AGE (2009) 31:353–363
DOI 10.1007/s11357-009-9108-1

# Cellular senescence: unravelling complexity

**João F. Passos · Cedric Simillion · Jennifer Hallinan · Anil Wipat · Thomas von Zglinicki**

Received: 17 March 2009 / Accepted: 19 June 2009 / Published online: 18 July 2009


**Abstract** Cellular senescence might be a tumour suppressing mechanism as well as a contributor to age-related loss of tissue function. It has been characterised classically as the result of the loss of DNA sequences called telomeres at the end of chromosomes. However, recent studies have revealed that senescence is in fact an intricate process, involving the sequential activation of multiple cellular processes, which have proven necessary for the establishment and maintenance of the phenotype. Here, we review some of these processes, namely, the role of mitochondrial function and reactive oxygen species, senescence-associated secreted proteins and chromatin remodelling. Finally, we illustrate the use of systems biology to address the mechanistic, functional and biochemical complexity of senescence.

**Keywords** Senescence · Oxidative stress · Mitochondria · Secretory phenotype · Systems biology · Interactomes

J. F. Passos (✉) · C. Simillion · J. Hallinan · A. Wipat · T. von Zglinicki
Ageing Biology Laboratories and Centre for Integrated Systems Biology of Ageing and Nutrition (CISBAN), Institute for Ageing and Health, Newcastle University, Newcastle upon Tyne NE4 5PL, UK
e-mail: Joao.Passos@ncl.ac.uk

## The classical view of cellular senescence

Cellular senescence is the ultimate and irreversible loss of replicative capacity first observed in primary somatic cell culture. Senescent cells, despite being unable to proliferate further, can remain alive in culture for a considerable period of time.

It was L. Hayflick who first found that embryo-derived fibroblasts can divide 50±10 times before arresting irreversibly (Hayflick and Moorhead 1961). Senescence has since intrigued scientists and has been the subject of numerous studies.

A major breakthrough occurred when it was suggested that the shortening of telomeres, the ends of linear chromosomes, could function as a replicometer (counting the finite number of cell divisions) and act as a trigger of replicative senescence in normal diploid cells (Olovnikov 1971; Watson 1972). Telomere shortening was proposed as a counting mechanism, which could explain two distinct observations, namely the reproducibility of the "Hayflick limit", and the fact that cells frozen at a certain population doubling level (PDL) would retain a memory of their PDL and, when thawed, undergo the expected maximum number of divisions (Hayflick 2000).

Only recently have we begun to understand the mechanisms underlying the recognition of telomeres by cells undergoing senescence. It has been found that when telomeres become too short, they are perceived as DNA double strand breaks (DSBs), eliciting a DNA damage response that culminates in irreversible cell cycle arrest.

The DNA damage response is regarded as an intrinsic mechanism for cells to prevent replication of damaged DNA and to allow sufficient time for repair (Shiloh 2006). If cells are unable to repair the DNA damage, then the arrest will become permanent. Over the last 5 years, several important studies have shed light on telomere biology and its role in senescence. Firstly, it has become apparent that factors other than mere length play an essential role in telomere signalling. Telomeres are normally in a 'capped' state, which is not recognised by DNA damage response and repair enzyme complexes (Blackburn et al. 2000). Structurally, telomeres form terminal loops that are stabilised by a number of telomere binding proteins. Two of these proteins (TRF-1 and -2) bind to double-stranded telomeric DNA, and POT-1 binds to single-stranded telomeric DNA. This complex of proteins has been called shelterin, since it "shelters", i.e. protects, chromosome ends (de Lange 2005). It is believed that telomere shortening destabilises telomeric loops (Griffith et al. 1999) and consequently increases the probability of telomere uncapping. Recent studies have shown that uncapping of telomeres, whether by inhibition of TRF2 or telomere shortening, activates the same signalling pathways (DNA damage response) as do DSBs (Fagagna et al. 2003; Takai et al. 2003). An additional factor that might play a role in senescence is loss of telomeric overhang. Loss of overhangs as cells approach senescence has been observed in two human fibroblast strains as they progress towards senescence (Stewart et al. 2003) but not in a number of other fibroblast strains (Keys et al. 2004).

Moreover, we also began to understand why telomere shortening is unlikely to be a counting mechanism. It has been observed that individual cells from clonally derived populations show heterogeneous division potential (Smith and Whitney 1980) and that a large heterogeneity in telomere length exists between chromosome ends both within individual cells and between cells (Baird et al. 2003; Lansdorp et al. 1996; Zou et al. 2004). Moreover, it has been shown, using a variety of markers including BrdU labelling (Kill et al. 1994), Ki67 staining (Thomas et al. 1997), p53-reporter assay (Bond et al. 1994) and staining for γ-H2AX, a marker for senescence-associated DNA damage foci (Fagagna et al. 2003), that the fraction of senescent cells present in a mass population increases progressively with population doublings.

Several hypothetical models have been put forward to explain heterogeneity in replicative senescence, including the random inheritance of the 'long' and 'short' strands of telomeres (Levy et al. 1992), abrupt telomere shortening (Rubelj and Vondracek 1999), and stochastic telomere damage caused by oxidative stress (Sozou and Kirkwood 2001). In accordance with such studies, senescent cells in 'young' cultures show short telomeres and activation of telomere-induced DNA damage signalling (Martin-Ruiz et al. 2004; Passos et al. 2007a; von Zglinicki et al. 2003). This indicates that the lifespan of an individual cell lineage is controlled not solely by telomere shortening but also by stochastic factors upstream of telomere shortening. These factors have been shown to be related to cell-to-cell variation in oxidative stress (Passos et al. 2007a) as discussed further below.

Telomeres are not the sole mechanisms responsible for induction of senescence. Several other processes, including oncogene activation, agents that alter chromatin structure, oxidative stress or non-telomeric DNA damage have been shown to induce senescence (Campisi and d'Adda di Fagagna 2007). Moreover, recent studies have shown that several other factors, such as senescence-specific secreted factors may play an important part in the maintenance of senescence (Kuilman and Peeper 2009).

In the remainder of this paper, we shall review in more detail the various mechanisms involved in the induction and maintenance of senescence, and demonstrate how its mechanistic complexity can be tackled using recent developments in systems biology and more precisely, interactome analysis.

## Why is senescence important? Does it happen in vivo?

Classically, senescence has been described in somatic cells grown in vitro; however, whether senescence is simply an artefact of cell culture or whether it exists to any significant extent in vivo, has been the subject of longstanding debate (Ben-Porath and Weinberg 2005). For a number of years the lack of unconditional evidence for senescence in vivo has lead sceptics to question why it ought to be studied in the first place. The absence of specific markers exclusive to senescent cells represents the main obstacle to their detection in living tissues. Until recently, the best available marker

was a histochemical assay for β-galactosidase activity (senescence-associated β -galactosidase) (Dimri et al. 1995), whose specificity has since been questioned (Cristofalo 2005). Using this assay, an age-dependent increase in Sen β-Gal stained fibroblasts in human skin has been observed (Dimri et al. 1995); however, others have failed to reproduce this data (Severino et al. 2000).

In recent years, significant advances have been made that have reinforced the view that senescence plays an important role in the in vivo context. One such step forward was the observation that senescent fibroblasts containing telomere-induced foci (TIF) increase with age in the skin of baboons (Herbig et al. 2006; Jeyapalan et al. 2007)—animals that possess a similar telomere length to humans and absence of telomerase activity.

TIF detection is probably the best marker of senescence to date; however, it is only able to detect cells that senesce in a telomere-dependent manner. This might be an issue when studying certain experimental models. For instance, laboratory mice have much longer telomeres than long-living mammals, concomitantly with high telomerase activity in many tissues during adulthood. Therefore, it is generally assumed that telomere-dependent senescence does not play a major role in the ageing of mice. However, a recent study has reported age-dependent telomere shortening in stem cell compartments in a variety of mouse tissues, and this seems to parallel stem cell dysfunction (Flores et al. 2008); however, this telomere loss is not sufficient to generate TIFs in mice (Wang et al. 2009). On the other hand, mouse models where telomere function has been compromised strongly support a role for senescence (and telomeres) in the ageing process. Telomerase knock-out (mTERC−/−) mice carrying a homozygous deletion of the RNA component of telomerase (Blasco et al. 1997) show a progressive generation-dependent telomere shortening, which results in both cell cycle arrest and apoptosis (Lee et al. 1998). Telomere shortening in telomerase knock-out mice has been shown to limit stem cell function, regeneration, organ homeostasis and lifespan (Wong et al. 2003).

Moreover, genetic manipulation of the classical senescence pathways has been shown to impact on organismal ageing. Genetic activation of p53 shortened mice lifespan despite suppression of tumour formation (Maier et al. 2004; Tyner et al. 2002), while a p21 knock-out rescued at least some accelerated aging phenotypes in telomerase (mTERC) knock-out mice (Choudhury et al. 2007), and p16 knockout rescued various mice stem cell systems from age-related decline (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006).

While cellular senescence appears to have a detrimental role as far as cellular regeneration is concerned, it might have a positive role alongside apoptosis as a tumour suppression mechanism, as recent studies suggest (Bartek et al. 2007; Ramsey and Sharpless 2006). Oncogene-induced senescence shares a great number of similarities with replicative senescence, including increased Sen β-Gal activity and condensation of heterochromatic bodies (Narita et al. 2003). Moreover, similar signalling cascades such as INK4A-RB and p53 pathways are activated in both instances.

Though the mechanisms responsible for the activation of senescence have been identified, it is still unclear how a cell "commits" to becoming irreversibly arrested. Recent studies have revealed that, apart from the so called "classical senescence pathway", which encompasses activation of p53, p21 and p16, several other changes, including secretion of proteins, mitochondrial alterations and chromatic remodelling might contribute to the reinforcement of the senescent growth arrest.

## Complexity of a phenotype

### Senescent-associated secretory phenotype

Regardless of the inducing mechanism, significant changes in gene expression, including increased expression of secreted proteins, have been observed in senescence (Coppé et al. 2008). Senescent fibroblasts have been shown to secrete growth factors, cytokines, extracellular matrix, and degradative enzymes.

It has long been recognised that the primary function of secreted factors is to allow inter- and intra-cellular communication. One proposed hypothesis for the role of secreted proteins in senescence is that they contribute to detrimental alterations in the surrounding tissue microenvironment. In fact, consistent with this hypothesis, both cell culture experiments and studies involving co-transplantation of senescent and cancer cells in recipient mice, have shown that senescent fibroblasts stimulated hyper-

proliferation of cancer cells, neoplastic progression and tissue damage (Krtolica et al. 2001; Liu and Hornsby 2007). However, it has also been proposed that secreted proteins act in an autocrine way and help reinforce the senescence phenotype.

Several studies have reported that IGF signalling, which has been implicated in ageing of several organisms including mammals, nematodes and fruit flies, has a causal role in senescence. The secreted proteins insulin-like growth factor binding protein 3 and 5 (IGFBP3 and IGFBP5) have both been implicated in regulation of senescence in human umbilical vein endothelial cells (HUVEC; Kim et al. 2007a, b). Expression of both these proteins is increased in senescence of different cell types and has been shown to be dependent on p53 (Kim et al. 2007a, b).

A recent study has rekindled interest in this family of proteins in senescence. IGFBP7 has been identified in a RNA interference screen to identify genes involved in oncogene BRAF-induced senescence in both human primary fibroblasts and melanocytes (Wajapeyee et al. 2008). This study also revealed that administration of IGFBP7 to mice melanoma xenografts inhibited tumour growth significantly, supporting the idea that secreted proteins in senescence have both autocrine and paracrine effects.

Similar unbiased high-throughput RNA interference approaches have reported a role for other secreted proteins in senescence. Recently, it has been shown, as a result of two independent screens, that activated chemokine receptor CXCR2 (also known as IL-8RB; Acosta et al. 2008) and IL6 receptor (Kuilman et al. 2008), both belonging to the interleukin receptor family, play a role in the senescence of various cell types. Acosta and colleagues have shown that inhibition of CXCR2 delayed the onset of both replicative and oncogene-induced senescence and led to decreased activation of the DNA damage response (Acosta et al. 2008).

Transforming growth factor beta (TGF-beta), another secreted protein, has also been implicated in senescence. It has been shown that inactivation of TGF-beta 1 secretion in mouse keratinocytes was enough to prevent oncogene-induced senescence (Tremain et al. 2000). In human fibroblasts, blocking antibody against the TGF-β1 receptor II has been shown to repress the UVB-and $H_2O_2$- induced increase of the proportion of cells positive for Sen β-gal activity (Debacq-Chainiaux et al. 2005; Frippiat et al. 2001).

Despite the increasing evidence for a role of secretory proteins in senescence, it remains unclear how to connect them with the classical senescence pathway, involving activation of p53, p21 and p16. Studies have revealed that some of these proteins are dependent upon p53 activation, such as CXCR2 (Acosta et al. 2008), IGFBPs (Kim et al. 2007a, b); however, others, such as IL6R (Kuilman et al. 2008) and TGF-beta 1 (Tremain et al. 2000), are not. However, the timing of the secretory response appears to be distinct from that of the initial stimulus as revealed by a recent study. Coppe and colleagues have shown that the senescent secretory phenotype takes 7–10 days to develop after induction of a DNA damage response (Coppé et al. 2008). A recent paper has suggested that autophagy plays a role in the establishment of senescence. Knock-down of autophagy proteins ATG5 or ATG7 was shown to delay production of secretory cytokines IL6 and IL8 during oncogene-induced senescence (Young et al. 2009).

However, the mechanisms by which secreted proteins contribute to the reinforcement of the senescent phenotype remain largely unknown.

## Free radicals, mitochondria and senescence

An emerging challenge to our understanding of the senescent phenotype is the involvement of mitochondria in this process. Mitochondria, apart from providing the energy necessary for the survival of cells, are also the major source of detrimental reactive oxygen species (ROS) responsible for damage to proteins, nucleic acids and lipids. Countless studies have reported a correlation between ROS generation, mitochondrial function and the ageing process (Balaban et al. 2005). However, genetic interventions affecting both mitochondrial function and oxidative stress in animal models have given rise to conflicting results (Muller et al. 2007).

Studies in cellular senescence have revealed that mitochondrial function changes as cells reach the end of their replicative lifespan, leading to metabolic inefficiency and increased generation of ROS (Allen et al. 1999; Hutter et al. 2004, 2002; Passos et al. 2007a; Zwerschke et al. 2003). Increased ROS leads to accumulation of oxidation products, such as protein carbonyls and lipofuscin, which have been shown to

occur in senescent fibroblasts (Sitte et al. 2001, 2000). Moreover, mitochondrial dysfunction has been shown to induce the retrograde response—a pathway that signals electron transport chain disruption to the nucleus, thus causing wide-ranging changes in gene expression. This adaptive process to mitochondrial dysfunction appears to be part of replicative aging in both budding yeast (Jazwinski 2005) and mammalian cells (Passos et al. 2007a; Passos and von Zglinicki 2008).

Interventions affecting both mitochondrial function and ROS generation have been shown to impact on telomere-dependent senescence in vitro. Treatment with free radical scavengers (von Zglinicki et al. 2000), low ambient oxygen concentrations (Forsyth et al. 2003; Richter and von Zglinicki 2007), over-expression of antioxidant enzymes (Serra et al. 2003), and mild chronic uncoupling (Passos et al. 2007a) have been shown to decelerate telomere shortening and to extend the lifespan of cells in culture. On the other hand, factors contributing to increased ROS generation such as severe mitochondrial depolarisation (Liu et al. 2002), and high ambient oxygen concentrations (von Zglinicki et al. 1995) have been shown to accelerate telomere shortening, and to lead to early induction of senescence.

Mechanisms involved in mitochondrial turnover and morphology also seem to play a role in senescence. For instance, mitochondrial elongation by knock-down of the mitochondrial fission protein hFis1 has been shown to induce senescence, possibly through increased ROS generation and activation of a DNA damage response (S. Lee et al. 2007). Moreover, evidence suggests that mitochondrial ROS generation in senescence might be due to increased mitochondrial density, which can occur as a consequence of mitochondrial dysfunction (Passos et al. 2007a, b). In fact, overexpression of PGC-1$\alpha$—the master regulator of mitochondrial biogenesis—has been reported to induce senescence in human fibroblasts (Xu and Finkel 2002).

However, mitochondrial dysfunction and ROS generation in senescent cells might be more than random consequences of age-dependent decline. Several recent studies suggest that these processes might be part of a tightly regulated program, with important consequences for the fate of cells. Furthermore, activation of key players mediating the classical senescence pathway has been related to ROS generation.

Over-expression of activated RAS has been shown to induce senescence and increase ROS levels (Lee et al. 1999). Over-expression of the tumour suppression gene p53 has been shown to be associated with an increase in ROS (Macip et al. 2003). Moreover, the p53 transcriptional target p21, when over-expressed can also induce senescence with a concomitant rise in intracellular ROS. Treatment with antioxidant N-acetyl cysteine (NAC) has been shown to prevent p21-induced growth arrest (Macip et al. 2002).

The role of p21 and p53 on ROS generation is largely unknown. An association between p53 and transcriptional activation of genes involved in mitochondrial apoptosis has been shown (Polyak et al. 1997). In addition, a connection has been established between p53 expression and mitochondrial cytochrome *c* oxidase (COX) assembly and activity. It has been reported that p53 knock-out mice exhibit decreased mitochondrial respiration and also that p53 expression influenced expression of the gene SCO2, which is required for the assembly of the mitochondrial DNA-encoded COX II subunit (Matoba et al. 2006).

Another study has shown that induction of senescence via the p16/Rb pathway establishes a ROS-generating positive feedback loop that is responsible for irreversibly blocking cytokinesis (Takahashi et al. 2006).

The described role of ROS appears to add an extra layer of complexity to the intricate senescence phenotype. However, it might be that ROS is in fact the missing link between the secretory phenotype and its proposed role in the reinforcement of the senescent phenotype. A couple of studies support such a notion. One study showed that interferon beta ($\beta$-IFN) leads to senescence through ROS activation and subsequent activation of a DNA damage response. Importantly, the DNA damage signalling pathway was inhibited by adding the antioxidant NAC, showing that the sensor of ROS production was general DNA damage (Moiseeva et al. 2006). Inhibition of CXCR2 in both replicative and oncogene-induced senescence has been shown to result in both decreased ROS and DNA damage response activation (Acosta et al. 2008).

## Senescence-associated heterochromatic foci

Another factor that contributes to senescence is a drastic rearrangement of chromatin. In several cell types, senescence arrest is accompanied by formation

of senescence-associated heterochromatic foci (SAHF), which appears to be dependent on the p16/Rb pathway (Narita et al. 2003). SAHF-positive cells in RAS induced senescence has been shown to occur with the same kinetics as other markers of senescence, such as Sen-β-Gal, p16 expression and Rb hypo-phosphorylation. Moreover, during senescence, SAHFs have been shown to accumulate on the promoters of the cell cycle genes responsible for silencing genes involved in proliferation (Narita et al. 2003; Rastogi et al. 2006).

Interestingly, the occurrence of SAHFs correlates strongly with the irreversibility of the senescent phenotype (Narita et al. 2003) and might be the mechanism that defines the point of no return in terms of growth arrest.

Therefore, senescence is a multi-layered and multi-temporal process and in order to fully understand it, we will need to fully characterise the kinetics of the interactions between its components and also find strategies to integrate the myriad pathways involved (Fig. 1).

## The future: systems biology and understanding senescence

It is clear from the above that senescence is a complex phenomenon governed by different complicated processes. More precisely, senescence is not a characteristic of individual cell components but of the cell as a whole. In other words, senescence is what is called, in the world of systems theory, an emergent property. As a result, senescence can only be completely understood by studying the cell as a complete system (Csermely and Soti 2006; Kirkwood and Kowald 1997). The latter requirement makes the many genome and proteome-wide techniques such as microarrays (Sherlock 2000), two-hybrid and other proteomics methods (Titz et al. 2004), as well as synthetic lethality screens that have become available in the last few years, especially appealing to researchers in the field of senescence. Promising as they are, the advent of these new methodologies also raises new challenges. First and foremost, computational methods are needed to analyse and organise the large amount of data generated by an individual experiment before biologists can infer knowledge from them. At least for microarray data, this issue has been addressed through the development of many software tools to analyse expression over the past years (Sherlock 2000).

On a higher level, researchers are faced with the problem of integrating the data from many different experiments of different types (Ng et al. 2006). A very common approach to address this problem is to construct functional gene networks. Whenever an experiment indicates a functional link between two or more genes or their products, a link between those genes is made in the network. Many public databases [BioGrid (Breitkreutz et al. 2008); Intact (Kerrien et al. 2007); KEGG (Kanehisa et al. 2008) and others (reviewed in Ng et al. 2006)] already exist that construct such networks either by collecting experimental data from different sources, conducting in silico predictions of interactions, literature curation or any combination of these.

To deal with the high proportion of false-positive results of many high-throughput techniques, statistical methods have been developed that assign a confidence value to each link in the network (Lee et al. 2004; I. Lee et al. 2007). Additionally, these methods also up-weight interactions that are detected by multiple, independent experiments. Adding these confidence scores to a network results in what is referred to as a probalistic integrated functional network (PIFN). Although already very useful by itself, the value of a PIFN can be increased drastically by capturing additional information about the type of links it contains. For instance, one would want to discriminate between protein–protein interactions and transcriptional activation networks. Additionally, including the directionality of a link, e.g. in a signalling pathway, will also increase the amount of knowledge a biologist can extract from a network. For this reason, ontologies such as the molecular interaction (MI) ontology (Hermjakob 2006) have been developed to annotate links in PIFNs.

The use of PIFNs is illustrated in Fig. 2. Figure 2a shows an example of a "traditional" interactome using information from BioGRID of the possible links between TP53 (a major player in the DNA damage response) and senescence-associated secreted factors (SASFs) as discussed above. While such a network does integrate a large amount of data, it is visually impenetrable, making it difficult to extract meaningful biological data.

As a demonstration of how relevant information can still be extracted from the network in Fig. 2a, we

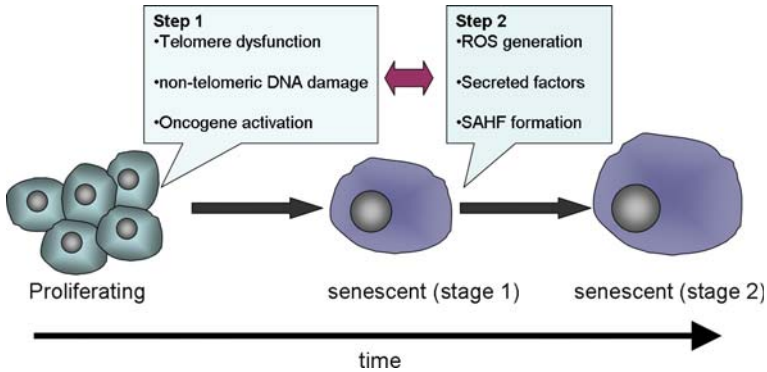


**Fig. 1** Senescence as a multi-component phenotype. Initially, stressors such as oncogene activation, telomere dysfunction and non-telomeric DNA damage can lead to activation of a DNA damage response (DDR) and cell cycle arrest. Following DDR, p53, p21 and p16 activation has been shown to induce generation of reactive oxygen species (ROS), which can contribute to the permanence of the growth arrest phenotype. Finally, secreted factors (a response delayed by 7–10 days following DDR) and formation of senescence-associated heterochromatic foci (SAHF) might contribute to the irreversibility of growth arrest

developed the following strategy: firstly, we used the BioGRID database in combination with the Phospho.ELM database (Diella et al. 2008). The latter provides information regarding pathway directionality of kinases and their targets. Secondly, confidence values were assigned to each link in the network using the scoring method described by Lee et al. (2004). By averaging the confidence scores of all links, the most probable pathways between TP53 and several SAFS (see legend to Fig. 2b) were calculated. By focussing only on these pathways, the size of the network under consideration could be reduced dramatically (Fig. 2b). Finally, we added information from a comparison between senescent and young microarrays (Passos et al. 2007a). The validity of the resulting information can now be more easily tested by experimentalists (Fig. 2b).

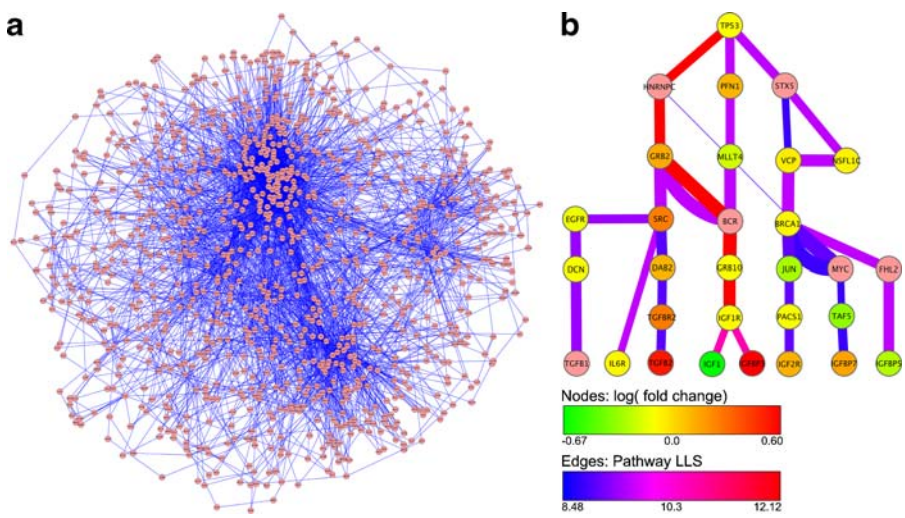


**Fig. 2 a,b** Interactome visualisation of the connection between TP53 and senescence-associated secreted factors (SASFs). **a** "Traditional" interactome with no-weighted links and data from BioGRID. **b** Probabilistic integrated functional network (PIFN): most probable pathways connecting TP53 to SASFs (TGFβ1,2, IGF1, IGF2R, IGFBP3,5,7 and IL6R), using data from BioGRID and Phospho.ELM databases. Edge thickness indicates the log likelihood scores (LLS) for any individual interaction, edge colour gives the pathways LLS, and node colour indicates the average log fold change in mRNA levels in senescent vs young MRC5 human fibroblasts (Passos et al. 2007a). In pink coloured nodes, no expression data was available

In conclusion, the above example shows that a multi-disciplinary approach involving a close collaboration between in-silico and experimental scientists will contribute greatly to a better understand of the complexity of senescence and, subsequently, the ageing process.

**Acknowledgement** Work leading to this paper was supported by BBSRC (CISBAN) and Research into Ageing UK.

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