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Research Article

The effect of copy number on α-synuclein's toxicity and its protective role in Bax-induced apoptosis, in yeast

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Apoptosis is a form of programmed cell death which is essential for the growth of dividing human cells whereas, in contrast, it is deleterious for post-mitotic cells such as neurons. Bax and α-synuclein are two human proteins which play a role in the induction of neuronal apoptosis in neurodegenerative diseases like Alzheimer's and Parkinson's. Human Bax and α-synuclein also induce cell death when expressed in baker's yeast, *Saccharomyces cerevisiae*. Quite unexpectedly, the human α-synuclein gene had been identified as an inhibitor of pro-apoptotic Bax using a yeast-based screen of a human hippocampal cDNA library. Plasmids were constructed with different promoters, which allow expression of wildtype and Parkinson's disease (PD)-related mutant α-synuclein genes, from (i) multi-copy 2μ (episomal) plasmids and (ii) integrative plasmids that compel expression of genes from chromosomal sites in varying copy numbers (1–3). All α-synuclein-containing plasmids were introduced, through transformation, into a yeast strain which already contained a chromosomally integrated copy of Bax. It is for the first time that it was observed that, depending on gene dosage, only wildtype α-synuclein is anti-apoptotic while mutant α-synuclein is not. The results also indicate that wildtype α-synuclein has a remarkable ability to manifest two contrasting effects depending on its level of expression: (i) normally, it would negate apoptosis but (ii) when overexpressed, it tends to induce apoptosis which is probably what happens in PD.

Introduction

Parkinson's disease (PD) is a severe neurodegenerative disorder which is categorised by bradykinesia (slow movement), postural instability, muscular rigidity and resting tremors [1,2]. Many PD cases are from an obscure cause, though some are known to be caused by missense mutations in the α-synuclein gene [3,4]. In PD, α-synuclein accumulates into protein aggregates that show, both in *in vitro* and *in vivo*, many features of amyloid formation [4,5]. α-synuclein is water-soluble, and it is made up of 140 amino acid residues. It contains seven 11-residue repeats in the positively charged N-terminal area, followed by a vastly acidic 40-residue C-terminal tail. α-synuclein is known to be associated with mitochondrial function [6,7]. However, it was also found in red blood cells that lack mitochondria [8–10]. α-synuclein is present predominantly in the presynaptic region of neuronal cells. However, the actual function of α-synuclein has not yet been defined.

Apoptosis serves as a mechanism that helps to restructure tissues through development, considering this definition/description, it was assumed that apoptosis could not be found in modest eukaryotes, i.e. yeast. However, when apoptosis started gaining link with the removal of damaged cells, major regulatory proteins involved in programmed cell death were recognised in yeast [11]. The α-synuclein protein is conspicuously expressed in the central nervous system [12], and functions in vesicular tracking

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and lipid metabolism [10,13]. It physically interacts with vesicular membrane proteins [3]. Overproduced α-synuclein inhibits phospholipase D, thereby inducing accumulation of lipid droplets which eventually adversely affects vesicle trafficking [3]. Overexpression of α-synuclein is associated with PD and also multiple system atrophy which is a rare neurological disorder [14]. Yeast is useful in the study of human apoptosis; in yeast, apoptosis can be induced by both intrinsic and extrinsic factors [15].

When overexpressed in yeast, α-synuclein protein migrates to the periplasmic membrane, just like in human cells. As in human cells, it forms cytoplasmic inclusion bodies (which are referred to as Lewy bodies in human cells) when overexpressed in yeast, resulting in the display of apoptotic features in yeast [5,16]. The process of apoptosis in human cells is complex. It is regulated by the Bcl-2 family of proteins which have both pro- and anti-apoptotic members [17]. It can occur dependent or independent of the tumour suppressor protein, p53 [17]. The absence of yeast homologues of mammalian Bcl-2 family members and also p53 initially undermined yeast studies linked to human apoptosis. However, identification of a yeast caspase (named metacaspase) made further studies, with human proteins like α-synuclein, plausible [18]. In human cells, activation of caspases underlines a commitment to apoptosis.

Overproduction of reactive oxygen species (ROS) controls the initiation of the apoptotic pathway and is thought to be a hallmark of mammalian apoptosis. Intracellular ROS is produced during the process of electron transport across the endoplasmic reticulum (ER) or nuclear membranes [18,19]. Like human cells, yeast cells' response to ROS varies with the amounts of cellular ROS that are present [20]. When the amounts of ROS are high, some antioxidant proteins, such as Msn2, Yap1 and 4p transcription factors, are activated within yeast cells [18,20]. Activation of these proteins results in a delay in yeast cell division. In the presence of even higher doses of ROS, some, if not all, cells undergo apoptosis [19]. α-synuclein overexpression in yeast is known to induce high levels of ROS.

α-synuclein is usually present in the cytosol and nucleus. It is associated with outer membranes of neurons affected by PD [21]. As noted above, periplasmic membrane association is also observed in yeast during α-synuclein overexpression that leads to its aggregation/misfolding and which culminates in yeast cell death [3]. Pathogenic neuronal cell demise stemming from misfolding/aggregation of α-synuclein involves DNA degradation triggered by the mitochondrial endonuclease G. The translocation of endonuclease G from mitochondria to the nucleus results in cell death [21]. Nuclear localisation of endonuclease G is cytotoxic to dopaminergic neurons of patients with PD. In flies and nematodes, endonuclease G is also crucial for α-synuclein-induced degeneration of dopaminergic neurons [21].

Besides α-synuclein, other proteins are linked to PD. They include Parkin, DJ-1 and PINK1. Like α-synuclein, they are all linked to mitochondrial function [13,22]. α-synuclein has been shown to interact physically with neuronal mitochondrial membranes. Consequently, overexpression of α-synuclein interferes with the normal function of mitochondria resulting in neuronal apoptosis [23–25]. It is, therefore, reasonable to say that one or more mitochondrial factors trigger α-synuclein-induced apoptosis in neuronal cells.

Mitochondrial dysfunction plays a major role in PD [26]. A systemic shortage of mitochondrial complex I activity has been reported in PD patients [16]. Hence, it has been proposed that the normal function of α-synuclein involves sequestration of cytochrome *c* within mitochondria. Only during its overexpression are Lewy bodies formed through α-synuclein aggregation, and the release of cytochrome *c* from mitochondria to the cytosol leading to neuronal apoptosis [16]. Apoptosis, which controls the overall turnover of cells without damaging the cellular environment, was discovered as having a vital role in the maintenance of cell number and function by Wyllie and colleagues [27]. The process of apoptosis, like all biochemical processes, is coordinated.

Human Bax protein-induced cell death in the yeast *Saccharomyces cerevisiae* shows the characteristics of apoptosis, which include membrane blebbing, DNA fragmentation, phosphatidylserine externalisation at the cytoplasmic membrane surface, marginalisation and condensation of chromatin, and vacuolisation of cytoplasm [28]. Bax expression also causes the release of cytochrome *c* from the mitochondria and decreases levels of cytochrome *c* oxidase [29]. As the mitochondria are interconnected and elongated [30], damaged mitochondria are removed through fission via a conserved mechanism [31]. Cyclin C, the activating partner of the cell cycle kinase Cdk8, translocates in response to stress to the mitochondria from the nucleus, suggesting that cyclin C may have a role to play in programmed cell death and mitochondrial fission [32].

α-synuclein produces a three-way complex with anionic lipids, like cytochrome *c* and cardiolipin. The complex induces peroxidase activity that leads to the enhancement of hetero-oligomerisation of α-synuclein with cytochrome *c* ultimately forming a huge molecular weight aggregate [16]. The aggregate induces activation of caspases and formation of the apoptosome, which represents a commitment to apoptosis [16]. Pro-apoptotic factors are released via damage to presynaptic mitochondria which serves as a threat to the survival of all neurons [33].

α-synuclein can halt the oxidative chain reaction, thereby hypothetically playing a vital handy role in averting brain lipid oxidative damage [8]. It has been claimed that aggregation of α-synuclein protein could be inevitable, but the circumstances which warrant this aggregation in cells is not yet well understood [9,34]. This could be due to the poor

understanding of α-synuclein's true function, although it is known that it is associated with vesicular membranes, and other membrane interactions [9,34].

The present study's aim was to study the characteristics of two pro-apoptotic human proteins, Bax and α-synuclein, in the baker's yeast *S. cerevisiae*. The two pro-oxidant human proteins, Bax and α-synuclein, which are deeply involved in the induction of apoptosis in human cells, were used as apoptotic triggers in yeast and were co-expressed.

Materials and Methods

Yeast strains

The yeast strain W303-1A Mat*a* (ATCC #208352), is auxotrophic for the genes *ADE2*, *HIS3*, *LEU2*, *TRP1* and *URA3*. New yeast strains were derived from W303-1A by transforming integrative plasmids (Supporting Information, Sections 1 and 2) which would express α-syn from the *MET25* or *GAL1* promoter.

Yeast transformation

Plasmids bearing α-syn gene expression cassettes under the control of either the methionine-repressible *MET25* or galactose-inducible *GAL1* promoter (*MET25*p/*GAL1*p; see Supporting Information, Sections 1 and 2) were used for genomic integration at the *HIS3*, *TRP1* and *URA3* chromosomal loci of the yeast strain to yield strains that contain 1–3 copies of α-syn. Similarly, plasmids bearing Bax-α gene expression cassettes under the control of galactose-inducible GAL1 promoter was used for genomic integration at the *LEU*3 chromosomal locus of the yeast strain. The integrative transformation was carried out using a published protocol [35,36].

Rhodamine 1 2 3 staining

Rhodamine 1 2 3 is a cationic fluorescent, cell-permeable dye. It is readily sequestered within active mitochondria. Rhodamine 1 2 3 dye from the yeast mitochondrial staining kit was used (Molecular Probes, Y7530), after the expression of protein or proteins of interest. A total of 1×10^6 cells were suspended in 1 ml of 50 mM sodium citrate buffer, containing 2% w/v glucose, at pH 5. A final concentration of 50 μM of Rhodamine 1 2 3 was added to cells and incubated at room temperature for 30 min. Fluorescent cells were visualised using a fluorescence microscope.

Detection of dead cells with Phloxine B dye

Cell death was assessed by staining cells with the red dye Phloxine B (Sigma, P-4030-25G) [37]. Live cells expel the dye, whereas it is accumulated in dead cells. This can be observed by fluorescence microscopy. Staining experiments were performed as published earlier [36].

Detection of ROS with dihydroethidium

AAT Bioquest Fluorimetric Intracellular Total ROS Activity Assay Kit (#22901) was used for measuring ROS. Experiments were performed as published earlier [36].

Quantifying mitochondrial membrane potential with the JC-10 dye

AAT Bioquest JC-10 Mitochondrial Membrane Potential Assay kit (#22800) uses water-soluble JC-10 to determine mitochondrial membrane potential (MMP) quantitively. Experiments were conducted as per the published protocol [36].

Assessing nuclear DNA fragmentation via the TUNEL assay

AAT Bioquest TUNEL Apoptosis Assay kit (#22844) was used for the detection of nuclear DNA fragmentation (NDF). The assays were performed as described earlier [36].

Western blotting

Western blotting was carried out using standard protocols (Haar, 2007), using primary antibodies specific to α-syn (Proteintech; #10842-1-AP), c-Myc mouse monoclonal antibody (Thermo Scientific, #MA 1-980) or β-actin (Proteintech; #60008-1-Ig) [38].

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Results and Discussion

The effects of increasing copy numbers of the GAL1 and MET25 promoters driven HA and eGFP-tagged α-synuclein expression cassette on yeast cells

The result of the toxicity mediated by wildtype human α-synuclein protein from two different inducible promoters; and mutant human α-synuclein proteins (A30P and A53T) when defined copy numbers of its gene were expressed, were presented below. At first, yeast cells were transfected with an episomal plasmid containing α-synuclein on a PGK1p promoter (pSYE239/α-synuclein-HA), protein expression from the PGK1p can occur in media containing either glucose or galactose as a carbon source. The control plasmid pSYE239, which includes no α-synuclein gene, was also transformed into yeast cells (Figure 1).

Expression of α-synuclein from an episomal plasmid

Episomal plasmids, in theory, should provide multiple copies of the plasmid within yeast cells. However, it has been published that yeast strains bearing episomal plasmids that contain GAL1p-α-synuclein expression cassettes offer less than one copy because of α-synuclein's inherent toxicity in yeast [3]. Hence, the effect of increasing copy number was studied by integrating α-synuclein expression cassettes at different chromosomal loci so that defined 1 copy, 2 and 3 copies of the α-synuclein gene could be expressed. Also, mutant α-synuclein genes (A30P and A53T) were integrated into yeast cells in 1–3 copies. Alongside, various control strains were generated.

The results seen in Figure 1 (where α-synuclein protein expression is switched on in the presence of both galactose and glucose), clearly show that human α-synuclein, when expressed from an episomal 2-micron (2μ) plasmid, does not stop cell growth and therefore is not toxic to yeast. Compared with the control strain, there is no significant retardation of growth. Outeiro and Lindquist (2003) have reported that yeast cells expressing GFP-tagged α-synuclein from a 2-μ plasmid also grew when expressed from the galactose-inducible GAL promoter. Expression of α-synuclein, controlled by the MET25 promoter, from a 2-μ plasmid, also did not inhibit cell growth [22].

Defined copies of α-synuclein would allow control of its amount at a particular time. The results in Figure 2 show that in contrast to the expression of α-synuclein from an episomal plasmid, expression of a single integrative copy of the human α-synuclein gene from the galactose-inducible GAL1 promoter blocks growth of yeast cells, likewise two and three copies of α-synuclein. These results obtained on a solid agar plate, containing galactose as the sole carbon

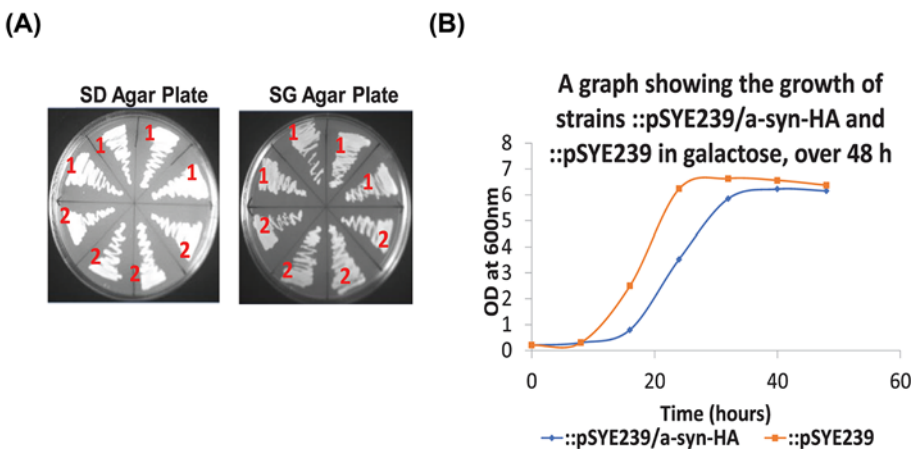

Figure 1. Expression of α-synuclein from an episomal plasmid

The results seen on plates (**A**), above (where α-synuclein protein expression is switched on in the presence of both galactose and glucose), growth of yeast cells, (harbouring the episomal plasmid, pSYE239/α-synuclein-HA) (1) and (harbouring the episomal control plasmid, pSYE239) (2), on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Clearly show that human α-synuclein, when expressed from an episomal 2-micron (2μ) plasmid, does not stop cell growth and therefore is not toxic to yeast. (**B**) Shows that a strain expressing α-synuclein from an episomal 2-μ plasmid also grows in liquid cultures. Compared with the control strain, there is no significant retardation of growth. Outeiro and Lindquist (2003) have reported that yeast cells expressing GFP-tagged α-synuclein from a 2μ plasmid also grew when expressed from the galactose-inducible GAL promoter. Expression of α-synuclein, controlled by the MET25 promoter, from a 2μ plasmid also did not inhibit cell growth [22].

source for growth, were corroborated with cells grown in minimal liquid medium containing galactose (Figure 2B). In Figure 2C,D, α-synuclein induced significant cell death in yeast cells. α-synuclein was then tagged with eGFP to allow the visible study of α-synuclein aggregation. Results from Figure 3 show that the expression of only three copies of α-synuclein-eGFP fusion protein retards/stop cell growth on galactose-containing SG agar plates. Figure 3B show the cells expressing three copies of α-synuclein-eGFP fusion protein have the most considerable retardation of growth. However, the growth retardation is not significant compared to cells carrying one copy and two copies of α-synuclein-eGFP; this could be due to the large size of eGFP fusion protein, the expressed protein appears to be aggregated (Figure 3C,D). It was seen that as the copy number of α-synuclein-eGFP gene increases, there was significantly more aggregation of α-synuclein. The results below indicate that, although α-synuclein-eGFP fusion protein does not block cell growth to any appreciable degree, it imposes apoptotic cell death which increases with increasing copy number. Figure 2E shows that expression of one copy, two and three copies of α-synuclein produces much more ROS than control cells which contain empty plasmid that bears no α-synuclein gene, similarly, this was observed in eGFP strains (Figure 3L). Figures 2F and 3E shows that there is a drop of MMP in yeast cells expressing one, two, and three copies of α-synuclein-HA and α-synuclein-eGFP compared with their respective control, indicating that both HA- and eGFP tagged α-synuclein expression has a deleterious effect on mitochondrial outer and inner membranes and thereby affected MMP which is a hallmark of apoptosis [39]. Figures 2G,H and 3H,I shows that DNA fragmentation was high in cells that express three copies of α-synuclein-(HA and eGFP) compared with strains expressing one copy and two copies but was very low in control cells which contained empty plasmids. The results in Figures 2 and 3 indicate that expression of HA-tagged α-synuclein, from a chromosomal locus, induces apoptosis in yeast.

The effects of increasing copy numbers of the GAL1 promoter-driven HA-tagged α-synuclein expression cassette (GAL1p-α-synuclein-HA) on yeast cells

The effects of increasing copy numbers of the GAL1 promoter-driven eGFP-tagged α-synuclein expression cassette (GAL1p-α-synuclein-eGFP) on yeast cells

Expression of mutant α-synuclein (A30P & A53T) gene in yeast cells bearing 1 to 3 copies of HA-tagged mutant α-synuclein gene, downstream of the GAL1 promoter, in galactose containing media

The mutant α-synuclein (A30P) harbouring strains did grow in galactose like the control strains that contained 1–3 copies of empty plasmids, without any α-synuclein gene. The results on plates show that the mutant α-synuclein (A30P) protein is non-toxic, the observations were corroborated in cells that were grown in a liquid medium; see Figure 4A,B. Contrary to the effects of wildtype α-synuclein expression, the A30P mutant showed no difference in growth and cell death between the strains expressing mutant A30P α-synuclein in 1–3 copies, and when compared with their respective controls. Mutant α-synuclein gene (A53T) α-synuclein in 1–3 copies did not grow in galactose. The results on plates show that like the wildtype α-synuclein protein, the mutant α-synuclein (A53T) protein is toxic. The observations were corroborated in cells that were grown in a liquid medium; see Figure 4G,H. Phloxine B staining of cells expressing 1–3 copies of the α-synuclein mutant (A53T) in Figure 4I,J, suggest that A53T mutant imposes toxicity on yeast cells while A30P mutant does not; wildtype α-synuclein and A53T mutant α-synuclein seems to have similar properties in terms of toxicity/cell death induction. Same results were obtained for yeast cells bearing α-synuclein on MET25p promoter, and α-synuclein with no tag (α-synuclein-No HA) (Figure 5).

The effects of expressing increasing copy numbers of untagged α-synuclein under the control of GAL1p, and HA-tagged α-synuclein on MET25p

Wildtype α-synuclein rescues yeast cells from Bax-induced cell death

α-synuclein is part of the SNARE complex at the synapses, and this is its physiological role. When it misfolds, α-synuclein becomes pathological, as seen in PD. In previous sections, the toxicity of wildtype and mutant (A53T) α-synuclein have been shown in different yeast strains. It was reported that only after increasing the dosage from one copy to two copies of α-synuclein does α-synuclein cause cell death in yeast [3,10]. However, we see that a single integrative copy of the wildtype α-synuclein (tagged or untagged) has already a profound effect on cell growth and death.

Having seen that yeast strains harbouring the GAL1 promoter-driven, chromosomally integrated copy of the Bax expression cassette did not grow on galactose containing media (Figure 6A–C), it was confirmed that pro-apoptotic Bax protein is toxic to yeast. The episomal plasmid containing α-synuclein (pSYE239/α-synuclein-HA) and the empty vector (pSYE239), as shown in (Figure 6D), was introduced into the yeast strain,:: Bax(LEU2), that already contains an integrated copy of the Bax expression cassette at yeast's LEU2 chromosomal locus, Figure 6D. Yeast cells were

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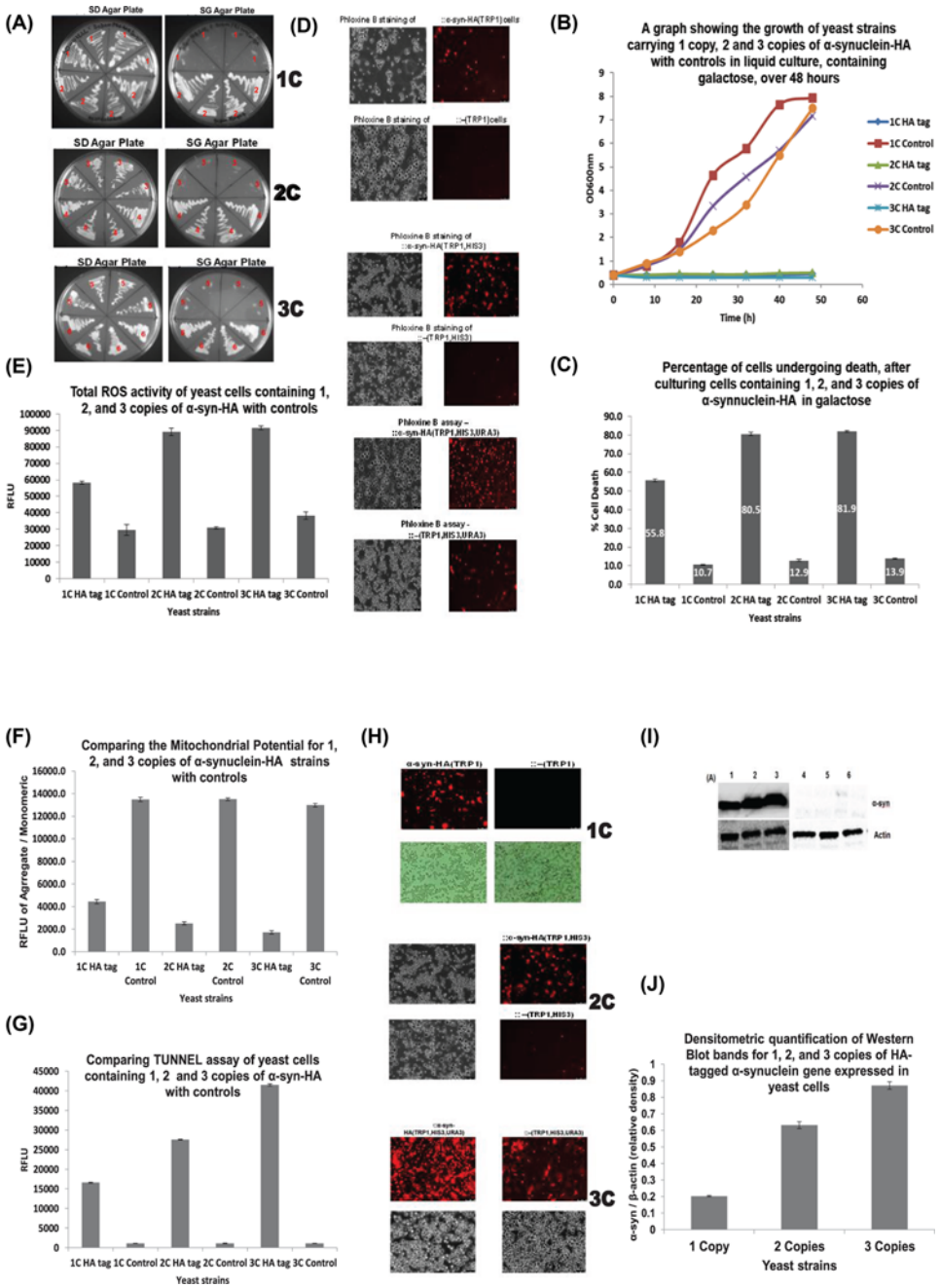

Figure 2. Effects of increasing copy numbers of HA-tagged wildtype α-synuclein

(**A**) Growth of yeast cells, ::α-syn-HA(TRP1) (harbouring the integrative plasmid) (1), ::–(TRP1) (harbouring the empty integrative plasmid) (2), ::α-syn-HA(TRP1,HIS3) (harbouring the two integrative plasmids) (3), ::–(TRP1,HIS3) (harbouring the empty integrative plasmids) (4), ::α-syn-HA(TRP1,HIS3,URA3) (harbouring the three integrative plasmids) (5), and ::–(TRP1,HIS3,URA3) (harbouring the empty integrative plasmid) (6) on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Plates were incubated at 30°C for 96 h. (**B**) A graphical representation of the growth of yeast strains (one, two, and three copies of α-syn-HA and respective control), over 48 h, in minimal selective medium with galactose as the sole carbon source for Growth. There was a significant difference between the three yeast strains with their respective controls ($P<0.05$); multiple comparisons also indicated that there was no significant difference between the Growth of the three strains expressing one copy, and two and three copies of α-syn ($P>0.05$). (**C**) Percentage of cells undergoing death upon expression of one, two, and three copies of HA-tagged α-synuclein from the GAL1 promoter (which is fully induced with 10 h), ANOVA test was performed, this revealed that cell death between all copy number and their respective controls were significant ($P<0.05$), moreover, the cell death due to copy number increase was significant between 1 and 2, and 1 and 3 copies of α-synuclein ($P<0.05$), but was not significant ($P>0.05$) between 2 and 3 copies of

←

α-synuclein. (**D**) Phloxine B staining image of yeast strains expressing 1, 2, and 3 copies of HA-tagged α-synuclein from the GAL1 promoter (**E**) The amount of ROS produced in the 1, 2, and 3 copies GAL1 promoter-driven HA-tagged α-synuclein expressing cells compared with the ROS produced in the strains containing empty plasmid, ANOVA test gave a statistically significant effect ($P<0.05$) from three independent experiments for ROS production. The difference between one and two copies ($P<0.05$), and one and three copies ($P<0.05$) was significant, but there was no significant difference between two and three copies ($P>0.05$). (**F**) Comparison of the MMP of yeast strains expressing 1, 2, and 3 copies of HA-tagged α-synuclein from GAL1 promoter and controls with empty plasmid. ANOVA tests gave a statistically significant effect ($P<0.05$) for mitochondrial potential between Yeasts strains carrying 1, 2, and 3 copies of α-synuclein and their respective controls. The difference between one, two, and three copies was not significant ($P>0.05$). (**G**) Comparison of the levels of NDF between yeast strains expressing 1, 2, and 3 copies of α-synuclein-HA from the GAL1 promoter and the controls containing empty plasmids, ANOVA test gave a statistically significant effect ($P<0.05$) for NDF through tunnel assay for both yeast strains carrying α-synuclein and their respective controls and between yeast strains carrying 1, 2, and 3 copies ($P<0.05$). (**H**) Microscopic representation of TUNEL assay of yeast strains expressing 1 , 2 and 3 copies of α-synuclein-HA with controls. (**I**) Western blot analyses of cells expressing 1–3 copies of HA-tagged α-synuclein protein with controls. Lanes 1, 2 and 3 were loaded lysates from cells that express 1, 2 and 3 copies of α-synuclein, while the lysates from cells with 1, 2 and 3 copies of empty plasmids were loaded on lanes 4, 5, and 6. The HA-antibody was used to probe for expression of α-synuclein-HA and the β-actin antibody for β-actin, which was used as a loading control. 10 μg of total cellular protein was loaded in each lane. (**J**) Densitometry quantification of Western Blot bands for cells expressing 1 copy, 2 and 3 copies of α-synuclein-HA in yeast. The values for β-actin were roughly the same.

rescued from death in cells co-expressing Bax (from an integrated copy) and α-synuclein (from a 2μ-plasmid) (see Figure 6D–G).

Expression of human Bax gene in yeast cells, downstream of the GAL1 promoter, in galactose containing media and Rescue of GAL1 promoter-driven Bax induced block of cell growth by PGK1 promoter-driven expression of α-synuclein from a 2μ plasmid

Copies of the α-synuclein-HA, α-synuclein-eGFP, A30P and A50T (mutant) genes expression cassette were also integrated into the yeast strain harbouring Bax(LEU2) at its TRP1, HIS3 and URA3 loci to create 1, 2 and 3 copies of the respective α-synuclein strains. Galactose allows expression of Bax and α-synuclein, both from the GAL1 promoter. It seems that cells which express one copy of HA-tagged α-synuclein struggle to overcome Bax-induced block in growth while the cells with the empty plasmid were completely blocked in Growth because of Bax protein's toxic effects. The results show that cells which express two copies of HA-tagged α-synuclein can overcome Bax-induced apoptosis while the cells with empty plasmid were completely blocked in growth or dead because of Bax's toxicity. These results have been corroborated by the growth of cells in SG liquid medium (Figure 7A,B). Three copies of HA-tagged α-synuclein could not overcome Bax-induced block in growth anymore. It behaved just like cells with one copy. The third copy seemed to increase the toxicity in the yeast cells.

Co-expression of two copies of HA-tagged α-synuclein with Bax results in nearly complete protection from death. The rescue by two copies of α-synuclein was significant. Although three copies of HA-tagged α-synuclein could not overcome blockage in cell growth induced by Bax, it seems that co-expression of 3 copies of HA-tagged α-synuclein with Bax in Bax(LEU2)::α-syn-HA(TRP1, HIS3, URA3) cells still protects cells from Bax induced death. This can be seen when percentage cell death (Figure 7C,D) is compared with control cells Bax(LEU2)::–(TRP1, HIS3, URA3) which express Bax alone but contain three empty plasmids that do not express any α-synuclein. There appears to be a significant reduction in ROS when two copies of HA-tagged α-synuclein were co-expressed with Bax. The results would suggest that two copies of α-synuclein protect cells from excess ROS generated through Bax expression in yeast (Figure 7E).

Figure 7F show that expression of one-copy of HA-tagged α-synuclein is protective of mitochondrial membranes damaged by Bax expression in yeast. It also indicates that there is a further increase in MMP in cells co-expressing Bax and two copies of α-synuclein-HA. This suggests that two copies of α-synuclein truly protects mitochondrial membranes from Bax-mediated mitochondrial damage. The TUNEL results (Figure 7G) show that expression of two-copies of HA-tagged α-synuclein prevents Bax-mediated cell death in yeast. The bar chart (Figure 7G) clearly indicates that two copies of HA-tagged α-synuclein rescue the majority of cells from Bax mediated apoptosis.

In Figure 8A,B, the 1-copy and 2-copies α-synuclein-eGFP containing strains grew upon full expression of the α-synuclein-eGFP fusion protein in the presence of Bax. The 2-copies strain was having more growth than 1-copy, while the 3-copy strain did not grow at all when both proteins, Bax and α-synuclein-eGFP, were fully expressed.

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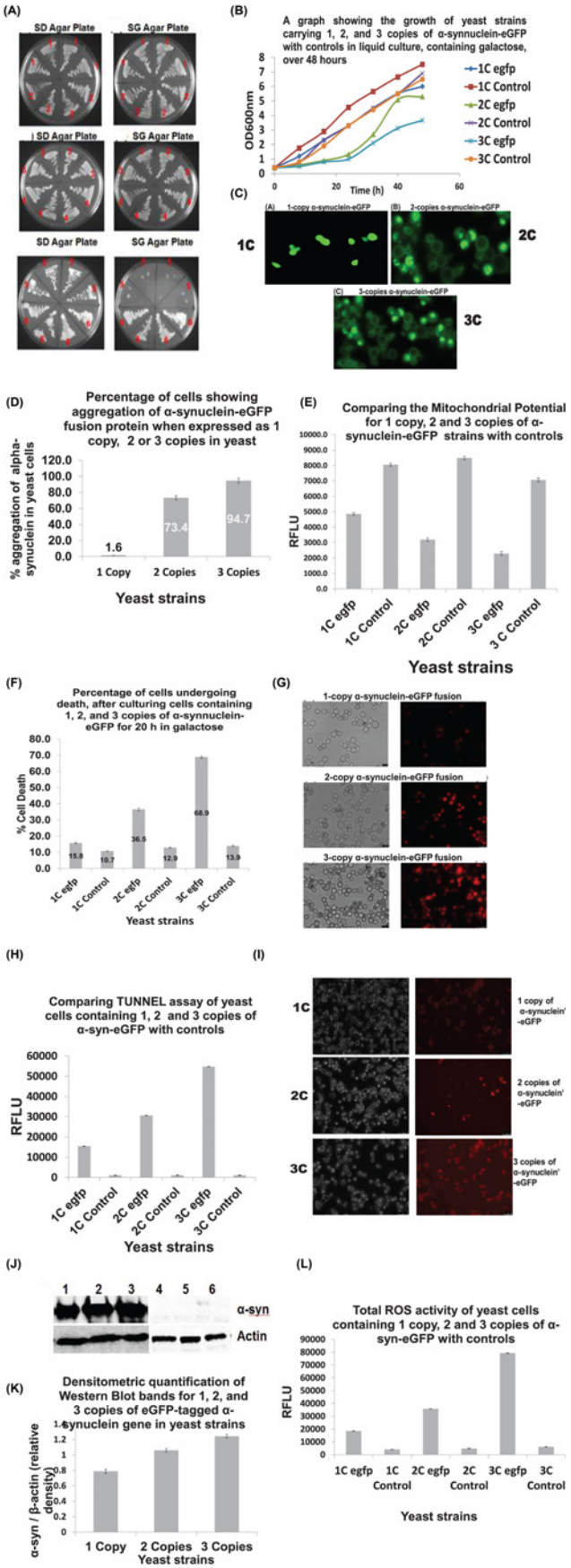

Figure 3. Effects of increasing copy numbers of eGFP-tagged wildtype α-synuclein

(**A**) Growth of yeast cells harbouring the 1, 2 and 3 yeast integrative plasmids that encode an α-synuclein-eGFP fusion gene, (harbouring the integrative plasmid) (1), (harbouring the empty integrative plasmid) (2), (harbouring the two integrative plasmids) (3), (harbouring the empty integrative plasmids) (4), (harbouring the 3 integrative plasmids) (5), and (harbouring the empty integrative plasmid) (6) on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Plates were incubated at 30°C for 96 h. (**B**) A graphical representation of the growth of strains BC300 expressing 1 copy, 2 or 3 copies of α-synuclein-eGFP fusion genes along with controls, over 48 h, in minimal selective medium with galactose. ANOVA test of yeast cell growth was statistically significant ($P<0.05$) between yeasts strains carrying 1, 2, and 3 copies of α-synuclein-eGFP and their respective controls, but the difference between 1, 2 and 3 copies was not significant ($P>0.05$). (**C**) Yeast cells expressing 1 copy, 2 and 3 copies of the α-synuclein-eGFP protein, as observed under a fluorescence microscope. (**D**) A bar chart depicting the percentage of cells that show α-synuclein-eGFP aggregation within yeast cells. α-synuclein-eGFP aggregation was significant among the three strains ($P<0.05$) (**E**) Comparison of the MMP of yeast strains expressing 1 copy, 2 or 3 copies of α-synuclein-eGFP fusion protein along with controls. ANOVA test gave a statistically significant effect ($P<0.05$) between copies of α-synuclein-eGFP and controls, the difference between 1 and 2, and 2 and 3 copies were not significant ($P>0.05$), but the difference between 1 and 3 copies was significant ($P<0.05$) (**F**) Percentage of cells undergoing death upon expression of 1 copy, 2 and 3 copies of α-synuclein-eGFP fusion protein monitored via Phloxine B staining. ANOVA test gave a statistically significant effect ($P<0.05$) for cell death assay (Ploxine B) of yeast cells expressing 1-3 copies of α-synuclein-eGFP. (**G**) Microscopic images of Phloxine B staining of cells expressing 1 copy, 2 and 3 copies of α-synuclein-eGFP. (**H**) Comparison of the amounts of NDF, as observed using the TUNEL assay, in yeast strains that express 1 copy, 2 and 3 copies of α-synuclein-eGFP along with controls. ANOVA test gave a statistically significant effect ($P<0.05$) for NDF (TUNEL) of yeast cells expressing 1–3 copies α-synuclein-eGFP. (**I**) TUNEL assay on cells expressing 1, 2 and 3 copies of α-synuclein-eGFP. (**J**) Western blot analyses of cells expressing 1–3 copies of the eGFP-tagged α-synuclein protein. Lanes 1, 2 and 3 have been loaded lysates from cells that express 1, 2 and 3 copies of α-synuclein while the lysates from cells with 1, 2 and 3 copies of empty plasmid were loaded on lane 4, 5 and 6. The HA-antibody was used to probe for expression of α-synuclein-eGFP, and the β-actin antibody for β-actin, which was used as a loading control. A total of 10 μg of total cellular protein was loaded in each lane. (**K**) Densitometric quantification of Western blot bands for 1 copy, 2 and 3 copies of the α-synuclein-eGFP gene in yeast strains. The densitometric values for β-actin were roughly the same. (**L**) The comparison of ROS produced in yeast strains expressing 1, 2 or 3 copies of the α-synuclein-eGFP fusion protein. ANOVA test showed that the difference between 1 and 2 copies ($P<0.05$), and 2 and 3 copies ($P<0.05$) was significant.

Hence, it suggests that only 1 and 2 copies of α-synuclein-eGFP can rescue the toxic effects of Bax. The results confirmed the earlier observations presented in Figure 7. The result shows that the percentage of cell death is least in the strain that co-expressed Bax, and two copies of α-synuclein-eGFP (Figure 8C,D), the difference in cell death is significantly low in the strain that co-expressed Bax and two copies of α-synuclein-eGFP.

The strain that co-expressed Bax and two copies of α-synuclein-eGFP produces the lowest amount of ROS with a significant difference from strains with 1 and 3 copies (Figure 8E). Cells in the strain which have two copies of α-synuclein-eGFP have the highest MMP, followed by strain with one copy and then strain with three copies of α-synuclein-eGFP (Figure 8F). Figure 8G,H shows that DNA fragmentation was high in the strain containing three copies of α-synuclein-eGFP, followed by the strain with one copy. The strain with two copies of α-synuclein-eGFP has the least DNA fragmentation. The presence of α-synuclein aggregation was seen in the strains expressing both 1-copy and 3-copies of α-synuclein-eGFP (Figure 8J). α-synuclein is barely seen in the strain that co-expressed 2-copies of α-synuclein-eGFP together with Bax.

In unstimulated cells, monomeric Bax-α (referred to as just Bax) protein, a pro-apoptotic member of the Bcl-2 family, usually resides in the cytosol and the periphery of intracellular membranes which include the mitochondria. Bax only inserts into mitochondrial membranes following a death signal [17]. Only homodimers of Bax are known to be pro-apoptotic. Malfunctioning of apoptosis can lead to diseases like viral infections, autoimmune diseases, cancer, while heightened apoptosis can lead to ischemic disease, neurodegenerative diseases, and AIDS. After co-expressing two human pro-apoptotic proteins α-synuclein and Bax, surprisingly, α-synuclein manifests an anti-apoptotic role in the presence of Bax, rescuing Bax-mediated cell death in baker's yeast.

Figure 9A–E shows co-expression of Bax with 1, 2 or 3 copies of mutant α-synuclein (A30P and A53T) genes. None of the yeast strains that co-express Bax and α-synuclein (A30P and A53T) proteins grew on expression in galactose. Hence, mutant α-synuclein (A30P and A53T) does not rescue yeast cells from Bax-induced cell growth block/death. It should be noted that independent of the copy number, yeast strains expressing mutant α-synuclein (A30P) alone are not restricted in cell growth and are therefore not toxic to yeast. In contrast, mutant α-synuclein (A53T) was as toxic as the wildtype (Figure 4).

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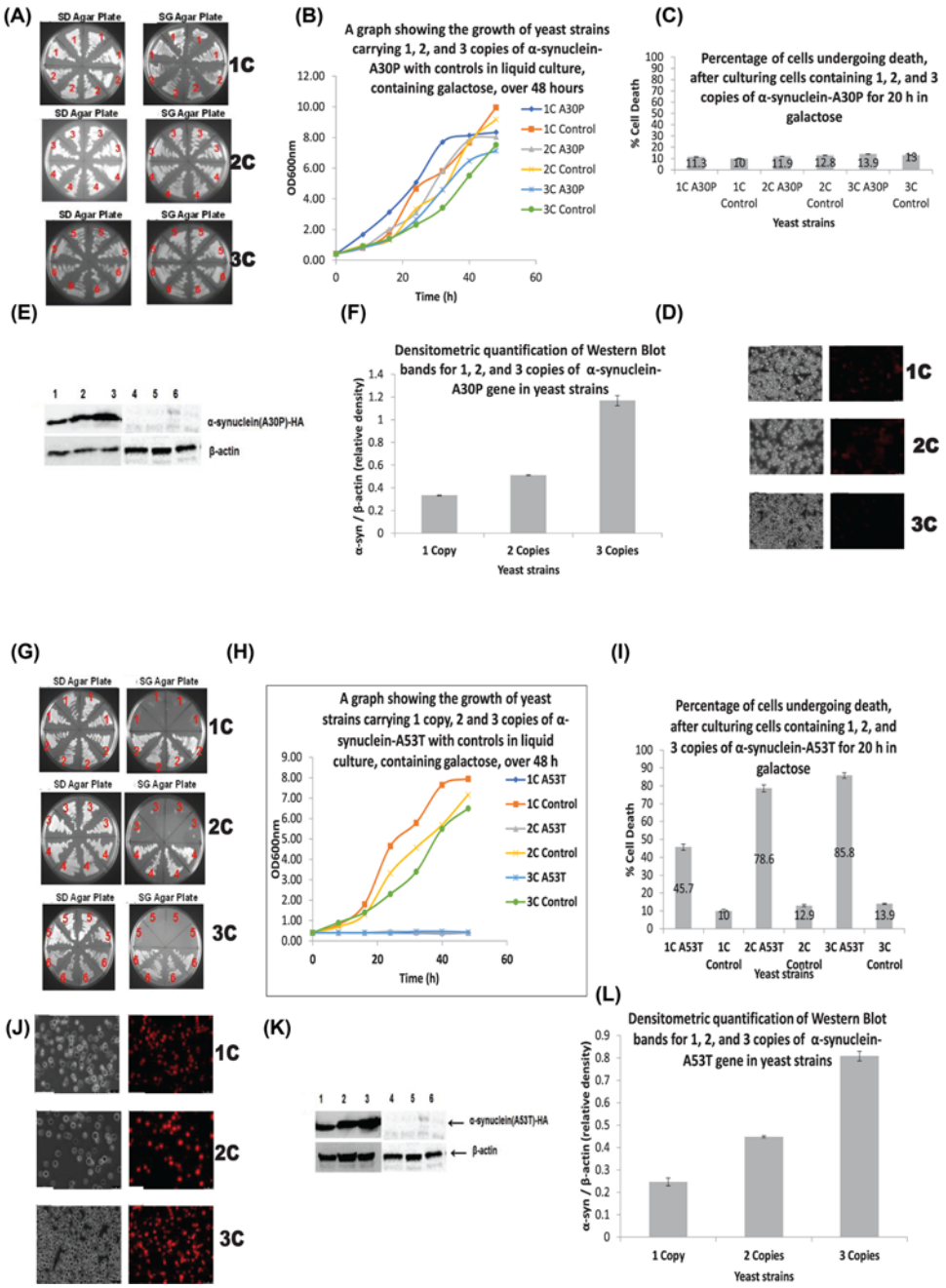

Figure 4. Expression of mutant α-synuclein (A30P and A53T) gene in yeast cells bearing 1–3 copies

(**A**) Growth of BC300 yeast cells harbouring the 1 copy of HA-tagged α-synuclein (A30P) gene (1) and 1 copy control (2), 2 copies of HA-tagged α-synuclein(A30P) gene (3) and 2 copies control (4) and 3 copies of HA-tagged α-synuclein(A30P) gene (5) and 3 copies control (6). The yeast transformants were grown on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Plates were incubated at 30°C for 96 h. (**B**) Growth of derived yeast strains expressing 1 (1C A30P), 2 (2C A30P) or 3 copies (3C A30P) of HA-tagged α-synuclein(A30P), from the GAL1p, in a minimal liquid medium (SG) where galactose is the carbon source along with controls. All three strains expressing 1–3 copies of HA-tagged α-synuclein (A30P) did grow over a period of 48 h. ANOVA tests statistically showed no significant difference in growth ($P>0.05$) between strains expressing mutant α-synuclein (A30P) and compared with control strains containing empty plasmids. (**C**) Phloxine B staining shows that there is no more death in cells expressing 1 copy, 2, and 3 copies of HA-tagged α-synuclein (A30P) protein than cells containing an empty vector. ANOVA tests showed; statistically, there is no significant difference ($P>0.05$) between α-synuclein (A30P) expressing strains and the control strains bearing empty plasmids. (**D**) Microscopic images of Phloxine B assay stained cells expressing 1, 2, and 3 copies of α-synuclein (A30P). (**E**) Western blot analyses of cells expressing 1–3 copies of HA-tagged α-synuclein (A30P) protein. On

lanes 1, 2 and 3 have been loaded lysates from cells that express 1 copy, 2 and 3 copies of α-synuclein (A30P)-HA while the lysates from cells with 1, 2 and 3 copies of empty plasmid were loaded on lanes 4, 5, and 6. The upper panel has been probed with an antibody that recognises the HA epitope and the lower panel has been probed with a β-actin antibody as a loading control, β-actin being a housekeeping gene. (**F**) Densitometry quantification of Western blot bands for 1, 2, and 3 copies of α-synuclein-A30P gene in yeast strains. The densitometric values for β-actin were roughly the same. (**G**) Growth of BC300 yeast cells harbouring 1 copy of HA-tagged α-synuclein(A53T) gene (1) and 1 copy control (2), 2 copies of HA-tagged α-synuclein(A53T) gene (3) and 2 copies control (4) and 3 copies of HA-tagged α-synuclein(A53T) gene (5) and 3 copies control (6). The yeast transformants were grown on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Plates were incubated at 30°C for 96 h. (**H**) Growth of BC300 derived yeast strains expressing 1, 2, or 3 copies of HA-tagged α-synuclein(A53T), from the GAL1p, in a minimal liquid medium (SG) where galactose is the carbon source along with controls. ANOVA tests gave a statistically significant effect ($P<0.05$) with controls but no significant effect between strains expressing different copies of mutant α-synuclein ($P>0.05$). (**I**) Phloxine B staining shows that there is more death in cells expressing 1, 2 and 3 copies of HA-tagged α-synuclein(A53T) protein than cells containing empty vector. There was a significant difference among the three yeast strains with their respective controls ($P<0.05$). The ANOVA test also revealed that there was a significant difference between cell death in strains expressing 1 copy, two or three copies of the mutant α-synuclein ($P>0.05$). (**J**) Microscopic images of Phloxine B stained cells expressing 1, 2, and 3 copies of α-synuclein (A53T). (**K**) Western blot analyses of cells expressing 1–3 copies of HA-tagged α-synuclein(A53T) protein. On lanes 1, 2 and 3 have been loaded lysates from cells that express 1 copy, 2 and 3 copies of α-synuclein(A53T)-HA while the lysates from cells with 1, 2 and 3 copies of empty plasmid were loaded on lanes 4, 5 and 6. The HA-antibody was used to probe for expression of α-synuclein(A53T), and the β-actin antibody for β-actin, which was used as a loading control. 10 μg of total cellular protein was loaded in each lane. (**L**) Densitometric quantification of Western blot bands of α-synuclein-A53T using ImageJ software. Densitometry of the β-actin bands shows roughly the same values for all three lanes.

When copies of α-synuclein were expressed, the α-synuclein protein seemed to localise to the mitochondria. It has been suggested that it is bonding to the mitochondrial outer membrane could affect the linkage of other proteins to the mitochondria, thereby causing modifications that eventually give way to permeability translocation pore dependent endonuclease G release [21]. Misfolded α-synuclein can spread from one neuron to another in the brain with the development of PD [40]. In a non-pathogenic nerve terminal, there is an equilibrium between monomeric, unfolded cytosolic α-synuclein and a multimeric, membrane-bound, α-helical form of α-synuclein which accompany SNARE complexes [7,40]. The relation between the conformation of the endemic form of α-synuclein and its aggregated pathological form is not entirely understood [14,40]. It is unclear if the aggregation of α-synuclein is from its membrane-bound oligomeric or cytosolic monomeric forms.

Rescue of Bax induced block of cell growth by α-synuclein-HA expressed from 1-3 copies of integrated plasmid, under the control of GAL1 promoter

Rescue of Bax induced block of cell growth by α-synuclein-eGFP expressed from 1-3 copies of integrated plasmid, under the control of GAL1 promoter

Co-expression of Bax and Mutant α-synuclein-HA (A30P & A53T) 1-3 copies of integrated plasmid, under the control of GAL1 promoter

It was observed that the yeast strain co-expressing one copy of both HA-tagged and eGFP-tagged α-synuclein, and three copies of eGFP-tagged α-synuclein with a copy of Bax produced relatively large amounts of both α-synuclein and Bax proteins. In contrast, the strains containing 2 and 3 copies of α-synuclein-HA and 2 copies of α-synuclein-eGFP produced much lesser amounts of both proteins. The β-actin loading control shows that equal amounts of proteins from cell lysates were indeed loaded; this suggests some form of protein–protein interaction in the two proteins.

From the Western blots, it would appear that protein degradation had occurred with the wildtype α-synuclein and Bax proteins when 2 and 3 copies of α-synuclein were co-expressed. The differences, between the levels of α-synuclein and Bax in the 1-copy α-synuclein strain on one hand and the 2 or 3-copy α-synuclein strains on the other, suggest that there is likely to be strong communication and/or interaction between the wildtype α-synuclein and Bax proteins during co-expression of 1–3 copies of α-synuclein and Bax. The strain co-expressing 2-copy α-synuclein-eGFP with Bax produces the least amount of both α-synuclein-eGFP and Bax proteins. This was also observed in yeast cell expressing α-synuclein under the control of MET25 promoter and α-synuclein untagged with HA (Figure 10).

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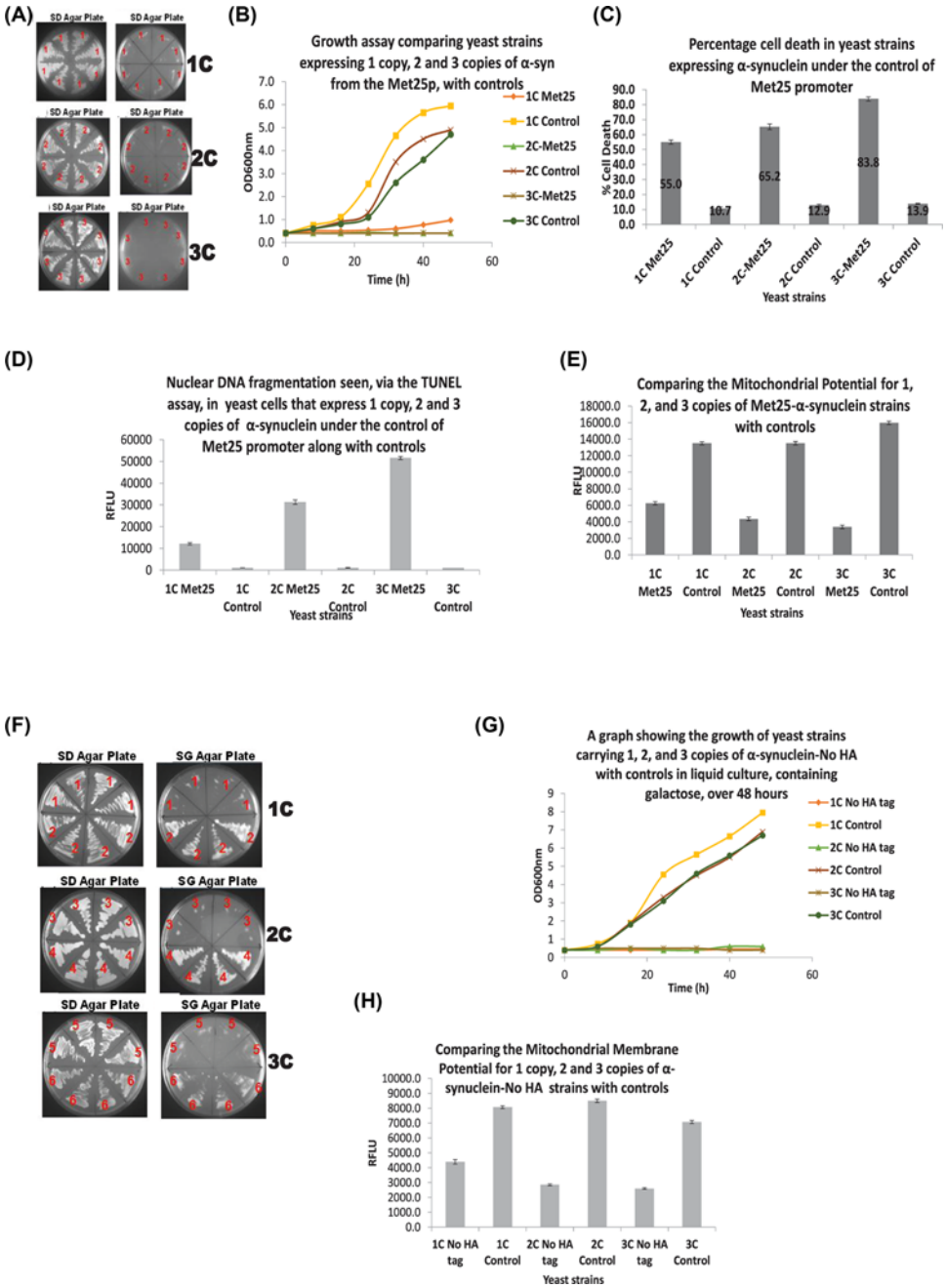

Figure 5. The effects of expressing increasing copy number of untagged α-synuclein on GAL1p, and HA-tagged α-synuclein on MET25p

(**A**) Growth of yeast cells harbouring 1, 2 and 3 yeast integrative plasmids that encode HA-tagged α-synuclein gene under the control of the MET25 promoter, one plate shows strains harbouring 1 copy, 2 and 3 copies of α-synuclein-HA, grown on SD agar plates containing 670 μM methionine. The other plate shows the same strains on SD agar plates that do not contain any methionine. Plates were incubated at 30°C for 96 h. (**B**) Growth of BC300 derived yeast strains expressing 1, 2, or 3 copies of α-synuclein-HA, from the MET25p, in a minimal liquid medium (SD) where glucose is the carbon source along with controls. Strains expressing 1–3 copies of α-synuclein-HA did not grow throughout 48 h. There was a significant difference between the three yeast strains with the respective controls ($P<0.05$). Multiple comparisons also indicated that there was significant difference in growth between 1 and 2, and 1 and 3 copies of α-syn ($P<0.05$), but no significant difference between 2 and 3 copies ($P>0.05$). (**C**) Percentage of cells undergoing death upon expression of 1 copy, 2 and 3 copies of α-synuclein under the control of MET25 promoter via Phloxine B staining. The 1-copy strain shows the least cell death. ANOVA test showed a statistically significant difference in cell death ($P<0.05$) between strains expressing increasing copies of α-synuclein when considering three independent experiments for

Phloxine B staining. (**D**) Comparison of the amounts of NDF, as observed using the TUNEL assay, in yeast strains that express 1 copy, 2 and 3 copies of α-synuclein under the control of MET25 promoter along with controls. ANOVA test showed a statistically significant difference in the levels of NDF ($P<0.05$) in the three strains expressing α-synuclein. (**E**) Comparison of the MMP of yeast strains expressing 1 copy, 2 and 3 copies of α-synuclein-HA from the MET25p, along with controls. The 1-copy strain has the highest MMP. In between sample ANOVA test was performed which revealed that differences in MMP between cells containing different copy numbers of α-synuclein and their respective controls were significant ($P<0.05$); there was significant membrane potential decrease with an increase in copy number, from 1 copy to 2 copies, and from 2 copies to 3 copies ($P<0.05$). (**F**) Growth of BC300 yeast cells harbouring the 1 copy of the untagged α-synuclein gene (1) and 1 copy control (2), 2 copies of the untagged α-synuclein gene (3) and 2 copies control (4) and 3 copies of the untagged α-synuclein gene (5) and 3 copies control (6). The yeast transformants were grown on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Plates were incubated at 30°C for 96 h. (**G**) Growth of cells harbouring (a) 1, 2, and 3 copies of untagged α-synuclein (b) 1, 2. and 3 copies of the empty plasmid in minimal medium with galactose as the sole carbon source; galactose induces GAL1 promoter. There was a significant difference in growth between the three α-synuclein expressing yeast strains and the corresponding controls ($P<0.05$), ANOVA test revealed that there was no significant difference in growth between the three α-synuclein expressing strains ($P>0.05$). (**H**) Comparison of the MMP of yeast strains expressing 1, 2 or 3 copies of α-synuclein-No HA from GAL1p, along with controls. There was a significant difference in the MMP of the three yeast strains expressing α-synuclein with their respective controls ($P<0.05$). ANOVA test revealed that there was also a significant difference between 1-copy and 2 or 3-copy α-synuclein strains ($P>0.05$).

Rescue of Bax induced block of cell growth by untagged α-synuclein (1-3 copies) of integrated plasmid, under the control of GAL1 promoter and α-synuclein-HA (1-3 copies) of integrated plasmid, under the control of MET25 promoter

With an increase in α-synuclein copy number, it was observed that there was a significant effect on cell growth, cell death, ROS, NDF and mitochondrial potential when α-synuclein is expressed in yeast cells from the GAL1 and MET25 promoters. This further confirmed that the toxicity of α-synuclein is proportional to an increase in gene dosage (i.e. an increase in copy number). However, the rescue of the yeast cell was seen when co-expressed with Bax. Figure 10I shows Rhodamine 1 2 3 stainings of cells from yeast strains that co-express Bax and one, two or three copies of untagged α-synuclein. Depending on the polarisation of the membranes (which includes mitochondrial membranes), the Rhodamine 1 2 3 stain can accrue on live cell membranes. This stain indicates the state of membrane structures. Intact membranes show durable stain in contrast with cells which have lost membrane polarisation and therefore stain very weakly or not all.

The results above clearly show that the untagged α-synuclein protein behaves similarly to the HA-tagged protein. Increase in untagged α-synuclein gene dosage showed a significant effect on cell growth and mitochondrial potential when α-synuclein was expressed from the GAL1 promoter, and this further confirmed that the toxicity of α-synuclein is proportional to an increase in copy number.

Can mutant α-synuclein proteins rescue yeast cells from the toxic effects of Bax? This was the question that came to our mind. The quantitative estimates of these Phoxine B staining results, as shown in Figure 9, clearly show that the mutant α-synuclein (A30P and A53T) has wholly lost wildtype α-synuclein's ability to protect cells from Bax-mediated death. Figure 9E,J depicts the expression of the two proteins Bax and α-synuclein (A30P and A53T), as monitored by Western blots, in the three different strains that express 1–3 copies of α-synuclein (A30P and A53T). In contrast with cells which co-express α-synuclein proteins that protect cells from death, cells expressing A30P and A53T mutants show undiminished levels of α-synuclein and Bax proteins on Western blots.

Contrary to its pathogenic role, α-synuclein exercises substantial neuroprotection in mice, overexpression of α-synuclein prevents rapidly progressive neurodegeneration mediated by deletion of CSPα, a presynaptic co-chaperone which is part of the SNARE complex [41]. Moreover, it has been suggested that α-synuclein cooperates with CSPα in preventing neurodegeneration [14]. Wildtype α-synuclein has also been reported to protect neurons from undergoing apoptosis through inhibition of caspase-3 [42]. It has been suggested that when misfolding of α-synuclein protein occurs; there is usually an increase in its toxicity and a decrease in its protective functions [43].

A schematic flow chart showing different apoptotic pathways and possible rescue mechanism

Bax forms oligomers at the outer membrane of the mitochondria which results in the release of cytochrome *c* (from the mitochondrial inter-membrane space) and other proteins (i.e. Nuc-1, Ndi-1, AIF, cytochrome *c*) from the mitochondria. Inhibitor of apoptosis protein (IAP) is also released into the cytosol. IAP typically suppresses caspases by

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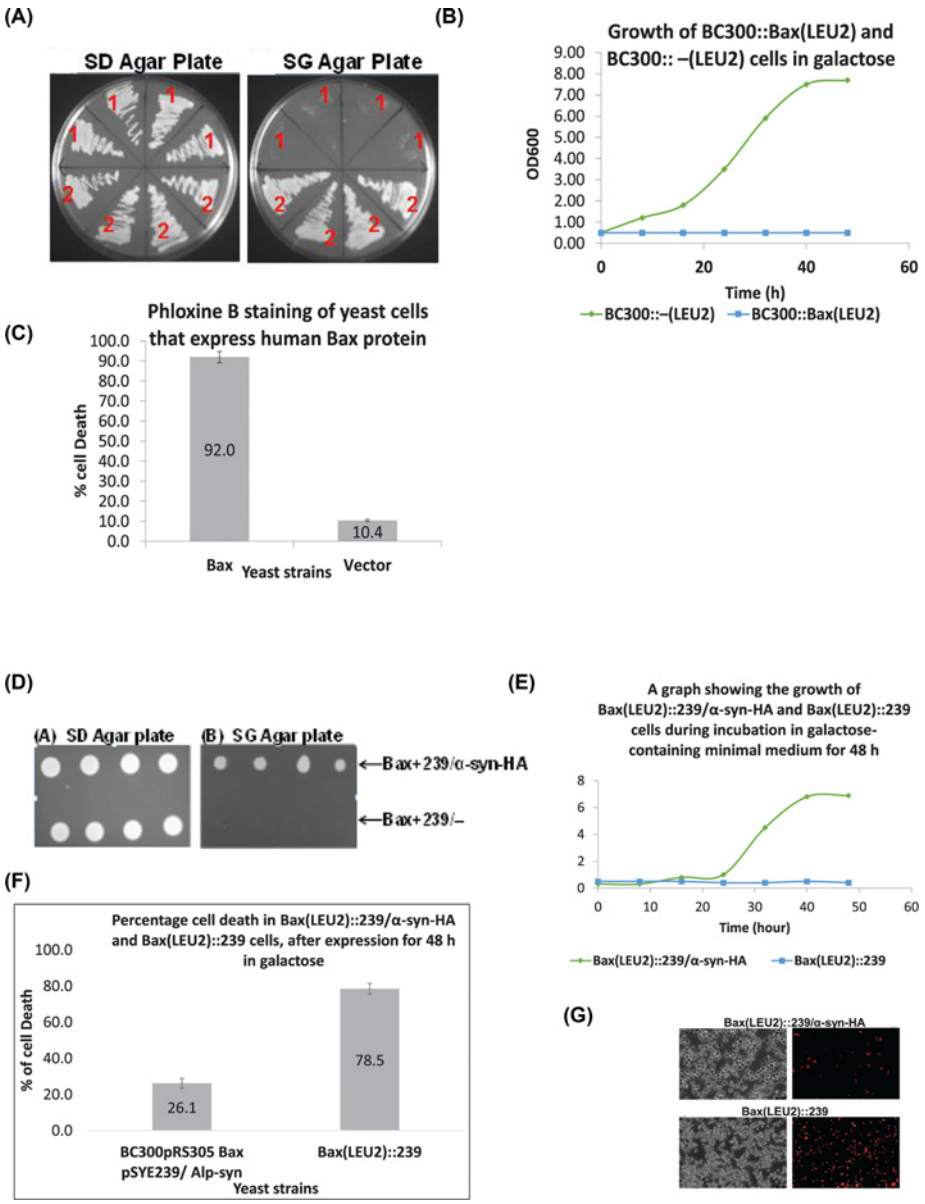

Figure 6. Expression of human Bax gene in yeast cells on GAL1p and rescue of Bax-induced block of cell growth by α-synuclein from a 2μ plasmid on PGK1 promoter

(**A**) Growth of yeast cells harbouring a copy of the C-terminal c-myc tagged human Bax gene, at the LEU2 chromosomal locus (1). The yeast transformants were grown on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Plates were incubated at 30°C for 96 h. The Bax harbouring strains (1) did not grow in galactose, whereas the control strains (with no Bax) (2) grew. The results on plates confirm earlier observations that the human Bax protein is toxic to yeast cells. These observations were corroborated by growing cells in a liquid medium; see (**B**) ($P<0.05$). (**C**) Phloxine B staining showed the percentage of death when human Bax gene was expressed in minimal medium containing galactose for 20 h. The ANOVA test revealed that the % difference in cell death was significant ($P<0.05$). (**D**) Five microlitres of cells (suspended in 0.9% NaCl) from four different transformants, from each of the two transformations [Bax(LEU2)::239/α-syn-HA and Bax(LEU2)::239], were spotted on to two minimal medium plates, SD-Agar (containing glucose, plate A) and SG-Agar (containing galactose; plate B). Plates were incubated at 30°C for 96 h. Galactose allows expression of both Bax and α-synuclein, from the GAL1 and PGK1 promoters. Cells with α-synuclein gene overcame Bax-induced block in growth while the ones with empty plasmid are blocked in growth because of Bax protein's toxic effects. These results have been corroborated by the growth of cells in SG liquid medium (**E**) ($P<0.05$). (**F**) The percentage of cell death was determined by staining with Phloxine B. Much less cell death was observed in Bax(LEU2)::239/α-syn-HA cells than in control Bax(LEU2)::239 cells. The results reflect the microscopic images in (**G**). ANOVA test revealed that there was a significant difference ($P<0.05$) in the % cell death between the two strains.

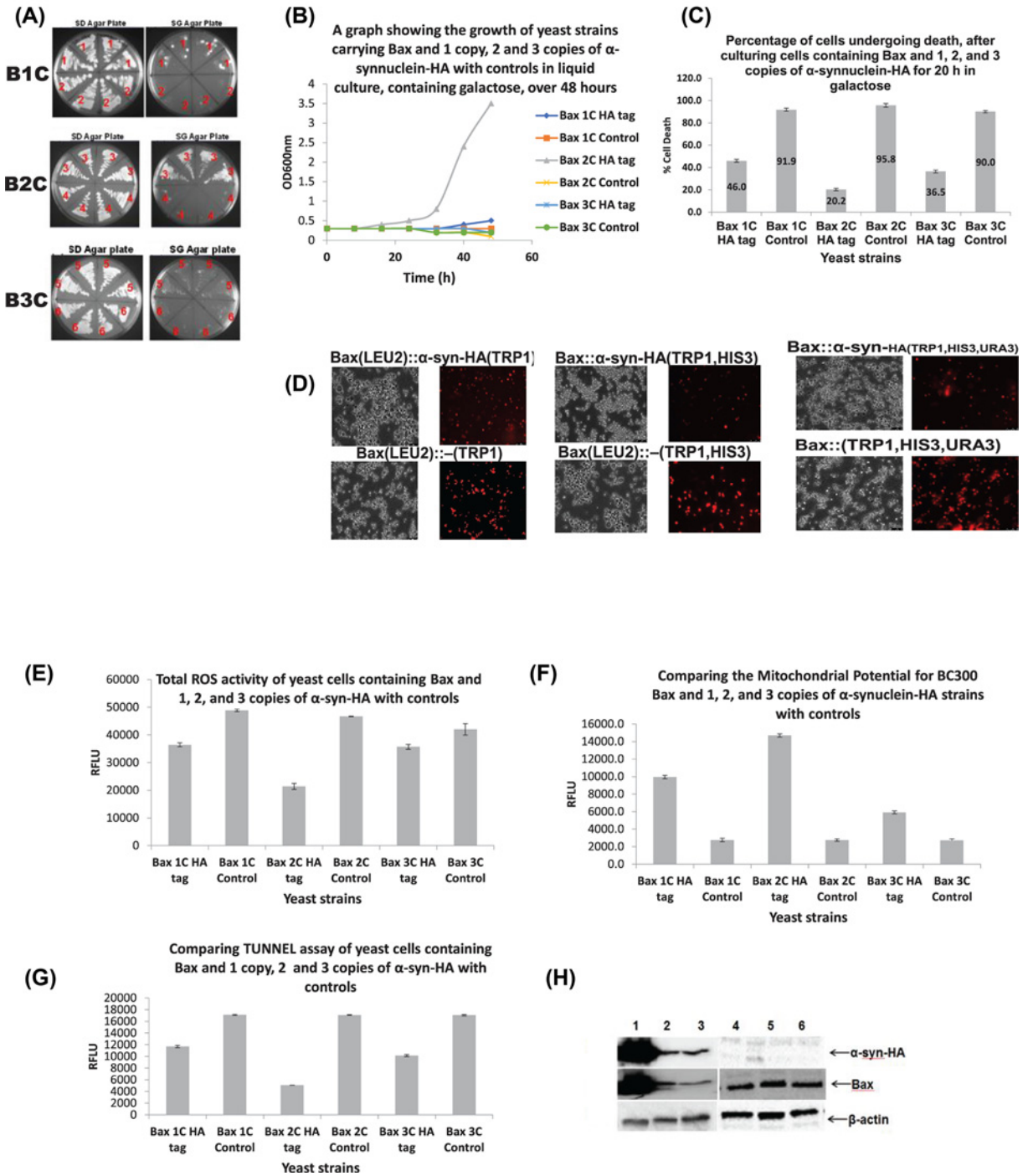

Figure 7. Rescue of Bax-induced block of cell growth by α-synuclein-HA expressed from 1 to 3 copies of integrated plasmid, under the control of GAL1 promoter

(**A**) Growth of BC300::Bax yeast cells harbouring 1 copy of α-synuclein-HA gene (1) and 1 copy control (2), 2 copies of the α-synuclein-HA gene (3) and 2 copies control (4) and 3 copies of the α-synuclein-HA gene (5) and 3 copies control (6). Cells were streaked out on to two minimal medium plates, SD-Agar (containing glucose, plate A) and SG-Agar (containing galactose; plate B); plates were incubated at 30°C for 96 h. (**B**) Growth of BC300::Bax cells containing 1 copy, 2 or 3 copies of α-synuclein-HA and controls. Cells were incubated in galactose-containing minimal liquid medium for 48 h. The results show that 2 copies of HA-tagged α-synuclein can rescue Bax-induced block in cell growth. ANOVA test was performed which revealed that the difference in cell growth between the 2-copy α-synuclein strain and the control Bax-containing strain was significant ($P<0.05$). In contrast, the difference in cell growth between 1 and 3-copy α-synuclein strains and the respective controls was insignificant ($P>0.05$). (**C**) The percentage cell death was determined by staining with phloxine B. Co-expression of 1, 2, and 3 copies of α-synuclein-HA with

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Bax results in much less death in cells with 2 copies of α-synuclein-HA compare to control cells which express Bax alone. There was a significant difference in cell death between the yeast strains that express different copies of α-synuclein and also their respective controls ($P<0.05$). (**D**) Microscopic images of BC300, co-expressing Bax and 1, 2, and 3 copies of α-synuclein-HA and control cells that express only Bax and contain 1, 2 and 3 copies of empty plasmids cells stained with phloxine B. (**E**) The amount of ROS produced in BC300, co-expressing Bax and 1, 2 and 3 copies of α-synuclein-HA was compared with the ROS produced in control cells that express only Bax and contain 1, 2, and 3 copies of empty plasmids. There is a reduction in ROS when 2 copies of HA-tagged α-synuclein was co-expressed with Bax. There was a significant difference among the three yeast strains with their respective controls ($P<0.05$). ANOVA test revealed that there was no significant difference in ROS levels between one and three copies ($P>0.05$), but there was a significant difference between one and two copies, and two and three copies ($P<0.05$). (**F**) Comparison of the MMP of BC300::Bax cells, which co-express 1, 2, and 3 copies of α-synuclein-HA and BC300::Bax cells, which co-express 1, 2, and 3 copies of empty plasmid, both controlled by GAL1 promoter. There was a significant difference between the yeast strains co-expressing Bax and 1-3 copies of α-synuclein and their respective controls ($P<0.05$) (**G**) Comparison of the levels of NDF between BC300::Bax cells, which co-express 1 copy, 2 and 3 copies of α-synuclein-HA and BC300::Bax cells, which co-express 1 copy, 2 and 3 copies of empty plasmid, via the TUNEL assay. The GAL1 promoter drove both Bax and α-synuclein gene expression. There was a significant difference in NDF in the yeast strains which co-express Bax and α-synuclein genes and which express Bax alone ($P<0.05$). (**H**) Western blots showing the presence of α-synuclein protein in cell lysates of yeast strains co-expressing Bax and one, two, or three copies of α-synuclein. 1-copy α-syn-HA (1); 2-copy α-syn-HA (2); 3-copy α-syn-HA (3), lanes 4, 5, and 6 were 1, 2, and 3 copies of an empty plasmid. 10 μg of total cellular protein was loaded in each lane. The HA-antibody was used to probe for expression of α-synuclein-HA, the c-myc antibody for Bax, and the β-actin antibody for β-actin, which was used as a loading control.

blocking caspase activities [44]. Once caspases are activated, they use multiple pathways to achieve apoptosis. Bcl-2 blocks the action of Bax typically, but p53 inhibits Bcl-2. Alteration in protein quality control (PQC) pathways has also been linked to mediate α-syn misfolding, accumulation, and aggregation [45].

Rescue of apoptosis could target some of the pathways stopping apoptosis from occurring (Figure 11), this could include the restoration of mitochondrial function which is essential, as it will stop every other downstream process. Restoration of mitochondrial function by an anti-apoptotic protein could also mean blocking pores made on the mitochondria, which would lead to the prevention of mitochondrial protein translocation (Figure 11B). Inhibiting/preventing the activation of caspases, for example, preventing the conversion of pro-caspase-3 to caspase-3 could also be an anti-apoptotic intervention. Similarly, interruption of AIF, NUC-1 and Ndi-1 may be necessary for the prevention of apoptosis. Other possible rescue pathways could involve protein-protein interactions between pro and anti-apoptotic proteins. Mopping up of oxidative stress or ROS in cells could be another channel for rescue.

Results of the present study show an interesting trend. With increasing copy number of α-synuclein, when co-expressed with Bax, there was a progression in rescue from one copy to two copies, but then rescue did not occur with three copies of α-synuclein. Protein–protein interaction could have led to degradation (as seen in two copies of α-synuclein when co-expressed with Bax), on the introduction of the third copy, rescue activity decreases significantly, owing to more or over the aggregation of α-synuclein, this suggests that the level of α-synuclein protein present at a point in time dictates its behaviour (pro or anti-apoptotic).

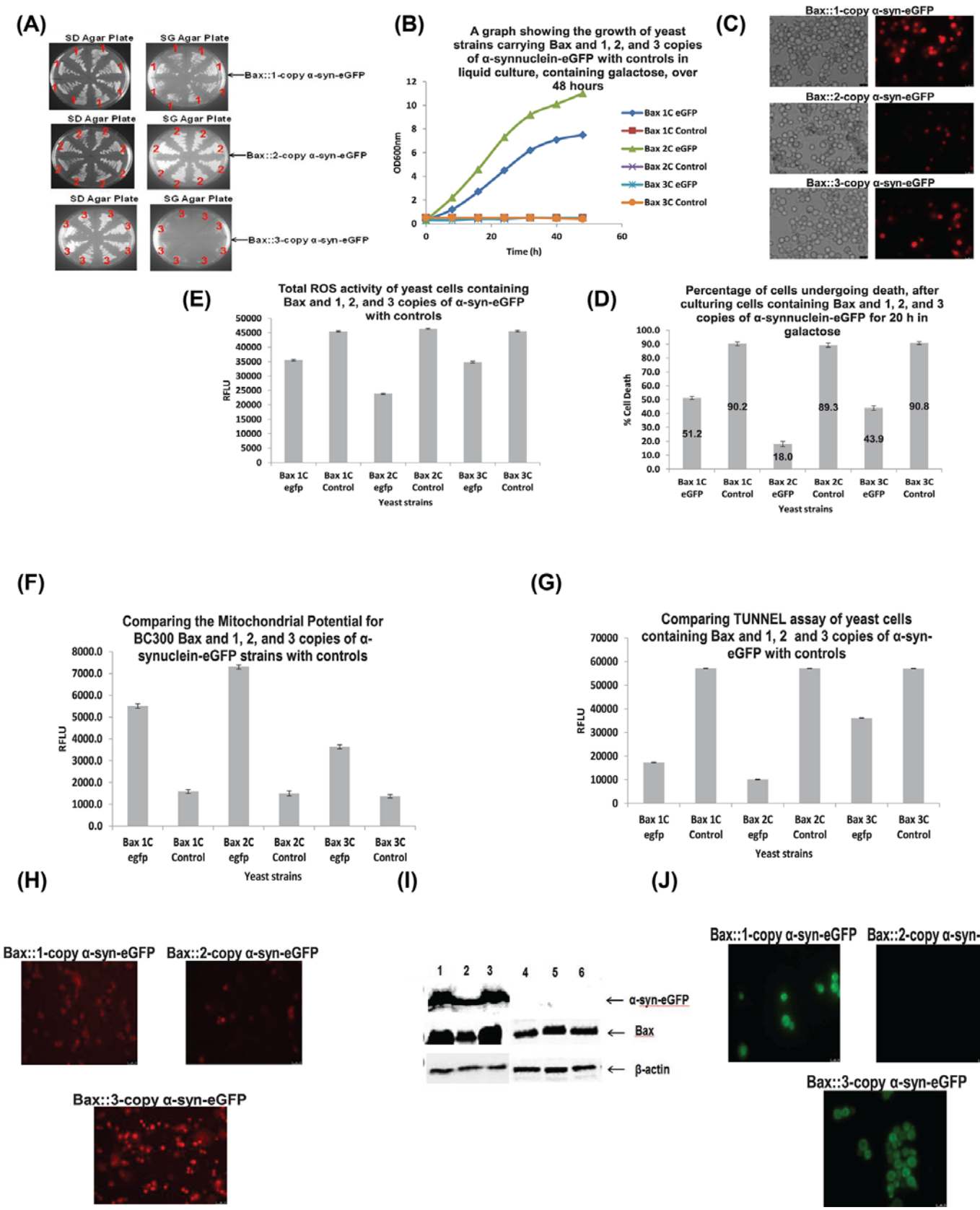

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Figure 8. Rescue of Bax induced block of cell growth by α-synuclein-eGFP expressed from 1–3 copies of integrated plasmid, under the control of GAL1 promoter

(**A**) Agar plates showing the growth of yeast cells when Bax gene is co-expressed with 1–3 copies of eGFP tagged α-synuclein gene. Bax(LEU2)::α-syn-eGFP(TRP1) (1); Bax(LEU2)::α-syn-eGFP(TRP1,HIS3) (2); Bax(LEU2)::α-syn-eGFP(TRP1,HIS3,URA3) (3). SD: glucose-containing minimal medium; SG: galactose-containing minimal medium. Plates were incubated at 30°C for 96 h. (**B**) Growth of cells co-expressing Bax and 1, 2 or 3 copies of the α-synuclein-eGFP fusion protein in galactose containing liquid SG minimal medium. The growth difference between one, and two copies with their own control was significant ($P<0.05$), while that of three copies was not significant ($P>0.05$). ANOVA test also revealed that there was no significant difference between strains co-expressing 1 copy and 2 copies of α-synuclein-eGFP with Bax ($P>0.05$), but there was a significant difference between 1-copy and 2-copy α-synuclein-eGFP co-expressing cells on the one hand and 3-copy co-expressing cells on the other ($P<0.05$). (**C**) The percentage cell death in the three different strains, co-expressing Bax and 1–3 copies of the α-synuclein-eGFP fusion protein, and their respective controls. This was determined by staining with Phloxine B. There was a significant difference between the yeast strains and their respective controls, and between strains expressing different copy numbers ($P<0.05$). (**D**) Representative microscopic images of Phloxine B stained dead cells showing an appreciable decrease in the death of cells expressing 2 copies of the α-synuclein-eGFP fusion protein. (**E**) Comparison of ROS produced in the three strains that co-express Bax and 1 to 3 copies of eGFP-tagged α-synuclein along with controls. There was a significant difference among the three yeast strains with their respective controls ($P<0.05$), ANOVA test revealed that there was a significant difference between one and two copies ($P<0.05$), and two and three copies ($P<0.05$), but there was no significant difference between one and three copies ($P>0.05$). (**F**) Comparison of the MMP of yeast strains co-expressing Bax and 1, 2 or 3 copies of α-synuclein-eGFP along with controls. There was a significant difference between the three yeast strains with their respective controls ($P<0.05$). ANOVA test revealed that there was no significant difference between 1 and 2 copies ($P>0.05$), and 1 and 3 copies ($P>0.05$), but there was a significant difference between two and three copies ($P<0.05$). (**G**) Measurement of NDF in the yeast strains that co-express Bax and 1, 2 or 3 copies of α-synuclein-eGFP along with controls via the TUNEL assay. There was a significant difference among the yeast strains both with their respective controls and between the copy numbers ($P<0.05$). (**H**) Shows a representative microscopic view of the TUNEL assay results of which were presented, in a bar-chart format. (**I**) Western blot of cell lysates co-expressing Bax and 1, 2 or 3 copies of the α-synuclein-eGFP fusion protein. 1-copy α-syn-eGFP (1); 2 copies α-syn-eGFP (2); 3-copies α-syn-eGFP (3), 1-copy control (4); 2-copies control (5); and 3-copies control (6). A total of 10 μg of total cellular protein was loaded in each lane. The HA-antibody was used to probe for expression of α-synuclein-eGFP, the c-myc antibody for Bax, and the β-actin antibody for β-actin, which was used as a loading control. (**J**) Monitoring of eGFP expression within cells, the strain expressing two copies did not show traces of aggregation.

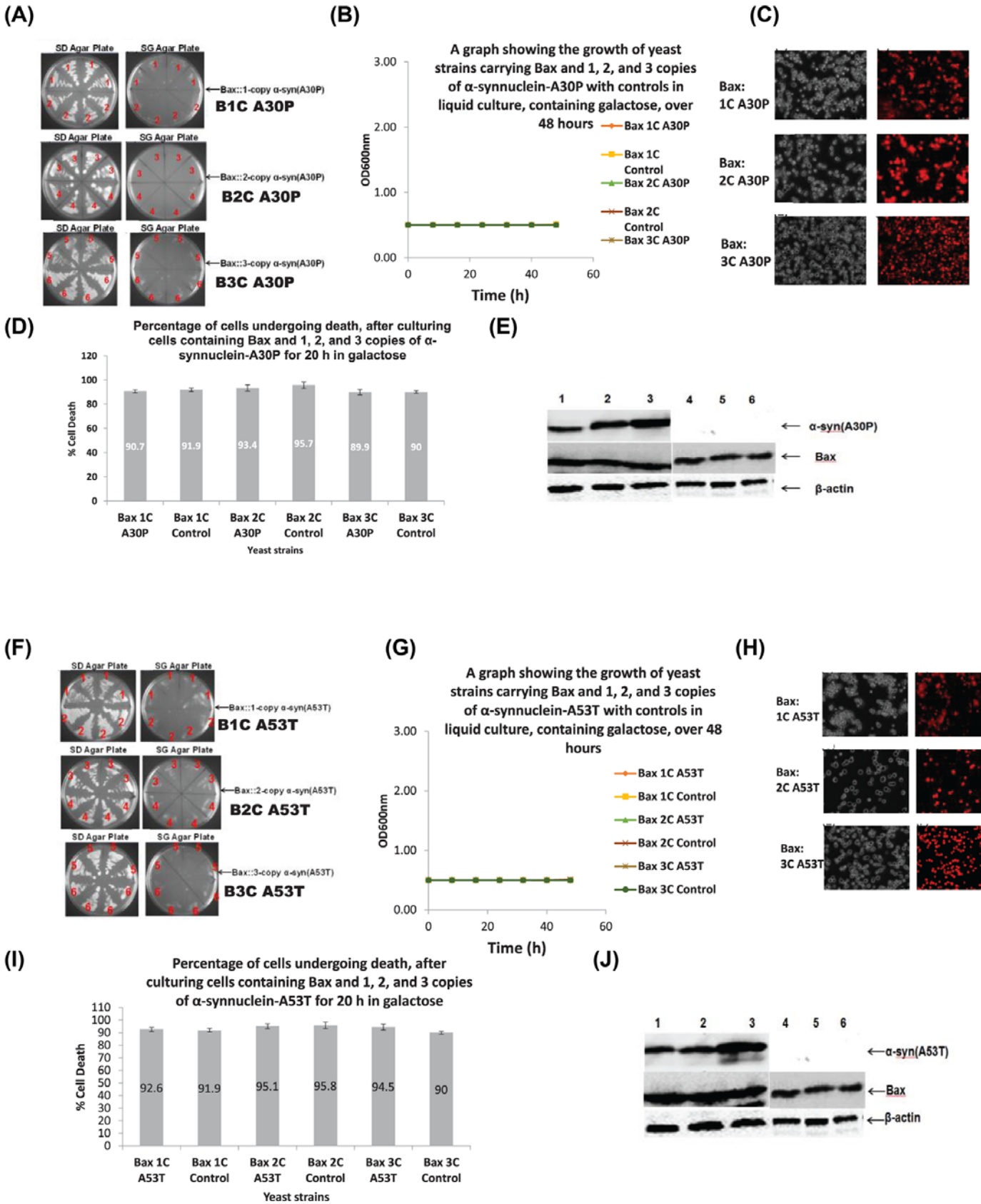

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Figure 9. Co-expression of Bax and mutant α-synuclein-HA (A30P and A53T) 1–3 copies of integrated plasmid, under the control of GAL1 promoter

(**A**) Growth of yeast cells co-expressing Bax with 1 (1), 2 (3) or 3 (5) copies of the mutant α-synuclein gene (A30P) on solid agar plates along with their controls expressing Bax with 1 (2), 2 (4) or 3 (6) copies of empty plasmids on SD and SG plate. Plates were incubated at 30°C for 96 h. (**B**) Growth of yeast cells that co-express Bax with 1, 2 or 3 copies of the mutant α-synuclein gene (A30P) in minimal liquid SG medium containing 2% galactose, along with control strains. There was no significant difference between the yeast strains and with their respective controls, and the copy numbers ($P>0.05$). (**C**) Microscopic images of Phloxine B staining of yeast cells bearing Bax and 1 copy, 2 and 3 copies of α-synuclein (A30P). (**D**) Percentage cell death in yeast cells co-expressing Bax and 1, 2, and 3 copies of α-synuclein-A30P along with empty plasmid, via staining with the fluorescent dye, Phloxine B. There was no significant difference among the yeast strains both with their respective controls and between the copy numbers ($P>0.05$). (**E**) Shows Western blots of lysates from cells co-expressing Bax and 1-3 copies of α-synuclein(A30P). 1-copy α-synuclein(A30P) (1); 2-copy α-synuclein(A30P) (2); 3-copy α-synuclein(A30P) (3). 1-copy control (4); 2-copies control (5); 3-copies control (6). (**F**) Growth of yeast cells co-expressing Bax with 1 (1), 2 (3), and 3 (5)copies of the mutant α-synuclein gene (A53T) on solid agar plates. SD plates: contain 2% dextrose/glucose; SG plates: contain 2% galactose. Along with control containing 1 (2), 2 (4), and 3 (6) copies of an empty plasmid. (**G**) Growth of yeast cells co-expressing Bax with 1, 2 or 3 copies of the mutant α-synuclein gene (A53T) in minimal liquid SG medium containing 2% galactose. Bax 1C A30P: Bax::1-copy α-syn(A53T); Bax 2C A30P: Bax::2-copy α-syn(A53T); Bax 3C A30P: Bax::3-copy α-syn(A53T). There was no significant difference among the yeast strains both with their respective controls and between the copy numbers ($P>0.05$). (**H**) Microscopic images of Phloxine B staining of yeast cells bearing Bax and 1, 2 or 3 copies of α-synuclein (A53T). (**I**) Percentage death in yeast cells co-expressing Bax and 1, 2, and 3 copies of α-syn(A53T) and empty plasmid, via staining with the fluorescent dye, Phloxine B. There was no significant difference among the yeast strains both with their respective controls and between the copy numbers ($P>0.05$). (**J**) Shows Western blots of lysates from cells co-expressing Bax and 1–3 copies of α-synuclein(A30P). 1-copy α-synuclein(A30P) (1); 2-copies α-synuclein(A30P) (2); 3-copies α-synuclein(A30P) (3); 1-copy control (4); 2-copies control (5); 3-copies control (6). The HA-antibody was used to probe for expression of α-synuclein(A30P), the c-myc antibody for Bax, and the β-actin antibody for β-actin, which was used as a loading control. A total of 10 μg of total cellular protein was loaded in each lane.

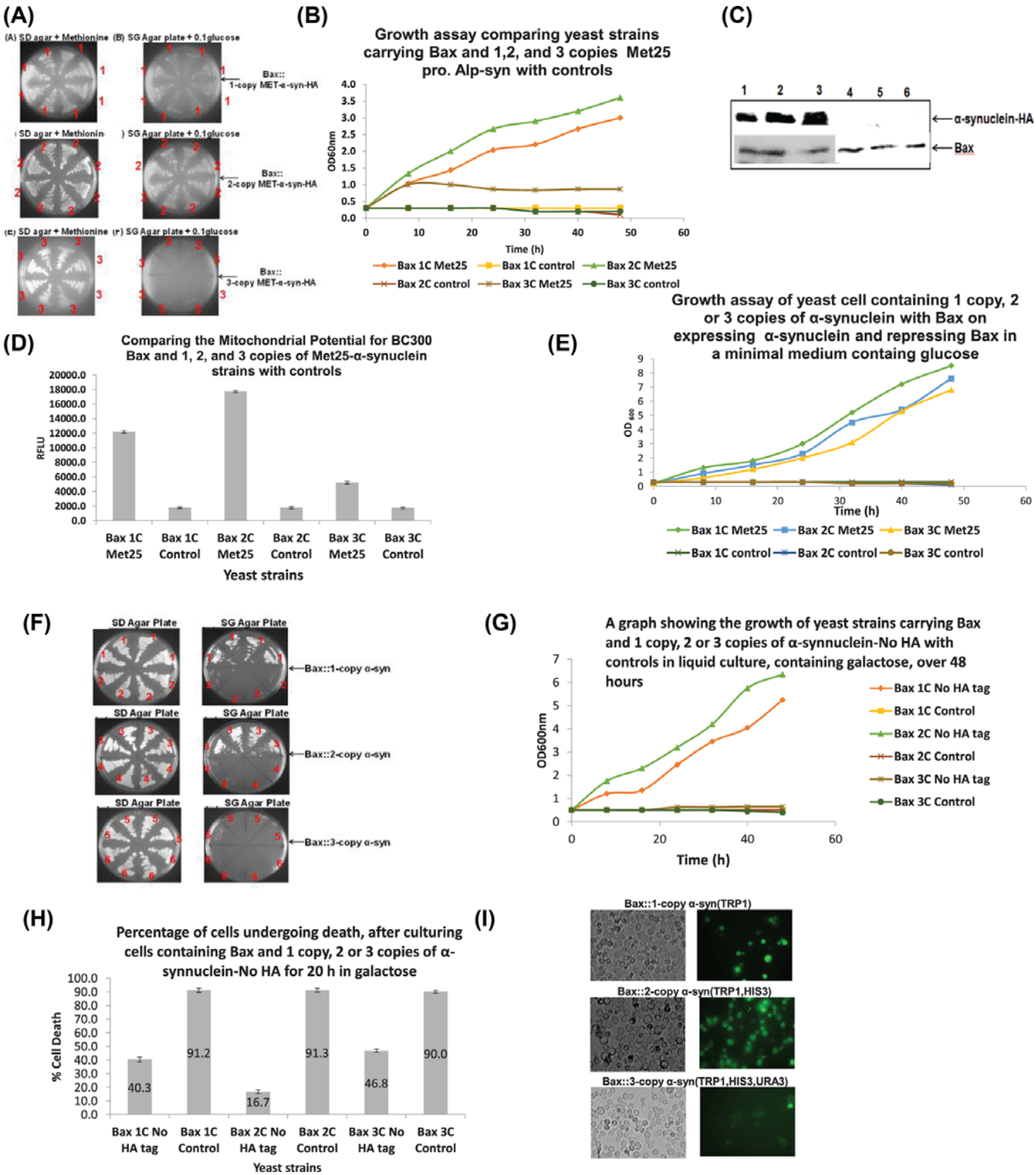

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Figure 10. Rescue of Bax induced block of cell growth by untagged α-synuclein (1–3 copies) on GAL1p and α-synuclein-HA (1–3 copies) on MET25p

(**A**) Growth on solid agar minimal medium plates of yeast transformants bearing one (1), two (2) or three (3) copies of the α-synuclein gene under the control of MET25 promoter (MET-α-syn-HA) and Bax gene under the control of GAL1 promoter (Bax). (**B**) Growth of yeast strains containing 1, 2 and 3 copies of α-synuclein-HA (MET25p) with a copy of Bax in a minimal liquid medium (SG + 0.1% glucose) along with controls. There was a significant difference among the three yeast strains with their respective controls ($P<0.05$) except three copies and its control ($P>0.05$), ANOVA test also revealed that there was no significant difference between one and two copies ($P>0.05$), but there was a significant difference between two and three copies ($P<0.05$). (**C**) Shows Western blots of cell lysates using 10 μg of total cellular protein. Lane 1: Bax::1-copy α-synuclein-HA; lane 2: Bax::2-copy α-synuclein-HA; lane 3: Bax::3-copy α-synuclein-HA; while Lane 4: Bax::1-copy control; lane 5: Bax::2-copy control; and lane 6: Bax::3-copy control. The upper panel was probed with an HA antibody, whereas the lower panel was probed with the c-myc antibody. (**D**) A comparison of the MMP of yeast strains containing 1, 2 and 3 copies of α-synuclein (expressed from the MET25 promoter) and a copy of Bax along with controls. There was a significant difference among the yeast strains both with their respective controls and between the copy numbers ($P<0.05$). (**E**) The graph shows the growth of cells during expression of HA-tagged α-synuclein from the MET25 promoter, in SG minimal medium (with 0.1% glucose), with no methionine. The cells contain 1, 2 or 3 copies of α-synuclein together with a single copy of the Bax gene integrated at the LEU2 locus. There was a significant difference among the yeast strains with their respective controls ($P<0.05$) but no significant difference between the copy numbers ($P>0.05$). (**F**) Growth of yeast cells co-expressing Bax and one (1), two (3) or three (5) copies of the untagged α-synuclein gene on solid agar plates along wi5th controls expressing Bax and one (2), two (4) or three (6) copies of empty plasmids on and SG plates. (**G**) Growth of yeast cells in galactose-containing SG medium. Co-expressing Bax and 1, 2, and 3 copies of α-synuclein-No HA along with controls. There was a significant difference among the three yeast strains with their respective controls ($P<0.05$), ANOVA test also revealed that there was no significant difference between one and two copies ($P>0.05$), and one and three copies ($P>0.05$), but there was a significant difference between two and three copies ($P<0.05$). (**H**) Percentage cell death in yeast cells, as monitored by staining with the fluorescent dye, Phloxine B. After co-expressing Bax and 1, 2, and 3 copies of α-synuclein-No HA along with controls. There was a significant difference among the yeast strains both with their respective controls and between the copy numbers ($P<0.05$). (**I**) Shows Rhodamine 1 2 3 stainings of yeast cells co-expressing Bax and 1 copy, 2 or 3 copies of untagged α-synuclein.

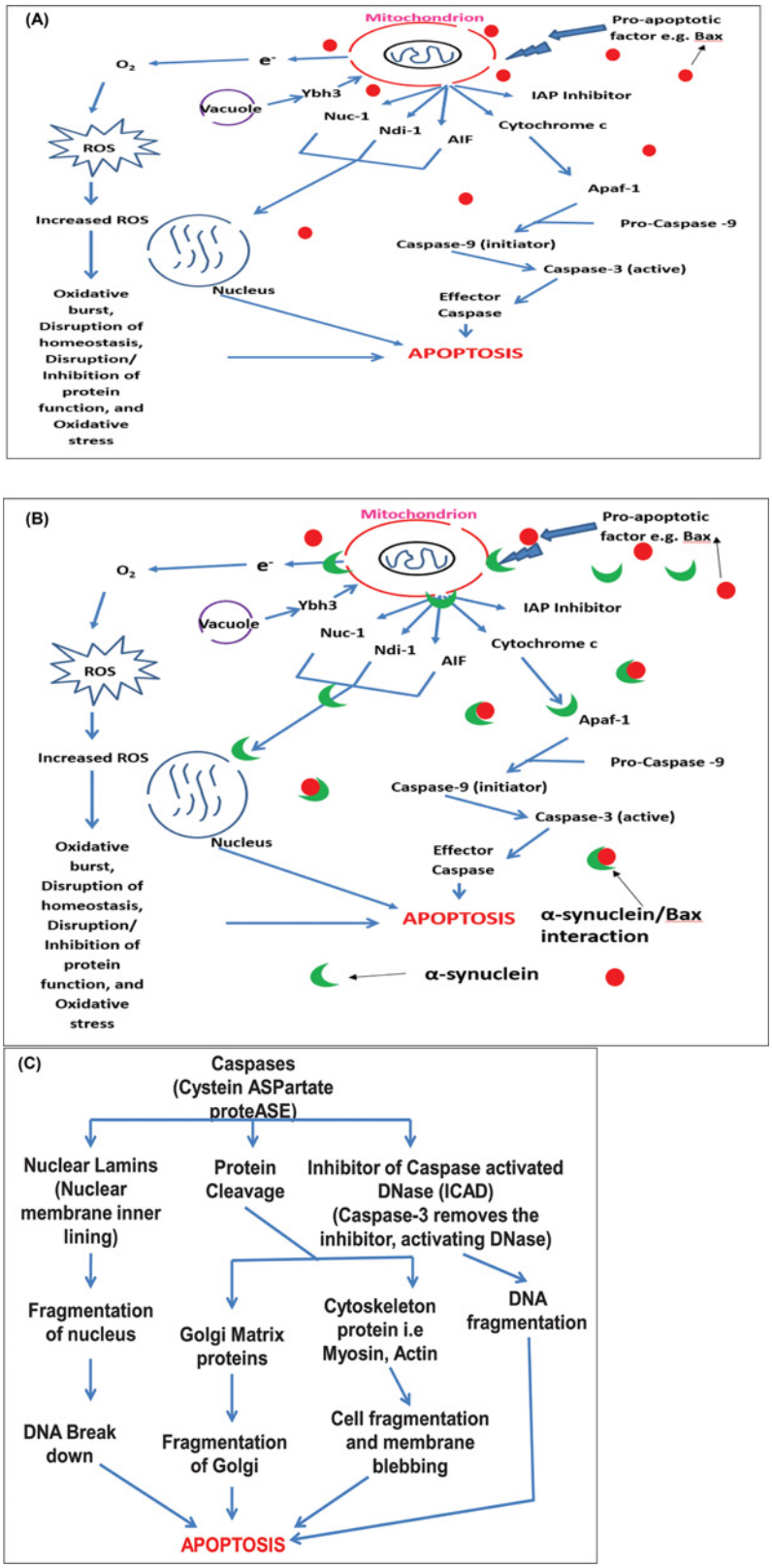

Figure 11. A schematic flow chart showing different apoptotic pathways and possible rescue mechanism

(**A**) A flow chart showing different apoptotic pathways induced by a pro-apoptotic protein, for example, Bax, through mitochondrial damage. (**B**) Show the hypothetical wildtype α-synuclein rescue pathway of Bax induced cell death (**C**) Flow diagram for caspase-activated pathways to apoptosis.

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Conclusions

Expression from episomal plasmids in yeast had failed to provide conclusive results regarding α-synuclein's toxicity in yeast, the effect of an increasing number of defined copies of wildtype α-synuclein is indeed toxic to yeast. Amongst the two α-synuclein mutants, A30P and A53T, A30P appears not to be toxic to yeast even when three defined chromosomal copies were expressed. In contrast, the A53T mutant was found to be toxic just like wildtype α-synuclein when expressed from chromosomal loci. The present study shows a very different aspect of wild type α-synuclein; it was quite interesting to see the dual nature of α-synuclein. It acts as a pro-apoptotic agent inducing blockage of cell growth and apoptosis (Figures 2 and 3). But α-synuclein seems to act as an anti-apoptotic agent when it is co-expressed with Bax, which is known to be a potent inducer of apoptosis both in dividing and non-dividing cells. Mutant α-synuclein proteins, A30P and A53T, when co-expressed with Bax, do not rescue yeast cells from Bax-induced apoptosis.

Overall, one can conclude that independent of the promoter, α-synuclein expressed from a 2μ plasmid does not have any toxic effects on yeast. It can be concluded that the mutant A30P and A53T α-synuclein proteins, in contrast with the wildtype α-synuclein protein, can not overcome Bax-mediated toxicity in yeast. The inability of these mutants to rescue Bax's toxicity somehow helps to maintain the levels of mutant α-synuclein proteins and Bax, as seen in Western blots. We would like to suggest that the mutant α-synuclein proteins are the causative agents of PD, responsible for the destruction of neuronal cells. In contrast, the wildtype α-synuclein exerts a protective influence on neurons deletion. However, we have also observed that, even in the course of protecting yeast cells by wildtype α-synuclein, overexpression of wildtype can be damaging too.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

D.D.A. performed all the experiments. B.C. coordinated the study. B.C. and D.D.A. wrote the manuscript.

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Abbreviations

GAL1p-α-synuclein-HA, GAL1 promoter-driven HA-tagged α-synuclein expression cassette; IAP, inhibitor of apoptosis protein; MMP, mitochondrial membrane potential; NDF, nuclear DNA fragmentation; PD, Parkinson's disease; ROS, reactive oxygen species; SNARE, SNAP REceptor; SNAP , soluble NSF attachment proteins; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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