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TOR complex 2 promotes proteasome dependent Pub1 (NEDD4 family E3 ligase) turnover in fission yeast.

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Abstract

Cells respond to alterations in their nutrient environment by adjusting the abundance of surface nutrient transporters and receptors. This can be achieved through modulation of ubiquitin-dependent endocytosis, which in part is regulated by the NEDD4 family of E3 ligases. Here we report four novel modes by which Pub1, a fission yeast *Schizosaccharomyces pombe* member of the NEDD4-family of E3 ligases, is regulated. Phosphorylation of the conserved serine 188 (an analogous site in human NEDD4L is phosphorylated but uncharacterized) provides resistance to extracellular canavanine, a toxic arginine analog, indicating S188 phosphorylation enhances Pub1's function to reduce canavanine uptake. Both Pub1 serine 188 phosphorylation and proteasomal turnover of Pub1 are inhibited by Gsk3 kinase. Thus, whilst Gsk3 kinase protects Pub1 protein levels it restrains Pub1 E3 ligase function by reducing serine 188 phosphorylation. Nitrogen stress stimulates Pub1 protein turnover by the proteasome, reducing protein levels by 60% and thereby increasing abundance of the amino acid transporter Aat1 at the plasma membrane. TOR complex 2 and Gad8 (AKT) signaling negatively regulates Pub1 protein levels, and the increased proteasomal Pub1 turnover upon nitrogen stress requires TORC2 signaling. In summary, environmental control of Pub1 protein levels to modulate the abundance of nutrient transporters is regulated by the major TORC2 nutrient-sensing signaling network and proteasomal dependent control of Pub1 protein levels.

Introduction

In all eukaryotic cells the external environment regulates cell fate. Highly conserved TOR (Target Of Rapamycin) signalling plays a key role in this control by responding to environmental cues, including stress and nutritional availability. This is achieved through TOR control of a series of metabolic processes, cell growth, cell migration, cell division and cell differentiation. TOR signalling is extremely sensitive to changes in the cellular nutrient environment. It is well established that reduced cellular energy levels and changes in amino acid concentrations are actively sensed by cells to modulate TOR pathway activity ¹.

Several nutrient acquisition pathways support TOR control of anabolic cell growth ². These include autophagy, which breaks down cellular components to generate nutrients for anabolism, uptake of nutrients via surface transporters and receptor-mediated uptake of macromolecular nutrients ^{1, 3-6}. Cells respond to alterations in their nutrient environment by regulating the abundance of surface nutrient transporters and receptors, in part through

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controlling their ubiquitin-dependent endocytosis.

Reciprocal regulation of TOR and nutrient pathways has been established, because nutrients activate TOR, whilst TOR activity promotes endocytosis and inhibits autophagy. The mechanisms of TOR's inhibition of autophagy to promote rapid cell proliferation in high nutrient environments is well-established ⁶. However, the impact of TOR stimulated endocytosis on nutrient utilisation is complex. On the one hand, TOR-controlled enhancement of endocytosis removes ion, carbohydrate and amino acid transporters from the plasma membrane, and may also reduce the surface population of macromolecular nutrient receptors, altogether reducing nutrient uptake ^{7, 8}. On the other hand, endocytosis is also vital for the uptake of macromolecular nutrients such as LDL ⁹.

TOR signalling is comprised of two structurally and functionally distinct multi-protein complexes. TOR kinases form TORC1 and TORC2 (TOR Complex 1 and 2), which are defined by unique subunits that are highly conserved across species. In mammalian cells the protein Raptor defines (mTORC1), while Rictor is exclusive to mTORC2 ¹. In the fission yeast *S. pombe* model, the focus of this study, Mip1 is the functional homolog of Raptor in TORC1, whilst Ste20 (Rictor homolog) defines TORC2 ¹⁰⁻¹² and Gad8, an ortholog of human AKT, is a substrate of TORC2.

Studies in both yeast and mammalian cells have established mechanisms of TOR regulated endocytosis and have documented both TORC1 and TORC2 dependent regulation of specific endocytic cargo ^{3-5, 13, 14}. For example, in *S. cerevisiae*, amino acid permeases such as Can1 are down-regulated under nutrient-replete conditions by their ubiquitination by Rsp5 (a NEDD4 family E3 ligase), a process requiring the Rsp5 adaptor Art1 ⁵. This process is conserved in mammalian cells ^{7, 15-17}. Ubiquitinated Can1 is then recognized by endocytic ubiquitin adaptors such as Ede1/Eps15 and Ent1/epsin and incorporated into clathrin-coated vesicles. TORC1 stimulates Can1 uptake pathway via the Npr1-dependent phosphorylation of Art1 and Can1, thereby enhancing Can1 ubiquitination ⁵. In mammalian neurons NEDD4 is a key regulator of neurite growth ¹⁷ and studies have identified NEDD4-1 mRNA as a prominent target of mTORC1 regulated translation ¹⁸. Human NEDD4-1 and NEDD4-2 (NEDD4L) also regulate ubiquitin-mediated autophagy, an mTORC1 controlled process ^{19, 20}. In fission yeast the abundance of the Aat1 and Cat1 amino acid permeases on the plasma membrane increases after nitrogen starvation, and a contribution of Pub1 (NEDD4 family of E3 ligases), Tsc1/2 ¹ (an upstream inhibitor of TORC1) and TORC1 pathway to this localization has been demonstrated by several laboratories ²¹⁻²⁸.

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Each member of the NEDD4 HECT E3 ubiquitin ligase family comprises an amino-terminal Ca²⁺-phospholipid binding domain (C2), WW domains for protein to protein interaction, and a carboxy-terminal HECT domain containing its ligase activity ^{17, 29, 30}. In the absence of Ca²⁺ binding to the C2 domain, conformational changes auto-inhibits NEDD4, whereas phosphorylation of NEDD4 on S347 and S348 by CK1 leads to its ubiquitination and degradation ¹⁷. NEDD4-2 can also exist in an inactive form, since AKT1- and SGK1-mediated phosphorylation of S342 and S428 promotes 14-3-3 binding to block NEDD4-2's interaction with its substrates, whilst in contrast AMPK and JNK phosphorylation at the carboxy-terminus is required for its activation ¹⁷.

NEDD4 is expressed in most mammalian tissues and regulates a number of key substrates. Therefore, not surprisingly, dysregulation of NEDD4 ligases gives rise to a variety of diseases including cancer, cystic fibrosis, respiratory distress, hypertension, kidney disease, nervous system dysregulation and epilepsy ^{17, 29}. In summary, NEDD4 ligase activity is regulated at multiple levels, including translation, phosphorylation, binding to accessory proteins and control of protein turnover, and consequently the molecular mechanisms of its regulation are complex and are still not fully understood. In the present study we used the fission yeast model system to gain further insights into the mechanism responsible for keeping the activity of this key E3 ligase under control.

Here we describe four novel modes of Pub1 regulation. We show that phosphorylation of the conserved serine 188 enhances Pub1 function and that Gsk3 kinase inhibits this phosphorylation. In contrast, proteasomal turnover of Pub1 is blocked by Gsk3. Nitrogen stress promotes Pub1 destruction, reducing protein levels by 60%. TOR complex 2 and Gad8 (AKT) signaling also reduce Pub1 protein stability, and the destruction of Pub1 upon nitrogen stress requires active TORC2 signaling. In summary, environmental control of Pub1 protein levels to modulate the abundance of nutrient transporters is regulated by the major TORC2 nutrient-sensing signaling network.

Results

Phosphorylation of serine 188 enhances pub1 function.

The cellular response to nutrient starvation is, in part, to increase the abundance of surface transporters to facilitate greater uptake of nutrients from the environment. In budding yeast, when nutrients are plentiful TORC1 inhibits Npr1 kinase to allow Rsp5 ubiquitin-dependent endocytosis of transporters ⁵. However, upon nutrient starvation when

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TORC1 activity is inhibited so is ubiquitin-dependent endocytosis, leading to higher levels of transporters at the plasma membrane ⁵.

We previously undertook a global quantitative fitness profiling study to identify those genes whose loss altered cell fitness in response to nitrogen stress or the addition of Torin1 to inhibit TORC1 and TORC2 signalling. Not surprisingly, deletion of Pub1 NEDD4-family E3 ligase increased cell fitness in response to both nutrient stress and the addition of Torin1 ³¹, presumably because cells were able to import higher levels of nutrients due to reduced ubiquitin-dependent endocytosis of nutrient transporters.

With the aim of increasing our understanding of how Pub1 itself is regulated by changes to the cellular nutrient environment we performed a quantitative, SILAC and label free mass spectrometry (MS) based analysis ³². Protein extracted from wild type fission yeast that had been treated with Torin1 (ATP competitor TOR inhibitor 1) for two hours to mimic nutrient starvation was mixed 1:1 with either SILAC labelled or label free solvent treated controls. This identified 5 phosphorylation sites on Pub1 (Supplementary table 1). Interestingly, Pub1 serine 188 (S188) phosphorylation, which was reported previously in global screens but not characterised further ^{33, 34}, appeared to be downregulated following Torin1 treatment (Supplementary Figure 1A, Supplementary table 1). Because this site is conserved in human NEDD4L (Serine 297; Figure 1) and a global phosphor-proteome study, based on human liver samples, identified phosphorylation on NEDD4L S297 ³⁵ but did not characterise the site further, we decided to study the role of this conserved phosphorylation.

To analyse the role of Pub1 S188 phosphorylation, we mutated the endogenous locus to generate an allelic series of serine point mutants. A phospho-blocking mutant substituted serine for alanine (A), whereas phosphomimetic mutants substituted serine for aspartic acid (D) or glutamic acid (E). We next used a simple well-established colony-forming growth assay to assess Pub1 function *in vivo* within these mutant strains. Transport of canavanine into cells, a toxic arginine analogue, is in part regulated by the amino acid transporter Can1 ³⁶, as the *can1.1* canavanine resistant mutant allele in contrast to wild type strain is able to form colonies when spotted from a serial dilution on agar-plates supplemented with canavanine. This is because the faulty transporter reduces the uptake of the toxic compound (Figure 1B) ³⁶. In contrast, it is well-established that cells deleted for the Pub1 E3-ligase (which independently have reduced growth rate even on EMM2 control) media are hypersensitive to canavanine (Figure 1B), since the block to ubiquitin-dependent endocytosis increases Can1 transporter abundance and therefore canavanine uptake ^{27, 36}. Interestingly, Pub1.S188D showed increased colony forming abilities in this

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assay, indicating enhanced Pub1 function of this phosphomimetic mutant, which would lead to reduced transporter abundance and therefore reduced uptake of the toxic compound (Figure 1B). On the contrary, Pub1.S188E and the Pub1 S188A phospho-blocking mutant did not change canavanine sensitivity when compared to wild type cells, implying that the level of S188 phosphorylation is relatively low in cells grown on minimal EMM2 media (Figure 1B) and that Pub1.S188E is a poor phosphomimetic for pSer. Whilst Pub1 S188 phosphorylation appears to increase Pub1 function by an unknown mechanism, it has no impact on Pub1 protein levels (Supplementary Figure 1B). Together, these data indicate that phosphorylation of the conserved Pub1 S188 (NEDD4L S297) is advantageous for the enzyme.

TOR complex 2 negatively regulates Pub1 protein levels.

Our MS experiments indicated that Pub1 S188 phosphorylation is reduced upon Torin1 treatment for 2hr to mimic nutrient starvation (Supplementary Figure 1A, Supplementary Table 1). Because Pub1 S188 phosphorylation appears to enhance Pub1 function (Figure 1B), reduced phosphorylation upon Torin1 treatment to mimic nutrient starvation is consistent with the view that reduced Pub1 E3 ligase activity when resources are sparse would act to increase surface transporter abundance.

To further validate our MS data, we generated phospho-specific antibodies to Pub1 S188 (Supplementary Figure 1C-E). The strain used for mass spectrometry was grown in EMMG supplemented with arginine and lysine media and treated with Torin1 or DMSO solvent control for 1 and 2 hours. Surprisingly, Pub1 total protein levels increased markedly after addition of Torin1 (Figure 1C) with Pub1 pS188 phosphorylation also increasing somewhat. Consistent with our MS data, the relative level of pS188 vs total Pub1 was reduced after 2 hr (Figure 1C). To assess whether longer Torin1 treatment would reduce relative pS188 levels further and to get further insight into the unexpected Torin1-mediated increase in Pub1 levels, we treated wild type cells with both Torin1 and rapamycin for 3hr. Only Torin1 promoted an increase in Pub1 and pS188 levels, though the decrease in relative pS188 levels seen at 2hr were no longer observed after 3hr treatment (Figure 1D), suggesting that pS188 phosphorylation is dynamic and is also not directly downstream of either TORC1 or TORC2. Because rapamycin only inhibits TORC1 while Torin1 inhibits both TORC1 and TORC2³⁷, our data suggest that it is the inhibition of TORC2 that results in increased Pub1 protein and therefore eventually also pS188 levels. Note that following Torin1 treatment a slower migrating form of Pub1 can be seen

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with both total and pS188 antibodies, indicating that TOR inhibition may facilitate unknown additional modification(s) of Pub1 (Figure 1C).

Deletion of the TORC2 specific component *ste20* (Rictor) also increased levels of Pub1 and pS188, though levels of pS188 relative to total Pub1 were unaffected by deletion of TORC2 activity (Figure 2A), in agreement with experiments using longer Torin1 treatments (Figure 1D). Elevated Pub1 levels were also observed in cells deleted of the TORC2 substrate *gad8* (AKT homolog) (Figure 2B) with no significant change to relative pS188 levels (data not shown). We next tested whether TORC2 control of Pub1 protein levels affected cells' sensitivity to canavanine. Cells deleted of *ste20* and *gad8* have very significant reduction of growth rate compared to wild type cells (data not shown), so are not ideal candidates to assess growth rates in our "canavanine-sensitivity" assay. We therefore took advantage of two other mutant strains to assess the consequences of increased or decreased TORC2/Gad8 activity. We previously showed that whilst a Gad8.T6D mutant (which reduces Gad8 (AKT) function, through reduced TORC2 binding to Gad8) has normal growth rates on EMM2 media (Figure 2C), Gad8 activity is reduced albeit not blocked³⁸, whilst in contrast TORC2 activity is modestly increased in a Tor1.I1816T mutant³⁹. Reduced Gad8 activity in Gad8.T6D cells resulted in somewhat larger colony size (increased cell proliferation) on canavanine plates when compared to wild type (Figure 2C), indicating that Pub1 function was modestly increased overall in this mutant. Importantly, Pub1 levels were also slightly increased in Gad8.T6D cells (Figure 2B), consistent with the modest increase in growth rate on canavanine plates. Notably, the opposite impact on growth rates was observed in cells with enhanced TORC2 activity (Figure 2C), as this mutant was sensitive to canavanine and exhibited a slight reduction in Pub1 protein levels. Together, these observations indicate that TORC2 and its downstream substrate Gad8 negatively impact on Pub1 protein levels and function.

Gsk3 protects Pub1 protein levels

Our data suggest that whilst TORC2 negatively regulated Pub1 protein levels, pS188 phosphorylation was maintained after addition of both Torin1 and rapamycin for 3hr treatment. We therefore concluded that neither TORC1 or TORC2 are responsible for this phosphorylation. With the aim to identify the Pub1 S188 kinase we submitted the pub1 sequence around S188 to (<http://www.cbs.dtu.dk/services/NetPhos/> and <http://gps.biocuckoo.cn/> for kinase prediction based on consensus sites. This identified

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CDK, GSK3, MAPK, CK2 and CK1 as potential kinases. No change to relative Pub1 pS188 levels was observed in mutants of yeast MAPKs Sty1, Pmk1 & Spk1, Cdc2 (CDK1), CK2 (orb5), CK1 (hhp1/2 and Cki1-3) or Gsk3 (data not shown). However, Pub1 protein levels were downregulated in a *gsk3.Δ* deletion strain (Figure 3A). Fission yeast Gsk31 is an ortholog of Gsk3. Whilst Pub1 levels remained unaffected in the *gsk31.Δ* deletion strain, Pub1 protein levels were further reduced in *gsk3.Δ gsk31.Δ* double deletion when compared to *gsk3.Δ* (Figure 3A). Although the growth rate of *gsk3.Δ gsk31.Δ* on minimal EMM2 media is reduced (Figure 3B) it is sensitive to canavanine. In agreement, deletion of *gsk3.Δ* alone reduced colony size when exposed to the toxic compound, suggesting that Pub1 function was reduced in mutants lacking Gsk3 (Figure 3B). This observation fits with the reduced Pub1 protein levels seen in the *gsk3.Δ gsk31* mutant (Figure 3A). However surprisingly, whilst Pub1 protein levels were reduced in the *gsk3.Δ gsk31.Δ* double mutant, relative pS188 levels were increased (Figure 3C). Together these observations suggest that Gsk3 is the major Gsk3-family kinase that positively regulates Pub1 protein levels. However, Gsk3 activity has a negative impact on S188 phosphorylation, which enhances Pub1 function, indicating that whilst Gsk3 protects Pub1 protein levels it also restrains its function.

Gsk3 activity is essential for Torin1 induced increase in pub1 Protein levels

Previous studies in fission yeast and human cells have shown that AKT inhibits Gsk3⁴⁰,⁴¹. We therefore asked whether the reverse impact of Gad8 (AKT) and Gsk3 on Pub1 levels was because Gad8 regulated Pub1 through inhibition of Gsk3 (in which case Pub1 levels in a double mutant are likely to resemble the levels seen in the *gsk3.Δ* mutant). However, the protein levels of Pub1 in the *gsk3.Δ gad8.Δ* double mutant were approximately half of those seen in *gad8.Δ* cells, and more than double that in *gsk3.Δ* cells (Figure 4A). These findings suggest that either the two kinases regulate Pub1 through independent mechanisms, or alternatively that Gsk3 kinase present in the *gsk3.Δ gad8.Δ* double mutant is hyperactivated due to *gad8* deletion, leading to elevated Pub1 protein levels compared to those seen in *gsk3.Δ*. Unfortunately, we were unsuccessful in generating a *gsk3.Δ gsk31.Δ gad8.Δ* triple deletion mutant to measure Pub1 protein levels and thus test this possibility. Nonetheless, in contrast to the situation in wild type cells (Figure 1D), Torin1 failed to increase Pub1 protein levels in the *gsk3.Δ gsk31.Δ* double mutant (Figure 4B), indicating that the TORC2/AKT dependent reduction in Pub1 protein levels occurs via modulation of Gsk3 activity. In summary, our observations suggest that

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Gsk3 activity protects Pub1 protein levels and that the reverse impact of TORC2/Gad8 and Gsk3 on Pub1 levels comes about because of conservation of TORC2/AKT mediated Gsk3 inhibition⁴¹ in fission yeast. Thus, lack of TORC2 activity enhances Gsk3 activity and consequently increases Pub1 protein levels.

Gsk3 prevents Pub1 degradation by the proteasome.

To get further insight into the mechanisms by which Gad8 and Gsk3 regulate Pub1 levels, we used quantitative PCR (QPCR) to assess the level of Pub1 mRNA in the two kinase deletion strains. Interestingly, whilst the Pub1 Protein levels are high in the *gad8.Δ* mutant, the mRNA levels are half that of wild type cells, whilst levels are unaffected in the *gsk3.Δ* *gsk31.Δ* double mutant (Figure 4C). These observations suggest that the impact on protein levels in both mutants is independent of transcription. Auto-ubiquitination of *S. Cerevisiae* Rsp5 and SCF mediated degradation of human NEDD4 have been reported^{42 43}. Interestingly, a block to proteasome function in the *mts3.1* proteasome mutant⁴⁴ increased Pub1 levels three-fold compared to wild type, to reach levels similar to that seen in the TORC2 mutant (Figure 2A & 4D). We conclude that Pub1 is degraded by the proteasome. Blocking proteasome function rescued Pub1 protein levels in the *gsk3.Δ* mutant (Figure 4D), demonstrating that Gsk3 activity is essential to prevent Pub1 degradation by the proteasome.

Nitrogen stress dependent down-regulation of Pub1 levels requires TORC2 and Gad8 (AKT) activities.

As mentioned above, the response to nutrient starvation is in part, to increase the abundance of surface nutrient transporters, implying that Pub1 family E3 ligase activities are down-regulated. Interestingly, the imposition of nitrogen stress, by changing the nitrogen source from good to poor, (here we changed from ammonia to proline - EMM2 to EMMP), resulted in a 60% decrease in Pub1 protein levels (Figure 5A). This reduction in Pub1 levels was maintained upon nitrogen-stress of *gsk3.Δ*, *gsk31.Δ* and *gsk3.Δ gsk31.Δ* mutants (Figure 5B, Supplementary Figure 2A). Whilst Gsk3 is dispensable for nitrogen stress induced Pub1 destruction, surprisingly lack of the proteasome blocked Pub1 protein turnover (Figure 5C), and blocking TORC2 signaling in *ste20. Δ* (Rictor) and *gad8.Δ* (AKT) mutants diminished Pub1 protein turnover (Figure 5D) relative to wild-type (Figure 5A), indicating that active TORC2 and AKT are required for proteasome mediated Pub1 destruction following nitrogen stress. To get further evidence of TORC2's role in Pub1

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turnover after nitrogen stress, we took advantage of our mutant in which we can inhibit TORC2 without affecting TORC1. Fission yeast Tor2 is the main kinase in TORC1, we previously identified the *tor2.G2040D* mutation, in which TORC1 is resistant to Torin1³⁷. When the *tor2.G2040D* mutant was nitrogen stressed and Torin1 was added simultaneously (to inhibit only TORC2) the destruction of Pub1 as a result of media change to proline was no longer observed (Figure 5C). Together, these observations indicate that the environmental control of Pub1 turnover after nitrogen stress is regulated by TORC2 and AKT signaling, but in this case does not involve Gsk3.

Aat1 amino acid transporter localization to the plasma membrane upon nitrogen stress requires TORC2 activity.

In fission yeast it is well-established that cells lacking Pub1 activity show increased abundance of the amino acid transporter Aat1 at the plasma membrane at cell tips^{21, 22}. To visualize this, wild type and *pub1::ura4⁺* deletion cells were grown in EMM2, and the wild type cells were stained for 45 min with FM-4-64, which accumulates in the vacuoles, to differentiate between the two cell types when mixed 1:1 and imaged for Aat1.GFP localization (Figure 6A). Wild type cells mainly had punctate cytoplasmic staining, previously attributed to localization at the Golgi⁴⁵. As expected, deletion of Pub1 increased Aat1 levels on the plasma membrane more than two-fold (Figure 6A) without changing overall levels of aat1.GFP protein (Supplementary Figure 3A). In contrast, despite the significant reduction in Pub1 protein levels in the *gsk3 gsk31* double mutant (Figure 3A), no increase in aat1.GFP at membranes was observed (Figure 6B). Importantly, this mutant has increased Pub1 pS188 (Figure 3C), which increases Pub1 function (Figure 1B). Presumably, such enhanced Pub1 activity, despite reduced Pub1 protein levels, keeps aat1.GFP off the plasma membrane.

Imposition of nitrogen stress, by shifting wild type cells from EMM2 into EMMP reduced Pub1 protein levels by 60% (Figure 5A). Consistent with such a fall in Pub1, localization of Aat1.GFP at the plasma membrane was increased in nitrogen stressed cells (Figure 7A), whilst total aat1.GFP protein levels remained unchanged (Supplementary Figure 3B). However, addition of Torin1 upon nitrogen stress to inhibit TOR signaling³⁷ and increase Pub1 protein levels (Figure 1C,D) abolished Aat1.GFP localization to the plasma membrane⁴⁵ (Figure 7B). Finally, nitrogen-stress of the *ste20* (Rictor) deletion to block TORC2 function and thus increase Pub1 levels (Figure 2A) also blocked Aat1.GFP localization at the plasma membrane (Figure 7C). In summary, Aat1.GFP localization at

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the plasma membrane in poor nutrient environments correlates with TORC2 regulation of Pub1 levels.

Discussion

Here we show for the first time that the fission yeast NEDD4 family of E3 ligase Pub1 is regulated by the nutrient environment and the major nutrient sensing TORC2 pathway, to control the levels of amino acid transporter on the plasma membrane and thus nutrient uptake. Previous studies have established mechanisms of both TORC1 and TORC2 dependent regulation of specific endocytic cargo and membrane transport in both yeast and mammalian cells ^{3-5, 13, 14}. However, we now show that TORC2 and its downstream substrate the Gad8 (AKT) kinase promote Pub1 protein degradation via the proteasome.

In addition, we demonstrate for the first time that Gsk3 protects Pub1 from proteasomal degradation, as blocking proteasomal function in the *mts3.1* mutant restores Pub1 protein levels in cells lacking Gsk3 activity (Figure 4D). Thus, whereas TORC2/AKT promotes Pub1 degradation, Gsk3 blocks it. In both fission yeast and human cells, it is well established that AKT inhibits Gsk3 ^{40, 41}. The reverse impact of AKT and Gsk3 on Pub1 protein levels is consistent with a model in which TORC2/AKT regulates Pub1 levels via its inhibition of Gsk3. The increase in Pub1 levels upon TOR inhibition with Torin1 is abolished in cells lacking Gsk3 activity (Figure 4B), which is consistent with this model. Thus, our findings suggest that TORC2/AKT promotes Pub1 protein turnover, in steady state cells grown in a good nutrient environment, through its inhibition of Gsk3. This model is summarised in Figure 8.

Our previous investigation of global quantitative fitness to detect genes whose deletion altered cell fitness in response to nitrogen stress or inhibition of TOR signalling identified Pub1 ³¹. Deletion of *pub1* enhanced cell fitness, presumably because cells lacking *pub1* are able to import higher levels of nutrients due to reduced ubiquitin-dependent endocytosis of nutrient transporters. In this screen the deletion of *gsk3* also enhanced fitness ($p = 0.108$) of cells grown on minimal media ³¹, which is consistent with our observation that Pub1 protein levels are reduced in the *gsk3.Δ* mutant (Figure 3A). Increased viability upon nitrogen starvation of cells deleted of *gsk3* has also been reported in an independent genome wide screen ⁴⁶. A role for Gsk3 in protein stability is well-established, though in contrast to the protective role of Gsk3 on Pub1 protein stability we describe here, Gsk3 is known to prime many substrates for proteasome degradation, with

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over 25 substrates identified in human cells that are degraded in a Gsk3 dependent manner⁴⁷.

Whether Pub1 is a direct substrate of Gsk3 is unclear. Whilst Gsk3 can phosphorylate Ser-Pro sites it commonly phosphorylates a primed sequence S/T-X-X-X-S/T(P) pre-phosphorylated by another kinase⁴⁸. Pub1 Serine 188 matches this Gsk3 consensus sequence, as well as being a Ser-Pro site (Figure 1A), prompting us to look at Gsk3. However, Gsk3 blocked rather than promoted S188 phosphorylation, as relative levels of pS188 increased in cells lacking Gsk3 activity (Figure 3C). Furthermore, Pub1 S188 phosphorylation had no impact on Pub1 protein levels (supplementary figure 1B). Human NEDD4 is also degraded by the proteasome, as phosphorylation of NEDD4 on S347 and S348 by CK1 leads to SCF mediated ubiquitination and degradation¹⁷. However, the SCF phospho degrons DSGXXS or T-P-P-X-S are not conserved in Pub1, and deletion of CK1 activity in fission yeast does not increase Pub1 levels (data not shown). NEDD4L is the closest homolog of Pub1, and to the best of our knowledge no mechanism of NEDD4L down-regulation by the proteasome has yet been reported. How Gsk3 protects Pub1 from proteasomal degradation is currently unclear.

GSK3 negatively regulates glucose homeostasis⁴⁹. However, insulin and growth factor signalling in human cells that activates mTORC1, mTORC2, AKT and S6K will inhibit GSK3 activity⁴⁰ and thereby increase glycogen synthesis. In contrast, NEDD4 enhances insulin and growth factor signalling^{50, 51}. Our observation suggests that reduced GSK3 activity as a result of insulin signaling may decrease NEDD4 levels and thus put a brake on insulin and growth factor signalling through a negative feedback loop. However, in human cells NEDD4 can directly bind to and ubiquitinate AKT which is prior phosphorylated on pS473, to degrade active AKT⁵². Thus, decreased GSK3 activity and therefore reduced NEDD4 levels would increase active AKT pS473, providing a positive feedback for insulin and growth factor signalling and glucose uptake to counteract the negative feedback and establish a steady state. Therefore, if conserved the mechanism described here would most likely only impact hormone signalling and glucose uptake when this pathway is interacting with other signalling pathway(s) that alter the steady state.

TORC2/AKT stimulated Pub1 degradation is not limited to cells in steady state (Figure 8). When cells experience changes to their nutrient environment they respond by increasing the abundance of surface nutrient transporters, in part through down regulating their ubiquitin-dependent endocytosis. In agreement, we demonstrate TORC2/AKT-dependent Pub1 protein turnover through proteasomal degradation following nitrogen stress (Figure

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5A,D, 8), which in turn results in increased Aat1 amino acid transporter abundance on the plasma membrane (Figure 7). Surprisingly, nitrogen-stress induced Pub1 degradation occurs in the absence of Gsk3 activity (Figure 5B). How TORC2/AKT targets Pub1 for proteasome degradation when nutrient are limited is unknown.

For the first time we demonstrate that phosphorylation of Pub1 S188, a site conserved in NEDD4L (Figure 1A), enhance protein function, as evidenced by increased resistance to uptake of a toxic arginine analogue in cells expressing a Pub1 S188D phosphomimetic mutant (Figure 1B). In the search for the kinase responsible for Pub1 S188 phosphorylation, we assessed a role for TOR, AMPK, AGC, MAPK, Cdk1, CK1, CK2 or GSK3, however Pub1 S188 remain phosphorylated, when the activity of all of these kinases are compromised. Phosphorylation of NEDD4L S297 (the analogous site in humans) has been demonstrated in human liver samples ³⁵, though this site remains uncharacterised in human cells. Pub1 S188 phosphorylation has no impact on protein levels (supplementary figure 1B), therefore, considering the close proximity of Pub1 S188 and NEDD4L S297 to their WW domains phosphorylation may regulate protein-protein interactions important for function.

In summary, here we have characterized the conserved Serine 188 in Pub1 and shown that it is important for Pub1 E3 ligase function. We show that whilst Gsk3 protects Pub1 protein levels it also restrains Pub1 function through its inhibition of S188 phosphorylation. Finally, we provide the first evidence of NEDD4 family E3 ligase being regulated by nitrogen stress and TORC2 signalling, to reduce ubiquitin-dependent endocytosis and thus increase the abundance of amino acid transporters on the plasma membrane when nutrient levels are challenging.

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Materials and Methods

Yeast cell cultures

Table 1 Strains used in this study

	Genotype	Source
JP350	<i>h⁺</i>	Lab stock
JP598	<i>gad8::ura4⁺ ura4.d18</i>	From ³⁸
JP1379	<i>ste20::KanMx</i>	Lab stock
JP1563	<i>h⁺ Tor1.I1816T</i>	Lab stock
JP367	<i>h⁻ pub1::ura4⁺ ura4.d18</i>	NBRP Japan
JP3397	<i>h⁺ pub1.S188A</i>	This study
JP3411	<i>h⁺ pub1.S188D</i>	This study
JP3412	<i>h⁺ pub1.S188E</i>	This study
JP2102	<i>h⁺ gad8.T6D</i>	From ³⁸
JP3237 (FY18665)	<i>h⁻ can1.1</i>	NBRP Japan
JP3508	<i>h⁺ gsk3::ura4⁺ ura4.d18</i>	This study
JP3516	<i>gsk31::ura4⁺ ura4.d18</i>	This study
JP3484	<i>gsk3::ura4⁺ gsk31::ura4⁺ ura4.d18</i>	This study
JP3538	<i>gsk3::ura4⁺ gad8::ura4⁺ ura4.d18</i>	This study
JP1156	<i>mts3-1</i>	Lab stock
JP3519	<i>gsk3::ura4⁺ ura4.d18 mts3-1</i>	This study
JP1669	<i>h⁺ tor2-G2040D</i>	Lab stock
JP3295	<i>h⁺ aat1.GFP.KanMx</i>	This study
JP3393	<i>h⁻ pub1::ura4⁺ aat1.GFP.KanMx ura4.d18</i>	This study
JP3529	<i>gsk3::ura4⁺ gsk31::ura4⁺ ura4.d18 aat1.GFP.KanMx</i>	This study
JP3460	<i>aat1.GFP.KanMx ste20::KanMX</i>	This study
JP1791	<i>h⁺ lys1-131 car2::natMX6 arg3-D4</i>	Lab stock

All cultures were grown at 28°C and cultured in log phase for 48 h. Cells were inoculated in in Edinburgh minimal media (EMM2-N) (ForMedium) ⁵⁶ supplemented with NH₄Cl (EMM2)⁵⁷. Media change to (EMM2-N) (ForMedium) ⁵⁶ supplemented with proline (EMMP)⁵⁷, was done by filtering cells, followed by resuspension in to prewarmed EMMP.

SILAC labeling and harvesting culture for mass spectrometry

SILAC labeling: Cells were inoculated in Edinburgh minimal media (EMM2-N) (ForMedium) ⁵⁶ supplemented with 20 mM L-Glutamic acid (EMMG) ⁵⁷ and 75 mg/l of either light [L-arginine monohydrochloride (Sigma) and L-lysine monohydrochloride (Sigma)] or medium [lysine-L, 2HCl 4.4.5.5-D4 (Cat code DLM-2640, Eurisotop), arginine-L, HCl, U-13C6 99%13C (cat. no. CLM-2265, Eurisotop)] amino acids. Cells were cultured

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in log phase for 30 h to ensure complete incorporation of labelled amino acids into the proteome. Early log phase cultures at 3.5×10^6 cell/ml were treated with 15 μ m Torin1 or DMSO control. Cells were collected by filtration (MF-Millipore™ Filter, 1.2 μ m pore size Cat. # RAWP04700, Millipore) washed with 15 ml TBS, resuspended in an appropriate volume of ice cold sterile ddH₂O and dropped directly into liquid nitrogen to produce frozen cell droplets.

Mass spectrometry

SILAC mass spec analysis of samples processed using a SPEX Sample Prep LLC 6850 Freezer Mill in presence of liquid nitrogen, were performed as described previously ³². Data was analysed with MaxQuant ⁵⁹ (v1.6.0.9) using the Andromeda search engine ⁶⁰ to query a target-decoy database of *S. pombe* from UniProt (September 2019 release).

Drug treatment

L-Canavanine sulfate salt (Cat. # C9758, SIGMA) was added to EMM2 agar plates at a concentration of 6 ug/ml, Torin1 (Cat. # 4247, TOCRIS) was used at a concentration of 15 μ m and 25 μ m. Rapamycin (Cat. #R0395, SIGMA) was used at a concentration of 300 ng/ml.

Generation of *pub1.S188X* mutants

The *pub1* gene (954 bp upstream and 892 bp downstream of the open reading frame) was amplified by PCR from genomic DNA and cloned into the pGEM-3 vector. *pub1* Serine 188 point mutations were generated by site directed mutagenesis. Transformation of a *pub1::ura4+* deletion and selection on YES-FOA plates ⁵⁷ were used to select for integration of *pub1.S188X* point mutation into the genome.

Western blotting

TCA precipitation protocol was followed for *S.pombe* total protein extracts ⁵⁸. The following dilutions of antibodies were used in this study: 1/500 Pub1 and 1/250 Pub1 pS188 (custom made by Thermo Scientific) in PBS buffer, 1/500 anti-GFP (Cat. # 11814460001, Roche) in TBS buffer, skim milk was used as blocking agent. Alkaline phosphatase coupled secondary antibodies were used for all blots followed by direct detection with NBT/BCIP (VWR) substrates on PVDF membranes.

Fluorescent Microscopy

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Staining of vacuoles: SynaptoRed™ C2 (Equivalent to FM®4-64) (Cat. # 70021, Biotium) was added to the growth media of cells (1×10^6 cells/ml) at a concentration of $1.5 \mu\text{M}$ for 45 min. Cultures of Stained and unstained cells were mix 1:1 and collected by filtration onto MF-Millipore™ Membrane Filter, $1.2 \mu\text{m}$ pore size (Cat. # RAWP04700, Millipore). Cells were resuspended in the original growth media of the FM®4-64 stained cells and subjected to live cell imaging immediately. Images of cells were obtained using a CoolSNAP HQ2 CCD camera. ImageJ were used to measure fluorescent intensities of Aat1.GFP. The relative fluorescence intensity of GFP was quantified as: intensity at cell ends vs nuclear background signal. Statistical significance was calculated using Unpaired t test.

RNA extraction and qPCR

RNA was extracted using TRIzol™ Reagent (Cat. # 15596026, ThermoFisher Scientific). In short, 1×10^7 cells in early log phase were collected by centrifugation. Cell pellets were snap-frozen in liquid nitrogen. 1 ml of Trizol and 200 μl of glass beads (Cat. # 11079105, Biospec) were added to the cells. Cells were disrupted by a FastPrep-24™ (MP) at 5 m/s for 60 seconds for 3 cycles in a cold room. Cell lysate was processed according to manufacture's instructions. RNA pellets were resuspended in 50 μl of RNase free water. 1000 ng of RNA subjected to DNA digestion by TURBO DNA-free™ Kit (Cat. # AM1907). First-strand cDNA were synthesized from 500 ng of RNA by using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Cat. # M3683, Promega). DNase treated RNA, 500 ng Oligo (DT)₁₅ (Cat. # C1101, Promega) and 100 ng random hexamer (Cat # C1181, Promega) and heated to 70°C for 5 minutes, cooled to 4°C , and incubated on ice for 5 minutes. For reverse transcription, RNA, primers, dNTP mix (Cat # N0446S, Bio New England Lab), M-MLV RT (H-) Point Mutant were used. 1: 4 diluted first-strand cDNA were used for second strand synthesis of cDNA and qPCR using Power SYBR™ Green PCR Master Mix (Cat. # 4367659, ThermoFisher Scientific). Reactions were run in Rotor-Gene Q (Qiagen) with initial activation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, 58°C for 1 minute. Comparative Quantitation Analysis from Rotor-Gene Q series software produced Representative Takeoff vale from triplicates of each sample. $2^{-\Delta\Delta\text{CT}}$ method was used to calculate pub1 gene expression relative to housekeeping gene act1. Primers to amplify pub1 gene: Forward: CCCTTATTGGAATGAGACTTTTG; Reverse: GGGTCAACATTTCATCACCTC. Forward and Reverse Primers to amplify the control act1 gene was a described ⁶¹.

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Statistics

GraphPad Prism 6.07 was used for data analysis. one-way ANOVA with Dunnett's multiple comparisons test or student T-tests were used as indicated. 95% confidence of interval was used for calculating significance.

Figure legends

Figure 1: Pub1 serine 188 phosphorylation enhances pub1 function.

A) Sequence alignment of Pub1 S188 and human homolog NEED4L. **B)** Growth characteristics of Pub1 S188 mutants on EMM2 and EMM2 + 6 µg/ml Canavanine. For all growth assays similar results was obtained for three independent biological repeats **C)** Elevated Pub1 and Pub1 pS188 when Torin1 is added to SILAC strain cells at 25 µm. **D)** Wild type cells were treated with Torin1 at 25 µm or Rapamycin at 300 ng/ul. Lysates were prepared from indicated cells and immunoblotted for Pub1 and Pub1 pS188, Ponceau S was used to stain total protein. Bars indicates fold change in levels vs. DMSO ± s.e.m., $n = 3$. Statistical significance was calculated using one-way ANOVA with Sidak's multiple comparisons test for **C**, and one-way ANOVA with Dunnett's multiple comparisons test for **D**. n represent biological independent experiments. Representative immunoblots are shown.

Figure 2: TORC2 negatively regulates Pub1 protein levels and cells sensitivity to canavanine.

A-B) Elevated Pub1 and Pub1 pS188 in mutants of TORC2 and Gad8 (AKT). Protein extracts were prepared from indicated cells and immunoblotted for Pub1 and Pub1 pS188, Ponceau S is used to stain total protein. Bars mean fold change in levels vs. wild type ± s.e.m., $n = 3$. Statistical significance was calculated using Unpaired t test for **A** and one-way ANOVA with Dunnett's multiple comparisons test for **B**. n represent biological independent experiments. Representative immunoblots are shown. **C)** Growth characteristics of indicated strains on EMM2 and EMM2 + 6 µg/ml Canavanine. For all growth assays similar results was obtained for three independent biological repeats.

Figure 3: Gsk3 positively regulates Pub1 protein levels but inhibits Pub1 S188 phosphorylation.

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A & C) Pub1 and Pub1 pS188 levels in mutants of Gsk3. Protein extracts were prepared from indicated cells and immunoblotted for Pub1 and Pub1 pS188, Ponceau S is used to stain total protein. Bars indicate fold change in levels vs. wild type $\pm$ s.e.m., $n = 3$. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test for **A** and Unpaired t test for **C** n represent biological independent experiments. Representative immunoblots are shown. **B)** Growth characteristics of indicated strains on EMM2 and EMM2 + 6 μ g/ml Canavanine. For all growth assays similar results was obtained for three independent biological repeats.

Figure 4: Gsk3 blocks proteasomal dependent Pub1 degradation.

A, B - D) Protein extracts were prepared from indicated cells and immunoblotted for Pub1 and Pub1 pS188, Ponceau S is used to stain total protein. Bars indicate fold change in levels vs. wildtype $\pm$ s.e.m., $n = 3$. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test for **A** and Unpaired t test for **B** and **D**. n represent biological independent experiments. Representative immunoblots are shown. **D)** Wild type and *mst3.1* cells were grown at 36 °C for 6 hours to inactive Mst3 before harvesting and protein extraction. **C)** Levels of specific RNA was measured by qPCR $n = 3$. Statistical significance was calculated using one-way ANOVA with Dunnett's multiple comparisons test. n represent biological independent experiments.

Figure 5: Nitrogen stress and TORC2 promote proteasomal dependent Pub1 turnover

A-D) Pub1 and Pub1 pS188 levels in indicated before and after nitrogen stress. Protein extracts were prepared from indicated cells and immunoblotted for Pub1 and Pub1 pS188, Ponceau S is used to stain total protein. Bars indicate fold change in levels vs. EMM2 $\pm$ s.e.m., $n = 3$. Statistical significance was calculated using unpaired Student t test except for *tor2.G2040D* in **D**, in which one-way ANOVA with Dunnett's multiple comparisons test was used. **C)** *mst3.1* cells were grown at 36 °C for 3 hours to inactive Mst3 before nitrogen stress. **D)** Torin1 was added at 15 μ m to EMMP medium of *tor2.G2040D* cells. n represent biological independent experiments. Representative immunoblots are shown.

Figure 6: Localization of the amino acid transport Aat1 to the plasma membrane is enhanced in the *pub1* deletion but reduced in cells lacking Gsk3 activity.

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A-B) Live cell imaging of Aat1.GFP of indicated strains. Wild type cells were strained with FM-4-64 (indicated by a star) for 45 min, which accumulates in the vacuoles, to differentiate between the two cell types when mixed 1:1 and imaged for Aat1.GFP localization. The relative fluorescence intensity of GFP was quantified as: intensity at cell ends vs nuclear background signal. Scale bar = 3 microN.

Figure 7: Torin1 and the *ste20* TORC2 deletion block Aat1 localization to the plasma membrane following nitrogen stress.

A-C) Live cell imaging of Aat1.GFP in indicated strains. To differentiate between the two cell types/treatments (which were mixed at a ratio 1:1) control cells were initially strained with FM-4-64 (indicated by a star) for 45 min (accumulates in the vacuoles), before they were combined with unstained cells for immediate imaging. The relative fluorescence intensity of GFP was quantified as: intensity at cell ends vs nuclear background signal. Scale bar = 3 microN. **A-B)** Unstained cells in EMM2 medium was treated prior to media shift for 1 hour with Torin1 at 25 μ M (A) or DMSO (B), before they were filtered into EMMP supplemented with Torin1 or DMSO respectively and treated for a further 90 min before live cell imaging together with stained cells **C)** 90 minutes before imaging, wild type and *ste20* deletion cells were shifted from EMM2 to EMMP medium.

Figure 8: Schematic of TORC2 and Gsk3 dependent control of Pub1.

Steady state: the reverse impact of TORC2/Gad8 and Gsk3 on Pub1 levels comes about because of conservation of TORC2/AKT mediated Gsk3 inhibition in fission yeast. Thus, lack of TORC2 activity enhances Gsk3 activity and consequently increases Pub1 protein levels. Likewise, the opposite impact on Pub1 S188 phosphorylation of TORC2 and Gsk3 suggests TORC2 regulates Pub1 S188 phosphorylation through its inhibition of Gsk3. Nitrogen stress: TORC2 and AKT independently promote Pub1 destruction through the proteasome following nitrogen stress.

Supplementary Figure 1. Pub1 S188 phospho-specific antibodies

A) Chart illustrating changes to phosphorylation of Pub1 S188 following Torin1 treatment for 2hr by mass spectrometry (see supplementary Table 1). **B-E)** Protein extracts were prepared from indicated cells and immunoblotted for Pub1 and Pub1 pS188 and anti-HA. Ponceau S is used to stain total protein. **E)** Pub1 S188 is phospho-specific. Indicated

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Ponceau S stained PVDF membranes were cut and incubated with or without lambda phosphatase for 1 hour, to dephosphorylate proteins prior to immuno-blotting.

Supplementary Figure 2. Gsk3 activity is not required for nitrogen-stress induced Pub1 turnover.

Protein extracts were prepared from indicated cells and immunoblotted for Pub1 and Pub1 pS188, Ponceau S is used to stain total protein. Bars indicate fold change in levels vs. EMM2 $\pm$ s.e.m., $n = 3$. Statistical significance was calculated using unpaired t test. n represent biological independent experiments. Representative immunoblots are shown.

Supplementary Figure 3. Aat1.GFP levels are unaffected by Pub1 and growth media.

Protein extracts were prepared from indicated cells and immunoblotted for GFP to visualize Aat1.GFP, Ponceau S is used to stain total protein. **A** Bars mean fold change in levels vs. EMM2 $\pm$ s.e.m., $n = 3$. Statistical significance was calculated using unpaired t test n represent biological independent experiments. Representative immunoblots are shown.

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Wang et al. Figure 1

Steady state:

Nitrogen stress:

Wang et al. supplementary figure 1

Wang et al. supplementary figure 2

Wang et al. supplementary figure 3

Supplementary Table S1

Gene Name	Modified Residue	UniProt ID	DMSO (LF)	TORIN 1 (LF)	DMSO (SILAC)	TORIN 1 (LF) DMSO (LF)	TORIN 1/DMSO (SILAC) ReQuant ON	TORIN/DMSO (SILAC) ReQuant OFF	Localization Probability	Proteins	Positions within proteins
Row 1	PUB1	S188	116829976	67248040	96940048	0.575506048	0.57550001	0.57550001	0.998777	sp Q92462 PUB1_SCHPO	188
Row 2	PUB1	S178	687300224	645309852	520640180	0.938905478	0.650606989	NaN	0.9945	sp Q92462 PUB1_SCHPO	178
Row 3	PUB1	S199	NaN	87404984	35219884	NaN	2.693098976	2.693098976	0.990906	sp Q92462 PUB1_SCHPO	199
Row 4	PUB1	S239	307189656	238989820	134140032	0.777987659	NaN	NaN	0.95343	sp Q92462 PUB1_SCHPO	239
Row 5	PUB1	S174	376456840	326960112	415350240	0.886592262	0.938220024	0.938220024	0.858552	sp Q92462 PUB1_SCHPO	174

Leading proteins	Charge state	PEP	Score	Delta score	Score for localization	Mass error [ppm]	Sequence window	Multiplicity	Number of Phospho (STY)	Protein group IDs	Amino acid	Position	Unique identifier
Row 1	sp Q92462 PUB1_SCHPO	2	3.17E-11	126.45	115.63	0.071694	SSRAGSPTRDNAPASPASSEPRTFSSFEQQ	2	2	2091	S	188	UID17894
Row 2	sp Q92462 PUB1_SCHPO	2	8.07E-34	173.09	163.04	0.071694	QSSSVSRNPASRAGSPTRDNAPASPASSE	1	1,2	2091	S	178	UID7022
Row 3	sp Q92462 PUB1_SCHPO	2	6.28E-07	124.12	104.76	1.4737	APAASPASSEPRTFSSFEQYGRLLPGWERR	1	1	2091	S	199	UID7033
Row 4	sp Q92462 PUB1_SCHPO	3	1.50E-134	297.33	282.4	1.0403	VDHNRSTTWIRPNLSSVAGAAAELHSSAS	2	2	2091	S	239	UID17988
Row 5	sp Q92462 PUB1_SCHPO	3	8.73E-09	92.817	89.29	-0.62565	PQSSQSSSVSRNPASRAGSPTRDNAPASP	2	2	2091	S	174	UID17997