Title

PAN deadenylase is required for mitosis in response to microtubule stress in yeast and humans

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Summary

The Poly(A) Tail Length (PATL) of mRNAs of certain cell-cycle regulatory genes undergo significant trimming during M-phase, however the functional importance is unknown. The Ccr4-Not and PAN complexes account for the majority of cytoplasmic poly(A) deadenylation, but their potential roles in regulation of cell-cycle mRNA PATLs has not been investigated. We find that under conditions of microtubule stress in yeast, loss of PAN deadenylase activity leads to arrest in M phase, defective spindles, and increased cell death. PAN consists of the catalytic subunit Pan2 and the RNA binding subunit Pan3. Consistent with a role in mitosis, *PAN2* interacts genetically with tubulin genes, prefoldin complex genes and the cyclins *CLB1* and *CLN3*. *PAN2* knockdown in human cultured cells disrupts mitosis and results in spindle fragmentation leading to abnormal cell division, while expression of human *PAN2* in yeast rescues *pan2*Δ cell-cycle phenotypes. Hence, we reveal an important highly conserved role for PAN in ensuring proper mitosis when cells are under microtubule stress. We propose PAN regulates PATLs of mRNAs of key cell-cycle/mitotic proteins in response to defective spindles.

Key words:

Poly(A) Tail Length (PATL), PAN complex, deadenylation, microtubule stress, cyclins, abnormal cell division

Introduction

In eukaryotes, poly(A) tails are present on the 3' end of almost every mRNA and regulate mRNA stability and translation (1–6). Poly(A) tail length (PATL) varies greatly from as short as ~20 nucleotides to as long as ~250 nucleotides and is governed by poly(A) deadenylases, which trim or remove the tails completely (7–10). Two protein complexes, PAN (PolyA Nuclease) and Ccr4-Not, carry out the majority of deadenylation, where PAN initiates trimming of longer tails while Ccr4-Not removes the remainder (11–14). Poly(A)-Binding Proteins (PABs) bind poly(A) tails promoting deadenylation by recruiting PAN complex to poly(A) tails. Genes such as *CDK1*, *TOP2A* and *FBXO5*, which orchestrate timing and coordination of mitotic events in somatic cells, exhibit reduced PATLs specifically in M-phase (15), suggesting deadenylation of specific mRNAs is important in cell-cycle regulation. However, roles for specific deadenylases in the cell-cycle are poorly defined.

The structure of PAN complex is well understood. It comprises the catalytic subunit Pan2 and an asymmetric homodimer of the RNA-binding regulatory subunit Pan3 (16–18). Pan2 contains a C-terminal DEDD-family exoribonuclease domain that is responsible for its poly-A deadenylase activity. Adjacent to the exoribonuclease domain is a ubiquitin-specific protease (USP) domain, which in human cells is functional (19,20). *Saccharomyces cerevisiae* Pan2 has this domain also, but it lacks critical residues in the catalytic triad indicating yeast Pan2 lacks deubiquitinase activity (16,17). Pan3 contains a CCCH-type zinc finger domain and a Poly(A) interacting motif 2 (PAM2) motif that interacts with PABs facilitating RNA binding. Pan3 also contains a pseudokinase domain (21). Pan2 is a substrate of Cdk1 phosphorylation (22). Pan3

phosphorylation regulates binding to PABs which regulates PAN activity (23), and Pan3 is a substrate of Cdk5 phosphorylation (24,25).

The biological relevance of PANs deadenylase activity is poorly understood. In human cells, knockdown of *PAN2* leads to an increase in mRNAs with long poly(A) tails (> ~150 nt), but does not alter mRNA half lives (14). Deletion of either *PAN2* or *PAN3* in yeast leads to longer mRNA poly(A) tails, but does not completely abolish deadenylation (11,12). This suggests that PAN's role may be more specialized than regulating stability of bulk mRNAs, which has been shown for *HIF1A* regulation (26). In yeast, a protein serine/threonine kinase involved in replication fork protection and the DNA damage checkpoint, Dun1, interacts with Pan2 and Pan3 and regulates the composition and activity of post-replication repair complexes. The *dun1*Δ*pan2*Δ strain is hypersensitive to replication stress and has elevated levels of an important regulator of error-free post-replication repair, Rad5 (27). This suggests PAN plays a role in post-transcriptional regulation of DNA repair and replication stress although the mechanism and mRNA targets are unknown.

We investigate a novel role for PAN complex in cell-cycle regulation. High throughput data indicates that both yeast *PAN2* and *PAN3* exhibit strong aggravating genetic interactions with genes that encode tubulin and tubulin folding proteins (28), and Δ*pan2* and Δ*pan3* yeast are among the most sensitive in the yeast deletion collection to various microtubule biogenesis inhibitors, including nocodazole and benomyl (29). This suggested to us an important, yet uncharacterized function for PAN in mitosis in yeast in response to microtubule stress. We found that nocodazole caused PAN-deficient cells to arrest in M phase, which was likely due to

defective spindle assembly, and resulted in increased cell death. Transcriptional profiling of cells expressing deadenylase-dead Pan2 in the presence of nocodazole revealed changes in key cell-cycle regulators further supporting a function for PAN in mitosis. Knockdown of *PAN2* in human cells caused spindle fragmentation and defective mitosis upon treatment with nocodazole and colchicine, indicating this function for PAN was conserved to metazoans.

Results

Yeast deletion mutants *pan2*Δ and *pan3*Δ are sensitive to nocodazole

On querying for information on the effects of small molecules on the growth of *pan2*Δ and *pan3*Δ yeast deletion mutants, we noticed that homozygous deletion of *PAN2* or *PAN3* sensitizes yeast to multiple drugs that disrupt tubulin biogenesis (29). These include nocodazole, benomyl, and a number of other small molecules that belong to the response signature 'tubulin folding and SWR complex'. Importantly, both *pan2*Δ and *pan3*Δ are ranked in the top 20 out of over ~4,800 yeast deletion mutants for nocodazole and benomyl sensitivity (29) (**SFig 1A**). Other homozygous deletion strains which occupied top ranks are *pac2*Δ*, cin1*Δ*, cin2*Δ*,* and *tub3*Δ. *TUB3* encodes α-tubulin, which associates with β-tubulin (*TUB2*) to form tubulin dimers, which polymerize to form microtubules. Pac2 is an alpha tubulin-binding protein, Cin1 is a beta-tubulin-binding protein, and Cin2 is a GTPase activator involved in tubulin complex assembly (30–33). Hence, Pac2, Cin1, and Cin2 are involved in protein folding and assembly of the tubulin complex. This suggested a role for PAN complex in microtubule related functions.

The nocodazole sensitivity screen described above was performed at 6 µM, while the typical concentration used for inducing G2/M arrest is 40 µM (34). Since the screen produced a

phenotype at 6µM (29), we treated log phase cells of *pan2*Δ, *pan3*Δ, and their isogenic wild-type strain with 6 µM nocodazole to mimic the screen and measured yeast growth. While no difference in growth rate was observed for WT, *pan2*Δ and *pan3*Δ grew substantially slower in the presence of nocodazole (**Fig 1A**), but not in the absence of nocodazole (**SFig 1B**). Since growth dynamics were monitored immediately upon the addition of nocodazole, we observed a lag period of approximately 100 minutes (~ 1 1/2 hours) before the effect of the drug was noticeable. For this reason, exponential growth rates were not determined for these experiments.

Expressing WT *PAN2* from a CEN plasmid eliminated the nocodazole sensitivity of *pan2*Δ cells (**Fig 1B**). In contrast, expression of a catalytic-dead mutant of *PAN2* with a mutation within the active site DEDD motif (D1020A) that is unable to perform in-vitro deadenylation on model-polyA RNAs (35) was unable to eliminate the nocodazole sensitivity (**Fig 1B**). To verify that the D1020A mutant was stably expressed, we tagged *PAN2* and the D1020A mutant with GFP and examined protein expression and function. We found no difference in the level of GFP-Pan2 and GFP-Pan2(D1020A) protein (**SFig 1C**). GFP-Pan2 largely eliminated nocodazole sensitivity of *pan2*Δ cells whereas GFP-Pan2(D1020A) did not, confirming that the nocodazole sensitivity is dependent upon Pan2 catalytic activity (**SFig 1D**).

To determine if the role of Pan2 in nocodazole sensitivity is conserved in mammalian cells, we codon-optimized the human *PAN2* gene for expression in yeast (**SFile 1**) and expressed the gene in the *pan2*Δ strain from a high copy 2-micron plasmid (2µ-HsPan2; **Fig 1C**). As was the case with the WT yeast Pan2, expression of HsPAN2 eliminated sensitivity to nocodazole (there was no difference in growth with or without nocodazole in cells expressing HsPan2). Interestingly,

overexpression of HsPAN2 in the *pan2*Δ strain caused a decrease in growth rate (**Fig 1C**). We hypothesized that this reduction in growth would be Pan3 dependent if this phenotype was a result of PAN activity. Indeed, expression of HsPan2 in the *pan3*Δ strain did not not slow growth and did not prevent nocodazole sensitivity (**Fig 1D**). We found a similar slow-growth phenotype with overexpression of yeast Pan2 (see **Fig 5D**). Thus, human Pan2 complemented the function of yeast *pan2*Δ in response to nocodazole, indicating this function of PAN was highly conserved.

PAN2 and _PAN3_ genetically interact with tubulin and genes governing tubulin folding

To better understand how PAN function was related to nocodazole sensitivity, we examined known common genetic interactions of *PAN2* and *PAN3 (28)* (**SFig 2A**). Gene-ontology (GO) analysis indicated roles for PAN in "chromosome segregation" (*APC1, BUB3, CBF1, MPS1, SLI15, SMC3, TUB2,* and *TUB3*), "cytoskeletal protein binding" (*ARP2, CIN1, GIM3, GIM5, PAC10, RBL2,* and *YKE2*), "secretion" (*ACT1, SEC1, SSO2,* and *VPS27*), and "RNA polymerase-II-specific transcription factor binding" (*CBF1, GCR2,* and *SWI1*) (**SFig 2A**). Within the "chromosome segregation" network, multiple functions relating to microtubules were identified including "spindle", "microtubule cytoskeleton", and "mitotic spindle assembly checkpoint signaling", consistent with a role for PAN in spindle function during mitosis.

To validate the genetic interactions described above and expand the interaction landscape to include microtubule stress from nocodazole treatment, we performed synthetic genetic array (SGA) double mutant screens with a *pan2*Δ query and the non-essential deletion collection of ~4800 strains in the absence and presence of nocodazole (1.5μM nocodazole as this does not lead to differential growth between WT and *pan2*Δ in liquid growth assays, **SFig 2B**). In the

absence of nocodazole using a cut-off ratio of 0.7 for aggravating genetic interactions (three standard deviations, 99.7% confidence interval) and p-value of less than 0.05 in 3/3 replicates, we found a total of 52 genetic interactions. For deletion mutants in the array whose growth was rescued upon deleting *PAN2* in them (referred to as alleviating genetic interactions from here onwards), we chose a cut-off ratio of 1.3 and found 27 genetic interactors (**SFile 2**). The 4 genes in common with the previously identified genetic interactors (28) were *TUB3, BUB3, RPL12B,* and *RPL14A.* Surprisingly, all the genes with functions related to chromosome segregation were not identified in the absence of nocodazole, but instead required addition of low levels of nocodazole (see below).

For SGA screens in the presence of nocodazole, we used a low nocodazole concentration (1.5 µM) anticipating that the genetic interactors themselves might be nocodazole sensitive, and that this dose would not produce a growth phenotype in the *pan2*Δ query strain (**SFig 2B**). Using the same cut-offs as the no-nocodazole screens, we identified a total of 30 aggravating and 21 alleviating genetic interactions (**SFile 2**). Of these, 7 genetic interactors were in common with the PAN interactions from the CellMap (**Fig 2A**). These genes were *CIN1, GIM3, MDM32, PAC2, TUB3, VPS68* and *YKE2*. *CIN1, GIM3, PAC2,* and *YKE2* are involved in tubulin folding and tubulin complex assembly. *CIN1* encodes for tubulin folding factor D involved in beta-tubulin (Tub2) folding (36). *GIM3*, *PAC2,* and *YKE2* encode for subunits of the prefoldin co-chaperone complex involved in tubulin folding (37). *TUB3* encodes alpha-tubulin which associates with beta-tubulin (Tub2) to form the tubulin dimer. It is unclear why nocodazole was required to detect genetic interactions with microtubule related components in our screens, but not those performed by the Boone lab (28) however, screen conditions (query strain background,

media composition, temperature, array density) varied between our labs, suggesting perhaps that our screens had reduced microtubule stress, which prevented identification of the interactions, or that their screens had increased stress mimicking nocodazole addition.

To confirm genetic interactions of *PAN2* with genes involved in microtubule function, we assessed the sensitivities of single and double knock-out strains in liquid growth assays using the same low dose of nocodazole used in our screens (1.5 µM), which did not affect the growth of *pan2*Δ single mutant cells (**SFig 2B**). We confirmed slow growth phenotypes in double mutants with *CIN1, GIM3, PAC2,* and *TUB3* in the presence of 1.5 µM nocodazole (**Fig 2B**), indicating aggravating genetic interactions with these genes. There was no difference in growth of the single and double knockouts in the absence of drug (**SFig 2C**), consistent with our screen results. We also expanded our test set to include other genes involved in tubulin folding including *CIN2* (GTPase activating protein, tubulin folding cofactor C), *CIN4* (GTPase involved in beta-tubulin folding, regulated by *CIN2*), and *GIM4* (prefoldin co-chaperone complex). These all showed aggravating genetic interactions with *PAN2* in the presence of 1.5 µM nocodazole but not in the absence. Since PAN regulates mRNA stability, we were intrigued to test the mRNA levels of tubulin genes *TUB1, TUB2,* and *TUB3*, in the presence and absence of nocodazole, but found no significant changes in transcript abundance (**SFig 2D**). Thus, the genetic interactions between *PAN2* and *PAN3* and genes governing tubulin folding provided strong genetic evidence for a role for PAN in microtubule function.

G2/M delay in *pan2*Δ cells treated with nocodazole

Upon establishing the nocodazole sensitivity of *pan2*Δ and *pan3*Δ and genetic interactions between *PAN2* and *PAN3* and genes governing tubulin folding in yeast, we were interested in understanding this phenotype further. We performed flow cytometry to determine the distribution of cells in G1/S and G2/M phases. Log phase cells of *pan2*Δ and WT were treated with either DMSO or 6 µM nocodazole and collected at different time points after treatment (3, 6 and 10 hours). They were fixed in ethanol and stained to analyze DNA content by flow cytometer. In the absence of drug treatment, we saw that both *pan2*Δ and WT cells have typical distributions for asynchronous log phase yeast cultures of 1N and 2N cells at all the time points, suggesting normal cell-cycle progression (**Fig 3A**). However, upon treatment with nocodazole, we found that there was a shift from 1N to 2N and a broadening of the 2N peak in *pan2*Δ as compared to WT cells at 3 hours, indicating a G2/M delay (**Fig 3A**). We did not observe a discrete 1N peak and the DNA content extended beyond 2N, suggesting genomic instability or an extensive G2/M arrest in *pan2*Δ cells after 6 hours of treatment with nocodazole (and also at 10 hours) (**Fig 3A**).

Analysis of the gates during flow cytometry revealed that *pan2*Δ cells upon 3 hours of treatment with nocodazole, had a different forward scatter than WT cells, suggesting that *pan2*Δ cells upon treatment with nocodazole for 3 hours were larger than WT cells (**SFig 3A**), while there was no difference in cell-size upon DMSO treatment (**SFig 3A**). This trend was consistent for the next two time-points as well (6 and 10 hours post treatment with nocodazole), so we determined the cell morphology of *pan2*Δ upon treatment with nocodazole. On treatment of log phase cells with 6 µM nocodazole for 6 hours, *pan2*Δ exhibited a significant increase in cell diameter (~1.75 times), relative to the WT cells, while there was no significant difference in size upon treatment

with DMSO (**Fig 3B**). *pan2*Δ cells treated with nocodazole were predominantly large-budded, and together with their increased cell size, were consistent with our FACS data indicating a G2/M delay. Upon expression of WT *PAN2* in *pan2*Δ cells, we were able to eliminate the large cell phenotype, whereas expression of deadenylase-dead *PAN2* did not, indicating the deadenylase activity of Pan2 was required to prevent the G2/M delay (**Fig 3C**).

Spindle defects in *pan2*Δ cells treated with nocodazole

We were intrigued to determine if there was a difference in spindle morphology in *pan2*Δ cells upon nocodazole treatment. Thus, we used (Tub1+3'UTR)-yomWasabi cassette (38) to first tag tubulin. Upon testing the sensitivity of *pan2*Δ-(Tub1+3'UTR)-yomWasabi yeast and WT-(Tub1+3'UTR)-yomWasabi yeast, similar to *pan2*Δ yeast, we found that *pan2*Δ-(Tub1+3'UTR)-yomWasabi yeast was sensitive to nocodazole, and that WT-(Tub1+3'UTR)-yomWasabi yeast exhibited a mild slow-growth phenotype compared to WT (**SFig 4A**). We set up an assay to observe spindle morphology as well as measure the total number of cells, dead cells and cell area using live-cell imaging of yeast (see Methods). In untreated cells, cell growth appeared to be normal and we did not observe significant differences in cell number or number of dead cells in WT vs *pan2*Δ cells over 8 h of imaging (**Fig 4A, left panels; also see movies in SFiles 4 and 6**). Cell area was largely unchanged in both WT and *pan2*Δ cells over the time-course (**Fig. 4B**). Treatment with 6μM nocodazole did not result in a significant difference in total cells or dead cells between WT and Δ*pan2* cells, and cell area remained largely unchanged (**SFig 4B-4C**). This was different from what we observed in liquid growth assay and we suspect was due to the different growth conditions during live-cell imaging (no shaking or aeration), which altered the level of effective drug exposure. Hence, we tested increased drug concentrations. At 12 μM

nocodazole we observed a general slowing of growth and a significant difference in total cells ($p<0.0001$) and dead cells ($p<0.0001$) between WT and *pan2*Δ. For WT treated with nocodazole, the mean total cell number was 120 at t=0 and 344.5 at t=480 minutes, while the mean number of dead cells increased from 0.75 to 19.75 (**Fig 4A, top right panel; also see movie SFile 5**), whereas for *pan2*Δ treated with nocodazole the mean total cell number was 125 at t=0 and 199 at t=480 min and the mean number of dead cells increased from 0.25 to 27.75 (**Fig 4A, bottom right panel; also see movie SFile 7**). We also observed a steady increase in cell area of both WT and *pan2*Δ until ~360 min, which then leveled off (**Fig 4B**). This increase in cell area was much greater for *pan2*Δ (~13 μm^2 to ~23 μm^2) than for WT (~12 μm^2 to ~17 μm^2) (**Fig 4B**). Taken together the decreased cell growth, increased cell death and increased cell area of *pan2*Δ cells treated with nocodazole were consistent with these cells having a defective cell-cycle.

Now next we examined cell morphology and tubulin-tagged spindles in detail. In untreated WT and *pan2*Δ cells, budding and cell division appeared normal with a cell-cycle duration of ~75 min (**Fig S4D**). Spindles appeared normal, with the spindle pole body being clearly visible at the time of bud emergence (15 min, S phase) and then migrating to the bud neck (30 min, G2 phase) before elongating through the neck and into the bud (55 min, M phase). For nocodazole treated cells, we chose to examine cells after 75 min of treatment with 12µM nocodazole because this time corresponded to approximately one round of cell division and there was little cell death at this time point. The WT cell shown had an elongated cell-cycle of ~90 min (compared to ~75 min for untreated cells), however cell morphology and spindles appeared normal (**Fig 4C**; times indicated are relative to the start of the cell-cycle for the given cell). This was consistent with our quantification, which indicated that over this time period cell number increased, there were few

dead cells, and there was only a marginal increase in cell size. In contrast, the *pan2*Δ cell shown did not have clearly detectable spindle pole bodies or spindles, and although bud growth progressed normally for ~90 min, cell division failed to complete and there was continued mother and bud growth leading to very large cells (see 270 min). For this cell, at 300 min the mother died and the now orphan "daughter" failed to grow or divide (as late as 380 min). This was consistent with our quantification, which indicated that cell number did not increase substantially after nocodazole addition, that the number of dead cells increased and there was a substantial increase in cell size. Thus, in *pan2*Δ cells treated with nocodazole, the spindle was compromised which led to defective mitosis and a failure to complete the cell-cycle.

Transcriptome analysis of yeast with deadenylase dead *PAN2* treated with nocodazole

Because deadenylase dead PAN2 did not eliminate the nocodazole sensitivity of *pan2*Δ cells, we were interested in the transcriptional profile of these cells and if there were altered expression of cell-cycle genes. Therefore, we performed an RNA-sequencing experiment on nocodazole-treated populations of *pan2*Δ + WT Pan2 and *pan2*Δ + D1020A Pan2 cells to identify differences in the transcriptome. Log phase cultures of the above genotypes in duplicate were treated with 6 µM of nocodazole for 6 hours and mRNA was collected and sequenced. Spearman correlations for expression of all the genes were plotted for the two replicates of the two samples (**SFig 5A**).

Out of the 6,031 ORFs analyzed, we chose an absolute z-score cutoff of 2 (equivalent to a log2 fold change cutoff of 1.182 and -1.0554). For a p value < 0.01 and log2 CPM > 1 (counts per million), we identified 129 upregulated and 39 downregulated genes (**Fig 5A**) (**SFile 3**). These genes were enriched in GO terms related to replication fork protection complex, arginine

biosynthetic process, carboxylic acid metabolic process, ascospore wall assembly, thiamine biosynthetic process, hexitol metabolic process and glyoxalase III activity (**Fig 5B**). These processes are known to be cell-cycle dependent and regulated at the transcriptional level, with histone mRNAs (replication fork complex genes) being synthesized exclusively in S phase (39). Hence, the change we see in the expression of these genes was consistent transcriptionally with nocodazole-treated PAN-deficient cells having a G2/M arrest. The transcriptional upregulation of hexitol metabolic process and glyoxalase III activity in *pan2*Δ + D1020A Pan2 compared to *pan2*Δ + WT Pan2, indicates that the cells are likely undergoing a metabolic shift and producing energy differently (hexitol metabolism) and are responding to the oxidative stress in the environment (glyoxalase III activity). While ascospore wall assembly and thiamine biosynthetic processes were also found to be upregulated, since these are haploid cells, it is likely that upregulation of ascospore wall assembly mRNAs is consistent with increased demand for cell wall synthesis during bud growth in G2/M and the increased cell-size of PAN-deficient cells. Some of the genes involved in sporulation might have alternative functions in conditions of nutrient limitation, environmental stress or even mutations that disrupt normal cell-cycle.

Two genes, *CLN3* and *CLB1*, with functions related to regulation of cyclin-dependent protein serine/threonine kinase (CDK) activity, were significantly different between WT and D1020A Pan2 treated with nocodazole. *CLN3* encodes a CDK regulatory subunit that regulates cell-cycle passage through START by regulating transcription at the G1/S transition. *CLB1* encodes a B-type cyclin involved in the regulation of the G2/M transition of both the mitotic cell-cycle and first meiotic division (40). *CLB1* is also involved in mitotic spindle assembly and spindle pole body separation through its interaction with CDK1 (41). By qPCR we found that *CLB1* mRNA

levels decreased ~2.5 fold in cells with WT Pan2 in response to nocodazole, whereas in cells with D1020A Pan2, *CLB1* decreased ~5.6 fold (**Fig 5C**). There was no difference in *CLB1* expression between cells with WT or D1020A Pan2 in the absence of nocodazole. Similarly, for *CLN3* there was no difference between cells with WT or D1020A Pan2 in the absence of nocodazole, however in the presence of nocodazole *CLN3* mRNA levels increased ~1.7 fold in cells with WT Pan2 and ~3.5 fold in cells with D1020A Pan2 (**Fig 5D**). Thus, PAN was required for regulation of *CLB1* and *CLN3* mRNA levels in response to nocodazole.

Given the changes we observed in *CLB1* and *CLN3* expression in cells with D1020A Pan2, we tested for functional associations between PAN and *CLB1* and *CLN3*. We constructed double mutants between *PAN2* and *CLB1* and *CLN3* and tested for genetic interactions in the absence and presence of nocodazole. Growth of the double mutants was the same as the *PAN2* single mutant both in the absence and presence of nocodazole, indicating deletion of either *CLB1* or *CLN3* did not modify the Δ*pan2* nocodazole sensitivity (**Fig S5B**). Next we tested overexpression of *PAN2* in the absence of *CLB1* or *CLN3*. Similar to HsPAN2, overexpression of yeast *PAN2* caused a slow growth phenotype in WT yeast (**Fig 5D**). This phenotype was enhanced in cells lacking *CLB1* and *CLN3* (**Fig 5D**), indicating a functional association between PAN and both *CLB1* and *CLN3*. Thus, these data supported that the role for PAN in response to microtubule stress involved regulation of Clb1 and Cln3 function.

HEK293 PAN2KD cells are sensitive to both nocodazole and colchicine

To determine if the function of Pan2 in regulating the cell-cycle upon microtubule stress is conserved from yeast to mammalian cells, we implemented a human cell culture system to examine sensitivity to microtubule destabilizing drugs and spindle integrity. We generated a population of HEK293 cells where Pan2 was stably knocked down using an shRNA targeting the ORF of *PAN2* (**see Methods**). Specifically, we observed ~ 75% reduction in protein levels of Pan2 by western blot in this population (**Fig 6A**).

To determine if this reduction in Pan2 reduced viability and/or slowed the growth of cells treated with the microtubule inhibitors nocodazole or colchicine, we used a colorimetric MTT (3-(4, 5-dimethylthiazol-2)-2, 5-diphenyltetrazolium bromide) assay for live, metabolically active cells that reads out as absorbance at 562 nm (42). We seeded wells at 1,000 cells per well, added drug 16 h later and at a further 0, 24, 48 and 72 h we performed MTT assay (**Fig 6B**). We observed a dose-dependent decrease in cell viability as measured by A562 in both control and Pan2KD cells treated with nocodazole or colchicine over the time course (**Fig 6C &D, left panels**). We compared the viability of Pan2KD and control cells at the endpoint of the assay on day 3. For nocodazole treatment, we observed a significant decrease in viability of Pan2KD compared to control cells at 40, 60 and 80 nM nocodazole (**Fig 6C, right panel**). For colchicine we observed significant decreases at all concentrations (**Fig 6D, right panel**). This suggested that, as was the case in yeast, depletion of Pan2 increased the vulnerability of mammalian cells to microtubule depolymerization.

HEK293 PAN2KD cells exhibit defective spindle phenotypes upon treatment with colchicine

We were curious to determine the cause of increased sensitivity of HEK293 Pan2KD cells to microtubular depolarization and how this related to the cell-cycle. Thus, we live-imaged microtubules in the control and Pan2KD cells using a tubulin staining dye, CellLight™ Tubulin-RFP BacMam 2.0. Upon treatment with a low dose of 10 nM of colchicine, we imaged cells for a period of 24 hours to capture the spindle morphology, as well as to determine the duration of different stages of mitosis (**Fig 7A**). In the absence of drug treatment, progression through the cell-cycle appeared normal for both control and Pan2KD cells (**Fig 7B &C; also see movie SFile 8**). We observed no differences in metaphase duration or spindle morphology. In contrast, upon treatment with 10 nM colchicine, as compared to control cells, Pan2KD showed abnormal progression through the cell-cycle (**Fig 7B; ; also see movie SFile 9**). Pan2KD cells spent longer time in metaphase (**Fig 7C**) and had a higher propensity to form multipolar spindles (~75% in Pan2KD vs ~40% in Ctrl) (**Fig 7C**; also see 100 min image in **Fig 7B**). This indicated that Pan2 played a role in spindle integrity during microtubule stress and was consistent with the decreased viability we observed in Pan2KD cells treated with nocodazole and colchicine.

Discussion

Since the role of PAN in response to microtubule stress was dependent on its deadenylase activity, we propose that PAN regulates the PATLs of mRNAs required for cellular survival of microtubule stress (**Fig 8**). PAN activity during microtubule stress would lead to translational repression of key proteins involved in either sensing microtubule stress and/or responding

to/recovering from formation of defective spindles. Some obvious targets are proteins involved in the spindle assembly checkpoint, however we did not find expression of any of these to be altered in our mRNA seq screen. We also did not detect altered expression of genes with functions directly related to microtubule biogenesis, including the tubulin genes themselves, making it unlikely that PAN regulates microtubules directly. However, it is possible that our screen missed these targets because it did not identify mRNAs with altered poly-A tail lengths. Altered poly-A tail length could lead to altered translation, independent of mRNA stability, hence screening for altered poly-A tail length would likely produce novel insights into PAN's mechanism of action (15,43). It is interesting that cells lacking PAN appear to have a normal cell-cycle, normal spindles, and show no decrease in fitness under regular growth conditions. This further supports a specific role for PAN in response to microtubule/spindle stress and perhaps explains its elusive role in the cell-cycle until now.

We did identify a functional connection between PAN and two important cell-cycle regulators, Clb1 and Cln3, although the significance remains obscure. Decreased expression of *CLB1* in response to nocodazole is consistent with failure of Δ*pan2* cells to complete mitosis, however because *CLB1* is decreased in Δ*pan2* cells it is unlikely to be a direct target. The fact that *CLB1* expression increases in response to nocodazole in WT yeast (which complete mitosis apparently normally) supports that *CLB1* is part of the microtubule stress response. This is also supported by the genetic interaction we observed between PAN and *CLB1*. It is possible that the low expression of *CLB1* in Δ*pan2* cells treated with nocodazole prevents completion of mitosis, however we might have expected to see loss of *CLB1* enhance the nocodazole sensitivity of Δ*pan2* cells, which wasn't the case. It is possible that *CLB1* expression is already limiting, in

which case overexpression of *CLB1* should reduce the nocodazole sensitivity of Δ*pan2* cells if Clb1 is playing an important role in the response. *CLN3* expression was found to be upregulated in response to nocodazole which was enhanced in Δ*pan2* cells. Since Cln3 is not known to play roles in mitosis it is unclear why Cln3 is being impacted. If *CLN3* was a direct target of PAN and its elevated expression was preventing completion of mitosis, we would have expected loss of *CLN3* to suppress the nocodazole sensitivity of Δ*pan2* cells, which wasn't the case. Because we identify a genetic interaction between PAN and *CLN3* it is likely Cln3 is functionally relevant, perhaps indirectly by affecting cell-cycle dynamics or by regulating PAN activity outside of M phase.

There is evidence for regulation of cell-cycle progression through regulation of PATLs. For example, genes such as *CDK1, TOP2A, KPNA2, UBE2C* and *FBXO5*, which orchestrate timing and coordination of mitotic events in somatic cells, have been found to have reduced PATLs in M-phase (15). Transcription of these genes peaks at G2/M phase and is repressed at G1 phase (44). In contrast, other well-known mitotic regulators which are also transcriptionally upregulated in G2/M phase, such as *CCNA*, *CCNB*, and *CENP-E*, do not show poly(A) length changes (44). This suggests that a subset of genes are selectively deadenylated to shut down once cells enter M phase. Specifically, *CDK1* displayed the most extreme change, whose median poly(A) tail length changed from 77 nt in S phase to 2 nt in M phase and displayed translational repression (15), unlike the previous report showing translational upregulation of *CDK1* in M phase (45). Analysis of gene ontology for genes exhibiting shortened poly(A) tails during the M phase identified enriched terms such as "regulation of mitotic cell-cycle", "microtubule cytoskeleton", and "regulation of ubiquitin-protein ligase activity". This implies that

deadenylation during the M phase may be linked to regulatory functions within the cell cycle. It is currently unknown which deadenylases are responsible for which modifications and our results suggest that PAN may play an important role. Ccr4-Not may also play a role, however, the fact that loss of Ccr4-Not activity in yeast does not lead to sensitivity to microtubule destabilizing drugs implies a prominent function for PAN (29).

Post-transcriptional regulation of mRNAs plays a critical role during the cell cycle and can be achieved through their localization to specific subcellular compartments (46–48). A high-throughput smFISH (single molecule Fluorescence *In Situ* Hybridization) screen of 602 proteins with centrosomal functions identified 8 human mRNAs (*NIN, CEP350, PCNT, BICD2, CCDC88C, ASPM, NUMA1,* and *HMMR)* that are locally translated at the centrosome (49). Seven of these are translated during mitosis (*CCDC88C* is translated only during interphase). *CEP350, ASPM, NUMA1,* and *HMMR* are microtubule binding proteins with functions in centrosome/spindle maturation and *NIN, PCNT* and *BICD* contribute to microtubule anchoring at the centrosome (50–56). Hence, these proteins are potential PAN targets in response to microtubule stress. However, we did not find altered levels of yeast spindle pole body mRNAs in our RNA-seq screen, suggesting either we missed detecting such changes or that PAN acts via other targets. Given that the yeast spindle pole body shares no structural similarity to the centrosome and that RNAs have yet to be localized to spindle pole bodies further suggests that PAN targets components other than the centrosome/spindle pole body in yeast and humans.

The spindle phenotypes we observed in Pan2KD cells treated with colchicine coupled with their decreased cell viability suggested these cells could have undergone abnormal chromosome

segregation. Cells with mis-segregated chromosomes often have abnormal nuclei and have increased cell death (57–59). An imaging based whole-genome siRNA screen in HeLa cells found that the absence of *PAN2* led to irregular nuclear shape and chromosome mis-segregation (60). Upon chromatin extraction during G2, Pan2 and Pan3 were found to be part of a soluble complex containing the spindle checkpoint proteins Bub1 and Mad1l1, the kinetochore proteins Ndc80 and Nuf2, and 265 other proteins enriched for the molecular functions poly(A)-specific ribonuclease activity, poly(A) binding, translation initiation factor activity, mRNA 3'UTR binding, RNA-helicase activity, ribosome binding, microtubule motor activity and gamma-tubulin binding (60). Hence, this study provides evidence that PAN's role in responding to microtubule stress may involve direct associations between PAN complex and key spindle regulatory proteins, which is required for proper chromosome segregation.

Previous studies have shown that the PAN localizes to P-bodies in yeast cells. P-bodies serve as sites of mRNA storage, decay, and translational repression. Sweet et al. find that treating yeast with the microtubule depolymerizing drug benomyl induces formation of P-bodies containing tubulin (61). However, according to the HIPHOP chemical genetics database (29), none of the P-body protein mutants are sensitive to nocodazole or benomyl, suggesting the role for PAN in microtubule stress is independent of P bodies. Additionally, many groups have reported an increase in P-body numbers during S phase, the disappearance of PB structures before mitosis, and their reassembly during cytokinesis in mammalian cells, further supporting a non-P body role for PAN during mitosis (62,63).

Finally, if the role of PAN is restricted to responding to microtubule stress in M-phase, this raises the question of whether PAN activity is regulated in a cell-cycle dependent manner and/or in response to stress. Pan3 has previously been identified as a substrate of the CDK Pho85 (24). Different groups have identified 9 phosphorylation sites on Pan3, one in the pseudokinase domain and the rest in unstructured regions. Holt et al. identified residue 415 in the linker domain of Pan2 as a substrate of CDK1 phosphorylation (22). Phosphorylation of Pan3 in mammalian cells has previously been shown to decrease its interaction with PABPC1 and reduce PAN activity (23). Together this suggests PAN activity is regulated by phosphorylation by CDKs in a cell-cycle dependent manner. Interestingly, our SGA screen identified an alleviating genetic interaction between *PAN2* and *PHO85*, supporting a functional connection between PAN and this important cellular stress response regulator. The need for cell-cycle dependent regulation of PAN might also explain our finding that having too much Pan2 in the cell leads to reduced cell growth, although the cause of this fitness defect has yet to be elucidated.

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Author contributions

JV, CDR and CJRL designed the experiments. JV performed the majority of the experiments. ZH collected live-cell imaging data for HEK293 cells, under the supervision of CDM. JARB collected the flow-cytometry data for cell-cycle analysis in yeast cells, under the supervision of LJH. PD confirmed HEK293 Pan2KD GFP positive cells by flow cytometry. BPY performed the initial testing of sensitivity of *pan2*Δ to nocodazole. SF performed the bioinformatics analysis on RNA-seq data. JV, ZH, JARB, PD, BPY, CDM, CDR and CJRL analyzed the data. JV and CJRL wrote the manuscript. CDM and CDR edited the manuscript.

Competing interests

The authors declare no competing financial interests.

Figures and Figure captions

Figure 1

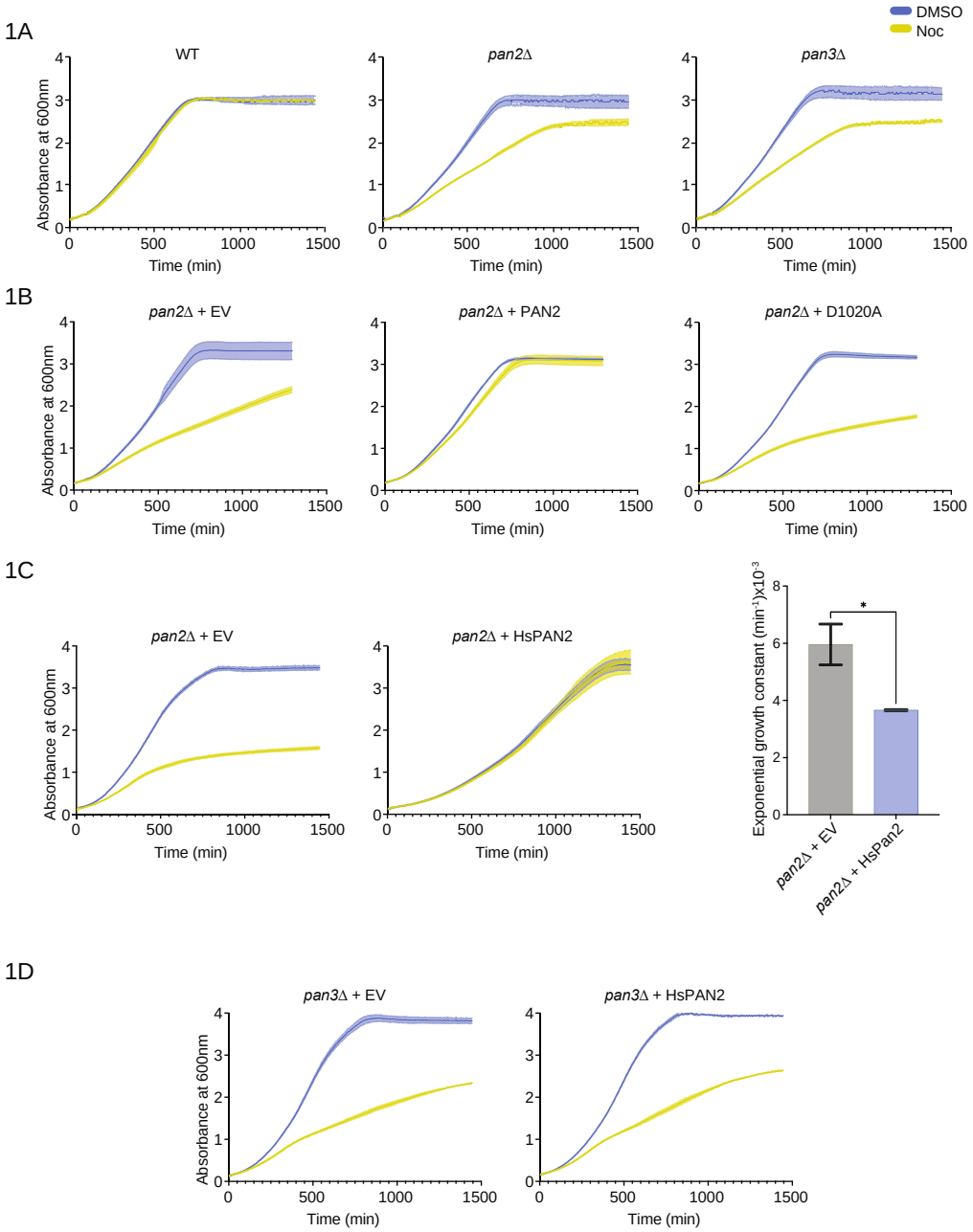
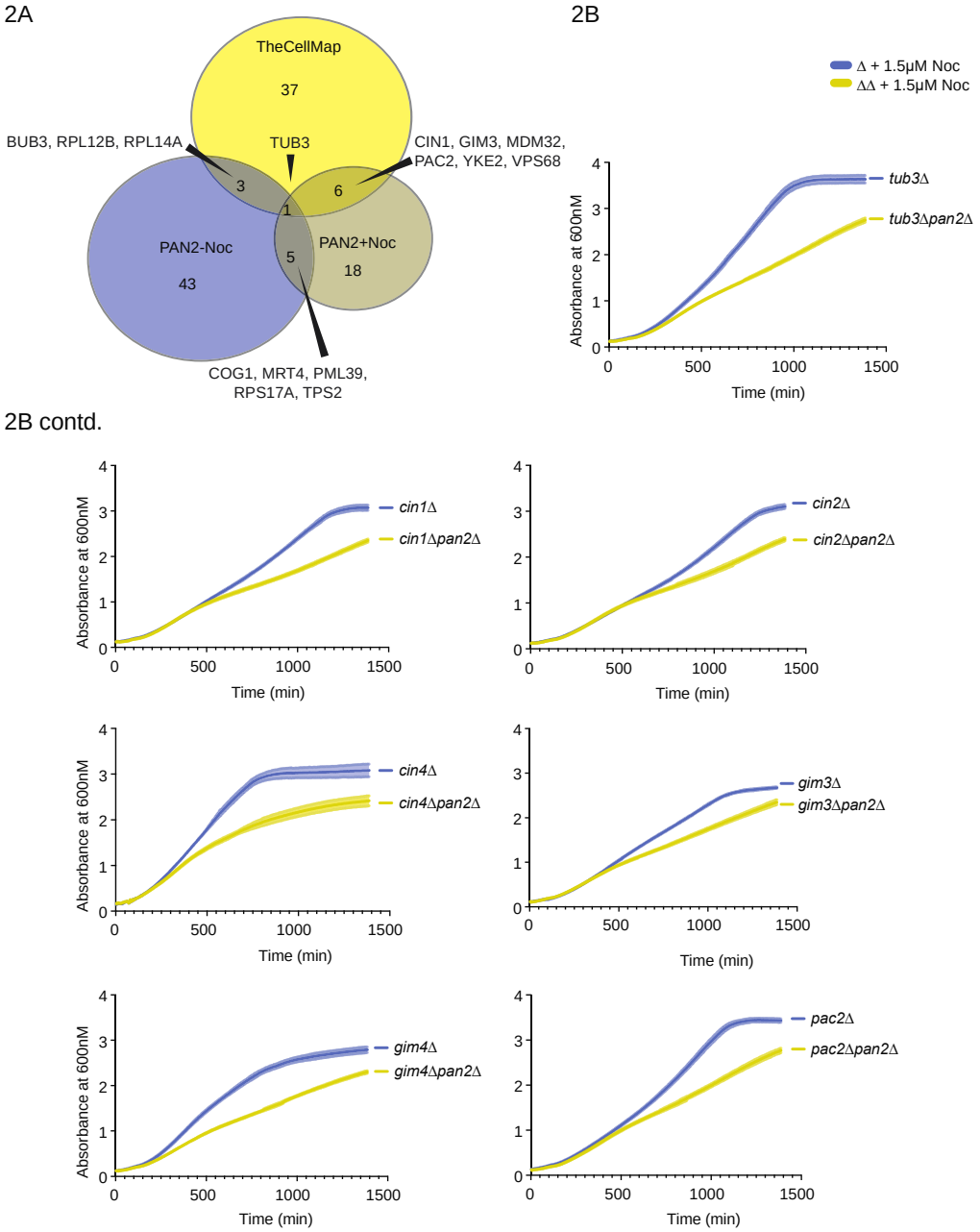


Fig 1: Yeast deletion mutants *pan2*Δ and *pan3*Δ are sensitive to nocodazole. (**A-D**) Growth curves of yeast strains in SC media with DMSO (blue) or 6 µM nocodazole (yellow) at 30°C for 24 hours. Absorbance plotted with SD (n=6). Normalized exponential growth constants have been represented in **Fig 1C**. Error bars are +/- SD from three separate experiments (n=6). Significant differences between different strains were tested using paired Student's *t*-test.WT, wild type; min, minutes; SC, Synthetic Complete media; SD, standard deviation; EV, empty vector; D1020A, deadenylase dead Pan2; Hs, *Homo sapiens.*

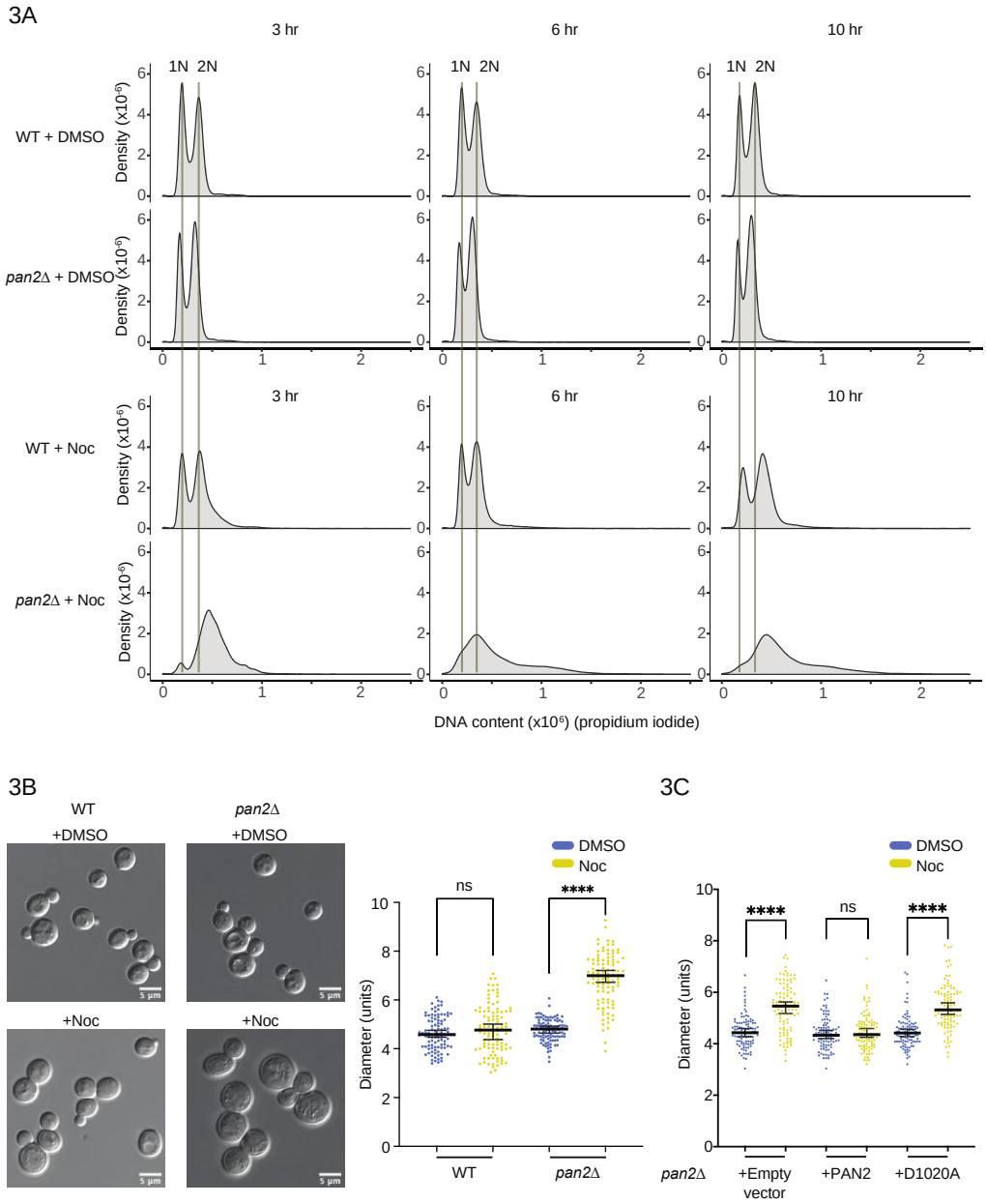
714 Figure 2



715

Fig 2: PAN2 and PAN3 genetically interact with tubulin and genes governing tubulin folding. (**A**) Venn diagram shows overlap between aggravating genetic interactions for PAN2 according to TheCellMap (yellow), PAN2 SGA screen in either absence (blue) or presence of nocodazole (light brown). **(B)** Growth curves of single knockout (blue) or double knockout (yellow) yeast strains in SC media with 1.5 µM nocodazole for 24 hours at 30°C. Absorbance plotted with SD (n=6). min, minutes; SC, Synthetic Complete media; SD, standard deviation.

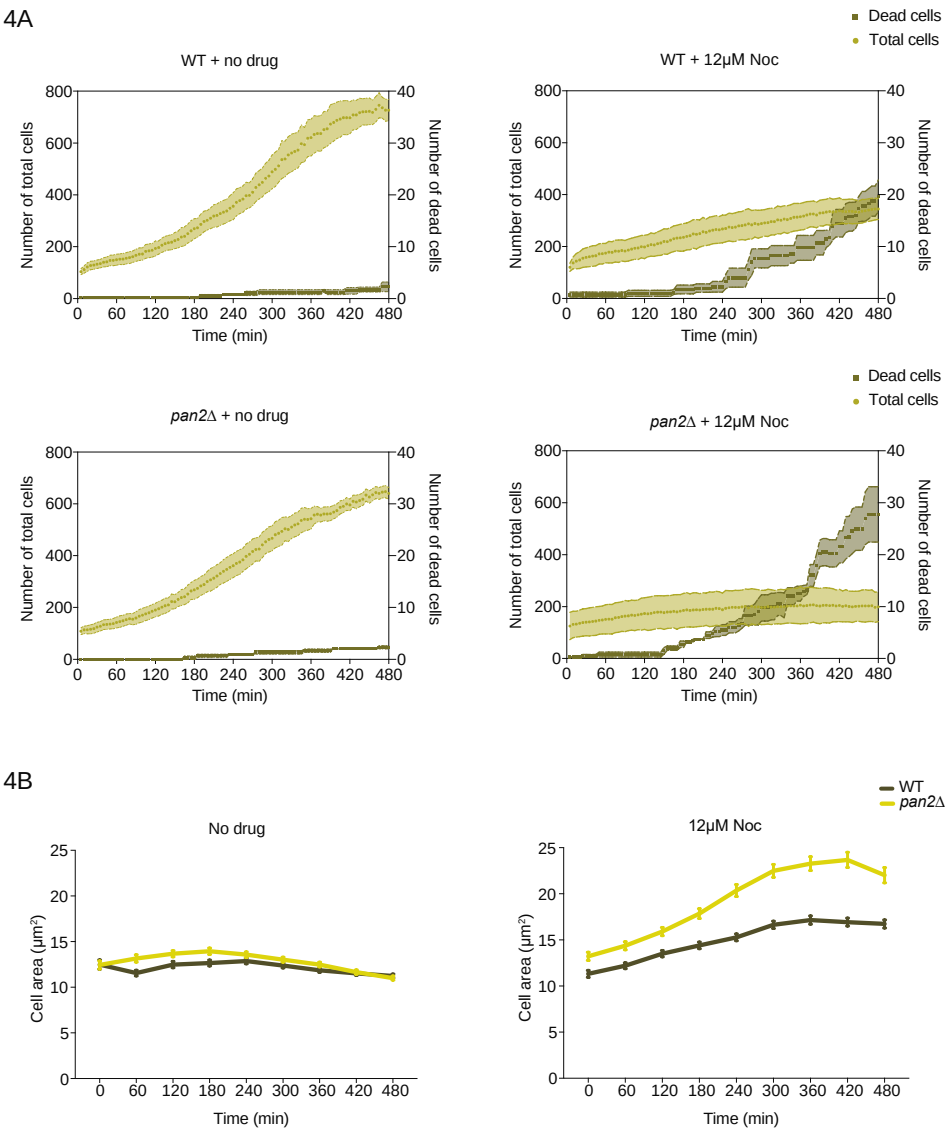
723 Figure 3



724

Fig 3: G2/M delay in *pan2*Δ cells treated with nocodazole. (A) FACS profiles post treatment with DMSO or 6 µM nocodazole. 1N and 2N respectively represent DNA content. Green lines represent the position of 1N and 2N DNA peaks in untreated cells. FACS, fluorescence activated cell sorting; hr, hours; WT, wild type; Noc, nocodazole. **(B-C)** Live imaging of yeast cells treated for 6 hours. Mother cell size quantified (n > 100). Scale bars: 5 µm. Paired Student's t-test for significant differences. WT, wild type; ns, not significant, ****, p<0.0001.

Figure 4


4A
Dead cells
Total cells
WT + no drug
WT + 12μM Noc
pan2Δ + no drug
pan2Δ + 12μM Noc
Number of total cells
Number of dead cells
Time (min)
4B
WT
pan2Δ
No drug
12μM Noc
Cell area (μm²)
Time (min)

4C

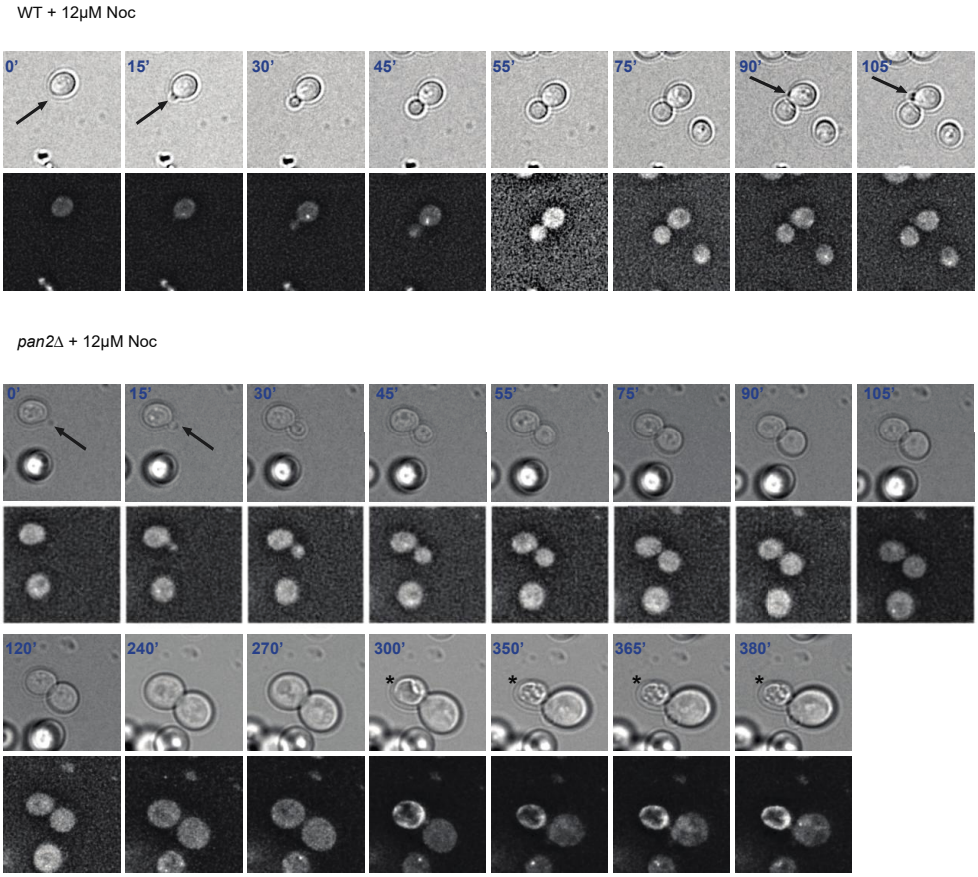
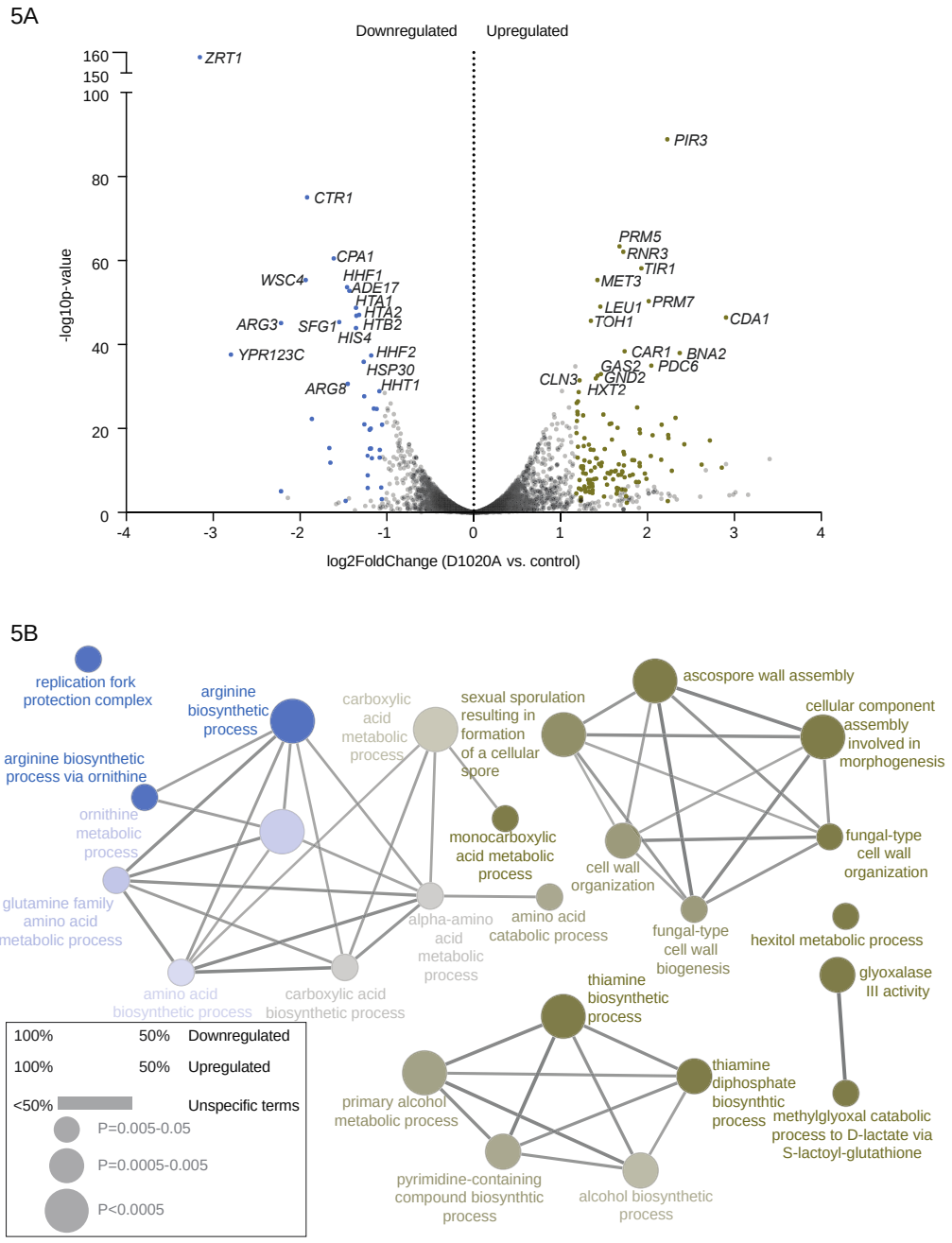


Fig 4: **Spindle defects in *pan2*Δ upon treatment with nocodazole. (A)** Number of total cells (left y-axis) and number of dead cells (right y-axis) upon treatment of WT or *pan2*Δ with DMSO (left) or 12μM nocodazole (right) for every 5-minutes over a total time interval of 480 minutes. **(B)** Cell area of individual cells of WT (brown) or *pan2*Δ (yellow) is represented with 95% confidence interval upon treatment with DMSO (left) or 12μM nocodazole (right) over a total duration of 480 minutes. Each time point represents the cell area of 500-2500 cells recognised by cell segmentation. **(C)** Live imaging of log phase yeast cells expressing (Tub1+3'UTR)-yom-

Wasabi WT (above) or *pan2*Δ (below). The cells are treated with DMSO or 12 µM nocodazole for ~75 minutes prior to imaging. The time-stamps are indicated on top left of each snapshot. WT, wild-type; DMSO, dimethyl sulfoxide; µM, microMolar; Tub1: alpha-tubulin; UTR, untranslated region.

Figure 5


5A
Downregulated
Upregulated
-log10p-value
log2FoldChange (D1020A vs. control)
ZRT1
CTR1
CPA1
WSC4
HHF1
ADE17
HTA1
HTA2
HTB2
ARG3
SFG1
HIS4
YPR123C
HHF2
HSP30
ARG8
HHT1
PIR3
PRM5
RNR3
TIR1
MET3
LEU1
PRM7
TOH1
CDA1
CAR1
BNA2
GAS2
PDC6
GND2
CLN3
HXT2
5B
replication fork protection complex
arginine biosynthetic process
arginine biosynthetic process via ornithine
ornithine metabolic process
glutamine family amino acid metabolic process
amino acid biosynthetic process
carboxylic acid biosynthetic process
carboxylic acid metabolic process
alpha-amino acid metabolic process
sexual sporulation resulting in formation of a cellular spore
monocarboxylic acid metabolic process
amino acid catabolic process
ascospore wall assembly
cellular component assembly involved in morphogenesis
cell wall organization
fungal-type cell wall organization
fungal-type cell wall biogenesis
hexitol metabolic process
glyoxalase III activity
methylglyoxal catabolic process to D-lactate via S-lactoyl-glutathione
thiamine biosynthetic process
thiamine diphosphate biosynthtic process
primary alcohol metabolic process
pyrimidine-containing compound biosynthtic process
alcohol biosynthetic process
100% 50% Downregulated
100% 50% Upregulated
<50% Unspecific terms
P=0.005-0.05
P=0.0005-0.005
P<0.0005

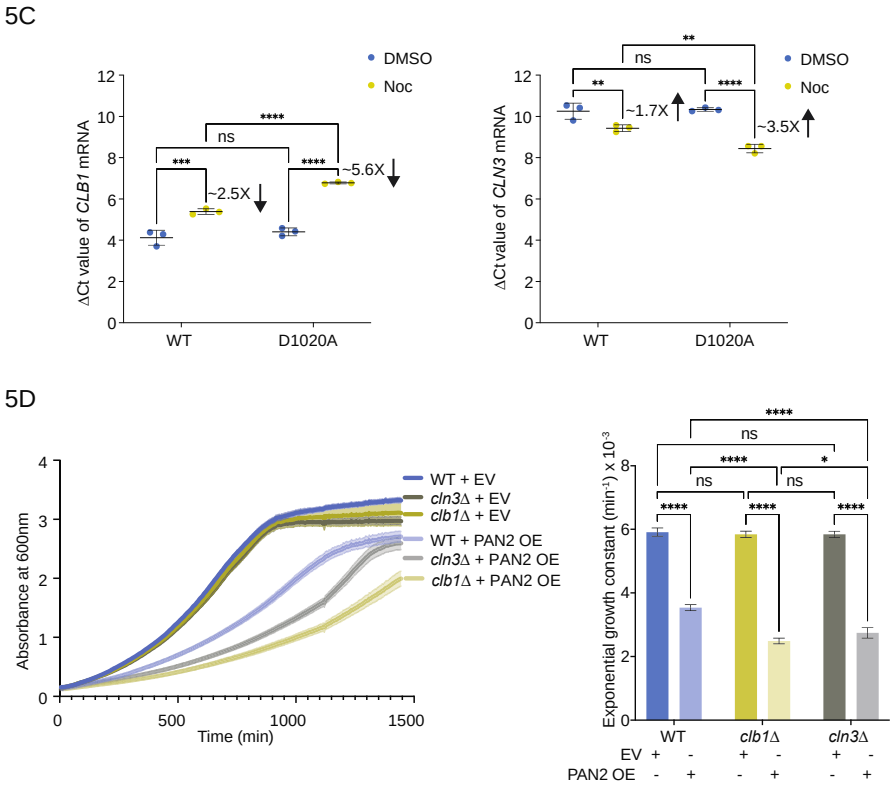


Fig 5: **mRNA levels of *CLB1* and *CLN3* are altered in deadenylase dead mutant of *PAN2* in nocodazole. (A)** Fold change and p-value of ~6000 ORFs analyzed in nocodazole treated Control and D1020A cells by RNA-sequencing. Each closed circle represents a single ORF, categorized into upregulated (green), downregulated (blue) or not-significantly different (gray) (**B**) Functional enrichment of the upregulated and downregulated genes identified in (**A**). Node size indicates statistical significance of enriched GO terms and edges indicate statistically significant associations between terms. Node color indicates the % of aggravating or alleviating interactions in each term. Unspecific terms in gray. (**C**) Gene expression changes in *pan2*Δ+WT-PAN2 or *pan2*Δ+D1020A-PAN2 post 6 hour treatment with DMSO or 6 µM nocodazole

assessed by qRT-PCRs. The ΔCt values are normalized to actin. Data mean +/- S.D. 0.4X and 2X indicate fold-change in *CLB1* and *CLN3* mRNA levels respectively. n.s., not significant; **** $p < 0.0001$; *** $p < 0.001$; **, $p < 0.01$. Two-way ANOVA for significant differences. Ct, cycle threshold; µM, micromolar; qRT, quantitative reverse transcriptase; ANOVA, Analysis of Variance. (**D**) Growth curves of yeast strains grown in liquid SC media and monitored for 24 hours at 30°C. Absorbance adjusted for baseline correction is plotted on the y-axis and time (in minutes) is plotted on the x-axis. An average of 6 replicates have been plotted with SD. min, minutes; SC, Synthetic Complete media; SD, standard deviation; WT, wild type; EV, empty vector; OE, overexpression. Base 2 exponential growth constants calculated from growth of different yeast strains. Error bars are +/- SD from three separate experiments. Each experiment has 6 replicates. Significant differences between different strains were tested using two-way ANOVA comparison.

Figure 6

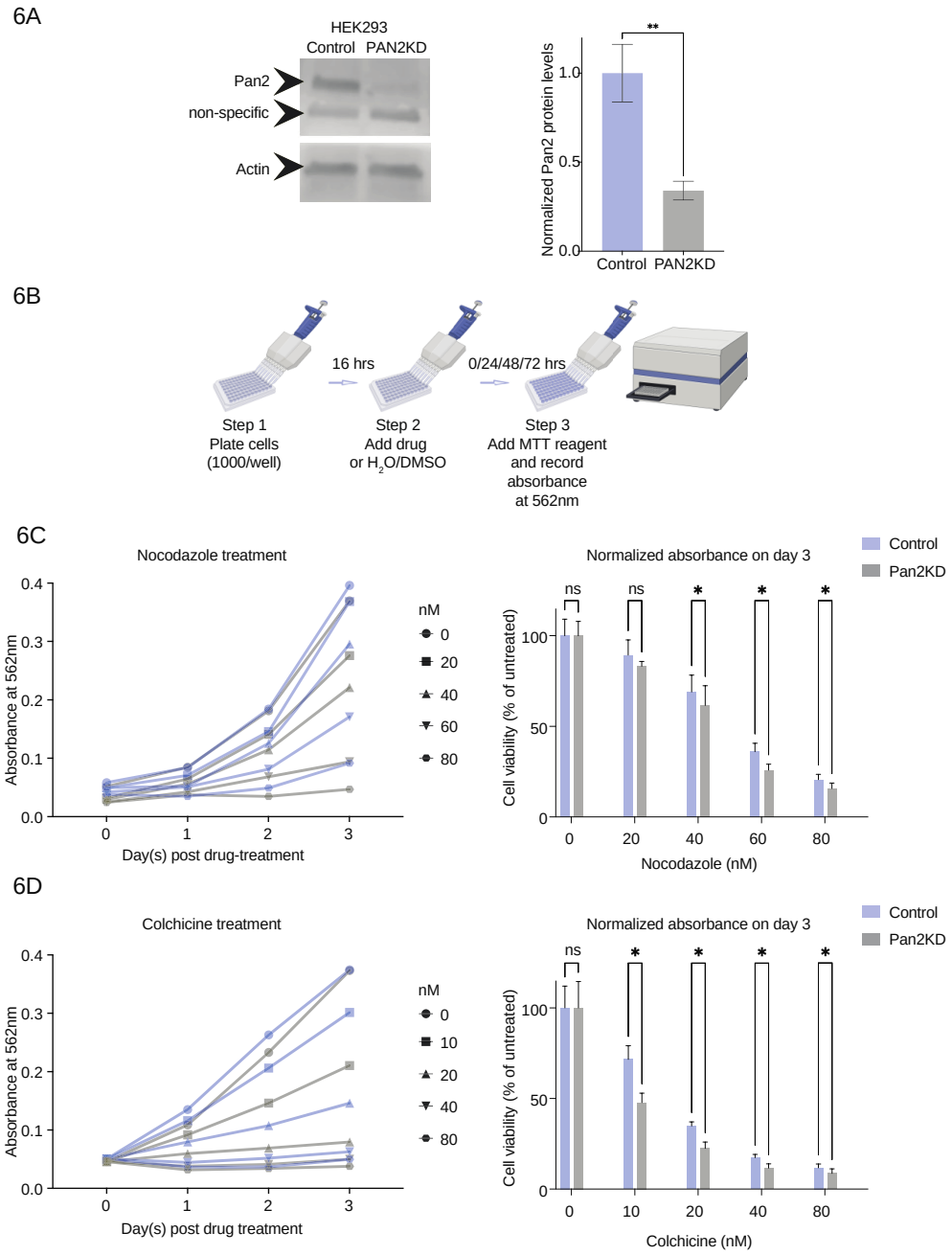
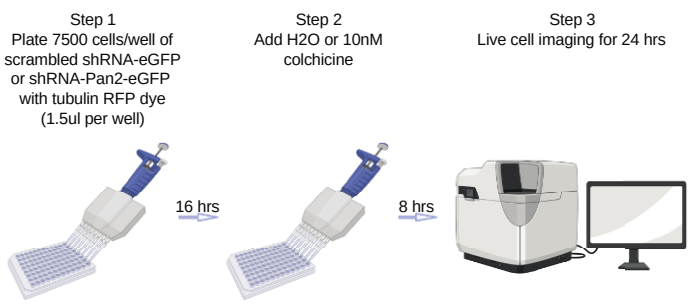

6A
HEK293
Control PAN2KD
Pan2
non-specific
Actin
Normalized Pan2 protein levels
1.0
0.5
0.0
**
Control PAN2KD
6B
16 hrs
0/24/48/72 hrs
Step 1
Plate cells
(1000/well)
Step 2
Add drug
or H2O/DMSO
Step 3
Add MTT reagent
and record
absorbance
at 562nm
6C
Nocodazole treatment
Absorbance at 562nm
Day(s) post drug-treatment
nM
0
20
40
60
80
Normalized absorbance on day 3
Control
Pan2KD
ns
ns
*
*
*
Cell viability (% of untreated)
Nocodazole (nM)
6D
Colchicine treatment
Absorbance at 562nm
Day(s) post drug treatment
nM
0
10
20
40
80
Normalized absorbance on day 3
Control
Pan2KD
ns
*
*
*
*
Cell viability (% of untreated)
Colchicine (nM)

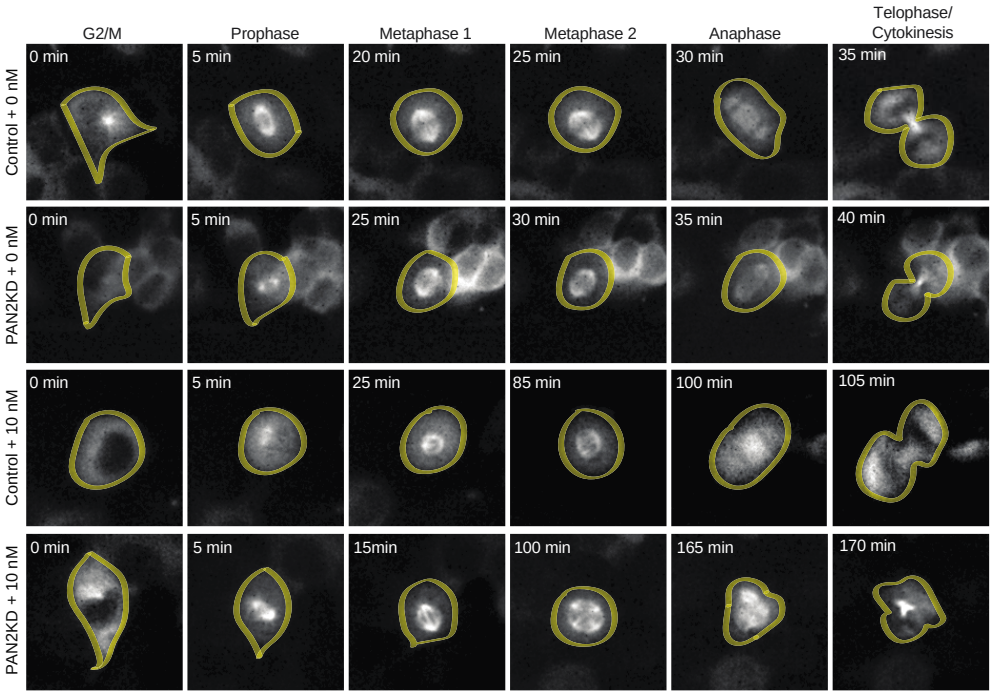
Fig 6: HEK293 PAN2KD cells are sensitive to nocodazole and colchicine. (**A**) Cell lysates from HEK293 Control and Pan2KD cells were analyzed on an SDS gel and probed for Pan2 and Actin levels, quantification for which is shown on right, n=3. Significant differences between the 2 strains were tested using paired Student's *t*-test. **, $p < 0.01$. (**B-D**) Protocol for recording MTT activity of cells over multiple days. Absorbance at 562 nm (y-axis) and days post treatment (x-axis) with nocodazole **(C)** or colchicine **(D)** was plotted for control (blue) and Pan2KD (gray) at various concentrations of drug. Absorbance of each treated condition was normalized to the absorbance of the untreated condition for each cell line on the day 3 post-treatment to plot cell viability (% of untreated). Average of 6 replicates has been plotted. Mean +/- S.D. Significant differences between Control and Pan2KD cells at each concentration was tested using multiple Student's *t*-test. nM, nanomolar; n.s., not significant; *, $p<0.05$.

Figure 7

7A



7B



7C

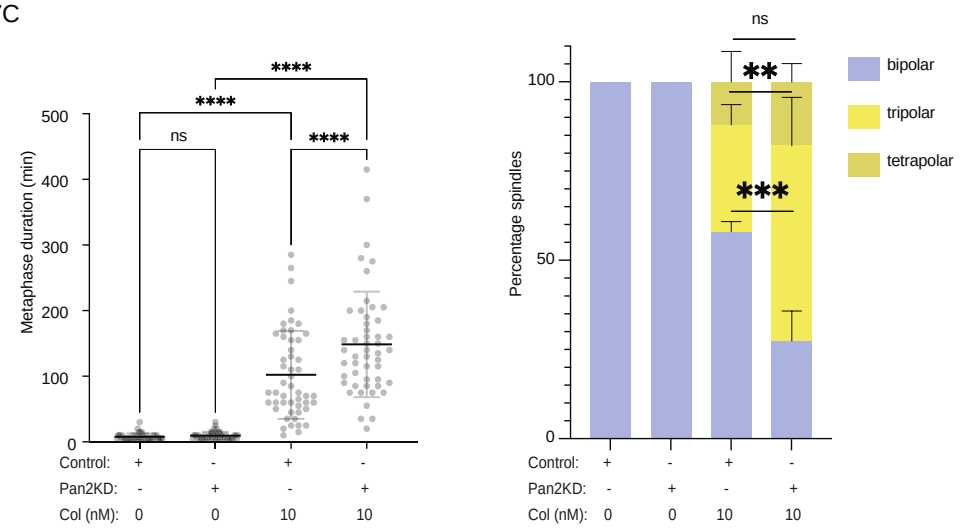


Fig 7: **HEK293 PAN2KD cells exhibit defective spindle phenotypes upon treatment with colchicine** (**A**) Protocol for live-cell imaging of colchicine treated cells using tubulin staining dye. (**B**) Representative images of HEK293 Control and Pan2KD cells with or without treatment with 10 nM colchicine. These cells are shown undergoing mitosis at the transition from G2 to M and different phases of M-phase (prophase, metaphase 1, metaphase 2, anaphase and telophase). The time taken for each stage of mitosis is indicated on top left of each snapshot. nM, nanomolar.

(**C**) Metaphase duration (in minutes) and percentage of cells having 2, 3 or 4 spindles is plotted for colchicine treatment of HEK293 Control and Pan2KD cells. Each closed circle represents one cell. 50 cells were counted for each condition. Mean +/- S.D. has been plotted. nM, nanomolar; min, minutes; ns, not significant; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$.

Figure 8

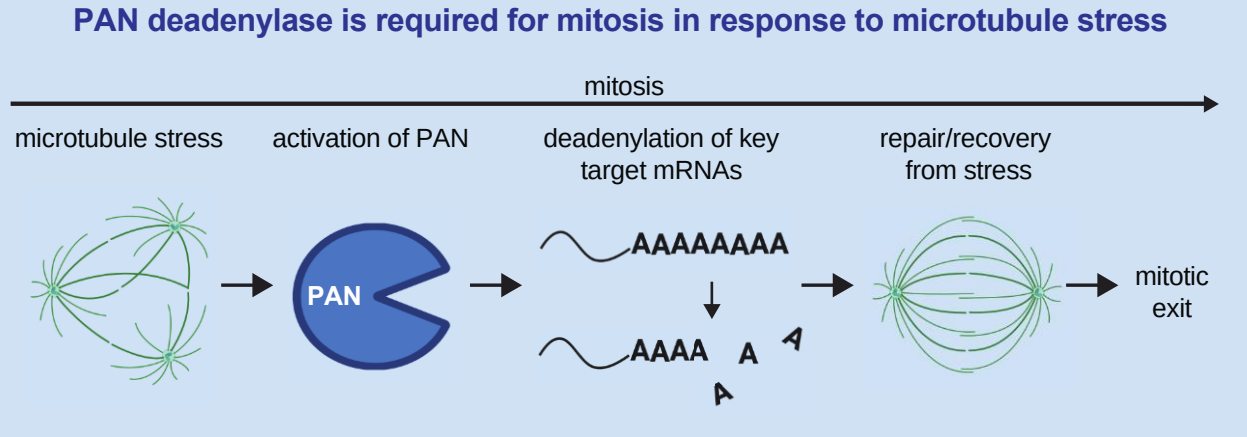


Fig 8: **Model schematic of PAN regulating the polyA tails lengths (PATLs) of mRNAs required for cellular survival of microtubule stress** (created using BioRender).

Supplementary figures and legends

Figure S1

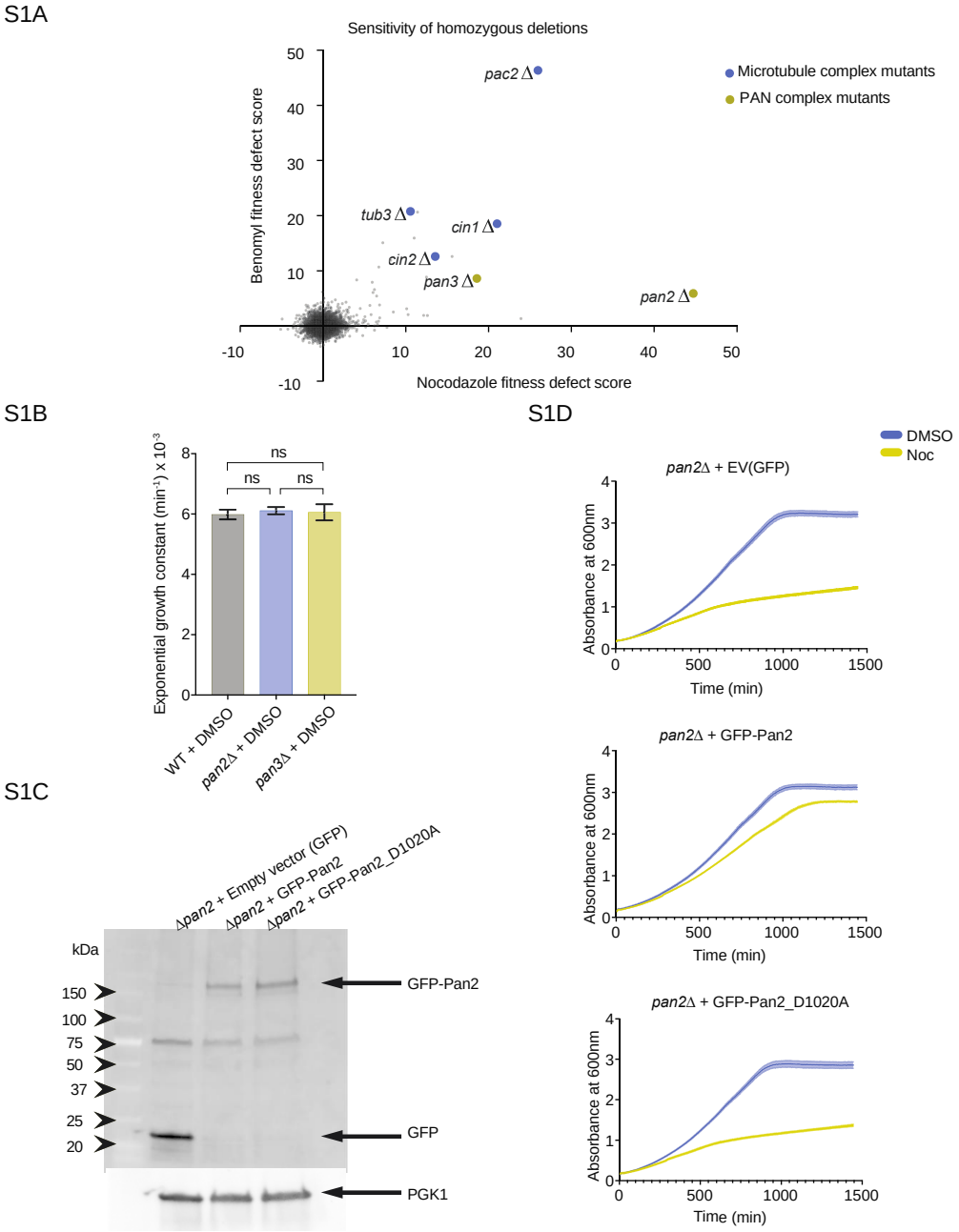
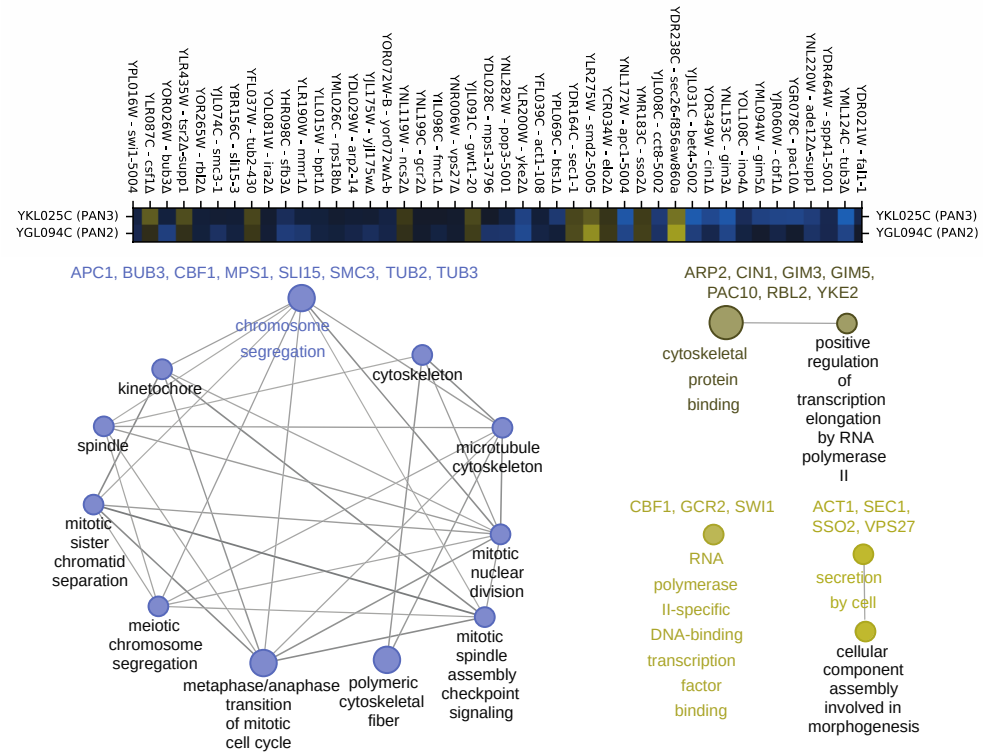


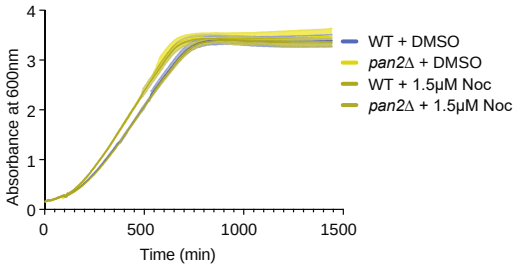
Fig S1A: Sensitivity of yeast homozygous deletion strains to nocodazole and benomyl. Ratio of fitness defect scores for over ~4,800 mutants (x-axis: nocodazole; y-axis: benomyl). Blue circles: microtubule complex mutants; green circles: PAN complex mutants. Data from A. Y. Lee et al. 2014. **Fig S1B**: Exponential growth constants for WT, *pan2*Δ, and *pan3*Δ with DMSO. Error bars: +/- SD (n=6, three experiments). Paired Student's *t*-test for strain differences. **Fig S1C**: GFP-Pan2 on plasmid assesses Pan2 expression in D1020A. Upper band: Pan-GFP; lowermost band: free GFP. PGK1 - loading control. CEN, centromeric; GFP, green fluorescent protein. **Fig S1D**: Growth curves of yeast strains with DMSO (blue) or 6 µM nocodazole (yellow) at 30°C for 24 hours. Absorbance plotted with SD (n=6). min, minutes; SC, Synthetic Complete media; SD, standard deviation; EV, empty vector; D1020A, deadenylase dead Pan2.

Figure S2

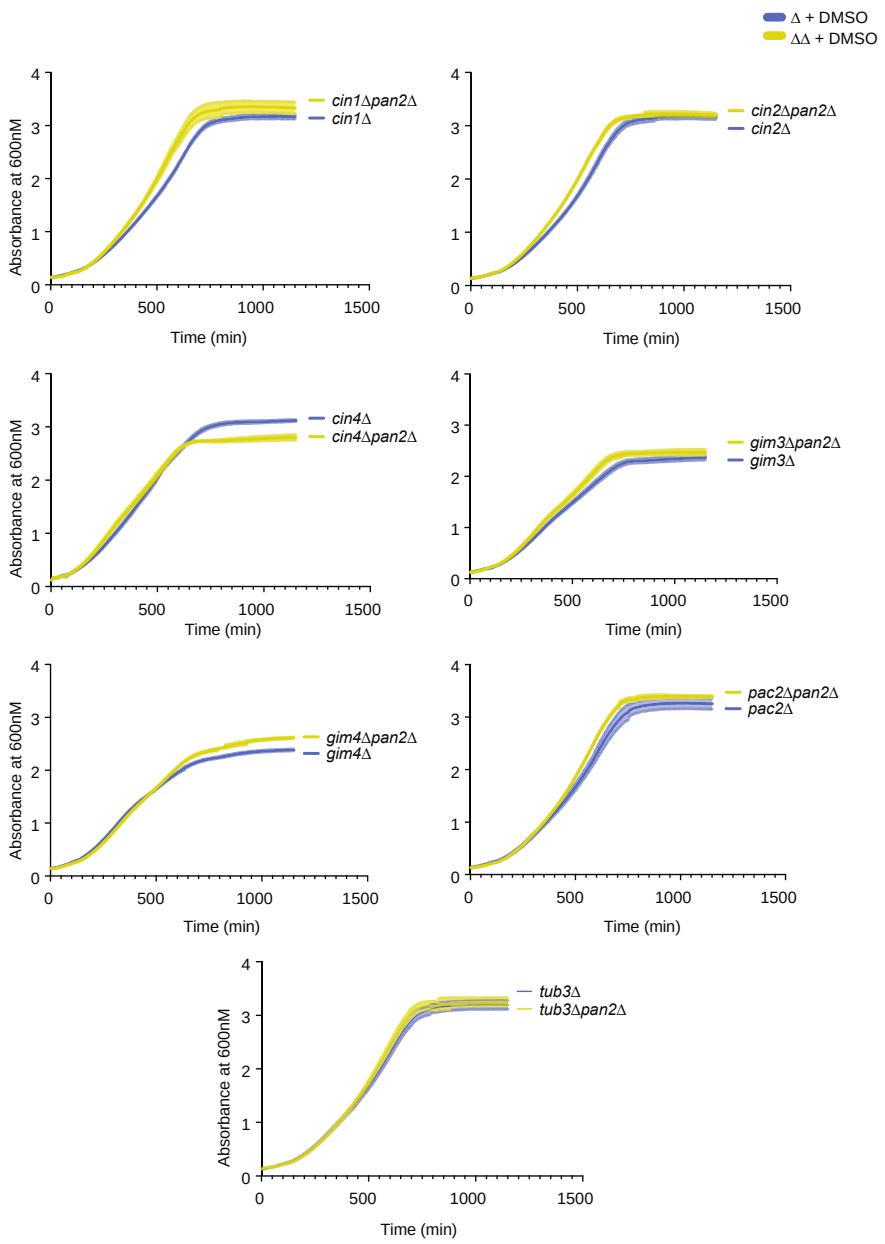
S2A



S2B



S2C


Δ + DMSO
ΔΔ + DMSO
cin1Δpan2Δ
cin1Δ
cin2Δpan2Δ
cin2Δ
cin4Δ
cin4Δpan2Δ
gim3Δpan2Δ
gim3Δ
gim4Δpan2Δ
gim4Δ
pac2Δpan2Δ
pac2Δ
tub3Δ
tub3Δpan2Δ
Absorbance at 600nM
Time (min)
0
1
2
3
4
500
1000
1500

822

S2D

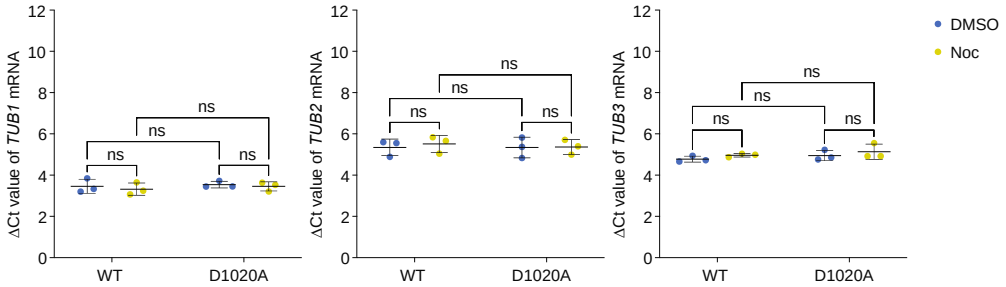


Fig S2A: Heatmap and Cytoscape analysis of genetic interactions of PAN2 and PAN3 by Costanzo et al., 2016. In the heatmap, darker shades of blue represent strong aggravating genetic interactions and darker shades of yellow represent strong alleviating genetic interactions. In the functional enrichment, node size indicates statistical significance of enriched GO terms and edges indicate statistically significant associations between terms. **Fig S2B-C**: Growth curves of yeast strains with DMSO or 1.5 µM nocodazole (B) and single or double knockout strains in DMSO (C). Absorbance plotted with SD (n=6). **Fig S2D**: Gene expression changes in *pan2*Δ+WT-PAN2 or *pan2*Δ+D1020A-PAN2 post 6 hour treatment with DMSO or 6 µM nocodazole assessed by qRT-PCRs. The ΔCt values are normalized to actin. Data are mean +/- S.D. n.s., not significant. Significant differences between all pairwise combinations of groups was detected using two-way ANOVA comparison. Ct, cycle threshold; µM, micromolar; qRT, quantitative reverse transcriptase; ANOVA, Analysis of Variance. min, minutes; SC, Synthetic Complete media; SD, standard deviation; WT, wild type.

Figure S3

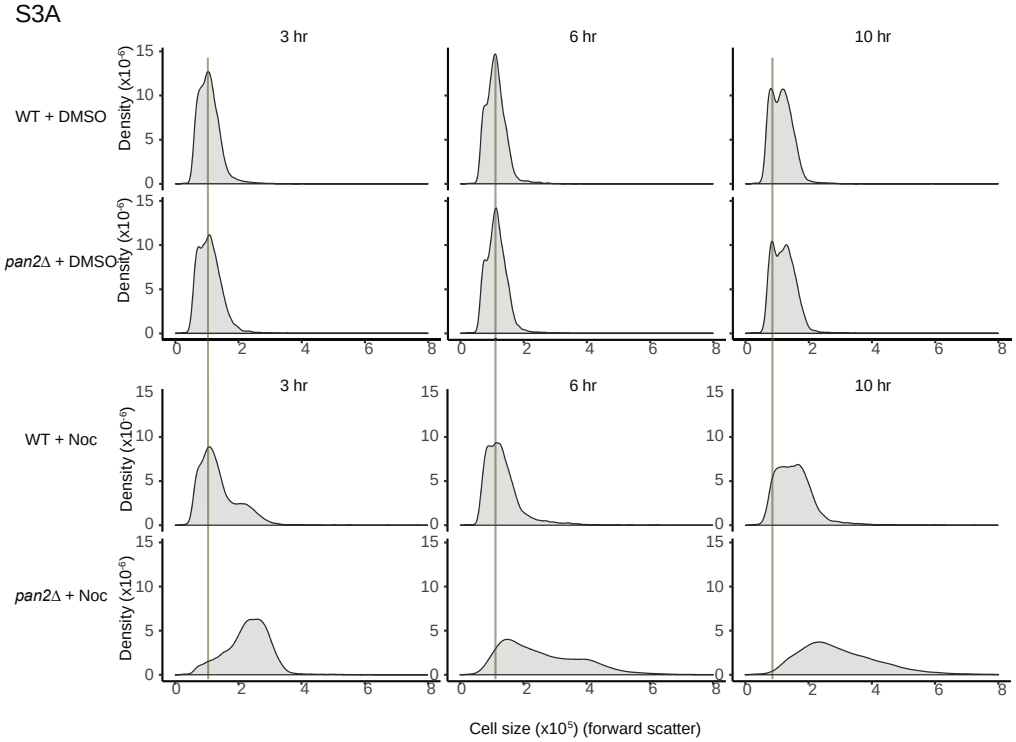
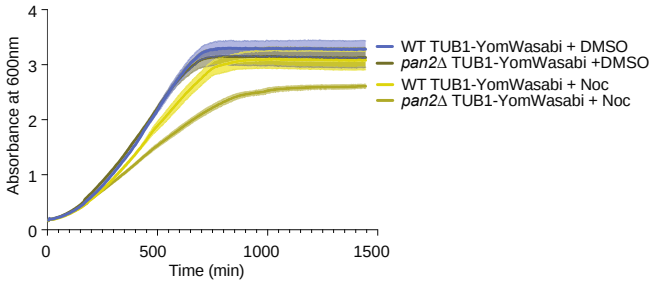


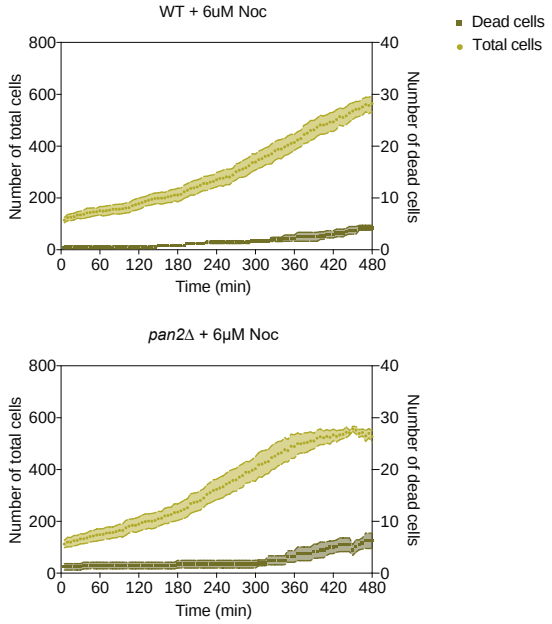
Fig S3A: FACS profiles post DMSO or 6 µM nocodazole treatment. FACS, fluorescence activated cell sorting; hr, hours; WT, wild type.

Figure S4

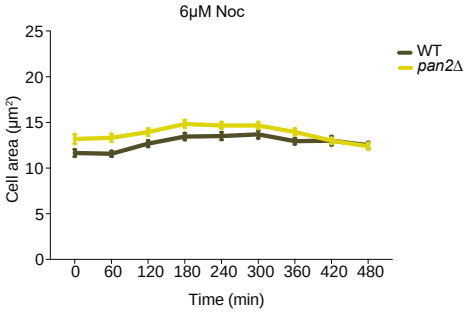
S4A



S4B



S4C



S4D

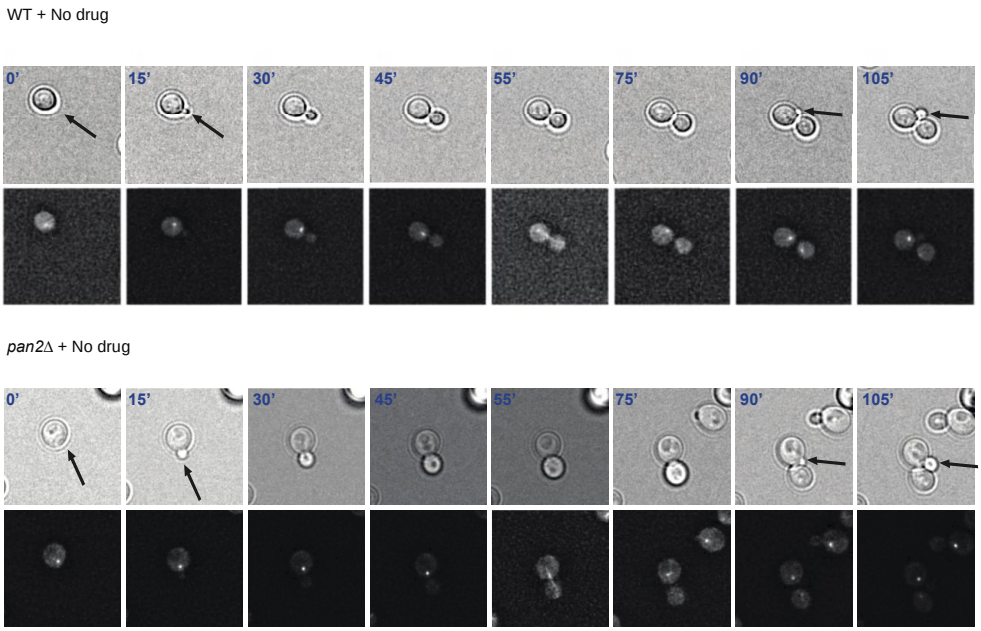
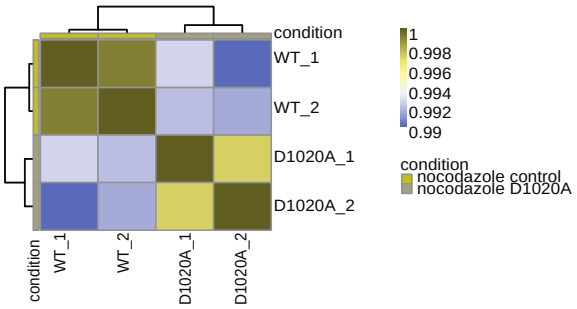


Fig S4A: *pan2*Δ Tub1-yW yeast is sensitive to nocodazole and WT-Tub1-yW exhibits a mild slow-growth phenotype compared to WT. Growth curves of yeast strains grown in liquid SC media with or without 6 µM nocodazole and monitored for 24 hours. An average of 6 replicates has been plotted with SD. Absorbance adjusted for baseline correction. µM, micromolar; SC, Synthetic Complete media; SD, standard deviation. **Fig S4B:** Number of total cells (left y-axis) and number of dead cells (right y-axis) upon treatment of WT (top) or *pan2*Δ (below) with 6µM nocodazole for every 5-minutes over a total time interval of 480 minutes. **Fig S4C:** Cell area of individual cells of WT (brown) or *pan2*Δ (yellow) is represented with 95% confidence interval upon treatment with 6µM nocodazole over a total duration of 480 minutes. Each time point represents the cell area of 500-2500 cells recognised by cell segmentation. **Fig S4D:** Live

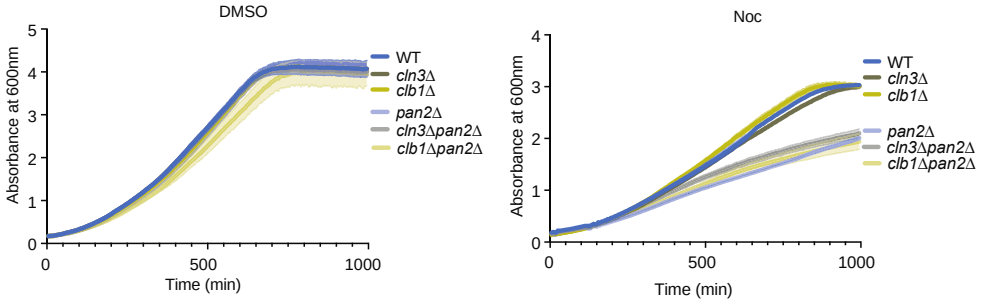
imaging of log phase yeast cells expressing (Tub1+3'UTR)-yom-Wasabi WT (above) or *pan2*Δ (below). The cells are treated with DMSO for ~75 minutes prior to imaging. The time-stamps are indicated on top left of each snapshot. WT, wild-type; DMSO, dimethyl sulfoxide; µM, microMolar; Tub1: alpha-tubulin; UTR, untranslated region.

Figure S5

S5A



S5B



S5C

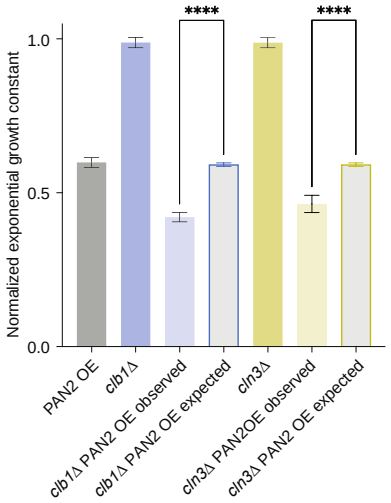


Fig S5A: Spearman correlations for expression of all the genes were plotted for the two replicates per sample of nocodazole treated Control and D1020A samples. **Fig S5B**: Growth curves of yeast strains in SC media with DMSO (left) or 6 µM nocodazole (right) at 30°C for 24 hours. Absorbance plotted with SD (n=6). min, minutes; SC, Synthetic Complete media; SD, standard deviation; WT, wild type. **Fig S5C**: Normalized exponential growth constants (data from **Fig 5D**). Error bars are +/- SD from three separate experiments (n=6). Significant differences between different strains were tested using paired Student's *t*-test. OE, overexpression.

Materials and Methods

Yeast strains, growth conditions, and manipulations

Deletion and tagged strains were constructed in JHY716 or BY4741 (derivatives of S288C) backgrounds using homologous recombination of PCR-generated linear fragments amplified from pKT209, pHIS3p:yomWasabi-Tub1+3'UTR::HPH, and pFA6a-kanMX6 (38,64,65), with successful recombination assessed by colony PCR. All yeast strains were maintained on standard yeast growth medium of either yeast extract, peptone, and dextrose (YPD) media or synthetic complete (SC) media consisting of Yeast Nitrogen Base (YNB) supplemented with appropriate amino acids and 2% dextrose. Yeast cultures were grown at 30°C. A complete list of all the strains used in this study is provided in **Table 1**.

Table 1: List of strains

Strain	**Genotype**	**Source**	**Parental strain**	**Notes**
BY4741	MAT**a**; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; S288C background	Lab collection		Wild type
BY4742	MAT*α*; *his3Δ1*; *leu2Δ0*; *ura3Δ0*; S288C background	Lab collection		Wild type
Y7092	MAT*α*; *his3*Δ*1*; *leu2*Δ*0*; *met15Δ0*; *ura3*Δ*0*; LYS2+; *can1Δ*::STE2pr-Sp_his5; *lypΔ1*; S288C background	Kind gift from Dr. Charlie Boone		SGA query strain background
JV1	*pan2*Δ::*uraMX*	This study	Y7092	PCR knock-in
JV2	*pan3*Δ::*uraMX*	This study	Y7092	PCR knock-in
JV3	*HIS3pr*:yomWasabi-Tub1+3'UTR::HPH	This study	Y7092	PCR knock-in

JV4	*pan2*Δ::*uraMX HIS3pr*:yomWasabi-Tub1+3'UTR::HPH	This study	Y7092	PCR knock-in
JV5	*pan2*Δ::*uraMX cin1*Δ:: kanMX6	This study	SGA background	SGA
JV6	*pan2*Δ::*uraMX cin2*Δ:: kanMX6	This study	SGA background	SGA
JV7	*pan2*Δ::*uraMX cin4*Δ:: kanMX6	This study	SGA background	SGA
JV8	*pan2*Δ::*uraMX gim3*Δ:: kanMX6	This study	SGA background	SGA
JV9	*pan2*Δ::*uraMX gim4*Δ:: kanMX6	This study	SGA background	SGA
JV10	*pan2*Δ::*uraMX pac2*Δ:: kanMX6	This study	SGA background	SGA
JV11	*pan2*Δ::*uraMX tub3*Δ:: kanMX6	This study	SGA background	SGA

Plasmids and constructs

CEN plasmids were taken from the MoBY-ORF collection (66). Galactose-driven overexpression plasmids were obtained from the FLEX overexpression library (67) generously provided by Tejomayee Singh from the laboratory of Dr. Phil Hieter. All plasmids were sequenced to confirm their identity (Plasmidsaurus). The coding sequence of *Saccharomyces cerevisiae PAN2* gene, along with its native promoter and 3'UTR sequence, and the codon optimized version of *Homo sapiens PAN2* (sequence is provided in **SFile 1**) were purchased from Twist Biosciences to insert into various plasmids for tagging or overexpression using the Gateway cloning kit (ThermoFisher Scientific). These plasmids were pAG426*GAL*-ccdB, pAG426-*GAL*-GFP-ccdB, pAG425-*GAL*-ccdB, pAG415-*GPD*-ccdB, and pAG425-*GPD*-ccdB. Details of these plasmids are provided in **Table 2**.

Table 2: List of plasmids

Plasmid name	Description	Source
pKT0209/pKT209	pFA6a–link–yEGFP–Ca*URA3*; used for URA amplification for making gene deletion cassettes	Addgene (64)
pHIS3pr:yomWasabi-Tub1+3'UTR::HPH	*pHIS3pr*:yomWasabi-Tub1+3'UTR::HPH: used for tagging alpha-tubulin	Addgene (38)
pFA6a-kanMX6	for one-step PCR deletion of gene of interest	Addgene (65)
pAG426-*GAL*-Pan2	URA3 marker, multi-copy, GAL promoter	This study
pAG426-*GAL*-Pan2-D1020A	URA3 marker, multi-copy, GAL promoter	This study
pAG426-*GAL*-GFP-Pan2	URA3 marker, multi-copy, GAL promoter, N-terminal GFP-tagging	This study
pAG426-*GAL*-GFP-Pan2-D1020A	URA3 marker, multi-copy, GAL promoter, N-terminal GFP-tagging	This study
pAG425-*GAL*-Pan2	LEU2 marker, multi-copy, GAL promoter	This study
pAG425-*GAL*-Pan2-D1020A	LEU2 marker, multi-copy, GAL promoter	This study
pAG425-*GAL*-HsPan2	LEU2 marker, multi-copy, GAL promoter	This study
pAG415-*GPD*-Pan2	LEU2 marker, low copy, GPD promoter	This study
pAG415-*GPD*-Pan2-D1020A	LEU2 marker, low copy, GPD promoter	This study
pAG425-*GPD*-Pan2	LEU2 marker, multi-copy, GPD promoter	This study
pAG425-*GPD*-Pan2-D1020A	LEU2 marker, multi-copy, GPD promoter	This study
pFLEX-Pan2	*GAL1-10pr, URA3*	A gift from Dr. Phil Hieter (67)

The deadenylase dead mutation (D1020A) in *PAN2*'s coding sequence was generated by site-directed mutagenesis (Q5 Site Directed Mutagenesis Kit from NEB E0554) with primer pairs 5' GGTTTGAATAATGCCTTCAAACACATTAATATTAATGTC 3' and 5'

ATGACCAACAAATACATTATTCAAACCATGACCAAC 3'. These PCR products were recombined in DHFpir bacteria which was a generous gift from Dr. J.T. Beatty.

For tagging Tub1 with yomWasabi-Tub1+3'UTR, the strategy used has been described in Markus et al., 2015 (38). Briefly, the yomWasabi-Tub1 tagging plasmid was altered by adding an additional 618 nucleotides corresponding to the region downstream of the *TUB1* locus. This sequence was chosen because it extends well beyond the last potential polyadenylation sequence (AATAAA, 145 nt downstream of the stop codon; ATTAAA, 540 nt downstream of the stop codon). Following transformation with the BsaBI-digested plasmid (*pHIS3p*:yomWasabi-TUB1+3'UTR), both the endogenous, untagged *TUB1* and the tagged *TUB1* genes are immediately followed by the 3'UTR sequence.

Quantitative liquid growth assays

Yeast were grown overnight in liquid SC media supplemented with appropriate amino acids, diluted into fresh medium, then grown until reaching logarithmic growth phase (OD_{600} 0.2-0.5). Yeast cells were then transferred to liquid media supplemented with an appropriate concentration of nocodazole (Sigma) or equivalent percentage (<0.5%) of DMSO (ThermoFisher). Strains were normalized to the same OD_{600} then diluted to an OD_{600} value of 0.005-0.010 in 96-well plates in triplicate wells to a volume of 200 µL. Plates were then incubated at 30°C with orbital shaking at 600 RPM in a Biotek Instruments Epoch 2 Microplate Spectrophotometer. Data was log_2 transformed and appropriate time points were fitted to a linear equation, with exponential growth constants derived from the slopes of the linear equation (except in drug treatments where yeast showed a non-exponential growth pattern). Statistical analysis was performed in GraphPad

Prism 10, and significant differences between pairs were detected using Student's *t*-test or between pairwise combinations of groups using a one-way ANOVA with *post-hoc* multiple comparisons corrections using Tukey's method.

Western blotting

Yeast cell lysates were prepared using the TCA (Trichloroacetic acid) precipitation method (68). The following primary antibodies were used: chicken anti-GFP (1:5000 of Abcam ab13970) and mouse anti-PGK1 (1:5000 of Abcam ab113687). The following secondary antibodies were used: donkey anti-chicken Alexafluor 488 (1:10,000 of Jackson ImmunoReserach Lab 703-545-155) and goat anti-mouse DyLight 800 (1:15,000 of Invitrogen SA5-10176). The experiments were performed in triplicates, and the results were quantified using the Image Lab software (BioRad).

Mammalian cells were lysed using RIPA (Radio Immuno Precipitation Assay) buffer (50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 5 mM EDTA) supplemented with cOmplete protease inhibitors (Roche). The following primary antibodies were used: rabbit anti-PAN2 (1:1000 of Proteintech 16427-1-AP) and mouse anti-actin (1:5000 of Abcam ab8226). The following secondary antibodies were used: goat anti-mouse HRP (Jackson 115-035-003) and goat anti-rabbit HRP (Jackson 111-035-003).

Synthetic Genetic Array Analysis

Creation of the genome-wide array of double mutants with the *pan2*Δ deletion strain was undertaken as previously described (69,70). Briefly, *PAN2* was knocked-out in the SGA query strain background Y7092 by genomic integration of the *URA3* cassette at the *PAN2* genomic locus amplified from pKT209. This *pan2*Δ query strain was mated to an array of non-essential deletion mutants marked with kanMX6 using a Singer RoToR HDA robot. Diploids were selected for and induced to undergo sporulation. From spores, *MAT***a** cells were obtained by pinning onto haploid selection media (SC-His/Arg/Lys supplemented with 100 µg/mL canavanine and 100 µg/mL thialysine). Control plates were obtained by re-pinning to 5-FOA media to counter select for the *PAN2* deletion, while experimental plates were generated by plating on media lacking uracil. These were then separately pinned onto plates containing 1.5µM of nocodazole. Plates were imaged using a CanoScan flatbed scanner and were analyzed using Balony software (69). Gene Ontology (GO) term enrichment and protein-protein interaction network analysis was performed using the GeneMANIA web server, with GO term enrichment presented as Benjamini-Hochberg FDR-adjusted p values (q values) from hypergeometric tests (71,72).

FACS analysis of DNA content

Yeast cells were cultured under specified conditions, fixed in 70% ethanol, and stored at 4°C. Fixed cells were rehydrated in phosphate-buffered saline, incubated with 100 µg/mL RNase A (ThermoFisher) for 3 hrs, and stained with 5 µg/mL propidium iodide (Invitrogen) for 1 hour. Cells were analyzed using a Cytoflex LX Analyser, and yeast cells were separated from debris

by gating with forward scatter and side scatter. The experiments were repeated three times using freshly grown cultures.

Confocal microscopy

Log phase (OD_{600} 0.2-0.5) yeast cells were centrifuged at 2,000 g and imaged in sterile filtered SC liquid medium (yeast nitrogen base without amino acids and with ammonium sulphate, amino acid mix, dextrose). Squashes of yeast were made and imaged on the Leica SP5 system with brightfield imaging. Images were analyzed using the Fiji distribution (73) of ImageJ software (74).

Sample preparation for live-cell imaging of yeast and cell-segmentation

Live-cell imaging of yeast was conducted in Concanavalin A (ConA) (Sigma L7647) coated 96-well microscope dishes using the Cell Discoverer 7 imaging system. To prepare the ConA-coated dishes, a stock solution of ConA at a concentration of 10 mg/mL was initially prepared and stored in aliquots at -20°C for a maximum of 6 months. A working solution was then prepared by diluting the stock to a final concentration of 1 mg/mL and stored at 4°C for several weeks. The ConA solution was evenly applied to each well of the 96-well dishes (Cellvis, P96-1.5H-N). Following a 5-minute incubation on the benchtop, excess solution was aspirated, and the dishes were allowed to dry for 5 minutes. For sample preparation, yeast cultures were grown overnight in SC medium (sterile filtered). The following day, yeast cultures were diluted and allowed to grow to log phase. Cells were adhered to the ConA-coated plates by applying 20 μL of the cell suspension to each well. After 10 minutes of incubation to ensure proper adhesion, the media

was removed, and 40 µL of fresh media without cells was added to eliminate potential optical artifacts. The CellDiscoverer7 (Carl Zeiss) microscope was set up prior to starting the live-cell imaging to reach 30°C and allow the objectives to acclimate to that temperature. An imaging time course was set up using ZEN Blue software (Carl Zeiss) to take images at 2 evenly dispersed, non-overlapping locations per well. For each condition, 2 biological replicates were imaged in separate wells. Images were taken in brightfield (TL LED lamp) and GFP (LED module 470nm) channels. The Plan Apochromat water immersion 50x/1.2 objective and 1x Tubelens were used for a final magnification of 50x, with an effective NA of 1.2. Images were taken every 5 minutes for 480 minutes using definite focus. The individual images were deconvolved using the constrained iterative algorithm and max intensity images were generated using extended depth of focus with an effective depth of ~1µm (9 z-stacks of ~0.125µm). Images were analyzed using the ZEN Blue software (Carl Zeiss). GFP segmentation was performed to collect data for individual cells after Gaussian smoothing, sigma=2, using iso data thresholding for light regions. For each GFP segmented cell, area and GFP mean intensity were measured. Data for the cells in each of the 4 images per well were compiled to generate the total cell number, number of dead cells (brighter, GFP mean intensity > 8000) and cell area.

RNA isolation and qRT-PCR analysis

Total RNA was isolated from cells using the RNeasy kit from Qiagen (74104) following the manufacturer's instructions. Quantitative reverse-transcriptase PCR was performed using Power SYBR® Green RNA-to-CT™ 1-Step Kit from ThermoFisher (4389986). All data were normalized to *ACT1* transcript levels and are presented as ΔCt values. Where a statistically

significant change was observed, fold-increase against control was indicated in the plots. The primers used in this study are listed in **Table 3**.

Table 3: RT-PCR primers

Primer	Sequence
Sc_Act1_qFwd	TTTGCCGGTGACGACGCTCC
Sc_Act1_qRev	ACAGGGTGTTCTTCTGGGGCA
Sc_Clb1_qFwd	CAGTCTAGGACGTTAGC
Sc_Clb1_qRev	GTCGTGAATAGTAGATCC
Sc_Cln3_qFwd	TCAGCGCTGCCTCATGTC
Sc_ Cln3_qRev	ATGGCCCGCCTCTTTTG
Sc_ Tub1_qFwd	GGCCACTGTGGATAGGGCCG
Sc_ Tub1_qRev	TCAGCGTATGAGTCGGCACCC
Sc_ Tub2_qFwd	CGTCGATCTAGAACCTGGGA
Sc_ Tub2_qRev	GACGCTGTCTACAAGCTCAG
Sc_ Tub3_qFwd	TGGCCGACCAATGTGACGGT
Sc_ Tub3_qRev	AGGCGCAGGATAAACGGCGA

RNA sequencing and analysis

Sample quality control was performed using the Agilent 2100 Bioanalyzer. Qualifying samples were then prepped following the standard protocol for the Illumina Stranded mRNA prep (Illumina). Sequencing was performed on the Illumina NextSeq2000 with Paired End 59bp × 59bp reads by BRC-sequencing facility. As previously described (75), multiple differential expression analysis pipelines were applied, and the results combined. The pipelines used the reference genome and transcriptome R64-1-1 downloaded from Ensembl (www.ensembl.org, Release 107).

Generation of stable PAN2 knockdown mammalian HEK293 cell populations

HEK293A cells were cultured in DMEM/F12 medium (Sigma) supplemented with 10% FBS and grown in an incubator at 5% CO_2 and 37°C and transfected with Dharmacon pGIPZ shRNA set (RHS4531-EG9924; shRNAs fused to GFP) targeting PAN2 ORF/3'UTR using Lipofectamine 2000 according to the manufacturer's protocol (ThermoFisher Scientific 11668027). Puromycin selection was initiated 48 hours post-transfection at a concentration of 1 µg/mL to generate stably integrated shRNA-mediated knockdowns. After 14 days of selection, the puromycin-resistant cells were subjected to FACS to isolate a stably transfected population of GFP-positive cells. Pan2 knockdown efficiency was then determined by Western blotting (1:1000 of Proteintech 16427-1-AP against human Pan2).

Mammalian cell viability assessment using MTT

HEK293 cells were plated in 96-well plates at 10,000 cells/well. The number of viable cells was quantified using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M2128, Sigma) assay at 0, 24, 48, and 72 h. After the designated treatment period, the culture medium was removed, MTT was added to each well at 1 mg/mL in regular growth media for 2 h to allow the formation of formazan crystals (purple precipitate). Following incubation, the MTT solution was aspirated, and the formazan crystals were solubilized using 100 µL of DMSO per well. The plate was gently agitated to ensure complete dissolution of the formazan crystals. The absorbance was measured at 560 nm using a spectrophotometer plate, with DMSO serving as the blank control. Cell viability was calculated as the percentage of viable cells relative to the

untreated control group, and the results were expressed as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed in GraphPad.

Live-cell imaging of mammalian cell spindle formation and mitosis

For spindle analysis of living cells, cells were grown in a 96-well plate (Corning) and a tubulin staining dye, CellLight™ Tubulin-RFP BacMam 2.0 was added prior to imaging the cells in a 37°C and 5% CO_2 environmental chamber (ImageXpress Micro XL). Images were taken using a 40X 0.75 NA dry objective with the MetaXpress 5.0.2.0 software on the ImageXpress Micro XL epifluorescence microscope (Molecular Devices Inc.). Images were taken every 15 minutes using 100-ms exposures, 2x2 binned resolution, with 25% of full lamp intensity per channel. Movies of the fluorescence channels were made in the MetaXpress 5.0.2.0 software (Molecular Devices Inc.).

Supporting information captions

SFile 1.docx: Sequence of human PAN2 codon optimized for yeast

SFile 2.xlsx: Synthetic Genetic Analysis (SGA) data for *PAN2* in presence or absence of nocodazole

SFile 3.xlsx: RNA-seq data for *pan2*Δ + WT Pan2 and *pan2*Δ + D1020A Pan2 in presence of nocodazole

SFile 4.avi: Yeast WT with DMSO

SFile 5.avi: Yeast WT with nocodazole

SFile 6.avi: Yeast *pan2*Δ with DMSO

SFile 7.avi: Yeast *pan2*Δ with nocodazole

SFile 8.avi: HEK293 cells - bipolar spindle formation

SFile 9.avi: HEK293 cells - tetrapolar spindle formation