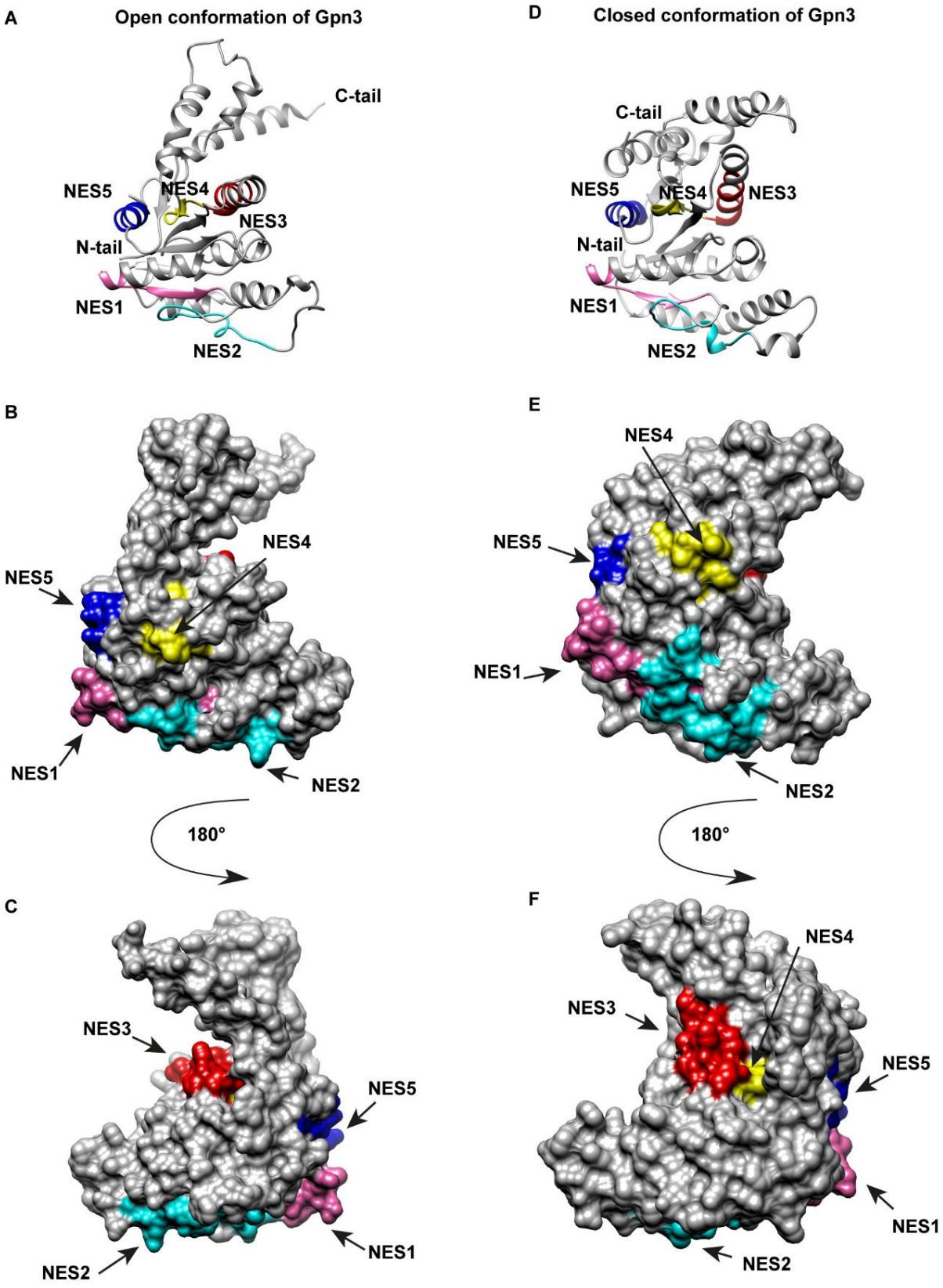

A
Open conformation of Gpn3
C-tail
NES5
NES4
NES3
N-tail
NES1
NES2
D
Closed conformation of Gpn3
C-tail
NES5
NES4
NES3
N-tail
NES1
NES2
B
NES4
NES5
NES1
NES2
180°
C
NES3
NES5
NES2
NES1
E
NES4
NES5
NES1
NES2
180°
F
NES4
NES3
NES5
NES1
NES2

Figure 4

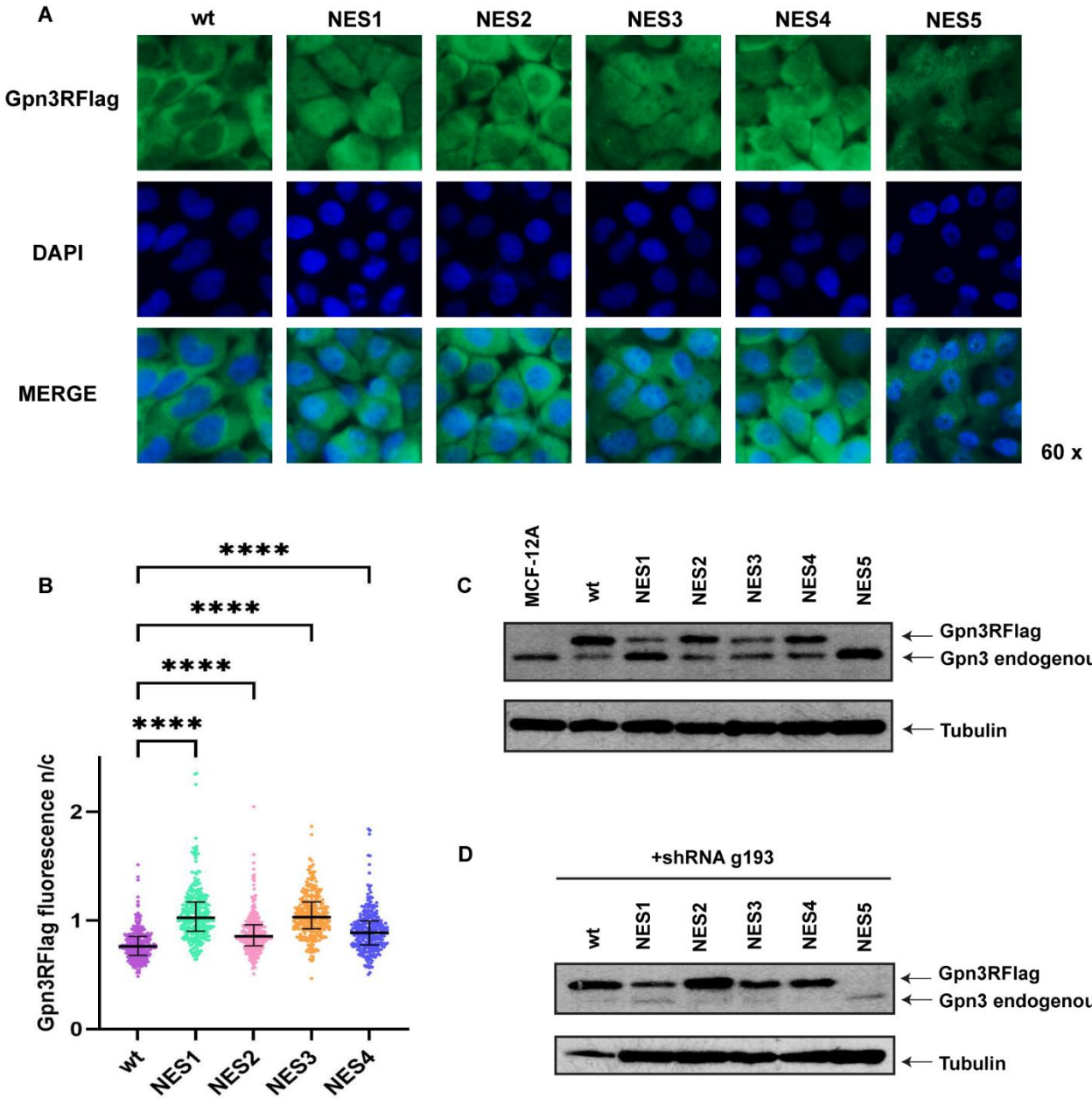

A
wt
NES1
NES2
NES3
NES4
NES5
Gpn3RFlag
DAPI
MERGE
60 x
B
****
****
****
****
Gpn3RFlag fluorescence n/c
2
1
0
wt
NES1
NES2
NES3
NES4
C
MCF-12A
wt
NES1
NES2
NES3
NES4
NES5
Gpn3RFlag
Gpn3 endogenous
Tubulin
D
+shRNA g193
wt
NES1
NES2
NES3
NES4
NES5
Gpn3RFlag
Gpn3 endogenous
Tubulin

Figure 5

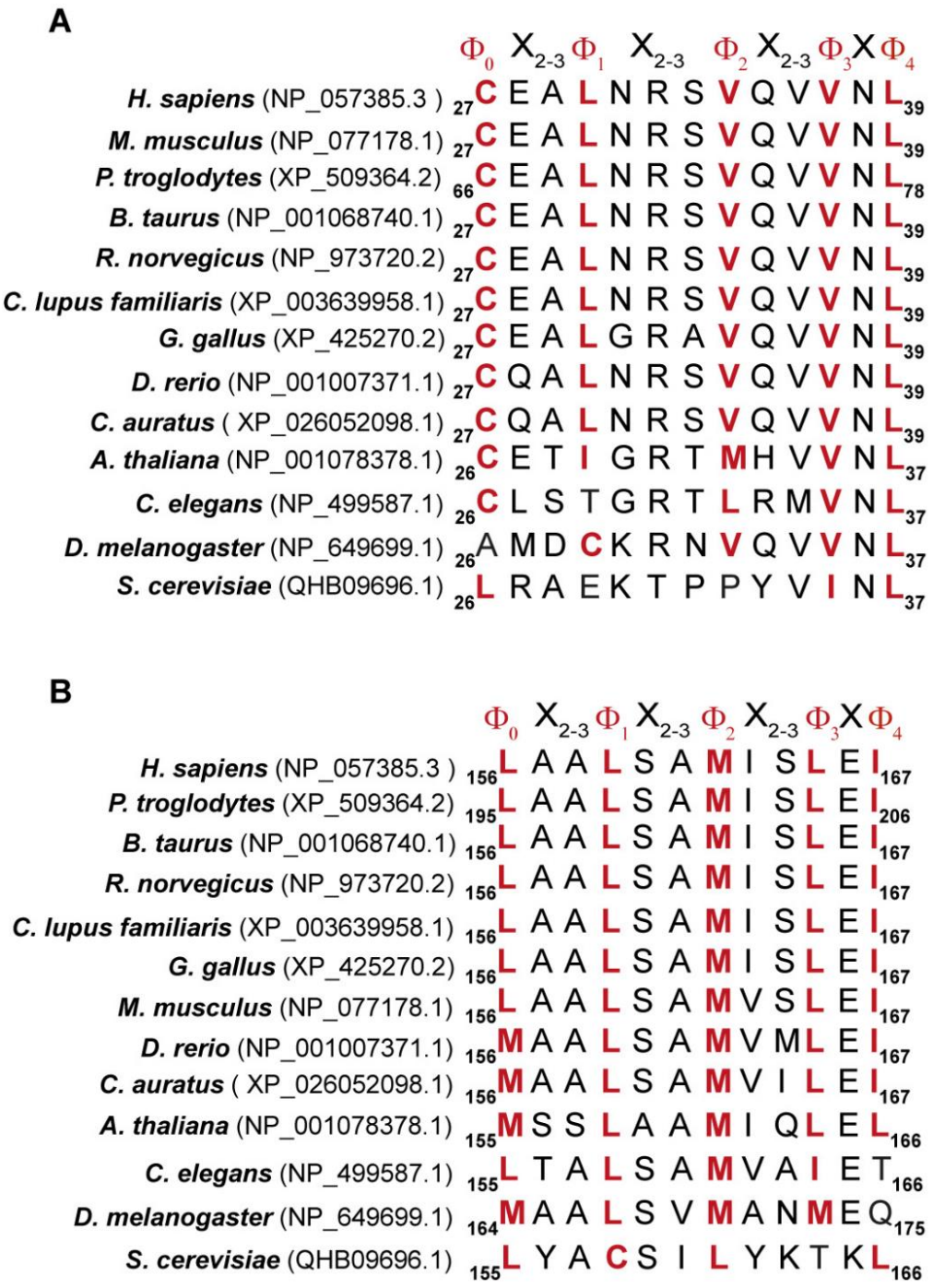

A
Φ0 X2-3 Φ1 X2-3 Φ2 X2-3 Φ3 X Φ4
H. sapiens (NP_057385.3) 27 C E A L N R S V Q V V N L 39
M. musculus (NP_077178.1) 27 C E A L N R S V Q V V N L 39
P. troglodytes (XP_509364.2) 66 C E A L N R S V Q V V N L 78
B. taurus (NP_001068740.1) 27 C E A L N R S V Q V V N L 39
R. norvegicus (NP_973720.2) 27 C E A L N R S V Q V V N L 39
C. lupus familiaris (XP_003639958.1) 27 C E A L N R S V Q V V N L 39
G. gallus (XP_425270.2) 27 C E A L G R A V Q V V N L 39
D. rerio (NP_001007371.1) 27 C Q A L N R S V Q V V N L 39
C. auratus (XP_026052098.1) 27 C Q A L N R S V Q V V N L 39
A. thaliana (NP_001078378.1) 26 C E T I G R T M H V V N L 37
C. elegans (NP_499587.1) 26 C L S T G R T L R M V N L 37
D. melanogaster (NP_649699.1) 26 A M D C K R N V Q V V N L 37
S. cerevisiae (QHB09696.1) 26 L R A E K T P P Y V I N L 37
B
Φ0 X2-3 Φ1 X2-3 Φ2 X2-3 Φ3 X Φ4
H. sapiens (NP_057385.3) 156 L A A L S A M I S L E I 167
P. troglodytes (XP_509364.2) 195 L A A L S A M I S L E I 206
B. taurus (NP_001068740.1) 156 L A A L S A M I S L E I 167
R. norvegicus (NP_973720.2) 156 L A A L S A M I S L E I 167
C. lupus familiaris (XP_003639958.1) 156 L A A L S A M I S L E I 167
G. gallus (XP_425270.2) 156 L A A L S A M I S L E I 167
M. musculus (NP_077178.1) 156 L A A L S A M V S L E I 167
D. rerio (NP_001007371.1) 156 M A A L S A M V M L E I 167
C. auratus (XP_026052098.1) 156 M A A L S A M V I L E I 167
A. thaliana (NP_001078378.1) 155 M S S L A A M I Q L E L 166
C. elegans (NP_499587.1) 155 L T A L S A M V A I E T 166
D. melanogaster (NP_649699.1) 164 M A A L S V M A N M E Q 175
S. cerevisiae (QHB09696.1) 155 L Y A C S I L Y K T K L 166

Figure 6

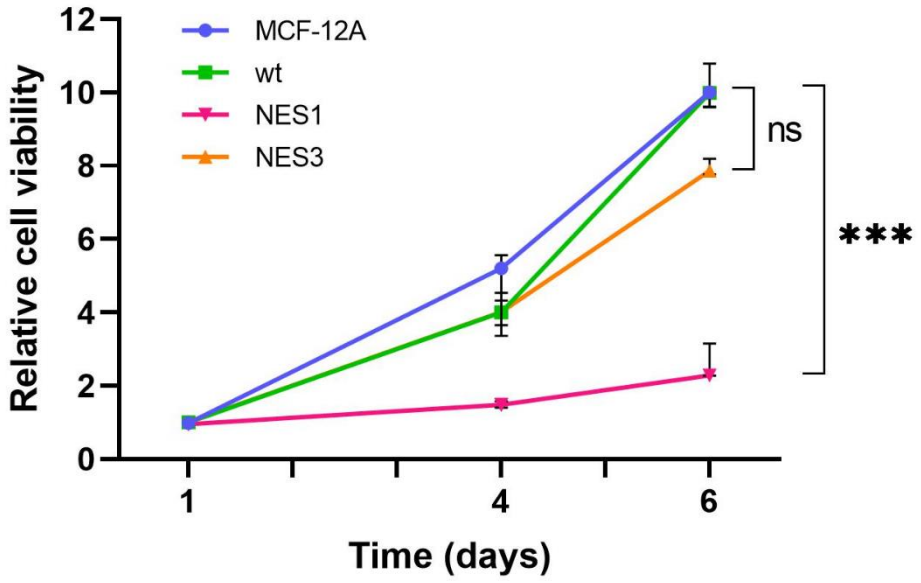


Figure 7

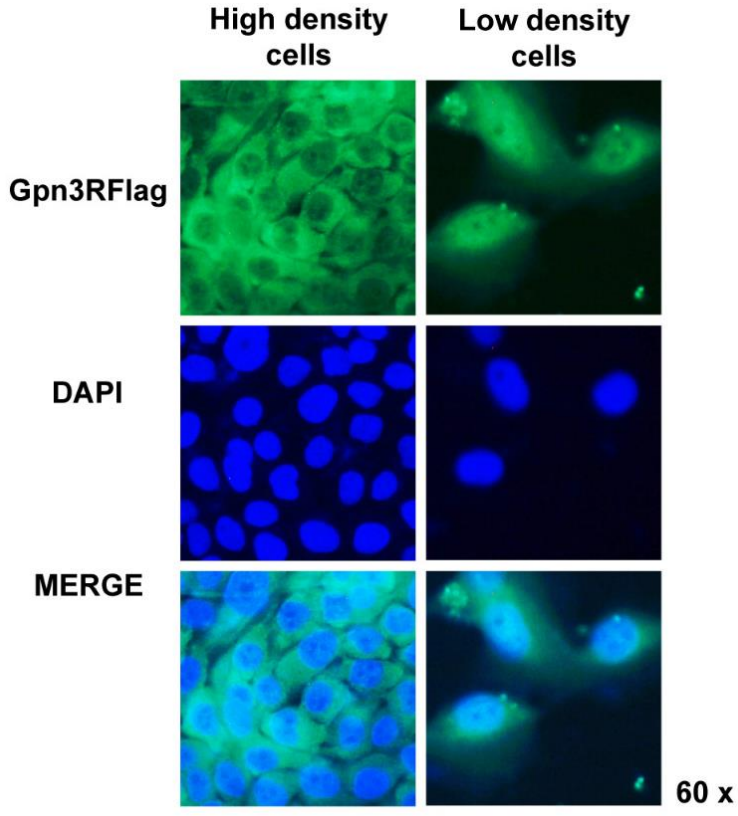
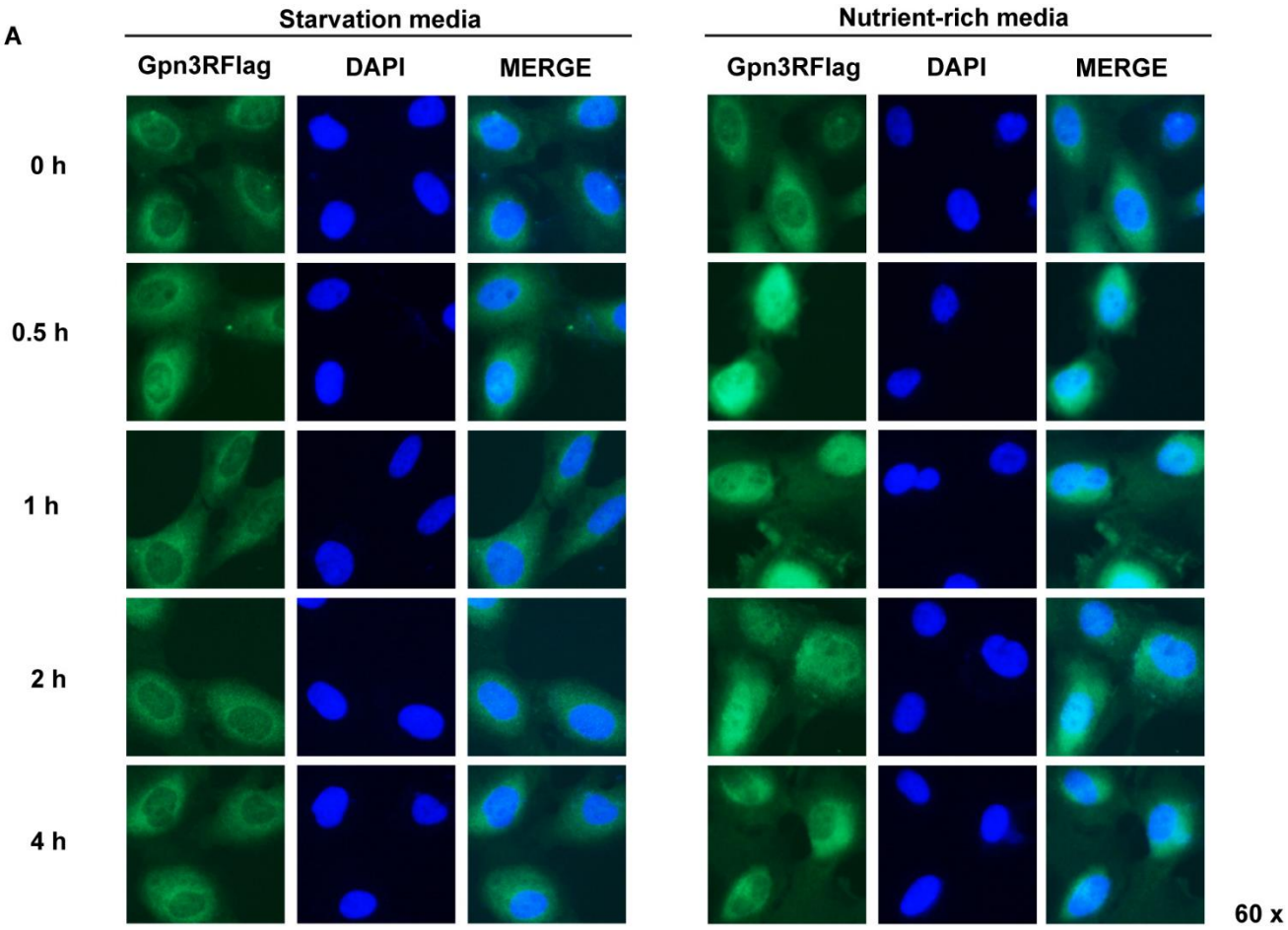


Figure 8


A
Starvation media
Nutrient-rich media
Gpn3RFlag
DAPI
MERGE
Gpn3RFlag
DAPI
MERGE
0 h
0.5 h
1 h
2 h
4 h
60 x

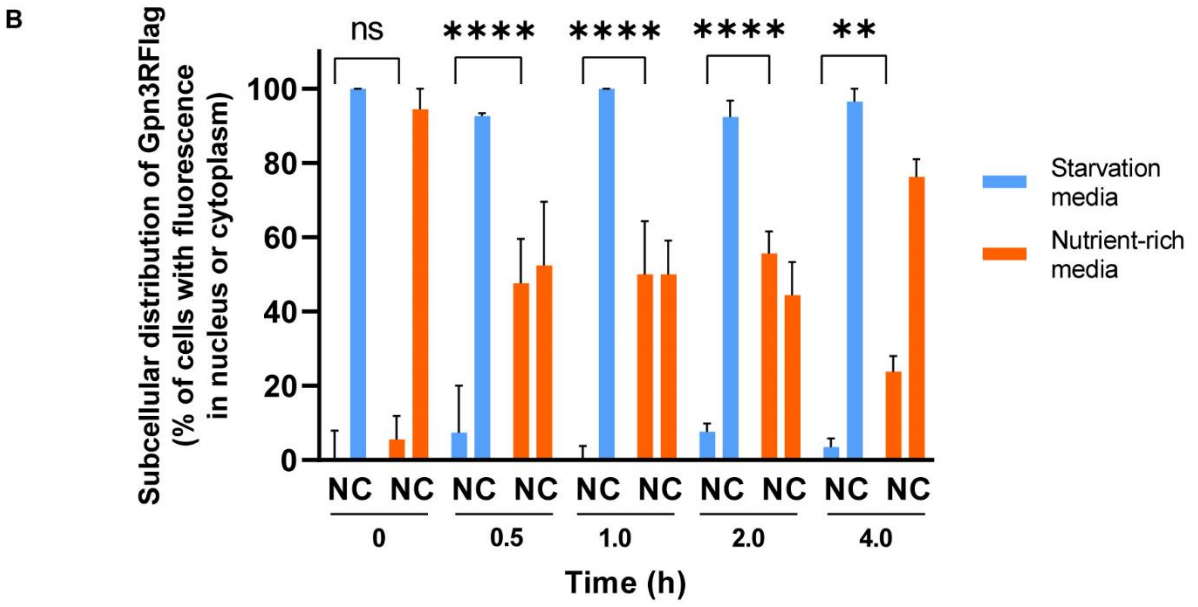

B
Subcellular distribution of Gpn3RFlag (% of cells with fluorescence in nucleus or cytoplasm)
ns
****
****
****
**
100
80
60
40
20
0
NC NC NC NC NC NC NC NC NC NC
0
0.5
1.0
2.0
4.0
Time (h)
Starvation media
Nutrient-rich media

Figure 9

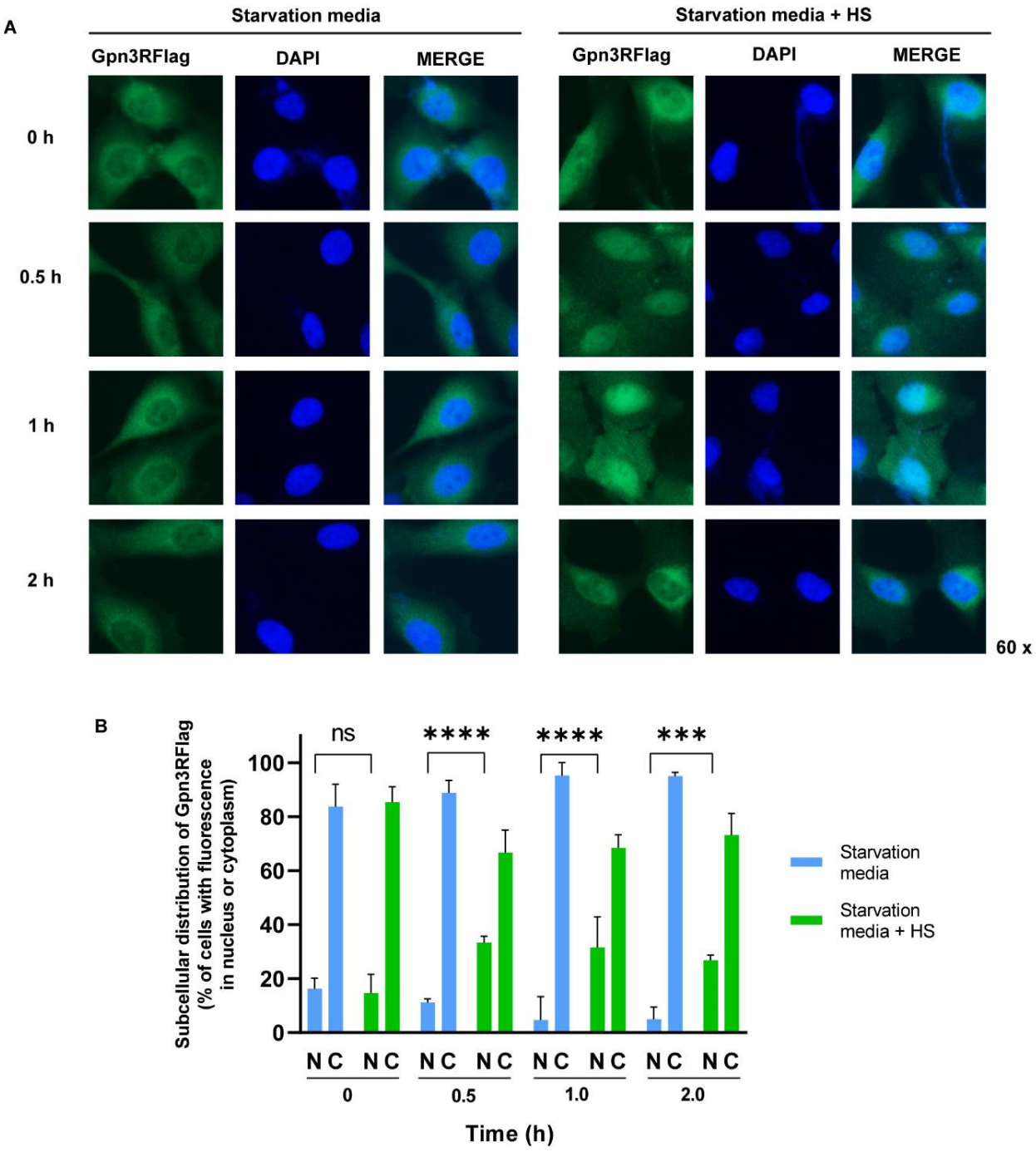

A
Starvation media
Starvation media + HS
Gpn3RFlag
DAPI
MERGE
Gpn3RFlag
DAPI
MERGE
0 h
0.5 h
1 h
2 h
60 x
B
Subcellular distribution of Gpn3RFlag
(% of cells with fluorescence
in nucleus or cytoplasm)
ns
****
****
***
100
80
60
40
20
0
N C N C N C N C N C N C N C N C
0
0.5
1.0
2.0
Time (h)
Starvation
media
Starvation
media + HS

Table 1.

Structural accessibility of the potential nuclear export sequence motifs in the GPN-loop GTPase Gpn3 in both, the GTP-bound (open) state and in the GDP-bound (closed) state. Surface accessibility to the solvent, and by extension to Crm1, for the five nuclear export sequence motifs in Gpn3 studied here was calculated in both, the GDP-containing, closed conformation, and GTP-containing, open conformation, with DSSP and Surface racer. Results obtained with both programs were very similar and values are expressed in A^2. Analysis was performed in the structural model of Gpn3 shown in Figure 3, which we generated based on the experimentally available structure of Npa3, the ortholog of human Gpn1.

Gpn3	Residues	DSSP (A^2)	Surface racer (A^2)
NES1 – GDP/closed	27-39	44.8	46.7
NES1 – GTP/open	**27-39**	**53.8**	**55.3**
NES2 – GDP/closed	46-60	53.3	54.3
NES2 – GTP/open	**46-60**	**59.8**	**61.0**
NES3 – GDP/closed	155-167	40.7	41.3
NES3 – GTP/open	**155-167**	**46.6**	**47.0**
NES4 – GDP/closed	167-178	27.2	28.1
NES4 – GTP/open	**167-178**	**17.6**	**17.25**
NES5 – GDP/closed	241-252	27.2	28.9
NES5 – GTP/open	**241-252**	**42.1**	**44.0**

Asunto: BBAMCR-23-545 - Confirming your submission to BBA - Molecular Cell Research
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De: "BBA - Molecular Cell Research (ELS)" <em@editorialmanager.com>
Destinatario: Roberto Sánchez-Olea <rsanchez@ifisica.uaslp.mx>
Responder a: "BBA - Molecular Cell Research (ELS)" <support@elsevier.com>

Article Title: Nucleocytoplasmic shuttling of the GPN-loop GTPase Gpn3 is regulated by serum and cell confluence in MCF-12A mammary cells
Article Type: Regular Paper

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This is to confirm that the above-mentioned manuscript has been received for consideration in BBA - Molecular Cell Research.

It has been assigned the following manuscript number: BBAMCR-23-545.

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https://www.editorialmanager.com/bbamcr/

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UASLP
Universidad Autónoma de San Luis Potosí

FACULTAD DE MEDICINA

Posgrado en Ciencias Biomédicas Básicas

Regulación de la función celular de la GTPasa Gpn3 a través de la modulación de su localización subcelular en células humanas

Examen que presenta:
M. en C. Sonia Griselda Peña Gómez

Para obtener el grado de:
Doctora en Ciencias Biomédicas Básicas

UASLP
Instituto de Física

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3

INTRODUCCIÓN

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Familia de GTPasas Gpn

- Las proteínas Gpn3 poseen los motivos característicos de las GTPasas.
- Tienen el motivo invariable glicina-prolina-asparagina (GPN).
- Están presentes en todos los organismos eucariotas y algunas arqueas.

Matte-Tailliez et al., 2000. Trends Genet, 16: 533-536; Gras et al., 2007. EMBO Rep, 8: 569-575.

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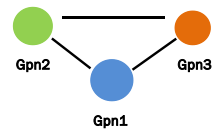
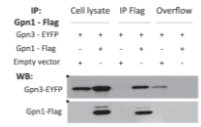
Familia de GTPasas Gpn





- Las proteínas Gpn interactúan entre sí.
- Son proteínas esenciales.
- Son proteínas muy conservadas durante el proceso de evolución.

Giaever et al., 2002. Nature, 418: 387-391; Alonso et al., 2013. Cell Cycle,12: 463–472; Minaker et al., 2013. Genetics, 193: 853–864; Méndez-Hernández et al., 2014. FEBS Letters, 588: 3823-3829.

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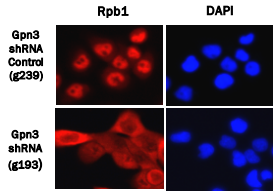
Las proteínas Gpn son necesarias para la acumulación nuclear de la RNAPII



Calera et al., 2011. BBA 1813: 1708–1716

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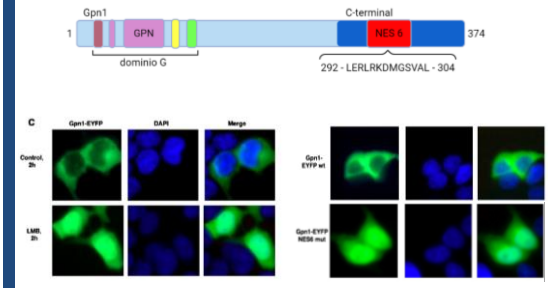
Gpn1 posee una señal de exportación nuclear (NES)



Reyes-Pardo et al., 2012. BBA, 1823: 1756-1766.

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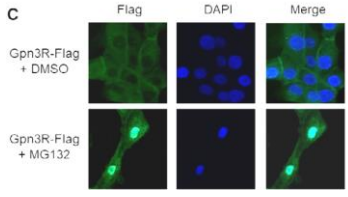
La inhibición del proteasoma por el MG132 induce la acumulación nuclear de Gpn3



Méndez-Hernández et al., 2017. FEBS Letters, 591: 3757–3770.

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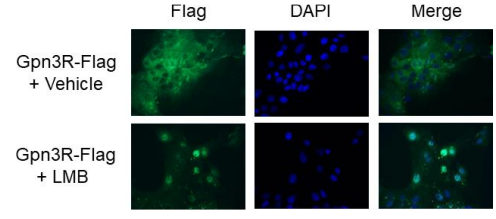
La inhibición de la exportina Crm1 con leptomicina B resulta en la acumulación nuclear de Gpn3



Méndez-Hernández et al., 2017. FEBS Letters, 591: 3757–3770.

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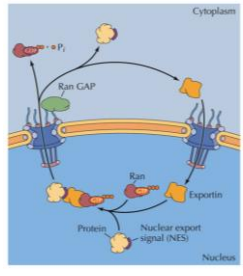
La exportación nuclear activa de proteínas cargo requiere de secuencias señal



Cooper, G. M. and Hausman, R. E., (2007). The cell: A molecular approach, Washington, DC, Sinauer Associates Inc.

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11

Señal de exportación nuclear tipo clásica (NES)

- Secuencia de 10 – 15 residuos.
- Secuencia consenso: $\Phi_0 X_{2-3} \Phi_1 X_{2-3} \Phi_2 X_{2-3} \Phi_3 X \Phi_4$

  Aminoácidos hidrofóbicos: Leu, Val , Ile, Phe o Met
- Es reconocida por la exportina Crm1.

Lee et al., 2019. Scientific Reports, 9:6627.

12

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JUSTIFICACIÓN

- Gpn3 se acumula en el núcleo celular en presencia de leptomicina B, un inhibidor de Crm1. Sin embargo, se desconoce las secuencias de exportación nuclear reconocidas por esta exportina.
- Se desconoce las condiciones fisiológicas en las que Gpn3 se moviliza entre el núcleo y el citoplasma.

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HIPÓTESIS

El transporte núcleo-citoplásmico de la GTPasa Gpn3 está regulado por secuencias de exportación nuclear reconocidas por la exportina Crm1.

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OBJETIVO GENERAL

Evaluar la funcionalidad de las secuencias de exportación nuclear presentes en Gpn3 e identificar las condiciones fisiológicas que modulen el transporte núcleo-citoplásmico de esta GTPasa en células humanas de mama MCF-12A.

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15

OBJETIVOS ESPECÍFICOS

1. Identificar los motivos en la secuencia primaria de Gpn3 que se ajusten a la secuencia consenso de unión a la exportina Crm1.

2. Generar derivados de la línea celular MCF-12A que expresen mutantes de Gpn3 en las diversas secuencias de exportación nuclear en ausencia de Gpn3 endógena.

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3. Determinar la localización subcelular de Gpn3 en los derivados de la línea celular MCF-12A generados en el objetivo anterior.

4. Evaluar la importancia de la exportación nuclear de Gpn3 en la proliferación celular.

5. Identificar las condiciones fisiológicas que regulan el transporte núcleo-citoplásmico de Gpn3.

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METODOLOGÍA Y RESULTADOS

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18

Células MCF-12A

- Células epiteliales humanas de mama no tumorogénicas (inmortalizadas espontáneamente).
- Cariotipo: rango de número de cromosomas 65 -71.
- Provenientes de un tejido tomado de una mamoplastía de reducción en una mujer de 60 años.
- Incubación: 37°C.
- En un ambiente con 5% de CO_2.

medio de crecimiento para células MCF-12A
DMEM F12
suero de caballo (HS)
insulina
factor de crecimiento epidérmico (EFG)
toxina del cólera
hidrocortisona
antibióticos: penicilina, estreptomicina

Debnath et al., 2003. Methods, 30: 256 – 268.
https://lifescience.invitro.com.au/products/s/ATCCRL10782/MCF12A-Mammary-Gland-Human/

19

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1. Identificar los motivos en la secuencia primaria de Gpn3 que se ajusten a la secuencia consenso de unión a la exportina Crm1.

$\Phi_0\ x_{2\text{-}3}\ \Phi_1\ x_{2\text{-}3}\ \Phi_2\ x_{2\text{-}3}\ \Phi_3 x\ \Phi_4$

NES1	27	CEA - L NRSVQV - V N L	39
NES2	46	F NYSV MAD I REL I E V	60
NES3	155	L AA - L SA - M IS - L E I	167
NES4	167	I PQ - V NI - MTK - M D L	178
NES5	241	M NIV L QH - I D - - F A I	252

elm.eu.org

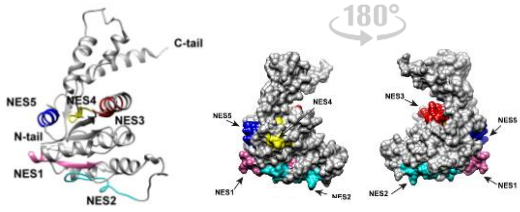
The Eukaryotic Linear Motif resource for *Functional Sites in Proteins*

20

20

Las secuencias de exportación nuclear de Gpn3 se localizan en la superficie de la proteína

conformación abierta - unida a GTP

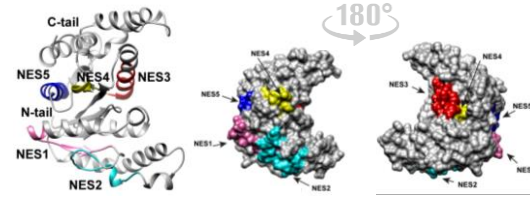


21

21

Las secuencias de exportación nuclear de Gpn3 se localizan en la superficie de la proteína

conformación cerrada - unida a GDP



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El área de exposición al solvente de la mayoría de las NESes en Gpn3 es mayor cuando la proteína une GTP comparado con GDP

Gpn3	Residues	DSSP (A^2)	Surface racer (A^2)
NES1 – GDP/closed	27-39	44.8	46.7
NES1 – GTP/open	**27-39**	**53.8**	**55.3**
NES2 – GDP/closed	46-60	53.3	54.3
NES2 – GTP/open	**46-60**	**59.8**	**61.0**
NES3 – GDP/closed	155-167	40.7	41.3
NES3 – GTP/open	**155-167**	**46.6**	**47.0**
NES4 – GDP/closed	167-178	27.2	28.1
NES4 – GTP/open	**167-178**	**17.6**	**17.25**
NES5 – GDP/closed	241-252	27.2	28.9
NES5 – GTP/open	**241-252**	**42.1**	**44.0**

23

23

2. Generar derivados de la línea celular MCF-12A que expresen mutantes de Gpn3 en las diversas secuencias de exportación nuclear en ausencia de Gpn3 endógena.

META 1: Diseñar oligonucleótidos para mutar los aminoácidos hidrofóbicos ϕ_3 y ϕ_4 de las 5 secuencias de exportación nuclear anteriormente identificadas.

$\phi_3, \phi_4 \rightarrow A$

$\Phi_0\ x_{2\text{-}3}\ \Phi_1\ x_{2\text{-}3}\ \Phi_2\ x_{2\text{-}3}\ \Phi_3 x\ \Phi_4$

NES1	27	CEA - L NRSVQV - V N L	39
NES2	46	F NYSV MAD I REL I E V	60
NES3	155	L AA - L SA - M IS - L E I	167
NES4	167	I PQ - V NI - MTK - M D L	178
NES5	241	M NIV L QH - I D - - F A I	252

24

24

	oligonucleótido
NES1 (V37A, L39A) fwd	5'-ccctcaaccggtctgtccaagttgcaaacgcggatccagcagcagaacac-3'
NES1 (V37A, L39A) rev	5'-gtgttctgctgctggatccgcgtttgcaacttggacagaccggttgaggg-3'
NES2 (I58A, V60A) fwd	5'-gatggctgacatccgggaactggccgaggcggatgatgtaatggaggatg-3'
NES2 (I58A, V60A) rev	5'-catcctccattacatcatccgcctcggccagttcccggatgtcagccatc-3'
NES3 (L165A,I167A) fwd	5'-ccctgagtgccatgatctctgcagaagctccgcaagtcaacatcatgac-3'
NES3 (L165A,I167A) rev	5'-gtcatgatgttgacttgcggagcttctgcagagatcatggcactcaggg-3'
NES4 (M176A, L178A) fwd	5'-gtcaacatcatgacaaaagcggatgcgctgagtaaaaaagcaaaaaagg-3'
NES4 (M176A, L178A) rev	5'-ccttttttgcttttttactcagcgcatccgcttttgtcatgatgttgac-3'
NES 5 (F250A, I252A) fwd	5'-cattgcattgcagcatattgatgctgccgctcaatatggagaagacc-3'
NES 5 (F250A, I252A) rev	5'-ggtcttctccatattgagcggcagcatcaatatgctgcaatgcaatg-3'

QuickChange Primer Design — Agilent Technologies

Multiple Primer Analyzer — ThermoFisher SCIENTIFIC

Síntesis de oligonucleótidos SIGMA-ALDRICH

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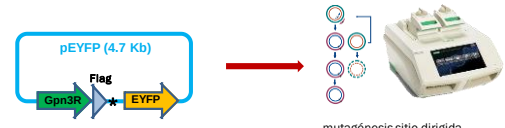
25

META 2: Generar mutantes de Gpn3RFlag y sus versiones mutantes en las NES mediante mutagénesis sitio-dirigida.



codón de paro

mutagénesis sitio-dirigida Mediante PCR

LANBAMA
LABORATORIO NACIONAL DE BIOTECNOLOGÍA AGRÍCOLA, MÉDICA Y AMBIENTAL
IPICYT

secuenciación

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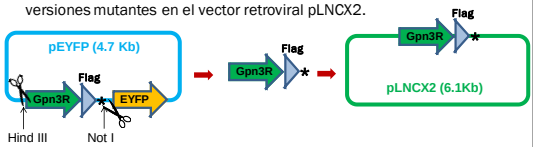
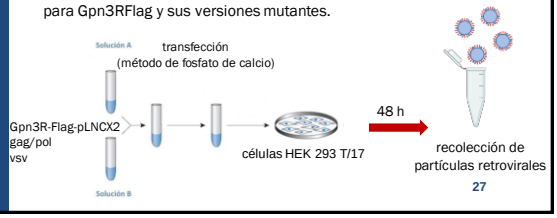
META 3: Subclonar la secuencia codificante de Gpn3RFlag y las versiones mutantes en el vector retroviral pLNCX2.



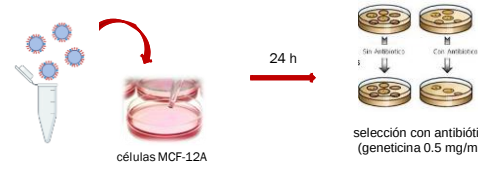
META 4: Generar partículas retrovirales que contengan la secuencia para Gpn3RFlag y sus versiones mutantes.

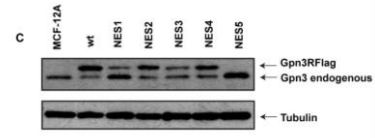


27

27

META 4: Generación de líneas celulares que expresen establemente Gpn3RFlag y sus versiones mutantes.

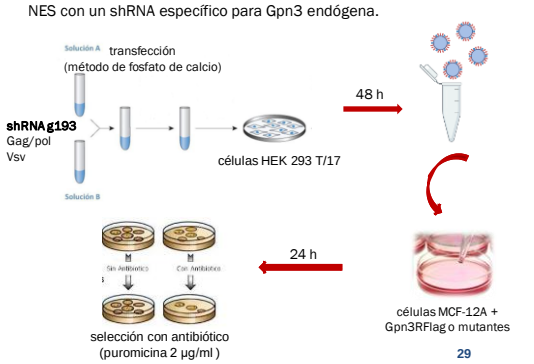




28

28

Meta 5: Suprimir la expresión de Gpn3 endógena en células que expresan establemente Gpn3RFlag y sus versiones mutantes en las NES con un shRNA específico para Gpn3 endógena.



29

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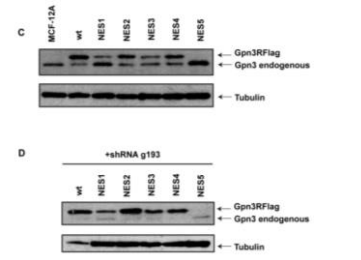
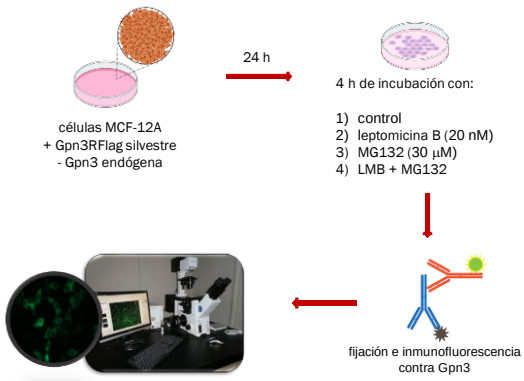
El shRNA g193 suprimió exitosamente la expresión de Gpn3 endógena pero no de Gpn3RFlag silvestre o sus versiones mutantes



30

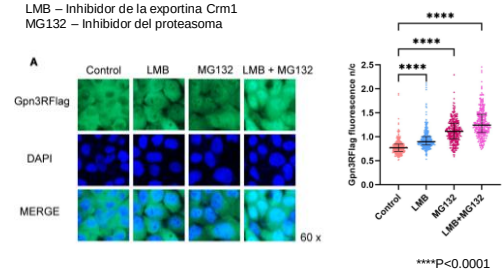
30



31

Gpn3RFlag se acumula en el núcleo celular en presencia de leptomicina B y/o MG132

LMB – Inhibidor de la exportina Crm1
MG132 – Inhibidor del proteasoma



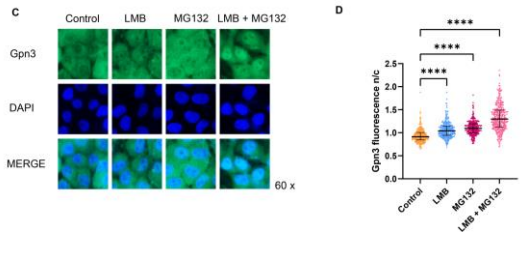
****P<0.0001

La LMB y el MG132 tienen un efecto aditivo en la acumulación nuclear de Gpn3RFlag.

32

32

La proteína Gpn3 endógena se concentra en el núcleo celular al igual que Gpn3RFlag en presencia de LMB y/o MG132

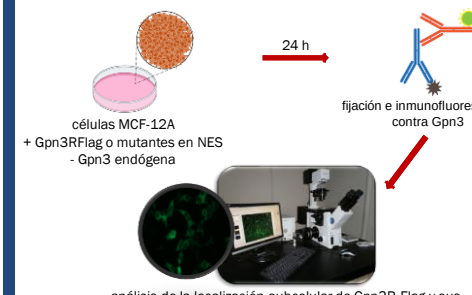


****P<0.0001

33

33

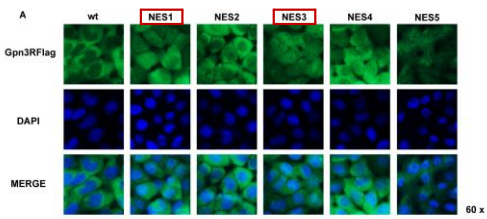
3. Determinar la localización subcelular de Gpn3 en los derivados de la línea celular MCF-12A generados en el objetivo anterior.



34

34

Gpn3 contiene varias secuencias de exportación nuclear funcionales que se unen a Crm1

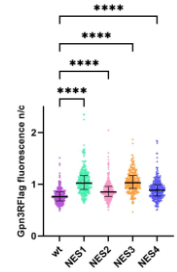


La NES1 y NES3 son señales de exportación nuclear funcionalmente importantes que movilizan a Gpn3 fuera del núcleo celular.

35

35

Las mutantes de Gpn3RFlag en las NES se acumulan en el núcleo celular

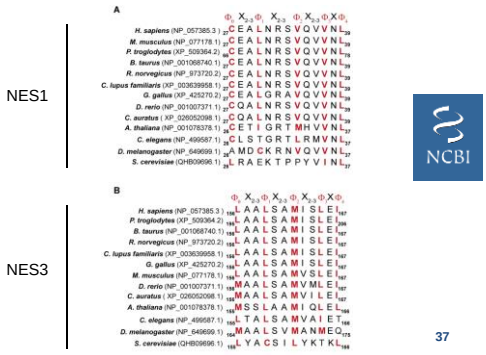


****P<0.0001

36

36

Las secuencias de exportación nuclear NES1 y NES3 están evolutivamente conservadas



37

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4. Evaluar la importancia de la exportación nuclear de Gpn3 en la proliferación celular.

Niveles de ATP
Kit de ensayo de luminiscencia

siembra de 1,000 células MCF-12A /pozo

lector de placas VICTOR 4X

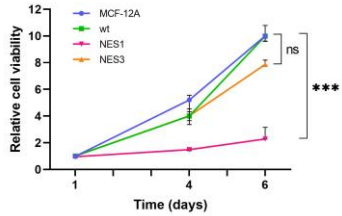
líneas celulares
MCF-12A
MCF-12A + Gpn3RFlag wt
MCF-12A + Gpn3RFlag NES1
MCF-12A + Gpn3RFlag NES3

medición de la luminiscencia a los días 1, 4 y 6 post-siembra

38

38

La exportación nuclear de Gpn3 es importante para la proliferación celular



***P = 0.0001

39

39

5. Identificar las condiciones fisiológicas que regulan el transporte núcleo-citoplásmico de Gpn3.

META 1: Efecto de la densidad en la localización subcelular de Gpn3

siembra de células MCF-12A + Gpn3RFlag wt – Gpn3 endógena

alta densidad

baja densidad

2 x10^5 células/pozo

9 x10^4 células/pozo

48 h

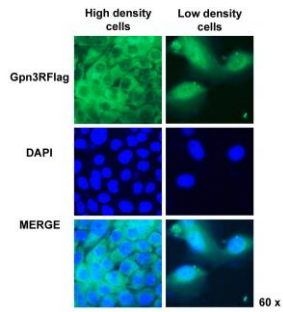
24 h

fijación de las células con paraformaldehído al 3.7% e inmunofluorescencia contra Gpn3

40

40

La localización subcelular de Gpn3 es sensible a la densidad celular



41

41

5. Investigar las condiciones fisiológicas que regulen el transporte núcleo-citoplásmico de Gpn3.

META 1: Efecto de los factores de crecimiento del medio de cultivo en la localización subcelular de Gpn3.

24 h

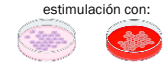
incubación con medio de ayuno

siembra de células MCF-12A + Gpn3RFlag wt

4 h

baja densidad
9 x10^4 células/pozo

estimulación con:



medio de ayuno / medio completo por 0, 0.5, 1, 2 y 4h

fijación e inmunofluorescencia contra Gpn3

42

42

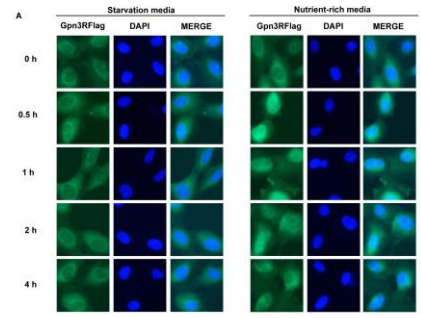
componentes	medio completo	medio de ayuno
DMEM F12	+	+
suero de caballo (HS)	+	–
insulina	+	–
Factor de crecimiento epidérmico (EFG)	+	–
toxina del cólera	+	+
hidrocortisona	+	+
antibióticos: penicilina, estreptomicina	+	+

Debnath et al., 2003. Methods, 30: 256 – 268.

43

43

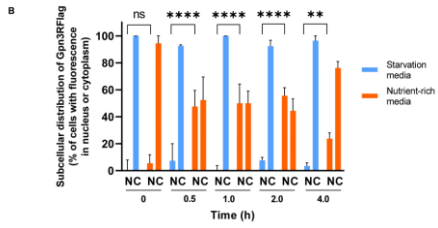
Gpn3RFlag se acumula en el núcleo celular después de la estimulación con medio de crecimiento completo en células MCF-12A



44

44

Gpn3 se acumula en el núcleo en cerca del 50% de las células entre los 30 min y 2 horas después de la estimulación con medio completo



**P=0.0038; ****P<0.0001.

45

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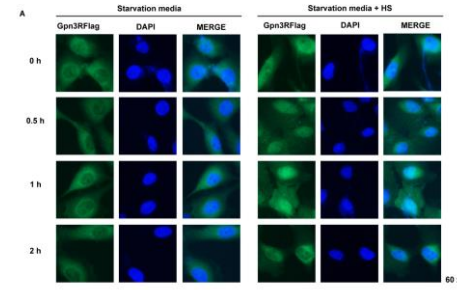
Gpn3 se transloca al núcleo en células MCF-12A en respuesta a la estimulación con suero de caballo



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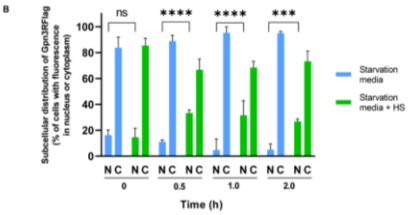
La acumulación nuclear de Gpn3 inducida por suero de caballo ocurre solo en una fracción de las células



. ****P<0.0001; ns, non- significant.

47

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RESUMEN

- La NES1 (aa 27-39) y NES3 (aa 156-167) son señales de exportación nuclear funcionales en Gpn3.
- La NES1 y la NES3 son secuencias evolutivamente conservadas.
- La NES1 y la NES3 se encuentran en la superficie de Gpn3, por lo tanto, las hace accesibles a la exportina Crm1.

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- Gpn3 tiene una distribución homogénea entre el núcleo y el citoplasma en células crecidas a baja densidad y una localización citoplásmica en células confluentes.
- La estimulación de células MCF-12A con suero de caballo induce la acumulación nuclear de Gpn3.

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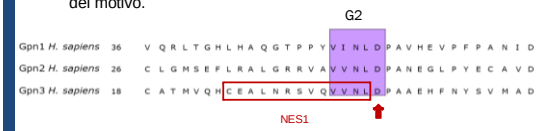
DISCUSIÓN

- LA NES1 de Gpn3 incluye al motivo G2 de GTPasa.
- El D40 del motivo G2 de Gpn1 en *S. cerevisiae* forma un enlace de coordinación con el Mg^{+2} y es el único aminoácido relevante del motivo.



- El motivo G2 no está bien conservado en otras GTPasas.

Anand et al., 2006. Nucleic Acids Res, 34: 2196–2205.
Niesser et al., 2015. Mol. Cell. Biol., 5:820-831.

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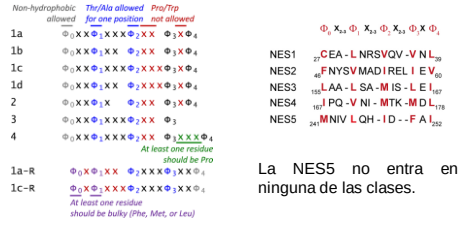
DISCUSIÓN

- Según la clasificación de Lee *et al*., 2019. La NES1 de Gpn3 pertenece a la clase 1a, mientras que la NES3 es de clase 1b.



La NES5 no entra en ninguna de las clases.

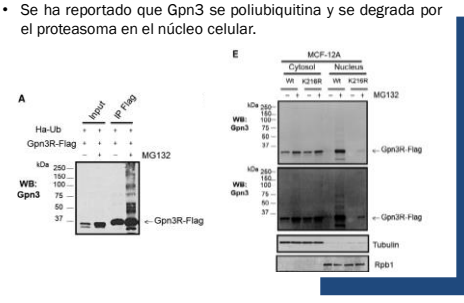
Lee et al., 2019. Scientific Reports 9:6627.

51

51

DISCUSIÓN

- Se ha reportado que Gpn3 se poliubiquitina y se degrada por el proteasoma en el núcleo celular.



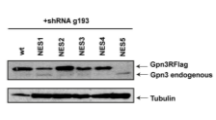
Méndez Hernández et al., 2017. FEBS Letters, 591: 3757–3770.

52

52

DISCUSIÓN

- Los bajos niveles de expresión de Gpn3RFlag-NES1 y Gpn3RFlag-NES3 pueden deberse a que estas proteínas se encuentran en el núcleo celular, lugar donde se ha reportado que Gpn3 se degrada por el proteasoma.



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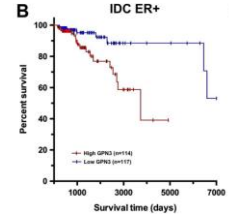
DISCUSIÓN

- Una expresión elevada de GPN3 disminuye el periodo de sobrevida en pacientes con cáncer de mama.



Lara-Chacón et al., 2019. TCRT, 18: 1- 11.

54

54

CONCLUSIONES

- En este proyecto se identificaron dos secuencias de exportación nuclear funcionales (NES1 y NES3) en la GTPasa Gpn3.
- La proliferación de células MCF-12A depende del movimiento núcleo-citoplásmico de Gpn3 modulado por la NES1.
- Los niveles proteicos de Gpn3 podrían regularse por la NES1 y la NES3, ya que Gpn3 se ubiquitina y se degrada en el núcleo.

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CONCLUSIONES

- La reducción en el periodo de sobrevida causada por una expresión elevada de GPN3 en pacientes con cáncer de mama, podrían deberse a un defecto en la localización subcelular de esta proteína.
- La regulación subcelular de Gpn3 por suero y por la densidad celular sugiere que la función nuclear de esta proteína se relaciona positivamente con el crecimiento y/o la proliferación celular.

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PERSPECTIVAS

- Evaluar si en células MCF-12A confluentes se induce la acumulación nuclear al estimularlas con medio completo y HS.
- Determinar si la mutación en la NES1 de Gpn3 afecta la actividad de GTPasa de Gpn3.
- Determinar si la exportación nuclear de Gpn3 ayuda a la exportación nuclear de otras proteínas.

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PERSPECTIVAS

- Evaluar el efecto de diferentes factores de crecimiento y estudiar las vías de señalización que podrían regular la traslocación de Gpn3 hacia el núcleo celular.

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Publicaciones en revistas internacionales indexadas


FEBS Letters

The Gpn3 Q279* cancer-associated mutant inhibits Gpn1 nuclear export and is deficient in RNA polymerase II nuclear targeting

Protein Expression and Purification

Human Gpn1 purified from bacteria binds guanine nucleotides and hydrolyzes GTP as a protein dimer stabilized by its C-terminal tail

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AGRADECIMIENTOS

- A la Dra. Mónica R. Calera Medina
- Al Dr. Roberto Sánchez Olea
- A la Dra. Gema R. Cristóbal Mondragón
- A Cristhian Raúl Vega Palomo
- A mi padre.
- A mis hermanos.
- A mis compañeros y amigos del laboratorio (Gpn team).






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GRACIAS POR SU ATENCIÓN

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