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Memoria do Traballo de Fin de Grao

Coordination of TOR signalling and the Greatwall-ENSA pathway in the differentiation response in fission yeast

Cordinación da ruta de señalización TOR e da vía Greatwall-ENSA na resposta de diferenciación no lévedo de fisión

Coordinación de la ruta de señalización TOR y de la vía Greatwall-Ensa en la respuesta de diferenciación en la levadura de fusión.

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UNIVERSIDADE DA CORUÑA

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REPORT:

That this work realized by Marcos Veloso Carril, has been realized under our direction and, considering it finished, we agree that is ready for being presented to the Qualifying

A Coruña, 21 of June de 2018

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ABSTRACT

Cell cycle and growth is controlled by a highly conserved signaling network among eukaryotic cells, the TOR signaling pathway. This signaling network must be accurately coordinated in order to being able to properly respond to the changes in the surrounding medium. In Schizosaccharomyces *pombe*, two different TOR complexes mediate the integration of nutritional cues to cellular cell cycle. How the interplay between the two TOR complexes takes place is poorly understood. Here we focused in the crosstalk of the two TOR complexes through the interaction of two of its effectors: Igo1(ENSA) from TORC1 and Gad8 from TORC2. We found that Gad8 can interplay the two TOR complexes through the interaction with Igo1 (ENSA). More signaling modules to coordinate TOR functioning have been previously revealed, being this one step more in the comprehension of the pathway. Our results demonstrate that Gad8 inhibits Igo1 (ENSA) in the first 30 minutes after nitrogen depletion, and that this interaction is a requisite to obtain the maximum Igo1 (ENSA) level of phosphorylation later on and a proper and coordinated expression of the mating genes when switching from a mitotic life cycle into a meiotic one after nitrogen depletion.

Key words: TOR signaling pathway, coordination, Schizosaccharomyces *pombe*, Igo1 (ENSA), Gad8, mating genes, cell cycle, nitrogen.

RESUMEN

El ciclo y el crecimiento celular se encuentran regulados por una vía de señalización altamente conservada entre los eucariotas; la vía de señalización TOR. Esta vía de señalización debe estar coordinada con precisión para poder responder adecuadamente a los cambios del medio circundante. En Schizosaccharomyces *pombe*, dos complejos TOR diferentes median la integración de las señales nutricionales con el ciclo celular. El modo en el que se produce la interacción entre ambos complejos TOR es poco conocido. Aquí nos centramos en la diafonía entre los dos complejos TOR a través de dos de sus efectores: Igo1 (ENSA) del TORC1 y Gad8 del TORC2. Hemos encontrado que Gad8 puede conectar la acción de los dos complejos TOR a través de la interacción con Igo1 (ENSA). Previamente se han revelado más módulos de señalización TOR, siendo este un paso más en la comprensión de la ruta. Nuestros resultados demuestran que Gad8 inhibe a Igo1 (ENSA) en los primeros 30 minutos después de que se agote el nitrógeno, y demuestra también que esta interacción es necesaria para que más tarde se pueda producir el máximo nivel de fosforilación de Igo1 (ENSA), así como, una adecuada y coordinada expresión de los genes de apareamiento cuando se produce el cambio del ciclo celular mitótico al meiótico, provocado este por el agotamiento del nitrógeno.

Palabras clave: vía de señalización TOR, coordinación, Schizosaccharomyces *pombe*, Igo1 (ENSA), Gad8, genes de apareamiento, nitrógeno.

RESUME

O ciclo e crecemento celular encóntranse regulados por unha vía de sinalización altamente conservada entre os eucariotas; a vía de sinalización TOR. Esta vía de sinalización debe estar coordinada con precisión para ser quen de responder aos cambios no medio circundante. En Schizosaccharomyces *pombe*, dous complexos TOR diferentes median a integración das sinais nutricionais co ciclo celular. O modo no que se produce a interacción entre ambos complexos TOR é pouco coñecido. Aquí centrámonos na diafonía entre os dous complexos a través de dous dos seus efectores: Igo1 (ENSA) do TORC1 e Gad8 do TORC2. Atopamos que Gad8 pode conectar a acción dos dous complexos a través da súa iteración con Igo1 (ENSA). Previamente reveláronse máis módulos de sinalización TOR, sendo este un paso máis na comprensión da ruta. Os nosos resultados demostran que Gad8 inhibe a Igo1 (ENSA) nos primeiros 30 minutos despois do esgotamento do nitróxeno, e demostra tamén que esta interacción é necesaria para que máis tarde se acade o máximo nivel de fosforilación de Igo1 (ENSA), así como, unha adecuada e coordinada expresión dos xenes de apareamento ao producirse o cambio do ciclo celular mitótico ao meiótico, provocado este polo esgotamento do nitróxeno.

Palabras chave: vía de sinalización TOR, coordinación, Schizosaccharomyces *pombe*, Igo1 (ENSA), Gad8, xenes de apareamento, nitróxeno.

INTRODUCTION

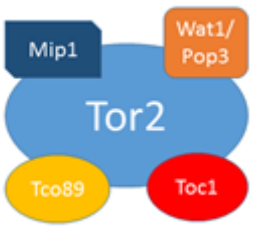
1. TOR signaling pathway

The target of rapamycin (TOR) is the central node of a highly conserved signaling network that regulates cell growth in response to a plethora of environmental cues including nutrients, growth factors, and cellular energy. TOR can be found in two functionally different complexes, which regulate different aspects of eukaryote growth, TOR complex 1 (TORC1) and Tor complex 2 (TORC2) (1). Whereas TORC1 regulates cell volume and/or mass by influencing protein synthesis and turnover, TORC2 regulates cell surface area by influencing lipid production and intracellular turgor. TORC effectors can also function as upstream regulators, suggesting that these complexes function in homeostatic feedback loops (2).

2. Model organisms for studying signaling pathways: *Schizosaccharomyces pombe*

Model organisms are chosen on the basis that they are relatively simple to grow and are easily amenable to experimental manipulation. They are easy to manipulate and grow and have a short cell cycle. Fission yeast (Schizosaccharomyces *pombe*) is a popular model organism and it has been particularly useful in the field of cell cycle research. Fission yeast is genetically tractable, can be easily maintained in haploid or diploid state and the classical genetic methods can be easily performed in it. Moreover, there is an extensive collection of molecular tools available for different studies. Human cells are more complicated than simple yeast cells, but the basic cell cycle machinery is conserved throughout evolution from yeast to human. S. *pombe*, is an excellent model system for analysis of the TOR complexes involved in cell cycle regulation. It appears to be that fission yeast possesses the same regulatory mechanisms for TORs that are conserved among eukaryotes including mammals. In addition, their amino acid sequences share 52% overall identity, and each share 42–44% identity with human TOR kinases (5).
In fission yeast, where nutritional sensing is intimately linked to sexual differentiation, both TOR complexes play opposite roles in the mating response (3). Under the presence of a rich nitrogen source TORC1 is active and its activity impedes the expression of genes required for differentiation (4). On the contrary, when a rich nitrogen source is not present, TORC2 is activated and thus mating activity is prompted (5). The two TOR complexes are linked through the protein phosphatase PP2A-B55^{Pab1} (3).

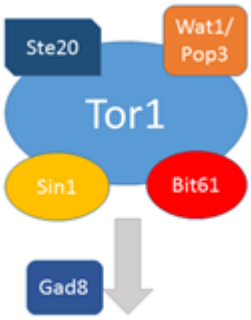
3. *S. pombe* TORC1



The major TOR kinase in TORC1 is Tor2. Tor2 is associated with different proteins: Mip1 is a suppressor of ectopic meiosis induced by *mei2*, an RNA binding protein required for meiosis (6); Wat1/Pop3 is the common subunit of TORC1 and TORC2 and it is in charge of the localization of F-actin (7); finally, Tco89 and Toc1 were identified by mass spectroscopic analysis, their functions are currently unknown (8).

Fig 1: Major TORC1 components.

4. S. pombe TORC2



The major TOR kinase in TORC2 is Tor1. Tor1 is also associated with different proteins: Ste20, which is involved in G1 arrest and sexual development under stress conditions (9); Wat1/Pop3, which is the common subunit of TORC1 and TORC2 as mentioned above; Sin1, which controls sexual development and the response to osmotic and high temperature stress conditions (10); and finally, Bit61 which was identified by mass spectroscopic analysis, its function is currently unknown (8).

Fig2: Major TORC2 components and its major effector Gad8

5. Regulation and coordination of TOR complexes

TORC1 and TORC2 work in a coordinated manner to promote cell growth and proliferation. Both complexes have opposite roles in certain processes and cell types, so its regulation must be accurate. Multiple mechanisms of feedback and crosstalk between the two TOR complexes have been revealed in recent studies (3, 11)

Under nitrogen-rich medium conditions, TORC1 leads to the inhibition of Ppk18, which is the main Greatwall kinase that phosphorylates Igo1 (ENSA) in *S. pombe*. Thus, when Ppk18 is not able to phosphorylate Igo1(ENSA) it is not able to carry out its function, which is to trigger the inactivation of PP2A-B55^{Pab1}, which links the two TOR complexes. Active PP2A-B55^{Pab1} prevents meiotic entry by reverting the phosphorylation on Ser546 of Gad8, the major effector of TORC2 (12).

When cells sense nutrient deprivation, for example by experimentally switching the rich medium into a no-nitrogen poor medium, a decrease in TORC1 activity prompts the reactivation of Ppk18. Ppk18 is now able to phosphorylate Igo1(ENSA) into its phosphorylated active form. In this situation, Ppk18 triggers the inactivation of PP2A-B55^{Pab1}, which now can neither exert its repressive role on mitotic entry, nor prevent phosphorylation of Gad8. On the other hand, the Ryh1 GTPase activates the TORC2 signaling pathway in some stress conditions and initiates the TORC2-dependent phosphorylation of the Gad8 kinase (24). This results in the phosphorylation of the downstream gene *fkh2* which leads to the induction of the expression of mating genes and therefore sexual differentiation (3, 11) [Scheme in Fig3].

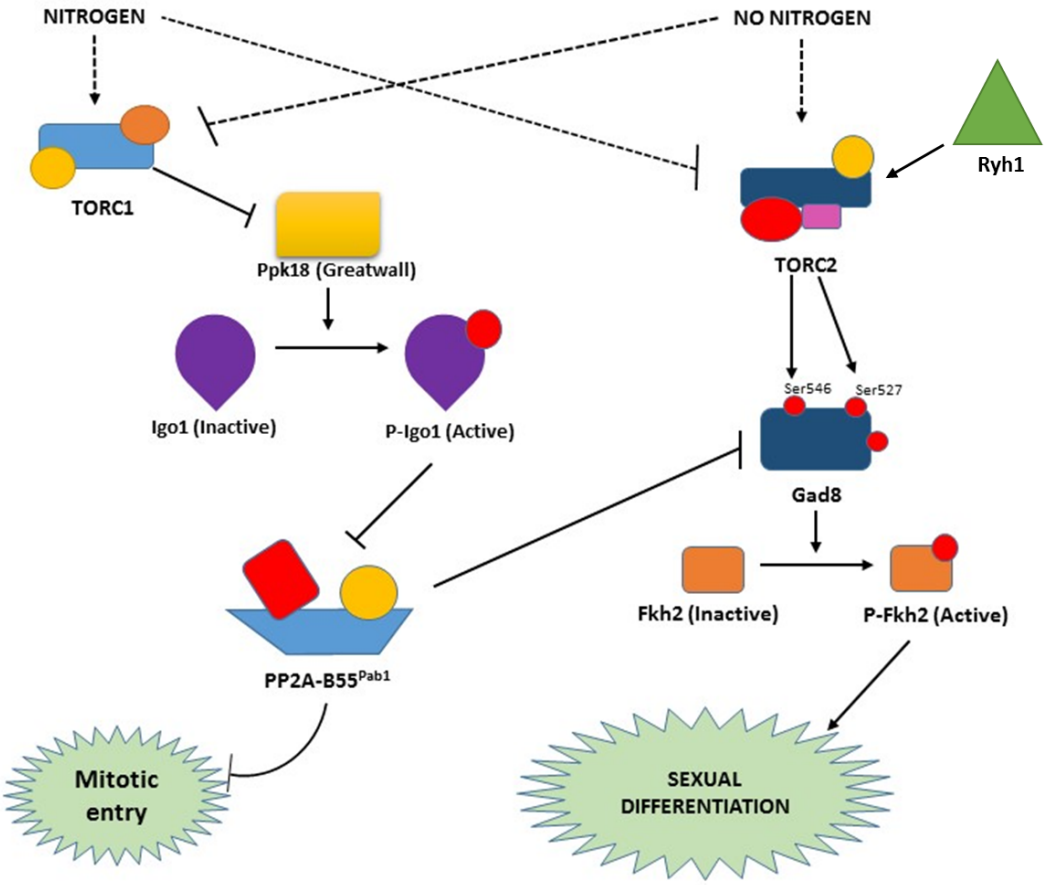


Fig3: Scheme illustrating the functioning of the TOR complexes under both nitrogen and no-nitrogen conditions.

Gad8

Gad8 is the only known effector of TORC2 (12), and together they play important roles in the cell, not only in processes related with sexual differentiation but also in other processes such as telomere maintenance and stress response reactions (13).
Gad8 carries three phosphorylation sites: Thr387 in the activation loop, which is phosphorylated by Ksg1 (Pdk) , Ser527 in the turn motif and Ser546 in the hydrophobic motif, both phosphorylated by the TOR complex 2 (14). Phosphorylation at Ser546 and Thr537 are produced under stress conditions such as nitrogen depletion. This process is necessary to produce a normal expression of the mating genes and start the process of cellular differentiation (3, 14). PP2A-B55^{Pab1}, which is normally repressing Gad8 phosphorylation, is inactivated under nitrogen starvation conditions by Igo1 (ENSA), so phosphorylation of Gad8 is now maintained.

Although both Ser546 and 527 are phosphorylated by TORC2, experiments carried out by Sandra Lopez's group, showed that mutation of Ser546 into Ala was alone sufficient to reduce Gad8 activity to a great extent in the differentiation process (11). If we take both factors into account we can conclude that phosphorylation of Gad8 can be used as a readout of TORC2 signaling, and also that its mutation in Ser456 into Ala can simulate a loss of TORC2 activity in the mating response.

PP2A phosphatase

PP2A is one of the major serine/threonine phosphatases in all eukaryotic cells. It is involved in the regulation of a plethora of cellular processes such as cell cycle regulation, cytokinesis, stress response or morphogenesis (26). PP2A is a holoenzyme formed by three different subunits: a catalytic (C) subunit, a structural (A) subunit and a regulatory (B) subunit. The regulatory subunit confers substrate specificity and regulates the subcellular localization of the PP2A complex. By changing B and C subunits the cell is provided with a set of distinct PP2A complexes of variable substrate specificity (27).
PP2A is a mediator of the two TOR signaling complexes. By preventing the untimely activity of the TORC2-Gad8 signaling module in the presence of nitrogen but not in its absence, the crosstalk between the two TOR complexes is produced.

Ppk18 (Greatwall) and Igo1 (ENSA)

In fission yeast, phosphorylation of the Igo1 endosulfine (ENSA in mammals) by the Greatwall kinase Ppk18 couples the nutritional environment to the cell cycle by inhibiting PP2A-B55^{Pab1} and thus promoting entry into mitosis upon nitrogen deprivation. Therefore, Igo1Δ mutants are not able to accelerate entry into mitosis after the shift from a rich nitrogen medium into a non-nitrogen medium (15). The same has been shown for the mating process (3)

Interconnection Gad8/Igo1

In a WT strain, in high nitrogen medium, basal phosphorylation of Gad8 is low. Nitrogen starvation led to a fast drop in TORC1 activity and subsequent phosphorylation of Igo1 (ENSA), however, the hyperphosphorylation of Gad8 is not immediately produced. Actually, Gad8 phosphorylation, and therefore TORC2 activity, drops before it started to increase as Igo1(ENSA) phosphorylation became apparent and reached a maximal level after 2 hours. This phosphorylation in no-nitrogen medium is a direct consequence of the inhibition of TORC1 through the repression of the PP2A-B55^{Pab1} by the kinase Ppk18 and its substrate Igo1 (ENSA) in fission yeast (In *igo1Δ* phosphorylation of Gad8 only marginally increases) (3, 16, 17).
This seems to show an interrelation between Gad8 and Igo1(ENSA) further than its connection through PP2A-B55^{Pab1}. In recent years it has been shown that Gad8 can also regulate TORC2 through the phosphorylation of Tor1 Thr1972 in a negative feedback loop (18). Maybe this type connection can be also happening in a way that Gad8 regulates the activity of Igo1 (ENSA), and this can explain basal Gad8 phosphorylation and why there is this initial drop in Gad8 phosphorylation in the first steps after nitrogen depletion.

OBJECTIVES

Here we have tried to understand and clarify, based on previous findings that showed how the phosphorylation of Gad8, and thus its activity, is correlated with the phosphorylation of Igo1 (ENSA) in the nitrogen starvation response that leads to sexual differentiation in S. *pombe*, the mechanisms involving the interrelation between Gad8 and Igo1 (ENSA).
We have hypothesized that Gad8 is inhibiting Igo1 (ENSA), and that an initial drop in Gad8 activity is necessary in order to lead Igo1 (ENSA) achieve the maximum level of phosphorylation.

To test the hypothesis, we have aimed at:

- Study the dependence of Igo1 (ENSA) phosphorylation on Gad8 activity in nitrogen starvation.
- Study the expression of mating genes dependent on Gad8.

MATERIALS AND METHODS

CELL CULTURE AND GROWTH

All the strains used were prototroph so that they could be able to be grown in minimal media without supplements (commonly adenine, uracil, leucine, lysine and histidine), which contain nitrogen. All the experiments in nitrogen medium were carried out using early exponential cells grown in EMM containing NH_4cl 93.5 mM as the source of nitrogen, no supplemented amino acids were used in the experiments (19). When no-nitrogen experiments were performed, cells were collected by filtration and washed with 5 volumes of EMM without NH_4Cl (EMM-N) before resuspending them in EMM-N. The first control points were taken directly from the culture just before filtration took place.

STRAIN CONSTRUCTION

For the construction of the strains, the target gene was deleted using a cassette amplified by PCR from pFA6a derivative plasmids containing an antibiotic resistance gene (29). The oligos used for this reaction share 20 nucleotides that are homologous to those we find in the plasmid. It also shares 80 nucleotides of homology with 3'UTR or 5'UTR of the gene of interest. This strategy allows the integration of the resistance cassette in the yeast genome by homologous recombination.
The transformation of the cells with the PCR cassette was performed in overnight cultures grown in YES media (glucose 3% and yeast extract 0.5% and 225 mg/l adenine, histidine, leucine, uracil and lysine hydrochloride). They were back-diluted to OD595=0,1 and incubated at 30 °C with shaking at 170 rpm. At an optimal OD595=0,5, 20 ml of the culture were collected and spun for 2 min at 2000 rpm. They were then washed with 10 ml of H_2O and subsequently the pellets were resuspended with 1 ml of Lithium Acetate 1M pH 4.9 in TE Buffer (LiAC/TE) and transferred to an eppendorf. The cultures were washed with 100 µl of LiAc/TE and transferred to a new eppendorf, whith 3 µl of salmon sperm and 7µl of PCR reaction (1 µg precipitated DNA); everything was incubated for 5 min at RT. Then, 280 µl of PEG 4000 50 %/LiAc 1 M in TE were added to each sample and incubated at 32 ºC for 30 min. Subsequently, 48 µl of DMSO were added, the mix was incubated at 42 ºC for 7 min and washed with mq-H_2O twice. The pellet was resuspended in 100 µl of mq-H_2O and the transferred onto YES-plates. Finally, Coliroller plating beads were used to spread the liquid. The plate was incubated at 32 ºC for 2 days and then replicated to a plate with the corresponding selective antibiotic (usually G418, nourseothricin or hygromycin)
The presence of the antibiotic cassette in the locus was confirmed by PCR amplification with oligos matching the antibiotic cassette and the 3' or 5' regions of the deleted gene.

The strains used in this work were already available in the laboratory collection. To get training in the construction of strains, other deletions were done in collaboration with the group.

TCA EXTRACTS

Collected samples were resuspended in 20% TCA and then washed with 1M TRIS (no pH adjusted) in order to neutralize de TCA. The pellet was resuspended in sample buffer 2x 10% DTT and boiled for 4 minutes at 95ºC with glass beads. Cells were broken down with Fast-Prep (level 6, 4 times x 30 sec). Tubes were pierced with a hot needle, placed in a 15

ml falcon and centrifuged for 5 min at 3000 rpm. The sample was transferred to a 1.5ml Eppendorf and centrifuged again at 12000 rpm to get rid of any left debris. The amount of protein was quantified using the Bradford method.

WESTERN BLOT

30-40 µg of the purified protein were loaded into the wells of the SDS-PAGE gel, along with molecular weight marker. The gel was run during 1-2 hours with running buffer. The transfer was performed in a Trans-Blot® Turbo™ Transfer System (BioRad) for 30 minutes. The membranes were blocked with milk 5 % in Tris Buffer Saline (TBS) plus 1% Tween 20 (TBS-T) for 1 h and incubated with the corresponding primary antibody overnight.

ANTIBODIES

Polyclonal anti Gad8S546-P antibodies were produced in rabbit by Eurogentec and used in 1:250-1:1000 dilutions for Western blotting.
The following commercial antibodies were used for WB: anti-PSTAIR (Abcam) 1:1000, anti-phospho-S6K (T389) mouse monoclonal antibody (clone # 1A5) (Cell Signaling) 1:1000 was used to detect phosphorylated Psk1; Phospho-ENSA (Ser67) (Cell Signaling) 1:1000; HRP-conjugated anti mouse IgG (SIGMA) 1:10000 and HRP-conjugated anti rabbit IgG (SIGMA) 1:10000.

RNA EXTRACTION AND QUANTITATIVE PCR (qPCR)

For qPCR experiments 20 ml samples were collected by centrifugation and pellets were washed in DEPC-treated water before freezing them in dry ice. Total RNA preparation was performed with MasterPureTM Yeast RNA Purification Kit (Epicentre) following the manufacturer instructions. 1 µg of RNA was used for cDNA
synthesis using SuperScript® III Reverse Transcriptase (Invitrogen). qPCR was performed with the corresponding oligos, *mei2*: AAGAAACTCCCACTGCTGCT and CTGGAGATGATTCAGTGCGT and *act1*: CAAATCCAACCGTGAGAAGA and CATCACCAGAGTCCAAGACG and *wtf20* with SYBR® Select Master Mix (Applied Biosystems). Analysis was done using the ΔΔCt method.

RESULTS

1. Inhibition of Gad8 prompts an increase in Igo1 (ENSA) phosphorylation in the early response to nitrogen starvation

In order to clarify the points above described of whether Gad8 could be inhibiting Igo1 (ENSA) in a negative feedback loop, we carried out experiments under no nitrogen conditions. We used strains already available in the lab simulating the inhibition of Gad8 such as:, Gad8S546A, *tor1Δ* and *gad8Δ*. Collected samples in no nitrogen experiments were prepared for phosphorylation analysis involving precipitation of protein using trichloroacetic acid (TCA).
According with the results obtained by our group and others (3) Gad8 phosphorylation was expected to show an initial drop before it started to increase until it reaches a maximal level after 105-120 min of nitrogen removal experiment. As we hypothesized before, this initial drop could have something to do with a feedback loop regulation involving the Igo1 endosulfine (ENSA). In order to clarify this point an experiment in which phosphorylation of Gad8 and Igo1 (ENSA) were followed simultaneously was carried out using a Wild Type strain and a Gad8 S546A strain, which is a Gad8 phospho null mutant on Ser546 which is regulated by Tor1 upon Nitrogen starvation (3).

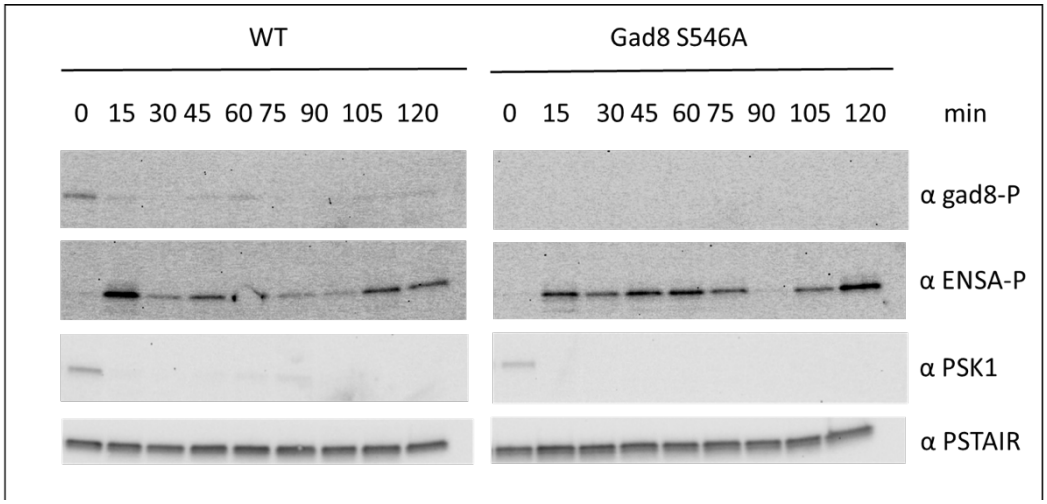


Fig4: Homothallic WT and Gad8 S546A cells were incubated at 25ºC in the absence of nitrogen. Samples were collected at the indicated time points. Gad8-Ser456 and ENSA phosphorylation were followed over time course by western blot. Phosphorylation of Cdc2 (PSTAIR) served as loading control. Psk1 served as control of TORC1 activity.

Confirming the previous finding in our lab, in the wild type strain the initial drop in Gad8 phosphorylation is seen, and it tallies with a high increase in ENSA phosphorylation. At later points, we can observe that Gad8 phosphorylation increases again and this coincides with a small drop in ENSA phosphorylation.
When focusing in the Gad8S546Ala we can observe, as expected because is a null mutant, that no Gad8 phosphorylation is observed all over the experiment. Interestingly, we can also see that Igo1 (ENSA) phosphorylation is higher in Gad8S546A in comparison with the WT and is maintained during the experiment. The fact that phosphorylation of Igo1 (ENSA) is higher when Gad8 phosphorylation is absent supports our hypothesis that Igo1 (ENSA) is negatively regulated by Gad8.

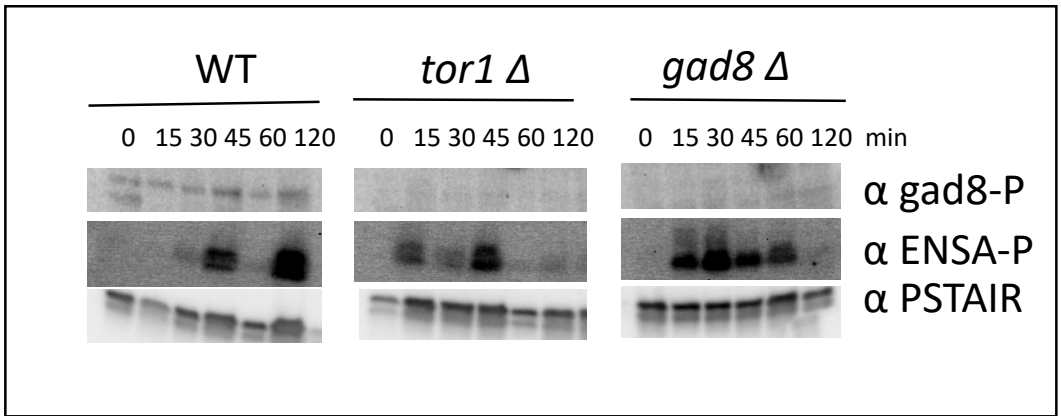


Fig5: Homothallic WT, Tor1 Δ and Gad8 Δ cells were incubated at 25ºC in the absence of nitrogen. Samples were collected at the indicated time points. Gad8-Ser456 and ENSA phosphorylation were followed over time course by western blot. Phosphorylation of Cdc2 (PSTAIR) served as loading control.

The same type of experiment was performed by eliminating both Gad8 (*gad8Δ* strain) and the major regulation center of its kinase, Tor1 (*tor1Δ* strain). In these two strains a similar result is expected, this is due to the fact that Tor1 is the kinase subunit of the TORC1 and its major effector is Gad8, which is going to be inactive due to the deletion of its activator. The only difference is that in the *tor1 Δ* strain Gad8 is going to be present although inactive. Under this situation we can observe that Igo1 (ENSA) starts its activation earlier in the mutated strains than in the wild type strain. However, phosphorylation of Igo1 (ENSA) at later points seems to be higher in the wild type strain than in *gad8* or *tor1* deletions. This situation is susceptible of being produced, following our hypothesis, because Gad8 negatively regulates the activity of Igo1 (ENSA) . Nevertheless, we can also observe that at later points Igo1 (ENSA) reaches a higher level of activity in the wild type strain, this hints that Gad8 regulates in some way the activity of Igo1 (ENSA) and that this process is required to obtain an effective nitrogen starvation response.

2. Hyperactivation of Gad8 changes Igo1 (ENSA) phosphorylation dynamic upon nitrogen starvation

In the next experiment, the activity of Ryh1, a Rab-family GTPase, important for stimulation of TORC2-Gad8 signaling under stress conditions (20) was stimulated by using the the Ryh1 QL mutant strain This mutation hyper activates Ryh1. Expression of Ryh1QL is sufficient to promote interaction between TORC2 and its major effector Gad8 and to induce Gad8 hyperphosphorylation (28). Following our hypothesis, as Gad8 negatively regulates Igo1 (ENSA), then a lower amount of Igo1 phosphorylation would be seen in the Ryh1 QL strain.

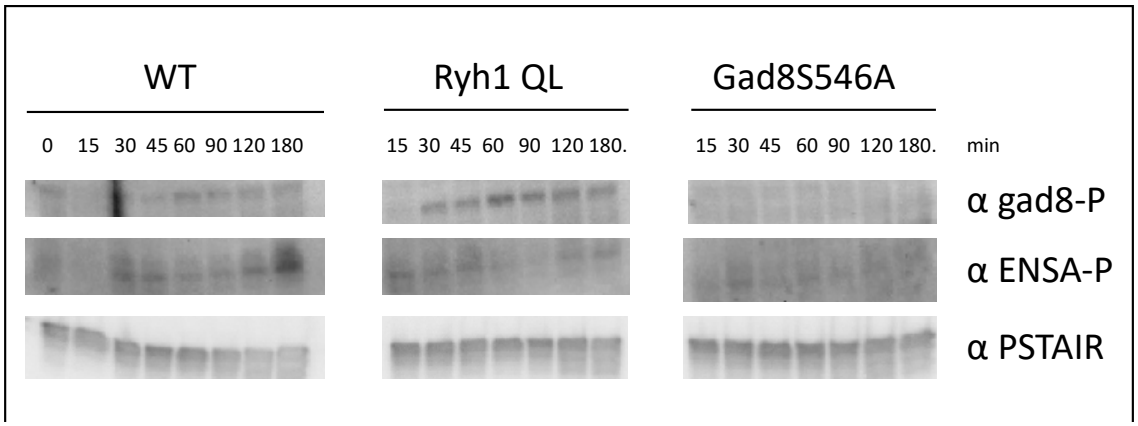


Fig6: Homothallic WT, Ryh1QL Δ and Gad8 S546A cells were incubated at 25ºC in the absence of nitrogen. Samples were collected at the indicated time points. Gad8-Ser456 and ENSA phosphorylation were followed over time course by western blot. Phosphorylation of Cdc2 (PSTAIR) served as loading control.

As we can observe in Figure 6, phosphorylation of Gad8 is more conspicuous in the Ryh1 QL strain than in the Wild Type strain, and this phosphorylation is absent in the Gad8S546A strain. When focusing in the phosphorylation of Igo1(ENSA) we are able to see that the lowest phosphorylation rates are found in the Ryh1 QL strain, and the highest phosphorylation of Igo1 (ENSA) is found in the wild type after 3 hours of nitrogen starvation. However, phosphorylation of ENSA is higher in the first points of the Gad8S546A strain than in the wild type strain.
This phenomenon can be once again explained by the hypothesis that Gad8 negatively regulated Igo1 (ENSA) in the early response to nitrogen starvation but that this regulation is necessary for achieving the maximum rates of ENSA expression under stress conditions.

3. The dynamic of Gad8 phosphorylation correlates with the expression of mating genes

The expression pattern of the mating genes is upregulated by the Gad8-Fkh2 signaling module. Under stress conditions such as nitrogen depletion, Gad8 is phosphorylated by the TORC2, its activation leads to the phosphorylation of the transcription factor Fkh2, which is subsequently activates de transcription of the mating genes.
How nitrogen depletion affects the pattern of expression of the mating genes have been studied in wild type, *tor1Δ* and g*ad8Δ* strains.
mRNA expression of two mating genes: Mei2 a well known meiotic gene which is involved in switching from mitotic to meiotic cell cycle and a central player in starvation response (21), and Wtf20 a negative regulator of ascospore formation in fission yeast which is induced upon nitrogen starvation and is regulated negatively by Tor1; were determined by qPCR in the early time points of nitrogen starvation in WT, *gad8* and *tor1* deletion strains. The expression values of these genes were related to actin expression and to the expression of the gene in the wild type strain at time 0.

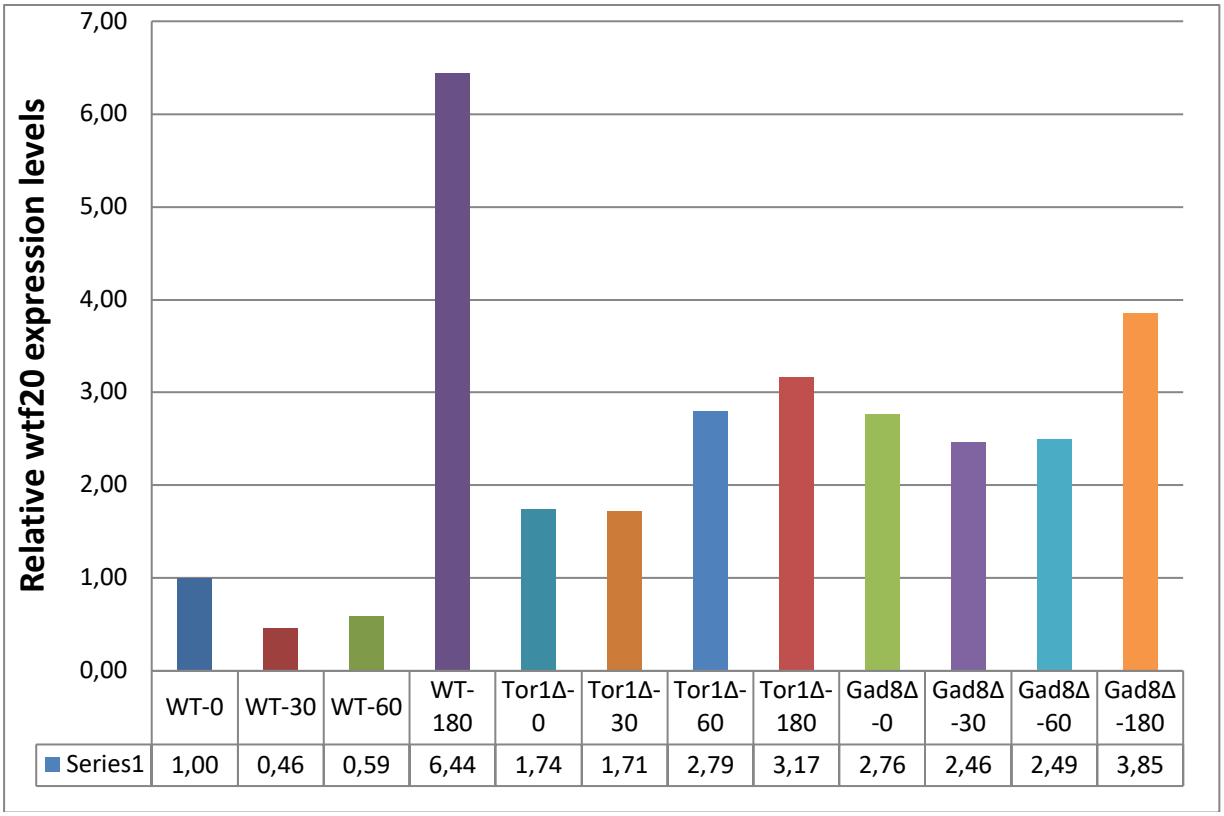


Fig 7: mRNA expression of wtf20 in Wild type, tor1Δ and gad8Δ after incubation for 180 minutes in EMM (control) and EMM-N. Expression is relative to expression of the gene in the wild type at time 0 and was determined by qPCR.

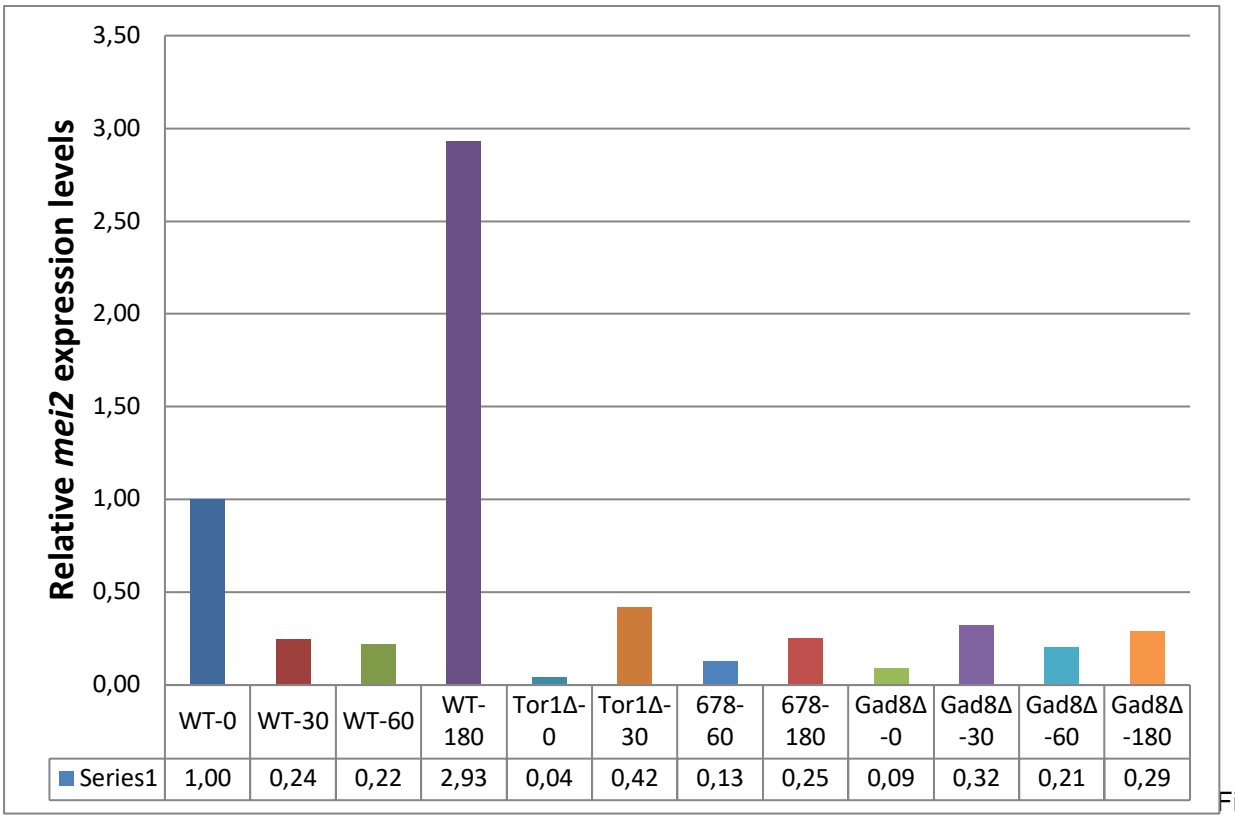


Fig 8: mRNA expression of *mei2* in Wild type, tor1Δ and gad8Δ after incubation for 180 minutes in EMM (control) and EMM-N. Expression is relative to expression of actin and to the gene in the wild type at time 0 and was determined by qPCR.

Levels of *wtf20* were lower in the wild type than in the *tor1Δ* and g*ad8Δ* mutated strains, about 2 and 3 fold respectively. 30 and 60 minutes after nitrogen starvation, *wtf20* expression levels in the wild type strain dropped to a minimum of 46 and 49% of the initial expression, however, these levels increased in the *tor1Δ* strain and decreased only a little (from 2.76 to 2.46-2.49) in the *gad8Δ* strain. Finally, there is final peak in the wild type strain multiplying more than 6 times the initial expression of the gene. This initial drop in the expression for subsequently achieving a maximum after three hours correlates with the expression of Gad8.
As this phenomenon does not happen in the mutated strains, this gene is most likely upregulated by the TORC2-Gad8 signaling module.
When focusing in the mei2 expression pattern, we can observe that in the wild type strain the expression of the gene correlates with the expression of wtf20. Nonetheless, the expression of the gene is always low in the mutant strains.

DISCUSSION

As both TOR complexes play opposite roles in the cell cycle by mediating stress responses such as nutrient deprivation, they must be accurately coordinated. In order to allow this coordination, the effector of one of the TOR complexes may take part in the regulation of the activity of the effectors of the other TOR complex. One of the best examples is how the effector of the TORC1 PP2A-B55^{Pab1} enables a crosstalk between the two TOR modules by actively dephosphorylating the major effector of the TORC2, Gad8. This interaction between the two effectors is required to engage in the differentiation program in *S. pombe* (11).

The importance of this pathway in the cell cycle regulation and how the effectors of both TOR complexes interact among them, suggest that additional levels of control between the two TOR modules may exist. In this project we carried out some experiments to discern if the initial drop in Gad8 activity after nitrogen starvation is an additional feedback loop in the control of the two TOR complexes. It has been already shown that the drop in Gad8 phosphorylation is related to the inhibiting action of Gad8 towards Tor1 (18), here we have explored a second feedback loop towards ENSA phosphorylation (Fig. 9).

In figures 4, 5 and 6 we can observe how the activity of Igo1 (ENSA) is lower in wild type strains than in *gad8* and *tor1* mutants in the first points after nitrogen starvation, nevertheless, it becomes higher as the experiment advances through time, suggesting that Gad8 must firstly inhibit Igo1 (ENSA) in order to finally achieve higher expression levels at the end of the stress response. The inhibition of Igo1 (ENSA)by Gad8 is also observed in the Ryh1QL strain of figure 6, where an increment of Gad8 phosphorylation leads to a decrease in Igo1 (ENSA) phosphorylation.

As Gad8S546A, *gad8Δ* and *tor1Δ* are all defective in the TORC2-Gad8 pathway and Igo1 (ENSA) is downstream the TORC1 which is inhibited upon nitrogen deprivation, the changes in Igo1 (ENSA) phosphorylation in the mutant strains might be a reflection of the lack of regulation between Ppk18 or Igo1 (ENSA) with some effectors of the TORC2. Moreover, among these mutants, *gad8Δ* and Gad8S546A are only defective in Gad8 activity, thus, Gad8 must be the responsible of the process involving Igo1 (ENSA) regulation. Why and how this interaction between Gad8 and Igo1 is produced is still unknown. Gad8 may be inhibiting Ppk18 Greatwall kinase directly by phosphorylation and therefore inhibiting Igo1 or it could be though another protein. In the future a thorough characterization of common interactors may be interesting to answer this question.

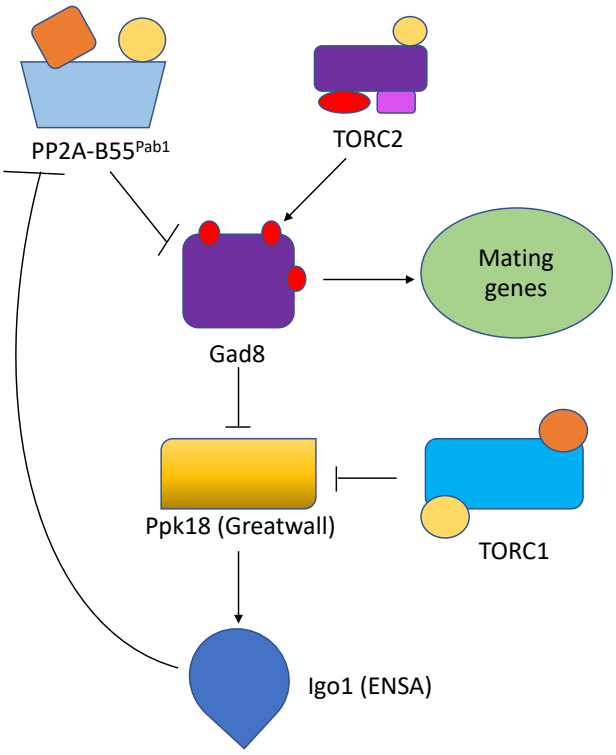


Fig 9 Working Model: Just after nitrogen depletion, TORC1 is inactivated and Gad8 represses the activity of Ppk18-Igo1(ENSA), which is not able to inhibit PP2A-B55^{Pab1}. The now active PP2A-B55^{Pab1} can subsequently dephosphorylate Gad8, causing part of the drop in Gad 8 phosphorylation seen in the first points after nitrogen starvation in Wild Type strains. The drop in Gad8 phosphorylation relieves the inhibition of ENSA, which blocks PP2A-B55^{Pab1} activity and Gad8 is phosphorylated.

In addition to cellular stress response to nutrient starvation, TORC2 and Gad8 are also required to responses related with DNA damage, gene silencing, telomerase maintenance and the expression of mating genes (22, 23). As in fission yeast nutritional sensing is intimately linked to sexual differentiation, the differentiation program switching from a mitotic cell cycle into a meiotic cell cycle and therefore the expression of the mating genes, is going to take place during nitrogen starvation (3). In this project, the activity of two mating genes have been checked by qPCR: *wtf20* and *mei2*. We could observe how in wild type strains the expression of these two genes is accurately coordinated and correlates with the Gad8 activity pattern, while in *gad8Δ* and *tor1Δ* the expression of *wtf20* is erratic and *mei2* seems to present only basal transcription. This fact stands out how Gad8 is a requisite for the expression of *mei2* and how *wtf20* cannot be properly expressed in its absence. Moreover, the initial drop in Gad8 phosphorylation seen in the first points after nitrogen starving is a requisite to avoid a premature meiosis, which in *S. pombe* takes place after 6-8 hours of nitrogen depletion. If this delay in meiosis is not produced, cells will not be able to arrest in G1, meiotic genes would not be expressed at the proper time and therefore meiosis would be erratic.

CONCLUSIONS

The TOR complexes are key mediators of stress responses, cell cycle regulation and cell metabolism. For this reason, different control mechanisms and crosstalk between the two TOR modules participate in its coordination. One of this control points is the regulation of Igo1 (ENSA) by Gad8. –

- Under no nitrogen conditions, an initial drop in Gad8 phosphorylation before maximum levels of its activity are produced is crucial to achieve maximum levels of Igo1 (ENSA) activity at later points. If Gad8 is not present, Igo1 (ENSA) cannot reach its highest level of expression at the end of the stress response.
- Normal activity of the Gad8 kinase is a requisite for a normal expression of the mating genes. The pattern of expression of *wtf20* and *mei2*, two of the genes involved in the mating response have the same expression pattern as Gad8 phosphorylation in Ser546. If Gad8 is absent, its expression is erratic or null.

CONCLUSIONES

Los complejos TOR son mediadores clave en la respuesta a estrés y en la regulación del ciclo y metabolismo celular. Por esta razón, participan en su coordinación diferentes mecanismos de control e interacción entre ellos. Uno de los puntos de su regulación es el ejercido por Gad8 sobre Igo1 (ENSA).-

- En ausencia de nitrógeno, es necesaria, para alcanzar el máximo nivel de fosforilación en Igo1 (ENSA), una bajada en la fosforilación de Gad8 antes de alcanzar su máximo nivel de actividad. Si Gad8 no está presente, Igo1 (ENSA) no puede alcanzar su máximo nivel de expresión al final de la respuesta a estrés.
- La adecuada actividad de Gad8 es necesaria para una expresión normal de los genes de apareamiento. El patrón de expresión de *wtf20* y *mei2*, dos genes participes en el apareamiento de S. *pombe*, presentan el mismo patrón que la fosforilación de la Ser546 de Gad8. Si Gad8 no está presente, su expression es errática o inexistente.

CONCLUSIÓNS

Os complexos TOR son mediadores chave na resposta a estrés e na regulación do ciclo e metabolismo celular. Por esta razón, participan na súa coordinación diferentes mecanismos de control e interacción entre eles. Un dos puntos de regulación e o exercido por Gad8 sobre Igo1 (ENSA).-

- En ausencia de nitróxeno, é necesaria para acadar o máximo nivel de fosforilación en Igo1 (ENSA), unha baixada na fosforilacion de Gad8 antes de acadar o seu máximo nivel de actividade. Se Gad8 non está presente, Igo1 (ENSA) non pode acadar o seu máximo nivel de expresión ao final da resposta a estrés.
- Unha adecuada actividade de Gad8 é necesaria para unha expresión normal dos xenes de apareamento. O patrón de expresión de *wtf20* e de *mei2*, dos xenes participes no apareamento de S. *Pombe*, presentan o mesmo patrón de expresión que o da fosforilación da Ser546 de Gad8. Se Gad8 non está presente a súa expresión é errática ou inexistente.

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