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Caveolar Vesicles in Cellular Senescence

Keith Wheaton
*Department of Biology, York University, Toronto*
Canada

1. Introduction

The replicative limit of human fibroblasts has long provided a model to assess the molecular mechanisms which underlie cellular aging. In culture, fibroblasts which reach the end of their proliferative lifespan acquire profound molecular changes that limit their response to growth factors, and cause permanent exit from the cell cycle. Part of the senescence programme is due to a well established link between telomere attrition which occurs with each population doubling and the subsequent activation of the p53 tumour suppressor. Critical shortening of telomeres is thought to cause a form of DNA damage, that leads to the activation of caretaker proteins ATM, ATR or DNA-PK which activate p53, leading to the initiation of senescence through p53 effector genes. In addition, p53 mediates senescence by many other stimuli including oxidative stress, DNA damaging agents and oncogenic activation.

Caveolins are the main scaffolding proteins driving the formation of caveolae (50-100 nm wide cave like invaginations at the plasma membrane) from lipid rafts and allows the organization of many signalling cascades. This compartmentalization concentrates receptors, proteins with lipid anchors, and the lipids from which second messengers are derived. In this capacity caveolin has also been shown to bind and inactivate many key components of mitogenic pathways through the caveolin scaffolding domain (CSD) and thus is often considered a tumour suppressor. About a decade ago, the original investigations into the relationship between caveolae and senescence showed elevated levels of the caveolin proteins during replicative senescence. Both the ectopic expression or endogenous upregulation of caveolin was shown to lead to p53 mediated senescent arrest. However, this upregulation has not been proven to lead to an increase of caveolae at the cell surface, but does lead to increased number of internalized structures. The mislocalized caveolar vesicles likely influence the regulation of mitogenic signals that normally are integrated through caveolae. In addition the caveolin protein has been shown to regulate many signalling cascades that have an impact on senescence, and on the activation of p53.

1.1 Fibroblast model of senescence

Primary human diploid fibroblasts (HDFs) have traditionally served as an experimental model to investigate cellular and molecular aspects of aging. Normal fibroblasts derived from human donors are neither immortal or transformed and are limited in the number of times they are capable of dividing *in vitro* and *in vivo*. This finite replicative lifespan terminates with the acquisition of a phenotype having distinct morphological and biochemical characteristics termed replicative senescence (Hayflick, 1965a; Hayflick and

Moorhead, 1961). This is a cell fate distinct from apoptosis or differentiation since the cells remain viable but are refractory to mitogenic signals. Senescing fibroblasts progressively acquire a flattened morphology with an increased cellular volume, an irregular shape, and increased accumulation of debris (Hayflick, 1965a; Hayflick and Moorhead, 1961).
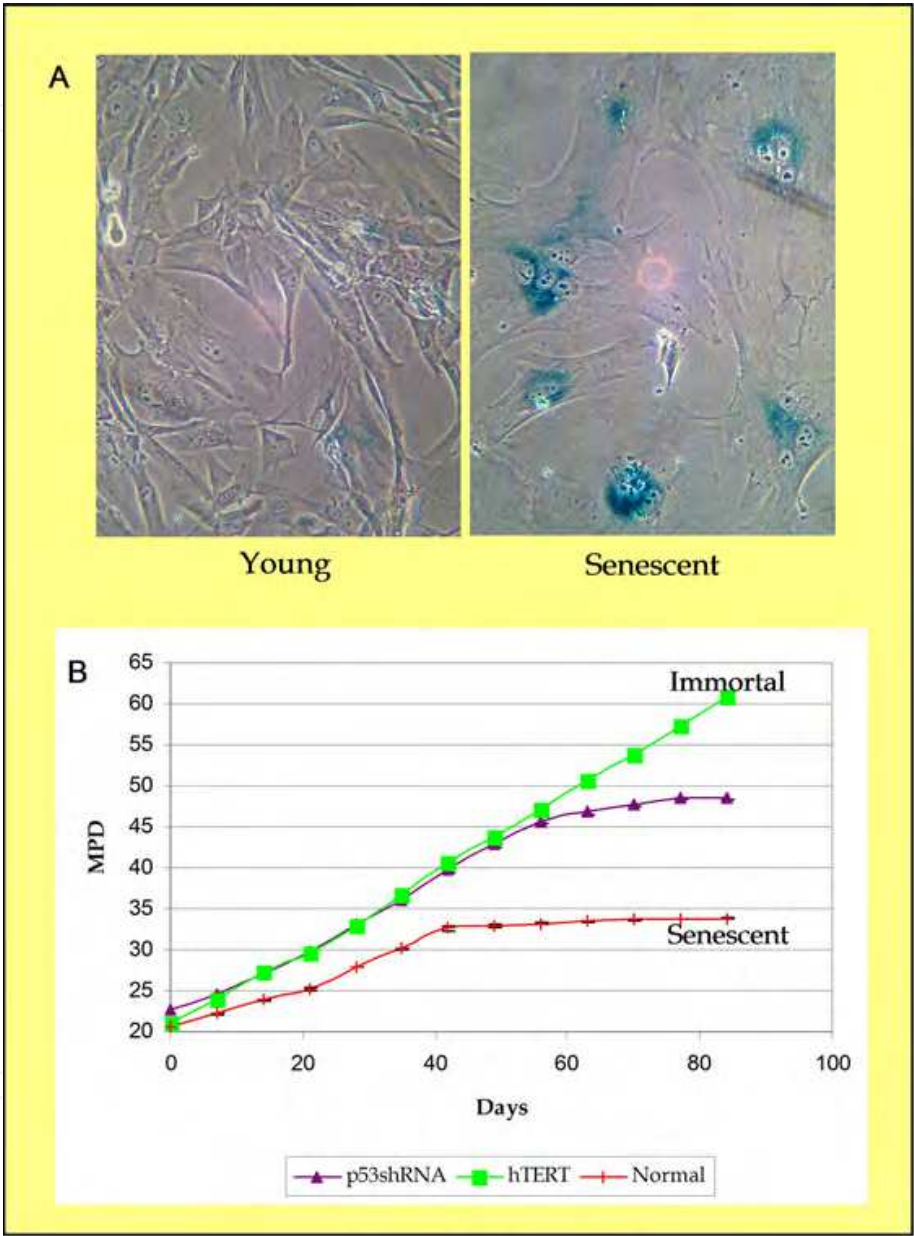
Fig. 1. Cellular Senescence
A) Young and senescent primary fibroblasts prepared with an acidic β-galactosidase assay, staining senescent cells blue. Note the enlarged and flattened morphology of senescent cells compared to young fibroblasts. **B)** A growth curve of AG08470 primary fibroblasts virally transfected with empty vector (Normal), human telomerase (hTERT) or p53 short hairpin RNA (p53 shRNA). This strain of fibroblasts undergo replicative senescence at a mean population doubling (MPD) of 34 (Normal). The knockdown of p53 protein levels using p53 shRNA extends the proliferative lifespan by 13 MPDs. The ectopic expression of hTERT immortalizes the fibroblast strain.

Additional phenotypic changes include increased actin stress fibers and an increase in cells containing irregular and multiple nuclei (Wheaton et al., 1996). These morphological changes were further shown to be dependent on the innate property of the number of mean population doublings of a particular cell strain, and were not influenced by proximity to lower passage cells, splitting ratio, viral contamination, media composition, or periods of storage in liquid nitrogen (Hayflick, 1965b; Hayflick and Moorhead, 1961). Senescent cells can be distinguished from low passage fibroblasts by the innate property to stain blue (Fig. 1A) when processed with an acidic β-galactosidase (β-gal) assay (Dimri, 1995).

Although HDFs in culture are separated from their normal cellular environment, several observations support the use of HDFs grown *in vitro* as a valid model of biological aging. These include: 1) Fibroblasts isolated from young individuals are reproducibly able to undergo more mean population doublings (MPDs) then cells from old donors of the same species (Martin et al., 1970). 2) Fibroblasts from longer lived species undergo more population doublings than short lived species, indicating a relationship between the maximum lifespan of a species and the proliferative capacity of their fibroblasts grown in culture (Goldstein and Singal, 1974; Rohme, 1981). 3) Fibroblasts from individuals with premature aging syndromes such as progeria and Werner's syndrome undergo fewer population doublings in culture than normal cells (Brown, 1990). 4) Normal human fibroblasts in tissues stain with β-gal, indicating senescence *in vivo* (Dimri et al., 1995). These and other observations regarding biochemical differences that accompany senescent HDFs suggest that the HDF model of cellular aging reflects the normal biological processes that occur during *in vivo* aging. In addition to this evidence it has become increasingly apparent that senescence is a natural barrier to oncogenic transformation in many different tumour types (Collado and Serrano, 2010).

2. The post-receptor block

As a result of the refractory nature of senescent fibroblasts to mitogenic stimuli, many of the original studies exploring the molecular details of replicative senescence focused on various growth factor signalling pathways. The growth factors which have been reported to give optimal proliferative response in human fibroblasts are the epidermal growth factor (EGF), insulin like growth factor 1 (IGF-1) and dexamethasone (Phillips and Cristofalo, 1988). Although these tyrosine kinase receptors (RTKs) have many potential down stream mediators, the classical example is a cascade through the small GTPase Ras that leads to the mitogen-activated protein kinase (MAPK) cascade. Early studies investigating the loss of mitogenic response of senescent human diploid fibroblasts focused upon the growth factor receptor:ligand interactions (Gerhard et al., 1991; Phillips et al., 1983; Sell et al., 1993). In the cases of EGF, dexamethasone and IGF-1, it was found that the number of receptors remained the same per unit surface area in senescent cells and that the ligand affinity remained unchanged (Sell et al., 1993). Similarly, the binding kinetics of the glucocorticoid receptor and the insulin receptor have been shown to remain unaltered in senescent cells (Chua et al., 1986). The degradation process of EGF receptor has been shown to be largely unchanged with fibroblast age (Phillips et al., 1984). Studies have also looked at tyrosine autophosphorylation of EGF and PDGF and found no significant differences (Chua et al., 1986; Gerhard et al., 1991). Thus, the general consensus is that decrease in mitogen response is not due to alterations of receptor function or processing (Rattan and Derventzi, 1991).

However, it has been established that the downstream responses of growth factors, such as the induction of the immediate early genes *c-fos* and *egr-1*, are blunted in senescent cells (Meyyappan et al., 1999; Riabowol et al., 1992; Seshadri and Campisi, 1990). Thus, the senescence-specific growth block is thought not to be due to alterations in growth factor-receptor engagement or processing, but to a postreceptor block which leads to reduced immediate early gene responsiveness. Many studies have attempted to identify the points where signalling cascades are modified in senescent cells to account for this block.

2.1 EGFR-Ras-MAPK cascade in senescence

The signal transduction pathway through the epidermal growth factor receptor (EGFR) (and other RTKs) begins with ligand engagement, which initiates dimerization and autophosphorylation within the cytoplasmic domains of the receptors (Fantl et al., 1993). This, in turn, is followed by Src homology 2 domain (SH2) containing molecules interacting with the receptor and recruiting other factors including adaptors. The classic adaptor molecule is Grb2, which binds to the growth factor receptor directly or indirectly through Shc and is associated constitutively with the son of sevenless (SOS) protein. The SOS guanine nucleotide exchange factor catalyses the transformation of inactive GDP-ras to active GTP-ras (Boguski and McCormick, 1993). Activated Ras recruits Raf1 to the membrane allowing its activation (Moodie and Wolfman, 1994). Appropriately localized and activated Raf1 then initiates a kinase cascade through the MAPK cascade. Raf 1 phosphorylates MEK (MAP kinase kinase) which activates Erk1 and Erk2 (MAPK) and leads to MAPK nuclear localization. In the nucleus MAPK phosphorylates transcription factors such as Elk-1 & serum response factor (SRF), stimulating cell proliferation (reviewed in (Wheaton et al., 1996)).

Many studies have attempted to elucidate the role of signalling cascades in senescence, and investigations into the signalling pathways downstream of the EGFR are the most common. It is unclear whether these pathways are compromised in senescence, or whether they need to be intact to promote senescence. Considering that EGFR has been localized to caveolae it is of interest to evaluate studies that have seen a differential regulation in senescent cells. First, it has been found that the EGFR is cleaved to a 100 kD fragment in non-ionic detergent isolates from senescent but not from young primary fibroblasts. This activity appears to be confined to a specialized region of the plasma membrane in senescent cells and was independent of receptor turnover (Carlin et al., 1994). However, it is unclear whether this proteolytic processing is an artefact of the isolation procedure or is physiologically relevant. Second, several studies have reported an age dependent decline in mitogenic stimulation of rat hepatocytes which may be caused by a reduced association between Shc and the EGFR (Hutter et al., 2000; Palmer et al., 1999). Although total tyrosine phosphorylation levels on EGFR appear equivalent after serum stimulation in young and senescent cells, there is a specific reduction of phosphorylation on tyrosine 1173 (a known phosphotyrosine binding (PTB) domain that interacts with Shc (Okabayashi et al., 1994)) in senescent cells. The 1173 residue is known as an autophosphorylation site (Downward et al., 1985), but can also be targeted by Src kinase *in vitro* (Wright et al., 1996) and also is recognized by the SHP-1 phosphatase (Keilhack et al., 1998). This site was subsequently shown to be dephosphorylated by an upregulation of SHP-1 and other phosphatases in senescent human fibroblasts (Tran et al., 2003). Thus, the EGFR in senescence is regulated by differential

interaction of adapter molecule Shc at the plasma membrane, which likely compromises its downstream signalling. Interestingly, different splice forms of Shc may have a role in cellular senescence and longevity (Migliaccio et al., 1999).

Studies have also focused on the downstream effectors of EGFR in senescence, particularly on the MAPK (erk 1/2). MAPK is pivotal in transactivation of various transcription factors that drive the cell through proliferation. Although a general reduction in MAPK (Erk1/2) activation has not been seen in senescence (Kim et al., 2000; Tresini et al., 2001), there is a dramatic reduction in localization of activated Erk to the nucleus of senescent fibroblasts (Bose et al., 2004; Kim et al., 2000; Lim et al., 2000; Tresini et al., 2001). However, it has also been found that a phosphatase MKP-2 is stabilized and acts on Erk in senescent cells (Torres et al., 2003), explaining the reduction in active MAPK. This reduced MAPK activity leads to a lack of Elk-1 activation, which loses its ability to bind to the adjacent ets region of the serum response element in *c-fos* promoter. These studies also concede that the reduction in binding of elk-1 alone cannot explain the entire loss of binding at the *c-fos* promoter, or the repression of its transcription. Interestingly, there is an equal amount of non-phosphorylated MAPK (activated) in the nucleus of young and senescent cells, and although reduced, there is still some localization of phospho-MAPK to the senescent cell nucleus. Therefore, these studies raise the question of whether the reduction in MAPK and Elk-1 represents a significant threshold to suppress *c-fos* transcription and explain the post receptor block. This was confirmed when SRF, the primary transcription factor of c-fos and Egr-1, was shown to be hyper-phosphorylated in senescent cells by PKCδ, and suppress immediate early gene transcription (Atadja et al., 1994; Wheaton and Riabowol, 2004). It was shown that immediate early genes could be restored in senescent cells by blocking PKCδ activity independent of MAPK. However, even though immediate early genes could be restored, this was insufficient to allow senescent cells to re-enter the cell cycle (Wheaton and Riabowol, 2004). Not surprisingly, other factors are clearly involved in senescent arrest besides the refractory mitogenic response.

Premature senescence can be induced by the introduction of oncogenic Ras (Serrano et al., 1997) or Raf (Zhu et al., 1998) or constitutively active MAPK (Lin et al., 1998) in primary fibroblasts. In every case, there is an upregulation of p21 and p16 and an induction of senescence which depends on p53 activity. Also the inducible levels of the *c-fos* transcript were found to be repressed in oncogenic Ras induced senescence (Serrano et al., 1997). This oncogenic Ras/Raf/MAPK pathway has been shown to impinge on the p38^{MAPK} stress activated kinase, which is required for the induction of premature senescence (Debacq-Chainiaux et al., 2010). The p38 activity likely impinges directly on p53, or the Rb/p16 pathway to lead to this arrest. The induction of premature senescence likely represents a natural mechanism to block oncogenesis when there is an inappropriately sustained mitogenic signal. Thus, the perturbations of the cellular system as a whole induced by hyperactive Ras/Raf/MAPK are important to inducing premature senescence and only indirectly produce the post receptor block.

2.2 Protein kinase C activity during senescence

Conventional PKCs (α, β, & γ isoforms) and novel PKCs (δ, ε, η, & θ isoforms) are activated by the lipid second messenger 1,2 diacylglycerol (DAG) which is generated downstream of

many G-protein-coupled receptors and tyrosine-kinase receptors (Dempsey et al., 2000; Newton, 2001). PKC family members are activated in response to a large number of extracellular signals, and regulate a large number of effectors including receptors, kinases, cytoskeletal components and transcription factors. The outcomes of these signals include changes in proliferation, gene expression, differentiation, permeability, migration, hypertrophy, apoptosis and exocytosis. Each outcome depends on the cellular context, the originating signal and the particular isoform activated (Dempsey et al., 2000). Altered PKC activity has been observed in senescent fibroblasts where a change in serum-induced translocation of PKC from the cytosol to the plasma membrane was reported (De Tata et al., 1993). In contrast, exogenously added phorbol 12-myristate 13-acetate (PMA, a DAG analog) stimulated this translocation identically in young and old cells, implying that the difference in response to serum was due to changes in the production of DAG (De Tata et al., 1993). A similar differential response to serum vs. PMA was also reported for the induction of the *c-fos* gene (Riabowol et al., 1992). Although old fibroblasts have a higher basal level of DAG (Vannini et al., 1994; Venable et al., 1994), the response due to serum induction was reported to be much lower in senescent cells (Chang and Huang, 1994). It should be noted that the specific isoforms were not noted in these studies, but are likely PKC α or β. Attenuated PKC response could contribute to the senescent phenotype in several ways. For example, decreased phosphorylation of substrates that are downstream of PKC could affect the expression of many growth-regulatory genes such as *c-fos* or other MAPK substrates. Alternatively, the higher basal amount of DAG in the resting state of senescent cells compared to young cells may also allow constitutive activation of some kinases. One such kinase, PKCα was found to be required in oxidative stress induced senescence in human fibroblasts. PKCα was shown to induce the senescent phenotype by acting on erk1/2 and Sp1, which converge on and up regulate transcription from the p21 promoter (Kim and Lim, 2009). Interestingly the knockdown of PKCα restored proliferative capacity of senescent cells, implying that the increased basal levels in DAG in senescence constitutively activate PKCα.

The PKCδ isoform has a multifunctional role in various processes including growth inhibition, differentiation, apoptosis and tumour suppression (Basu and Pal, 2010). Many groups have reported that PKC δ activation leads to growth inhibition, including CHO cells, smooth muscle, NIH 3T3 fibroblasts, human glioma cells, and capillary endothelial cells (reviewed in (Gschwendt, 1999)). Studies using ectopic PKCδ implicate p27 as part of the cellular arrest phenotype (Ashton et al., 1999). PKCδ also assists in the sustained up-regulation of p21 message and protein levels in a p53-independent manner (Zezula et al., 1997). However, PKCδ is also capable of phosphorylating and activating p53 in response to genotoxic stress (Johnson et al., 2002; Yoshida et al., 2006). The activity of PKCδ is substantially higher in senescent cells, and has been shown to hyper-phosphorylate SRF, which prevents it from binding to DNA and transactivating the immediate early genes *egr-1* and *c-fos* (Wheaton and Riabowol, 2004). Similarly, increased PKCδ activity during senescence was shown to indirectly inactivate WARTS, a kinase required to exit cytokinesis, and arrest cells after nuclear fission but not before division (Takahashi et al., 2006). A subsequent report also established that constitutively active PKCδ can induce senescent phenotype when introduced to young fibroblasts (Katakura et al., 2009). Interestingly, the activation of PKCδ by PMA is known to be localized to caveolae in cardiomyocytes (Rybin

et al., 2008). PKCδ has also been found in the caveolar membrane microdomains of human fibroblasts (Wheaton, 2002). The high activity of PKCδ in senescence may be due to the loss of Src activity in caveolae, which normally inactivates PKCδ through degradation (Blake et al., 1999).

2.3 Phospholipase D activity during senescence

Phospholipase D (PLD) activity is required for an integrated mitogenic response from a variety of receptors including RTKs (reviewed in (Exton, 1998)). PLD catalyses the hydrolysis of phosphatidylcholine (PC) to yield phosphatidic acid (PA) and choline. PA is an important second messenger in many biological processes (Exton, 1997) such as in a membrane localization of Raf-1 (Ghosh et al., 1996). PA can be modified further to the mitogenic lyso-PA by phospholipase A2 or to DAG by PA phosphohydrolase (McDermott et al., 2004). Activation of PLD is complex, involving phosphatidylinositol 4,5-bisphosphate (PIP$_2$), PKCα/β, Rho family GTPases and ARF GTPase (Exton, 1999). PLD activity has been correlated with mitogenic activity (reviewed in (Foster and Xu, 2003)) and has been shown to be reduced in senescent cells (Venable et al., 1994), possibly due to the reduction of PKC activity or inhibition by ceramide (Venable and Obeid, 1999).

Ceramide acts as a second messenger in a variety of biological processes after being generated from membrane associated sphingomyelin by neutral sphingomyelinase (reviewed in (Foster and Xu, 2003)). The ceramide levels remain stable with fibroblast age, but rise 2-4 fold relative to young cells upon the onset of senescence and are accompanied by an increase in the neutral Mg^{2+} dependent sphingomyelinase activity. The addition of a synthetic, cell permeable analog of ceramide to young cells led to growth arrest and cellular senescence (Venable et al., 1995). These cells showed cellular arrest, lack of Rb phosphorylation, no AP-1 production (Venable et al., 1995) and stained positive with the β-gal test for senescence (Mouton and Venable, 2000). Ceramide acts to inhibit the activation of PLD, by possibly three mechanisms, inhibiting activation by PKC, translocation to the membrane and gene expression, but none of these has been clearly defined (Exton, 1999). Exogenous ceramide inhibits PLD activity in primary fibroblasts (Venable et al., 1994) and this inhibition was shown to depend on PKC interaction but not on phosphorylation or translocation in cell free systems (Venable et al., 1996). However, ceramide has been shown to disrupt the liquid order of lipid rafts, which leads to PLD inhibition (Gidwani et al., 2003). To this end, recent work has identified that the deficiency in PLD activity is specific to the senescent membrane fractions, and not to changes in the cytosol (Webb et al., 2010). The increase in ceramide in senescent membranes likely changes the properties of lipid rafts, which no longer supports an integrated PLD response. Thus, the elevated ceramide levels and PKC regulation are important factors in reduced PLD activity in senescent cell membranes.

2.4 The phosphoinositide 3-kinase pathway during senescence

Due to the role of IGF-1 in the optimal proliferative response of primary fibroblasts (Phillips and Cristofalo, 1988), a common downstream effector of this pathway, phosphoinositide 3-kinase (PI3K) was assessed for its role during senescence. PI3K consists of a p85 regulatory subunit and a p110 catalytic subunit which selectively phosphorylates the 3-OH position of

phophoinositides. The enzyme is implicated in both mitogenic and survival signals which are propagated through down stream effectors such as PKB/Akt, IRS-1 and p70 S6K (reviewed in (Vanhaesebroeck et al., 1997). Application of a specific PI3K inhibitor (LY294002) to young primary fibroblasts resulted in cell arrest and the senescent phenotype. Conversely, in the same study, inactivation of the Ras/Raf/MAPK pathway by PD98059 also arrested cell growth, but no induction of senescence was noted (Tresini et al., 1998). Another group using the same approach showed that p27 was highly up-regulated, but that the presumed mediators of senescence, p21, p16, and p53 were all down-regulated. They additionally showed that p27 was the only mediator of the response, since p27-/- mouse embryonic fibroblasts did not become senescent with application of the drug (Collado et al., 2000). Overall, the role of PI3K seems to be in maintaining continued growth, and its inhibition leads to senescence through a non-traditional mechanism of p27 up-regulation.

One of the main effectors of the PI3K pathway is the mammalian target of rapamycin (mTOR), a kinase that integrates signals from multiple mitogenic pathways. The mTOR protein is activated by PI3K through the pathway of PKB/Akt leading to TSC1/TSC2 inactivation, which normally acts as a guainine exchange protein for the small GTPase Rheb. Rheb-GDP holds mTOR in an inactive state, until it is activated through PI3K-Akt-TSC1/2, allowing GTP exchange (Li et al. 2004). The increased mTOR activity has a global effect on translation and cell growth, which is required for rapid cell proliferation (Guertin and Sabatini, 2007). However, it has been shown that serum mitogens are required for the full development of the senescent phenotype (Satyanarayana et al., 2004). The mitogen based induction of senescence has been shown to involve the activation of mTOR, because its inactivation by rapamycin prevents senescence, even in the presence of DNA damage (Leontieva and Blagosklonny, 2010). Recently, treatment with rapamycin has even been shown to extend the proliferative lifespan of human fibroblasts (Cao et al., 2011). These effects were shown to depend on increased autophagic degradation of proteins that enforce senescence, but may also involve a disconnection between mitogen induced translational control and promotion of the cell cycle. Thus, the roles of PI3K and mTOR in senescence would seem to oppose each other, even though they are in the same pathway. These divergent results are difficult to reconcile, but possibly can be explained by other downstream PI3K targets that promote proliferation over senescence. This is supported by the finding that transcriptional targets of the PI3K/PKB are not properly regulated in replicative senescence (Lorenzini et al., 2002). Conversely, in stress or oncogenic induced senescence the PI3K pathway has been shown to have a positive role in the onset of senescence (Binet et al., 2009; Matuoka et al., 2003), consistent with a role for mTOR.

3. P53 and telomere attrition

Replicative senescence is widely accepted to be triggered by critically short telomeres. Stress-induced senescence (also called premature senescence) occurs when cells are exposed to sub-lethal cellular stress such as DNA damage (Di Leonardo et al., 1994; Resnick-Silverman et al., 1998), oxidative stress (Chen and Ames, 1994), or oncogenic stress (Serrano et al., 1997). Both replicative and stress-induced senescence result in the accumulation of cells that are incapable of further divisions and arrest primarily in the G1 phase of the cell cycle. Thus, senescence is thought to represent an intrinsic barrier to oncogenesis by limiting proliferation.

The p53 tumour suppressor protein is a transcription factor that under normal cellular conditions is unstable and inactive. In response to many forms of cellular stress, p53 becomes activated by post-translational modification and is able to transactivate target genes that regulate diverse cellular processes including, cell cycle progression, senescence, DNA repair, metabolism and cell survival (Appella and Anderson, 2001; Vousden and Lane, 2007). Active p53 protein promotes cell cycle arrest through transcriptional activation of many genes including: *p21WAF1* (el-Deiry et al., 1993), *GADD45α* (Kastan et al., 1992), *BTG2* (Rouault et al., 1996), *REPRIMO* (Ohki et al., 2000), *14-3-3σ* (Hermeking et al., 1997), and Prl-3 (Basak et al., 2008). Different subsets of p53-responsive genes regulate DNA repair, metabolism, survival and apoptosis. The p53 protein has been implicated as one of the key mediators of both the onset (Hara et al., 1991; Shay et al., 1991) and maintenance (Beausejour et al., 2003; Gire and Wynford-Thomas, 1998) of cellular senescence. The transcriptional targets of p53 shown to initiate replicative senescence include *p21WAF1* (Brown et al., 1997; el-Deiry et al., 1993; Noda et al., 1994), BTG2 (Wheaton et al., 2010), GADD45α (Jackson and Pereira-Smith, 2006) and PAI (Kortlever et al., 2006). When p53 is knocked down by shRNA (or other method) in fibroblasts it extends the proliferative lifespan by 10-20 population doublings, by avoiding p53 induced senescent program (Fig. 1B).

Various chemical agents and gamma irradiation can cause DNA double stranded breaks, which also lead to premature senescence. The biological process that occurs at a doubled stranded break involves hundreds of H2AX histones becoming phosphorylated (γH2AX) as the signal propagates from the break site. This DNA damage can visualized in the nucleus of a cell as foci by immunofluorescence using a γH2AX phospho-specific antibody (Rogakou et al., 1999; Rogakou et al., 1998). The kinase Ataxia telangiectasia mutated (ATM) acts as a sensor of DNA damage, becomes activated at the γH2AX foci and phosphorylates p53 (Bakkenist and Kastan, 2003). This phosphorylation stabilizes and activates p53, and allows it to transactivate its effector genes.

Telomeres consist of a tandem repeat of 6 nucleotides (TTAGGG) that cap the ends of linear double stranded DNA in mammals. Human telomeres consist of 5-10 kilobase pairs at the end of every chromosome arm and have an overhang of single-stranded telomeric repeat of several hundred bases. If ends of chromosomes were not protected by telomeres the cell would detect the end of the DNA as a double stranded break and initiate a DNA damage response. Telomeres normally protect the double stranded chromosome ends by the formation of telomere-loops (t-loops) in which the telomeric single-stranded overhang hybridizes within the double-stranded telomeric region of a chromosome. T-loops are maintained and stabilized by a complex of proteins referred to as shelterin (de Lange, 1994). Telomeres are know to shorten with each round of DNA replication because of the "end-replication problem"; the inability of DNA polymerases to completely replicate the 3' end of linear DNA molecules (Harley et al., 1990). Telomeres will shorten to a critical length after a finite number of DNA replication cycles, and lose the ability to form the sheltern/t-loop cap at the ends of chromosomes. The DNA damage signal arising from eroded telomeres will activate ATM, leading in turn to p53 activation (Herbig et al., 2004). Thus, t-loop disruption as a consequence of telomere shortening exposes telomeric DNA that leads to p53 activation and the initiation of replicative senescence (Herbig et al., 2004; Karlseder et al., 2002; Li et al., 2003; Li et al., 2004; Stewart et al., 2003). This model is supported by studies that ectopically expressed telomerase (hTERT) allowing fibroblasts to bypass senescence (Figure 1B) and become immortalized by preventing telomere shortening (Bodnar et al., 1998; Vaziri and

Benchimol, 1998). Hence, critically short telomeres provide a physiological signal for activation of the p53-dependent replicative senescence program in human cells.

4. Caveolae

Caveolae are flask shaped invaginations which form at the plasma membrane from lateral assemblies of cholesterol and sphingolipids known as lipid rafts (reviewed in (Harder and Simons, 1997)). Lipid rafts are composed of sphingomyelin, glycosphingolipids and cholesterol which form microdomain rafts surrounded by the phosphatidylcholine rich plasma membrane. These rafts sequester a specific subset of transmembrane associated proteins (Simons and Ikonen, 1997). Thus, lipid rafts are organelle like environments which have been postulated to have specific functions in various cell types (Simons and Toomre, 2000). Lipid rafts are also found to form higher order structures, caveolae (little caves), when associated with the scaffolding transmembrane proteins (Figure 2A & B). Caveolae are distinct from other vesicle-like invaginations, such as clathrin coated pits, due to their small size and lipid raft composition (Kurzchalia and Parton, 1999). The catalyst of caveolar formation is the oligomerization of a class of sphingolipid and cholesterol binding proteins known as caveolins (Monier et al., 1996), which stabilize and induce the lipid rafts to coalesce (reviewed in (Harder and Simons, 1997)). Three caveolin genes have been identified encoding caveolin proteins 1, 2, and 3 (Glenney, 1992; Kurzchalia et al., 1992; Scherer et al., 1996; Tang et al., 1996 ; Way and Parton, 1996). Both the N- and C-termini of caveolins are exposed to the cytoplasm forming a hairpin structure in the membrane, and are palmitoylated (Dietzen et al., 1995). Caveolin monomers oligomerize into hetero- and homo-dimers and trimers as they are translated in the cytoplasm (Lisanti et al., 1993), traffic through the trans-Golgi network and associate at the plasma membrane (reviewed in (Rietveld and Simons, 1998)). Due to their unique lipid raft composition caveolae can be biochemically purified based on their insolubility in the detergent Triton X-100 as well as on their low buoyant density on sucrose gradients (reviewed in (Anderson, 1998)). The insolubility of caveolae is likely due to the enrichment in sphingolipids and cholesterol in contrast to the glycerophospholipid rich plasma membrane (Hanada et al., 1995).

Recent advances in the characterization of lipid rafts have identified a new class of proteins which are essential to the biogenesis and regulation of caveolae; cavins 1 to 4. Cavin 1 (or polymerase I and transcript release factor: PTRF) has been shown to be essential for the stability and organization of caveolar structures (Hill et al., 2008; Liu and Pilch, 2008). Cavins 1-4 are part of a heterologous complex which bind to mature caveolin proteins (Figure 2B) that have emerged as caveolae after being processed through the trans-golgi system (Hayer et al., 2010). This interaction is cavin 1 and caveolin dependent and is thought to be due to a cavin phosphatidyl-serine interaction domain in all cavin proteins; a lipid which is enriched in caveolae (Bastiani et al., 2009). Cavin-2 is thought to play a role in the curvature of caveolar structures (Hansen et al., 2009). Cavin-3 is known to be involved in caveolae endocytosis and coordinates internalization on microtubules (McMahon et al., 2009). Cavin-4 is specifically expressed in striated muscle and has been shown to have specific roles related to myogenesis (Tagawa et al., 2008) and contraction (Ogata et al., 2008). Caveolin and cavin expression are interdependent on one another, since the depletion of one leads to the suppression of the other. Thus, cavins have emerged as an essential component of caveolae regulation, stability, and biogenesis.
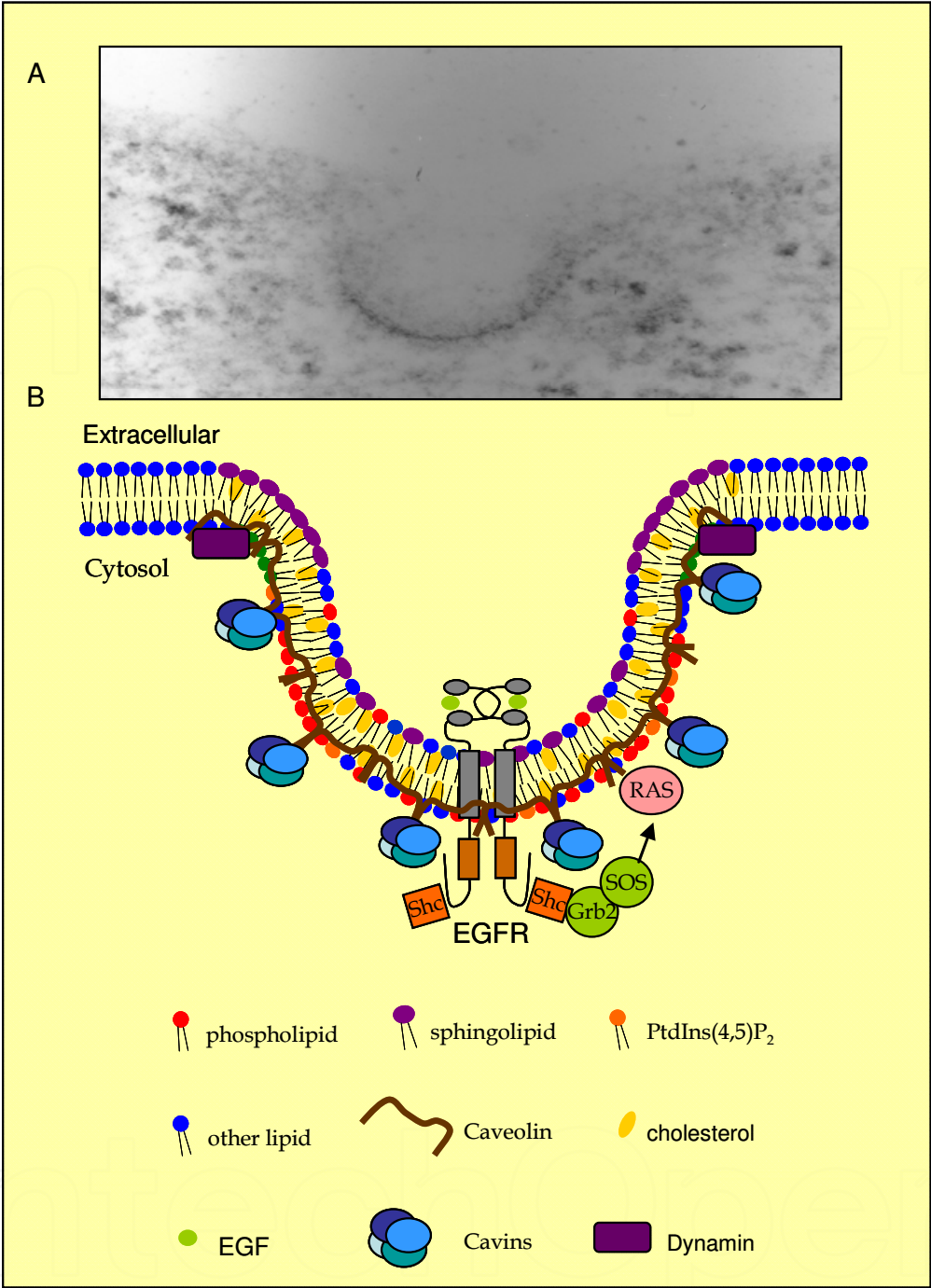
Fig. 2. Caveolae Structure
A) Electron micrograph of a caveolae at the membrane surface of 3T3 L1 fibroblasts.
B) Schematic diagram of the lipid and protein composition of caveolae. Phospholipids and PtdIns(4,5)P2 are concentrated on the inner leaflet, while sphingolipid is found in the outer leaflet of caveolae. Cholesterol is required for the caveolin proteins to bind and create a multimeric scaffold that forms the caveolar structure. Dynamin proteins surround the neck of the caveolae, and play a role in the fission of caveolar vesicles during endocytosis. Many receptor and signalling cascades are compartmentalized in caveolae, including the epidermal growth factor receptor (EGFR), the adaptors Shc, Grb & SOS, and Ras small GTPase. Cavins 1 to 4 bind to caveolin and regulate caveolae biogenesis, shape and trafficking.

Caveolae are a type of endocytic vesicle (much like clathrin coated pits) and can undergo internalization with particular stimuli. The process is dependent on dynamin (a GTPase involved in membrane fission) and cholesterol and leads to the production of enclosed vesicular caveolar structures with in the cytoplasm of the cell (Fig. 3A & B). For example, caveolae can be generated in transformed NIH 3T3 cells when a dominant negative dynamin is introduced, and is blocked in the presence of cholesterol sequestering agent (Li et al., 2001). This implies that even in cells that normally have no detectable caveolae, there is a steady state of caveolar invagination that is stabilized with dominant negative dynamin. Pinched off caveolae can be visualized as self contained vesicles (called cavicles) using electron microscopy (Mundy et al., 2002), and have been shown to fuse with a specialized endosome (that is caveolin positive) called caveosomes (Pelkmans et al., 2002). This pathway has been shown to be utilized by SV-40 virus, cholera toxin and albumin (reviewed in (Lajoie and Nabi, 2007)). Caveolae utilizing this pathway are known as type I (Fig. 3B) and this cavicle trafficking has been shown to depend upon transport along microtubles. These caveolar vesicles form into alternate morphologies in different cell types; in adipocytes, caveolar vesicles cluster into rosettes, and in endothelial cells, the vesicles elongate and fuse to form channels. A second population of caveolae (type 2) are involved in continuous rounds of fusion and invagination (Fig. 3B) but are restricted to remain near the cell membrane by the actin cytoskeleton (Pelkmans and Zerial, 2005). This cycle is highly regulated by various kinases (del Pozo et al., 2005) which can also shift these caveolae into long range type I transport. It is theorized that type 2 caveolae are largely responsible for the potocytosis of vitamins and ions (Anderson et al., 1992). Type 3 caveolae form elongated tubes from the cell surface in certain cell types and are not as well characterized. Several types of caveolae can be seen simultaneously in the same cell lineage (Fig. 3A & B), and often carry out specialized functions that depend on cell type (reviewed in (White and Anderson, 2005)).

In addition to the endocytic transport described above, it has recently been found that the EGFR has a functional role during trafficking through caveolar system. Under normal conditions, activated EGFR is recycled through clathrin coated pit mediated endocytosis and degraded or recycled through endosomes or lysomes respectively (reviewed in (Madshus and Stang, 2009)). However, in circumstances of sustained EGF signalling levels or the presence of oxidative stress or gamma irradiation, the caveolar system transports the EGFR through an endosomal endoplasmic route to the nucleus (reviewed in (Dittmann et al., 2009)). These functions have been shown to be dependent on active EGFR, which is capable of utilizing transport through the nuclear pore and becomes soluble in the nucleoplasm. This localization is thought to be very important in the resolution of DNA damage. To this end, nuclear EGFR has been shown to phosphorylate and activate DNA dependent protein kinase (DNA-PK), an important mediator of non-homologous end joining (Dittmann et al., 2005). In addition, nuclear EGFR has been shown to regulate transcription of cyclin D1, iNOS and B-myb, and regulate PCNA by phosphorylation, all of which correlate with entry into the cell cycle (Dittmann et al., 2009). Many tumours have been reported to have a nuclear population of EGFR, and its presence is linked to continued proliferation. Thus, this cellular transport system is the target of chemotherapy agents that either inactivate EGF signalling by inhibition of src kinase or antibody therapies that promote the uptake of EGFR through caveolae and thus block its activation (Dittmann et al., 2009).
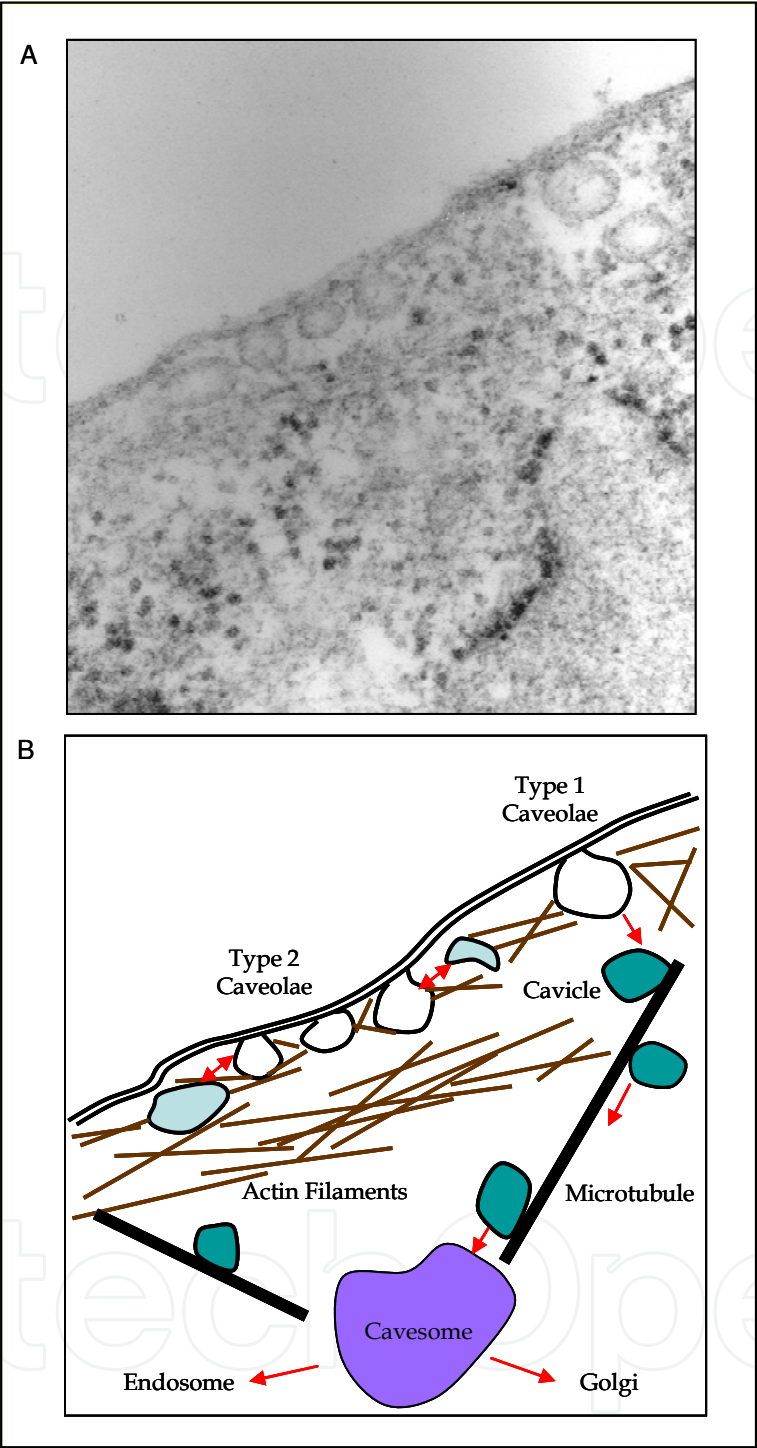
Fig. 3. Caveolar Endocytosis
A) Electron micrograph of caveolar structures at the membrane surface of differentiated 3T3
L1 fibroblasts. **B)** A schematic diagram showing caveolar endocytosis. Type I caveolae
invaginate in response to various stimulae and form caveolar vesicles (cavicles) that are
transported on microtubules. Cavicles can fuse together in cavesomes, where various
contents may be recycled, or are transported to endosomes or the Golgi. Type II caveolae
invaginate, fuse, and transport back to the cell surface. They are confined by the actin
cytoskeleton and are important for potocytosis.

4.1 Cell signalling through caveolae

There is significant evidence that many well-studied receptor signalling systems are localized and operate through caveolae. Caveolin protein has been shown to directly interact with many of these signalling molecules through a 20 amino acid domain known as the caveolin scaffolding domain (CSD: (Couet et al., 1997)). Interaction with this domain has been shown to hold the signalling molecules in an inactive conformation (Okamoto et al., 1998). The pepitide sequence is rich in aromatic amino acids and has been shown to bind to adenylyl cyclase (AC), heterotrimeric Gα and Gβγ, PI3K, endothelial nitric oxide synthase (eNOS), protein kinase A (PKA), PKC, ERK1/2 and Src family kinases. In each case, the CSD is associated with the inactivation of the particular pathway (reviewed in (Patel et al., 2008)). However, since the CSD also binds lipids, there is the possibility that there are two populations of caveolin, one integral with rafts and another associated with the membrane that is not part of caveolae (Parton and Simons, 2007). Alternatively, the CSD has been conjectured to release the signalling proteins it binds to upon conformational change with activation (Okamoto et al., 1998). Although the exact role of the CSD is controversial, it is clear that it is involved in regulating and possibly sequestering signalling molecules to the caveolae. The inhibitory signalling associated with caveolin proteins has been challenged by many studies which show a requirement for Caveolin 1 in activating signal cascades (Kurzchalia and Parton, 1999, White, 2005 #707). This theory is based on the caveolin driven generation of caveolae, which acts to compartmentalize many signalling cascades. Thus, the caveolar structures and caveolin protein act as a center of cross talk and integration of mitogenic responses in normal cycling cells.

Many proteins have been found to localize to caveolae either through direct interaction with caveolins or sequestered through the properties of the lipid microdomains (Zajchowski and Robbins, 2002). Localization of proteins to caveolae is also enhanced by palmitoylation. A significant number of these are membrane bound receptors and kinase cascades with their adaptor proteins. Lipid moieties in caveolae are often utilized as second messengers (figure 2B) or for docking to plasma membrane by signalling proteins. This allows caveolae to regulate the activation of signalling cascades by concentration or proximity of substrates and second messengers (Cohen et al., 2004). Many receptor signalling systems are localized to and operate through caveolae and include; Src family kinases, nitric oxide synthase, EGFR, PDGFR, PLCγ, PLD, PKCα & β, Ras, trimeric G-proteins, MEK and Erk2, among others. Some of these proteins can also be found to be concentrated in lipid rafts (reviewed in (de Laurentiis et al., 2007)).

The most thoroughly characterized RTK residing in caveolae is EGFR, which is also crucial to mitogenic reposnse in human fibroblasts. Whether the localization of EGFR in caveolae has a negative or positive role in EGFR signalling is controversial. There is strong evidence for both, and it may be difficult to reconcile the observations definitively. The first evidence of the MAPK pathway in caveolae found H-Ras, Grb2 and SOS in Rat-1 cells (Mineo et al., 1996). The EGFR was present in the caveolin fraction in unstimulated Rat-1 cells, but declined rapidly after stimulation by EGF. The same migration was found in human fibroblasts and depended on EGF engagement, active EGFR tyrosine kinase, and phosphorylation of the EGFR receptor (Mineo et al., 1999). These observations implied that the activation of EGFR occurred within the caveolae, and is required to migrate in order to attenuate the EGF response. This was supported by the finding that some common mutations in EGFR that are oncogenic are also incapable of migrating from caveolae when stimulated.

In addition to the MAPK pathway, EGFR has been shown to activate other downstream effectors such as phospholipase Cγ (PLCγ) through PI3K (Jang et al., 2001) and PLD1 (Han et al., 2002)) in caveolae. PLCγ hydrolyses phosphatidylinositol- 4,5-bisphosphate (PIP_2) to produce diacylglycerol and inositol 1,3,5,-trisphosphate (IP_3), which activates PKC and mobilizes intracellular calcium respectively. PLD catalyses the hydrolysis of phosphatidylcholine to yield phosphatidic acid. These second messengers lead to the activation of Akt and mTOR which promote cellular proliferation (reviewed in (de Laurentiis et al., 2007)).

5. Caveolae and cavicles in senescence

The first report examining caveolin protein in replicative senescence (Park et al., 2000) found that the protein was highly elevated as human fibroblasts reached high population doublings. This upregulation was correlated to an increased number of caveolar structures seen in the EM of senescent cells, which were thought to be caveolae. Additionally, the group found EGFR signalling was attenuated in senescence through the binding to caveolin. However, these studies did not address whether caveolin generated bona-fide functional caveolar structures or whether it represented a differential regulation of caveolar pathway. Unfortunately, the EM chosen to examine the senescent cells did not have a clear cross section of the outer membrane, and it was unclear whether the structures being scored were caveolae or cavicles. The paradigm then (as now) was that increased caveolin drives the formation of caveolae and the regulation of cavicle generation had not yet been fully elucidated. This lead to the interpretation that the number of caveolae increase in senescent cells. Additionally, the low numbers of caveolae counted in low passage fibroblasts was in direct contrast to what had been previously seen in human fibroblasts by EM (Rothberg et al., 1992) or by freeze fracture EM (Fujimoto et al., 2000). Since this initial report, other groups have visualized a similar elevation of caveolar structures, which appear to be vesicles, and not classical caveolae in senescent cells (Bai et al., 2011; Volonte and Galbiati, 2011). Figure 3A shows structures consistent with caveolae in differentiated 3T3 L1 fibroblasts. Note the flask like invaginations within the outer membrane and the opening to the extracellular space through a smaller pore like neck. Thus, classically defined caveolae do not increase during senescence, and potentially are lost, while cavicles consistent with increased endocytosis have been seen in all senescent cells examined so far (Figure 3A & B). Although our own work examining caveolae (Wheaton and Riabowol, 2004) originally appeared to radically differ from other published reports, it does support the idea of increased caveolar vesicles in senescent fibroblasts. A comparison of young and senescent cells indicated that senescent cells contained a higher total amount of caveolins 1 and 2, but had significantly less of both proteins in the caveolar fraction obtained by sucrose density centrifugation. Additionally, caveolar fractions from senescent cells completely lacked the tyrosine kinase activity associated with functional caveolae and the EGF response. Furthermore, old cells had little caveolar protein exposed to the outer plasma membrane as estimated by an *in vivo* biotinoylation assay and there was no caveolin 1 detected on the cell surface using immunofluorescence (Fig. 4A & B) and confocal microscopy. Together, these data suggest that a fundamental loss of signal integration at the plasma membrane of senescent cells is due to the loss of signalling competent caveolae. However, they do not rule out the possibility that increased caveolar vesicles are present within the cytoplasm, as has been conjectured above. In fact, the poor response of EGFR and tyrosine phosphorylation of

its targets in caveolae could be due the separation of these structures from the extracellular space. Therefore, the increase in caveolar structures in senescence may represent a misregulation of the normal functional caveolae during the aging process. This implies that some part of the endocytic process is blocked, leading to accumulation and stabilization of cavicles during senescence. This is supported by the finding that endocytosis is reduced in senescent cells and is correlated with an upregulation of caveolin 1 (Park et al., 2002).
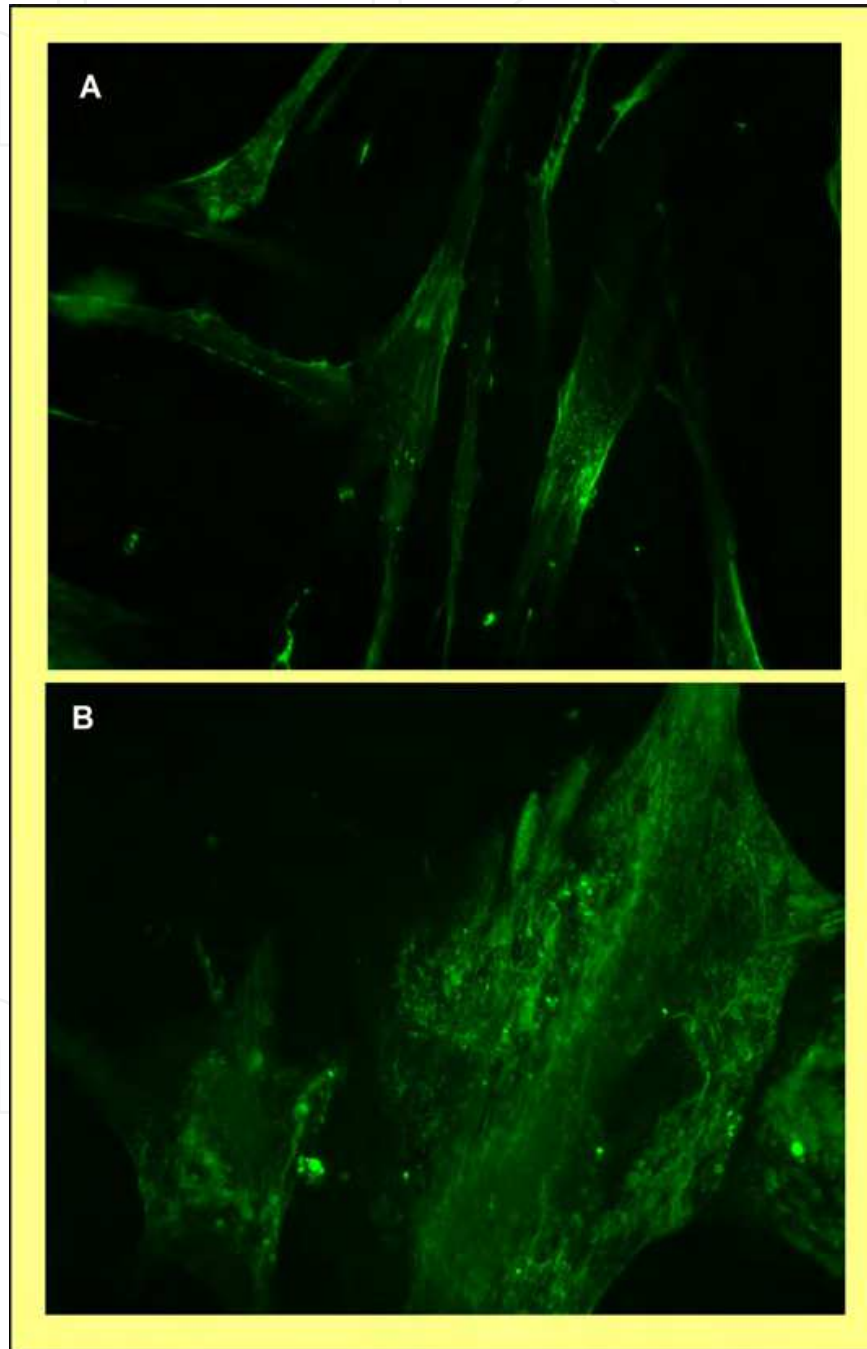

Fig. 4. Caveolin-1 Localization in Young and Senescent Fibroblasts
Young (A) and senescent (B) Hs68 fibroblasts were immuno-stained with anti-caveolin-1 and Alexa 488 flourescent secondary antibodies. Caveolin-1 is concentrated at the cell periphery in young cells (A) and is largely internalized in senescent cells (B).

These data suggest a model in which senescent cells are unable to organize and localize the components of functional caveolae to the plasma membrane. The pinched off caveolar vesicles observed in senescent cells may represent a unique misregulation of caveolae that could explain both the functional differences and the increase in caveolar structures seen previously (Wheaton, 2011). Thus, although increased expression of cavin-1 and caveolin proteins drive the biogenesis of caveolar structures, they unbalance the normal equilibrium between cavicles and cavaolae. This unbalance could promote invagination, but stall cavicle migration at some point in their migration along microtubles before they fuse with caveosomes or endosomes. Consistent with this idea, caveolin 1 staining is found at the cell periphery in young cells, but not at the membrane of senescent cells. However, there is very intense staining of caveolin within the interior of senescent cells (Fig. 4A & B). Using confocal microscopy with a deconvolution algorithm found intense caveolin-1 staining in senescent cells that was associated with fibre like structures (Fig. 5B). Confocal analysis of young cell caveolin -1 found it concentrated in regions of the cell periphery (Fig. 5A). This pattern of fibres is consistent with the caveolae associating with microtubules, and becoming stalled after internalization. The increased number of cavicles could represent a natural response to stress, in which cell arrest is maintained by sequestering key signalling receptors away from their ligands in the extracellular space. This would have a similar effect to EGFR antibody based chemotherapies which drive internalization of the EGFR through caveolae, but prevent its localization to the nucleus and activation (Dittmann et al., 2009). Considering internalization of EGFR through caveolae is a response to gentoxic agents, it is possible that DNA damage downstream of telomere attrition may do the same. Thus, the cell may respond to stress by down regulating mitogen activated cascades through internalization of caveolae. Recent studies in which caveolae are found to be redistributed by internalization during mitosis support this idea (Boucrot et al., 2011) . The caveolae in this case internalize through microtubules and could be a mechanism to block mitogenic signals after the commitment to divide.

5.1 Physical changes in senescent membranes and lipids

It is possible that some of the changes seen in caveolar structures can be explained by the unique properties and composition of the plasma membrane of old cells (Rutter et al., 1996; Schroeder et al., 1984). Rafts are comprised of a high concentration of sphingolipids and cholesterol, which have strong cohesive forces that counteract the entropic force inherent in a fluid mosaic membrane (reviewed in (Harder and Simons, 1997)). Examining senescent cells using proton magnetic resonance has shown that the ratio of cholesterol/ phosphatidylcholine increases as human fibroblasts age in culture, indicating an increased amount of mobile cholesterol (Rutter et al., 1996). Another early report sought to probe the lipid composition of these fibroblasts by using fluorescent probes. These labelled lipids can be used to determine the limiting anisotropy (fluidity) of membranes and showed that the microsomal fraction increased in fluidity with donor age (Schroeder et al., 1984). Lipid composition changes during fibroblast aging lead to higher lipid fluidity and may reflect the inability of significant raft domains to form with age, since rafts by nature are islands of less fluid lipid. This is in part is due to the levels of a species of phosphatidylcholine comprised of stearic acid and arachidonic acid becoming elevated in senescent fibroblasts (Naru et al., 2008). This dilutes the components of lipid rafts in the membrane of senescent cells.
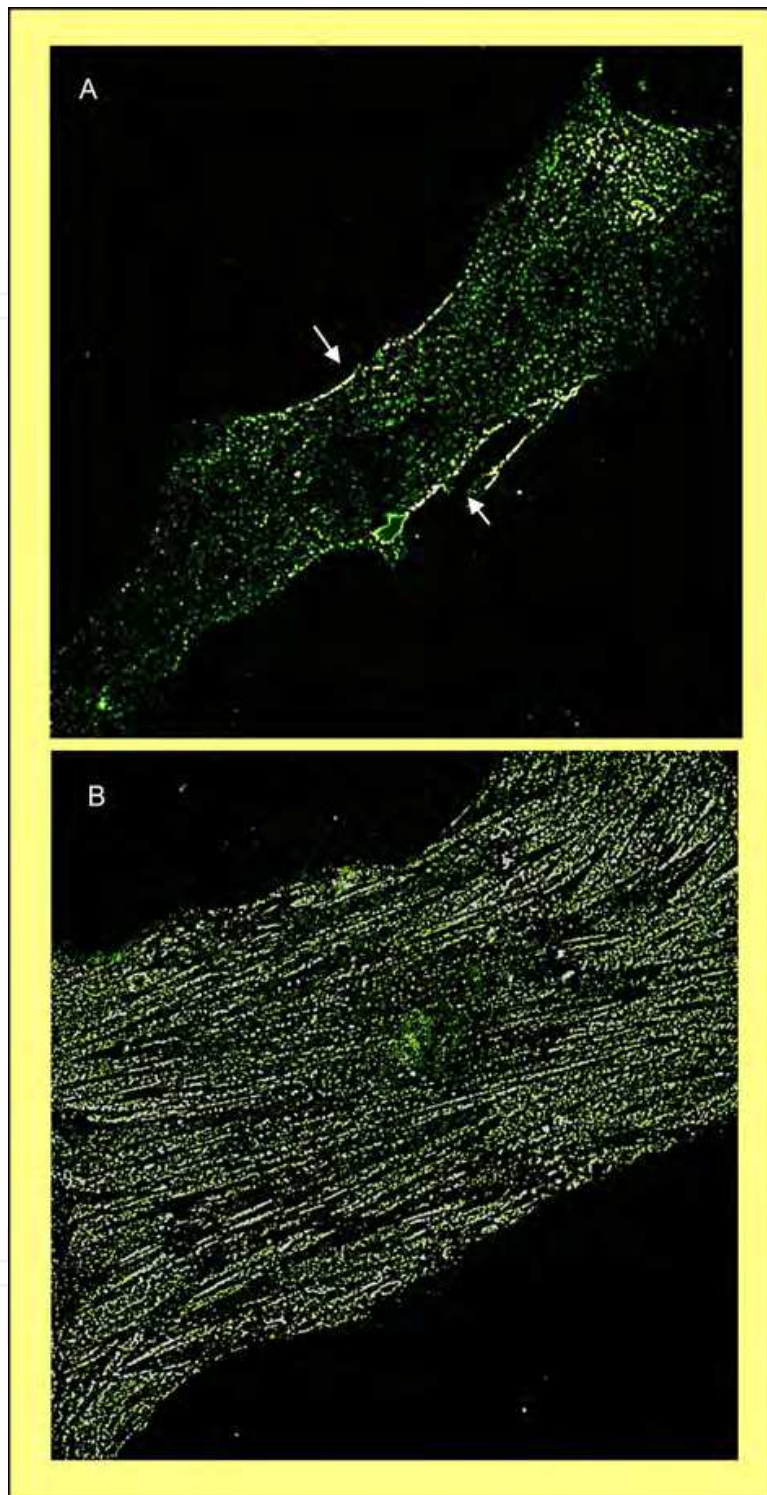
Fig. 5. Confocal Microscopy of Caveolin-1 Staining in Young and Senescent Fibroblasts. Young (A) and senescent (B) Hs68 fibroblasts were immuno-stained with anit-caveolin-1 and Alexa 488 fluorescent secondary antibodies. The images show a single confocal layer that was filtered through a de-convolution protocol to show the localization in sharper detail. The young cells have a high concentration of caveolin at the cell periphery, indicated by arrows. In senescent cells, caveolin is concentrated through out the cell on filaments consistent with microtubules.

In addition, senescent cell membranes are characterized by the specific loss of cholesterol from the microsomal fraction (Nakamura et al., 2003), even if the overall cholesterol may rise with age (Park et al., 2000). As previously mentioned, cholerterol is essential for lipid raft and caveolae formation (Fig. 2B). The altered cholesterol in the membrane also likely affects various signal transduction pathways (Fielding and Fielding, 2004). Alteration of the raft composition or dynamics may interfere with raft coalescence or its ability to sequester caveolin proteins. The loss of caveolae at the surface of the plasma membrane in old cells supports this model, since it would indicate that the proteins are unable to associate with rafts due to altered dynamics or composition, and subsequently they are not transported to the cell surface.

5.2 Upregulation of caveolin proteins and its effect on caveolar signalling

Key signalling pathways that operate through caveolar structures which are inactivated or misregulated could lead to aspects of the senescent phenotype. Since many growth receptors have been found to be localized within, and to signal through caveolar structures, it is possible that one or more of these may modulate the mitogenic response in primary fibroblasts. Many signalling pathways shown to be altered in senescence, including MAPK, PI3K, PLD and PKCδ (reviewed in (Caino et al., 2009)) are localized to caveolae. Since a significant post receptor block of mitogenic signalling is associated with the development of senescence, the existence of microdomains where receptors and signalling cascades are believed to be linked offers a promising functional connection to these observations. Indeed, the absence of a membrane population of caveolae may prevent mitogenic signal propagation. Thus, the post receptor block could be the result of receptors being either uncoupled from internalized caveolae or sequestered from access to ligands in the extracellular space. These kinase cascades would subsequently be unable to maintain integrated downstream responses to ligand and contribute to senescent arrest. Alternatively, the increases in caveolin protein itself may lead directly to the attenuation of many kinase cascades by virtue of the tumour suppressive function of CSD binding to key components of the cascade. Thus, a mechanism linking the post receptor block to caveolae is either through misregulated caveolar internalization or the inhibitory CSD of caveolin, both of which are characterized by increased caveolin-1 protein expression.

The initial observation linking caveolae to repressed signal propagation in senescence noted that EGF signalling was attenuated (Park et al., 2000). These first studies observed an increase in caveolin-1 & 2 proteins, and therefore the CSD domain (Park et al., 2000; Wheaton et al., 2001). Consistent with this observation, ectopic caveolin 1 expression in fibroblasts was shown to induce premature senescence and thus lead directly to cellular arrest (Volonte et al., 2002). This was further supported by the suppression of caveolin-1 using small inferring RNA or antisense oligos in senescent fibroblasts. Knockdown of caveolin in fibroblasts restores the response to EGF and the cells are capable of entering the cell cycle (Cho et al., 2003). It is thought that this response works primarily through the interaction of the caveolin CSD with erk1/2, leading to attenuation of kinase activity (Engelman et al., 1998). Supporting this, the erk1/2 kinase has been previously reported to be misregulated during senescence (see section 2.1).

Oxidative stress has been shown to cause irreversible growth arrest in NIH 3T3 cells and is dependent on the upregulation of caveolin-1 (Volonte et al., 2002). Such stress also leads to

tyrosine phosphorylation of caveolin-1 which depends on p38 and Src (Volonte et al., 2001) and drives the internalization of caveolae. The same stress (H_2O_2) was shown to cause premature senescence in human fibroblasts, with a relocalization of caveolin to the cytoplasm and nucleus that required p38 activity (Chretien et al., 2008). Thus stress induced senescence appears to upregulate caveolin and redistribute the protein (and likely caveolar structures). This is similar to what is seen in replicative senescence where caveolin protein is found in the cytoplasm (Wheaton et al., 2001) or when cavicles accumulate (Bai et al., 2011; Park et al., 2000; Volonte and Galbiati, 2011). Thus, p38 activated by stress may drive internalization of caveolae and causes receptor tyrosine receptor signalling to be attenuated.

Senescent cells have a flattened and enlarged morphology that is characterized by an increase in focal adhesions (Cho et al., 2004) and which lead to increased amount of actin stress fibres (Wheaton et al., 1996). Caveolin has also been reported to localize to focal adhesions through integrin and likely plays a role in mitogen signalling during cell adhesion. This interaction appears to be upregulated during senescence since caveolin-1 and paxillin (a focal adhesion marker) co-localize strongly in senescent cells (Cho et al., 2004). Furthermore, when caveolin-1 is knocked down in senescent cells, there is an inhibition of focal adhesion kinase, depolarization of anchored actin and reversion of the cells to a younger morphology (Cho et al., 2004). The origin of these morphological alterations is difficult to verify since caveolin repression affects other aspects of mitogenic signalling. However, it has been shown that breaking cell adhesion contacts influences the internalization of lipid rafts within transformed cells (del Pozo et al., 2004; del Pozo et al., 2005). In addition, the clustering of integrins is known to activate Fyn in a caveolin-1 dependent manner to allow anchorage dependent growth (Wary et al., 1998). Consistent with these studies it has been shown that localization of caveolin-1 to cholesterol enriched microdomains decreases in senescent fibroblasts when they are liberated from the substratum by scraping or trypsin (Inomata et al., 2006). This implies that internalization of lipid rafts occurs in senescent cells when there detached and reflects caveolae misregulation in senescent cells. The absence of caveolae when senescent cells are establishing new focal contacts could represent another way in which mitogenic signals are blocked.

In summary, inappropriate caveolin regulation in senescent cells may contribute to the senescence associated post receptor block in three ways. First, the CSD may bind and neutralize various signalling molecules, and prevent signal propagation. Second, caveolin drives internalization of caveolae which prevents receptors access to ligands. Third, caveolin may no longer be sequestered to lipid rafts preventing the development of caveolae to allow signal integration.

5.3 P53, DNA damage and caveolae

Although the most common interpretation in the literature is that telomere attrition is the origin of DNA damage in replicative senescence, a considerable amount of damage foci (γH2AX) are not localized to telomeres in senescent cells (Sedelnikova et al., 2004). The γH2AX-telomere foci are also dependent on whether these cells are cultured in normoxic (2% O_2) conditions (Herbig et al., 2004). Thus, the possibility exists that other forms of stress cause DNA damage in parallel with telomere erosion, such as elevated reactive oxygen species (ROS) or replication fork collapse. These forms of stress may result from the misregulation of caveolae or the upregulation of caveolin. The upregulation of caveolin

leading to a increase in caveolar vesicles causes both the attenuation of the EGFR signalling (de Laurentiis et al., 2007) and an increase in the activity of the p53 tumour suppressor through elevated ROS (Volonte and Galbiati, 2009a). Both of these mechanisms may lead to DNA damage that is not localized to telomeres. This is also consistent with studies in senescent cells which demonstrate higher p53 activity and p21 expression (see section 3).

Overexpression of caveolin 1 protein has been shown to cause G1 arrest, which is dependent on p21 expression induced by increased p53 activity. Mouse embryonic fibroblasts expressing caveolin-1 have a reduced proliferative lifespan and a senescent morphology (Volonte et al., 2002). The aberrant caveolin-1 levels lead to G0/G1 cell cycle arrest, activation of p53 and upregulation of p21 (Galbiati et al., 2001). This shows that caveolin-1 expression mediates premature senescence through a p53/p21 dependent pathway. This pathway is further enhanced by caveolin 1 mediated inactivation of MDM2 and PP2A-C which act as negative regulators of p53 and ATM, respectively (Bartholomew et al., 2009; Volonte et al., 2009). The MDM2 protein is an ubiquitin ligase which targets p53 for degradation and keeps p53 at basal levels in unstressed conditions. Upon activation by stress p53 is phosphorylated at residues which prevent MDM2 association and in turn stabilizes p53. It has been shown during oxidative stress induced senescence that MDM2 is neutralized by caveolin and stabilizes p53 (Volonte et al., 2009). It is thought that the activation of p53 is usually achieved by the kinase ATM, which is sensor of DNA damage (see section 3). Auto-phosphorylation of ATM occurs when it binds to regions of DNA damage and dissociates as active monomers (Bakkenist and Kastan, 2003). ATM is turned off after repair is completed by the phosphatase PP2A-C. When modeling pulmonary emphysema in murine fibroblasts, it was found that oxidative stress caused the sequestration and neutralization of PP2A-C into caveolar enriched microdomains (Bartholomew et al., 2009). Thus, there appears to be a role for CSD in caveolin for the activation of p53 at many levels. To further demonstrate a role for the CSD in caveolin, it was shown that introduction of the peptide of this domain was able to cause premature senescence by itself (Volonte et al., 2009).

The family of cavin proteins have recently been investigated for their potential role during senescence. Cavin-1 (PTRF) and Cavin-3 (SRBC) both appear to be elevated through protein stabilization during replicative senescence, but not quiescence (Bai et al., 2011; Cong et al., 2006) Additionally, it has been shown that cavin-1 is upregulated during oxidative stress induced premature senescence (Volonte and Galbiati, 2011). The ectopic expression of cavin-1 leads to activation of p53, a decrease in MAPK activity, and induced premature senescence. Interestingly, the cavin-1 expression also induced DNA foci that were visualized by γH2AX and did not colocalize with telomeres. The knockdown of cavin-1 extended proliferative lifespan, and reversed the γH2AX in high passage cells (Bai et al., 2011). Similarly, the knockdown of PTRF prevented oxidative stress from inducing senescence and was shown to prevent the MDM2:caveolin 1 interaction leading to p53 activation (Volonte and Galbiati, 2011). Cavin-1 expression is known to have a direct affect on the levels of caveolin (Hansen and Nichols, 2010) and therefore the upregulation of cavin-1 in these systems likely explains the upregulation of caveolin observed during senescence. Although these two studies focused on PTRF, much of the data can be explained by the elevation of caveolin and caveolar vesicles. What is most significant is the generation of DNA damage seen downstream of caveolar vesicles (Wheaton, 2011).
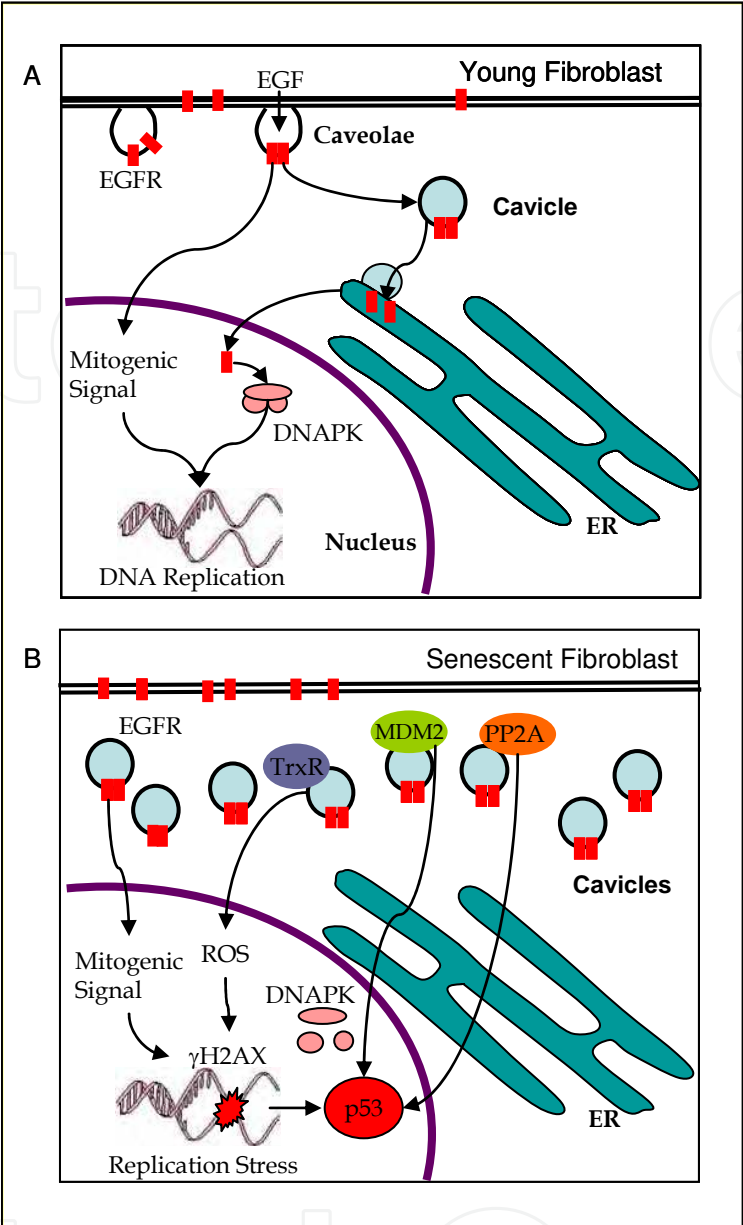
Fig. 6 Model for Cavicle Induced Senescence.
A) In young fibroblasts mitogenic signalling is achieved through a caveolae localized EGFR cascade that activates transcription of growth promoting genes. Signalling competent caveolae are capable of internalizing and traffic to the endoplasmic reticulum (ER) in response to cellular stress. EGFR is transported via cavicles to the ER followed by localization to the nucleus where it can activate DNA protein kinase (DNA-PK). DNA-PK is required to repair DNA damage that is generated by stress or during replication.
B) In senescence fibroblasts mitogenic signalling occurs through EGFR, but is unable to initiate DNA replication. The majority of caveolae are internalized as cavicles, and the increased caveolin protein levels inactivate many signalling molecules or negative regulators of the p53 tumour suppressor (MDM2 and PP2A-C). In theory the EGFR is no longer able to localize to the nucleus and therefore DNA damage generated by reactive oxygen species (ROS), replication stress, and cellular aging cannot be repaired. The activation of p53 through DNA damage causes cellular senescence.

The upregulation of caveolin was shown to block thioredoxin reductase I activity, and thus raise the ROS levels within fibroblasts (Volonte and Galbiati, 2009b). Elevated ROS production is well known to damage DNA, activate p53 and lead to senescence (Chen et al., 1995). Thus, DNA damage could be caused by the presence of ROS being produced in cells that over express caveolin 1 or reach replicative senescence. Furthermore, the negative regulation by caveolin 1 of many key regulatory proteins involved in the p53 mediated DNA damage response could be ensuring that damage signals that lead to a senescent outcome are reinforced.

The transfer of EGFR to the nucleus is well known to influence the resolution of γH2AX damage foci. As previously described (section 4), EGFR is internalized in response to gamma radiation or oxidative stress through a caveolar mediated endocytotic pathway. This pathway leads to EGFR localization to the nucleus and phosphorylates targets such as DNA-PK which facilitate the completion of DNA repair (Dittmann et al., 2005). This function depends on EGFR kinase activity and is blocked by a class of radio-sensitizing agents that work by antagonizing EGFR such as Cetuximab or Gefitinib. Such drugs enhance DNA damage and lead to apoptosis of malignant cells because of unrepaired DNA damage. The EGF signalling pathway is also upstream of the survival kinases MAPK and Akt which assist in the resolution of DNA damage (Golding et al., 2009). Additionally, EGFR activity was found to modulate non-homologous end joining after gamma radiation (Kriegs et al., 2010). Taken together, EGFR signalling plays a major role in controlling cell cycle arrest in response to cellular stress and DNA damage. In support of the role of caveolae in this model, EGFR was found to transport to the nucleus in a caveolin and Src dependent mechanism after oxidative stress (Khan et al., 2006). Similarly, gamma radiation causes Src induced association between caveolin-1 and EGFR leading to internalization. This leads to nuclear localization and to control of DNA-PK activity (Dittmann et al., 2008). Caveolin-1 expression has been demonstrated to be up regulated by ionizing radiation and is required for both homologous recombination and non-homologous end joining (Zhu et al., 2010). In this case, caveolin-1 is pivotal in forming the caveolar vesicles that allow transport of the EGFR, and nuclear DNA-PK activation. Lastly, Gefitinib (an EGFR kinase inhibitor) can generate premature senescence in non-small lung cancer cells (Hotta et al., 2007). Thus, the current evidence strongly suggests mitogenic signals and EGFR internalization to the nucleus are required to resolve DNA damage. Furthermore, blocked EGFR activity leads to apoptosis or senescence.

Although primary fibroblasts are genetically stable, they do undergo transient DNA damage foci as a result of mitogenic stimulation (Ichijima et al., 2005; McManus and Hendzel, 2005). These γH2AX foci occur as a natural part of the synthesis of DNA or division, and are resolved by the time the cell returns to G0 of the cell cycle. Thus, it is possible that a signal from the EGFR could assist cycling cells to resolve this DNA damage. Therefore, EGFR would promote both the initiation of the cell cycle and its continued signalling would be required to resolve DNA damage arising from progress through the cell cycle. However, in senescent cells, the up regulation of caveolin antagonizes EGFR signalling (see section 5.2). Thus, the attenuation of the EGFR pathway during senescence could perpetuate the normally transient DNA damage foci in fibroblasts. The blocking of EGF signalling in this case would prevent the resolution of damage induced by replication stress during normal growth. This could explain the DNA damage seen downstream of increased caveolar vesicles during aging as a form of replication stress (Fig. 6A & B).

6. Conclusion

The inhibition or reduction of components of mitogenic signaling cascades in senescent cell caveolar fractions is consistent with the loss of caveolae playing a causal role in the blunted growth response seen during cellular senescence. Collectively, these observations suggest that localization and integration of signalling cascades in caveolae are disrupted in senescent cells. The mitogenic pathways leading to MAPK, PLD, PKC isoforms and PI3K are all localized to caveolae and have been shown to have deficiencies in senescent cells. In the case of EGFR, the proper coordination with shc is disrupted, the CSD of caveolin blocks kinase activity, and inappropriate retention or internalization in caveolae occurs. It is unclear whether this difference influences the downstream localization of activated erk1/2 to the senescent nucleus, however several groups have noted a decreased erk stimulation by EGF with age. Evidence suggests that the composition of lipid rafts likely change in senescent cells and that this may influence how PI3K, PLD and PKC are regulated. Cholesterol levels are decreased in lipid raft fractions, PC levels increase in the plasma membrane, and ceramide levels increase with fibroblast age. These observations suggest an increase in fluidity of the membrane, which changes the properties of lipid rafts. The increases in ceramide disrupt PLD, and the increase in DAG constitutively activates PKCα in caveolae. The decrease in src kinase activity in caveolae may prevent the inactivation of the PKCδ and induce senescence. The loss of PI3K signal integration through caveolae may be a part of the induction of senescence, explaining why inhibitors of the pathway induce premature senescence. These examples indicate that signal transduction pathways rely upon caveolae for signal integration and propagation that they become disorganized in senescent cells. Collectively, these signalling changes may explain their reduced or absent response to mitogens in senescent cells.

There has been a shift in the understanding of how the DNA damage occurring in senescence is generated. Remarkably, the increase of caveolar vesicles observed in the senescent state can itself lead to the generation of DNA damage foci in parallel with the well known DNA damage localized to eroded telomeres. The exact mechanism by which this is achieved is still unknown, but likely involves the strong inhibitory activities of the scaffolding protein caveolin 1. The proteins theoridoxin, MDM2 and PP2A-C are all inhibited or sequestered by increases in caveolin protein during senescence. Inhibition of theoridoxin leads to an increase in ROS, which leads to DNA damage and p53 activation. The activation of p53 in turn is augmented by inhibition of the negative regulators of p53, MDM2 and PP2A-C by caveolin. The DNA damage that leads to p53 mediated senescence may also be generated through failure to resolve γH2AX foci that occur during normal replication. The increase of caveolin and cavin-1 proteins shift the balance of caveolar structures to internalized cavicles. These cavicles are no longer capable of integrating the EGFR cascade, which is required to transfer EGFR to the nucleus and resolve replication dependent DNA damage through proteins such as DNA-PK. The failure to repair DNA leads to replication stress, the activation of p53 and thus cellular senescence (Fig. 6A & B). Thus, the misregulation of caveolar vesicles interferes with the essential role of these structures in repairing DNA damage.

7. Acknowledgements

8. References

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Senescence

Edited by Dr. Tetsuji Nagata

The book "Senescence" is aimed to describe all the phenomena related to aging and senescence of all forms of life on Earth, i.e. plants, animals and the human beings. The book contains 36 carefully reviewed chapters written by different authors, aiming to describe the aging and senescent changes of living creatures, i.e. plants and animals.

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