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The Effect of Yeast *Saccharomyces cerevisiae* Red Pigment on the Expression of Cloned Human α-Synuclein

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Abstract—Two transgenic yeast strains expressing human α-synuclein were used to study the impact of yeast red pigment exhibiting antiamyloid properties. It has been demonstrated that the endogenous red pigment produced under special conditions in strains carrying an *ade1* mutation inhibits the expression of the hybrid protein α-synuclein-GFP. This was evident from the reduced mean value of GFP fluorescence and diminished number of cells accumulating cytoplasmic inclusions of α-synuclein-GFP. Exogenous forms of the purified red pigment (natural, synthetic and hydrolyzed derivatives) differ from the endogenous red pigment by their effect on α-synuclein. Exogenous red pigments increased the number of both cells expressing GFP fluorescence and those containing cytoplasmic inclusions. However, both endogenous and exogenous red pigments reduced the cloned α-synuclein toxicity and resulted in redistribution of the α-synuclein in cells. α-Synuclein content decreased in cell lysate pellets and increased in supernatants.

Keywords: amyloid, α-synuclein, red pigment, yeast

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INTRODUCTION

Protein misfolding and aggregation are associated with various diseases. Accumulation of amyloid aggregates in tissues is a common feature of many degenerative disorders, Alzheimer's and Parkinson's diseases in particular (Alberti, 2012). Neurodegenerative diseases are predominant in Europe and the United States, and their frequency is increasing with time. The biggest problem is the lack of effective drugs that are able to block amyloid deposition, with treatment being purely palliative instead.

Parkinson's disease is the second most common neurodegenerative disorder after Alzheimer's disease. In developed countries, it affects around 1–2% of people 65 years old or older (Vishnevskaya et al., 2007; Corti et al., 2011). In the overall human population, its frequency is also high (0.3%), amounting to 3–4% of people 80 years old or older. Limited motor activity (bradykinesia), rigidity of facial and skeletal muscles, and resting tremor are symptoms of Parkinson's disease. This disease, was known already in ancient India as Kampavata. In 1817, James Parkinson described six cases of this disease. After a long period of neglect, his work was remarked by the renowned neurologist Charcot, who named the disease after Parkinson.

The disease is accompanied by loss of dopaminergic neurons in substantia nigra pars compacta (SNpc) (Wales et al., 2013). Surviving neurons accumulate cytoplasmic inclusions known as Lewy bodies, which were named by K.T. Tretyakov in honor of Dr. Fritz Lewy (a colleague of Prof. Alzheimer), who was the first to describe these structures (Goedert et al., 2013). Lewy bodies in Parkinson's patients accumulate a large amount of α-synuclein protein in fibrillar aggregates (Spillantini et al., 1997, 1998; Baba et al., 1998). Transmission of α-synuclein pathology between neurons suggests that Parkinson's disease has prionlike features (Prusiner, 2013; Goedert, 2015).

α-Synuclein is composed of 140 amino acids. Under normal, nonaggregated conditions, this protein exhibits neuroprotective properties. It is located in presynaptic nerve terminals and, occasionally, in neuron nuclei. Its key function is the regulation of neuromediator release underlying the neuron impulse transmission. α-Synuclein aggregation results in damage to dopaminergic nerve cells. Details of protein aggregation and its role in the manifestation of Parkinson's disease are presented in reviews (Breydo et al., 2012; Stefanis, 2012; Wales et al., 2013).

[1] *Abbreviations:* Pig^+ and Pig^-—presence and absence of red pigment, respectively; (r) and (w)—red and white cells, respectively; GFP^+—positive fluorescence of green fluorescent protein; PI^+—positive staining with propidium iodide; Mn—mean value of fluorescence of GFP^+ cells; MeAIR—synthetic red pigment; P0 and P1—fractions of exogenous red pigment; PHyd—hydrolyzed derivative of P1 pigment.

Numerous studies have led to the appearance of a consensus on the disease pathogenesis. α-Synuclein first produces soluble oligomeric forms of different sizes (protofibrils). Later, they lose solubility and fuse into amyloid fibrils of Lewy structure. The process is regarded as a fundamental pathogenic factor and a promising target for developing therapeutic agents to cure Parkinson's disease (Stefanis, 2012).

It has been shown that L-dopamine (levodopa) helped patients with Parkinson's disease, but did not prevent the disease progression. Clinical research on dozens of drugs failed to find one that showed promise for medical practice (Racette and Willis, 2015).

Saccharomyces cerevisiae yeast with cloned genes of human α-synuclein is a widespread test system for agents affecting α-synuclein aggregation and amyloidization (Outeiro and Lindquist, 2003). α-Synuclein expressed in yeast produces cytoplasmic inclusions similar to Lewy bodies in higher organisms. It has been demonstrated (Soper et al., 2008) that production and aggregation of cytoplasmic vesicles induced by α-synuclein is indeed analogous to the formation of Lewy bodies in patients with Parkinson's disease. The yeast system is regarded as one of the most adequate Parkinsonism models (Franssens et al., 2010; Breydo et al., 2013).

Our research is based on the fact that, in a number of inherited cases, multiplication of the α-synuclein locus and resulting protein overproduction cause Parkinson's disease. The phenomenon is used to study the mechanisms of the pathogenesis. *S. cerevisiae* yeast with integrated plasmid containing two copies of the recombinant gene αSyn-GFP responsible for the synthesis of both α-synuclein and green fluorescent protein (GFP) (Outeiro and Lindquist, 2003; Miller-Fleming et al., 2008; Tenreiro and Outeiro, 2010; Tenreiro et al., 2013) was used as a model organism.

Expression of hybrid protein αSyn-GFP helps to track the accumulation and aggregation of α-synuclein in living cells under a luminescent microscope. Genes responsible for the synthesis of the hybrid protein are controlled by the galactose-driven promoter GAL1p. Protein expression is triggered only by galactose, but not with other carbon sources, such as raffinose or glucose.

We were provided with yeast strains constructed as described above. The effect of red pigment yielded by yeast cells with ade1 and ade2 mutations on amyloidogenesis has been previously studied nu the Group of Mitosis Genetics, Laboratory of Structural Dynamics, Stability, and Folding of proteins, Institute of Cytology, Russian Academy of Sciences. The red pigment was isolated and chemically characterized as a polymer of aminoimidazole ribotide in 1967 (Smirnov et al., 1967), but its impact on amyloids has been investigated only in recent years. We found that the red pigment inhibited aggregation and amyloidization of a number of cellular proteins in vivo and in vitro—yeast prion [PSI$^+$], in particular—as well as yeast-expressed human β-amyloid, which plays an important role in the pathogenesis of Alzheimer's disease. The red pigment reduces neurodegenerative lesions, restores behavior functions, and improves the memory in transgenic *Drosophila* receiving red yeast as food (Mikhailova et al., 2011; Nevzglyadova et al., 2011; Amen et al., 2012 Nevzglyadova et al., 2015).

Here, yeast cells transformed with human α-synuclein fused to GFP (αSyn-GFP) were used as a model to study the effect of endogenous exogenous red pigment on the expression and aggregation of this recombinant protein.

MATERIALS AND METHODS

Yeast strains and their cultivation. VSY71, VSY72, and VSY73 strains were kindly provided by Dr. T. Outerio at the Institute of Molecular Medicine, Lisbon University, (Portugal). They were constructed based on W303-1A (*MAT a can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1 ade2-1*) strain. Strain VSY71 (W303-1A *trp1-1::pRS304 TRP1+*; *ura3-1::pRS306 URA3+*) has two integrated copies of pRS304 vectors and is a control for VSY72 and VSY73 strains. VSY72 strain has two copies of cloned nucleotide sequence encoding human wild-type α-synuclein (W303-1A *trp1-1::pRS304 GAL1pr-SNCA(WT)-GFP TRP1+; ura3-1::pRS306 pRS306GAL1pr-SNCA(WT)-GFP::URA3+*). VSY73 strain carries S129A mutation, resulting in dephosphorylation of the amino acid in position 129 (W303-1A *trp1-1::pRS304 GAL1pr-SNCA(S129A)-GFP TRP1+; ura3-1::pRS306 GAL1pr-SNCA(S129A)-GFP::URA3+*). In both cases, α-synuclein gene is driven by GAL1 promoter and fused with the sequence encoding GFP. The culture media and yeast manipulation methods were as described (Sherman et al., 1986). α-Synuclein was induced with a selective medium containing 2% galactose. The control medium with 2% raffinose did not induce α-synuclein expression.

Isolation and modification of yeast red pigment. The pigment was isolated from 23S (MATα *ade2*) strain at the stationary growth phase. The strain was kindly provided by Dr. Zekhnov (St. Petersburg State University). The pigment was isolated according to the method described in (Smirnov et. al., 1967) with some modifications (Nevzglyadova et al., 2011). Cells with cloned α-synuclein were treated with natural pigment p derivatives P0 and P1 obtained by p elution with 0.1 M HCl on DEAE-sepharose (first, a more alkaline P0 fraction not requiring neutralization is eluted, and then a P1 fraction with low pH is released, which was neutralized before its purification on a Sephadex column). We also used the pigment PHyd resulting from p acid hydrolysis and its synthetic analog MeAIR. Isolation of various pigment derivatives was previously described in (Amen et al., 2012). All these pigment forms were used in approximately the same concentra-

tions (0.05 M water solutions). It is difficult to adjust the dose precisely, because the pigment is a mixture of varied in size AIR polymers with bound amino acids.

The culture growth was monitored with spectrophotometry by optical density registered at 660 nm (OD_{660}).

Flow cytometry. GFP^+ cell number, mean fluorescence intensity (Mn) of GFP^+ cells, and cell death were assessed with a Coulter EPICS XL cytometer (Beckman Coulter, United States) equipped with an argon laser (emission wavelength 488 nm) with a maximum flow rate of 1 μL/s. Dead cells were assayed with propidium iodide (PI). This fluorescent dye penetrates into dead but not live cells (Tenreiro et al., 2014b). The number of dead cells (%) was estimated with two-dimensional histograms showing GFP against PI fluorescence.

Microscopy. Cell number with cytoplasmic inclusions of hybrid protein αSyn-GFP was counted under an Axiovert Zoom (Zeiss, Germany) luminescent microscope using a 40×/0.75 objective, E10 filter, and DFC 420c, 2592 × 1944 camera. About 500 cells were counted in each of from four to eight repeats.

Preparation of pelleted and supernatant protein fractions from cell lysates. Yeast cells were grown in solid or liquid selective medium for 5 or 2.5 days, respectively. Crude cell lysate preparation and fraction separation after centrifugation at 1000 *g* was performed as described (Nevzglyadova et al., 2015). After isolation of two lysate fractions, the supernatant was precipitated with ethanol at −20°C for 12 h. Protein fractions were extracted with different buffers. To measure amyloid quantity from thioflavin T fluorescence, the pellet was suspended in sodium-glycine buffer (70 mM NaOH, 130 mM glycine). Thioflavin T was added as described in (Nevzglyadova et al., 2009). Pellets were prepared for Western blotting by suspension in a buffer composed of 5% SDS, 0.15 M Tris-HCl and 50 mM DDT, pH 6.8 (Laemmli, 1970).

Electrophoresis and Western blotting. Proteins from different fractions of crude lysates were resolved in 10% SDS-PAAG (Laemmli, 1970) and transferred onto PVDF membrane (Biorad, Hercules, United States) soaked in Towbin buffer (25 mM Tris-HCl, 192 mM glycine and 20% methanol, pH 8.3) (Towbin et al., 1979) together with BLUeye stained marker proteins (GeneDireX, United States). α-Synuclein was detected with primary anti-α synuclein MJFR1 antibodies and secondary goat antirabbit antibody HRP (ab138501 and ab6721, respectively) (Abcam, United States).

All experiments were performed in triplicate. The results are presented as mean values with confidence interval $p < 0.05$.

RESULTS

The effect of endogenous yeast pigment on α-synuclein expression in VSY72 and VSY73 strains. Experiments were performed on selective agar plates with various adenine concentrations.

With a low amount of adenine 10 mg/L, the cells accumulate red pigment for 5–6 days, whereas, at a higher adenine concentration 100 mg/L, the cells remain white. Red Pig^+ (r) and white Pig^- (w) cells were compared by number of cytoplasmic α-synuclein inclusions visible under a luminescent microscope. The percentage of GFP^+ and PI^+ (dead) cells and mean value of their fluorescence (Mn) were estimated with flow cytometry.

It was found that red pigment impeded α-synuclein aggregation in both stains. Under conditions promoting α-synuclein expression (galactose medium) white derivatives in 5–6 days form inclusions in 10 and 20% of cells (VSY72 and VSY73 stains, respectively), whereas the cell number with inclusions declined to 0.5 and 3%, respectively, in red derivatives (Fig. 1a).

Pig^+ and Pig^- cells of VSY72 and VSY73 strains grown under conditions inducing α-synuclein expression differ in mean GFP fluorescence and dead cell number (Figs. 2a, 2b). Pigment accumulation in stationary phase yeast cells diminished both parameters. Mn value and percentage of dead cells did not differ in both r and w cells grown on raffinose (no α-synuclein induction) and declined in both cases.

After of α-synuclein induction Mn value and percentage of dead PI^+ cells in white derivatives was higher in VSY73 strain than in VSY72. Pig^+ cells in both stains showed no difference in Mn value and number of dead cells.

Pigment effect on the aggregate size was assessed by redistribution of cellular amyloid between the pellet and supernatant from crude lysates of cells producing or not producing α-synuclein. Cells of both strains were cultivated for 5 days in the selective medium with galactose or raffinose. Crude cell lysates were adjusted for protein content and centrifuged at 1000 *g* for 30 min. Pellet proteins with aggregated molecules were compared with supernatant proteins of relatively low molecular weight. The amyloid amount in both fractions was quantified by cell staining with fluorescent dye thioflavin T specifically binding with amyloids (Figs. 3a–3d). The fluorescence intensity of pellet proteins from red cells of the VSY72 strain was lower than in white cells, and supernatant proteins differed antithetically (Fig. 3a). In cultures grown on raffinose (Fig. 3b), there was no difference between protein fractions from r and w cells. Similar results were obtained with VSY73 cells (Figs. 3c, 3d). This suggests that the amount of amyloid in r and w derivatives of VSY72 and VSY73 strains mostly depends on the quantity of amyloid form of the cloned α-synuclein.

More direct information on red pigment effect on aggregation of α-synuclein was obtained in experiments using blot hybridization. Proteins from pellet and supernatant fractions isolated from red and white cells of VSY72 and VSY73 strains were separated with PAGE and hybridized with antibodies to α-synuclein. Figure 3e shows that, in both strains, the amount of α-synuclein in a pellet (i.e., aggregated α-synuclein) is reduced in the presence of red pigment while weakly or not nonaggregated protein remains in the supernatant (Fig. 3f).

Effect of culture conditions and yeast exogenous pigment on α-synuclein. The influence of exogenous red pigment on α-synuclein was studied with VSY72 and VSY73 strains. Most experiments were done with natural pigment isolated as described (Nevzglyadova et al., 2011). It was of interest to compare its effect with that of other two forms of red pigment having a much lower molecular weight. One form was obtained with acid hydrolysis, the other one was a synthetic red pigment resulted from aminoimidazole ribotide polymerization (Amen et al., 2012).

Pigments were added into the liquid synthetic galactose medium simultaneously with inoculate. Yeast culture conditions were optimized for generation of α-synuclein inclusions. The cells were maintained in liquid medium on the flask surface at 30°C without agitation as a monolayer. After 12–20 h, about 30% cells in the VSY72 strain and 60% in VSY73 strain had α-synuclein inclusions (Fig. 1b). Inoculated cell number is a crucial factor. Cells used for inoculation were grown overnight on raffinose- or glucose-containing agar plates. Inoculate OD_{660} was adjusted to 30 arb. units, and 3 μL inoculate was used for 250 μL synthetic medium with galactose.

Figure 4 shows that cells from all three strains grown in monolayer and agitation reached the stationary growth phase after 24 h. VSY71 strain having a vector without α-synuclein encoding sequence exhibit the same growth dynamics with and without agitation. VSY72 and VSY73 cell growth was stimulated with agitation. Their growth rate was different and both strains differed from VSY71 control. These differences, supposedly, relate to various capacity of VSY72 and VSY73 strains to accumulate α-synuclein inclusions.

We tried to answer the question whether there was a positive correlation between accumulation of inclusions and cell death in VSY72 and VSY73 strains grown with and without agitation. We also used flow cytometry to determine the expression of α-synuclein, i.e., GFP^+ cell percentage and Mn value. Cells were cultured in 750 μL synthetic medium with or without agitation, and 60 μL were used for testing after 6, 12, 24, 36, and 48 h. Mean values from four repeats are presented graphically in Fig. 5. The Mn value in VSY72 strain grown with agitation declined for 6–24 h, but later the difference was not evident. It should be noted that, in the beginning and end of the growth.

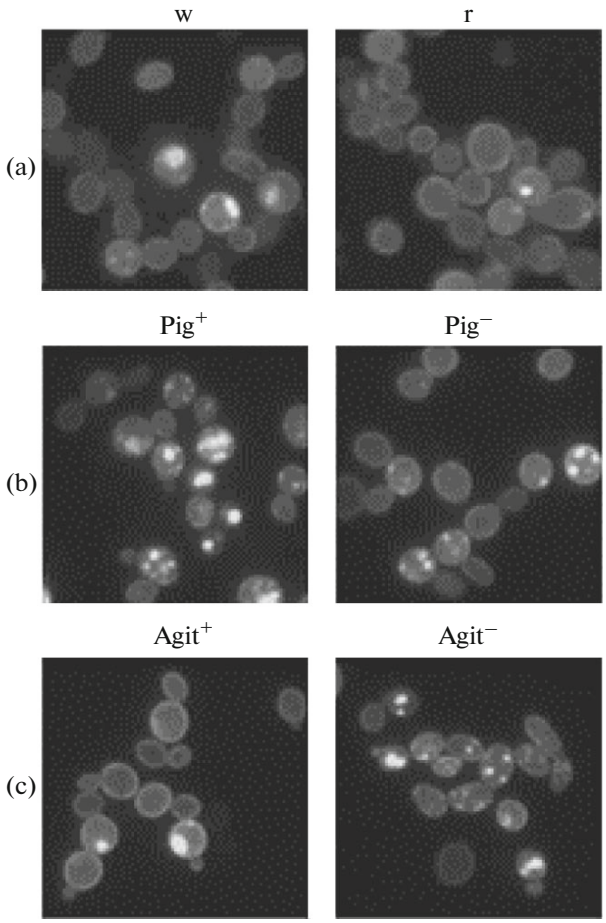

Fig. 1. Generation of α-synuclein inclusions in VSY73 yeast strain (a) containing endogenous pigment and (b) exposed to exogenous pigment or (c) grown without agitation. w—white cells; r—red cells (accumulated pigment); Pig^+—cells cultivated with P1, a red pigment derivative; Pig^-—cells grown without added pigment; $Agit^+$—grown with agitation; $Agit^-$—grown without agitation.

the cells exhibited nearly equal mean fluorescence (about 40 arb. units). VSY73 strain displayed the difference only after 36 h. As in VSY72 strain, agitation diminished the Mn value (Figs. 5a, 5b). Its maximum value was higher in VSY73 strain than in VSY72 under all tested growth conditions.

The number of GFP^+ cells was higher in VSY72 strain than in VSY73 both with and without agitation (Figs. 5c, 5d). After 12 h, their number was higher in cultures maintained with agitation. The percentage of cells with aggregates was higher in VSY73 strain than in VSY72 strain (Figs. 5e, 5f). In both strains, their quantity increased in cultures grown without agitation.

Figures 5g and 5h show that cell death depends on culture conditions. In both strains, more dead cells were observed in cultures grown without agitation. The strains differed in cell death, it being higher in VSY73 strain.

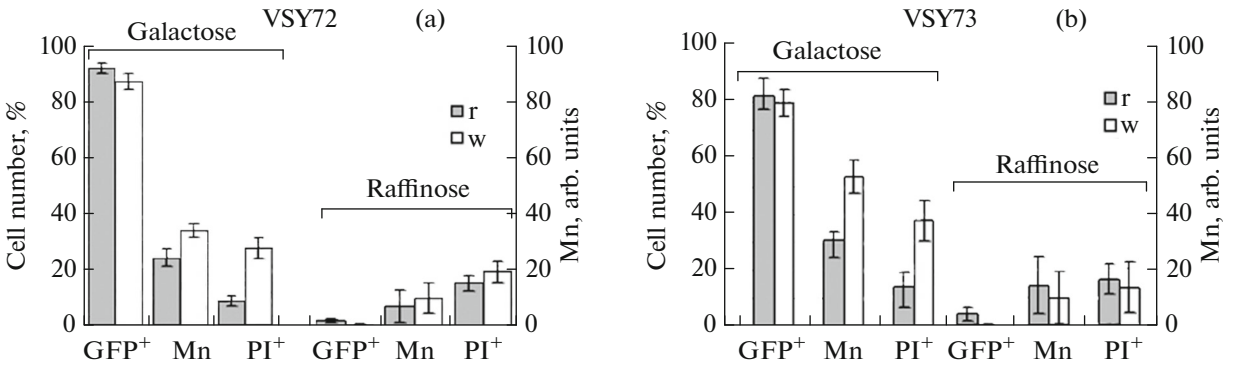

Fig. 2. The effect of endogenous pigment on fluorescence intensity (Mn), percentage of GFP^+ and PI^+ cells of (a) VSY72 and (b) VSY73 strains grown on a selective medium for 5 days.

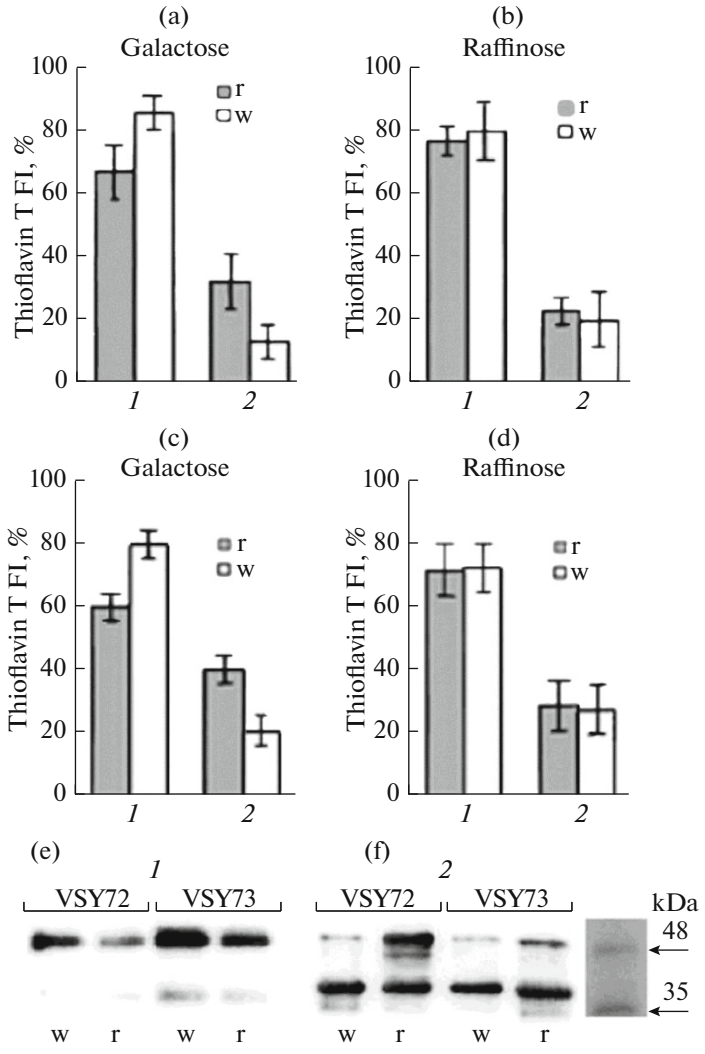

Fig. 3. Amyloid distribution between (*1*) pellet and (*2*) supernatant in cell lysates of r- and w-derivatives of (a, b) VSY72 and (c, d) VSY73 strains. Thioflavin T fluorescence intensity (FI) of VSY72 cells grown on (a) galactose or (b) raffinose; FI of VSY73 cells grown on (c) galactose or (d) raffinose. Calculations for pellet and supernatant were done relative to the total amount of cellular amyloids. (e, f) Western blotting of proteins from (e) pellet or (f) supernatant with antibodies to α-synuclein. Arrows point to molecular weight markers. w—white cells; r—red cells (accumulated pigment).

We monitored the link between cell death (Figs. 5g, 5h) and inclusion number (Figs. 5e, 5f) visually determined within the same time intervals. It was found that increased cell number with inclusions was accompanied with increased cell death for 12 h. Later, the inclusion number dropped, whereas the number of dead cells increased.

To elucidate whether culture conditions affected amyloid accumulation, VSY73 cells were grown in liquid medium containing galactose with and without agitation for 2.5 days. Cell lysates with adjusted protein concentration were centrifuged. Pellet proteins were suspended and incubated with thioflavin T. The dye was also added to supernatant proteins. The amount of amyloid (%), as well as the fluorescence intensity of the dye bound with amyloid proteins, was measured in both fractions (Fig. 6a). Amyloid content in a lysate pellet of VSY73 cells was higher in cultures grown without agitation. This suggests that agitation impedes α-synuclein aggregation (Figs. 5e, 5f). To confirm this idea, pellet and supernatant proteins were subjected to blot-hybridization with antibodies to α-synuclein. It is seen (Fig. 6b) that agitation decreases α-synuclein content in a pellet and increases it in a supernatant.

The effect of various forms of exogenous red pigment (P0, P1, MeAIR) on expression, aggregation, and toxicity of cloned human α-synuclein was tested in yeast cells cultured in a synthetic galactose-containing medium without agitation for 2.5–3 days. Pigment was added together with inoculate. Figures 7a and 7b show the influence of various pigment forms on Mn of α-synuclein-bound GFP in VSY72 and VSY73 strains. It is seen that PO decreases, whereas MeAIR increases, Mn in VSY72 strain. No difference was visible between control and pigment-treated VSY73 cells. Mn in these cells was slightly higher than in VSY72 strain.

α-Synuclein expression was monitored with flow cytocytometry counting the number of GFP^+ cells (Figs. 7c, 7d). It was found that pigment-treated VSY72 and VSY73 cells differed from control. However, no difference between various pigments was noted. GFP^+ cell percentage was higher in cultures incubated with pigment in both strains. The number of aggregate-containing cells depends on the pigment form (Figs. 7e, 7f). In VSY72 strain, only pigment P1 increased aggregation, whereas P0, P1, and PHyd enhanced aggregation in VSY73 strain. The proportion of aggregate-containing cells was higher in VSY73 strain than in VSY72.

Exogenous pigment affected α-synuclein toxicity. All pigment forms reduced the number of dead PI^+ cells (Figs. 7g, 7h). P0 and P1 were the most effective for VSY72 strain, whereas P0 and MeAIR were more effective with VSY73 cells. The influence of exogenous P1 on amount of amyloid was studied on VSY73 cells grown for 2.5–3 days without agitation. The fluores-

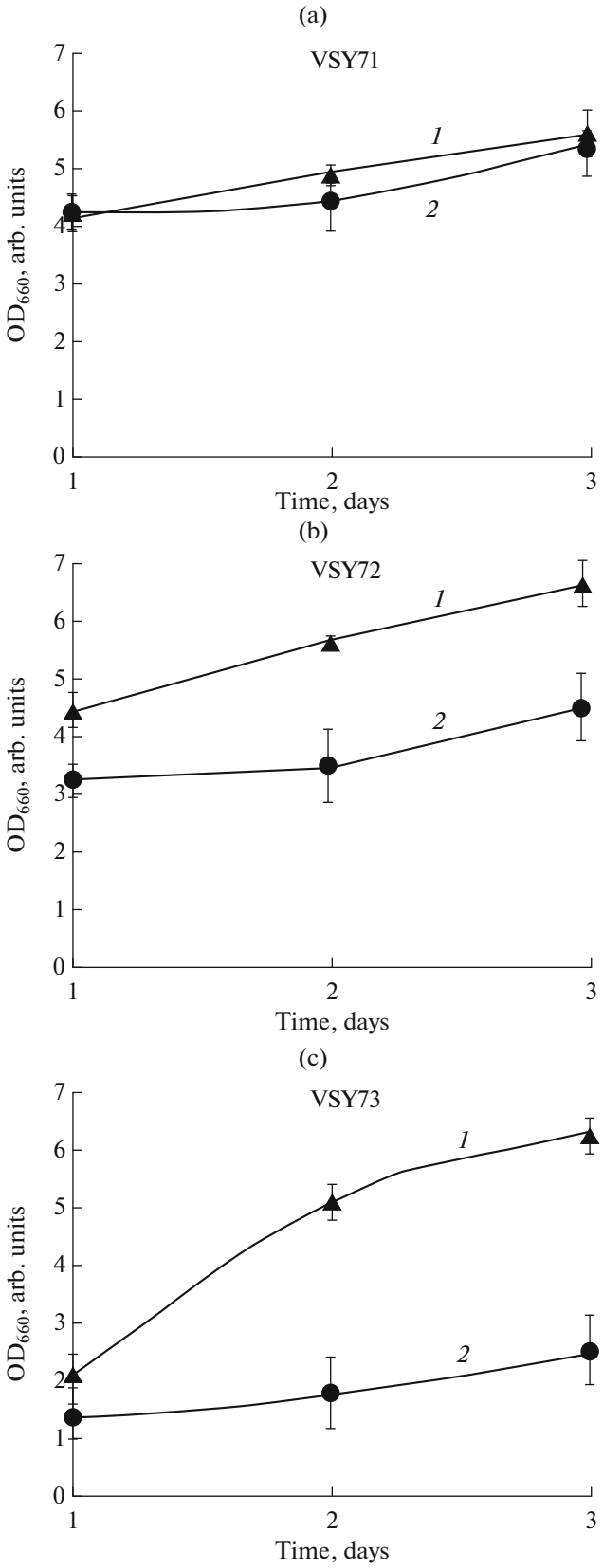

Fig. 4. Cell growth dynamics of various yeast strains grown in synthetic liquid medium with galactose for 3 days (*1*) with or (*2*) without agitation. OD_{660}—suspension optical density.

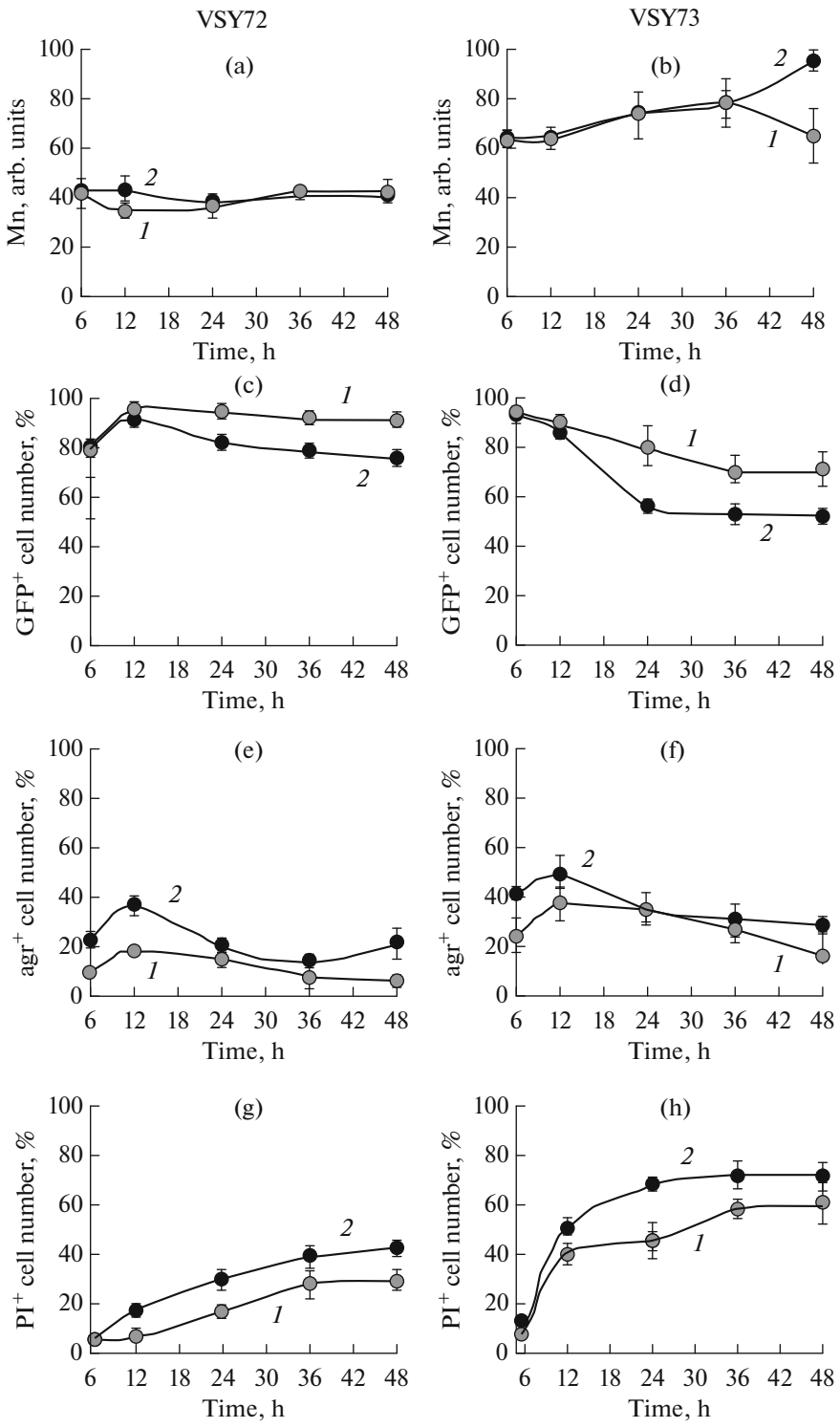

Fig. 5. Dynamics of α-synuclein-dependent effects in VSY72 and VSY73 yeast cells grown (*1*) with or (*2*) without agitation. (a, b) Mn value; (c, d) GFP^+ cell number; (e, f) cell number with aggregates (agr^+); (g, h) PI^+ cell number.

cence intensity of thioflavin T bound with amyloids in pellet was measured in cells cultured with galactose or raffinose. α-Synuclein was induced only in cells grown with galactose but not raffinose. Figure 8a shows that P1 decreases the amount of amyloid only in cells synthesizing α-synuclein. This indicates the essential contribution of α-synuclein aggregates to the total amount of cell amyloids in the strains.

Figure 8b shows the impact of red pigment on α-synuclein accumulation in cells. The results of blot-hybridization of cellular proteins with antibodies to α-synuclein show that the α-synuclein quantity in the pellet fraction of lysates containing large aggregates decreases after treatment with the red pigment at the expense of the supernatant fraction. It applies to both strains independently of culture conditions (with or without agitation).

DISCUSSION

The effect of endogenous and exogenous red pigment on expression and toxicity of hybrid α-synuclein was assayed in two yeast strains. VSY72 strain carries wild-type α-synuclein, whereas VSY73 strain has amino acid substitution (S129A) that results in a phosphorylation defect in this position (Sancenon et al., 2012; Tenreiro et al., 2014b).

Post-translational modifications influence protein folding, aggregation, and functioning, thereby playing a critical, albeit controversial, role in neurodegenerative diseases. A hallmark of Parkinson's disease is α-synuclein accumulation in Lewy bodies (Spillantini et al., 1997, 1998) and its phosphorylation in position 129 (Anderson et al., 2006). Phosphorylation effect was studied with mutants affecting the serine in this position. However, results obtained in various model systems are controversial (Sato et al., 2013; Tenreiro et al., 2014a).

In our experiments, mutation leading to dephosphorylation had no effect on red endogenous pigment action in VSY73 cells. The Mn value was about 25–30 arb. units, and the number of dead cells was near 10% in both VSY72 and VSY73 red cells. With an absence of red pigment (white cells), the strains differ in these properties (Fig. 2). Like other authors (Sancenon et al., 2012; Tenreiro et al., 2014b), we found that mutant α-synuclein was more toxic. However, red pigment eliminates this difference. The distinction between wild-type and S129A mutant has been demonstrated to result from the mutant's inability to activate protein quality control (Sontag et al., 2014), autophagy in particular (Rubinsztein et al., 2007). We suppose that the red pigment targets this pathway.

Endogenous pigment inhibited the Mn value, unlike all exogenous pigment forms (Figs. 7a, 7b). On the contrary, the percentage of GFP^+ cells depended on the exogenous, rather than the endogenous, pigment (compare Figs. 2 and Figs. 7c, 7d). All exogenous forms of the red pigment increased their proportion. The link between α-synuclein toxicity and percentage of cells containing cytoplasmic inclusions is particularly noteworthy, as is the dependence of this effect on red pigment. Toxic molecules are present at all steps of α-synuclein aggregation and amyloidization from monomer and dimer to various linear and looped oligomers, protofibrils, and, finally, amyloid fibrils and aggregates (Tenreiro et al., 2013). It has been demonstrated that an oligomer is a major toxic product (Outeiro et al., 2008; Brown, 2010; Winner et al., 2011). This does not exclude a correlation between toxicity and aggregate presence. Such a correlation is often found (Sancenon et al., 2012; Tenreiro et al., 2014a).

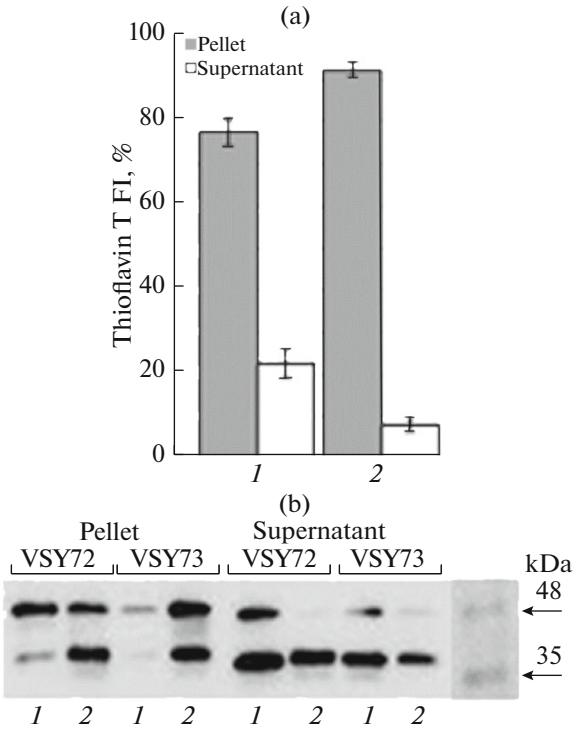

Fig. 6. (a) Amyloid and (b) α-synuclein content in pellet and supernatant of yeast lysates of VSY72 and VSY73 cells grown (*1*) with or (*2*) without agitation. (a) Thioflavin T FI in pellet and supernatant from VSY73 strain; (b) Western blotting of pellet and supernatant proteins from VSY72 and VSY73 crude cell lysates with antibodies to α-synuclein. Arrows point to molecular weight markers.

The presence of numerous aggregates in cells suggests the preceding accumulation of oligomers. Toxic oligomers trigger cell death, but the process, like aggregate generation, is also extended in time. Thereby, if "yesterday" oligomers were been abundant, then "today" numerous dead cells can be observed. Thus, the number of aggregates and dead cells seems to be interrelated, although factually they both depend on prior oligomer accumulation. Since there is no direct causal link between these values, the relationship between toxicity and aggregate-containing cell number depends on various factors, particularly on a experimental design. In our experiments, aggregate accumulation was correlated with toxicity in the initial stage of VSY72 and VSY73 cells growth on galactose medium (Sancenon et al., 2012; Tenreiro et

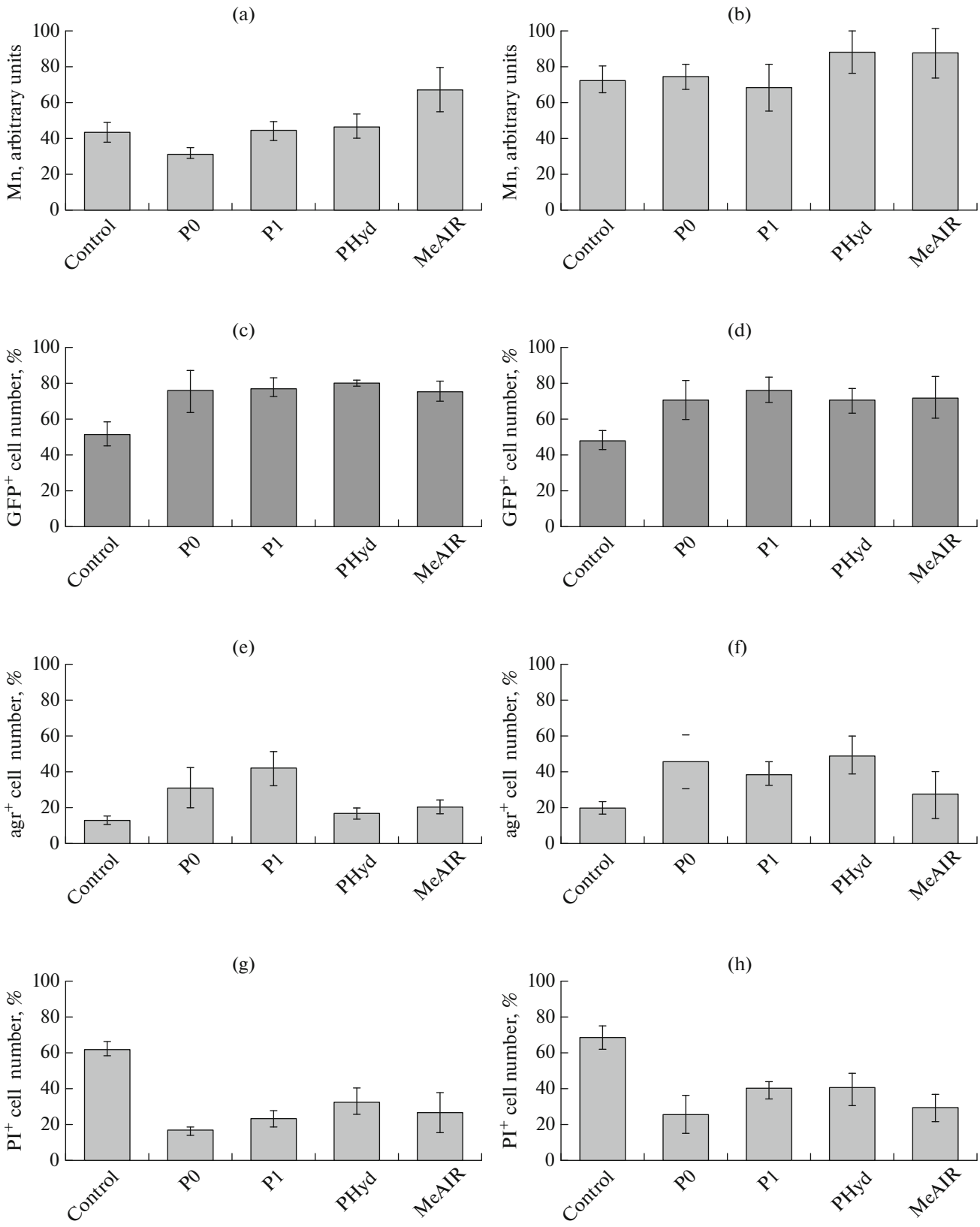

Fig. 7. α-Synuclein effects on (a, c, e, g) VSY72 and (b, d, f, h) VSY73 yeast strains exposed to P0, P1, PHyd, and MeAIR exogenous red pigment forms. (a, b) Mn value, (c, d) GFP^+ cell number, and (e, f) agr^+ number of cells with aggregates. (g, h)—dead PI^+ cell number.

al., 2014a). However, over time, this correlation is broken at later stages of growth.

Growth conditions strongly affect these parameters in both strains. The boost in the number of cells with aggregates is followed by cell death increased in cultures grown without agitation (Figs. 5g, 5h). A correlation between the number of cells with aggregates and cell death was observed in both strains containing endogenous red pigment, although the frequency of cells with inclusions was lower on solid selective medium (Fig. 1a).

It is known that endogenous red pigment is concentrated in vacuoles. Pigment polymerization occurs during aminoimidazole ribotide transport into vacuoles (Chaudhuri et al., 1997; Sharma et al., 2003). At the same time, amyloids accumulate in the insoluble protein deposit (IPOD) located on vacuoles (Kaganovich et al., 2008). We suppose that endogenous red pigment in vacuoles comes into contact and exchanges with nearby α-synuclein material in the IPOD. This permits the pigment to inhibit aggregate production or induce their remodeling or dissolution, which, in turn, diminishes the number of dead cells. Two variants of the idea are possible. First, that large aggregates exhibit toxicity, and, second, which is most probable, that endogenous pigment reduces the number of toxic oligomers. In last case, the IPOD may regulate the choice of one or another path of amyloid oligomerization. Endogenous pigment may also be present in a small amount in the cytoplasm of yeast cells and affects the initial stages of α-synuclein aggregation, directing the process into a nontoxic pathway. α-Synuclein aggregation via a nontoxic pathway has been demonstrated in experiments with gallate epigallocatechin (Ehrnhoefer et al., 2008). This was accompanied with a decline of the amount of α-synuclein β-sheet-rich amyloid. Mature α-synuclein aggregates have also been remodeled into an amorphous nontoxic material (Bieschke et al., 2010). This is in line with our findings, although some authors suggest that, unlike stress granules and JUNQ (juxtanuclear quality control compartment), other cytosolic sites, such as IPOD, are irreversible terminal "burials" of amyloid aggregates (Kaganovich et al., 2008; Amen and Kaganovich, 2015). It should be noted that these authors did not possess an agent such as red pigment to "attack" the IPOD "inside" from the vacuole. It is possible that the role of IPOD may be reexamined due to red pigment findings.

An alternative interpretation should also be considered. As it is commonly believed, the most toxic misfolded relatively mobile peptides enter the JUNQ (Weisberg et al., 2012), α-synuclein molecules caught in the IPOD are not so dangerous for the cell. In this case, the effect of red pigment on amyloids may depend on the pigment molecules outside a vacuole. This alternative should be kept in mind in explaining the mechanisms of exogenous pigment action.

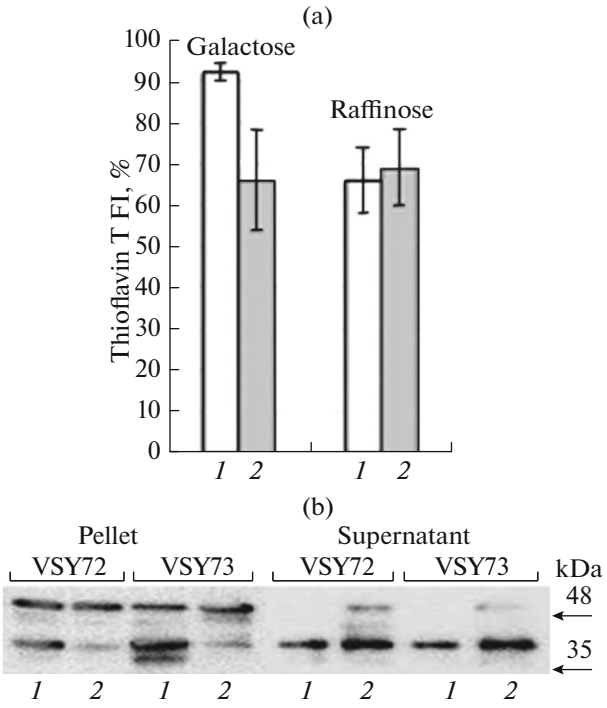

Fig. 8. The effect of exogenous pigment on (a) amyloid content and (b) α-synuclein accumulation in cell lysate fractions of VSY72 and VSY73 strains. (a) Amyloid content determined by thioflavin T FI in lysate pellet fraction of VSY73 cells grown with galactose or raffinose (*1*) without or (*2*) with P1 pigment treatment; (b) Western blotting of proteins from crude lysate pellet or supernatant fractions of cells (*1*) untreated or (*2*) treated with pigment P1. Arrows point to molecular weight markers.

We have demonstrated that both exogenous and endogenous red pigments reduce α-synuclein toxicity in yeast. Most likely, they decrease the content of a toxic intermediate. However, we revealed a difference between endo- and exogenous pigment effects. Endogenous red pigment diminished the number of visible aggregates whereas exogenous pigment exerted opposite effect and increased the percentage of cells with cytoplasmic inclusions. We suggest that the red pigment sticks small α-synuclein oligomers and/or protofibrils together and augments the number of visible aggregates. We did indeed observe clustering of β-amyloid fibrils due to red pigment using transmission electron microscopy, but, in this case, red pigment did not increase the number of aggregates visible by light microscopy (Nevzglyadova et al., 2015).

It still remains uncertain where the contact between exogenous red pigment and α-synuclein takes place. If exogenous pigment is mostly recruited into the vacuole, this process is not likely to be very effective. We have observed the penetration of red pigment in cells, but its inclusion into the vacuole was not obvious (Nevzglyadova et al., 2015). Most likely, the effective concentration of exogenous red pigment is higher

in cytoplasm and lower in vacuoles compared to endogenous red pigment. The decreased α-synuclein toxicity caused by exogenous pigment shows that the pigment targets a toxic form of α-synuclein. Reduced toxicity is not accompanied by a drop in number of cells with visible aggregates; thereby, it is possible to suggest that the toxic form is in fact oligomers. At the level of aggregates invisible under a microscope but identified by Western blotting in lysate pellets, there is no difference between exogenous and endogenous pigment action.

Significantly, the assay of pellet and supernatant fractions of cell lysates revealed similar redistributions of α-synuclein in cells exposed to either endogenous (Fig. 3) or exogenous (Fig. 8) pigments. Assessment of amyloid content with thioflavin T and Western blotting demonstrated that α-synuclein amyloid fraction was decreased in pellet and increased in the supernatant composed of smaller aggregates and oligomers. Reduced toxicity caused by both pigment types suggests that increased supernatant fraction was not enriched for α-synuclein toxic forms (oligomers or short fibrils). It implies that red pigment is able to cut back these forms and neutralize their toxicity.

Analysis of supernatant and pellet fractions in lysates of cells grown without pigment with or without agitation also demonstrated α-synuclein redistribution. Cells cultivated with agitation accumulate a large number of cytoplasmic inclusions (Figs. 5e, 5f), and it does not seem surprising that the lysate pellet fraction of these cells has increased α-synuclein content (Fig. 6). Cell death increased in cultures grown without agitation.

At a first approximation, agitation seems to model the action of red pigment. It reduces α-synuclein content in a pellet and decreases its toxicity. However, agitation prevents aggregate accumulation, whereas exogenous pigment facilitates it. We propose that cytoplasmic inclusions observed with and without pigment differ in their composition of oligomers and fibrils. Preliminary results of FRAP analysis of VSY73 strain confirmed the presence of molecules with modified mobility in aggregates in cells exposed to red pigment (data not shown).

In conclusion, using a model of β-amyloid (Nevzglyadova et al., 2015) and α-synuclein cloned in yeast (this study), we demonstrated a potential therapeutic effect of red pigment. Red pigment reduced both α-synuclein amyloid content and toxicity. β-Amyloid did not display toxicity in yeast but red pigment decreased its content and aggregation.

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