Effects of the Yeast RNA-Binding Protein Whi3 on the Half-Life and Abundance of CLN3 mRNA and Other Targets

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Abstract

Whi3 is an RNA binding protein known to bind the mRNA of the yeast G1 cyclin gene CLN3. It inhibits CLN3 function, but the mechanism of this inhibition is unclear; in previous studies, Whi3 made no observable difference to CLN3 mRNA levels, translation, or protein abundance. Here, we re-approach this issue using microarrays, RNA-Seq, ribosome profiling, and other methods. By multiple methods, we find that the whi3 mutation causes a small but consistent increase in the abundance of hundreds of mRNAs, including the CLN3 mRNA. The effect on various mRNAs is roughly in proportion to the density of GCAU or UGCAU motifs carried by these mRNAs, which may be a binding site for Whi3. mRNA instability of Whi3 targets may in part depend on a 3' AU rich element (ARE), AUUUUA. In addition, the whi3 mutation causes a small increase in the translational efficiency of CLN3 mRNA. The increase in CLN3 mRNA half-life and abundance together with the increase in translational efficiency is fully sufficient to explain the small-cell phenotype of whi3 mutants. Under stress conditions, Whi3 becomes a component of P-bodies or stress granules, but Whi3 also acts under non-stress condition, when no P-bodies are visible. We suggest that Whi3 may be a very broadly-acting, but mild, modulator of mRNA stability. In CLN3, Whi3 may bind to the 3’ GCAU motifs to attract the Ccr4-Not complex to promote RNA deadenylation and turnover, and Whi3 may bind to the 5’ GCAU motifs to inhibit translation.

Introduction

WHI3 was discovered in a screen for mutants that commit to cell division at an unusually small cell size (“Wee” or “Whi” mutants) [1]. By sequence, it is an RNA binding protein of the RRM type, and biochemical studies showed that it binds to the mRNA of CLN3 [2], which encodes a G1 cyclin, and which also affects cell size [3]. Binding of Whi3 to CLN3 mRNA depends on repeats of the motif GCAU in the CLN3 mRNA [4]. Just two-fold over-expression of CLN3 results in exactly the same small-cell phenotype as the whi3 mutant [1,5]. Thus, it was suggested that Whi3 might be an inhibitor of CLN3, and that in a whi3 mutant, CLN3 would be about two-fold hyperactive, allowing commitment to division at small cell size [1,2]. Indeed, while a whi3 single mutant has small cells, a whi3 cln3 double mutant has large cells, exactly like a cln3 single mutant [2]. The genetic result that whi3 has no effect on size in a cln3 mutant strongly supports the idea that the whi3 cell size phenotype is due to inhibition of CLN3 at some level.

However, it has been difficult to understand exactly how Whi3 inhibits CLN3. Northern analysis failed to show any difference in CLN3 mRNA levels between wild-type cells and whi3 mutants; Western analysis failed to show any difference in Cln3 protein levels; and polysome profiling failed to show any difference in the translation of Cln3 [2].

Another kind of idea is that Whi3 forms aggregates or foci [5,6], and that translation of bound CLN3 therefore occurs close to these foci, raising the local concentration of Cln3 protein. Wang et al. showed that Whi3 binds Cdc28 protein as well as CLN3 mRNA. In principle, Whi3 could therefore bind the complex between Cdc28 and the locally-translated Cln3, thus functioning as a cytoplasmic retention device for Cln3-Cdc28 complexes [5]. Since Cln3-Cdc28 acts in the nucleus, this cytoplasmic retention would inhibit Cln3 function. However, there are difficulties with this hypothesis. First, despite several genome-wide screens for protein-protein interactions, and despite multiple screens for protein-protein interactions involving Cdc28 or its homologs in other organisms, other workers have not seen physical interactions between Cdc28 and Whi3. Second, although Aldea and co-workers have shown cytoplasmic Cln3 (the Cln3 presumably retained in the cytoplasm by Whi3) [5,7], other workers have seen only nuclear Cln3 [8,9] (Zhao and Futcher, unpublished). Cln3 is a non-abundant protein, and accurate localization is difficult.

Third and most important, the whi3 mutant has many other phenotypes beyond small cell size. Some of these (poor sporulation; a lack of invasive growth; a lack of pseudohyphal growth) are probably also due to the inhibition of CLN3, since a cln3 mutant is epistatic to whi3 for these phenotypes [2]. On the other hand, many other phenotypes of whi3 have no apparent connection to CLN3. These phenotypes include sensitivity to oleate, Calcofluor...
white, and Congo Red, which cannot easily be explained by deregulation of Cln3 activity [4,10]. In addition, Colomina et al. have shown that Whi3 binds a large number of different mRNAs, suggesting it has targets beyond CLN3 [4]. The idea that Whi3 binds Cdc28 and so is a cytoplasmic retention device for Cln3-Cdc28 complexes could explain its inhibition of Cln3, but it seems difficult to generalize this model to dozens or hundreds of other targets. Therefore, with the recent availability of methods of higher accuracy and precision, we have re-investigated the effects of Whi3 on CLN3 mRNA and translation.

Whi3 has a homolog called Whi4 [1]. The whi4 mutant has little phenotype on its own, but the whi3 whi4 mutant has even smaller cells than the whi3 mutant. In addition, the whi3 whi4 mutant is somewhat sick and slow growing. Because the whi3 single mutant has obvious phenotypes, and yet is not sick, we have mainly investigated the single mutant. In selected situations, we have also studied the whi3 whi4 double mutant.

Whi3 is reasonably well conserved among fungi. There is high conservation in and around the RNA binding domain. This region includes a putative site for phosphorylation by cyclic AMP dependent protein kinase [11,12]; this site could regulate Whi3 function. In addition, Whi3 and Whi4 have a striking Q-rich domain, and a Q-rich domain is a prominent feature of many of the homologs. In some homologs the Q-rich domain has expanded dramatically (e.g., the homologs of S. bayanus, A. gossypii), while in other cases the Q-rich domain is reduced or perhaps absent (e.g., in sec1 of S. pombe). The Q-rich domain is involved in the formation of Whi3 aggregates in Abyaa gossypii, where the Q-rich domain is particularly large [6].

Results
Identification of mRNAs Bound by Whi3
Colomina et al. [4] immunoprecipitated Whi3 and obtained a list of 326 co-precipitating mRNAs. To confirm and extend these results, we likewise used RNA immunoprecipitation followed by microarray analysis (RIP-chip) to find RNA targets bound by Whi3. An epitope-tagged Whi3 protein (Whi3-TAP) expressed from its endogenous promoter was immunoprecipitated. As a control, the same experiment was performed with a mutant Whi3 protein lacking its RNA binding domain (the RRM). Multiple experiments were done with each of the wild-type and RRM-deleted Whi3 strains, and the 262 mRNAs whose net enrichment was at least 1.65 standard deviations above the mean (see Materials and Methods) were defined as putative targets of Whi3. CLN3 mRNA was one of these mRNAs, and ranked as the 70th most-enriched RNA. Our list of 262 mRNAs had 111 mRNAs over-lapping with the previous list of 326 mRNAs [4] (for lists, see Supplementary Tables S1 and S2); this overlap had a p-value of about 10^-77. The large overlap and small p-value suggest that the two independent studies are substantially in agreement, and are likely identifying real targets of Whi3.

Table 1 shows all 21 mRNAs from the joint list of 111 that are enriched four-fold or more in the immunoprecipitate, and 19 additional mRNAs of interest. Some of these genes are important for cell cycle (the G1 cyclins CLN3 and PCL2, CDC9, ADD1, NRT1), for protein synthesis (the eIF5A elongation factor homolog HIP2 and ANB1, and SNU13, RP414, TEF4), for cell wall synthesis and maintenance; for lipid metabolism; and for transport of small molecules. The presence of OEL1 (oleic acid requiring) on the list could be connected to the oleic acid sensitivity of whi3 [10]. Analysis of our 262 mRNA targets reveals a statistically-significant enrichment of mRNAs encoding proteins associated with membranes, particularly transporters; 91 out of 262 entries (34.7%, p-value = 4.3×10^-5) are annotated as membrane associated, showing a moderate enrichment compared to the background frequency of 21.3%. Among these 91 RNAs, 29 encode for proteins having transmembrane transporter activity (31.9%, p = 0.007). Similarly, the previous study of Colomina et al. found an enrichment of proteins involved with the cell wall, ER, or plasma membrane, and found transporters. Colomina et al. studied the cell wall defects of whi3 mutants, showing that whi3 mutants were sensitive to Calcofluor White and Congo Red [4]. However, while the enrichments of genes in these various functional categories are statistically significant, they are also modest, and many of the target mRNAs do not belong to any of these categories. Overall, it appears that Whi3 interacts with a broad variety of mRNAs, with no single strong, functional theme obvious to us.

RNA Sequence Motifs Associated with Whi3 Targets
Previous studies found that the mRNA targets of Whi3 are enriched with the motif GCAU [4,13] or UGCAU [14]. The algorithm FIRE [15], which uses mutual information including positional information, has some advantages over previously-used motif search algorithms. Application of discontinuous FIRE to our dataset identified two motifs that are enriched in the 3' UTRs of Whi3 targets. The first and most significant was, as previously, UGCAU, with a Z-score of 25.9 (i.e., 26 standard deviations from expectation). Despite this, the actual enrichment is modest; the Whi3 RIP-chip targets have on average 3.6 GCAU motifs per kb, while non-targets have 3.1. It is possible that the GGAU motifs are somewhat clustered in the targets, but we have not rigorously examined clustering. A direct interaction between Whi3 and the UGAU motif has not been demonstrated.

Second, FIRE found the additional motif AUUUUA, with a lower but still extremely high Z-score of 19.2. 29% of Whi3 targets have an AUUUUA in the 3' UTR, while only 17% of non-targets do.

In mammals, a class of repeats called “AU Rich Elements” (AREs) is associated with mRNA instability [16,17]. Although many distinct sequences can act as an ARE, the consensus sequence is AUUUA, which is quite similar to the AUUUUA motif found in the Whi3 targets. Interestingly, Vasudevan and Pelcz studied ARE-mediated mRNA decay in S. cerevisiae, and found that the 3' region of the TIF51a gene (aka HIP2), which encodes the translation factor eIF5A, contained a modular, transportable region specifying regulated mRNA turnover [18]. This module contained two repeats of AUUUUA, but of great interest to us, it also contained two repeats of GCAU [18]. In fact, both TIF51a/HIP2 and its homolog ANB1 appear high on our list of Whi3 targets (ranks 8 and 12 on the joint list). Thus, one possibility is that Whi3 binds at GCAU motifs, but potentiates mRNA turnover (see below) through AREs such as AUUUA or AUUUUA. Many of the potential Whi3 targets, including CLN3, do contain strong AREs downstream of the GCAU motifs.

Because these motifs are short, we wondered whether Whi3 might recognize structure as well as sequence. Kertesz et al. defined RNA secondary structures genome-wide by using ribonuclease specifically recognizing single stranded or double stranded RNA structures [19]; they then created the searchable PARS database of yeast RNA structures. We searched this structure profile to see if any structural feature was associated with the UGAU sites in Whi3 targets (Materials and methods), but did not find any such structure. For instance, the UGAU is typically in single stranded RNA, but only to the same extent that other sequences are also typically single stranded. The UGAU is not
adjacent to a region that is preferentially double stranded or single stranded.

Effects of Whi3 on RNA Expression and Half Life

Having found putative targets of Whi3, we next asked what effect Whi3 had on their expression. In principle, an RNA binding protein such as Whi3 could affect RNA stability; RNA storage; splicing (for those genes with introns); translation; and RNA localization. We first asked if deletion or over-expression of WHI3 altered the steady-state levels of its target transcripts. Since over-expressing WHI3 from the GAL promoter is lethal [1], we integrated three extra (four total) copies of WHI3 at its genomic locus to create a strain constitutively over-expressing WHI3. This strain displayed a large cell size and was confirmed to have around

Table 1. Partial joint list of Whi3 targets.

| Common Name | SysName   | IP Enrichment | rank | Function                                      |
|-------------|-----------|---------------|------|-----------------------------------------------|
| SNU13       | YEL026W   | 3.8           | 1    | RNA binding protein; rRNA processing, mRNA splicing |
| YGR146C     | YGR146C   | 3.0           | 2    | Unknown                                       |
| YMR122W-A   | YMR122W-A | 2.8           | 3    | Unknown                                       |
| FUR1        | YHR128W   | 2.6           | 4    | Uracil phosphoribosyltransferase              |
| MFB1        | YOR298C-A | 2.6           | 5    | Transcriptional coactivator, bridges Gcn4 and Spt15 |
| KAP95       | YLR347C   | 2.5           | 6    | Karyopherin beta, nuclear import             |
| YGR035C     | YGR035C   | 2.4           | 7    | Unknown                                       |
| ANB1        | YJR047C   | 2.4           | 8    | Translation elongation factor elf-5A         |
| YOR052C     | YOR052C   | 2.3           | 9    | Unknown                                       |
| SML1        | YML058W   | 2.3           | 10   | Ribonucleotide reductase inhibitor            |
| OLE1        | YGL055W   | 2.2           | 11   | Delta(9) fatty acid desaturase                |
| HYP2        | YEL034W   | 2.1           | 12   | Translation initiation factor elf-5A         |
| YEL033W     | YEL033W   | 2.1           | 13   | Unknown                                       |
| SAM1        | YLR1180W  | 2.1           | 14   | S-adenosylmethionine synthetase              |
| RPA14       | YDR156W   | 2.1           | 15   | RNA polymerase I subunit A14                 |
| PHO84       | YML123C   | 2.0           | 16   | High-affinity inorganic phosphate (Pi) transporter |
| TEF4        | YKL081W   | 2.0           | 17   | Translation elongation factor EF-1 gamma      |
| YKR075C     | YKR075C   | 2.0           | 18   | Unknown                                       |
| MRH1        | YDR033W   | 2.0           | 19   | Unknown                                       |
| MDM32       | YOR147W   | 2.0           | 20   | Mitochondrial inner membrane                 |
| NDD1        | YOR372C   | 2.0           | 21   | M-phase transcriptional activator            |
| BSC1        | YDL037C   | 1.9           | 22   | Cell surface flocculin                        |
| GPI18       | YBR004C   | 1.8           | 23   | Mannosyltransferase                           |
| IPT1        | YDR072C   | 1.7           | 24   | Inositolphosphotransferase, sphingolipid synthesis |
| GPA2        | YER020W   | 1.7           | 25   | G protein; glucose signalling                 |
| FEN1        | YCR034W   | 1.7           | 26   | Fatty acid elongase, sphingolipid synthesis   |
| HXT1        | YHR094C   | 1.7           | 27   | Low-affinity glucose transporter              |
| LCB1        | YMR296C   | 1.6           | 28   | Serine palmitoyltransferase, sphingolipid synthesis |
| NSG2        | YNL156C   | 1.5           | 29   | Regulation of sterol synthesis               |
| CDC6        | YJL194W   | 1.5           | 30   | Initiation of DNA replication                 |
| DHH1        | YDL160C   | 1.4           | 31   | DEAD/H-box helicase, mRNA decapping and decay |
| UTR2        | YEL040W   | 1.4           | 32   | Cell wall protein, transfer of chitin        |
| OST3        | YOR085W   | 1.3           | 33   | Oligosaccharyltransferase complex             |
| CLN3        | YAL040C   | 1.3           | 34   | G1 cyclin, activates Start                   |
| NRM1        | YNR009W   | 1.3           | 35   | Co-repressor of MBF transcription factor      |
| KRE1        | YNL322C   | 1.3           | 36   | Cell wall, beta-glucan assembly              |
| YE44        | YEL004W   | 1.3           | 37   | Required for chitin synthesis                |
| MUP1        | YGR055W   | 1.2           | 38   | High affinity methionine permease            |
| MUP3        | YHL036W   | 1.1           | 39   | Low affinity methionine permease             |
| PCL2        | YDL127W   | 0.9           | 40   | G1 cyclin                                   |

1 IP Enrichment is log2 of the enrichment in the RIP-chip (i.e., log2(Whi3-tagged/rrm whi3-tagged)).
2 rank is the rank of genes in the joint list.

doi:10.1371/journal.pone.0084630.t001
a 4 fold increase in WHI3 mRNA levels by Q-PCR. We called this strain “WHI3x4”. We then used microarrays to compare the levels of mRNAs in the WHI3 wild-type, the whi3 deletion, and the WHI3x4 over-expressor strain, and we did this in glucose medium and in YEP ethanol medium (Supplementary Table S3). Previously, Malcher et al. [20] also looked at whi3 mutants (but not over-expressors) using microarrays. Their data has a correlation coefficient of about 0.42 with ours, and we have incorporated some of their data into our analysis (as noted below).

We note that the whi3 mutant promotes Start, so whi3 cells have relatively short G1 phases, and over-expression of WHI3 delays Start, so WHI3 over-expressors have long G1 phases. Therefore, genes that are differentially expressed between the whi3 mutant and the WHI3 over-expressor should include cell cycle regulated genes, but for an indirect reason. We screened for genes that increased at least two-fold in the whi3 glucose experiment, and that also increased in either the whi3 ethanol experiment or the whi3 experiment of Malcher et al., and also decreased in at least one of the WHI3x4 over-expressor experiments (glucose and ethanol). There were 34 genes that exactly or nearly met these requirements (Table 2). Of these, 8 (24%) were Whi3 RIP-chip targets, about a six-fold enrichment over the random expectation. These 8 included YGR146c, MIB1, and SML1, ranked 2, 5, and 10, respectively, on our list of 262 Whi3 targets. Also among the 34 genes was PDR3, which is not a highly-ranked target, but does contain an unusually high density of GCAU motifs. PDR3 is a transcription factor, and the list of 34 genes also contains 10 genes that are normally induced by PDR3; possibly their abundance is increased in the whi3 mutant (and decreased in the over-expressor) because the abundance of their transcription factor is increased. Finally, there are other genes of uncertain significance.

We also looked for genes with the opposite behavior (i.e., low in the whi3 mutant, high in the over-expressor), finding just 6 (Table 2). None of these were scored as Whi3 targets. Again, these genes are of uncertain significance.

These results suggest that the direct effect of Whi3 on mRNA steady state levels is relatively small in most cases, and also that many of the differentially-expressed genes seen by microarray analysis are differentially expressed because of secondary effects; i.e., they may not be direct targets of Whi3, since, typically, they do not co-precipitate with Whi3. However, the effects are not always small, since eight of the RIP-chip targets, including three of the ten highest-ranked targets, were also seen as differentially expressed genes. All of the RIP-chip Whi3 targets that were differentially expressed were increased in abundance in the whi3 mutant and decreased in the Whi3 over-expressor, suggesting that Whi3 is reducing their abundance.

To better analyze any small effect Whi3 might have on RNA levels genome wide, we divided all the mRNAs into four quartiles based on their GCAU density (Materials and Methods) and plotted the fold change in expression obtained from the microarrays for whi3 and WHI3x4. A small but obvious trend was observed: in the whi3 mutant, mRNA levels increased with increasing GCAU density (i.e., GCAUs per kilobase of the gene including UTRs), while in the WHI3x4 mutant, mRNA levels decreased (see Fig. 1), consistent with the idea that Whi3 might be destabilizing mRNAs that contain GCAU. The same analysis for 3’UTRs or coding regions separately gave the same result, and the same analysis for maximum local GCAU density (Materials and Methods) gave essentially the same result. Note that the effect was seen even when comparing the lowest quartile with the second lowest quartile—this suggests that Whi3 can act in proportion to GCAU density even for genes where that density is low. This also implies that Whi3 may be working on a very large number of mRNAs—perhaps thousands—to a very slight extent. No trend in RNA levels was observed for the density of the control tetrancleotide CGUA (Fig. 1). These results suggest that Whi3 has weak but wide-spread effects, and slightly reduces mRNA levels for its targets. This is consistent with the highly polymorphic phenotypes of whi3.

Because these effects were small, we sought to confirm them using an assay other than the microarray. Therefore we used RNA-Seq to measure transcript abundance in a whi3 mutant and a WT strain. Genes with higher GCAU density again showed slightly higher abundance in the whi3 mutant strain than in the WHI3 WT strain (Fig. 2) exactly consistent with the microarray results, and consistent with the idea that binding by Whi3 reduces mRNA abundance by some indirect mechanism.

One explanation of the lower mRNA abundance of Whi3 targets would be that Whi3 decreases mRNA half-life. We attempted to examine this gene home-wide using the temperature sensitive RNA polymerase mutant rbt1-1 to turn off transcription, and then follow mRNA abundance with time using microarrays. In our first experiment, we did indeed see a statistically highly significant stabilization of many Whi3 target mRNAs in the whi3 mutant (not shown). However, we could not reproduce this in two subsequent experiments (not shown). We have no explanation for the discrepancy between the experiments, except to say that the half-life measurements were quite noisy.

**Whi3 Reduces CLN3 mRNA Levels**

The main known functional target of Whi3 is the CLN3 mRNA. Therefore we asked if Whi3 affects the abundance or stability of the CLN3 mRNA.

First, we tested Whi3’s effect on the abundance of CLN3 mRNA. We used Q-RT-PCR to measure CLN3 mRNA in total RNA isolated from a WT, whi3 or WHI3x4 strain. The ACT1 mRNA level was used for normalization. CLN3 mRNA levels showed a small but significant increase of 1.4 fold in whi3 mutants and a decrease of 0.8 fold in WHI3x4, compared to the level in WT cells. The same trend was observed for two other Whi3 target mRNAs, MTL1 and MID2, in these PCR assays. (Fig. 3) Previous studies [2] reported no change in CLN3 mRNA levels in whi3 mutant or Whi3 overexpression cells as assayed by northern blotting. The discrepancy can be explained by the small change and the higher precision provided by the Q-RT-PCR approach.

Although these changes are small, cell size is extremely sensitive to the dose of CLN3 [3], and a 1.4-fold increase in CLN3 abundance is phenotypically significant [3] (see Discussion). Because the Whi3-dependent differences in CLN3 mRNA were small, we also used a second approach, in which we compared the relative abundance of two different CLN3 transcripts in the same cell, where one transcript contained the GCAU motifs for binding Whi3, and the other was a mutant transcript that did not. Colomina et al. have previously shown that the gcau mutant form of CLN3 mRNA does not bind to Whi3 [4]. We constructed diploid strains that carried two, distinguishable forms of CLN3 mRNA, a long form and a short form (Materials and Methods). In addition, we mutated all 12 GCAU motifs (the putative binding sites of Whi3) in the long or the short forms of CLN3, while preserving coding capacity (Materials and Methods). Thus, we had four versions of CLN3: the fully wild-type form (“Long-GCAU”); the long form lacking GCAU motifs (“Long-gcau\textsubscript{mut}”); the short form with GCAU motifs (“Short-GCAU”); and the short form lacking GCAU motifs (“Short-gcau\textsubscript{mut}”). These four forms of CLN3 were inserted at the CLN3 locus, expressed from the CLN3 promoter, and characterized for function (Materials and Methods).
These haploids were then crossed to each other in various combinations so that alleles containing and lacking GCAU sites could be compared to each other when expressed in the same diploid cell. Using these diploids, in a single strain and a single RNA preparation, we could directly compare the abundance of the long form and the short form of CLN3 by RT-PCR, and see whether the relative abundance depended on the presence of the GCAU motifs, and/or the WHI3 gene.

Because the differences in abundance were small, we did 15 independent long:short ratio measurements in a WHI3 strain, and 12 independent long:short ratio measurements in an isogenic whi3 strain. Indeed, the ratios obtained depended on the presence or absence of the GCAU motifs; and also depended on the presence

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**Table 2. Differentially regulated genes in whi3.**

| Common Name  | SysName | whi3 YPD | whi3 YPE | whi3 Mal. | WHI3X4 YPD | WHI3X4 YPE | Target Rank |
|--------------|---------|----------|----------|-----------|------------|------------|-------------|
| Up-regulated |         |          |          |           |            |            |             |
| PHO84        | YML123C | 3.7      | 0.0      | 0.2       | −0.9       | −0.4       | 17          |
| RSB1         | YOR049C | 2.2      | 1.4      | 1.1       | −1.0       | −0.3       |             |
| PHM8         | YER0357W| 2.5      | −0.1     | 0.9       | −1.5       | −1.5       |             |
| YLR346C      | YLR346C | 2.7      | 1.2      | 0.0       | −0.5       | 0.7        |             |
| DSE4         | YNR067C | 1.4      | 0.9      | 1.3       | 0.0        | −0.5       | 212         |
| GTO3         | YMR251W | 2.0      | 1.1      | 0.6       | 0.0        | 0.3        |             |
| FIT2         | YOR382W | 2.2      | 0.2      | 0.3       | 0.5        | −0.6       |             |
| SPL2         | YHR136C | 1.9      | 0.4      | 0.3       | −0.7       | −1.1       | 203         |
| YGR146C      | YGR146C | 1.8      | 1.1      | 0.3       | 0.2        | −0.6       | 2           |
| BDH2         | YAL061W | 2.3      | 1.1      | −0.3      | −0.5       | 0.0        |             |
| CHA1         | YCL064C | 1.1      | 0.9      | 0.9       | −1.3       | −2.4       |             |
| ATG7         | YHR171W | 1.2      | 0.4      | 0.6       | 0.1        | −0.1       |             |
| CLB1         | YGR108W | 1.2      | 0.9      | 0.7       | −1.2       | −0.7       | 240         |
| HES1         | YOR237W | 1.5      | 0.2      | 0.3       | −0.2       | −0.1       |             |
| GLK1         | YCL040W | 1.6      | 0.1      | 0.2       | −0.1       | −0.2       |             |
| MPN1         | YJL066C | 1.0      | 0.7      | 0.7       | −0.3       | −0.1       |             |
| SML1         | YML058W | 1.0      | 1.1      | 0.7       | −0.7       | −0.4       | 10          |
| GDH3         | YAL062W | 1.4      | 0.6      | 0.3       | −0.4       | 0.2        |             |
| MBF1         | YOR298C-A| 1.1     | 1.0      | 0.5       | −0.5       | −0.5       | 5           |
| YHR140W      | YHR140W | 1.2      | −0.2     | 0.5       | −0.6       | −0.1       |             |
| AIM17        | YHL021C | 1.3      | 0.8      | 0.3       | −0.2       | 0.4        |             |
| ICY1         | YMR195W | 1.1      | 0.1      | 0.5       | −0.4       | −1.0       |             |
| GPD1         | YDL022W | 1.4      | 0.9      | 0.2       | −1.7       | −0.6       |             |
| ENO1         | YGR254W | 1.3      | 1.0      | 0.2       | −0.4       | −0.5       |             |
| YOR1         | YGR281W | 1.2      | 0.4      | 0.3       | −0.3       | −0.1       |             |
| HSPB2        | YPL240C | 1.1      | 0.3      | 0.4       | −0.6       | −0.2       |             |
| GLO4         | YOR040W | 1.2      | 0.4      | 0.2       | 0.2        | −0.6       |             |
| PDR3         | YBL005W | 1.2      | −0.1     | 0.2       | −0.1       | −0.1       |             |
| SNQ2         | YDR011W | 1.1      | 0.6      | 0.3       | −0.2       | −0.2       | 171         |
| PDR15        | YDR406W | 1.4      | 0.0      | 0.0       | −0.7       | 0.0        |             |
| ICT1         | YLR099C | 1.1      | 0.0      | 0.0       | −0.2       | 0.0        |             |
| VTC3         | YPL019C | 1.2      | −0.1     | −0.2      | −0.2       | −0.5       |             |
| CMK2         | YOL018C | 1.1      | 0.1      | 0.0       | −0.2       | −0.1       |             |
| YIL100W      | YIL100W | 1.0      | 0.7      | 0.0       | −0.8       | 0.7        |             |

| Down-regulated | | | | | | | |
| SAM3         | YPL274W | −1.4    | −0.5    | 0.1      | 0.8        | 0.4        |             |
| GDH1         | YOR375C | −1.4    | −0.2    | −0.1     | 0.3        | 0.9        |             |
| MMP1         | YLL061W | −1.4    | −0.4    | −0.1     | 0.6        | −0.1       |             |
| NDJ1         | YOL104C | −1.9    | NA      | −0.4     | 0.6        | 0.2        |             |
| JHD2         | YJR119C | −1.5    | −0.2    | −0.9     | 0.5        | 0.0        |             |
| POT1         | YIL160C | −1.5    | −0.6    | −1.1     | 0.8        | 0.6        |             |

doi:10.1371/journal.pone.0084630.t002
or absence of WHI3. The dependence on the presence or absence of Whi3 had a p-value less than 0.0001.

In a whi3 mutant, where there is no Whi3 to interact with the GCAU motifs, loss of the GCAU motifs nevertheless caused a statistically significant decrease in the amount of CLN3 mRNA (relative abundance of Long-gcaumut = 0.46), presumably because one or more of the mutations has some Whi3-independent effect that decreases mRNA abundance. This could be due to some Whi3-independent sequence effect on mRNA stability, or it could be related to the fact that the 5' GCAU motifs of CLN3 overlap with the DDE motifs thought to affect CLN3 expression [21]. In any case, this is a Whi3-independent effect, and the design of the experiment allows normalization for this effect.

In contrast, in a WHI3 strain, loss of the GCAUs caused a statistically significant increase in the amount of CLN3 mRNA (relative abundance of Long-gcaumut = 1.15), consistent with the idea that loss of Whi3 binding stabilizes CLN3. That is, when normalized against WT mRNAs containing GCAUs, the mutant mRNAs lacking GCAUs increased 2.5-fold in abundance in WHI3 strains vs whi3 strains (1.15 divided by 0.46), consistent with the idea that Whi3 destabilizes the mRNA by about two-fold via the GCAU motifs (normalization method 1).

The key comparison was the relative amount of Long-GCAU CLN3 mRNA in the whi3 mutant versus the WHI3 wild-type strain. This relative amount could be calculated by normalizing in each case to the amount of Short-gcaumut CLN3 (which should not be affected by the presence or absence of Whi3). This ratio was 1.50 (normalization method 2). That is, by this assay, fully wild-type CLN3 mRNA is 50% more abundant in a whi3 strain than in a wild-type strain. This is strikingly similar to the 1.4-fold increase seen in the straightforward quantitative PCR experiment (see above).
Motifs in the 5\'-UTR (4.4 min in WT, 2.9 min in whi3 mutant) by RNA-Seq.

Figure 2. mRNAs rich in GCAUs have increased abundance in whi3 mutants by RNA-Seq. Total RNA from whi3 or WT cells was extracted and sequenced using a HiSeq. As in Fig. 1, genes were divided into quartiles according to their GCAU density (Materials and methods). For each quartile, relative mRNA abundance is shown for whi3 mutants relative to wild-type as the log2 of the ratio of abundance. doi:10.1371/journal.pone.0084630.g002

Whi3 Destabilizes CLN3 mRNA

To directly measure CLN3 mRNA half life, we inserted a GAL promoter in front of CLN3. Cells were grown in medium with raffinose and galactose to mid log phase. The GAL promoter was then repressed by addition of glucose, and CLN3 mRNA levels were followed by Q-PCR. In WT cells, CLN3 mRNA decreased by 60% at 2 min, while in whi3 cells the decrease was only 40% (Fig. 4). We fit the data to a first order rate kinetic model of decay. The estimated half life for CLN3 was 1.9 min in WT cells (consistent with the 2 min half-life previously reported [22,23,24]) and 3.8 min in whi3 cells, suggesting a two-fold stabilization of CLN3 RNA in whi3 mutant cells. In contrast, the half-life of a control mRNA, GAL1, did not change in the whi3 mutant.

Whi3 has a homologue named Whi4. Although the whi4 mutant shows only a very small decrease in cell size, the whi3 whi4 double mutant is noticeably smaller than either single mutant, suggesting redundancy [1]. Therefore, we also measured CLN3 mRNA half-life in the whi3 whi4 double mutant. As shown in Figure 4, there was a slight increase in CLN3 mRNA half-life, to about 4.5 min. Although the various mRNA half-life differences are small, they were repeatable over multiple experiments (Fig. 4), and the 95% confidence interval for the half-life in the WT strain is clearly separated from the 95% confidence interval in the whi3 or whi3 whi4 strains (Fig. 4).

In the above GAL-CLN3 experiments, the 5'-UTR of CLN3 was replaced by the 5'-UTR of GAL1. However, the CLN3 5'-UTR contains cis translational regulatory elements which may also affect RNA stability as a side effect of translation [25,26,27]. Furthermore, of the 12 GCAU sites in CLN3 mRNA, six are in the 5'-UTR while the remaining six are towards the 3'-end of the coding region. Therefore, we made a construct in which the GAL promoter was inserted further upstream of the CLN3 ORF, preserving the endogenous CLN3 5'-UTR. The CLN3 RNA half life was measured as described above. Again, the CLN3 RNA was more stable in the whi3 mutant than in WT cells, this time by 1.5 fold (4.4 min in whi3 cells vs 2.9 min in WT). Thus, the GCAU motifs in the 5'-UTR may not be important for the destabilization of CLN3 mRNA, even though it is known that they can mediate binding [4] (see Discussion).

Whi3 physically interacts with Not1, Not3, Not4, Caf40, and Pop2 [20], which are all subunits of the Ccr4-Not complex, the predominant deadenylase in the cytoplasm [29,30,31]. Shortening of poly(A) tails of the mRNA is an initial step of RNA decay [32,33,34,35,36]. To see if Whi3 affects the half lives of its target RNAs at the step of poly(A) tail length, we measured the poly(A) tail length of CLN3 RNA in WT and whi3 cells using the ligation-mediated poly(A) test (LM-PAT) [37,38]. In this PCR based method, a shift in the mobility of the PCR product indicates a change in the length of the poly(A) tail. As shown in Figure 4c (lane 5–7), no difference in the poly(A) tail length of CLN3 was observed in the whi3 mutant, though accumulation of longer poly(A) tracts were detected in the positive control, the caf4 mutant, as expected.

It was possible that the inability to detect CLN3 mRNAs with very short tails was due to their extremely rapid degradation. Therefore, we repeated the experiment in an xrn1 background. XRN1 encodes the exonuclease degrading RNA from 5' to 3' [33,34,39,40]. Deletion of XRN1 blocks 5' to 3' degradation and allows accumulation of RNAs with short poly(A) tails. In the xrn1 background, all the RNAs tested showed a higher mobility band, indicating accumulation of short poly(A) tail RNA. However, the level of accumulation was similar in WT and whi3 mutant (see Figure 4c, lane 1–4). We therefore see no evidence that Whi3 destabilizes CLN3 mRNA by regulating poly(A) tail length. However, the assay was a qualitative one (i.e., looking at a gel).

We also tested CLN3 mRNA half life using the same galactose inducible system as described above in the xnl deletion background. In the xnl mutant, CLN3 mRNA was dramatically stabilized and its half life increased by around 12 fold to 23.5 min. This suggests that after deadenylation, CLN3 is degraded in a 5' to 3' direction through decapping. No difference was observed for the CLN3 mRNA degradation curve between the xnl single mutant and the whi3 xnl double mutant and the half life found in the double mutant was 23.2 min. Although several interpretations are possible, the most obvious is that CLN3 mRNA degradation requires the Xrn1 pathway, whether that degradation is mediated by Whi3 or not.
Ribosome Profiling

Ribosome profiling is a relatively new technique that footprint actively translating ribosomes, providing a record of their positions on all mRNAs [41,42,43]. The total number of footprints for a particular species of mRNA gives the number of ribosomes engaged in translating that mRNA.

Because the binding of Whi3 to an mRNA could alter translation, we used ribosome profiling on whi3 and wild-type strains. More than 14 million footprint reads were obtained for each strain. Full results are available at NCBI’s GEO database under accession number GSE51164.

The most striking observation was that in the whi3 mutant, there were 2.3 fold more ribosome footprints over CLN3 than in the wild-type (Fig. 5). That is, there were 2.3 fold more ribosomes passing over CLN3 mRNA in whi3 cells, suggesting that about 2.3 fold more Cln3 protein would be produced. This number was obtained by normalizing to all other ribosome footprints for all other genes, by the standard “reads per kilobase per million mapped reads” method (RPKM).

There were two contributors to this 2.3 fold increase. First, the RNA-Seq portion of the experiment showed that the CLN3 mRNA was 1.52 fold more abundant in the whi3 strain than in the wild-type strain (Fig. 5). That is, there were 2.3 fold more CLN3 mRNAs in each arm of the comparison.

Second, CLN3 had a higher translational efficiency (i.e., more ribosome footprints per CLN3 mRNA) in the whi3 strain than in the wild-type strain (Fig. 5). The translational efficiency was 1.53 (i.e., about 50% more ribosome footprints per CLN3 mRNA in whi3 than in WT). This measurement is also quite accurate, because of a large number of footprint reads. We calculated the relative translational efficiencies for all genes in whi3 vs wild-type (Fig. 6), and found that the CLN3 gene had the 19th highest relative efficiency in the genome. This was highly significant (p-value of 0.002 for a one-tailed test, ignoring genes with less than 200 sequence reads). Like CLN3, many of the other genes with a high relative translational efficiency in whi3 mutants had GCAU motifs close to the Start codon (in some, the “UG” residues of the AUG Start codon were part of a UGCAU motif); however, we have not yet calculated the statistical significance of this effect.

Thus, there were about 1.5 fold more CLN3 mRNAs in the whi3 mutant, and each of these mRNAs was translated by about 1.5 fold more ribosomes, leading to a total increase of about 2.3 fold.

Other observations from the ribosome profiling include the fact that there is no obvious correlation between the pattern of ribosome footprints (the peaks and valleys) and the position of GCAU motifs (Fig. 5).

Figure 4. CLN3 mRNA half-life is increased in the whi3 mutant. pGAL-CLN3 WHI3 (CYL104a), pGAL-CLN3 whi3 (CYL105a), and pGAL-CLN3 whi3 whi4 (CYL133a) cells were grown in YP medium with 2% raffinose and 2% galactose to log phase. 30 ml of culture was collected as t0. Glucose was added to a final concentration of 2% to stop CLN3 transcription. Cells were collected at 2, 5, 10 and 15 min. (A) CLN3 mRNA levels and (B) GAL1 mRNA levels were measured by Q-PCR and normalized using ACT1 for each sample. Lines show a first order rate fit of the decay data. Shaded regions indicate the 95% confidence interval of the fit. (black, WT; red, whi3; blue, whi3 whi4) Decay of GAL1 is a control. (C) LM-PAT analysis of CLN3 poly(A) tail length in xrn1 (lane 1), xrn1 whi3 (lane 2–4), WT (lane 5), whi3 (Lane 6) and ccr4 (lane 7). doi:10.1371/journal.pone.0084630.g004

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Other observations from the ribosome profiling include the fact that there is no obvious correlation between the pattern of ribosome footprints (the peaks and valleys) and the position of GCAU motifs (Fig. 5). We note that peaks and valleys generally like those in CLN3 are also seen in most other genes. Also, in the whi3 mutant, there is a pronounced peak of footprints at the upstream AUG at about -315 in the 5’ UTR, whereas this peak is almost completely absent in the WT strain. The upstream AUG has been implicated in translational control of CLN3 under certain nutritional conditions [26]. Although our results come from only one measurement, it is possible that Whi3 and the six GCAU motifs in the CLN3 5’ UTR are somehow inhibiting translation initiation at the CLN3 start codon, and this explains the change in translational efficiency and is somehow reflected in the preference for the upstream AUG versus the downstream start AUG. Additional experiments will be required to investigate this hypothesis.
Whi3 Co-localizes with P-bodies/Stress Granules

Gari et al. [2] used immunofluorescence to show that Whi3 has a somewhat lumpy cytoplasmic localization. This led to the hypothesis that cytoplasmic aggregates or foci of Whi3 were restraining Cdc28-Cln3 complexes from entering the nucleus, thereby inhibiting it [5]. Wang et al. [5] showed that Whi3 physically interacts with Ded1, an RNA helicase essential for translation initiation. Ded1 is a component of P-bodies [44], which are cytoplasmic granules involved in processing and degradation of mRNAs. Tarassov et al. [28] also found evidence of a physical interaction between Whi3 and several P-body components, including Ded1, Pub1, Scd6, Dh1, and Lsm4. We were interested in seeing the localization of Whi3 in live cells, and whether it had any relationship to P-bodies.

A Whi3-GFP fusion was created, which has fully wild-type function as assayed by cell size. When cells are grown in glucose, a condition in which the small cell phenotype of whi3 is manifested, Whi3-GFP is somewhat unevenly spread throughout the cytoplasm (Fig. 7). Although its distribution is not completely homogeneous, it does not appear lumpy, or to be in distinct foci. Its unevenness could be due to exclusion from intracellular compartments such as the nucleus and vacuole.

We asked if Whi3 co-localizes with P-bodies. To identify P-bodies, we used Dcp2-RFP (from Dr. R. Parker, Materials and Methods). Dcp2 is an RNA decapping enzyme shown to be a component of P-bodies [45,46]. Cells growing in medium containing 2% glucose showed neither Whi3-GFP foci nor Dcp2-RFP foci, consistent with the observation that few or no P-bodies are seen in glucose-fed, exponentially growing cells [47]. However, after glucose was depleted for 30 min, both Whi3-GFP and Dcp2-RFP foci became visible and Whi3-foci co-localized with Dcp2-RFP foci. (Figure 7a) We obtained the same result for another P-body marker, Edc3-mCherry, which also plays a role in RNA decapping [45,48]. Unlike Dcp2, Edc3-mCherry foci were sometimes visible even in the presence of glucose, but the number was typically 0 to 2 per cell. In response to glucose depletion, the number of Edc3-mCherry foci increased dramatically, and Whi3-GFP foci then became visible and co-localized with Edc3-mCherry foci. The co-localization was not perfect: there were occasional foci containing either Whi3 or the P-body marker, but not both, and in addition there were examples of incomplete overlap between the markers (e.g. Fig. 7b).

Stress granules are cytoplasmic foci related to P-bodies involved in storage and translation repression of RNA and induced under various stress conditions. Stress granules share some protein
components with P-bodies [49] [50]. We also asked if Whi3 co-localizes with stress granules using Pub1-mCherry as the stress granule marker under heat shock conditions (however, Pub1 may not be completely specific for stress granules) [51,52] [50]. As shown in figure 7a, both Whi3-GFP and Pub1-mCherry were distributed homogenously throughout the cytoplasm during exponential, unstressed growth. 10 minutes after a 46°C heat shock, both Whi3-GFP and Pub1-mCherry formed foci, and they

Figure 7. Whi3 co-localizes with P-bodies/stress granules. (A) Upper: Co-localization of chromosomal GFP tagged Whi3 (green) with P-body markers: Dcp2-RFP (pRP1155, magenta) and Edc3-mCherry (pRP1574, magenta) during log phase growth (+Glu) or after 30 min of glucose starvation (-Glu). Lower: Co-localization of chromosomal GFP tagged Whi3 (green) with stress granule marker Pub1-mCherry (pRP1661, magenta) after 10 min shift to 46°C. (B) Co-localization of Edc3-mCherry (magenta) and Whi3-GFP (green) after 30 min of glucose starvation. A partial co-localization is shown by the arrow. (C) Co-localization (or lack thereof) of GFP-tagged whi3 rrm or Q-domain mutants with P-body markers under stress. Upper: 30 min of glucose starvation. P-body markers are Dcp2-RFP (pRP1155, magenta) and Edc3-mCherry (pRP1574, magenta). Lower. 10 min of heat shock at 46°C. Stress granule marker is Pub1-mCherry (pRP1661, magenta). A scale bar of 2 μm is shown in each panel.

doi:10.1371/journal.pone.0084630.g007
usually (but not always) co-localized. These foci formed under heat shock were larger and more numerous than those formed under glucose starvation, and some foci were apparently connected to each other. We have not attempted to rigorously distinguish P-bodies from stress granules, but it appears Whi3 is associated with both.

Whi3 is not required for the formation of P-bodies/stress granules, since Dcp2-RFP foci still form normally in a whi3 mutant in response to glucose depletion (data not shown). Since Whi3 was not required for P-body/stress granule formation, we asked what recruits Whi3 to these foci. Whi3 protein has two prominent domains: the Q-rich domain (rich in glutamine) and the RRM (RNA-binding) domain [1]. It has been proposed that Q/N rich domains are prone-like and have a role in protein aggregation. Many known P-body components contain Q/N rich domains, and it has been shown that these domains either enhance the accumulation of the protein in the P-body or promote the formation of the P-body or aggregation [33,54]. To see if the Q-rich domain functions in the accumulation of Whi3 in the P-body we deleted amino acids 201–300 (the Q-rich region) in Whi3-GFP, to yield Whi3-dQ-GFP, and tested the co-localization of Whi3-dQ-GFP with P-bodies and stress granules as described above. Deletion of the Q-rich domain did not affect co-localization of Whi3 with P-bodies or stress granules. (e.g., Figure 7c).

Next, we deleted the C-terminal region of Whi3 from aa 539 onwards to delete the RNA binding domain (RRM) and asked if the RRM domain plays a role in recruiting Whi3 to cytoplasmic foci. After glucose depletion, the co-localization of Whi3-dRRM-GFP with a P-body marker was largely but not entirely disrupted. However, after heat shock, co-localization was still observed, but the Whi3-dRRM-GFP was apparently less concentrated in foci (Figure 7c). Finally, we deleted both the RRM and the Q-rich domains. Now, few if any distinct Whi3 foci were observed even after glucose depletion or heat shock, despite wild-type protein abundance (data not shown). To the extent that occasional concentrations of Whi3-dQ-dRRM-GFP were seen, they were diffuse, and not well co-localized with P-bodies or stress granules. This result suggests that the Q-rich domain and the RRM domain are redundant in localizing Whi3 to P-bodies/stress granules.

Since Whi3 selectively binds a subset of RNAs and co-localizes with P-bodies/stress granules, Whi3 might bring its bound RNAs to P-bodies/stress granules. To test this hypothesis, we examined the in vivo localization of CLN3 mRNA using the MS2 system [55]. We integrated 12 copies of MS2 loops between the coding region and the 3'UTR of CLN3 RNA and ectopically expressed NLS-MS2-GFP, which binds specifically to the MS2 loops. The NLS in MS2-GFP sequesters the unbound MS2-GFP in the nucleus so that the GFP background is reduced in the cytoplasm. (Reagents obtained from [55,56].) Cells containing CLN3 with 12 MS2 loops were wild-type for cell size, showing that the gene is functional. In cells carrying CLN3 with the MS2 loops, but not in control cells with CLN3 lacking the loops, one or two bright GFP foci (and sometimes additional less bright foci) were observed in the cytoplasm (Fig. 8). The much larger, brighter green spots are nuclei, which contain unbound NLS-MS2-GFP. We co-expressed Dcp2-RFP with NLS-MS2-GFP in the CLN3-MS2 tagged cells, and observed that, under glucose starvation, the GFP foci containing CLN3 mRNA co-localized with Dcp2-RFP foci, suggesting that some of the CLN3 mRNA was localized to P-bodies/stress granules (Fig. 8). Perhaps surprisingly, the co-localization of CLN3 mRNA and the P-body marker was preserved in the whi3 mutants and in the whi3 whi4 double mutant, which implied that neither Whi3 nor Whi4 is required for localizing CLN3 mRNA to the P-body. Additionally, we found that TUB2

and PGK1, which do not bind Whi3 as far as we know, also co-localized with the Dcp2 foci in response to glucose depletion (data not shown and Figure 8b). Thus, although Whi3 and its target CLN3 can both be found in P-bodies/stress granules under stress conditions, it is unclear what the role of Whi3 is, if any, in targeting CLN3 to this location.

Discussion

The Whi- Phenotype of whi3 Mutants is due to Stabilization, Increased Abundance, and Increased Translational Efficiency of CLN3 mRNA

The whi3 mutant was found because it has small cells. Nash et al. [1] found that a whi3 mutant has a mode cell volume of 35 femtoliters, versus 45 femtoliters for a WT cell. Interestingly, Nash et al. [3] found that a strain with two copies of the CLN3 gene also has a mode cell volume of 35 femtoliters, versus, in this case, 44
femtoliters for a WT cell. That is, a doubling of the CLN3 dosage gives exactly the same phenotype as the whi3 mutation. Here, six different experiments all showed that the whi3 mutation increases the abundance or stability of the CLN3 mRNA. The estimated increases were 1.4 fold (Q-PCR); 1.5 fold (long-short ratio measurement, normalization method 2); 1.5 fold (RNA-Seq); 1.5 fold (stabilization in GAL-CLN3 experiment with 5’ UTR); 2 fold (stabilization in GAL-CLN3 experiment without 5’ UTR) and 2.5 fold (long-short ratio measurement, normalization method 1). Over the six kinds of experiments, the median increase in CLN3 abundance in the whi3 mutant is 1.5 fold, and the mean increase is 1.7 fold, which is almost enough to explain the whi3 small-cell phenotype [3].

But in addition, it appears that Whi3 inhibits the translation of CLN3 (Fig. 5, 6), possibly because of the 5’ cluster of GCAU sites in the 5’ UTR (Fig. 5). The increased translational efficiency of CLN3 in the whi3 mutant is 1.5 fold. Together, the increased CLN3 mRNA abundance/stability (1.5 to 1.7 fold), and the increased translational efficiency (1.5 fold) should give about 2.3 fold more protein and therefore are fully sufficient to explain the whi3 small cell phenotype, which requires a two-fold increase in Cln3 amount [3]. Possibly the destabilization of CLN3 depends mostly on the 3’ GCAU motifs, and the inhibited translation of CLN3 depends mostly on the 5’ GCAU motifs; further experiments will be required to test this possibility.

**Whi3 Destabilizes Many mRNAs**

Microarrays, RNA-Seq, and other methods show that the Whi3 protein reduces the mRNA abundance of its RIP-chip targets and of many other genes that contain GCAU motifs. At least in the case of CLN3, reduced abundance is because of reduced half-life. The effects are probably very widespread. Figs. 1 and 2 show that the reduction is proportional to the density of GCAUs. On its own, this could mean that many, most, or virtually all mRNAs could be affected to varying degrees, or it could mean that a small number of mRNAs are affected very strongly, and these few mRNAs change the average. However, the second interpretation is less consistent with the fact that by microarray, only modest effects are seen. Furthermore, evidence from RNA-Seq shows directly that hundreds of mRNAs are being affected, with almost all of the effects quite small.

mRNA turnover is thought to begin with gradual deadenylation of the poly(A) tail [29,30,31]. This deadenylation is carried out by the Ccr4-Not complex, a large complex with many functions, but which includes two 3’ exo-ribonucleases, Ccr4 and Caf1 (in S. cerevisiae, Caf1 is thought to be catalytically inactive) [29,30,31]. By poorly characterized mechanisms, but potentially through interactions with sequence-specific RNA binding proteins, the Ccr4-Not complex is thought to be brought preferentially to some mRNAs, and thereby preferentially shortens the poly(A) tail of these mRNAs. Once the poly(A) tail has been shortened to some critically short length, the 5’ cap on the mRNA is removed by a decapping enzyme, and this allows rapid degradation from the 5’ end via a 5’ exo-nuclease of the Xrn1 family. In this model, mRNA half-life would be largely determined by the extent to which the Ccr4-Not complex is attracted to a specific mRNA.

Tarassov et al. used a Protein Complementation Assay to find yeast protein-protein interactions on a genome-wide scale [28]. Strikingly, they found that Whi3 interacts with five components of the Ccr4-Not complex, and Whi4 also interacts with four of these. Whi3 also interacts with four other proteins affecting mRNA turnover. All of the physical interactions found for Whi3 and Whi4 are shown in Table 3. These protein-protein interactions of Whi3 and Whi4 are supportive of a model in which the binding of Whi3 to an mRNA attracts the Ccr4-Not complex, which then accelerates mRNA degradation. We failed to observe the poly(A)

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**Table 3. Proteins Interacting with Whi3.**

| Bait | Interactor | Function |
|------|------------|----------|
| Whi3 | Whi3 | RNA binding protein |
| Pub1 | Whi3 | RNA binding protein involved in mRNA turnover |
| Puf4 | Whi3 | RNA binding protein involved in mRNA turnover |
| Not4/Mot2 | Ccr4/Not complex. Involved in mRNA turnover. Ubiquitin ligase. |
| Not1/Cdc39 | Ccr4/Not complex. Involved in mRNA turnover. |
| Not1/Cdc39 | Ccr4/Not complex. Involved in mRNA turnover. |
| Caf40 | Ccr4/Not complex. Involved in mRNA turnover. |
| Pop2 | Ccr4/Not complex. Involved in mRNA turnover. |
| Lsm4 | RNA binding complex involved in RNA turnover, decapping. |
| Dhh1 | RNA helicase, stimulates decapping; interacts with Ccr4/Not |
| Ded1 | RNA helicase needed for initiation of translation. |
| Scd6 | Binds elf4A; represses initiation of translation. |
| Def1 | Factor for degradation of RNA polymerase II |
| Mbf1 | Transcriptional co-activator; also found as mRNA target of Whi3 |
| Whi4 | Whi3 | RNA binding protein. |
| Not4/Mot2 | Ccr4/Not complex. Involved in mRNA turnover. Ubiquitin ligase. |
| Not1/Cdc39 | Ccr4/Not complex. Involved in mRNA turnover. |
| Not1/Cdc39 | Ccr4/Not complex. Involved in mRNA turnover. |
| Caf40 | Ccr4/Not complex. Involved in mRNA turnover. |

Interaction data is taken from Tarassov et al. [28], and shows all proteins found to be interacting with Whi3 or Whi4 baits.

doi:10.1371/journal.pone.0084630.t003
shortening expected from this model (Fig. 4), but the expected change is small and the assay was not quantitative.

Interestingly, Tarassov et al. also found an interaction between Whi3 and Ded1 and Scd6 [28]. Ded1 is needed for initiation of translation, and Scd6 is a direct inhibitor of initiation of translation. This interaction could help explain how Whi3 reduces translational efficiency of CLN3 and perhaps some other mRNAs.

Our overall model for Whi3 is that it binds to GCAU motifs on mRNAs. In many cases, particularly if the GCAUs are towards the 3' end of the mRNA, or if an AU Rich Element (ARE) is present, the bound Whi3 might attract the Ccr4-Not complex, which would increase the rate of deadenylation and mRNA turnover. On the other hand, if the GCAUs are near the start codon, there could be other effects. In CLN3, there are six GCAUs in the 5' UTR, and it appears that Whi3 inhibits translation. This could be by physical occlusion of the ribosome from the start codon; alternatively, the interaction of Whi3 with Ded1 or Scd6 could lead to inhibition of translation. We note that the function of another RNA binding protein, Mmi1, varies according to where it binds in its target mRNA [58]. Binding near the 3' end leads to rapid RNA degradation; binding further from the 3' end results in slower RNA degradation; and binding near a splice site inhibits splicing. Similarly, it is possible that Whi3 functions in different ways depending on the context of binding.

We have also shown that Whi3 is a component of P-bodies, but most of our experiments are in conditions where P-bodies are not seen. Accordingly, we have little idea of the role of Whi3 in P-bodies. However, the presence of Whi3 in P-bodies could possibly be connected to the previous finding of cytoplasmic foci containing Whi3 [2,5].

Small Effects

Perhaps surprisingly, the effects we have observed of Whi3 on target RNAs are all small, in the range of two-fold or less. Although for some mRNAs, such as CLN3, a two-fold effect is large enough to have major phenotypic effects [3], in general one has to wonder about the biological reasons for effects of this size (see below). At the same time, small effects are difficult to demonstrate rigorously. In the case of CLN3, we have used several different kinds of experiments to establish the effect of Whi3 (RNA abundance by Q-PCR; RNA abundance by RNA-Seq; translating RNA abundance by ribosome profiling; RNA abundance/half-life by comparison of wild-type and gcau mutant mRNAs; and measurement of CLN3 half-life by GAL-CLN3 shut-off experiments), all giving essentially the same result. In addition, we have repeated the experiments as many times as needed (27 experiments for the comparison of wild-type and gcau mutant mRNAs in WHI3 and whi3 strains) to drive down the standard error of the mean and obtain very small p-values. In addition, some of our experiments looked at hundreds or thousands of molecules (RNA-Seq, ribosome profiling). Although the effects are small, the p-values we have obtained in each of several very different kinds of experiments are very small, often less than 0.0001, and so we can reliably conclude that Whi3 is reducing abundance of the CLN3 mRNA.

Monkey on a Typewriter

What is the point of having an RNA binding protein that slightly destabilizes hundreds or thousands of mRNAs via a frequently-occurring four or five nucleotide motif? It is natural to suppose that such an RNA binding protein might be used to coordinate regulation of its targets, and this might be true. However, in the case of Whi3 and its targets, it is not easy to ascribe any overall function to the target mRNAs, and furthermore, despite some recent progress suggesting Whi3 could be regulated by phosphorylation [20,59] (Cai and Futcher, unpublished), there is no clear idea of when and how Whi3 activity is regulated, if indeed it is regulated at all. Furthermore, other investigations of yeast RNA binding proteins and their targets have likewise found an enormous number of targets for many of the RNA binding proteins, with no readily definable overall function for these targets. Here, as a speculation, or perhaps just as a null hypothesis, we would like to state the “monkey on a typewriter” hypothesis, which is the opposite of the “co-ordinate regulation” hypothesis. One can imagine that each mRNA in the cell would have an optimum average half-life, and of course this would vary gene by gene, according to the function of the gene. But what is the mechanism by which one mRNA comes to have a different half-life from another? We suggest that if there is an array of different RNA binding proteins, and some of these increase half-life and some decrease half-life, and if each RNA binding protein recognizes a short motif of four or five nucleotides, then each mRNA can evolve to contain a set of short motifs targeting it to some subset of the RNA binding proteins such that, overall, the mRNA achieves its optimal half-life. In this hypothesis, the clients of a particular mRNA binding protein need have no common function, and need not be co-ordinately regulated. They are independently seeking their optimum half-life. One could say that the RNA binding protein “regulates” its client’s abundance, but over evolutionary time. The fact that the binding motifs are short is consistent with this hypothesis—the shortness of the motif is evidence that the RNA binding protein is not highly specific. (A monkey on a typewriter will only rarely produce “Hamlet”, but will often produce “GCAU”–particularly if the keyboard has only four letters!) The ARE hypothesis—that mRNAs are destabilized by very short AU-rich elements [16]–is a branch of this hypothesis.

Materials and Methods

Yeasts Strains and Growth

Yeasts were grown in YP medium (1% yeast extract, 2% peptone and 40 mg/L adenine) supplemented with 2% glucose, if not otherwise specified. For transcriptional shut down in GAL-CLN3 experiments, cells were initially grown to log phase in YP medium with 2% raffinose and 2% galactose, and then glucose was added to a final concentration of 2% to shut off the GAL promoter. Cells for microscope imaging were grown in synthetic medium (5% ammonium sulfate, 1.7% yeast nitrogen base) with appropriate amino acids and carbon sources.

Yeasts strains are listed in Table 4. Unless otherwise stated, standard genetic techniques were used. Tagging, insertion and deletion was performed by PCR based homologous recombination using cassette described by Longtine et al. and Janke et al. [57,60]. To construct the WHI3x4 strain, pRN200b [1] was linearized with BstEI and incorporated into the genomic locus of WHI3. Cell sizes of isolated transformants were measured using a Coulter Counter Z2 and cells with sizes larger than average were selected and Q-PCR was used to quantify the Whi3 transcript levels. A plasmid containing CLN3 with 12 GCAU sites mutated was obtained from M. Aldape [4]. A CLN3 length polymorphism with a deletion of base pairs 1582–1650 was made by fusing the 5' fragment of CLN3 with the 3' fragment. The CLN3 allele with the length polymorphism and the GCAU mutations (“Short-gcau”) was generated by the same PCR reaction but using the GCAU mutant CLN3 as template. To truncate the wildtype CLN3 with these mutant CLN3 alleles, PCR products of these CLN3 alleles were transformed into a chs3::CaURA3 strain and cells were selected on 5FOA plates. Then haploid strains carrying different
CLN3 alleles were mated to obtain the diploids with two different versions of CLN3. Note that we differ from Aldea and co-workers with regard to the number of GCAU motifs in CLN3 (12 vs 14) because of a different definition of the 3' UTR.

Plasmids
Plasmids used in this study are listed in Table 5.

RIP-chip and Microarray
8L of yeast cells carrying Whi3 tagged with the TAP tag were grown in YP to log phase. Cells were harvested, resuspended in cold lysis buffer (10 mM Heps-Na, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT and protease inhibitors) and lysed by passing through a French press twice. KCl and Triton were added to the extract to a final concentration of 200 mM KCl and 1% Triton. After centrifugation at 4000 rpm for 5 min and 15000 rpm for 15 min at 4°C, aliquots of the supernatants were saved for assays of input protein and RNA, and the rest was passed through columns filled with 100 μl IgG Sepharose 6 Fast Flow (GE Healthcare) (The protein A moiety of the TAP tag binds to IgG). After three washes with IPP150 (10 mM Tris-Cl, pH 8, 150 mM NaCl, 0.1% Triton), the IgG beads were resuspended in 600 μl TES (10 mM Tris-HCl, pH 8, 10 mM EDTA, pH 8, 0.5% SDS). To isolate the RNA associated with the beads, an equal volume of acid phenol was added, vortexed and incubated at 65°C for 15 min. After two rounds of acid phenol extraction, the isolated RNA was ethanol precipitated from the aqueous phase for labeling.

Microarray
The microarrays for RNA-IP (RIP-chip) were performed on homemade arrays. Manufacture of these arrays was described in Spellman et al. [61]. 20–25 mg input RNA and all of the immunoprecipitated RNA was used for labeling by an aminoallyl labeling method adapted from The Institute for Genomic Research, Standard Operating Procedure SOP #M004. Briefly, the RNA was reverse transcribed with SuperScript II (invitrogen) and Oligo-dT, 2 mM dNTPs and 0.3 mM amino-allyl-dUTP (Ambion) was added for incorporation into the cDNA. The labeled cDNA was purified and coupled with the appropriate NHS-ester Cy-dye (Amersham). All the labeled cDNAs from immunoprecipitated RNA were hybridized against a total RNA normalization control containing 50 pmol of incorporated dye. Arrays were scanned using a GenePix 4000B (Axon Instruments)

Table 4. Strains used in this study.

| Name      | Genotype                        | Source                           |
|-----------|---------------------------------|----------------------------------|
| BY4741    | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 |                                  |
| BY4742    | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 |                                  |
| BY4743    | MATα/his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0 MET15 ura3Δ0/ura3Δ0 |                                  |
| W303a     | MATa leu2Δ3,112 trp1Δ1 can1Δ100 ura3Δ1 ade2Δ1 his3Δ1,15 | Wang et al 2004                     |
| HWL41     | W303a, WHI3-TAP-KanMX4          |                                  |
| CYL4      | W303a, whi3-dRRM-TAP-KanMX4     | this study                        |
| CYL21     | BY4741, whi3-URA3              | this study                        |
| CYL240    | BY4743, whi3-URA3/whi3-URA3    | this study                        |
| CYL81a1   | BY4741, Whi3Δ4-URA3            | this study                        |
| CYL104a   | BY4742, URA3-pGAL-CLN3         | this study                        |
| CYL105a   | BY4741, URA3-pGAL-CLN3 whi3ΔLEU2 | this study                        |
| CYL133a   | BY4741, URA3-pGAL-CLN3 whi3ΔLEU2 whi4ΔKanMX4 | this study                        |
| xrn1      | BY4742, xrn1-KanMX4            | Research Genectis deletion set    |
| ccr4      | BY4742, ccr4-KanMX4            | Research Genectis deletion set    |
| CYL178    | BY4743, CLN3/cln3-Short-gcaumut | this study                        |
| CYL179    | BY4743,cln3-Long-gcaumut/cln3-Short-GCAU | this study                        |
| CYL180    | BY4743, CLN3/cln3-Short-GCAU   | this study                        |
| CYL181    | BY4743, CLN3/cln3-Short-gcaumut whi3ΔURA3/whi3ΔURA3 | this study                        |
| CYL182    | BY4743, cln3-Long-gcaumut/cln3-Short-GCAU whi3ΔURA3/whi3ΔURA3 | this study                        |
| CYL183a   | BY4743, CLN3/cln3-Short-GCAU whi3ΔURA3/whi3ΔURA3 | this study                        |
| WHI3-GFP  | BY4741, WHI3-GFP-HIS3           | yeast GFP collection              |
| CYL99a    | BY4741, whi3-dQ-GFP-HIS3        | this study                        |
| CYL156a   | BY4741, whi3-dRRM-GFP-hphNT1    | this study                        |
| CYL169a   | BY4741, whi3-dQdRRM-GFP-hphNT1  | this study                        |
| CYL76a    | BY4742, CLN3-MS2L               | this study                        |
| CYL82a    | BY4742, CLN3-MS2L whi3ΔURA3    | this study                        |
| CYL93a    | BY4742, CLN3-MS2L whi3ΔURA3 whi4ΔKanMX4 | this study                        |
| CYL194a   | BY4742, PGK1-MS2L               | this study                        |
| CYL130c   | BY4741, whi3ΔLEU2 xrn1ΔKanMX4   | this study                        |

doi:10.1371/journal.pone.0084630.t004
Table 5. Plasmids used in this study.

| Name         | character                  | source                     |
|--------------|----------------------------|----------------------------|
| pRP1155      | Dcp2-RFP, CEN, LEU2        | Teixeira, et al 2005       |
| pRP1574      | Ecd3-mCherry, CEN,URA3     | Buchan, et al 2008         |
| pRP1661      | Pub1-mCherry, CEN,URA3     | Buchanan, et al 2009       |
| YCplac318-MS2_GFP | NLS-MS2CP-GFP, CEN, LEU2 | Gu, et al 2004             |
| pRP1155-LSY52| Dcp2-RFP, CEN, LYS2        | this study                 |
| pSH47        | pGAL-CRE, CEN, URA3        | Haim, et al 2007           |
| pLoxH55MS2L  | for integration of MS2L-loxP-Sphi5^-loxP | Haim, et al 2007 |
| pRN200b      | pRS306 with insertion fragment of DNA containing WHI3 for construct WHI3x4 | Nash, et al 2001 |
| pCYC2072     | cln3-GCAU<sup>mut</sup>, CEN, URA3 | Colomina, et al 2008       |

The RNA Binding Protein Whi3 Destabilizes mRNAs
controlled by GenePix Pro 5.1 software. Images were quantified using GenePix Pro. After background subtraction and loess normalization, the log ratios from multiple independent spots with the same probe were averaged. To obtain the list of Whi3 targets, a net log ratio was calculated by subtracting the ratio of the control experiment from that of the Whi3 IP experiment. The whole experiment was done independently twice, and the net log ratios were averaged. RNAs with net log ratios that were 1.65 standard deviations above the mean were defined as Whi3 targets.

By GenePix Pro 5.1 software. Images were quantified using GenePix Pro. After background subtraction and loess normalization, the log ratios from multiple independent spots with the same probe were averaged. To obtain the list of Whi3 targets, a net log ratio was calculated by subtracting the ratio of the control experiment from that of the Whi3 IP experiment. The whole experiment was done independently twice, and the net log ratios were averaged. RNAs with net log ratios that were 1.65 standard deviations above the mean were defined as Whi3 targets.

## Ligation-Mediated Poly(A) Test (LM-PAT)

Total RNA was prepared as described above. Ligation and reverse transcription was performed according to the protocol described by Salles and Strickland [38]. cDNA was PCR amplified and detected via 10% polyacrylamide gel electrophoresis and ethidium bromide staining.

### GCAU Analysis

Gene sequences were obtained from SGD. 5’UTRs and 3’UTRs were defined according to the RNA-Seq data from Nagalakshmi et al. [62]. For genes for which UTR information was not available, 200 nucleotides upstream or downstream of the ORF were used as the 5’UTR or 3’UTR respectively. If not specifically mentioned, GCAU density was calculated as the number of GCAUs per kb in the gene including both the 5’UTR and the 3’UTR. To calculate local maximum GCAU frequency, the maximum number of GCAUs in a sliding window of 300 nucleotides was recorded for each gene.

### Long/Short CLN3 GCAU Experiment

Semi-quantitative PCR was performed using a pair of oligos flanking the deleted region in the CLN3 length polymorphism to differentiate the long and short CLN3 mRNA species. Different numbers of PCR amplification cycles were tested to make sure the amplification reaction was in the linear range. PCR products were separated by gel electrophoresis and stained with ethidium bromide. The intensity of the two bands was quantified by ImageJ.

## Ribosome Profiling and RNA-Seq

1 liter of homozygous diploid WT or whi3 cells was grown in YPD to log phase. The culture was mixed with ice and cycloheximide at the same time (final CHX concentration was 100 μg/ml). Cells were washed once with ice cold water with 100 μg/ml cycloheximide. An ARTseq<sup>™</sup> Ribosome Profiling Kit was used to generate a ribosome footprint library and total RNA library. The given protocol was followed strictly except that an additional rRNA removal step using biotinylated oligos was performed before the end repair step.
Sequence Analysis for RNA-Seq and Ribosome Profiling

Sequence data from an Illumina HiSeq were initially trimmed and low quality reads were filtered using the FASTX-toolkit. The first 26 nucleotides of the sequencing reads were used for alignment using Bowtie2 with the end-to-end alignment method against S288C reference genome R64. Unaligned reads were recycled for additional alignment using coding region sequence as reference to retrieve exon junction reads. The position of the 5’ end in the read was used to assign reads to genomic features [43]. Reads mapped to the rDNA were discarded for following analysis. Reads per kilobase per million reads (RPKM) was calculated to quantify mRNA and ribosomal footprints. Briefly, after removal of genes with less than 7 reads, the count of reads for each gene was normalized by its length (in kb) and the total number of reads (in million). RNA-seq and ribosome profiling data can be found in the NCBI’s GEO database under accession number GSE51164.

Microscopy

For glucose starvation experiments, log phase cells were washed, resuspended with the same medium but lacking glucose, and incubated for 30 min before imaging. For heat shock experiments, log phase cells were collected by centrifugation, resuspended in the same medium prewarmed to 46°C and incubated at 46°C for 10 min before imaging. To prepare cells for microscopy, concentrated cell culture was mounted onto prewarmed agarose and pictures were taken immediately after the glucose starvation or the heat shock treatment. Observations were made using either a Zeiss Axiosplan2 microscope with a Zeiss mRM Axiocam (Carl Zeiss) or a Zeiss Observer.Z1 microscope with an attached Orca II ERG camera (Hamamatsu). Zeiss Axiovision 4.8 was used to acquire images. Z-stack images were made by a Z-series compilation of 4–7 images. P-body markers and stress granule marker were all ectopically expressed and the plasmids used are listed in Table 5. 12 copies of the MS2 loop were integrated between the ORF and 3’UTR by the method developed by Haim et al. [55]. To visualize RNA localization, cells tagged with MS2 loops were transformed a plasmid containing MS2CP-GFP which binds specifically to the MS2 loop of RNA [56].

Supporting Information

Table S1 Whi3 RIP-chip. This spreadsheet contains separate tabs for the full RIP-chip results (Whi3_RIP-chip) and the RIP-chip results for the 262 mRNAs identified as Whi3 targets in this study (Whi3_targets). In both datasets, net_IP_ratio is the averaged net IP ratio as described in the paper. The rank of the net_IP_ratio is also listed.

Table S2 Joint list of Whi3 targets. This table contains a set of 111 Whi3 target mRNAs common between this study and the study by Colomina et al. The net_IP_ratio and rank are the averaged net IP ratio and the rank of the net IP ratio from this study. IP_col and rank_col are the IP ratio and the rank of the IP ratio from Colomina et al. A description from SGD for each gene is also given.

Table S3 Microarray of whi3 and WHI4X4. This table contains the processed data from the microarray experiment on the whi3 and the WHI4X4 strain. whi3YPD and WHI4X4YPD are the log2 ratios of mutant over WT for whi3 and WHI4X4 cells grown in YP glucose medium. whi3YPD and WHI4X4YPD are the log2 ratios of mutant over WT for whi3 and WHI4X4 cells grown in YP ethanol medium.

Acknowledgments

We thank Marti Aldea, Jeff Gerst, Roy Parker and Robert H. Singer for providing reagents used in this study. We also thank Hongyin Wang, Hong Wang and Gang Zhao for technical assistance. We thank Steve Skiena for helpful discussions regarding the “monkey on a typewriter” hypothesis. We thank K. Holmes and M. Cyert for communicating results prior to publication. We thank an anonymous reviewer for insightful comments.

Author Contributions

Conceived and designed the experiments: YC BF. Performed the experiments: YC BF. Analyzed the data: YC BF. Contributed reagents/materials/analysis tools: YC. Wrote the paper: YC BF.

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