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## Apoptotic death of ageing yeast

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### Abstract

Yeast has been a valuable model to study replicative and chronological ageing processes. Replicative ageing is defined by the number of daughter cells a mother can give birth to and hence reflects the ageing situation in proliferating cells, whereas chronological ageing is widely accepted as a model for postmitotic tissue ageing. Since both ageing forms end in yeast programmed death (necrotic and apoptotic), and abrogation of cell death by deletion of the apoptotic machinery or diminishment of oxidative radicals leads to longevity, apoptosis and ageing seem closely connected. This review focuses on ageing as a physiological way to induce yeast apoptosis, which unexpectedly defines apoptosis as a pro- and not an anti-ageing mechanism.

### Introduction

Apoptosis is a highly regulated cellular suicide program crucial for metazoan development. Damaged, infected, or simply superfluous cells are homeostatically removed without an inflammatory reaction occurring. Conversely, necrotic cell death is characterized by cell lysis including the release of cytoplasmic material to the environment, ultimately triggering inflammation (Kroemer et al., 2007).

Like metazoan cells, yeast cells succumb to cell death showing typical apoptotic markers such as externalization of phosphatidylserine (PS) to the outer leaflet of the plasma membrane, chromatin condensation and DNA fragmentation (Madeo et al., 1997). The programmed death of yeast has also been linked to complex mitochondrial processes, such as cytochrome c (Ludovico et al., 2002; Manon et al., 1997), and AIF release (Wissing et al., 2004), channel opening upon human Bax expression (Pavlov et al., 2001), depolarisation of mitochondrial membrane potential, and mitochondrial fragmentation (Pozniakovsky et al., 2005).

Exogenously, yeast apoptotic scenarios can be induced by application of a variety of substances, such as acetic acid (chromatin condensation, DNA cleavage, PS externalisation) (Ludovico et al., 2001), sugar- (ROS, DNA degradation, nuclear fragmentation) (Granot et al., 2003) or salt-stress (nuclear fragmentation) (Huh et al., 2002; Wadskog et al., 2004), plant antifungal peptides (ROS) (Narasimhan et al., 2001), or hydrogen peroxide (chromatin condensation, DNA cleavage, ROS) (Madeo et al., 1999). Chronological ageing of yeast cultures (Fabrizio et al., 2004a; Herker et al., 2004) and replicative ageing of yeast mother cells (Laun et al., 2001) represent tools for endogenous, physiological forms of apoptosis induction.

Reactive oxygen species (ROS) are considered to be key regulators of ageing, but also of apoptotic execution, conserved during evolution (Madeo et al., 1999; Schulz et al., 1997). In recent years, several yeast orthologues of central metazoan apoptotic regulators have been identified, such as a yeast caspase (Yca1p) (Madeo et al., 2002), the apoptotic serine protease HtrA2/Omi (Nma111p) (Fahrenkrog et al., 2004) with its anti-apoptotic substrate Bir1p (Walter et al., 2006), the EndoG homologue Nuc1p (Buttner et al., 2007), Aif1p (Wissing et al., 2004), Ndi1p (AMID) (Li et al., 2006), Bax inhibitor BI-1 (Chae et al., 2003), and conserved proteasomal pathways such as Cdc6 destruction (Blanchard et al., 2002; Ligr et al., 2001).

The orthologue of the yeast Cdc48 protein, which has lead to the first discovery of yeast apoptosis (Madeo et al., 1997), could even be identified as a regulator of mammalian apoptosis, having antiapoptotic functions (Shirogane et al., 1999), which are particularly apparent during neuronal pathology (Kimura and Kakizuka, 2003). Furthermore, the mystery of single cell apoptosis has been resolved by Fabrizio, Herker and colleagues as being a mechanism that ensures that nutrient resources are spared only for the fittest individuals in stressful times (Fabrizio et al., 2004a; Herker et al., 2004).

Since the dysfunction of apoptosis also leads to several age-associated human diseases such as cancer, Alzheimer's and Parkinson's diseases, the finding of apoptosis in aged yeast cells is of great medical interest and will help to understand some of the still unknown molecular mechanisms at the core of neurotoxicity (Buttner et al., 2008; Outeiro and Lindquist, 2003; Outeiro and Muchowski, 2004).

### Apoptosis and ageing in yeast

Cellular ageing provides endogenous induction of an apoptotic scenario. Two forms of ageing have been described in yeast, both of which can be used as models for the ageing processes of mammalian cells. These two established model systems are largely different and need to be explained in more detail.

### Mother cell specific ageing

Yeast replicative life span, or 'mother cell specific ageing', is defined as the number of divisions an individual cell undergoes before dying (Bitterman et al., 2003). To observe ageing mother cells, daughter cells are removed by means of micromanipulation to avoid colony formation and to permit uni-cellular handling. The median life span of most *S. cerevisiae* strains used for research is about 25 generations. Replicative old yeast cells are much larger but less dense than young cells. Their cell cycle as well as protein synthesis is slowed down and the cell surface appears loose and wrinkled. For each daughter cell produced during life span a budding scar remains documenting the high replicative age (reviewed in Laun et al., 2006).

Accumulation of ROS, which is a characteristic marker of apoptosis, is observed in replicative old cells when dying (Laun et al., 2001). Furthermore, additional phenotypes of apoptotic death such as PS exposure to the outer membrane leaflet, nuclear DNA fragmentation, and chromatin marginalization occur in senescent yeast mother cells (Laun et al., 2001) indicating that replicative-old yeast cells finally die in an apoptotic fashion.

As *S. cerevisiae* divides asymmetrically, replicative age is not transfused to daughter cells, therefore leading to rejuvenation of the newborn daughter cell. Aguilaniu et al. demonstrated that oxidatively damaged proteins are asymmetrically inherited in a Sir2p-dependent fashion (Aguilaniu et al., 2003). More recently, Erjavec et al. showed that oxidatively damaged proteins associated with Hsp104p are retained in the mother cell during cell division by a Sir2 dependant mechanism (Erjavec et al., 2007). The mother cell retains most of its old and damaged contents (proteins, lipids, etc.) whereas the newborn daughter receives only “fresh material” and is therefore fitter. With cell-damage (protein-, lipid- and DNA oxidation) being largely linked to oxidative stress resulting from defective mitochondria, the inheritance of only properly functioning and non-leaking mitochondria could be a mechanism to guarantee the fitness of new daughter cells.

### Molecular Pathways of replicative ageing

Sir2 is an NAD dependant histone and protein deacetylase, able to modify chromatin structure and thereby modulating gene expression (Kaeberlein et al., 1999). Using NAD as a cofactor it links gene expression to the metabolic state of the cell. In yeast, the formation of extra chromosomal rDNA circles (ERCs) is described as a phenomenon occurring during replicative ageing. However, since this process is thought to be restricted to yeast it may not be a cause of ageing in higher eukaryotes. Nevertheless, other molecular pathways evolving from Sirtuins are conserved to higher eukaryotes. This might eventually indicate that ERC formation does not determine age but instead there is simply a correlative relationship between the two.

The clear interdependencies of Sir2, calorie restriction and replicative ageing are still controversial (Kaeberlein et al., 1999; Kaeberlein et al., 2005; Lin et al., 2000; Sinclair et al., 2006). Most recently, Sun et al. showed that deletion of SIR2 prevents apoptosis induced by valproic acid through accumulation of neutral lipids in yeast cells (Sun et al., 2007). This publication supports the hypothesis that Sir2 functions are multifaceted, and that ageing and apoptosis can be connected.

The nutrient dependant kinases Tor1/2 (target of rapamycin), PKA (protein kinase A) and Sch9 show effects on yeast longevity (Kaeberlein et al., 2005). These four proteins are major controllers of cell growth pathways and link nutritional intake to cellular growth responses via mostly unknown mechanisms. Yeast expresses the two Tor proteins Tor1 and Tor2, which are identical in 70 % of their amino acid sequence. Tor kinases may be inactivated by the inhibitor rapamycin. The phenotype of both rapamycin-treated and Tor knock out yeast strains is similar to starved cells: down-regulation of translation, alterations in the transcription pattern and an accumulation of storage carbohydrates (Schmelzle and Hall, 2000).

PKA activity is regulated by glucose via changes in cyclic adenosine triphosphate (cAMP) levels. High cAMP levels stimulate PKA activity by binding to its regulatory subunits (Bcy1p) and thereby releasing its catalytic active subunits (TPK1, 2, 3). Mutations in either TPK1, TPK2 and TPK3 all increase life span. Furthermore, the mutation of CDC25 and CDC35, encoding a GDP/GTP exchange factor or adenylate cyclase respectively, extend life span as well. In summary, various mutations in the PKA pathway lead to extended life span

(Lin et al., 2000), but if PKA activity is generally modified during replicative ageing, is still unclear.

Sch9 is a functional homologue of Akt/PKB, a serine threonine kinase making up the pro-ageing pathways in worms, flies and mice (Longo and Finch, 2003). Sch9, like its human homologues Akt-1/Akt-2/PKB, is implicated in insulin signalling, translocation of glucose transporters, apoptosis, and cellular proliferation (Fabrizio et al., 2001). Most recently, it could be demonstrated that the deletion of SCH9, provides protection against the age-dependent defects of yeast lacking the helicase Sgs1p by inhibiting error-prone recombination and preventing DNA damage and dedifferentiation (Madia et al., 2008). Moreover, Madia et al. speculate that alterations of the IGF-I-Akt-56K pathway could also protect against premature ageing syndromes in mammals (Madia et al., 2008).

What is still lacking, and what would support a more tight connection between replicative ageing and apoptosis, is the observation of a lifespan extension with a strain deleted in an important apoptosis regulator. Up to now, no data could confirm this. However, such a situation has been described in the filamentous fungus *Podospora anserina*, where deletion of the AMID homologue PaAmid1, the metacaspase PaMca2 and particularly of the metacaspase PaMca1 resulted in lifespan extension (Hamann et al., 2007).

### Chronological ageing in yeast

Yeast chronological life span is the length of time a population remains viable in the post-diauxic and stationary phases (Fabrizio and Longo, 2003). Chronological ageing studies in yeast are used as a model for oxidative stress and ageing of postmitotic tissues in higher eukaryotes. Chronologically aged yeast cells die exhibiting markers of apoptosis, such as accumulating oxygen radicals and caspase activation (Fabrizio and Longo, 2003). Allen et al. were able to discriminate and sort stationary phase yeast cells into two fractions based on their differential density in a sucrose gradient. The fraction with the higher density represents the fully quiescent daughter cells, which do not exhibit bud scars at all. They perform the best in survival tests, when separately grown after centrifugation. On the contrary, the first cells to undergo programmed cell death in this heterogeneous culture are the non-quiescent and replicative older ones. The result, once the first cells have died, is a culture only consisting of virgins and thereby having the highest potential to persevere and manage survival of the cohort (Allen et al., 2006). The mechanism of recognition or discernment of older cells from others still remains elusive but is a necessary assumption.

The benefit of old cells undergoing suicide seems obvious as nutrients are spared for fitter cells and nutrients released from dead cells represent an additional nutritional source for the ageing culture (Buttner et al., 2006).

### Molecular Pathways of chronological ageing

Chronological ageing in yeast is largely regulated by nutrients such as glucose. Severe calorie restriction or mutation in RAS2, CYR1/PKA, TOR or SCH9, which are all encoding downstream effectors of glucose signalling, extend the median yeast chronological life span up to 300% (Fabrizio et al., 2004a; Fabrizio et al., 2003; Fabrizio et al., 2004b; Fabrizio et al., 2001; Longo et al., 1997; Powers et al., 2006). This life span extension is mediated

through transcription factors involved in stress resistance (Msn2, Msn4), heat shock proteins or scavenger enzymes for oxidative stress such as, mitochondrial superoxid dismutase (SOD) and catalases (Fabrizio et al., 2003; Fabrizio et al., 2001). Msn2 and Msn4 represent transcription factors stimulating the expression of stress resistance proteins. Up-regulation of these transcription factors therefore leads to increasing SOD and catalase levels thereby minimizing oxidative stress and cellular damage (Gorner et al., 1998). A recent study by Wei et al. showed that the chronological life span extension in yeast caused by deficiencies in either Ras2, Tor1, Sch9, or by calorie restriction is dependant on the serine/threonine kinase Rim15 (Wei et al., 2008). Furthermore, the deletion of Msn2/4 and Gis1, which are positively regulated by Rim15, cause a major reversion of the life span extending effect of calorie restriction (Wei et al., 2008).

Quite a number of proteins in this ageing pathway including SOD, catalase and heat shock proteins are conserved between *S. cerevisiae* and *C. elegans*. Sch9 or Akt/PKB, respectively, is even conserved from yeast to worms, flies, mice and humans (Longo and Finch, 2003).

In contrast to replicative ageing, SIR2 deletion promotes longevity during chronological ageing under calorie restricting conditions (Fabrizio et al., 2005). Stress resistance is decreased with increased Sir2 levels in mitotically inactive cells. Furthermore, alcohol dehydrogenase (Adh1p) activity is connected to Sir2 activity (Longo and Kennedy, 2006), although the reasons for this are not clear. A recent study proposes that higher Sir2 activity relies on an elevated NAD<sup>+</sup>/NADH ratio as a consequence of increased Adh1 levels (Lin et al., 2000; Reverter-Branchat et al., 2007). During ageing Adh1p becomes oxidatively modified thereby reducing its activity (Reverter-Branchat et al., 2007). Furthermore, experiments by Reverter-Branchat et al. have shown that chronological ageing is prolonged with increased ADH1 expression (Reverter-Branchat et al., 2007).

In order to answer the question how chronological ageing is modified when cells may not enter apoptosis anymore, Herker et al. investigated the ageing properties of a yeast strain deleted in the YCA1 gene. During the early stages of chronological ageing the strain has a short-term survival advantage. However, it accumulates cells that are no longer able to rebuild a new cell population, thus damaged or less fit cells (Herker et al., 2004). Therefore, such a strain has a survival disadvantage in comparison to a wild type strain, in the long run.

A recent finding by Weinberger et al. is that ectopic expression of CLN3, a G1 cyclin, shortens chronological lifespan (Weinberger et al., 2007). The experimental setting makes the cells more frequently arrest in S phase instead of G1 phase during nutrient depletion. This also lead to the assumption that replication stress is an important determinant of chronological lifespan in budding yeast (Weinberger et al., 2007).

In summary, chronological life span in yeast is increased under growth-promoting and nutrient poor conditions or those conditions mimicking starvation. Additionally, protection against oxidative damage and other environmental stresses increases survival.

## Replicative versus chronological ageing – similarities and differences

**Similarities**—A number of similarities can be observed in the replicative and the chronological model-system. For example accumulation of ROS, increase of oxidative stress and detection of apoptotic and necrotic markers are hallmarks of both ageing models (Fabrizio et al., 2004a; Herker et al., 2004; Laun et al., 2001). The similarity of these features underlines the great general importance of oxygen stress and apoptotic and necrotic mechanisms during ageing.

Reduction of the glucose content in the yeast culture medium (a means of calorie restriction) leads to replicative and chronological lifespan extension as well. Consequently, in both systems the nutrient dependant kinases Tor1/2, PKA and Sch9 (Akt/PKB) are accelerators of the ageing process. The upstream regulating factor glucose is therefore a key-player in longevity regulation.

Additionally, a rise in Adh1p levels (2-fold expression) extends both chronological and replicative lifespan (Reverter-Branchat et al., 2007). As higher Adh1p levels are accompanied by an increasing activity of Sir2p (Reverter-Branchat et al., 2007), a dependence on Sir2 seems obvious at least for replicative ageing.

**Differences**—Despite some similarities a number of interesting differences remain for discussion. Firstly, differences concerning the phenotype are to be addressed. While replicative old cells are blotched with bud scars (Fig. 1A picture 3 and 4), chronologically aged ones are mostly virgin cells (Figure 1B) not providing a single bud scar. The replicative old cells are bigger and much more wrinkled than the globular, normal sized cells remaining at the end of chronological ageing studies. Moreover, they differ in their actin cytoskeleton, cell wall structure and metabolism, which is reviewed in more detail by Laun et al. (Laun et al., 2006).

Besides the microscopically observable phenotype, molecular differences need to be mentioned. While increased Sir2 activity probably enhances replicative lifespan (Kaeberlein et al., 1999), the reduction of Sir2 activity under nutrient poor conditions actually extends the chronological lifespan (Fabrizio et al., 2005). A recent study by Smith et al. establishes that the two ageing models differ under calorie restricting conditions. While life span extension in the replicative ageing model is suggested to be mediated by Sir2p, Sirtuins appear not to be major regulators of chronological lifespan and were not required for the lifespan extension (Smith et al., 2007). Interestingly, no additional benefit for lifespan extension was achieved through growth on non-fermentable carbon sources (thereby forcing the yeast to respire), when combined with calorie restriction (Smith et al., 2007). This hints to the idea that calorie restriction might increase chronological lifespan through a maximum increase in respiration.

Another crucial difference between the two ageing models could be the differing effective alcohol concentration, which is a result of the different cultivation techniques. Chronological ageing is performed in liquid culture with cell densities up to  $2 \times 10^8$  while mother cell specific ageing uses agar containing medium with only a single cell growing on it, which may not produce as much alcohol that it would affect the cell itself.



## The bitter end of ageing

At the very end of a yeast cell's life stands death. Both ageing forms end in apoptotic or necrotic death scenarios. Thus, the limited lifespan can be extended through abrogation of cell death pathways by deletion of the apoptotic machinery or diminishment of oxidative radicals (Fabrizio et al., 2004a; Herker et al., 2004; Piper et al., 2006). Consequently, apoptosis and ageing are tightly connected, proved by the discovery of complex pro and anti-death pathways: The deletion of Endonuclease G (Nuc1p) (a proapoptotic nuclease), or its release from mitochondria, leads to an improvement of chronological life span when respiration is high. Conversely, life span is diminished when respiration is low. Interestingly, deletion of Yeast EndoG diminishes apoptotic death when mitochondrial respiration is increased but enhances necrotic death when oxidative phosphorylation is repressed. This suggests both a lethal and a vital role for EndoG (Buttner et al., 2007).

Furthermore, disruption of AIF1, YCA1, NDI1 or NMA111 all delay the onset of age-induced cell death (Li et al., 2006; Madeo et al., 2002; Walter et al., 2006; Wissing et al., 2004). In parallel, a decrease in chronological viability is achieved through overexpression of YCA1 (Madeo et al., 2002), whereas overexpression of BIR1 (Walter et al., 2006) and YAP1 (Herker et al., 2004) lead to prolonged survival of chronologically aged yeast cultures (see Table 1).

Most recently, heterologous expression of alpha-Synuclein in yeast has been shown to induce apoptotic and necrotic cell death and to depend on chronological ageing (Buttner et al., 2008).

Concluding, cell death pathways provide cells with a pro-ageing tool. The altruistic behaviour of yeast to commit suicide during ageing is a selective advantage (Fabrizio et al., 2004a; Herker et al., 2004). Going even further, ageing could be regarded as nature's gift, which improves the overall survival of a species viewed from the point of evolution.

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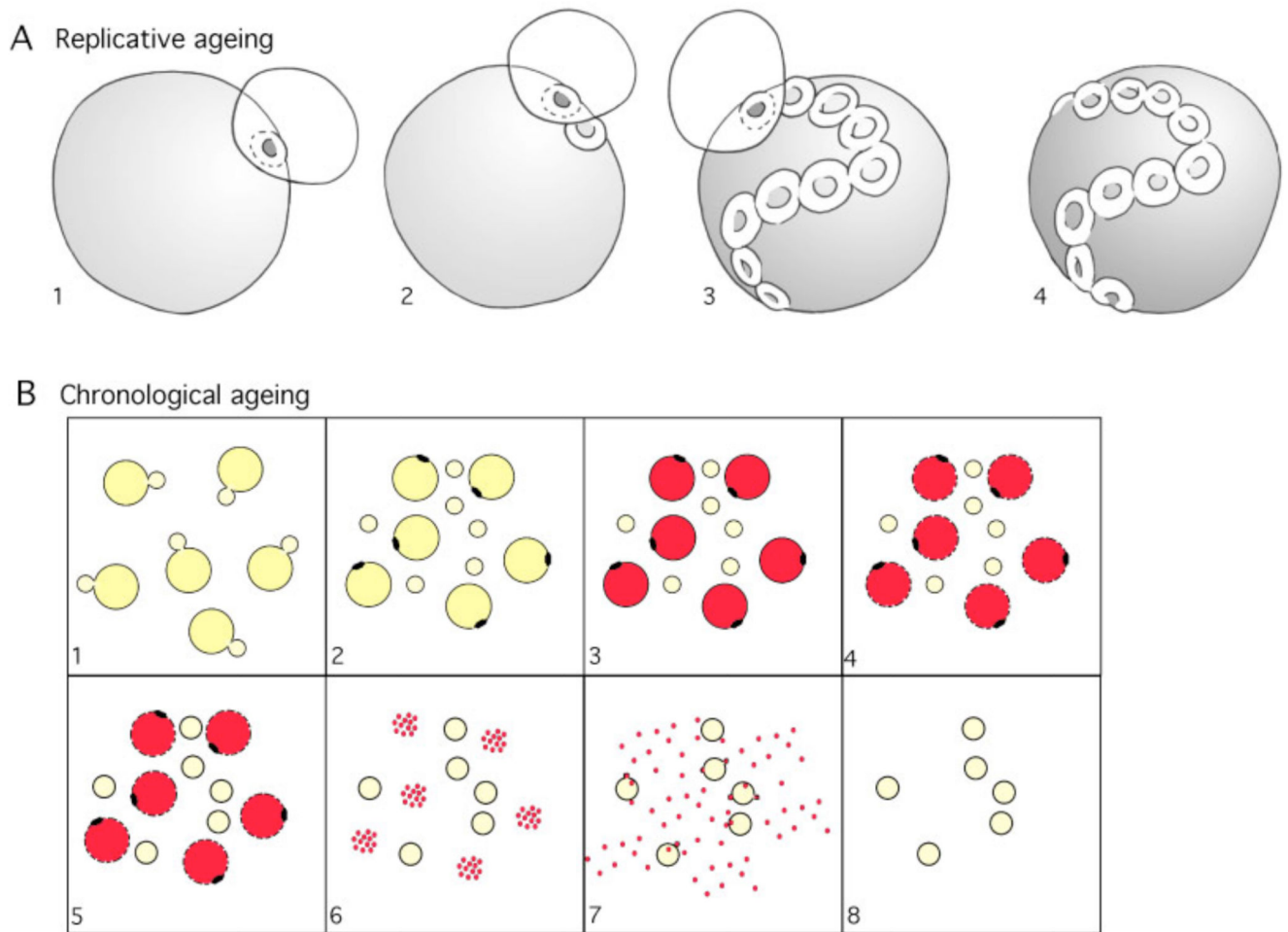
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**Figure 1. Cartoon of the two model systems.**

A. Series of a Yeast cell during replicative ageing. The virgin cell in picture 1 is budding for the first time. Overtime bud scars accumulate (2,3). Each bud-scar documents the amount of daughter cells produced. Finally the cell stops producing daughter cells at the end of its replicative life span (4).

B. Various stages of chronologically ageing yeast cells. Synchronously growing yeast cells (1) finish budding and the ageing process starts with virgin cells and cells mostly having only one bud scar (2). The first cells to show ROS accumulation or other markers of cell death are the replicatively older ones (3). Apoptotic processes occur leading to membrane disintegration (4,5) and to the formation of apoptotic bodies (6,7). The new population of virgin-only cells (8) now continues the chronological ageing process analogously.

**Table 1**

Apoptotic genes involved in chronological lifespan regulation

Yeast genes	Homologue	Knock out	Overexpression	Reference
<b>YCA1</b>	Caspase	Increase of survival rate in chronological ageing	Prolonged culture of YCA1-overexpressing cells reduces viability after 44 or 96 hr.	Madeo et al., 2002
<b>AIF1</b>	AIF	Delay of age-induced cell death	<i>No data</i>	Wissing et al., 2004
<b>NDI1</b>	AMID	Elongation of chronological life span	<i>No data</i>	Li et al., 2006
<b>NUC1</b>	Endo G	Improvement of chronological life span with high respiration rate. Diminishment of life span when respiration is low.	Decreased survival in chronological ageing. Death is independant from Yca1p and Aif1p.	Buttner et al., 2007
<b>BIR1</b>	Survivin	No significant acceleration of age induced cell death	Delayed onset of cell death in chronologically aged cells	Walter et al., 2006
<b>NMA111</b>	Omi/Htr2	Delayed onset of cell death in chronologically aged cells	<i>No data</i>	Walter et al., 2006
<b>YAP1</b>		<i>Deletion is not viable</i>	Prolonged survival of chronologically aged cultures.	Herker et al., 2004