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The ING1a model of rapid cell senescence

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ABSTRACT

Replicative capacity of normal human cells decreases as telomeric sequence is lost at each division. It is believed that when a subset of chromosomes reach a critically short length, an ATM-initiated and p53-mediated transcriptional response inhibits cell growth, promoting cell senescence. In addition to loss of telomeric sequence, senescence can be induced by other stresses including ionizing radiation, oxidative damage, chemical cross-linkers like the chemotherapeutic agent cisplatin, as well as overactivation of oncogenes and tumor suppressors. Our group found that the expression of an isoform of the **IN**hibitor of **G**rowth **1** gene called ING1a increases approximately 10-fold as fibroblasts approach senescence and that forced expression rapidly induces a senescent phenotype in primary diploid fibroblasts, epithelial and endothelial cells that resembles replicative senescence by most physical and biochemical measures. ING1a induces these changes through strongly inhibiting endocytosis to block mitogen signaling by inducing the expression of intersectin 2, a key scaffolding protein of the endosomal pathway. This, in turn increases the expression of Rb and of $p57^{Kip2}$ and $p16^{INK4a}$ that serve to maintain Rb is an active, growth inhibitory state. The ING1a model is currently being used to better understand the mechanism(s) responsible for activating Rb to enforce the senescent state.

1. Aging and senescence

Physiological aging has been of profound interest to humans for millennia, and has long been recognized by physicians as a factor promoting many disease states (Haber, 2004). Aging has been described as the progressive loss of physiological fitness, leading to impaired function and increased probability of death (López-Otín et al., 2013). Beyond the progressive decline in functioning, aging is a major risk factor for many human diseases including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases (Niccoli and Partridge, 2012). It is therefore important to develop relevant models to study and characterize the mechanisms responsible for transducing signals that enforce the cell aging (senescence) process and promote age-related diseases.

The phenomenon of cellular senescence was first described by Hayflick and Moorhead to characterize the irreversible growth arrest observed in human diploid fibroblast cells in culture after undergoing extensive passages (Hayflick and Moorhead, 1961). In their seminal paper, they went on to speculate that senescence acted as a cellular "stopwatch" that could underlie the process of organismal aging. Today, it is known that the phenomenon of replicative senescence is due to the progressive shortening of telomeres at the ends of chromosomes (Harley et al., 1990). Telomeres consist of tandem repeats of a short DNA sequence (5′-TTAGGG-3′$_n$ in vertebrates where n ranges from 800-3,500 in humans) and associated proteins at the end of linear chromosomes that protect the chromosome ends from degradation and recombination (d'Adda di Fagagna et al., 2004). Due to the intrinsic inability of the DNA replication machinery to copy the ends of linear molecules, a phenomenon termed "the end replication problem", telomeres become progressively shorter with each round of replication. When the telomeres, or a subset of telomeres in the cell reach a critical length, ATM-mediated DNA damage signaling is initiated activating p53 as a transcription factor (Atadja et al., 1995; Vaziri et al., 1997), which induces expression of the p21 cyclin-dependent kinase inhibitor, culminating in growth arrest and replicative senescence. Replicative senescence prevents the proliferation of cells that are genetically unstable and prone to fusion of short telomeres (Feldser and Greider, 2007; Harley et al., 1990). Other harmful stimuli such as oxidative damage (Parrinello et al., 2003), ionizing radiation (Suzuki et al., 2001) and inappropriate activation of oncogenes (Bartkova et al., 2006; Serrano et al., 1997) can also initiate growth arrest and induce a senescence-like phenotype. However, recent studies have indicated that there may be roles for senescence in many normal physiological processes including embryonic development, wound healing, tissue repair, and tumor suppressive activity (Muñoz-Espín et al., 2013; Storer et al., 2013).

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Although initially characterized in cultured cells, senescence is now considered a key hallmark of organismal aging, sharing signaling, genetic and cellular mechanisms that contribute to the overall process of aging itself (Baker et al., 2012, 2011). Impaired clearance of senescent cells by the immune system may result in the net accumulation of senescent cells in some but not all human tissues, notably in the skin, liver, lung and spleen (Herbig et al., 2006), and these senescent cells appear to impair normal organ function. Furthermore, senescence also appears to affect stem and progenitor cells, limiting the regenerative capacity of tissues despite stem cells showing detectable telomerase activity (Sousa-Victor et al., 2014).

2. Hallmarks of senescence

Senescent cells differ from other non-dividing cells, such as quiescent or terminally differentiated cells, in several distinct ways. *In vitro*, senescent cells develop large, flattened, vacuolized and, in certain cases, multinucleated morphologies (Matsumura et al., 1979). *In vivo*, however, the morphology of senescent cells is dependent on cell type and tissue architecture (Rubin, 1997). As senescence is associated with stable proliferative arrest, it follows that senescent cells show an absence of proliferative markers, such as Ki67 protein, or 5-bromodeoxyuridine (BrdU) incorporation (Sherwood et al., 1988), and down-regulation of cyclins and cyclin-dependent kinase (CDK) activity (Choi et al., 2005; Narita et al., 2003). Mediators of senescence, including the CDK inhibitors $p16^{INK4a}$, $p21^{Cip1/Waf1}$, $p15^{Ink4b}$, $p27^{Kip1}$, $p57^{Kip2}$ and the human double minute (HDM) inhibitor $p14^{ARF}$, it's target p53 and hypo-phosphorylated Rb are also used as canonical markers of senescence (Chicas et al., 2010; Quelle et al., 1995; Serrano et al., 1997; Stein et al., 1999). Another defining feature of senescence is the presence of senescence-associated heterochromatic foci (SAHF), which contain many hallmarks of heterochromatin, such as trimethylation at lysine 9 of histone 3 (H3K9me3), heterochromatin protein 1 homologue-γ (HP1γ) and macroH2A. SAHF are thought to be involved in the silencing of genes required for proliferation (Narita et al., 2003).

3. The senescence-associated secretory phenotype

Senescent cells secrete a number of soluble factors that are not secreted by replication-competent cells, which collectively make up the senescence-associated secretory phenotype (SASP) (Coppé et al., 2008). Among the secreted factors are transforming growth factor-β (TGFβ), insulin-like growth factor 1 (IGF1) binding proteins, plasminogen activator inhibitor 1 (PAI-1), and inflammatory cytokines and chemokines (Özcan et al., 2016), which have been shown to reinforce and propagate senescence in both autocrine and paracrine manners (Acosta et al., 2013; Nelson et al., 2012). Components of the SASP are thought to help recruit immune cells involved in clearance of senescent cells (Krizhanovsky et al., 2008; Xue et al., 2007). However, certain SASP factors also appear to induce deleterious effects such as the destabilization of neighboring cells during aging (Coppé et al., 2010).

The most widely used assay for the presence of senescent cells is the histochemical detection of β-galactosidase activity at pH 6.0, termed senescence-associated β-galactosidase (SA-βGAL) (Dimri et al., 1995). Increased β-galactosidase activity is due to increased lysosomal content of senescent cells, and the detection of lysosomal βGAL is most indicative of senescence when done at an acidic pH (Kurz et al., 2000). While none of the biomarkers noted above are completely unique to senescent cells, and all senescent cells typically do not exhibit all markers, senescent cells express many of them, making them useful and informative to identify senescent cells *in vitro* and *in vivo* (Muñoz-Espín and Serrano, 2014).

4. Senescence stressors and effectors

Senescence was originally identified as a response to progressive telomere shortening in cultured cells that is interpreted as a chronic DNA damage signal. Senescence has also been shown to act as a mechanism to block the propogation of other forms of intrinsic and extrinsic molecular damage. This type of senescence has been called stress-induced premature senescence (SIPS) and can occur independently of telomere length (Dierick et al., 2002). Cells in culture can undergo senescence in response to many stresses including suboptimal culture and media conditions (Killilea and Ames, 2008), high oxygen levels (Parrinello et al., 2003), and the absence of proper cellular microenvironments (Conboy et al., 2005). SIPS can also be induced experimentally by exposure to hydrogen peroxide and t-butylhydroperoxide (Chen et al., 2001; Duan et al., 2005), DNA damaging agents such as ionizing radiation (Suzuki et al., 2001) and chemotherapeutic agents (Elmore et al., 2002), oncogene hyperactivation (Bartkova et al., 2006; Di Micco et al., 2006; Serrano et al., 1997) and abnormal tumor suppressor levels. These observations suggest that like replicative senescence, SIPS can function as a protective mechanism to inhibit replication of cells harbouring potentially harmful genomic alterations.

Although diverse stimuli induce senescence, they appear to converge on either or both of two main pathways: the p53 or the $p16^{Ink4a}$–retinoblastoma protein (pRB) tumour suppressor pathways. Stressors that trigger a DNA damage response, such as telomere erosion or ionizing radiation, are thought to induce senescence primarily through the p53 pathway. p53 is negatively regulated by the E3 protein ubiquitin-ligase HDM2 which facilitates its degradation. However, in response to senescence-inducing stimuli, the alternate-reading-frame protein ($p14^{ARF}$) is expressed and inhibits the activity of HDM2 (Kamijo et al., 1997). Activated p53 directly induces the expression of $p21^{Cip1/Waf1}$, (Brown et al., 1997) a potent CDK inhibitor (CKI) which physically interacts with and inhibits, the activity of cyclin-CDK1, -CDK2, and -CDK4/6 complexes, thus functioning as a negative regulator of cell cycle progression during the G1 and S phases to enforce senescence. The critical role of this pathway is shown by the observation that reduction of p53, $p21^{Cip1/Waf1}$ or DNA Damage Response (DDR) proteins *in vitro* can inhibit telomere- or DNA damage-induced senescence (Brown et al., 1997; Di Micco et al., 2006), and in certain cells expressing little or no $p16^{Ink4a}$ or RAS it may even delay or temporarily reverse senescence growth arrest (Beausejour et al., 2003).

The $p16^{Ink4a}$-Rb pathway can be activated by various senescence-inducing stimuli including oncogenic RAS, CDKN2A locus de-repression, elevated levels of reactive oxygen species (ROS) and expression of ING1a (Rajarajacholan et al., 2013; Rajarajacholan and Riabowol, 2015a; Soliman et al., 2008). The cyclin dependent kinase (CDK) inhibitor, $p16^{Ink4a}$, is a transcriptional product of the INK4a locus. When expressed, $p16^{Ink4a}$ inhibits the activity of cyclin D- and E-associated CDK complexes, helping maintain pRb in its hypophosphorylated state, effectively keeping it tightly bound to the E2F family of transcription factors (Chicas et al., 2010). pRb-bound E2F is unable to initiate transcription of genes that are required for S-phase entry and cell cycle progression (Bracken et al., 2004). The $p16^{Ink4a}$–pRb pathway is also crucial for generating SAHF, which are thought to silence genes that are needed for proliferation (Narita et al., 2003).

5. The changing epigenetic landscape during senescence and aging

Widespread epigenetic remodeling has been observed at both the level of DNA and histone modification during organismal aging and as cells undergo senescence *in vitro*. DNA methylation of CpG dinucleotides in promoter regions is a mark of transcriptional silencing (Liu et al., 2016). Replicative senescence is characterized by a global decrease in DNA methylation, but focal hypermethylation occurs at specific chromosomal loci (Cruickshanks et al., 2013; Wilson and Jones, 1983), altering transcriptional control. The global hypomethylation of CpG dinucleotides observed during senescence is attributed to

mislocalization, decreased activity or expression of DNA methyltransferase 1 (DNMT1) (Smallwood et al., 2007). In contrast, senescence-associated focal hypermethylation is thought to be induced by the recruitment of DNMTs to focal sites through HP1 present in SAHF (Smallwood et al., 2007). A comparable change in CpG methylation is seen in aging human cells (Day et al., 2013). In fact, senescence-associated DNA methylation alterations are accurate predictors of cumulative cell population doublings (Koch et al., 2012). Changes in DNA methylation appear to be associated with replicative senescence but not with forms of SIPS as multiple rounds of DNA replication are required to accumulate significant changes in DNA methylation (Bielak-Zmijewska et al., 2014; Sakaki et al., 2017).

Cellular senescence is also accompanied by histone modification, variant histone expression and histone depletion, all of which have important regulatory roles in DNA replication, transcription and repair (Cheng et al., 2017). For example, global decreases in the histone modifications H4K16Ac, H3K4me3, H3K9me3 and H3K27me3 and global increases in H3K9Ac and H4K20me3 occur with cell age (Sanders et al., 2013). Significant changes in histone modification have also been observed in SIPS, however the nature and distribution of the various histone modifications differ depending on the particular senescence-inducing stimulus (Chandra et al., 2012). Telomere shortening during replicative senescence correlates with, and may trigger overall histone depletion and introduction of histone variants such as phosphorylated H2AX (γ H2AX), H3.3 and macroH2A.1.1 (Chen et al., 2015; Corpet et al., 2014).

These senescence-associated histone alterations may have significant functional consequences and contribute to the establishment and maintenance of senescence growth arrest. A well-studied example is the histone methyltransferase complex, polycomb repressive complex (PRC). PRC binds directly to the INK4/ARF locus and induces H3K27 trimethylation, resulting in repression of its transcriptional products, $p16^{Ink4a}$ and $p19^{ARF}$, the murine homolog of human $p14^{ARF}$. Derepression of this locus is a potent inducer of senescence (Martin et al., 2014). Sirtuins are another diverse class of histone-modifying proteins whose role in senescence and aging has been heavily studied. SIRT1, SIRT6 and SIRT7 are NAD+-dependent histone-deacetylases (HDACs) that catalyze modifications of both histones and non-histone proteins, such as transcription factors, and may regulate senescence *via* changes in gene transcription and genome instability (Chen et al., 2016; Luo et al., 2001). These senescence-associated histone alterations may also interact with other regulatory proteins to give rise to the SASP. The presence of histone variant macroH2A.1.1 is associated with an increase in the activity of the poly-ADP-ribose polymerase (PARP) 1 enzyme which induces SASP through the PARP-1/NF-κB signaling cascade (Ohanna et al., 2011).

6. Chromatin architecture

Both cell senescence and organismal aging are accompanied by significant changes in chromatin architecture. The “heterochromatin loss model of ageing” describes the decreased or inappropriate redistribution of heterochromatin and heterochromatin-silencing proteins that may have a causal effect on the cellular dysfunction observed with age (Tsurumi and Li, 2012). This model is consistent with various physiological and accelerated models of aging. The age-related phenomenon of chromatin remodeling has been attributed to interactions between the chromatin regulatory machinery and nuclear periphery proteins such as nuclear lamins, which establish nuclear microdomains with regions of active and repressed gene expression (Dechat et al., 2008). Senescing cells also develop SAHF while misregulation of chromatin architecture and disruption of interactions between chromatin machinery and proteins at the nuclear periphery may also contribute to senescence. For instance, reducing levels of lamin B1 in proliferating fibroblasts can induce senescence through locus-specific remodeling of H3K4me3 and H3K27me3 (Shah et al., 2013) and it promotes SAHF formation (Sadaie et al., 2013). In senescing cells chromatin accessibility also increases at retrotransposon insertion sites resulting in increased transcription and transposition of transposable elements, which might be reflected by the generation of circular extrachromosomal DNA containing repetitive elements, in senescing fibroblasts (Riabowol et al., 1985) and destabilization of the genome.

7. Cellular senescence: from physiology to aging

Research during the past decade has indicated that senescence, along with apoptosis, are essential mechanisms for neutralizing or eliminating damaged cells (Muñoz-Espín and Serrano, 2014). These processes are especially important in cancer and ageing, which are both associated with the accumulation of various forms of cellular damage. The anti-proliferative nature of senescence makes it an effective tumor suppressor mechanism. The observation of senescent-like growth arrest mediated by p53 and $p16^{Ink4a}$ after ectopic expression of oncogenic Ras in normal primary cells introduced the concept of oncogene-induced senescence as a mechanism that restrains the growth of potentially cancerous cells (Serrano et al., 1997). Oncogene-induced senescence has also been implicated as a tumor suppressive mechanism *in vivo* (Di Micco et al., 2006; Feldser and Greider, 2007). Consistent with this idea, many of the genes involved in inducing senescence are frequently inactivated in human cancers (Minamino et al., 2009). Further, many chemotherapeutic drugs currently used in the clinic act by inflicting severe DNA damage and trigger cellular senescence *in vitro*. These observations have led to the proposal that senescence could contribute to the success of chemotherapy. There are, however, conflicting reports where senescence was shown to inhibit chemotherapeutic effects, leading to cancer relapse. Therapy-induced senescent (TIS) cells were observed to persist in tissues and contribute to local and systemic inflammation through a form of SASP (Demaria et al., 2017). Accumulation of DNA damage-induced senescent cells promoting resistance to prostate cancer therapy through their secretory phenotype component WNT16B is an example of a potentially oncogenic role of senescence (Sun et al., 2012). The secretion of chemokines by senescent cells was shown to promote the emergence, maintenance and migration of cancer stem-like cells in multiple myeloma, and also confer drug resistance and malignancy upon them (Cahu et al., 2012).

8. Senescence in development

Although cellular senescence has been viewed primarily as a tumor suppressive cell-cycle arrest mechanism that inhibits uncontrolled cell division, recent insights have extended its known functions to other complex biological processes including development, tissue repair, ageing and age-related disorders. The presence of senescent cells has been detected in several embryonic structures such as mesonephric tubules during mesonephros involution, the interdigital webs, the endolymphatic sac of the inner ear, the apical ectodermal ridge (AER) of the limbs, and the closing neural tube (Muñoz-Espín et al., 2013; Storer et al., 2013). This has led to cellular senescence, as a mechanism that promotes tissue remodeling and embryonic growth and patterning, being suggested as a conserved feature of embryonic development across vertebrates. Beyond embryonic development, programmed senescence also occurs in adult organisms. Senescence has been observed in both megakaryocytes (Besancenot et al., 2010) and placental syncytiotrophoblasts (Chuprin et al., 2013) as part of their natural maturation process. Cellular senescence also appears to be an essential process in wound healing, specifically in restricting the initial fibrotic wound response (Jun and Lau, 2010).

Unlike the programmed nature of senescence in development and tissue repair, ageing-related senescence is more persistent and appears to be unscheduled and stochastic in nature (van Deursen, 2014). There is convincing evidence of senescent cell accumulation in aging tissues of humans, primates, and rodents (Jeyapalan et al., 2007; Wang et al.,

2009) and this likely occurs in most multicellular organisms. However, the mechanisms by which cellular senescence contributes to aging and age-related dysfunction is less clear, but it has been proposed that senescence contributes to the decreasing regenerative potential of aging tissue by depleting stem and progenitor cell populations. This is supported by several observations including that stem and progenitor cell pools of various tissue types are depleted and/or functionally compromised in late-generation telomerase knockout mice (Terc−/−) mice (Rudolph et al., 1999).

Senescence also contributes to the aging process and age-related dysfunction through SASP proteases such as MMP family proteases and tissue-type plasminogen activators, causing tissue dysfunction by cleaving membrane-bound receptors, signaling ligands, and extracellular matrix proteins, disrupting tissue structure and organization (Comi et al., 1995; Millis et al., 1992; West et al., 1989). The accumulation of senescent cells may also contribute to chronic inflammation *via* SASP-mediated secretion of proinflammatory growth factors, cytokines and chemokines such as GM-CSF, GROα, IL-1, IL-6, IL-8, macrophage inflammatory proteins (MIPs), and monocyte chemo-attractant proteins (MCPs). Chronic tissue inflammation that is associated with organismal aging is characterized by the presence of infiltrating lymphocytes and macrophages, abundant blood vessels, fibrosis and, often, tissue necrosis (Sarkar and Fisher, 2006).

The development of a transgenic INK-ATTAC mouse model harboring a senescent cell “suicide gene” has shed some light on the effect of clearance of senescent cells *in vivo*. Upon administration of the drug AP20187, apoptosis was induced through dimerization of FKBP-fused caspase 8 in $p16^{Ink4a}$-positive senescent cells (Baker et al., 2011). In a BubR1 progeroid mouse, life-long removal of $p16^{Ink4a}$-expressing cells was reported to delay the onset of age-related pathologies in adipose tissue, skeletal muscle and the eye. Further, late-life clearance of senescent cells attenuated the progression of already established age-related disorders (Baker et al., 2011). This model was also used to show that genetic depletion of senescent cells in the joint ameliorates osteoarthritis (Childs et al., 2016; Jeon et al., 2017) and senescent cell clearance mitigates fibrotic lung disease (Schafer et al., 2017). These results suggest pathological effects of senescent cell accumulation in this model and implicate senescence as a therapeutic target.

The multifunctional nature of senescence is likely due to antagonistic pleiotropy, the proposed relationship in which biological processes are selected to benefit the health and fitness of young organisms but also lead to manifestation of unselected deleterious effects in older organisms that contribute to the ageing process (Kirkwood and Austad, 2000). Thus, acute senescence has essential roles during embryonic development and acts as a potent tumor suppressor in the early part of an organism's lifespan, while chronic senescence later in life contributes to aging-associated phenotypes.

9. Senescence as a potential therapeutic target

Because of the multifunctional nature of senescence, both pro-senescence and anti-senescence approaches may be beneficial depending on the therapeutic context. Pro-senescence therapies may help limit the fibrotic response during ongoing tissue damage and act as an anti-cancer therapy (Efimova et al., 2010; Lin et al., 2010). Therapies that induce senescence in cancer cells should be accompanied by a complementary treatment to subsequently eliminate the senescent cells, preventing possible pro-tumorigenic effects of the SASP on neighboring cells (Muñoz-Espín and Serrano, 2014; Sun et al., 2012). In fact, senescence-inducing CDK4 inhibitors have made it to phase II clinical trials and have shown promising results in mantle cell lymphoma, breast cancer and liposarcomas (Dickson et al., 2013; Finn et al., 2009; Leonard et al., 2012).

Anti-senescence therapies have also proven to be beneficial in diseases of aging and in tissues where the accumulation of senescent cells has deleterious effects, largely through the action of the SASP. Senolytics are a class of drug used to specifically kill senescent cells. First-generation senolytics typically act by inhibiting pro-survival pathways used by senescent cells to resist apoptosis (Zhu et al., 2015). These compounds have shown positive results in the treatment of atherosclerosis, osteoarthritis and other age-related diseases (Roos et al., 2016; Takayama et al., 2014). Another potential form of senotherapy is the direct inhibition of SASP (Laberge et al., 2015), however, these therapies would likely require continuous dosing which is less practical.

10. Changes in the epigenome

The epigenome undergoes profound alterations as cells senescence, and it is thought that these epigenetic changes, including altered DNA methylation and histone modifications, contribute to senescence and the aging process itself (Lowe et al., 2016). Histone methylation and acetylation regulate chromatin conformation and transcription, and heterochromatic foci are a hallmark of senescent cells (Chen et al., 2016; Krishnan et al., 2011). We have reported that expression of the epigenetic regulator Inhibitor of Growth 1a (ING1a), a histone reader that targets Sin3A-HDAC complexes to the H3K4Me3 mark of active transcription, is dramatically upregulated in senescent cells (Rajarajacholan et al., 2013; Rajarajacholan and Riabowol, 2015a; Soliman et al., 2008), and that its ectopic expression induces a senescent phenotype in low-passage fibroblasts. In fact, expression of ING1a induces senescence *in vitro* much more rapidly than all other previously described senescence-inducing stimuli (Rajarajacholan and Riabowol, 2015b), making this a useful model to understand the temporal relationships between the genetic and biochemical pathways responsible for initiating and enforcing senescence and to devise strategies to modulate this process.

11. ING1a induces senescence

The INhibitor of Growth (ING) family is made up of five genes (ING1–5), each encoding multiple isoforms. ING proteins can be broadly classified as type II tumor suppressors as they are frequently downregulated and or misslocalized in cancers. The founding member of the ING family, ING1, was discovered by PCR-mediated subtractive hybridization using cDNAs from a normal mammary epithelial cell strain and several transformed breast cancer cell-lines, followed by an *in vivo* functional screen to identify differentially expressed factors that affect tumorigenesis (Garkavtsev et al., 1996; Toyama et al., 1999). Overexpression of ectopic ING1 was observed to promote cell cycle arrest or apoptosis, while inhibition of its expression with antisense RNA led to cellular transformation *in vitro* and tumor formation *in vivo*.

The other four members of the ING family (ING2-5) were identified largely based on sequence homology to ING1. Orthologs of ING proteins have been discovered throughout the animal kingdom and have emerged as a versatile family of phospholipid effectors, histone mark sensors, and growth regulators (Tallen and Riabowol, 2014). All five ING proteins share a highly conserved plant homeodomain (PHD) form of zinc finger through which they selectively bind the lysine 4 residue of histone H3, with affinity increasing with methylation state and H3k4me3 having the highest affinity. This epigenetic mark is preferentially located at promoters and downstream of transcription start sites (Matthews et al., 2007; Peña et al., 2006) and is a mark of active chromatin and gene expression (Santos-Rosa et al., 2002). ING proteins are stoichiometric members of histone acetyltransferase (HAT) and histone deacetylase (HDAC) complexes (Doyon et al., 2006), and through their PHD, they can direct HAT or HDAC activity to the immediate vicinity of the marks to affect chromatin structure and alter gene transcription. These multidomain proteins also encode one or more nuclear localization signals (NLS) that target them to the nucleus and to different chromatin domains where they exert their activity within the cell (Tallen and Riabowol, 2014).

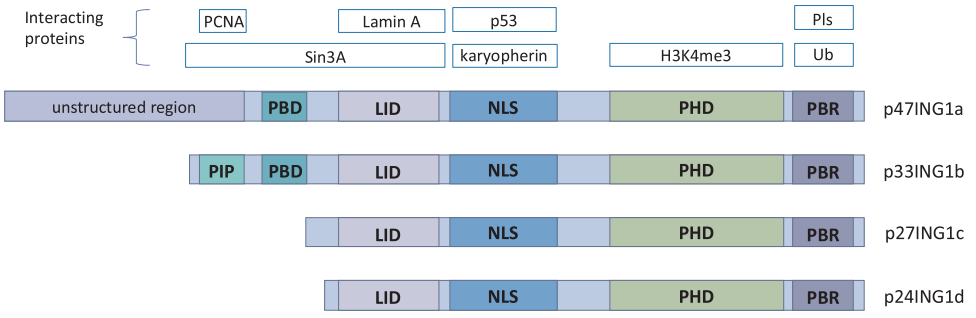

Fig. 1. ING1 protein isoform structure and interacting partners. ING1 encodes four isoforms through alternative promoter usage (ING1a and ING1b), internal initiation (ING1c) and splicing (ING1d). In addition to those shown, many additional interacting proteins have been reported, but they have not been mapped to specific regions of the ING proteins.

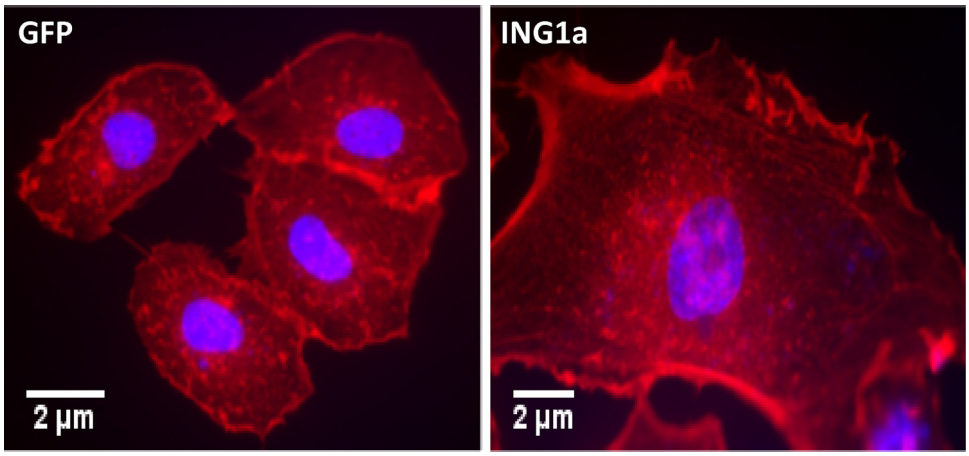

Fig. 2. Ectopic expression of ING1a induces a senescent phenotype in different cell types including endothelial cells. Phalloidin-TRITC staining of EA.hy926 primary endothelial cells shows that ING1a overexpression induces changes to the actin cytoskeleton and enlarged, flat morphology typical of senescent cells.

ING1 contains functional domains that are unique to this family member. Within the NLS of ING1 proteins are short, highly basic nucleolar translocation sequences (NTS), which are needed for translocation to the nucleoli in the event of DNA damage. Mutations within this motif result in the loss of nucleolar targeting in response to UV damage and subsequent reduced levels of apoptosis (Scott et al., 2001). ING1 has a partial bromodomain (PBD) which facilitates interaction with SAP30 of the Sin3-HDAC1 and HDAC2 complexes that modify chromatin architecture and regulate gene transcription (Kuzmichev et al., 2002). Both ING1 and ING2 contain a polybasic region (PBR) adjacent to the PHD through which they bind with high affinity to bioactive phosphoinositides such as phosphatidylinositol 5-phosphate (PtdIns(5)P) in the nucleus (Kaadige and Ayer, 2006). The phospholipids that specifically bind the PBR domain of ING1 and ING2 are stress-inducible and suggest that ING1 and ING2 proteins targeting HDAC complexes to chromatin are strongly induced in response to various forms of cellular stress (Tallen and Riabowol, 2014). This region also serves as a ubiquitin-binding region (as noted in Fig. 1) and so activated phospholipids compete with ubiquitin for binding to INGs 1&2 (Thalappilly et al., 2011).

ING proteins also contain a lamin interaction domain (LID), whose sequence is unique within the entire human proteome (Han et al., 2008). The LID domain has high affinity for lamin A, an essential component of the nuclear lamina and inner nuclear membrane. This interaction is thought to help tether ING1 preferentially to the inner membrane of the nucleus, thereby localizing its chromatin modifying activity to regions that typically display a heterochromatic character (Han et al., 2008). Mutation of the gene encoding lamin A results in several laminopathies, including Hutchinson-Gilford progeria syndrome (HGPS), a severe premature ageing disorder (Eriksson et al., 2003). HGPS cells show disruption of nuclear architecture as well as decreased expression and misslocalization of ING1. Disruption of ING1-lamin A interaction also manifests as an atypical nuclear phenotype similar to that of HGPS cells (Han et al., 2008). These findings suggest that ING proteins may contribute to the HGPS phenotype through alteration of the epigenetic status of lamin A mutant cells.

The ING1 gene encodes four potential variants, of which p33ING1b and p47ING1a are the best characterized (Fig. 1). All four isoforms share common domains: the LID, the NLS, the PHD, and the PBR, however, each isoform differs at the amino terminal end. The ING1a isoform has a unique amino terminal unstructured region which may have functional significance in its ability to rapidly induce cell senescence. While ING1a is expressed at modest levels in low-passage or “young” fibroblasts, levels of endogenous ING1a mRNA and protein increase as cells undergo senescence, during which time the expression of ING1b is downregulated. Alternative promoter usage within the ING1 gene in senescent fibroblasts alters the INGla:INGlb ratio by ~30-fold compared to low passage primary fibroblasts (Soliman et al., 2008). Consistent with a role in inducing senescence, knocking down ING1 expression in fibroblasts increases their replicative life span in culture by 10–20% (Garkavtsev and Riabowol, 1997).

The ectopic expression of the protein p33ING1b induces features of a stress-induced senescence-like phenotype, including SA-β-gal activity, increased expression of p16 and growth arrest (Li et al., 2011), through its interaction with p53 (Abad et al., 2007; Garkavtsev et al., 1998) and transcriptional silencing activity (Goeman et al., 2005). However, at high levels of expression, cells subsequently develop pyknotic nuclei and undergo apoptosis (Bose et al., 2013; Helbing et al., 1997).

Alternatively, when the p47ING1a isoform is ectopically expressed in different cell types, cell growth is arrested in a state resembling replicative senescence as shown in the endothelial cells in Fig. 2. Overexpression of this isoform promotes senescent cell morphology, high levels of SA-β-gal activity and induces the formation of structures resembling senescence-associated heterochromatic foci containing heterochromatin protein 1 gamma (Soliman et al., 2008). Compared to other canonical initiators of stress-induced premature senescence

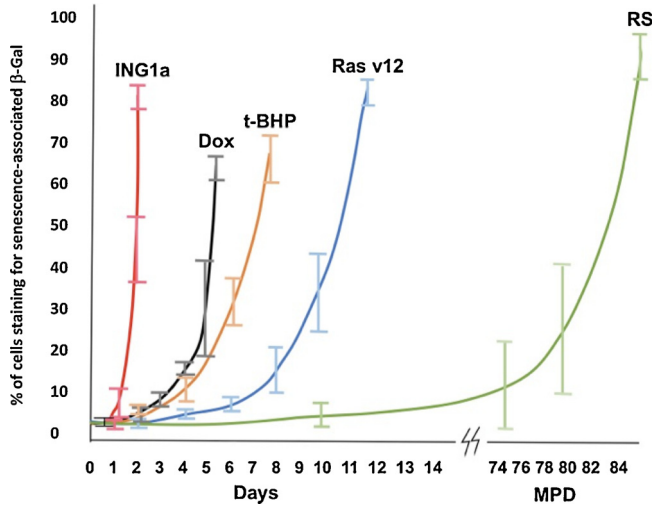

Fig. 3. Comparison of the time taken for the indicated agents to induce SIPS in Hs68 cells as estimated by SA-β-Gal staining. Oxidative stress was induced by t-butyl hydroperoxide exposure (1 h treatment everyday with 70 μM for 8–9 days). Genotoxic stress was induced by Doxorubicin exposure (100 ng/ml for 6–7 days). Ras v12 and ING1a were expressed using adenoviral expression constructs. While ING1a, Dox, Ras v12 and t-BHP induced telomere independent senescence in several days, telomere dependent replicative senescence requires several months of passaging before cells reach the end of their replicative life span. ING1a is the most rapidly acting agent and induces senescent phenotypes within 36–48 h. MPD refers to mean population doublings.

including oncogene activation, oxidative stress, and genotoxic stress which take 7–12 days to induce a senescent phenotype, ING1a-induced senescence occurs in 36–48 h (Rajarajacholan and Riabowol, 2015b) as noted in Fig. 3. Although the ability of ING1 to induce cellular senescence is clear, it is only recently that details regarding the molecular pathways underlying ING1a-induced senescence have emerged.

12. LinkING senescence and endocytosis

To identify the mechanism by which ING1a induces senescence, a microarray-based analysis in human diploid fibroblasts transfected with exogenous ING1a identified 242 up-regulated and 172 down-regulated genes (Rajarajacholan et al., 2013). Many of the genes whose expression was significantly altered by ING1a overexpression were known to function in endocytosis, vesicular trafficking, or growth factor signaling. Notably, Intersectin 2 (ITSN2), a key component of endocytosis and a direct target of ING1a, was most highly affected, showing an almost 30-fold increase. A similar increase in ITSN2 expression was also observed in cells undergoing normal replicative senescence (Rajarajacholan et al., 2013). ITSN2 is a multidomain 180 kDa scaffolding protein that facilitates the formation of clathrin-coated pits during clathrin-mediated endocytosis of growth factor receptors (Tsyba et al., 2011; Yamabhai et al., 1998). It exerts its function by interacting with epsin, a clathrin pit component, and with AP2, a clathrin adaptor complex, and binds to both dynamin and synaptojanin, two proteins involved in the pinching off of clathrin vesicles from the membrane surface. Overexpression of ITSN2 independently inhibits clathrin-mediated endocytosis and blocks transferrin (TR) (Pucharcos et al., 2000) and epidermal growth factor receptor (EGFR) (Martin et al., 2006) internalization and uptake, and subsequently induces senescence (Rajarajacholan et al., 2013). It has been proposed that the increased expression of ITSN2 disrupts endocytosis by causing the formation of constricted clathrin-coated pits, likely through interactions between its Src-homology-3 A (SH3A) domain and dynamin (McPherson et al., 1999).

Several major endocytotic pathways, including clathrin-mediated and caveolae-dependent endocytosis, are down regulated in cells undergoing replicative senescence (Wheaton et al., 2001) and it has been suggested that restoration of receptor-mediated endocytosis may lead to functional recovery of the senescent cells (Park, 2002). Moreover, independently disrupting endocytosis, either pharmacologically by treating with a dynamin inhibitor or genetically by manipulating the expression of endocytosis proteins, induces senescence markers (Olszewski et al., 2014; Rajarajacholan et al., 2013). Dysregulation of clathrin-mediated endocytosis results in attenuation of growth factor signaling pathways including EGFR, Akt and ERK and the subsequent hyper-activation of Rb through the induction of the p16INK4a and p57KIP2 CDK inhibitors. Upon either ING1a or ITSN2 expression, phosphorylation of Rb at residues S795, S780, S807, and S811 is blocked (Rajarajacholan et al., 2013). Phosphorylation at these sites inhibits Rb from tightly binding and inhibiting the transcription factor E2F. Increased expression of ING1a also increases overall Rb levels in an ITSN2-independent manner. Various transcriptional targets of E2F known to promote cell growth, cell cycle progression and proliferation are downregulated in ING1a-expressing cells. The ING1a–ITSN2 axis thus plays a causal role in senescence by dysregulating endocytosis and activation of Rb, and may also play a causal role in the functional decline associated with aging as endocytosis regulates many key cellular processes including nutrient uptake, growth factor sensitivity, and immune response. A model for the steps currently known by which ING1a affects the Rb axis to induce senescence is shown in Fig. 4.

13. ING1a-induced senescence as a model to study aging mechanisms

Because of the rapid and synchronous manner in which ING1a-induces cellular senescence in cultured cells (Fig. 4), it serves as a

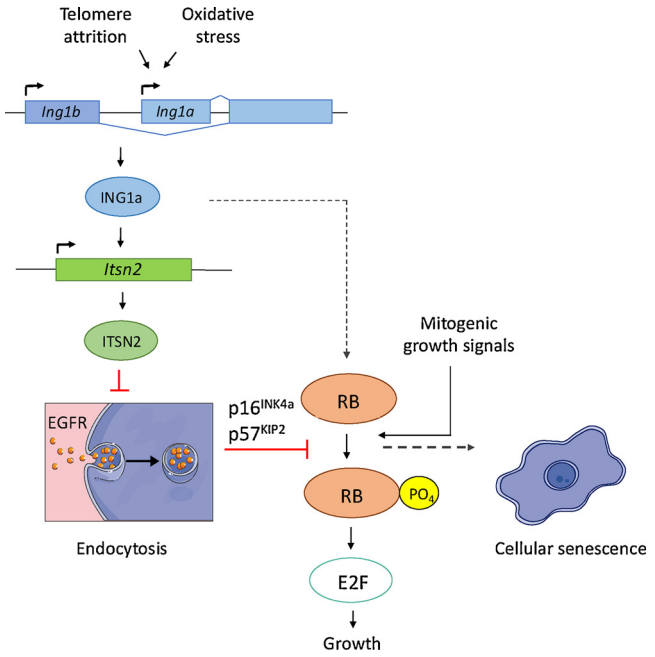

Fig. 4. A model for ING1a-induced senescence *via* ITSN2 and disruption of endocytosis. The shortening of telomeres and oxidative stress induce the expression of ING1a through the usage of alternative promoters. ING1a directly binds and activates the promoter of ITSN2, increasing its protein levels. This leads to disruption of endocytosis and a loss of signal transduction *via* clathrin- and caveolae-mediated endocytosis. Inhibition of endocytosis results in increased the levels of p16 and of $p57^{KIP2}$, which inhibit Rb phosphorylation, maintaining it in its hypophosphorylated, growth-inhibitory state. Inhibition of Rb phosphorylation as well as increase of Rb protein levels *via* an ITSN2 independent mechanism culminate in the induction of cellular senescence.

valuable model for studying the molecular pathways contributing to the Rb mediated induction and maintenance of senescence *in vitro*. ING1a has been shown to effectively induce many of the canonical makers of senescence including senescent cell morphology, SA-βGal staining, formation of SAHFs and stress-induced actin filaments, suggesting that all of these hallmarks of senescence may be linked to the ING1a-Rb-$p16^{Ink4a}$ axis. This has broad relevance since ING1a expression induces a senescence-like phenotype in diverse cell types such as fibroblasts, endothelial cells, keratinocytes, and vascular smooth muscle cells (Rajarajacholan and Riabowol, 2015a). Furthermore, it is able to do so in the absence of an activated p53-DNA damage signaling pathway. Given the increased expression of both ING1a and ITSN2 during replicative senescence, and in response to other forms of stress, it is possible that physiological aging, SIPS, and replicative senescence share many of the same components and signaling pathways, despite being initiated by different agents. Mouse *ING1* encodes three isoforms through differently regulated promoters (Zeremski et al., 1999), however, an ortholog to human ING1a has not yet been identified. Therefore no mouse model for ING1a-induced senescence currently exists. We expect that identifying more genes that act in the ING1a-induced senescence pathway will shed additional light on both the epigenetic and the cytoplasmic signaling networks underlying senescence.

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