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ATP Mediates Yeast Cell-Cell Communication

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Abstract

Yeast secrete ATP in response to glucose, a property with previously unknown functional consequence. In this report, we show that extracellular ATP is a signal for growth of surrounding cells. The ATP signaling behavior was uncovered by finding reduced toxicity of an inducible, dominant-lethal form of alpha tubulin (tub1-828) in cells grown at high, compared to low cell density. Reduced cell death at high cell density resulted because the rate of chromosome loss/cell division was lower (18-fold) in a cultures inoculated with a high density (350,000) compared to a low density (5,000) of cells. The sparing effect of growth at high cell density could be replicated by growing together 3440 cells that express tub1-828, with 2.3 E6 cells that do not express the mutant protein. Toxicity was reduced at high cell density apparently because a secreted signal induces growth, so that the mutant protein is rapidly diluted by synthesis of wild-type α-tubulin. Further, fluorescence-activated cell sorting (FACS) analysis after DNA staining showed that the rate of the G1-G2 transition was faster with cells at high density. ATP replaced the need for high cell density for resistance to tub1-828, and stimulated the transition from G1 to G2 in cells at low density. Cells lacking the enzyme nucleoside diphosphate kinase did not respond to nucleotide stimulation of growth during expression of mutant tubulin, suggesting that NDP kinase has a regulatory role in growth stimulation. This newly discovered quorum sensing response in yeast, mediated by ATP, indicates that yeast decision-making is not entirely autonomous.

Introduction

A search for a suppressor of a GTPase defect in a yeast lethal tubulin mutant (tub1-828, Anders and Botstein, 2001) that forms abnormally stable microtubule was surprisingly complicated by our finding that the toxicity of the mutant tubulin depended on cell density. This apparently results from cells secreting a growth promoter that induces synthesis of wild-type TUB1

to dilute and mitigate the effect of the mutant tubulin (Spellman et al., 2000, Fig. 1). The growth promoter was identified as ATP, which had previously been found to be secreted by yeast. Although extracellular ATP binding to purinergic receptors plays an important role in signaling in multicellular organisms, because yeast lack these receptors, its glucose-dependent secretion of ATP (Boyum and Guidotti, 1997; Esther et al., 2008) would appear to waste energy. However, ATP stimulation of neighboring cells will increase a colony's population and diminish food for rival organisms. and thereby increase its likelihood of survival.

Results

DBY3363 cells that had been modified to express tub1-828 from a tet-on promoter (Belli et al., 1998) were used for the studies reported here. This haploid strain contains a multiply marked supernumerary copy of Chromosome III, and cells that lose one of the extra chromosomes form red colonies that are resistant to cryptopleurine (Hoyt et al., 1990); cells that lose any other chromosome are not viable. This property was used to determine the effect of tub1-828 on chromosome loss.

Effect of TUB1-828 expression: Although the tubulin dimer containing tub1-828 could be isolated when this was expressed from a plasmid containing the gene with an inducible gal or tetracycline promoter (Fig. 2), the mutant sequence *was only detected soon* after galactose or tetracycline induction, apparently because the mutant tubulin resulted in inaccurate distribution of the plasmid containing the coding sequence, from the mitotic spindle. Expression of mutant tubulin also resulted in chromosome loss (see below).

The non-native GFP-TUB1 or GFP-tub1-828 proteins constituted <10% of the alpha tubulin (Fig. 3) in induced cells, despite the fact that the tetracycline promoter is ordinarily highly efficient (Belli et al, 1998). This low concentration, which probably results from the GFP moiety enhancing degradation, results in a high TUB1/tub1-828 ratio. This is important since the toxic stabilization of microtubules by tub1-828 is expected to be proportional to the 13th power its mole fraction of the total tubulin (Caplow and Shanks, 1996).

Both GFP-TUB1 and GFP-tub1-828 were incorporated into microtubules (Fig. 4), and fluorescent microscopy of microtubules containing GFP-TUB1 showed normal dynamic instability (results not shown).

Effect of tub1-828 expression: The toxicity of tub1-828 was studied with cells grown in the presence of tetracycline in both liquid and solid media. Most cells grown on agar formed microcolonies by dividing 2-12 times in 48 hours. Surprising was formation of 1 mm diameter macrocolonies (Fig. 5A, B), from a highly variable fraction of cells that had divided about 20 times.

Formation of macrocolonies indicates that cell division can occur despite tub1-828's dominant lethal phenotype (Anders and Botstein, 2001). This behavior was reproducible and observed with cells taken from a colony on a refrigerated plate, and with cells from a liquid culture in early and late log phase, or at a stationary-state for 48-360 hours. Fewest macrocolonies were formed with log phase cells. Most cells within macrocolonies were not viable (i.e., did not grow on YPD without tetracyclin); the fraction of dead cells varied widely and in many cases was c. 90%. Sequencing of genomic DNA from viable cells in macrocolonies revealed that tub1-828 had not undergone reversion to TUB1. Proof that the tub1-828 gene sequence had been retained also came from our finding that re-plated viable cells from a macrocolony formed both microcolonies and macrocolonies (Fig. S1, Table S1).

The behavior of cells in liquid YPD-tetracycline media was also unexpected: growth was greater in cultures started with a large inoculum (Fig. 6A). This was observed in approximately 25 experiments and was not observed in the absence of tetracycline. Most surprising was finding fewer viable cells after 20 hours of growth of a culture started with a small inoculum (5000 cells/5ml), compared to a large inoculum (350000 cells/5ml; Table 1). This reflects the 18-fold greater loss of a chromosome/cell division with cells grown from a small inoculum (Table 1).

A few studies were done in SCD-tetracycline media where the impact of tub1-828 expression was much greater: with a 17400 cell inoculum of stationary state cells grown for 20 hours only 1.7% of cells were viable and 50% of cells had lost the supernumerary copy of chromosome III; with a 870,000 cell inoculum 11% of cells were viable and 0.3% of cells had lost

chromosome III. It is proposed that slower growth in SCD prevents rapid accumulation of wild-type Tub1 to dilute the toxic tubulin.

Growth from an inoculum of log phase cells was also dependent on the inoculum size (Fig. 6B); however, the number of cells/5 ml to observe stimulation was >125000, compared to 10000 with stationary-state cells. The dependence of growth on cell density suggested that cells secrete a factor that mitigates tub1-828 toxicity by promoting growth-associated TUB1 synthesis (Fig. 10); the different behavior of log-phase cells may result because they secrete lesser amounts, or are less sensitive to this factor. In support of the latter, log-phase cells grow poorly on a tetracycline plate, with only 0.7% of cells able to form a microcolony and this was unchanged with 100 uM ATP, and only increased to 1.5% with 100 uM CTP. In contrast, these nucleotides enhance growth of stationary-state cells plated on tetracycline media (see below).

Growth promotion by cells not expressing mutant tubulin: Evidence that cells communicate via a growth factor that promotes resistance to tub1-828 was obtained by comparing the growth from a small inoculum of stationary-state cells that express tub1-828 (3440 cells/5 ml), when this was supplemented with 2.3 million stationary-state cells that were otherwise identical, except for lacking the URA^+-tub1-828 genomic insert. These cells could not form colonies on agar lacking uracil, so enhanced growth could be determined from the increased number of URA^+ colonies with aliquots from these cultures at high cell density (Table 2). With cells grown for 6 hours, which is sufficient for three cell doublings, there was approximately one additional doubling in the culture grown at high cell density. Growth was analyzed for only 6 hours since the growth-promoting effect of high cell density is maximal before media exhaustion reduces cell growth.

<u>Effect of cell density and ATP on the G1-G2 transition:</u> FACS analysis *(Haas and Reed, 2002*) showed the rate of the transition from stationary phase to growth was more rapid with cells grown at high cell density (Fig. 7, Left Panel), and with cells grown with ATP (Right Panel). In this experiment 1.06 E6 stationary-state cells were transferred to 5 ml (high density growth), and 2.12 E5 cells were transferred to 250 ml fresh media (low density growth). Whereas a significant fraction of cells grown at high

density duplicated their DNA in 4.5 hours, cells grown at low density were either in G1 or in the process of duplicating their chromosomes, and only a small fraction of cells were in G2. The enhanced growth produced by high cell density was reproduced with cells grown at low density in the presence of ATP (Fig. 7, Right Panel, C and D).

Nucleotide Effects On Growth on Solid Media: Nucleotides also stimulate growth on tetracycline/agar (Fig. 8, 9). The extent of nucleotide-stimulation was determined from analysis of a histogram that described the number of cells that had divided n-times, calculating the total number of cells that would be contained in 100 colonies (Figs. S2; Table S2). Both ATP and CTP increased the rate of growth with cells expressing tub1-828, with measurable stimulation at 1 uM. ATP was without effect on cells grown without expression of the mutant protein (no tetracycline).

Secretion of ATP: We confirmed earlier results (Boyum and Guidotti, 1997; Esther et al., 2008) showing that cells secrete ATP (Fig. 10). A relatively high concentration of cells generated 20 nM ATP in an hour, corresponding to a rate of about 250 molecules/s/cell; this is similar to that for α-factor (700/s/cell, Goncalves-Sa and Murray, 2011).

Role of Nucleoside Diphosphate Kinase: To determine whether growth stimulation by CTP and GTP (Figs. 7, 8) actually resulted from ATP generated by the membrane-associated nucleoside diphosphate kinase's conversion (Zhang et al, 1995) of ADP to ATP, ynk1 cells were analyzed. Results were surprising: whereas it was expected that the nul mutant might form additional microcolonies in response to ATP, but not to CTP, instead, virtually every cell formed a macrocolony in the presence and absence of nucleotide. This was totally unlike the bimodal distribution of colony size with cells with an intact YNK1 locus (where 48% of cells formed microcolonies, with 14-or fewer divisions, Fig. 5B), with only 2% of ynk1 cells forming microcolonies (red in Fig. 11). The predominance of macrocolonies in cells lacking NDP kinase can be accounted for if this enzyme/signal-transducer represses early TUB1 synthesis, thereby allowing early TUB1 synthesis so that 5 predominate. Also, growth stimulation by nucleotides (compare red with green and purple in Fig. 11) would be unnecessary to increase the TUB1/tub1-828 ratio. In any case, the altered behavior of ynk1 cells indicates that NDP kinase regulates cell growth.

Nucleotides were also without effect with quiescent ynk1 cells taken from a refrigerated agar plate (Fig. S3). However, unlike growth with stationary state cell (Fig. 11) only 3% of viable cells formed macrocolonies both with and without nucleotides (ATP or CTP). The lack of an effect of ATP is consistent with a mechanism in which NDP kinase is part of the signaling path that promotes the transition to cell growth and TUB1 synthesis.

Although ynk1 cells did not respond to added ATP or CTP, they were responsive to the size of the inoculum (Fig. 12). As noted above, assay of cell growth as a function of the inoculum size is sufficiently sensitive to detect stimulation of 1 out of 10000 cells, so that a small stimulatory effect of a secreted growth promoter can be detected by measuring the number of cell divisions before cell death. Such a minuscule stimulatory effect by nucleotides cannot be detected from counting the number or size of colonies on tetracycline plates.

Discussion

Evidence for secretion of a growth substance: It was not a surprise to find that the lethality of mutant tub1-828 results from its disrupting the mitotic spindle to allow inaccurate chromosome distribution (Table 1). What was surprising was that this effect was dramatically reduced in cells grown at high density (Table 1). This presumably results because high cell density produces a sufficient concentration of a secreted signal molecule that stimulates growth. The growth-associated synthesis of TUB1 dilutes tub1-828 in the mitotic spindle to mitigate the mutant protein's toxicity. Support for this survival mechanism is the normal growth of cells that constitutively express tub1-828, when these also contain a plasmid to provide supplemental expression of TUB1 to dilute the toxic protein (Fig. 1).

Additional evidence for secretion of a signal molecule came from the sparing effect of a high density of cells that do not produce tub1-828, on the survival of a low density of tub1-828-expressing cells (Table 2). Similarly, the G1-G2 transition was accelerated in cells at high density (Fig 7); the transition is expected to be accompanied by TUB1 synthesis.

Mechanism for resistance to tub1-828 toxicity: Secreted ATP (Boyum and Guidotti, 1997, Esther et al., 2008; Fig 10) is suggested to be the signal molecule for enhanced cell growth. Support for this thesis comes

from finding that added ATP reduced tub1-828 toxicity (Fig. 8, 9) and accelerated the G1-G2 transition (Fig. 7).

It is postulated that ATP induces rapid growth and earlier than normal TUB1 synthesis (which ordinarily peaks at the entry to mitosis; Spellman et al., 2000) to dilute quickly the tetracycline-regulated toxic protein (Belli et al., 1998). Cells that can generate and sustain a high TUB1/tub1-828 ratio will have greater survival in liquid culture (Fig 6A) and form macrocolonies on agar (Fig. 5, 13). An increase in the TUB1/tub1-828 ratio can come about if ATP alters the subunit-induced suppression of tubulin mRNA degradation that coordinates TUB1 and TUB2 synthesis (Cleveland et al., 1981), or if ATP-signal induces premature initiation of TUB1 synthesis.

The wide variation in cell survival (both micro and macro colonies are formed) is presumed to result from cells being randomly distributed in the cell cycle when they are exposed to tetracycline. Also, the large (30%, Spellman et al., 1998) standard error for TUB1 expression suggests that there are significant variations in TUB1 synthesis during the cell cycle. Cells that are at the extreme in synthesizing TUB1 will resist tub1-828 toxicity and form macrocolonies.

Evidence that cell death is random and precipitous, occurring in the last few divisions, was the fact that macrocolonies were always perfectly spherical (tens of thousands of colonies were measured); if death were progressive, colonies would have been scalloped and misshapen. Also, if death were progressive it would not be possible in 24 hours to generate million-cell-macrocolonies in which 50-90% of cells were dead; persistent cell division is require to generate a million cells. In summary, both the growth of cells that go on to form macrocolonies, and the precipitous death of cells in a microcolony appear to be random events.

Unlike macrocolonies, which were invariably spherical, about 25% of microcolonies had a shape that suggested that death occurred in an early point, so that a large portion of the sphere was missing. The basis for early and precipitous cell death from tub1-828 is discussed next.

Cooperativity for tub1-828 toxicity: Cell survival in the presence of tub1-828 is on a knife edge. This results from the mechanism by which the mutant protein's defective GTP hydrolysis generates abnormally stable

microtubules (Anders and Botstein, 2001). Accordingly, the behavior of the mutant protein it is assumed to be equivalent to that of tubulin-GTP subunits, which cap and transiently stabilize microtubules in the mitotic spindle (Desai and Mitchison, 1997). Although the size of the stabilizing GTP-cap is not known, in vitro studies of microtubules with tubulin subunits containing a nonhydrolyzable GTP analogue (GMPCPP) demonstrated that stabilization requires 13 or 14 contiguous tub1-828 subunits at a microtubule end (Drechsel and Kirschner, 1994; Caplow and Shanks, 1996). This means that microtubules stabilization by tub1-828 subunits, and the resultant toxicity will be proportional to the 13th or 14th power of the mole fraction of tub1-828 subunits in the cell's pool of alpha-tubulin. Therefore, a slight decrease in TUB1 expression, or a small stochastic increase in tub1-828 expression in individual cells (Raser and O'Shea, 2005; Bar-Even et al., 2006) will, after a variable number of cell divisions, result in loss of one of the cell's 16 chromosomes, and cell death.

Our finding increased cell survival (Fig. 6A) at cell densities that form low concentrations of ATP (Fig. 10) may be accounted for by a requirement for 13 contiguous tub1-828-GTP subunits to generate a abnormal microtubule cap that allows chromosome loss. Since the probability for 13 contiguous tub1-828 subunits is proportional to the 13th power of the mole fraction of mutant subunits, a minimal stimulation of cell growth is likely to generate sufficient TUB1-GTP subunits to disrupt the continuity of a 13-subunit tub1-828-GTP cap. Thus, the dependence of cell survival on ATP concentration does not reflect a Kd for ATP binding, but rather a reflection of its ability to stimulate TUB1 synthesis to disrupt the tubu1-828 cap. Finally, low concentrations of secreted ATP are likely to be especially effective in stimulating cell growth on agar, since limited diffusion will increase the local concentration.

ATP Stimulation of Growth: Although yeast in synthetic media generate 1-2 uM ATP after 40 hours, in a path regulated by the EGO complex (Peters et al., 2016), the lesser amount under our experimental conditions (Fig. 10) appears to be discrepant with finding that ≥1 uM ATP concentrations was required to generate a detectable stimulation of growth on agar (Figs. S2, 3; Table S2). However, with cells growing on agar the ATP concentration proximate to growing colonies may be significantly

increased since dilution is decreased by limited diffusion. On the other hand, for growth in liquid media, stimulation by <1 uM ATP is likely to be detected because the growth promotion is amplified: For example, whereas stimulation that promotes prolonged exponential growth of an individual cell generates a single macrocolony, this same growth in liquid can significantly change the cell count. To illustrate this point: if growth of 999 of 1000 cells is limited to 10 divisions (yielding 1 million cells), and ATP allows 1 cell to divide 20-times (yielding 1 million cells) the ATP stimulation results in an approximate doubling of the number of cells from the inoculum, corresponding to one extra cell division. Were this mixture plated on agar the results would be less convincing: there would be one additional macrocolony. This analysis agrees with our finding that with a cell concentration where there is one extra cell division (i.e., with 100,000 cells/5 ml, Fig. 6A) the ATP concentration after one hour would be about 0.03 nM. Since 1 uM ATP has a detectable effect using an enormously less sensitive assay (Fig. S3, Table S2), it is likely that 0.03 nM ATP would produce the results in Fig. 6A. Finally, our finding an effect of extracellular ATP concentrations well below the approximately 2.5 mM intracellular concentration (Ozalp et al., 2010) was also seen for growth of S. pombe mutants with reduced TORC1 signaling (Forte et al., 2019). Our, and the earlier results suggest that extracellular ATP signaling occurs before ATP entry into the cell.

Role of nucleoside diphosphate kinase in growth stimulation: Cells made null for nucleoside diphosphate kinase (ynk1) have an altered growth response to tub1-828 and an altered response to nucleotides. Induction of tub1-828 from stationary state cells resulted in rapid growth, without formation of microcolonies. This can be taken to indicate that NDP kinase negatively regulates early TUB1 synthesis and growth so that in a nul mutant sufficient TUB1 is synthesized to offset tub1-828 toxicity; therefore, NTP stimulation of TUB1 synthesis is not required for growth. The very different results with quiescent ynk1 cells, which are sensitive to induced tub1-828, allowed test of an hypothesis that NDP kinase is required for CTP stimulation of growth: results supported this hypothesis (Fig. 11). Although deletion of NDP kinase eliminates a requirement for nucleotide-dependent growth, it did no eliminate the effect of inoculum size on growth (Fig. 12). However, as noted above, assay for growth stimulation with a high inoculum is enormously more sensitive to growth stimulation

measured with a cell plating assay (Fig 11) : enhanced growth by 1 cell in 1000 can be detected. (see above). Our finding that NDP kinase has a regulatory role for cell-cell communication and growth is in accord with observations that this protein regulates tumor metastasis and drosophila development and sexual development in S. pombe (Izumiya and Yamamoto, 1995; Woolworth et al. 2009).

Yeast Signaling: Discovery of an extracellular ATP signaling pathway for stimulating yeast growth adds to the S. cerevisiae signaling paths for: quorum sensing and generation of an undifferentiated multicellular species (Chen and Fink, 2006), for the dimorphic transition induced by ammonia (Vopálenská et al., 2010), and for sexual reproduction. Although yeast lack a purinergic receptor, evidence that this does not necessarily preclude an ability to respond to ATP comes from studies showing that plants, which like yeast lack purinergic receptors, use ATP to regulate growth, development and the response to stress (Choi et al., 2014).

The signaling pathway for ATP stimulation of the transition from stationary-state to rapid growth is unknown, but may involve activation of the Yck1p and/or the YCK2 membrane-associated casein kinases that mediate the transition from quiescence to rapid growth in starved cells (Zaman et al., 2008; Wand et al., 1996). The ATP-binding domain for these enzymes may require extracellular ATP for activity. Alternatively, the ATP signaling path may involve YNK1. Evidence for this was the absence of an ATP effect on growth on tetracycline plates with cells that are nul for this enzyme-signaling protein (Izumiya and Yamamoto, 1995; Woolworth et al. 2009).

Our studies indicate that yeast decision-making for adapting to a new environment is not entirely autonomous. Information provided by the ATP growth-promoting signal is somehow integrated into the complex regulatory mechanisms that induce the vast changes in protein expression that allows adaptation to new conditions (Gasch et al., 2000; Gelade et al., 2003).

Experimental Methods

Creation of Plasmids for Insertion of TUB1 and GFP-tub1-828 Into the Genome: To produced plasmids for integration of TUB1 and GFP-tub1-

828 into the yeast genome, the following modifications were made to the episomal plasmid pCM190 (obtained from Euroscarf). First, the tet-off promoter of pCM190 (5) was replaced with a tet-on promoter, contained in a BamHI/EcoRI fragment from plasmid pCM252 (from Euroscarf). Second, the TUB1 and GFP-tub1-828 coding sequences (from DBY2963, obtained from Dr. David Botstein) were inserted at the BamHI site to place them under the control of the tet-on promoter. Third, after a cytosine residue that flanks and prevents cleavage at the Cla1 site in pCM190 had been deleted, the pCM190 plasmids containing TUB1 and GFP-tub1-828, respectively, were PCR-amplified away from the ClaI and HpaI sites that bracketed the 2-micron element; the resulting products were phosphorylated and blunt-end ligated. Sequencing and digestion studies of the product revealed that there were 126 rather than 1347 bp between the Cla1 and Hpa1 site, confirming deletion of the 2-micron element. Fourth, the 2 micron-less plasmid was used as a template for PCR with a primer containing 50 bp of 5'-untranslated TRP1 sequence and a primer contained 50 bp immediately following the TRP1 step codon; the resultant linear PCR product was used to transform DBY3363 cells that had been modified as follows. The tet-on TUB1 and GFP-tub1-828 pCM190 plasmids were used to transform yeast as described next.

Insertion of TUB1 and GFP-tub1-828 Into the Genome: Yeast strain DBY3363 is a haploid strain containing a multiply marked supernumerary copy of Chromosome III that enables chromosome loss to be observed easily on various media. Two modifications to DBY3363 were made before introducing into its genome the tet-on TUB1 and GFPtub1-828 PCR products (see above). First, we disrupted the ura-3-52 allelle in DBY3363 with a hygromycin resistance gene (from pAG2). The disruption prevented repair of ura3-52 and enabled selection of the URA3-containing tet-on TUB1 and GFP-tub1-828 pCM190 plasmids. Second, it was necessary to introduce into DBY3363 a gene from plasmid pCM242 that encoding the reverse tetracycline-controlled transcriptional activator (rtTA), which activates the tetracycline responsive elements only in the presence of doxycycline. The LEU2 gene of pCM242, flanked by Bsp106 and EcoRV sites, was replaced with LYS2 (derived from pRS317); the LYS2 gene of DBY3363 was disrupted by insertion of a nourseothricin resistance gene, thus providing a means to select for genomic insertion of the LYS2-containing pCM242 plasmid.

To insert the tet-on TUB1 construct into the genome of modified DBY3363, the tet-on TUB1 pCM190 plasmids was amplified by PCR with a forward primer containing 50 bp of 5'-untranslated TRP1 sequence and a reverse primer containing 50 bp immediately following the TRP1 stop codon; the resultant linear PCR products were used to transform DBY3363, selecting for URA^+ cells.

GFP-tub1-828 was also inserted into the genome of modified DBY3363 in the form of a tandem repeat of TUB1 (with its native promoter) and GFP-tub1-828 (with the tet-on promoter). This insertion was generated by transformation with the product of a BstEII digestion within the tub1-828 sequence at position 1014 of the GFP-tub1-828-containing pCM190 plasmid. Because the tub1-828 mutation lies between 870 and 881, transformation with the BstEII digestion product placed the TUB1 sequence upstream of 1014 at the 5' of TUB1 (i.e., with the native promoter), followed by the sequence containing the tet promoter and the GFP-tub1-828 sequence. This cell line was designated as MCY005. MCY002, in which the tub1-828 in MCY005 had reverted to TUB1, was isolated as a revertant and characterized by sequencing. MCY047 cells, in which the genome of DBY3363 was not otherwise modified, contained the tet-off promoter that is normally found in pCM190, and tub1-828, rather than GFP-tub1-828, inserted into TRP1. The nucleoside diphosphate kinase gene YNK1 in MCY046 was inactivated by insertion of TRP1. Unless stated otherwise, studies were done with MCY046.

Analysis of Cell Behavior: Stationary-state cells were generated by growth of a large inoculum for 24-72 hours in YPD liquid media in the absence of doxycycline; log-phase cells were obtained from overnight growth of a small inoculum. Induction of tub1-828 was in liquid or on agar plates with doxycycline (5 ug/ml), and the extent of growth in liquid media was determined with a hemocytometer. DNA in cells was stained as described (Haas and Reed, *2002*), and images were collected with a BD LSR II Flow Cytometer. FACS analysis was performed in the absence of tetracycline, so that formation of tub1-828 did not influence the results. The effect of tub1-828 expression was determined in liquid YPD-tetracycline or on agar plates with tetracycline and, in some cases, with nucleotide; these substances were added after autoclaving and they did not

alter the pH of the media. Comparisons of cell growth were performed with plates prepared on the same day and stored under identical conditions. The number of cell divisions after growth on agar was estimated from measurement of colony diameter using the reticle in a low-power microscope, assuming that the colony and individual cells (5 µm diameter) were spherical, with cells packed so that they occupied 71% of the volume; this value is between the 63% for random close packing and 74% for hexagonal close packing. Evidence that this method was accurate was finding a similar doubling time when growth was measured with log-phase DBY3363 cells plated on YPD-agar (1.77 h doubling time) and when a hemocytometer was used to analyze the same cells grown in liquid YPD (1.55 h doubling time).

ATP secretion was measured with a LB953 AutoLumat luminometer, using the ApoSENSOR ATP Cell Viability Kit for analysis of samples after removing cells by centrifugation (3).

Plasmids:

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pMC1 pCM190 (from Euroscarf) with the tet-off promoter converted to tet-on promoter: The BamH1/EcoR1 fragment from pCM252 was inserted into similarly cut pCM190.
pMC2 pMC1 containing GFP-tub1-828, derived from DBY2963 (obtained from Dr. David Botstei).
pMC3 pMC1 containing TUB1.
pMC4 pMC2 with the 2 micron element deleted. The pcr product from primers starting at the Cla1 and the Hpa1 sites in pMC2 (from Euroscarf) was blunt end ligated and the product after cutting position 1014 in the tubulin sequence with BstEII was used for transformation. This generates a tandem repeat of TUB1 with the native promoter and tub1-828 with the tet-on promoter in Chromosome XIII.
pMC5 pCM242 with LEU2 replaced by LYS2. The inhibitor in pCM242, which is synthesized in the absence of tetracycline, prevents expression from the reverse tTA tetracycline activated promoter. LYS2 was cloned from pRS317 and inserted into the vector after cutting with Bsp106 and EcoRV.

Cells:

MCY001 DBY3363 with URA disrupted by hygromycin and LYS2 sequence disrupted by nourseothricin (from pAG25).
MCY002 MCY001 containing the inhibitor of the tetracycline promoter. pMC5 was linearized with BamH1 and used for transformation.
MCY005 MCY002, with a tandem repeat of TUB1 (with its native promoter) and GFP-tub1-828 (with the tetracycline promoter).
MCY006 MCY002, with a tandem repeat of TUB1 (with its native promoter) and GFP-TUB1 (with a tetracycline promoter), isolated as a MCY005 revertant.
MCY046 MCY002 with GFP-tub1-828 expressed from the tet-on promoter. The pcr product generated with primers from the non-coding sequence of TRP1 was used for transformation. Unless stated otherwise, all studies were done with this cell.
MCY047 Identical to MCY046 except containing the tet-off promoter normally found in pCM190 and tub1-828 rather than GFP-tub1-828.
MCY048: YNK1 disrupted by insertion of a TRP1 marker into MCY046.

Materials: Cryptopleurine was a generous gift from Dr. Paul Savage at CSIRO and was used as described (Hoyt et al., 1990).

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Fig. 1 tub1-828 is only toxic at high concentration. DBY6596, a diploid heterozygote with TUB1 and tub1-828 is viable as long as it contains pRB326, a CEN plasmid containing TUB1 expressed from a native promoter.

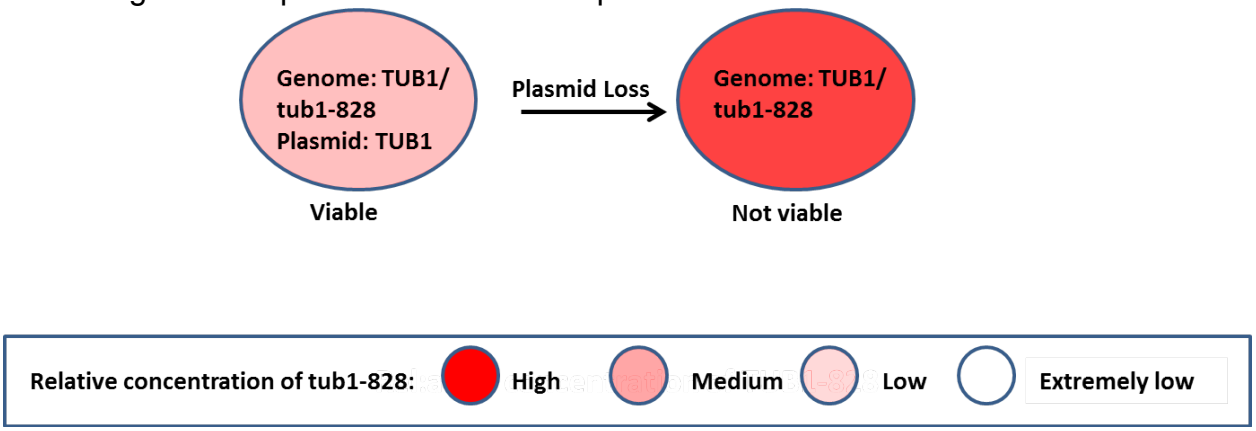

***Fig. 2*. *Mass spectroscopy of tryptic peptides from purified tub1-828*.** *Tubulin was purified as described (Davis et al., 1993) from MGY1(ura) containing pMC1/Tub1-828-His. This gave a tryptic peptide containing the mutant ALNA sequence at 2336.2124; there was no evidence of the corresponding wild-type peptide containing the DLNE sequence.*

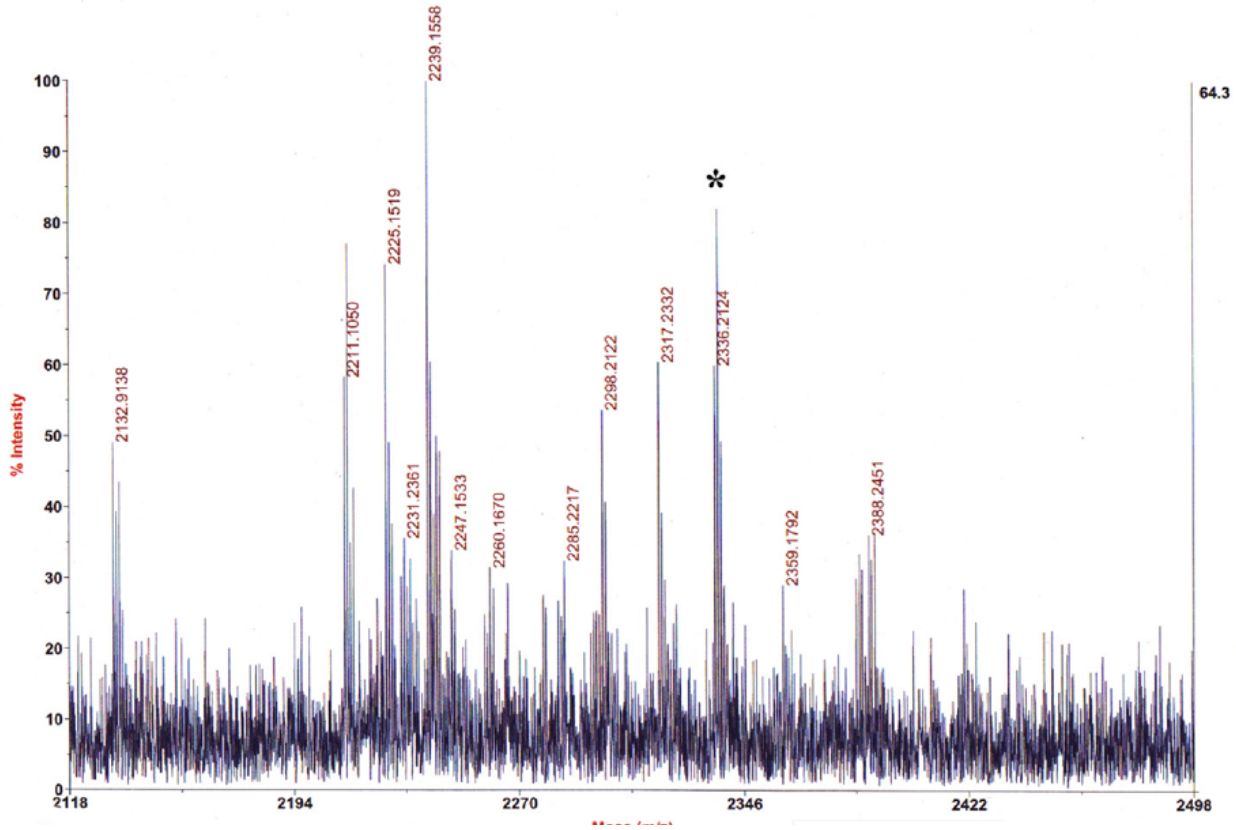

Fig. 3 The concentration of GFP-tubulin in cells is very low. An extract from cells expressing either GFP-TUB1 (MCY006), or GFP-tub1-828 (MCY005), was probed with an antibody (DM1-α) to α-tubulin to determine the relative concentrations of TUB1 (50 KDa) and GFP-TUB1 or GFP-tub1-828 (77 KDa) (left panel). Induction of GFP-TUB1 (lanes 1 and 2) and GFP-tub1-828 (lanes 3 and 4) for 4 and 16 hours show no signal other than that for TUB1. The right-hand panel shows that TUB1 and GFP-TUB1 would be readily separated by sds gel electrophoresis since porcine tubulin (lanes 5 and 6) was readily separated from BSA (66 KDa; lane 7) in a mixture of the two proteins (Lane 8). It is estimated that the concentration of GFP-tubulin is <10% that of TUB1 by assuming that this concentration would have been detected were it present in the results shown in Lanes 1-4.

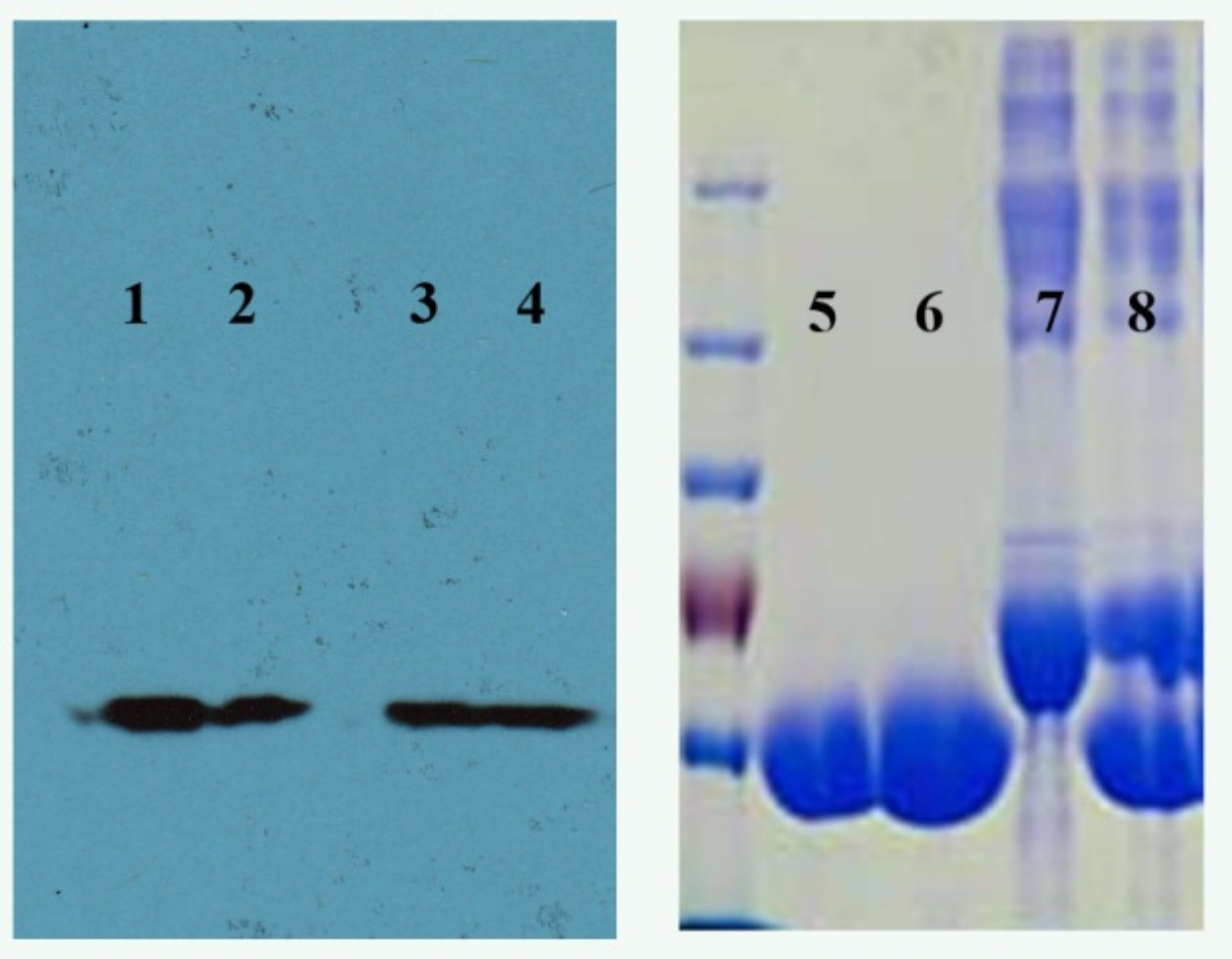

Fig. 4 **GFP-tubulin is incorporated into microtubules**. Cells expressing GFP-TUB1 (A, MCY002) or GFP-tub1-828 (B, MCY005) were grown in the presence of tetracycline. Adjacent images were recorded using dic and fluorescence illumination. Note that GFP-protein is not detected in every cell because: cells have not yet formed a mitotic spindle in which microtubules are bundled and therefore have a higher density of fluorescent protein; GFP-microtubules are not in the plane of focus; insufficient GFP-protein has been formed to be detected by the camera; GFP-tub1-828 has been photobleached so that can no longer be detected.

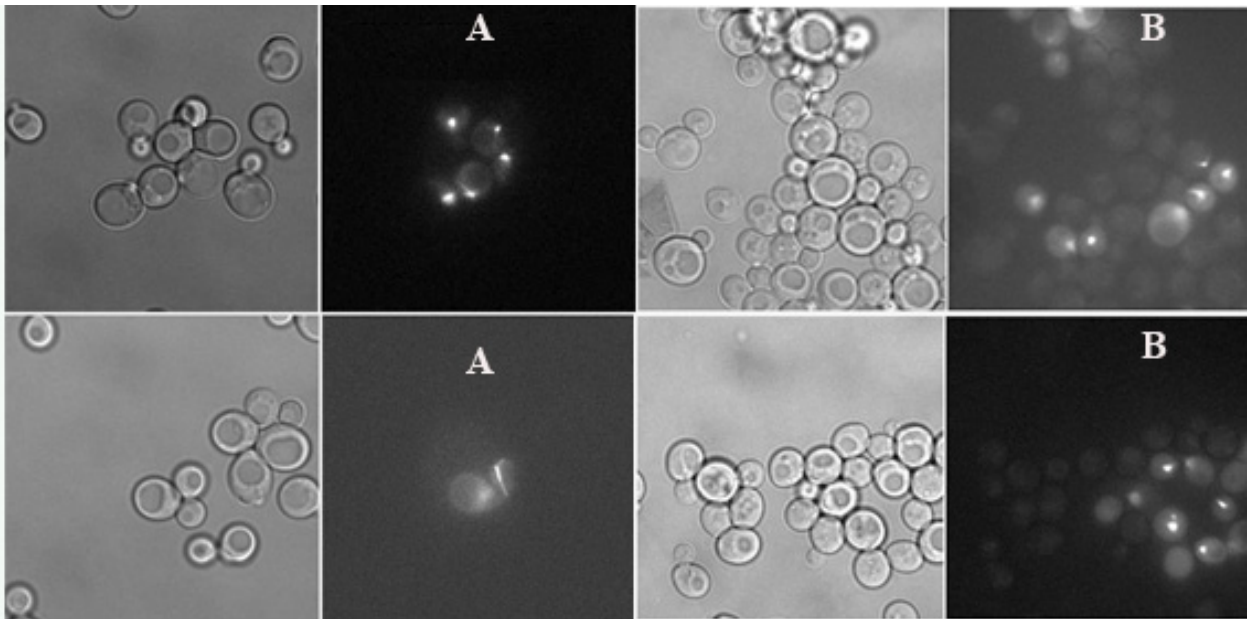

Fig. 5A. **Formation of micro and macrocolonies with cells (MCY046) expressing tub1-828**. Colonies with a 1 mm diameter colonies contain about one million cells. Note the variation in the size of the three colonies.

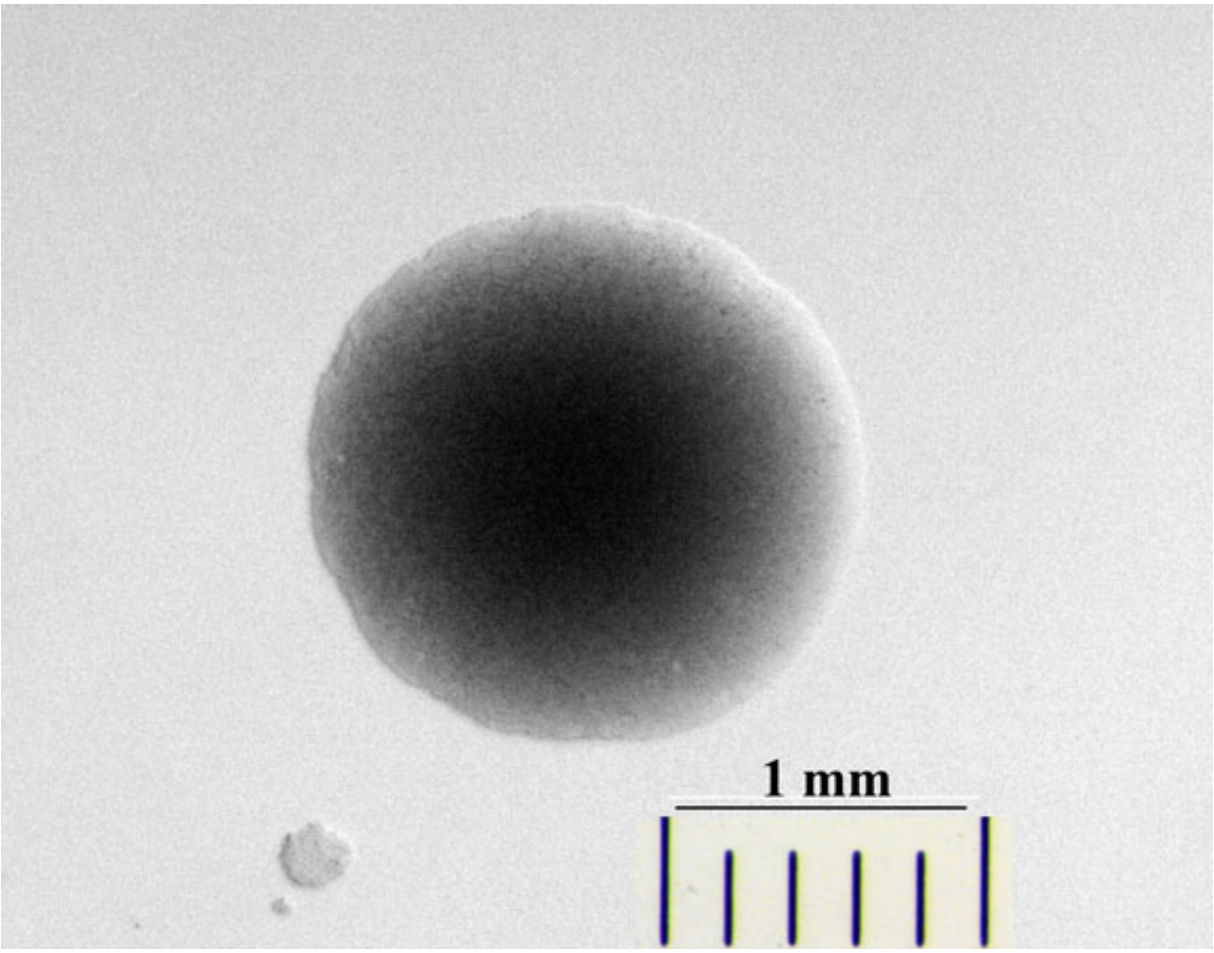

Fig. 5B. Formation of micro and macrocolonies from cells expressing tub1-828. The number of cell divisions in 48 hours was calculated from the diameter of colonies formed from stationary state cells that had been plated on YPD/tetracycline.

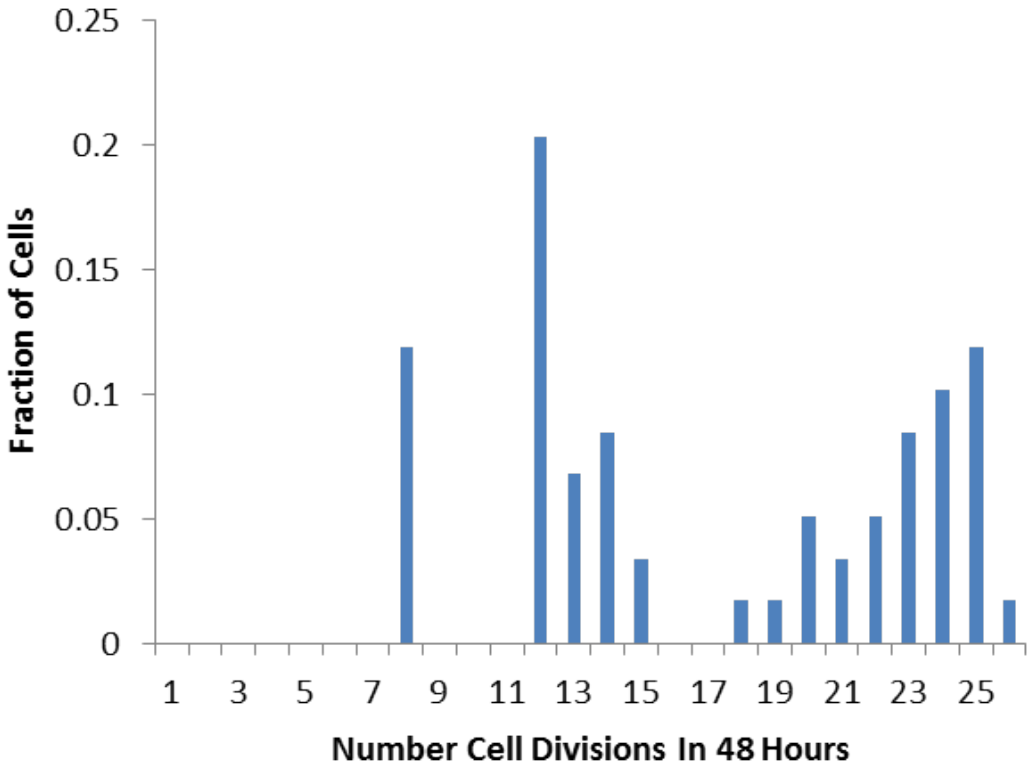

Fig. 6A Effect of inoculum size on cell growth of stationary state cells in the presence of tetracycline. Cells grown for 72-360 hours were regrown for 19 hours in the presence and absence of tetracycline; the number of apparent cells division was determined with a hemocytometer. Growth was limited by media exhaustion in all of the reactions without tetracycline.

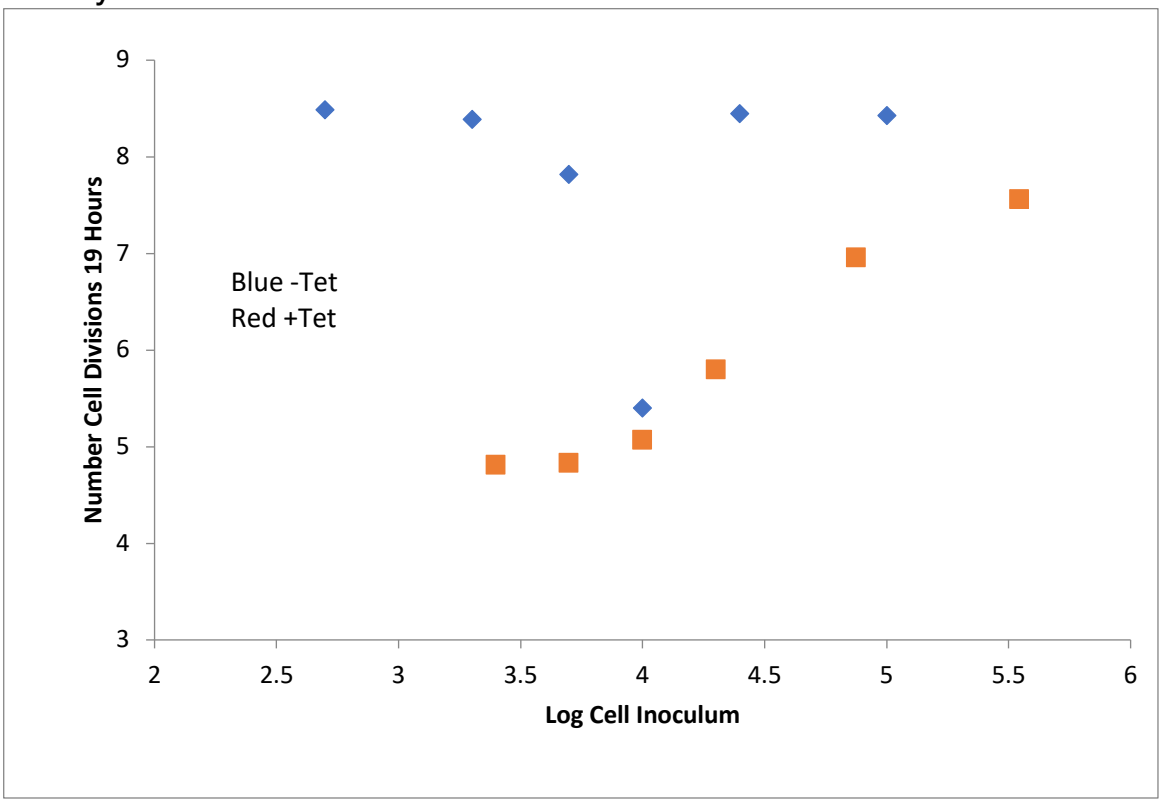

Fig. 6B Effect of inoculum size on growth of log phase cells in the presence of tetracycline. Results from three experiments are shown. In the experiment described with green symbols the supernumerary copy of Chromosome III was lost at a rate of 1/343 cells/division and 1/910 cells/division in the reactions at the highest and next-to-highest cell inoculum.

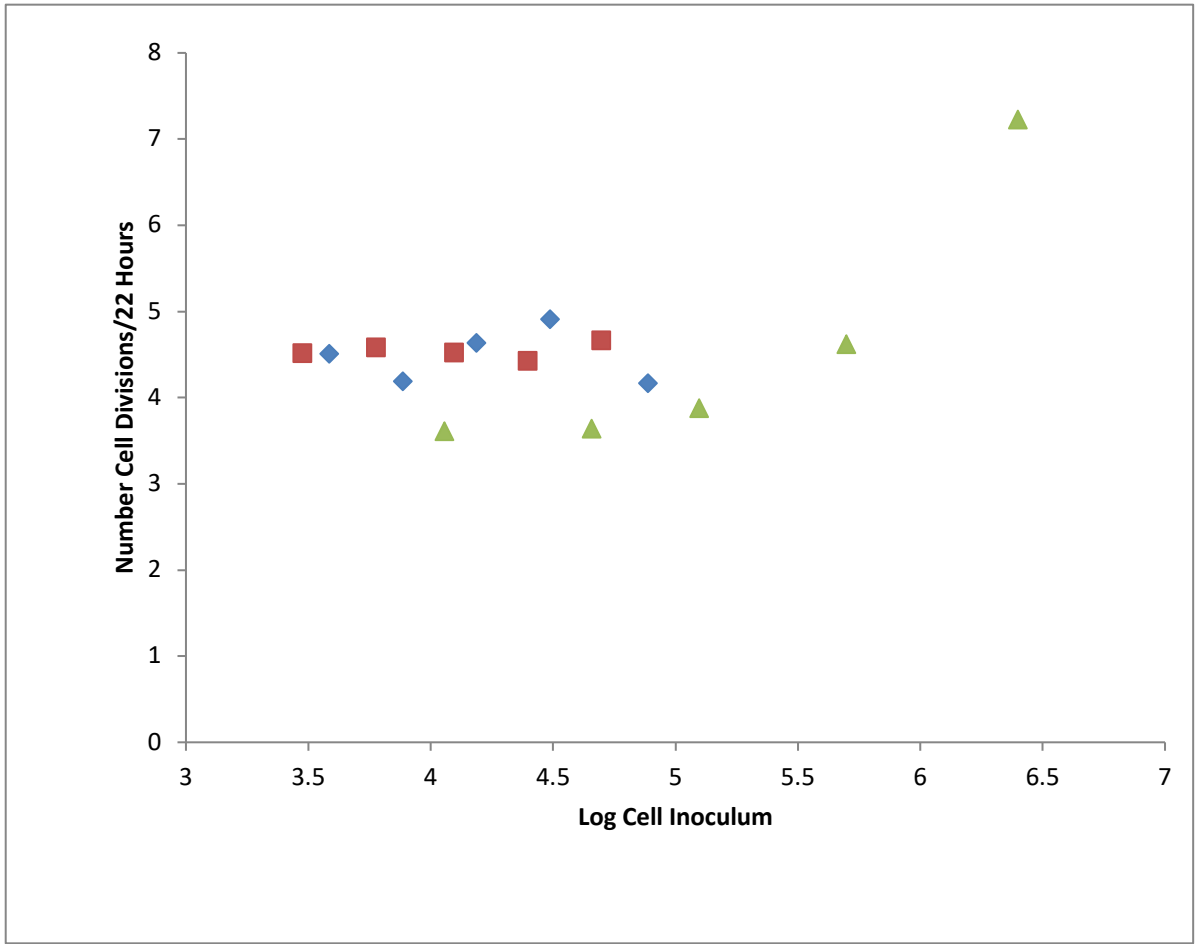

Fig. 7 Left DNA profile of stationary-state cells grown at low and at high cell density. *The left-hand and right-hand peaks from the cell sorting correspond to cells in G1 and G2; cells that fall between these peaks are in the process of chromosome replication.*

Fig. 7 Right Effect of nucleotides on the G1/G2 transition. Stationary state cells (A: zero-time) were grown in the absence of nucleotide for 5 hours at low density (B) and at high density (C). Growth at low density in the presence of 100 uM ATP (D) was similar to that at high density in the absence of nucleotide (C). Results with CTP (curve not shown) were identical to that for ATP.

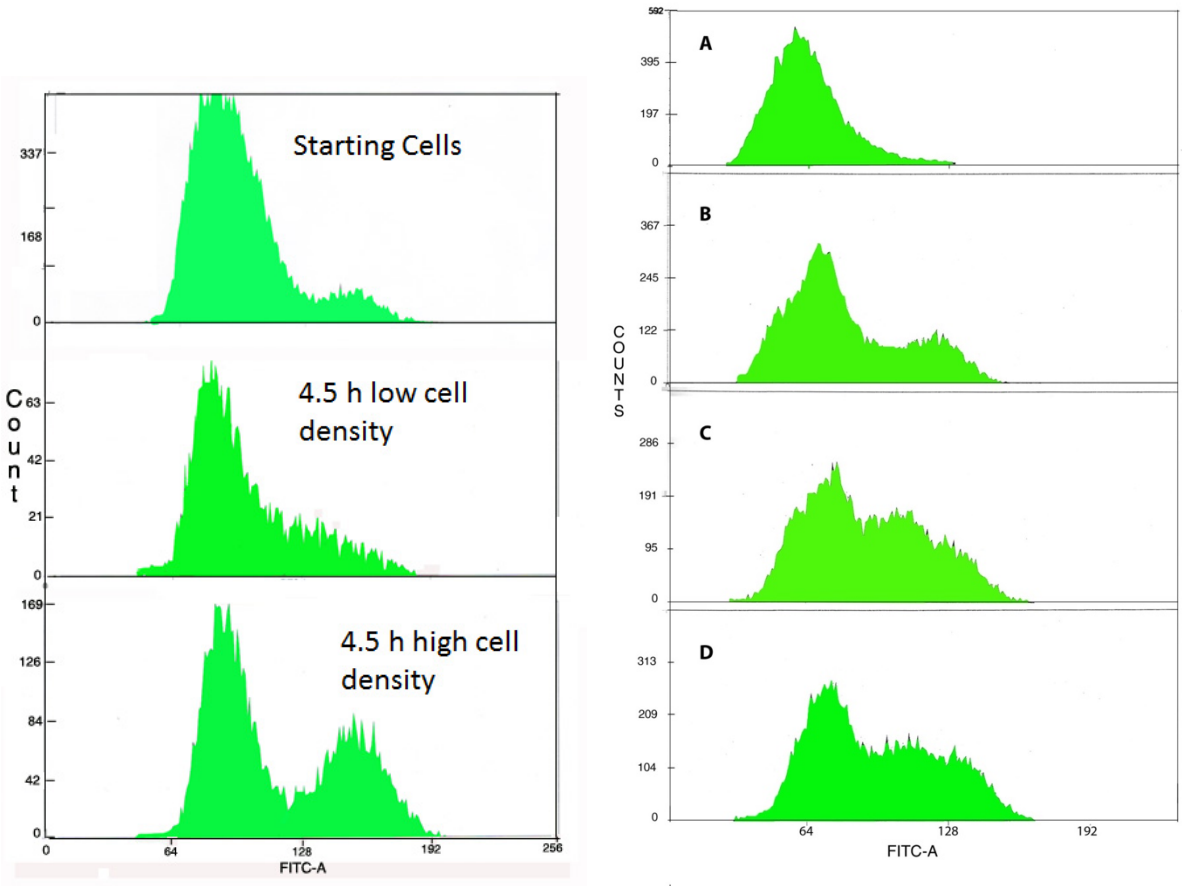

Fig. 8A Growth of cells on YPD with and without tetracycline and 100 uM ATP and ADP. The behavior of cells with CTP and GTP and tetracycline was similar to that with ATP.

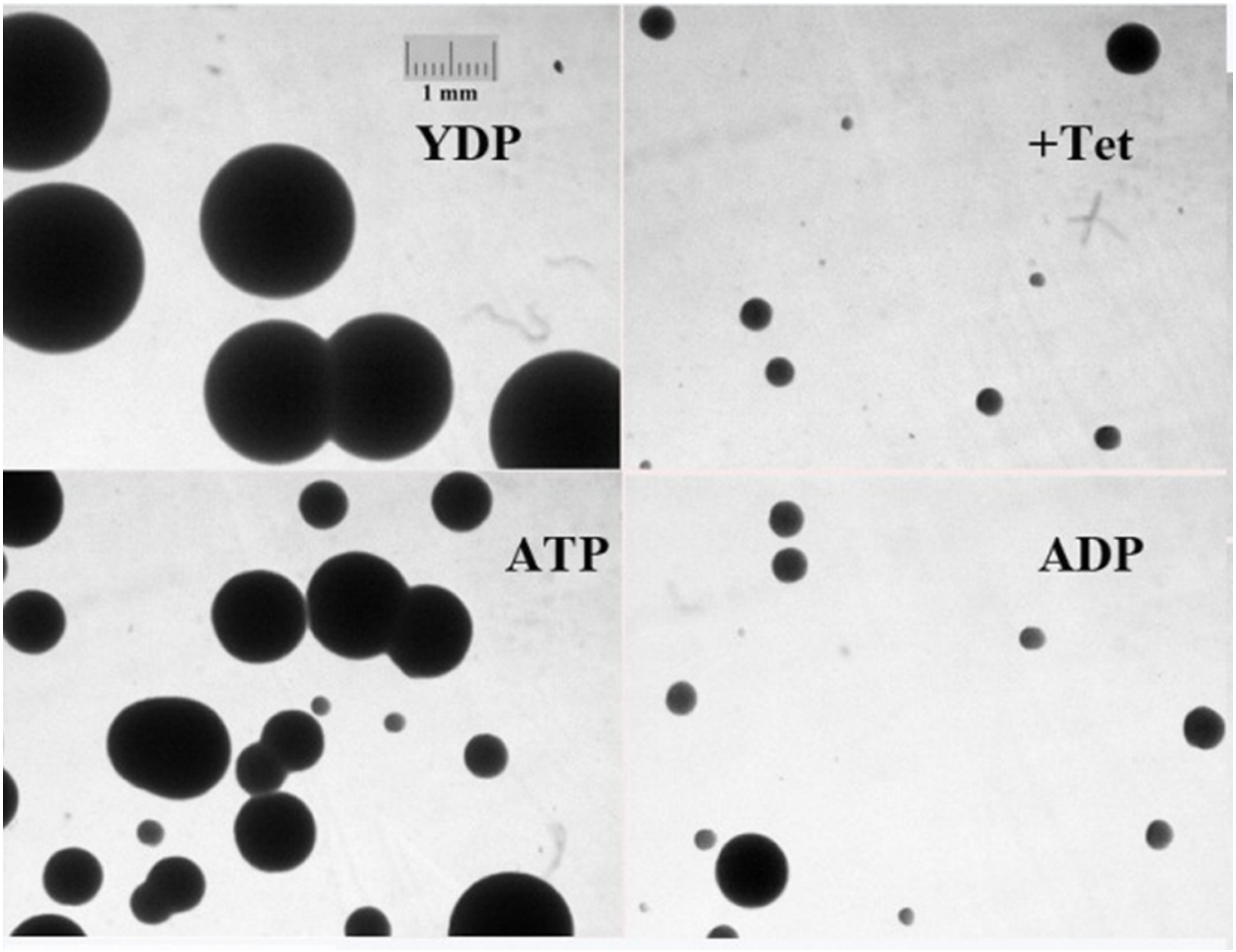

Fig. 8B Effect of 100 uM ATP and ADP on growth of cells expressing tub1-828.

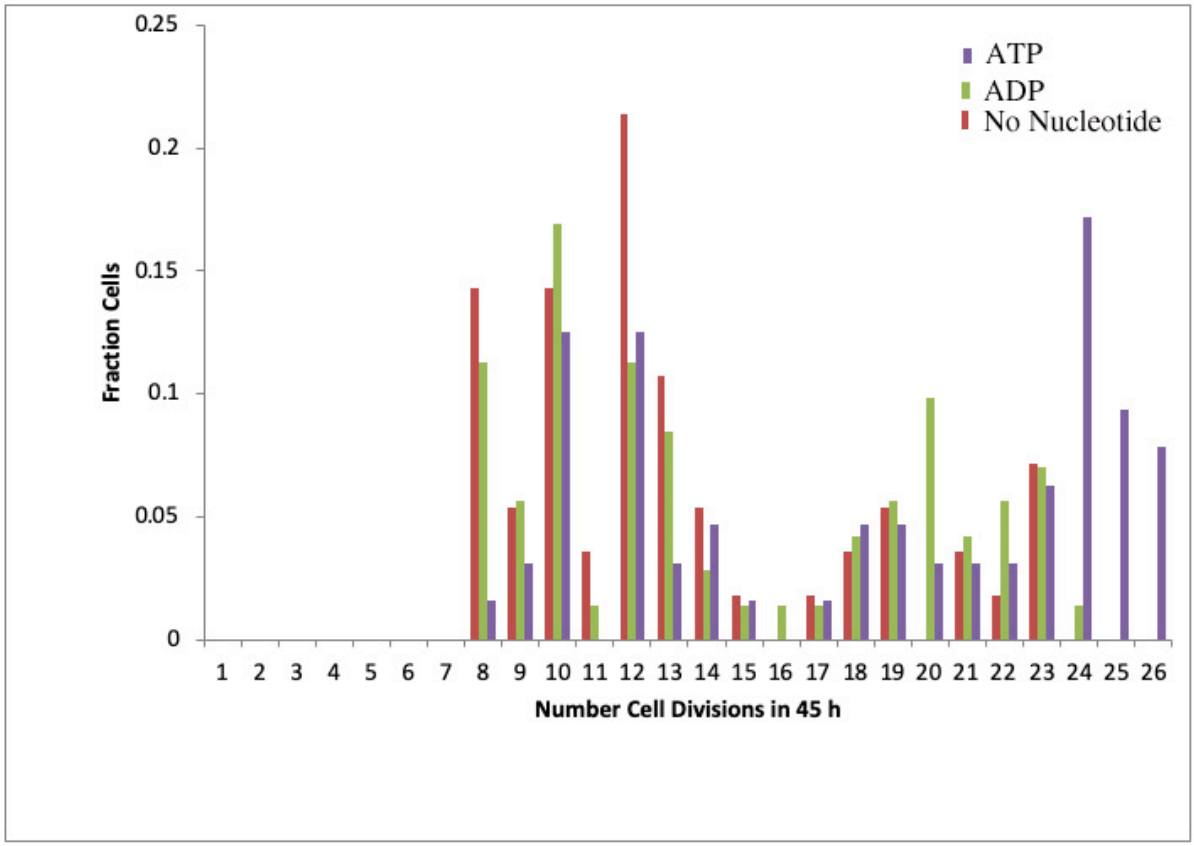

Fig. 8C Effect of ATP and CTP on Growth of Cells Expressing tub1-828.

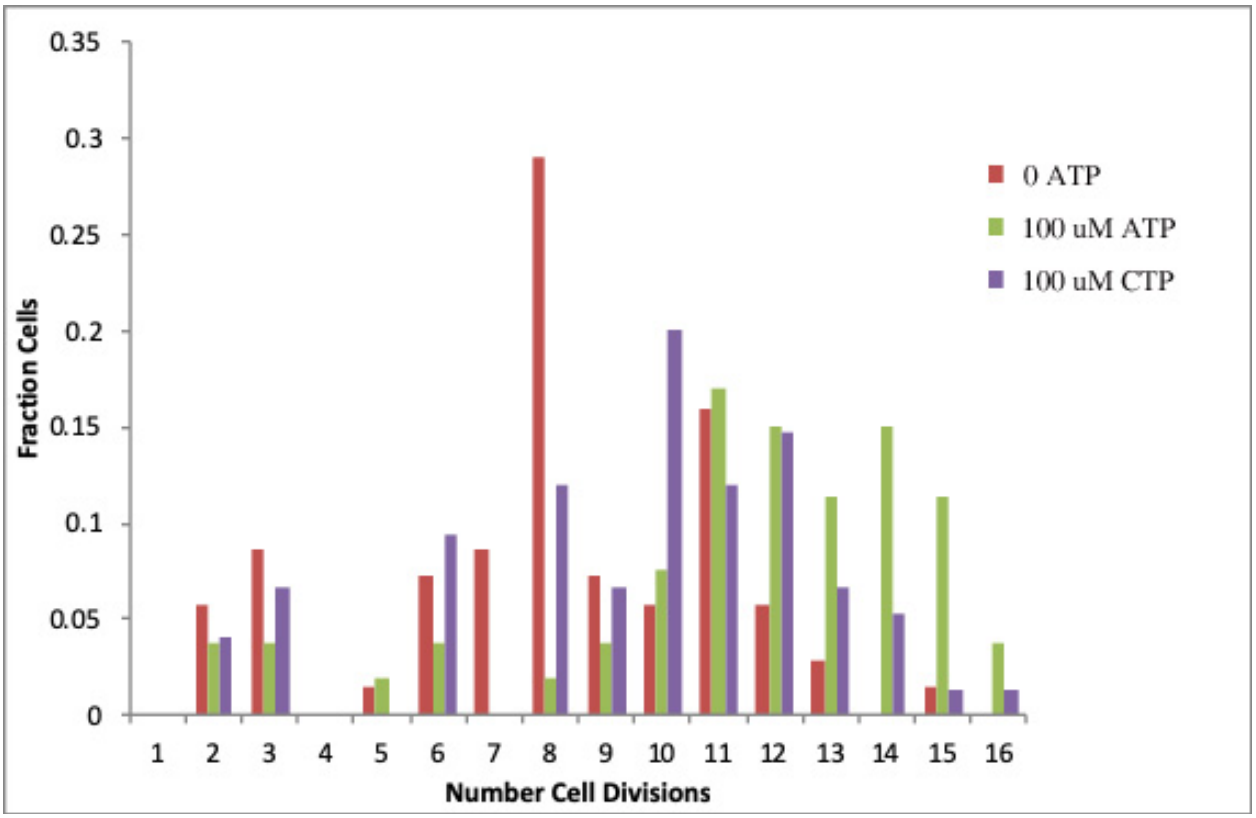

Fig. 9 Effect of ATP on growth of cells expressing tub1-828. 1129 cells that formed colonies on YPD generated 68 macrocolonies (>300 um diameter) on a YPD/Tet plate without ATP (left) and 159 with ATP (right). An additional experiment with ATP, GTP and CTP is below

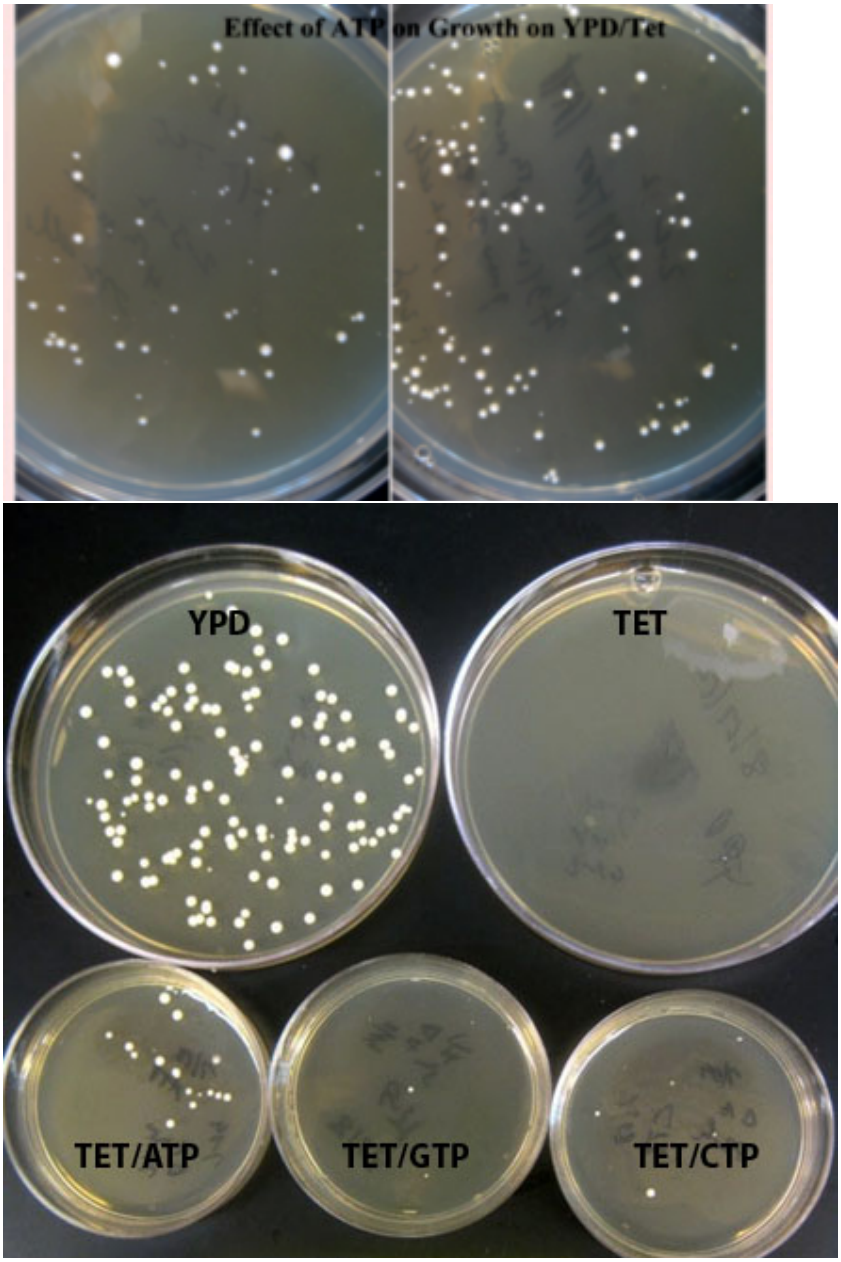

Fig. 10. Yeast ATP Secretion. The supernatant from centrifugation of cells (c. 3.7 x 10^7 /ml in TK buffer (2)) was assayed for ATP as described (2). A comparable rate was seen in YPD in the presence of tetracycline. The rate is approximately 250 molecules/cell/second.

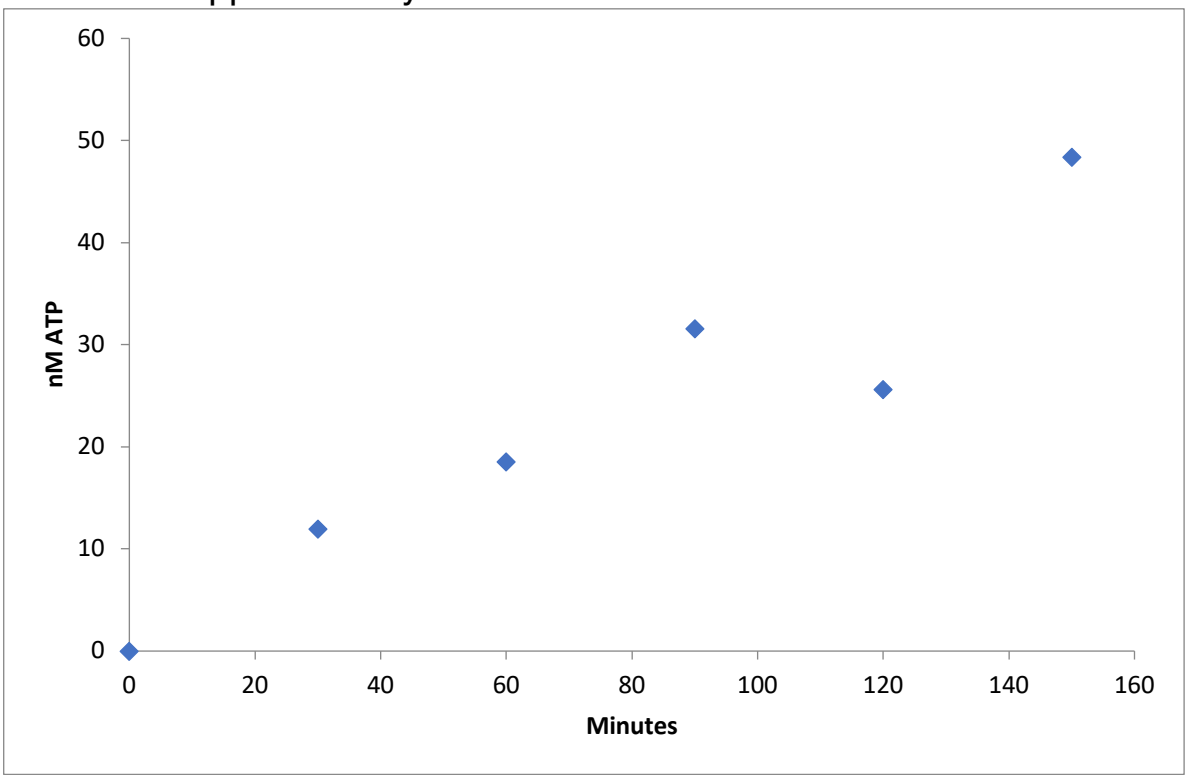

Fig. 11. Growth of ynk1 cells in the presence and absence of nucleotides. Cells from a saturated culture divided 19.19, 19.91 and 19.77 times on plates containing without nucleotide and with ATP and with CTP, respectively.

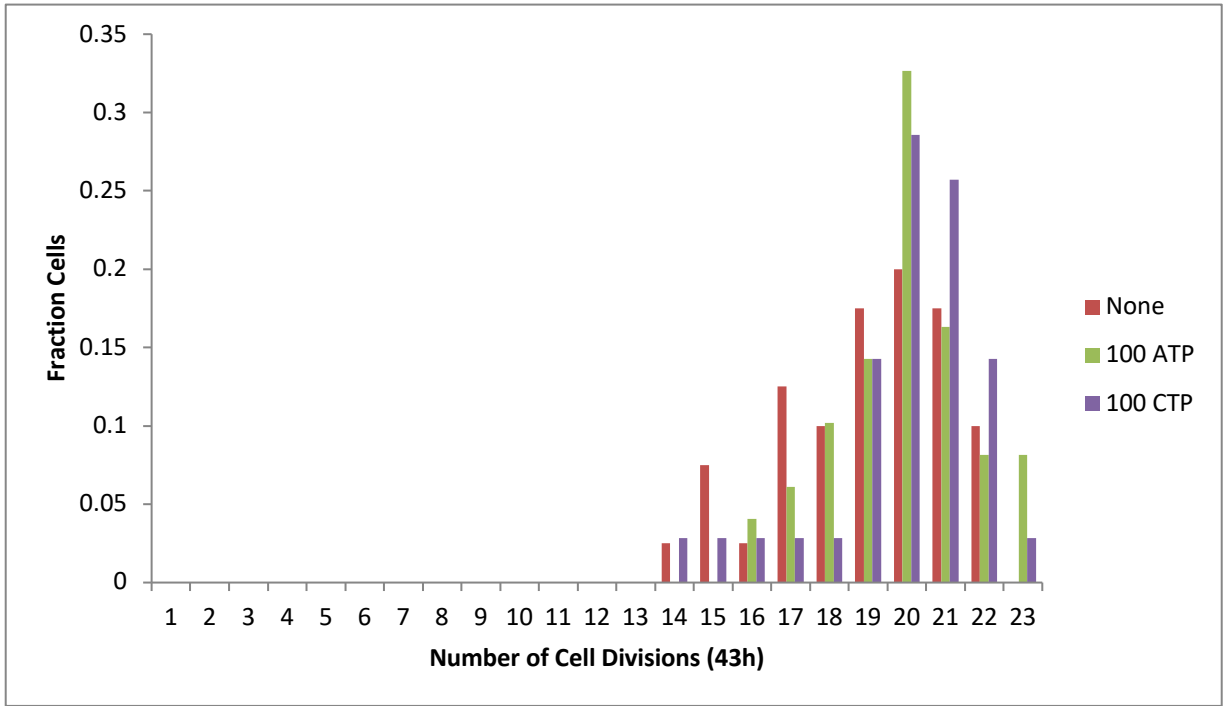

Fig. 12 Effect of inoculum size on growth of ynk1 cells (MCY048) in liquid media. Results from two experiments are given.

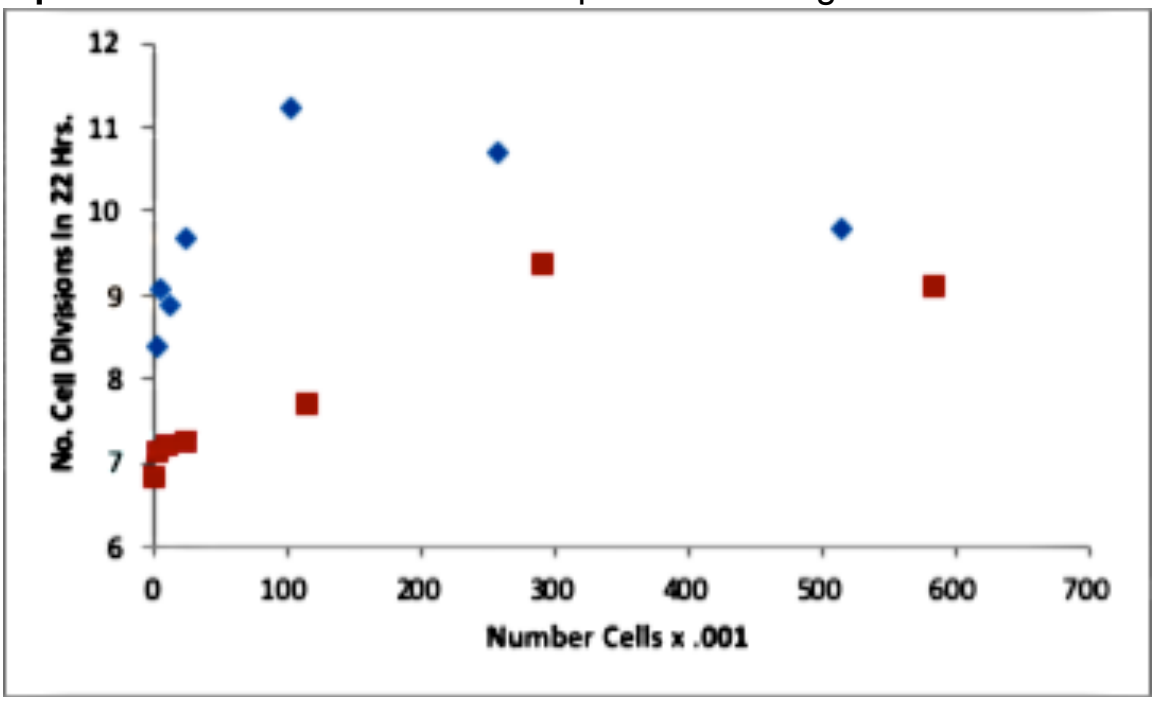

Fig. 13. Path for formation of macro and microcolonies during expression of tub1-828. Macrocolonies form if growth (and TUB1 expression) start immediately so that tub1-828 is diluted during the early phase for cell growth.

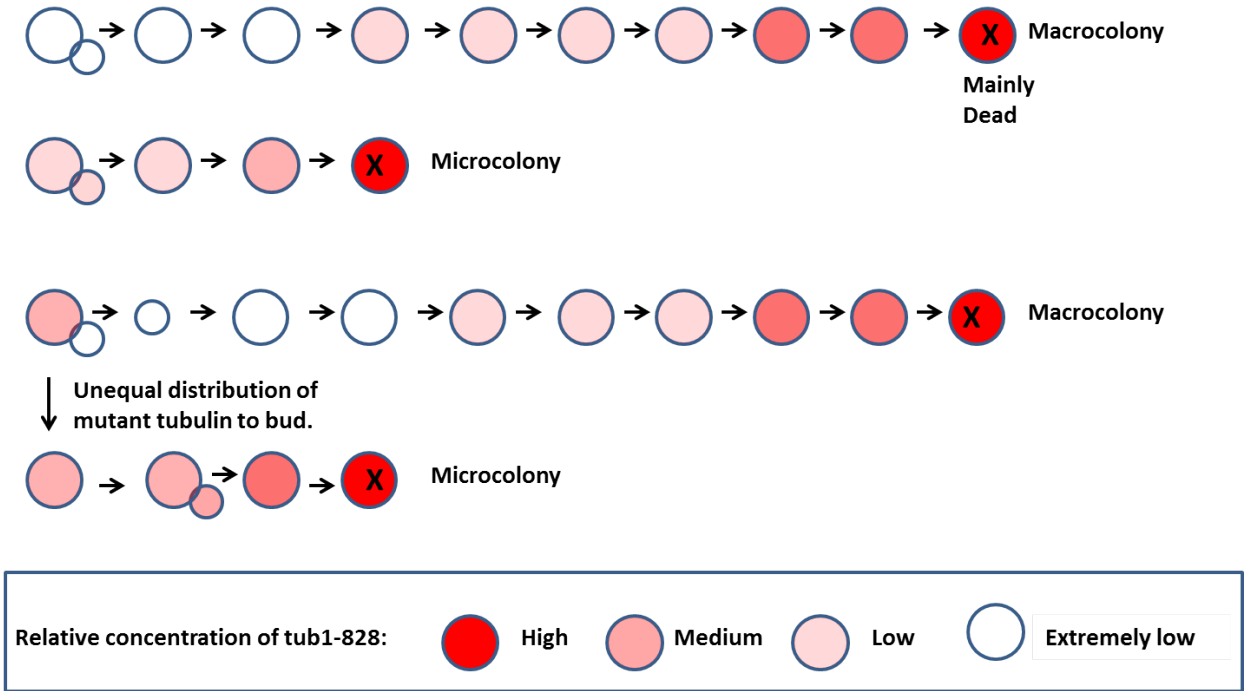

Table 1
Effect of Cell Density During Expression of tub1-828 on the Loss of Chromosomes

Inoculum 5 ml	Fold cell Increase in 20 hours	% Cells Viable	Predicted Cells Viable[1]	% Chr.III lost at 20 hours	% Chromosome loss/cell division[2]
350,000	188	83	85	.061	1.95
75,000	124				
20,000	56	11	16	.64	20.5
10,000	34				
5,000	28	2.8	2.9	1.12	35.8
2,500	28				
870,000		11		0.3	
17,400		1.7		50	

\

Cells from a stationary state culture were grown for 20 hours in the presence of tetracycline and the fraction of cells that were viable and fraction of viable cells that had lost a copy of chromosome III were determined by plating on YPD and on YPD with cryotopleurine, respectively. It was confirmed that red colonies were resistant to cryptopleurine. Results from two experiments are provided.

1. Calculated by assuming that cells divided 8-times in the 20 hours. For example, with cells that die at 1.95%/division (see results with a 350000 cell inoculum), it is expected that the fraction of viable cells will be equal to $(1-0.0195)^8 = 0.91$; the observed fraction viable cells was 0.83. For growth with

a 20,000 cell inoculum it is calculated that the fraction of viable cells after 20 hours would be 0.16.

2. The rate of chromosome loss/cell division was determined by multiplying the observed rate of loss of Chromosome III by two, since loss of the unmarked Chromosome III is not scored in the assay; this rate is multiplied by 16, the total number of chromosomes. Cells that lose chromosomes other than Chromosome III are not viable.

Table 2
Effect of Heterologous Cells on the Initial Rate of Growth of Cells Expressing tub1-828

Reaction	MCY046 Cells	ur Cells	Colonies
1	3440	2360000	670 ± 100
2	3440	0	346 ± 120

A small inoculum (3440 cells) of stationary-state MCY046 cells (URA, tet-tub1-828) was grown in 5 ml YPD/tet media with (Reaction 1) and without (Reaction 2) addition of 2360000 cells that were otherwise identical, except that they were ura-minus and did not express tub1-828. Cells were isolated by centrifugation. To be sure that there was equal pelleting of the low number of MCY046 cells in the two reactions 2360000 ura-cells were added to Reaction 2 immediately before cells were isolated by centrifugation. Pelleted cells were washed three times (to remove YPD) before plating on media without uracil. The colonies counted did not include those from ura-minus cells since no colonies were formed when 2360000 ura-minus cells were plated alone.