A Comparative Analysis of Stress Granule Assembly in Replicative and Stress-Induced Premature Senescence

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ABSTRACT

It has been well-established that primary human cells possess a limited proliferative capacity, undergoing an irreversible cell cycle arrest after a set number of population doublings known as replicative senescence. However, other senescence programmes can also be activated following exposure of cells to subcytotoxic oxidative stress and genotoxic stresses such as ionising radiation. Senescent cells have been linked through a multitude of studies to organismal ageing, and the dysfunction of these senescent cells can negatively affect tissue function through extracellular matrix remodelling and inflammatory protein secretion, resulting in the onset of a number of age-related diseases. Furthermore, it has been previously proposed that this release of inflammatory proteins, termed the Senescence-Associated Secretory Phenotype, may be exacerbated by aberrations in the cellular stress response.

The formation of cytoplasmic aggregates of RNA-binding proteins known as stress granules, which repress translation and modulate signalling pathways to promote cell survival, is a widely established response to cell stress. Stress granules are known to form more readily following stress in replicative senescent cells compared to in proliferating cells, but whether this is true of all senescence programmes remains unknown. Unexpectedly, this study demonstrates that prematurely senescent cells possess a far more limited granule-forming potential than both proliferating and replicative senescent cells, indicating that increased granule formation is not universal to all senescence phenotypes but differs between senescence programmes. In attempting to determine potential mechanisms through which this differential formation could be established it was further discovered that exposure of fibroblasts to X-ray irradiation did not induce the formation of stress granules, and that X-rays ablate the granule-forming capacity of these cells in the hours immediately following exposure. However, whist ultimately not conclusive, a subsequent investigation also suggested that the Wnt signalling pathway was likely not responsible for this loss of granule formation.

This study has therefore discovered two novel circumstances in which stress granule formation is inhibited. Whilst the mechanisms through which this abrogation of stress granule assembly is brought about remain unknown, both of these scenarios occurred following exposure to X-ray irradiation, both in the short- and long-term. Considering a common response to X-ray exposure is the activation of apoptosis, it is possible that stress granule ablation is a response intended to push cells towards cell death over survival. However, further mechanistic studies are required to better elucidate the signalling pathways responsible for this alteration of granule formation before the functional consequences of this granule inhibition can be fully determined.

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ACKNOWLEDGEMENTS

I wasn't quite sure when I began writing these acknowledgements whether I wanted them to be curt and professional, or whether I wanted them to be a heartfelt salute to everyone who has supported me and stood by me over the past year, and beyond. In the interests of procrastination, I have chosen the latter as my acknowledgement of choice.

First and foremost, it is both my duty and my honour to thank my family for everything they have ever done for me. Mum and Dad, I find it difficult to put into words how grateful I am for everything you have ever done for me. You cherished and loved me when I was younger, and supported me in every way you could and knew how. You raised me to be inquisitive, and to question the world around me at every possible opportunity (which I'm sure made me great fun as a child). Without you I would not be here on this Earth, but more than that I would not be sitting here now, finishing my Masters degree, ready to move forward to a PhD. And to Michael, thank you for being so indefatigably caring and unwaveringly loyal as both a brother and a friend. I may not always say it, but I truly appreciate everything you do for me. There is so much I could thank you all for, enough to fill a thesis by itself, so I shall end by saying thank you for being you, and for helping me be me.

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Knowing my luck, the thesis will be deemed unsatisfactory and I'll have to rewrite this in six months with slightly different wording to avoid Turnitin detecting it as plagiarism.

INTRODUCTION

It has been readily established that human primary cells grown in culture maintain only a limited ability to proliferate. Hayflick & Moorhead (1961) were the first to observe changes in the rate of cell division during serial culture passage and determined that, following a logarithmic growth phase in early population doublings, cell division slows and eventually stops; this state of proliferative arrest was later shown to be related to the number of divisions undergone by a cell, and was termed cellular senescence (Hayflick, 1965).

Senescence has since been shown to play important roles in a number of bodily processes, and has a variety of effects on tissues containing senescent cells. A large body of evidence from both in vitro and in vivo sources has determined that this stoppage of cell growth acts as a safeguard against the development of cancer, effectively halting the proliferative capacity of cells before tumorigenesis can occur (Cosme-Blanco et al., 2007; Feldser & Greider, 2007; Prieur & Peeper, 2008). However, in contrast to the apparent benefits of senescence in an anti-oncogenic role, strong correlations have emerged between the presence of senescent cells and the onset of a number of age-related diseases including atherosclerosis (Minamino & Komuro, 2007; Wang & Bennett, 2012) and osteoarthiritis (Price et al., 2002), making studies of senescent cell function clinically relevant. Furthermore, Krtolica & Campisi (2002) have proposed that senescent cells may in fact establish the ageing phenotype due to their deleterious effects on tissue function brought about by secretion of inflammatory factors (Davalos et al., 2010) or through the loss of structural integrity brought about through remodelling of the extracellular matrix by differential transcription of matrix proteins (K. E. Yang et al., 2011) and an upregulation of matrix-degrading enzymes (Benanti et al., 2002). Understanding the altered function of senescent cells is therefore becoming an increasingly important aspect of ageing research, and may lead to novel treatments for age-related diseases.

A subsection of senescence research has focused on understanding how senescent cells respond to external stresses such as oxidative stress. Previous researchers have

observed that, although senescent cells activate apoptotic cell death far less readily in response to genotoxic stress than their proliferative counterparts (Rochette & Brash, 2008; Sanders et al., 2013), senescent cells are less able to respond to stress than young cells (Bruunsgaard & Pedersen, 2000). The first conclusive studies revealing this focused on inflammatory responses of different age groups following exercise, finding that blood plasma of older age groups tended to contain higher levels of the protein Creatine Kinase, associated with a greater level of muscle damage due to a dampened stress response (Cannon et al., 1994). However, subsequent studies have shown corroborating results, with senescent fibroblasts also less able to remove damaged proteins via the proteasome in cells undergoing peroxide-induced oxidative stress (Merker, Sitte, & Grune, 2000), and old hearts less able to recover following periods of stress (Rosenfeldt et al., 2004). Whilst some studies have attempted to elucidate the stress responses employed by senescent cells and how they differ from proliferating cells (Gallouzi, 2009; Lian & Gallouzi, 2009), this field remains poorly understood. The present study sought to further characterise these senescent stress responses through the study of cytoplasmic aggregates of RNA-binding proteins known as stress granules, which form in response to external assaults on cells (Kedersha & Anderson, 2002).

Senesence Programmes

Since the work of Hayflick and Moorhead (1961) established that diploid fibroblasts are only able to undergo a finite number of population doublings prior to the arresting of cell division, many studies have sought to discern the mechanisms through which this growth arrest is established. Considering the first observation of this senescence onset was as a result of serial cell division, the majority of early research focused on alterations undergone by cells at higher population doubling numbers, and therefore the first mechanism proposed and subsequently causally linked to replicative senescence was that of telomeric shortening (Cooke & Smith, 1986; Olovnikov, 1973a), wherein the progressive attrition of chromosomal end repeats dictates the number of times each cell is capable of dividing. Senescence induced due to this shortening of telomeres is known as replicative senescence, and

is the senescent programme most well-linked to organismal ageing (Smith & Kipling, 2004). However, other stimuli were later discovered that could induce a growth arrest with some morphologically and biochemical similarities to replicative senescence, including subcytotoxic oxidative stress, DNA damage, and oncogene activation (Collado, Blasco, & Serrano, 2007).

Replicative Senescence

Telomeres are comprised of tandem repeats at the end of chromosomes, providing an effective buffer zone against loss of genetic information from coding regions of DNA (O'Sullivan & Karlseder, 2010). Telomeres are heavily protected by an array of dedicated proteins known as the Shelterin complex, which ensures that the ends of the DNA strands are not recognised as a double strand break by DNA repair machinery (de Lange, 2005). However, as a consequence of DNA polymerases uniformly traversing strands in a 5' to 3' direction, DNA replication is inherently asymmetric. This results in a fully replicated leading strand but an incomplete lagging strand (Olovnikov, 1973b). Therefore as somatic cells undergo repetitive cell division telomeres progressively shorten and eventually reach a threshold length below which they are no longer protected by telomere-binding proteins. This results in the recognition of the DNA ends as a double strand break and the activation of the DNA Damage Response (DDR) (Reaper, Fagagna, & Jackson, 2004).

A number of DDR-associated proteins, including phosphorylated histone H2AX, 53BP1, and MDC1 subsequently coalesce at these exposed telomeric ends in cells approaching senescence (d'Adda di Fagagna et al., 2003). Considering the role of MDC1 as a regulator of cell cycle checkpoints (Stewart et al., 2003), the persistent DDR that occurs due to critical telomere shortening results in a continuous arrest in G1 or G2 phases (Kim et al., 2012). Further to this MDC1-associated checkpoint control, telomeric strand-break recognition induces activation of ATM and ATR kinases (Bakkenist & Kastan, 2003), which in turn phosphorylate and activate the checkpoint kinases Chk1 and Chk2, leading to activation of the tumour suppressor p53 and proliferative arrest (Herbig et al., 2004).

A second pathway through which shortened telomeres transduce the senescent phenotype lies in the activity of the tumour suppressor Retinoblastoma protein (Rb), a mediator of transcription factor activity for the E2F family (Ikeda, Jakoi, & Nevins, 1996). At the end stages of telomere shortening and usually therefore during late-stage senescence progression, the inhibitor of CDK4 and CDK6 known as $p16^{INK4A}$ (hereinafter referred to as p16) is expressed (Alcorta et al., 1996; Brenner, Stampfer, & Aldaz, 1998). In proliferating cells, CDK4 and CDK6 associate with Cyclin D (Morgan, 1995) in order to phosphorylate Rb, inhibiting binding to E2F transcription factors and maintaining various gene loci in a transcriptionally active euchromatin form to allow cell cycle progression (Korenjak & Brehm, 2005). The expression of p16 results in senescence due to the protein's binding to CDK4 and CDK6 (Shapiro et al., 1995; Shapiro et al., 1998), blocking Cyclin D interaction and thus eliminating their kinase activity. This leaves Rb hypophosphorylated and therefore active, resulting in association with the E2F family, blocking their transcription factor activity and recruiting proteins to condense large chromatin tracts to transcriptionally inactive heterochromatin, inducing a cell cycle arrest and senescence (Narita et al., 2003; Siddiqui et al., 2007). Both signalling pathways have been proven vital for senescent induction to different extents depending on the cell line that is examined (Dannenberg et al., 2000; Dirac & Bernards, 2003), and the protein $p21^{waf1/cip1}$ (hereinafter referred to as p21), activated by p53, provides a method of crosstalk with the p16-Rb pathway by deactivating a separate inhibitor of Rb activity to further suppress E2F activity (Ben-Porath & Weinberg, 2005).

Stress-Induced Premature Senescence

In addition to telomere shortening, senescence can be induced before cells reach their Hayflick limit through exposure to adverse culture conditions, or to sublethal doses of toxic compounds such as hydrogen peroxide which can create oxidative stress within the cell and also cause DNA damage (Chen & Ames, 1994). Oxidative stress is primarily driven by the accumulation of reactive oxygen species within the cell, leading to protein damage, organelle dysfunction, changes in cellular signaling,

and in extreme cases the activation of cell death pathways (Dixon & Stockwell, 2014; Lushchak, 2014). The correlation between high-oxygen environments and cellular senescence was first made by Packer & Fuehr (1977), who found that culturing of fibroblasts in a high-oxygen environment of around 20% resulted in early senescence onset, whereas culturing at concentrations below physiological conditions (2%) resulted in an extended proliferative lifespan. Considering aberrant cellular function, protein damage, and altered metabolism are all pathways which can lead to the onset of tumour growth, stress-induced premature senescence is also considered an anti-tumorigenic response to ensure that oxidative stress does not result in the development of cancer (Collado et al., 2005).

Whilst oxidative stress has previously been implicated in the acceleration of telomeric shortening (von Zglinicki et al., 1995; von Zglinicki, 2002), it is now known that stress-induced premature senescence (SIPS) occurs independently of telomere length, emerging even in cells with long telomeres or rejuvenated telomeres through telomerase activity (de Magalhaes et al., 2004), and also utilises subtly different signalling pathways to achieve a result that is morphologically indistinguishable from replicative senescence (Ben-Porath & Weinberg, 2005). Specifically, although SIPS also appears to utilise the p53-Rb crosstalk signalling pathways for senescence induction of cell cycle arrest upon oxidative stress – evidenced by a greater proportion of p21-expressing cells in high-oxygen culture conditions as opposed to a greater proportion of p16-expressing cells, where p21 acts as a nexus between the p53 and Rb pathways (Itahana et al., 2003) – there is also ample evidence to suggest that oxidative stress can activate a dedicated signalling pathway that feeds into the p16-Rb pathway via the p38-MAPK stress kinase (Debacq-Chainiaux, Boilan et al., 2010; Iwasa, Han, & Ishikawa, 2003), and the p16-Rb pathway was first identified as the key determinant of SIPS progression (Ramirez et al., 2001; Robles & Adami, 1998). The use of shared activation pathways for SIPS and replicative senescence makes sense when considering that oxidative stress has long been established as a genotoxic stress, activating DNA damage repair machinery similar to that used during telomere shortening as a result of DNA strand breaks (Barzilai & Yamamoto, 2004; Honda et al., 2001).

A third broad category of senescence, known as oncogene-induced senescence, also results in a stoppage of the cell cycle, and is induced as a result of the activation of a number of genes which signal, either directly or indirectly through myriad signalling pathways, through to the common p53 and Rb signals discussed above (Courtois-Cox, Jones, & Cichowski, 2008). However, due to the complexity of the signalling pathways involved in oncogene-induced senescence onset (Yaswen & Campisi, 2007), and due to the omission of this senescence programme from the scope of this study, the mechanisms involved in regulation of this senescence model shall not be discussed here. For an adequate review of oncogene-induced senescence, see Kuilman et al. (2010).

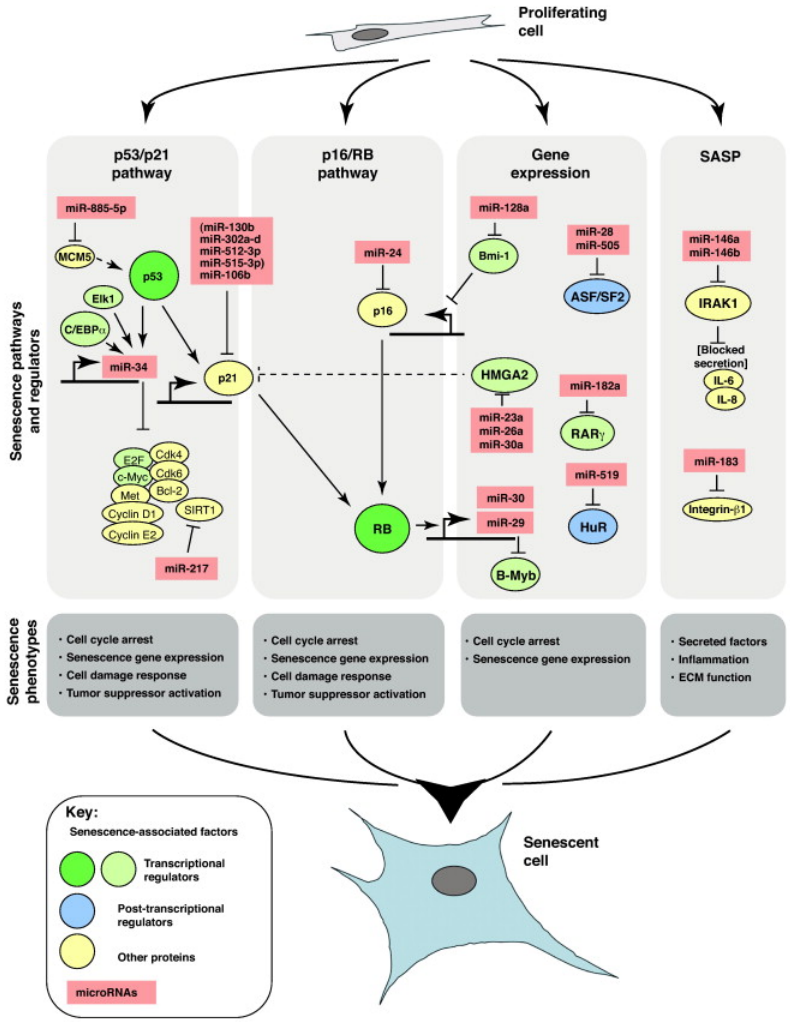

Figure 1. An overview of pathways contributing to senescence onset (Gorospe & Abdelmohsen, 2011).

Stress Granules as Translational Regulators

Following transcription, gene expression can be effectively modulated once mRNA translocation to the cytoplasm has been completed, primarily through the regulation of RNA stability and translation. In order to bring about this regulation, RNA is often localised to dense, cytosolic aggregates of RNA-binding proteins. The first of these granules is known as the P-body, and contains an assortment of proteins associated with RNA degradation, including decapping enzymes which prime RNA for cleavage (van Dijk et al., 2002) as well as ribonucleases such as XRN1 (Bashkirov et al., 1997). P-bodies are also capable of associating closely with, and exchanging mRNAs with,

the second RNA granule known as the stress granule (Wilczynska et al., 2005). Stress granules were first observed following exposure of chicken embryonic fibroblasts to heat stress, containing aggregates of small heat shock proteins (Collier & Schlesinger, 1986). However, stress granules have since been shown to form as a rapid response to a number of other external assaults including oxidative stress (Souquere et al., 2009), hypoxia (van Der Laan et al., 2012) and UV radiation (Moutaoufik et al., 2014). Since their discovery, much of the mRNA and protein composition of these granules has been characterised (Anderson & Kedersha, 2006), with the primary constituents including subunits of stalled preinitiation complexes including the initiation factors eIF2α and eIF4E (Kimball et al., 2003), along with proteins previously established to play roles in RNA stability and metabolism, including G3BP1 (Matsuki et al., 2013; Tourrière et al., 2003), TIA1 and TIAR (Kedersha et al., 1999). The major component of these granules being a precursor to translation complexes is crucial, as this allows for RNAs to be bound and held in a translationally repressed state, and therefore stress granule formation is generally associated with a global suppression of mRNA translation, with the exception of several specific genes associated with stress recovery, which are tightly regulated to allow for their continued translation (Buchan & Parker, 2009). Transcripts encoding genes crucial for cell survival and recovery from stress, such as Hsp70, are excluded from these granules, allowing their translation to continue unimpeded (Kedersha & Anderson, 2002)

The primary mechanism associated with the formation of stress granules is through the phosphorylation of the initiation factor eIF2α by activated stress kinases such as PERK (Teske et al., 2011). This phosphorylation results in the stoppage of eIF2α dissociation from factors which catalyse GTP-GDP exchange during translation (Kedersha & Anderson, 2002). As a result, the level of the ternary complex, required to initiate methionine ribosomal loading, is severely reduced, preventing the assembly of the 48S ribosomal pre-initiation complex (Srivastava, Kumar, & Kaufman, 1998). This stalls complex assembly, and these stalled complexes are shuttled into stress granules through the prion-like aggregation of TIA1 and TIAR (Gilks et al., 2004) as well as through the action of the endoribonuclease G3BP1 (Tourrière et al., 2003). However, hydrogen peroxide stress is known to induce stress granules independently

of eIF2α phosphorylation, suggesting that other as-yet unknown mechanisms may play a role in granule assembly (Emara et al., 2012).

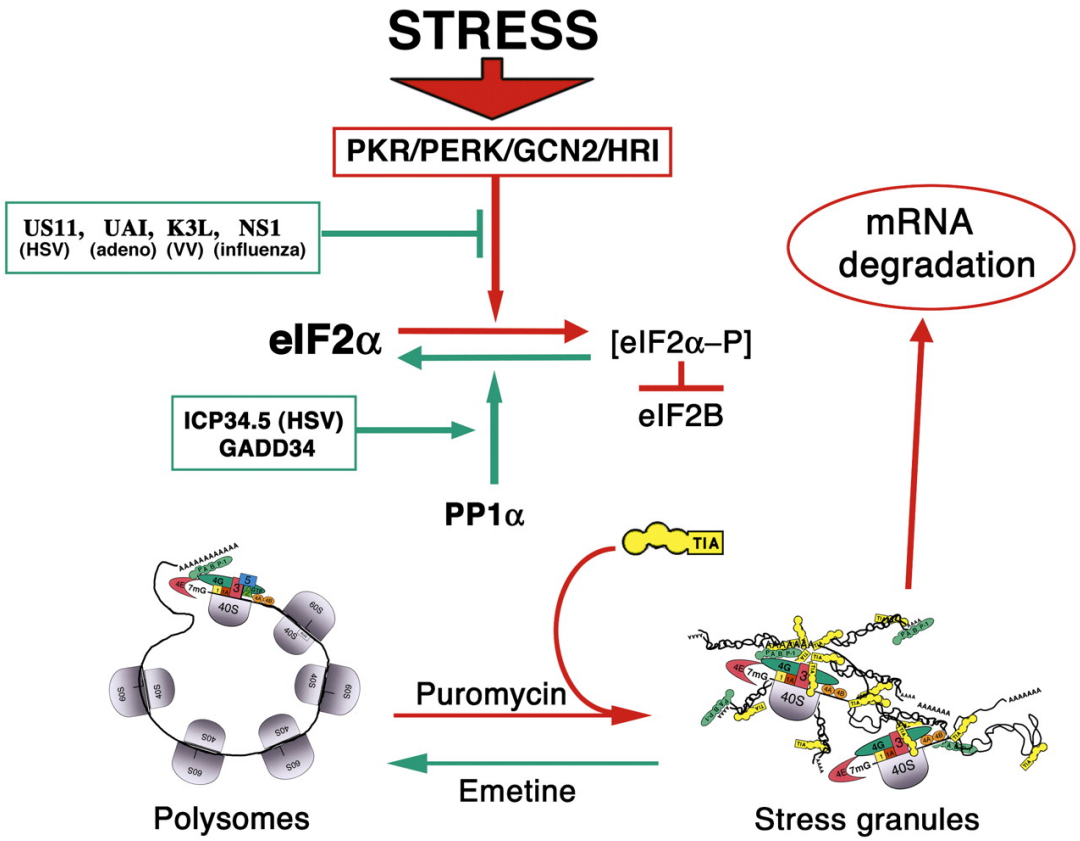

Figure 2. The currently understood schematic for stress granule assembly (Anderson & Kedersha, 2002).

Since their discovery, the importance of stress granules in cell responses to extracellular conditions has been increasingly uncovered. Stress granules are known to modulate the inhibitory abilities of G3BP1 against the protein USP10, which has a known antioxidant activity during oxidative stress, resulting in a decrease in reactive oxygen species and consequent decrease in apoptosis (Takahashi et al., 2013). Granule formation is also correlated with enhanced survival following heat shock (Groušl et al., 2009) and cold shock (Hofmann, Cherkasova, Bankhead, Bukau, & Stoecklin, 2012). These granules also function to regulate a number of signalling pathways including the MAPK and mTOR pathways, in doing so enhancing the likelihood of successful recovery from stress (Kedersha, Ivanov, & Anderson, 2013). Understanding how these granules function in different cellular contexts is therefore crucial to understanding how altered cellular stress responses differ dependent on the environment.

Stress Granule Function in Senescence

Recent studies have revealed that oxidative stress can induce inflammation in nearby cells through the secretion of inflammation-related proteins in proliferating macrophages (Salzano et al., 2014). Whilst the results of this study are yet to be replicated in other cell lines, they underscore the importance of understanding how responses to stress can affect diverse cellular processes. However, despite the knowledge that senescent cells possess a more limited capacity to recover from a variety of stresses compared to proliferating cells (Rochette & Brash, 2008), the stress response of senescent cells remains relatively uncharacterised. Considering stress granules are a crucial mechanism through which cells modulate the stress response (Kedersha et al., 2013), the examination of their formation upon stress onset could help to elucidate why stress responses differ in these senescent cells. Lian and Gallouzi (2009) were the first to examine stress granules in the context of senescence, and found that upon treatment with sodium arsenite, senescent fibroblasts formed a significantly greater number of stress granules than proliferating fibroblasts, with these stress granules assembling faster during stress and disassembling slower after removal of stress in the senescent population. Furthermore, the granules that formed were shown to have differential function, rapidly inhibiting the translation of p21 in late senescent cells but not in proliferating cells. Gallouzi (2009) later posited that the altered dynamics of these granules could explain the more limited capabilities of senescent cells to respond to stress, suggesting that altered heat shock protein expression may be responsible for these altered kinetics, and further proposed that this aberrant granule function may play a role in inducing the senescence-associated secretory phenotype.

However, there are several limitations to the study that require the validation of this study prior to any further research based on their conclusions. Firstly, whilst the major finding of the study, that stress granule number is increased in stressed senescent cells, is replicated in two cell lines, much of the work of Lian and Gallouzi

(2009) relies on the use of the IDH4 cell line as a senescence model. The IDH4 line is a fibroblast cell line immortalised through infection with the simian virus 40, and induces senescence when deprived of dexamethasone in media (Wright, Pereira-Smith, & Shay, 1989). Whilst this model is useful logistically, due to the faster turnaround to senescence, it is possible that the infection itself could alter the overall function of these cells. Secondly, whilst Gallouzi (2009) does appear to show that heat shock proteins are delayed in their expression in senescent cells, which may correlate with the longer recovery period of senescent cells, this result would not explain why senescent stress granules form more rapidly than granules in proliferating cells, and therefore more research into the mechanisms surrounding assembly and disassembly in senescent cells is required.

Therefore, the initial findings of Lian and Gallouzi (2009) were tested again in the fibroblast DD1 cell strain, discussed further in Materials and Methods, to confirm that stress granule increase can be found across multiple replicative senescent fibroblast cell strains. However, whilst initial studies believed that there was no distinguishable difference between replicative and stress-induced premature senescent cells due to their morphological similarities (Ben-Porath & Weinberg, 2005), it is being increasingly established that different senescence programmes possess distinct expression profiles in relation to genes implicated in growth, morphogenesis and metabolism (Pascal et al., 2005). Notably, this study showed that stress-induced premature senescence results in the differential regulation of a smaller number of genes than replicative senescence. This suggests that stress-induced senescence is a more targeted response to a specific form of toxicity, due to the lower number of genes that undergo altered transcription, compared to the more sweeping changes to the transcriptional landscape undergone following telomeric shortening (Dierick et al., 2002; Kalume et al., 2002). This was further supported by their finding that certain stress-related proteins, such as apolipoprotein J (Patrick Dumont et al., 2000), were upregulated to a greater extent in stress-induced senescence than in replicative senescence. Considering these phenotypic differences, and stress-related transcriptional differences, between replicative and premature senescence, the possibility was considered that different senescence systems could also respond

differently when exposed to stress. As a result, the stress response of stress-induced senescent fibroblasts was also examined in order for comparisons to be drawn between different senescence phenotypes. Exposure of cells to ionising radiation such as X-rays is known to induce a persistent DNA damage response in cells, associated with growth arrest and the onset of premature senescence (Fumagalli et al., 2012; Kim et al., 2013), and was therefore used to induce senescence in otherwise proliferating fibroblasts.

Materials and Methods

Chemicals

All chemicals were sourced from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

Cell Strains and Tissue Culture

The human dermal fibroblast cell strain DD1, derived from the foreskin of a healthy adolescent male, was utilised in the present work due to its use in several recent studies relating to fibroblast stress responses and senescence (Gibbs-Seymour et al., 2015; Pekovic et al., 2011). Due to the recent derivation of this cell strain minimising the effects of genetic drift, as well as the health of the donor, this strain was used to ensure that stress experiments were not carried out on cells which were already unhealthy or stressed. These cells were grown from initial batches of passage number 12 and 13. DD1 cells were cultured via serial passage at 37^{o}C in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (Gibco, Paisley, UK) supplemented with 1% (v/v) penicillin-streptomycin and 10% foetal bovine serum (LabTech). Once cultures reached 90% confluence, they were washed twice in warm PBS prior to incubation in Trypsin for 5 mins at 37^{o}C. When passaging into flasks, a 1:2 split ratio was maintained consistently. In order to induce oxidative stress and subsequent stress granule formation, cells were incubated with sodium arsenite (AS) diluted to 0.5mM in complete media for 30 mins prior to further processing. All experiments were performed on cells plated to 90% confluence.

Culture Irradiation

Cells were exposed to ionising radiation in a 320kV high-stability X-ray chamber irradiator (Gulmay Inc., Byfleet, UK) at an irradiation rate of 0.24 Gy/min, with exposure time adjusted to produce the required dosage. In order to discount the possibility of confounding factors producing false positive results, control treatments were taken to the irradiator but were not exposed to X-ray irradiation. If cultures were maintained for more than 24h post-irradiation, such as during stress-induced

premature senescence experiments, cells were washed once in warm phosphate-buffered saline (PBS) after 24h, and media replaced, to ensure that cells were not continuously kept in an environment rich in reactive oxygen species.

Fixation and Immunofluorescence

DD1 cells were seeded at a density of 1.8 x 10^4 cells per well of a 24-well dish and grown to 70% confluence on glass coverslips, treated as required, and washed in warm PBS prior to fixation for 10 minutes in warm 4% (w/v) paraformaldehyde. Cells were permeabilised in 0.5% Triton-X100/PBS for 5 minutes, washed twice in PBS, and subsequently incubated in a blocking solution of 1% (v/v) newborn calf serum (NCS) diluted in PBS for 30 minutes prior to a further two washes in PBS. Primary antibodies were diluted in this blocking solution (see Table 1) and applied to coverslips for 1h at room temperature. Coverslips were then washed five times in PBS before the application of secondary antibodies for 1h diluted in blocking solution to the following concentrations: Mouse FITC, 1:500; Rabbit FITC, 1:500; Mouse TRITC, 1:500; Rabbit TRITC, 1:500; Mouse AF488, 1:100; Rabbit Cy3, 1:100. The nuclear marker 4',6-diaminido-2-phenylindole (DAPI) was also applied to the coverslips for the final 10 minutes of this incubation at a dilution of 1:1000. Following a further five PBS washes performed in a dark chamber, coverslips were mounted in Vectashield Mounting Medium (Vector Laboratories, Peterborough, UK) and stored in the dark at 4^{o}C until imaging. In order to distinguish between genuine signal and background noise when examining slides, secondary antibody controls were performed by following the above protocol without the addition of a primary antibody.

Table 1. A list of primary antibodies utilised during immunofluorescent staining, with dilutions used during course of study.

Antibody Species, Target	Dilution	Company
Rabbit G3BP1	1:200	ProteinTech
Mouse γH2AX	1:200	Millipore
Rabbit β-catenin	1:250	Abcam
Rabbit TIAR	1:200	Cell Signalling
Mouse G3BP1	1:100	BD Transduction

Fluorescent and Confocal Microscopy

Stained samples were imaged with an Axioskop 40 inverted microscope loaded with the Axiovision Rel. 4.8 programme (Carl Zeiss Ltd., UK) with epifluorescence optics using a 40x/1.3 oil immersion lens. Where slides required a confocal microscope for further resolution, the LSM 880 (Carl Zeiss Ltd., UK) was utilised equipped with a C-Apochromat 40x/1.2 W Corr objective. Images were then exported from Zen Blue 0.2 and figures collated in Microsoft Powerpoint.

Protein Extraction

For creation of whole cell lysate protein extracts, DD1 cells were seeded at a density of 8.1 x 10^4 cells per well of a 6-well dish and grown to 70% confluence before being washed twice in ice-cold PBS, scraped, and centrifuged at 4000 rpm for 5 mins at 4^{o}C. The resulting pellet was then lysed in ice-cold RIPA buffer [50mM Tris-HCl, pH 7.4, 150mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, 50mM NaF, 2mM EDTA] supplemented with 1x protease inhibitor cocktail and 1x phosphatase inhibitor (G Biosciences, St Louis, MO, USA), and suspended on ice for 30 mins prior to centrifugation at 14000x *g* for 15 mins at 4^{o}C. The supernatant was then stored at -80^{o}C until use. Protein isolation was confirmed through coomassie blue staining.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was carried out broadly following the specifications established by Laemmli (1970). A 12% separating gel [2.5ml 1.5M Tris-HCl pH 8.8, 3.33ml 30% (w/v) Bis/Acrylamide, 100µl 10% (w/v) SDS, 50µl 10% (w/v) APS, 5µl TEMED, 4ml dH_2O] was prepared using a casting frame (BioRad, Herts, UK), with water-saturated butanol pipetted onto the gel before setting for 30 mins. Once the gel was set, this butanol was removed with tissue and dH_2O. A 4% stacking gel [1.25ml 1.5M Tris-HCl pH 6.9, 0.67ml 30% (w/v) Bis/Acrylamide, 50µl 10% (w/v) SDS, 75µl 10% APS, 5µl TEMED, 3ml dH_2O] was then poured onto the separating gel to the top of the casting apparatus with a 12-well comb inserted. Once set, the comb was removed and gels were placed in a clamp and electrophoresis tank containing 1x running buffer [0.1% (v/v) SDS, 192mM glycine, 25mM Tris pH 8.3]. Samples were then loaded using a fine-tipped pipette alongside 2µl of the PageRuler Prestained Protein Ladder Plus (Fermenta, UK) molecular weight marker. Following loading, gel electrophoresis was performed at a constant 100V for 2 hours or until samples had migrated entirely through the separating gel. Following resolution with SDS-PAGE, gels were first transferred to a staining chamber and stained with 0.1% Coomassie Blue R250 in 50% methanol, 40% water and 10% glacial acetic acid for 2h, followed by destaining overnight in 50% water, 40% methanol and 10% glacial acetic acid, to confirm equal protein loading between wells. Once confirmed, replicate gels were run to allow for western blotting to occur.

Western Blotting

Whilst gel electrophoresis was run, 18 pieces of Whatman 3MM Chr papers were cut to dimensions of 7.5cm x 6.5cm. 6 pieces were placed into buffer A [0.3M Tris, 20% (w/v) methanol], 3 pieces into buffer B [0.025M Tris, 20% (w/v) methanol] and 9 pieces into buffer C [0.025M Tris, 14mM glycine, 20% (v/v) methanol]. Following removal from electrophoresis tanks, gels were placed into buffer B. Once soaked the gel, Whatman paper and a 0.45µm pore size nitrocellulose membrane were placed into a Transblot SD Semi-Dry Transfer Cell (BioRad, Herts, UK), with papers from

buffer A placed first, followed by papers in buffer B, nitrocellulose membrane soaked in buffer B, gel, and papers from buffer C. To elicit transfer of proteins to the membrane, the transfer machine was run at constant 0.8mA/cm^2 for 90 mins. Membranes were then removed and washed in dH_2O for 10 mins prior to blocking at room temperature for 2hr in blocking solution [5% (w/v) milk powder in TBS-T (0.1% (w/v) Tween-20, 25mM Tris-HCl pH 7.4, 150mM NaCl)]. Following blocking, membranes were washed in TBS-T three times, for five minutes each wash. Membranes were then cut where necessary to allow for multiple stainings, and incubated in a falcon tube containing primary antibodies (Rabbit G3BP1, ProteinTech; 1:500, Rabbit β-catenin, AbCam; 1:1000; Rabbit GAPDH, 1:10,000) diluted in 5% (w/v) milk powder in TBS-T overnight at 4°C. Membranes were again washed three times in TBS-T for 5 minutes prior to incubation in secondary antibodies (Rabbit IgG.HRP, Sigma; 1:2000) diluted in 5% (w/v) milk powder in TBS-T. Following a further five washes, membranes were developed in the absence of light for 5 minutes using a mixture of ECL1 [1ml 1M Tris-HCl pH 7.5, 44μl coumaric acid and 100μl luminol, 8.85ml dH_2O] and ECL2 [100mM Tris-HCl pH 8.5, 0.02% (v/v) H_2O_2] in equal proportions, and imaged under the chemiluminescence filter of the LAS-1000plus Fuji luminescent image analyser (FujiFilm, Japan).

Senescence-Associated β-Galactosidase Assay

Following fixation as described above, coverslips were washed twice in PBS and incubated at 37°C overnight in a staining solution comprised of 8.8ml SA- β-Gal solution [150mM NaCl, 200mM $MgCl_2$, 40mM citric acid, 12mM sodium phosphate, adjusted to pH 6], 1ml Potassium Solution [5mM potassium ferrocyanide, 5mM potassium ferricyanide] and 0.2ml 5-bromo-4-chloro-3-indolyl-βD-galactopyranoside (X-gal) 20mg/ml stock solution. Coverslips were then washed three times in PBS, mounted in Vectashield Mounting Medium, and imaged using a bright field microscope.

RESULTS

Characterisation of senescence

In order to examine how the progression of senescence programs affected the capacity for human fibroblasts to form stress granules, it was firstly necessary to confirm that the cell strain to be used was capable of reaching senescence, and to briefly characterise the extent of this senescent phenotype. Whilst the DD1 fibroblast cell strain has been used in previous studies of progeria and ageing, being cultured to senescence via serial passage (Pekovic et al., 2011), their use as a model for senescence remains less common than other cell strains such as MRC5 fibroblasts, and as a result the extent to which the common markers for senescence are exhibited in senescent DD1 cells is unknown.

Prior to the quantification of the extent of replicative senescence in culture, a population of DD1 cells were firstly cultured by serial passage to confluence, prior to counting using a haemocytometer. These cells were then split 1:2 to ensure approximately equal cell numbers were seeded into new flasks with each subsequent passage and to avoid excessive perturbation of the cells through higher split ratios, which is known to affect proliferation. The population size of each confluent flask was then used to plot a population growth curve, providing an initial indication of DD1 population dynamics. As seen in Fig. 3B, DD1 cells exhibit a marked slowing of growth at later passages, taking longer periods to reach confluence. This is indicative of a gradual progression towards full senescence, with a greater number of cells becoming senescent with each progressive passage number due either to telomeric shortening or to premature senescence activation due to bystander effects. Whilst this curve is not enough to confirm that DD1 cells display senescent characteristics at later passages, it shows that they exhibit population dynamics that would be expected during senescence onset. To confirm that this slowing of cumulative population number was due to senescence, several methods were used to validate a senescent phenotype.

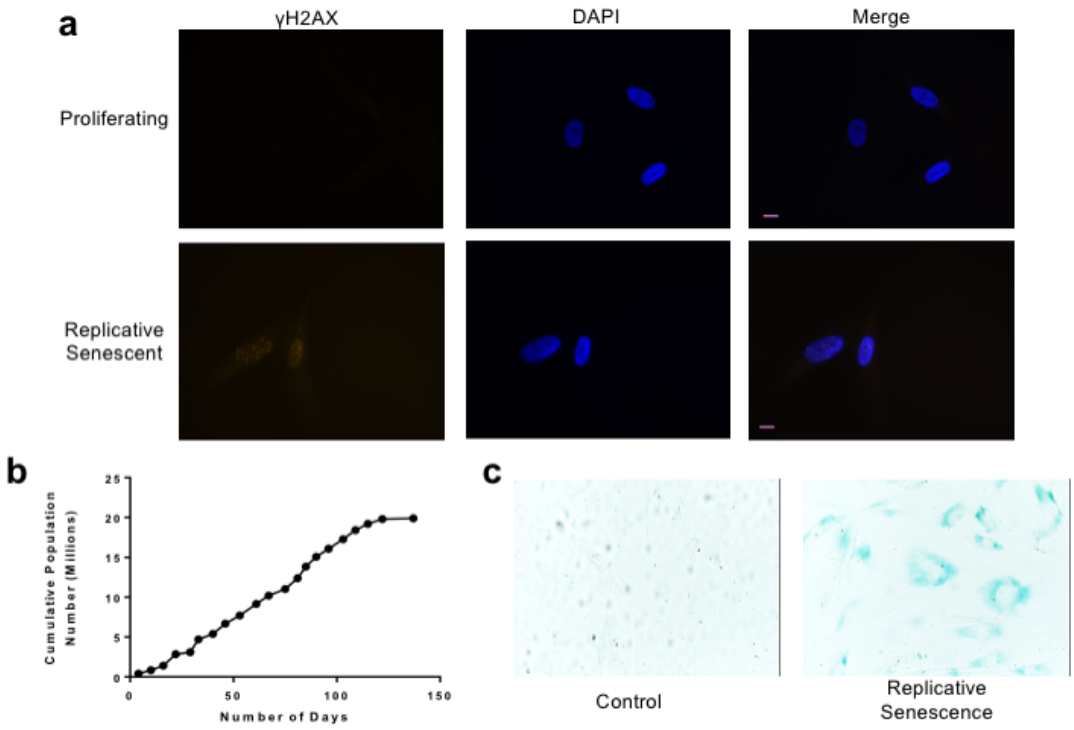

Figure 3. DD1 fibroblasts reach replicative senescence following serial passage. A) Immunofluorescent stain for phosphorylated H2AX, imaged at 40x. DAPI is nuclear stain. Scale bar=10μm. B) Population growth curve for DD1 fibroblasts. C) Sen-β-gal staining of proliferative and senescent coverslips.

The most commonly-used method for the detection of senescence is the examination of senescence-associated beta-galactosidase (Sen-β-gal) activity. This marker is based around the observation that only cells that are undergoing the transformation to a senescence phenotype stain blue following a pH 6.0 β-galactosidase assay, initially proposed to be the result of a hydrolase capable of catalysing the hydrolysis of β-galactosides that would only be expressed during the onset of senescence (Dimri et al., 1995). Whilst it is now known that the β-galactosidase that is responsible for this staining is actually lysosomal in origin as opposed to a novel protein (Kurz et al., 2000; Lee et al., 2006), the assay itself remains a readily-used marker for senescence, and is widely considered to be a reliable indicator of senescence in culture. Considering the robustness of this assay, Sen-β-gal activity was determined, as detailed in Materials and Methods, to validate the occurrence of DD1 replicative senescence.

Cells cultured to passage numbers x+4 and x+19 were plated onto glass coverslips in 24-well plates, and considered to be representative of the general population of DD1 cells at their respective passage numbers in terms of the proportion of senescent cells. When stained, cells maintained at passage x+19 showed a significant level of blue-stained cells (Fig. 3C), indicating higher β-galactosidase activity. Considering the majority of cells stained this colour, compared to almost no cells possessing blue staining in the x+4 sample, this suggests that cells used in passage numbers of x+19 or above are largely senescent in nature and suitable for use as representative replicative senescent samples.

Further to late passage cells staining positive for β-galactosidase activity, the morphology of the stained cells was then examined to determine whether any gross changes had occurred. Bayreuther et al. (1988) demonstrated that fibroblast morphology alters as cells become post-mitotic and senescent, with cells tending towards an enlarged and more rounded cell shape at later stages of senescence. Further studies also confirmed that fibroblasts can be consistently characterised using these different morphologies (Toussaint, Houbion, & Remacle, 1992), and that different fibroblast classes also differ in terms of gene expression, therefore pointing to functional differences underlying these classes (Rodemann et al., 1989). Therefore, analysing which classes of cells are most prevalent in cultures of different passage numbers may lend further support to the evidence that the cells at x+19 have become post-mitotic. Fibroblasts from the x+19 sample displayed a greater proportion of type F V and F VI fibroblast morphologies that correspond to the later senescent phenotypes, whereas cells at x+4 remain largely fibrous in shape, possessing largely F I and F III morphotypes, which are characteristic of normal cell function proliferation (Bayreuther et al., 1988). Whilst these observations on cell shape are qualitative, and the exact morphotype of each cell remains open to interpretation and therefore is not reliable in itself as a senescence marker, this further supplements the findings of the Sen-β-gal assay. When considered in conjunction with the population growth curve's findings (Fig. 3B) indicating the plateauing of DD1 cell growth at higher passage numbers, this data provides a

convincing case for the onset of senescence, with the process beginning to occur at approximately passage x+19.

Whilst the above data provides a convincing case to suggest that the DD1 fibroblasts had undergone senescence by passage x+19, there are inherent problems with these markers that mean that in isolation they are not robust enough to confirm senescence. Sen-β-gal activity at pH 6.0 has been shown to be induced through a number of culturing conditions (Severino et al., 2000), and can also occur in some quiescent cell cultures (Yegorov et al., 1998), which would also produce the stoppage of the population growth represented in Fig. 3B. Whilst the alteration of morphotype discussed above lends credence to the potential for senescence induction, it was concluded that a further marker should be used to independently verify this phenotype.

One method often used to detect senescence *in vitro* is to examine the formation of foci of phosphorylated histone H2AX (γH2AX) in the nuclei. As a component of the DNA damage response apparatus, γH2AX localises to strand breaks, comprising a large complex that is visible via immunofluorescent staining. It has been previously established that the number of γH2AX foci present in the nucleus is directly correlated to ionising radiation dosage following exposure, and therefore is regarded as a reliable marker for DNA damage (Hernández et al., 2013). As discussed previously in the introduction, the pathways leading to replicative senescence are induced following shortening of chromosomal telomeres past a critical length, after which cells detect further degradation as DNA damage and activate mechanisms to halt any further mitosis (Rai & Chang, 2011; Takai, Smogorzewska, & de Lange, 2003). The result of this degradation past the critical length is that DNA damage response foci assemble to attempt to repair this perceived damage. However, whilst most foci are known to disassemble up to 24h following initial damage, γH2AX foci detected in senescent cells are known to persist. Therefore, a greater number of γH2AX foci in the nucleus under standard culture conditions is being increasingly used to confirm the senescent phenotype.

In the putative senescent population, a greater proportion of cells exhibited γH2AX foci than in their proliferating counterparts, indicating a greater recognition of DNA damage occurring in the late passages consistent with telomere-associated foci that form during the onset and maintenance of senescence (Fig. 3A). Furthermore, when comparing cells which stained positive for foci in proliferating versus senescent populations, the number of foci per nucleus was significantly greater in senescent cells, with many of these foci appearing more defined than in controls. Taken together, this data suggests a greater number of cells have activated DNA damage response mechanisms in the later passage, and that this response is more pronounced than in proliferating cells. Whilst is should be noted that several cells at low population doublings do possess a small number of foci, previous studies have investigated such foci in lung fibroblast liness and have found them not to be associated with telomeric DNA and therefore are not an indicator of senescence, instead appearing to be an inherent characteristic of the cell line (Nakamura et al., 2008). Assuming that the same is true of the foci present in proliferating DD1 cultures, the increasing γH2AX staining in later passages corroborates the findings of the Sen-β-gal assay. It should be noted, however, that this increase in γH2AX foci formation at later passages has not been confirmed to be due to telomere shortening in DD1 cells as no colocalisation has been demonstrated between telomeric DNA and γH2AX foci. Whilst this can be confirmed through the use of other methods such as staining for telomere probes alongside DNA repair apparatus (Herbig et al., 2004) or through the use of DNA microarrays following immunoprecipitation with γH2AX to confirm the location on the chromosomes that foci are binding (d'Adda di Fagagna et al., 2003), and these would provide more evidence for replicative senescence occurring with increasing passage number, the confirmation of increasing H2AX phosphorylation coupled with a progressive slowing of cell division in Fig. 3B and positive sen-β-gal staining (Fig. 3C) was considered to provide sufficient evidence of senescence for experiments to continue in earnest.

Considering the aims of this project require the comparison of replicative and stress-induced premature senescence, it was also necessary to confirm that senescence was induced in early passage DD1 cultures following exposure to 10Gy ionising radiation, as opposed to other mechanisms that arrest the cell cycle such as quiescence. In order to do so, the Sen-β-gal assay and immunostaining for persistent γH2AX foci were also performed on cultures that were maintained for 14 days following radiation exposure. Similar to replicative senescent cultures, DD1 cells exposed to ionising radiation also tested positive for senescence in Sen-β-gal assays, with almost all cells exhibiting some level of blue colouration following staining. Furthermore, many of these cells had begun to adopt morphologies indicative of post-mitotic fibroblasts, supporting the stoppage of the cell cycle as a response to ionising radiation exposure (Fig. 4B).

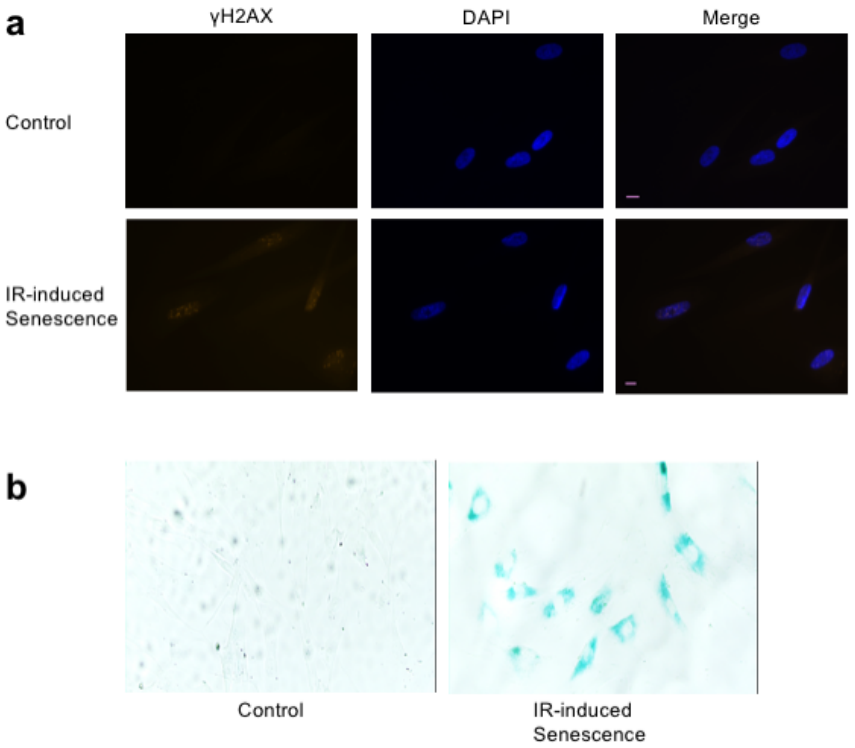

Figure 4. DD1 fibroblasts reach senescence 14 days after X-ray exposure. A) Immunofluorescent stain for phosphorylated H2AX, imaged at 40x. DAPI is nuclear stain. Scale bar=10μm. B) Sen-β-gal staining of proliferative and senescence coverslips.

Staining for γH2AX also demonstrated a marked increase in foci, with a majority of cells maintaining persistent foci 14 days post-exposure, with cells staining positive for foci containing a significantly higher number than in unirradiated cells (Fig 4A). Whilst persistent γH2AX foci in replicative senescent cells represent the activation of the DNA repair machinery due to critical telomeric shortening, the foci present in irradiated cells instead represent sites of double strand breaks in DNA, and therefore are not entirely analogous to those found in replicative senescence. Furthermore, the link between high DNA damage and senescence activation is less elucidated than that between telomere length and senescence. However, both Marková et al. (2011) and Fumagalli et al. (2012) have previously demonstrated that a persistent DNA damage response, as represented by long-term γH2AX foci, occurs following exposure to high doses of ionising radiation, and Fumagalli et al. (2012) further demonstrated that inactivating the damage response resulted in continuing proliferation as opposed to the development of senescence. Considering this data suggests that a consistent DDR is heavily associated with and is necessary for the induction of senescence following ionising radiation exposure, the observation of γH2AX foci up to 14 days post-irradiation in DD1 cells, coupled with the stalling in cell division, supports the onset of senescence as opposed to quiescence. As a result, it was assumed from hereon that cells cultured for 14 days after irradiation could be used to represent stress-induced premature senescence.

Having established with confidence that both culture by serial passage and exposure to X-ray irradiation are capable of inducing a state of proliferative arrest, and further linking this growth arrest to replicative and stress-induced premature senescence respectively, subsequent experiments were undertaken to examine the assembly of stress granules during these different senescence programmes.

Replicative and Stress-Induced Premature Senescence Differentially Form Stress Granules

Prior to examining the potential for different senescence phenotypes to form stress granules, it was firstly necessary to confirm the presence of bona fide stress granules in the DD1 cell strain, and to determine an appropriate concentration of sodium arsenite for use in further experiments. Sodium arsenite is a well-characterised and widely used inducer for stress granule formation due to the strong oxidative stress cells undergo during treatment. In order to provide cells with adequate time to form stress granules, and in line with other studies, cells were exposed for 30 minutes to varying concentrations of sodium arsenite and fixed as detailed in Materials and Methods. In order to confirm stress granule formation had occurred in response, the colocalisation of two different stress granule markers was analysed using immunofluorescence. G3BP1 is a universal marker for stress granules, and plays a crucial role in the signalling pathways facilitating protein aggregation and subsequent nucleation of stress granules, and is thus a commonly used marker for stress granule formation. TIAR is a nuclear protein, closely related to the stress granule nucleating protein TIA1, which has been demonstrated to shuttle from the nucleus to the cytoplasm following the onset of oxidative stress where it acts as a stress granule component.

In order to quantify the effect of different sodium arsenite concentrations on cultured cells, blind sampling was used to locate a field of view containing 100 cells with a 20x objective, and the proportion of cells forming granules was measured. In cells not treated with sodium arsenite, G3BP1 remained diffuse within the cytoplasm, not assembling into any detectable granule structures. Furthermore, TIAR remained nuclear in unstressed cells and therefore did not colocalise with G3BP1 (Fig. 5A). Following treatment with sodium arsenite at all concentrations, G3BP1 assembled into granules, colocalising with TIAR, therefore confirming that DD1 cells are capable of forming stress granules and are therefore a suitable cell strain for study. 84% of cells treated with 0.25mM sodium arsenite formed stress granules, indicating that even comparatively low doses of stress granule inducers were capable of eliciting a

robust stress response in the majority of cells. However, this was still a lower percentage of granule-positive cells than was observed in cells incubated with higher concentrations of sodium arsenite; 92% of cells exhibited granule formation following a 0.5mM arsenite dose, whilst 96% and 98% of cells formed granules after treatment with 1mM and 2mM sodium arsenite, respectively. Considering the difference in percentage of granule-positive cells was significant between the 0.25mM and 0.5mM treatments, but not between 0.5mM and either of the two higher concentrations, it was determined that a concentration of 0.5mM was sufficient for future treatments, and is also consistent with the body of work regarding arsenite-induced stress granules.

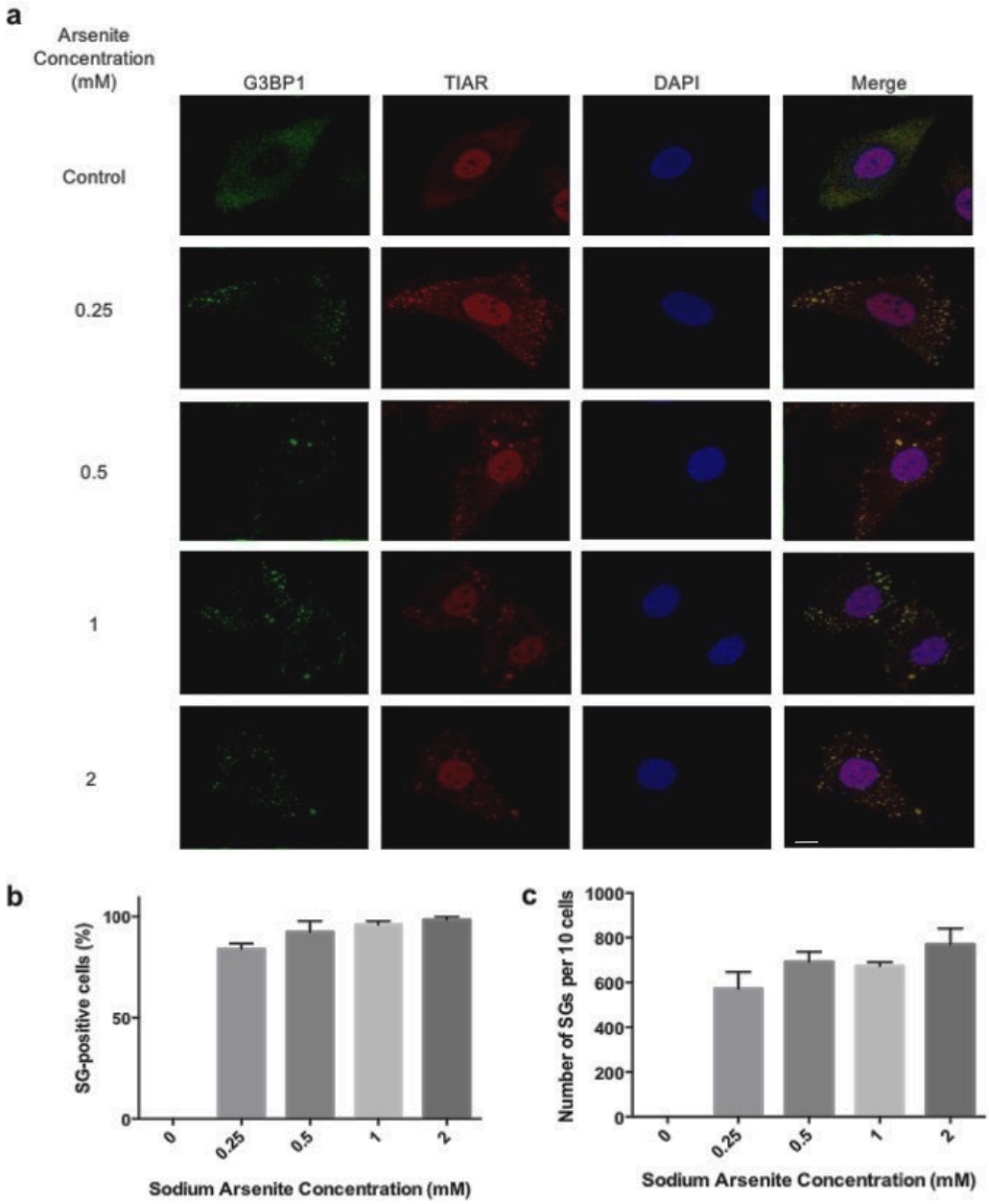

Figure 5. An arsenite titration to determine optimum concentration for granule formation. A) Confocal images of stress granule markers G3BP1 and TIAR at 63x. DAPI is nuclear stain. Scale bar=10μm. B&C) A graph depicting the percentage of cells staining positive for stress granules, and the number of stress granules respectively, at differing concentrations. Error bar=1SD.

A second metric used for quantification of stress granule formation in response to oxidative stress is the number of stress granules formed by each cell following arsenite exposure. This factor likely differs heavily dependent on the nature of the cell liness utilised, and it was therefore necessary to establish the parameters of DD1 stress granule formation before comparative analysis could occur with senescent

cells could occur. Considering that fibroblasts in particular are capable of forming several distinct cell shapes, with different sizes and likely altered cytoskeletal networks, it was necessary to collect data from multiple cells per replicate to ensure that any perceived differences in granule numbers were not artificial as a result of analysing different cell shapes. As a result, blind sampling with a 63x oil objective was used to locate a field of 10 cells, and the number of granules formed in response to sodium arsenite exposure was counted from each cell and summed to give an overall indication of stress granule formation across the field of view. This cumulative number of stress granules was collected in triplicate, and analysed to see whether a linear dose-response is observed with respect to stress granule formation at different sodium arsenite concentrations. Treatment with all concentrations of sodium arsenite resulted in the formation of granules, and cells treated with 2mM sodium arsenite formed significantly more stress granules than those treated with 1mM, with means of 769 and 571 granules per 10 cells respectively (Fig. 5C) However, 0.5mM-treated cells formed 691 granules on average, which was not significantly higher than 0.25-treated cells, and a further doubling of sodium arsenite to 1mM resulted in a mean granule number of 673, representing very little alteration in formation. Furthermore, whilst these means were both lower than the mean for 2mM-treated cells, the differences between them were not considered statistically significant due to the inherent variation in results. Therefore, whilst small differences in stress granule number may result from large changes in concentration, many of these were not significant and there does not appear to be a linear relationship between dosage and granulation in DD1 fibroblasts. Considering the limited effect that a higher dose had on the number of granules formed per cell, it was again decided that 0.5mM concentrations of sodium arsenite should be used, as there would be no benefit from using any higher concentration in terms of visualisation of stress granules.

Having confirmed that DD1 cells form bona fide stress granules in response to oxidative stress, determined that 0.5mM was the optimal concentration of sodium arsenite for usage in further stress experiments, and parameterised the number of stress granules formed by cells following varying severities of stress, a comparative

study of the response of proliferating and senescent cells to oxidative stress was performed. Populations of proliferating cells, as determined by passage number compared against the population growth curve, as cells cultured to replicative senescence, were plated on glass coverslips and treated with 0.5mM sodium arsenite. A further population of proliferating cells were exposed to 10Gy of X-ray radiation and cultured for a further 14 days to stress-induced premature senescence prior to treatment.

Whilst all three populations of cells contained significantly higher percentages of G3BP1 granule-positive cells following arsenite treatment compared to their untreated counterparts which did not form granules (t-test; $p<0.01$), there was also a significant difference in the percentage of cells forming stress granules between the proliferative and senescent populations, as seen in Fig. 6B (One-way ANOVA; $p<0.01$). With an average of 97% of cells forming stress granules, replicative senescent cells appear to form stress granules more readily than their proliferative counterparts, where only 92% of cells contained granules (Tukey HSD; $p<0.05$). However, both the proliferative (Tukey HSD; $p<0.01$) and replicative senescent (Tukey HSD; $p<0.01$) populations contained a far greater proportion of stress granule-positive cells in comparison with the stress-induced senescent population, where only 7% of cells on average formed granules 30 minutes post-stress, indicating a severe abrogation of stress granule formation. Concomitantly, the number of granules formed by cells in the prematurely senescent population was significantly lower than in both the proliferative and replicative senescent populations (Tukey HSD; $p<0.01$), with post-irradiation cells forming only 57 granules per 10 cells on average, compared to 691 granules in the proliferative population (Fig. 6C). In contrast, replicative senescent cells formed an average of 1546 granules per 10 cells, indicating a significantly larger granule-forming potential in these cells compared to both proliferative and prematurely senescent populations (Tukey HSD; $p<0.01$).

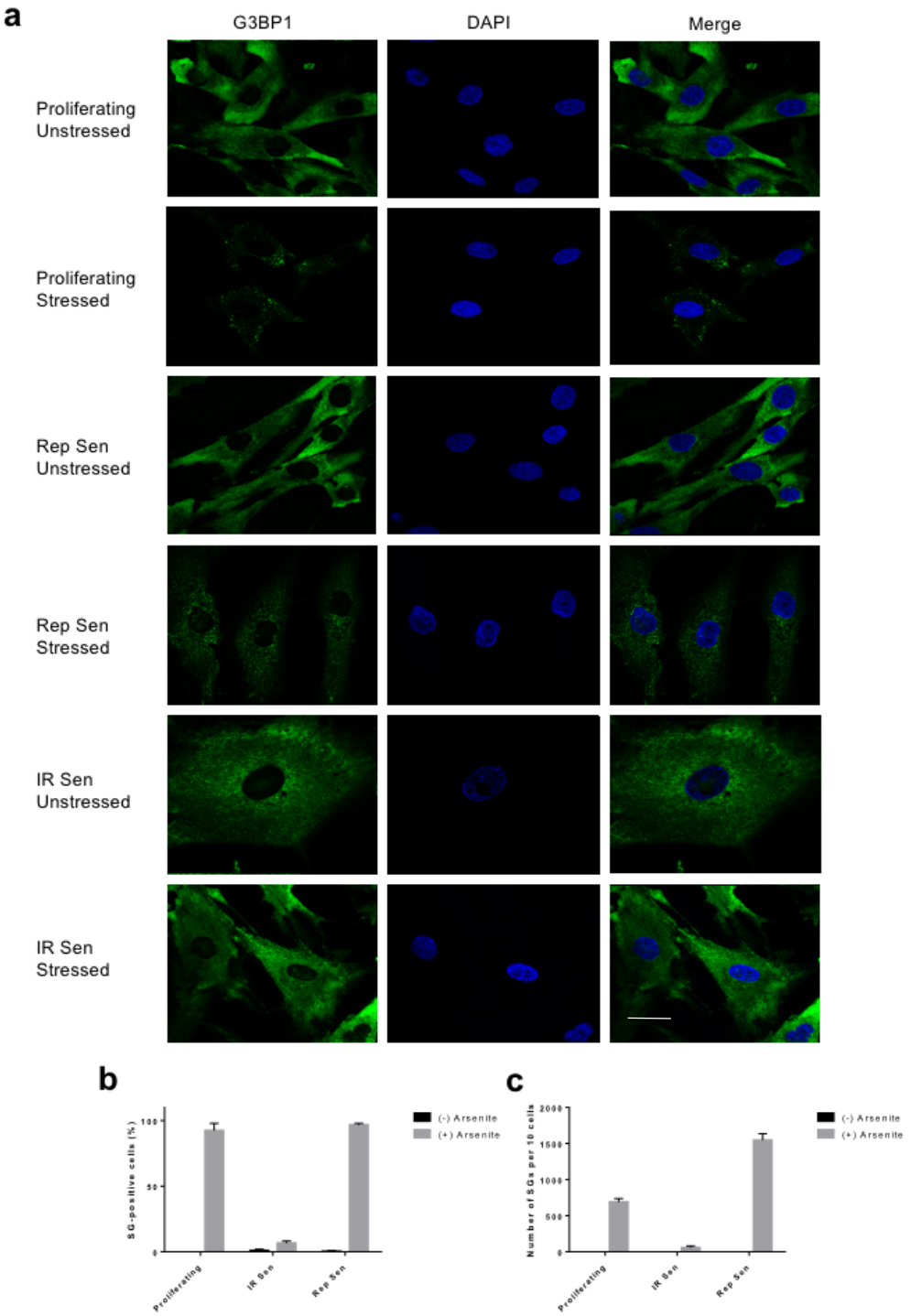

Figure 6. Differing senescence programmes possess a differential capacity to form stress granules. A) Confocal images of G3BP1-stained cells imaged with a 63x objective. Nuclear stain is DAPI. Scale bar=10μm. B&C) A graph depicting the percentage of granule-positive cells, and number of granules formed per cell, in proliferating or senescent populations exposed to 0.5mM sodium arsenite. Error bar=1SD.

The above findings that replicative senescent cells form stress granules more readily following exposure to sodium arsenite is heavily consistent with the work of Lian & Gallouzi (2009), and strengthens the evidence in favour of differential stress responses between proliferating and senescent cells (Merker et al., 2000; Servais et al., 2005). However, whilst no discernable change in granule formation would have been significant in itself, the lack of stress granules following arsenite treatments in stress-induced premature senescent cells is even more unexpected, and is in stark contrast with the replicative senescent phenotype. This finding also suggests that other aspects of the stress response regulated by stress granules may differ between the two senescence programmes. For example, this could indicate that stress-induced prematurely senescent cells would be more likely to undergo apoptosis following exposure to oxidative stresses, due to a loss of granules which are known to reduce levels of reactive oxygen species (Takahashi et al., 2013).

Whilst a number of functional studies could have been undertaken to examine how a lack of granule formation affects the stress responses in both the short- and long-term in prematurely senescent cells, it remained important to determine through which mechanisms these different phenotypes were established. It is well-established that stress granule formation is heavily dependent on shuttling of components along the cytoskeleton (Rajgor & Shanahan, 2014). The majority of studies have focused on the roles of the microtubule network in the assembly of stress granules, with live imaging studies confirming that stress granules move across microtubules (Nadezhdina et al., 2010), and disruption of microtubule polymerisation using the drug nocodazole has been shown previously to abrogate the formation of stress granules upon treatment with sodium arsenate (Ivanov, Chudinova, & Nadezhdina, 2003). Other cytoskeletal components are also known to affect formation of stress granules. Ivanov et al. (2003) demonstrated that disruption of the actin cytoskeleton through latrunculin B treatment enhanced the speed of stress granule formation. When considered in conjunction with observations that filamentous actin can sequester the stress granule component Elongation Factor 1α (G. Liu et al., 2002), these results suggest that the actin skeleton may act to negatively modulate stress granule dynamics. However, following their observations that

replicative senescent cells form a greater number of stress granules, Lian & Gallouzi (2009) undertook an examination of the actin cytoskeleton but found no alterations in the network itself during senescence, or in the morphology of the cells following stress induction, suggesting that alterations in actin filaments were not responsible for the observed changes. There is also little to no evidence to suggest that the microtubule cytoskeleton is altered during senescence and, whilst the body of evidence supporting a role for microtubules in assisting stress granule formation makes their network worthy of further investigation in the context of senescence, priority in study was instead given to a cytoskeletal network with better-established roles in senescence and stress responses.

Intermediate filament proteins are a family of over 70 cytoskeletal components (Szeverenyi et al., 2008; Zimek, Stick, & Weber, 2003) which can assemble into filaments which are 10nm in diameter on average, which possess visoelastic properties that are notably resistant to mechanical stress (Janmey et al., 1998; Kreplak et al. , 2005). Vimentin is a type III intermediate filament protein expressed in cells with a mesenchymal origin that assembles into cytoskeletal networks (Steinert et al., 1981). Vimentin networks play a number of functional roles within the cell including maintenance of organelle distribution within the cytosol (Chang et al., 2009) and the preservation of cell integrity upon mechanical stresses (Goldman et al., 1996). However, vimentin networks also interact with other cytoskeletal components, including a close association with microtubules in parallel arrays (Chang & Goldman, 2004; Goldman, 1971) as well as interactions with the F-actin network, though it is not yet known whether this binding is direct or via an actin-binding protein (Kim & McCulloch, 2011). Furthermore, a recent study has shown that the rate of transport of vimentin through the cell is influenced by both the actin and microtubule networks in concert (Robert et al., 2014). These studies demonstrate that vimentin is capable of linking actin and microtubules together, helping to integrate the separate networks into a cohesive cytoskeleton. It is therefore possible that vimentin could influence stress granule formation and dynamics both through direct interactions, or through alterations in the actin and microtubule cytoskeletons.

A number of studies have also suggested that vimentin may play roles in the induction or maintenance of senescence. Firstly, Nishio et al. (2001) observed that senescent fibroblasts possessed up to four times as much vimentin protein than proliferating fibroblasts, and that this increase in expression was likely the result of an increase in vimentin mRNA. Furthermore, when vimentin was transfected into cells to induce overexpression, the cells undertook shape changes that recapitulated the senescent morphotype. A subsequent study also showed a strong colocalisation between vimentin and p53 – a key regulator of cell proliferation and senescent induction - in senescent cells, indicating that vimentin may play a role in the cytoplasmic anchorage of p53 (Nishio & Inoue, 2005), and cleavage of vimentin was found to release p53 into the cytosol where it underwent translocation (X. Yang et al., 2005). Furthermore, vimentin is also specifically targeted for modification through glycation in both senescent primary human fibroblasts (Ahmed, et al, 2010), which was associated with a loss of contractility due to network disruption (Kueper et al., 2007). These findings all suggest that the alteration of vimentin networks during the onset of senescence are more pronounced than changes to other cytoskeletal components, and therefore vimentin is a prime candidate for further study in the context of senescence.

Furthermore, studies of vimentin have found that it possesses various roles in response to cell stress. Firstly, the vimentin network undergoes a strong reorganisation in response to heat shock, mediated by the small GTP-binding protein Rac1 (Lee et al., 2001), and is also hyperphosphorylated (Cheng & Lai, 1994), indicating that vimentin networks have a bona fide response to heat stress. A similar rearrangement of the vimentin cytoskeleton can occur in response to oxidative stress, induced by covalent binding of a family of lipids known as cyclopentenone prostaglandins which are produced more abundantly during oxidative challenges (Stamatakis, Sánchez-Gómez, & Pérez-Sala, 2006). Recent research has also shown that mutation of the cysteine residue required for the binding of zinc also significantly reduced the capability of vimentin to respond to a variety of electrophilic and oxidative stresses, suggesting that as-yet unidentified signalling pathways exist to regulate vimentin networks (Pérez-Sala et al., 2015). Vimentin, along with other

intermediate filament proteins, can also be both upregulated and downregulated during heat stress depending on tissue type, further supporting its role in cellular responses to stress (Bechtold & Brown, 2003; Fisher, Heredia, & Brown, 1996; Vilaboa et al., 1997).

Whilst vimentin is increasingly becoming implicated in stress responses, no studies have yet demonstrated a functional link or association between vimentin networks and stress granule formation. However, considering the previously discussed rapid reorganisation of vimentin cytoskeletons in response to stress, it is possible that one function of this is either to assist in the maintenance of stress granules, or to aid in their disassembly following removal of stress. This hypothesis is further supported considering vimentin also associates with a number of small heat shock proteins such as HSP27 and HSP90 (Perng et al., 1999; Zhang et al., 2006) which are known to shuttle into granules during certain cellular stresses and have been implicated in the formation of granules themselves (Kedersha et al., 1999; Matsumoto et al., 2011). In order to perform a preliminary study of whether vimentin could help to explain the difference between the stress responses of different senescent phenotypes, coverslips were co-stained with G3BP1 and vimentin, both before and after treatment with sodium arsenite, before immunofluorescent analysis.

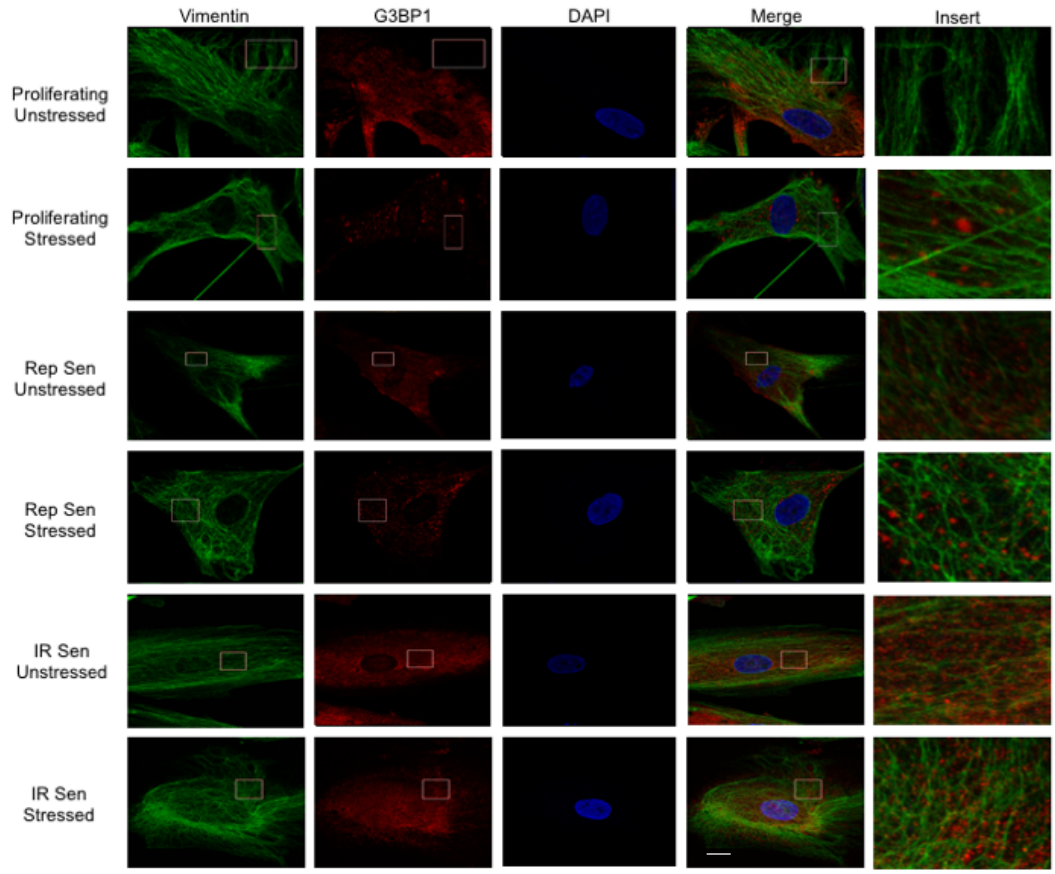

Figure 7. G3BP1 may associate with the vimentin network during granule formation. Confocal image of costained vimentin (AF488) and G3BP1 (Cy3) taken with a 63x objective. Scale bar=10μm.

Fig. 7 shows that the vimentin network and diffuse G3BP1 do not tend to align in unstressed cells. The insert from the untreated proliferating fibroblast in particular indicates a point in the randomly sampled cell where the vimentin network is particularly dense with no concordant increase in G3BP1 density or signal. However, upon stress, there appears to be colocalisation between stress granules and vimentin. with many granules forming adjacent to filaments and occupying spaces between vimentin filaments. This result can be observed best in proliferating cells, as indicated by the insert, but appears to be less pronounced in replicative senescent cells, where there are a much greater number of granules that do not align as clearly with holes in the vimentin network. However, it should be noted that the larger granules in the replicative senescent sample appeared to form in areas where the vimentin network was less dense, potentially indicating that vimentin constrains the size of granules under normal conditions. Given that the vimentin network is

particularly dense throughout the cytoplasm in these senescent cells, this would provide some explanation for the large number of granules if they were less able to shuttle across the cytoskeleton and merge with adjacent granules. However, this thick and bundled vimentin cytoskeleton apparent in the replicative senescent samples was also present in the prematurely senescent samples, despite the much larger size and altered morphotype observed in the majority of these cells. Therefore, the overall structure of the vimentin cytoskeleton is unlikely to contribute to the loss of granule formation in a significant manner. One observation that can be made from Fig. 7 is that, in both stressed and unstressed proliferative and replicative senescent cells, some vimentin filaments accumulate into a thin perinuclear ring, with a curving of the vimentin skeleton around the nucleus. Whilst the vimentin cytoskeleton at this ring is not as thick as some other bundles observed towards the periphery of the cell, which may indicate sites where vimentin is contributing to cell adhesion (Ivaska, Pallari, Nevo, & Eriksson, 2007), it is most notable in unstressed cells in the replicative senescent image, followed by the proliferating image. In arsenite-treated cells, an apparent increase in vimentin bundles surrounding the nucleus may suggest a thickening of this perinuclear ring in response to oxidative stress. However, in both the treated and untreated stress-induced senescent cells this ring is far less apparent, with no such bundling of filaments immediately surrounding the nucleus. Such vimentin rings have been previously observed in several studies, as a feature of the normal network (Helmke, Goldman, & Davies, 2000), as well as a component which can be induced through signalling and treatment (Palladini, Finardi, & Bellomo, 1996). Whilst their purpose remains unknown, it should be noted that, both in this study and in prior studies, many stress granules cluster in a perinuclear fashion (Hinton et al., 2010; Thomas et al., 2005), and this has previously been linked to mature granules. It is possible, therefore, that such a ring could assist granule formation through providing nuclear anchorage for granules, or potentially through propagation of nuclear signalling into the cytoplasm (Georgatos & Blobel, 1987) which could assist in stress granule formation. However, given that no further data regarding vimentin function, expression, or network modulation was collected in the project, the above discussion of perinuclear rings remains purely speculative.

Whilst these changes to the vimentin network are unlikely to explain the wholesale abrogation of granule formation, considering this cytoskeletal component appears to change conformation as opposed to being wholly disrupted, this alteration could be a contributing factor towards a lower potential for granule formation, which could play an additive role in blocking their assembly, and conversely a thickened vimentin network could be implicated in the increased granule formation observed in replicative senescent cells. Whilst this study was ultimately preliminary due to time constraints, the pilot data demonstrating that stress granules appear to interact with the vimentin cytoskeleton in granule-positive cells may identify intermediate filaments as novel factors influencing granule assembly. In order to further test to what extent vimentin regulates granule formation, it is recommended that live imaging with fluorescent constructs for vimentin and stress granule markers be performed to observe whether granules are transported along filaments, following the protocols of Nadezhdina et al. (2010). Furthermore, disruption of the vimentin cytoskeleton through treatment with the anti-filament antibody (Lieber & Evans, 1996; Pruss et al., 1981) could also help to determine to what extent vimentin functions in granule maintenance, assembly, and transport.

Stress Granule Formation is Inhibited Following X-ray Exposure

In order to determine potential factors underlying the differential dynamics of stress granule formation between proliferating, replicative senescent and stress-induced premature senescent cells, it was necessary to establish whether stress granule formation occurred as an initial response to ionising radiation exposure. It is becoming increasingly established that stress granules act as a nexus for several signalling pathways (Kedersha et al., 2013), and their formation has recently been linked to inhibition of the mTOR (Thedieck et al., 2013; Wippich et al., 2013) and JNK pathways (Arimoto et al., 2008), as well as alteration of RhoA/ROCK1 signalling (Tsai & Wei, 2010). It is therefore possible that the formation of stress granules in response to ionising radiation may induce alterations in signalling that are responsible for the abrogated stress granule formation observed in SIPS cells.

A number of studies have demonstrated that exposure to non-ionising radiation such as UV results in the cytoplasmic shuttling of established stress granule components such as hnRNP A1 and B1 (Guil, Long, & Cáceres, 2006; van der Houven van Oordt et al., 2000), and the subsequent formation of *bona fide* stress granules albeit with non-canonical disassembly kinetics (Moutaoufik et al., 2014). However, no published work has yet demonstrated such cytoplasmic shuttling or stress granule assembly in response to exposure to ionising radiation (Haley et al., 2009), leading some to conclude that stress granules do not form in response to ionising radiation exposure and to categorise stresses depending on their capacity to induce stress granules (Arimoto et al., 2008; Takahashi et al., 2013). Despite the lack of evidence supporting radiation-induced stress granule formation, it remained important to examine their potential role in the radiation response, as stress granule induction appears to differ depending on a number of factors including the cell strain examined and the stress itself; this phenomenon is exemplified by the inconsistent results regarding the extent to which stress granules form in response to hydrogen peroxide treatment, wherein some papers observed stress granule formation (Emara et al., 2012; Isabelle et al., 2012) where others did not (Takahashi et al., 2013). It is therefore possible that, although previous studies did not observe radiation-induced granules, their

formation could still occur albeit under non-canonical conditions. Furthermore, recent research examining post-translational protein modification in response to ionising radiation found that a number of proteins involved in stress granule formation, including the Initiation Factor EIF5A, underwent acetylation following exposure to a 2 Gy dose, and further detected an upregulation in the histone acetyl transferase KAT2B (Barjaktarovic et al., 2015). Considering KAT2B has previously been implicated in stabilising stress granules following formation

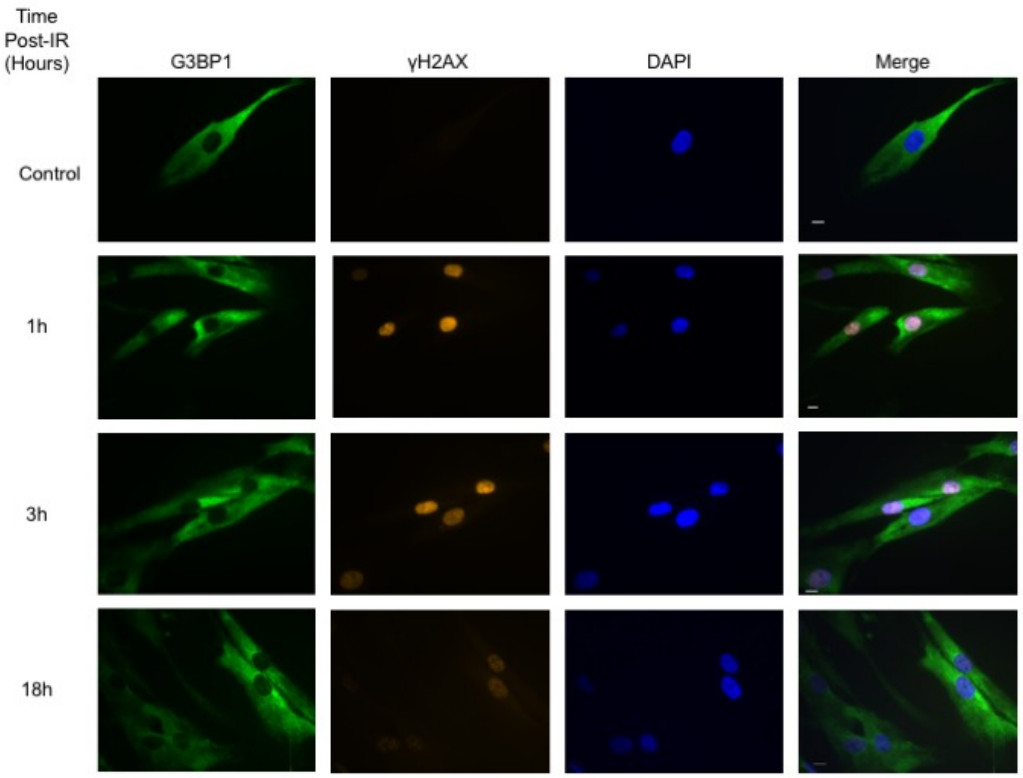

Figure 8. Initial characterisation of stress granule formation in response to 10Gy X-rays. Fluorescent microscope image (40x) showing a large increase in phosphorylated H2AX (TRITC), but no concordant G3BP1 (FITC)granulation. Nuclear stain is DAPI. Scale bar=10μm.

In order to determine the extent to which ionising radiation doses induce stress granule formation, the response of the DD1 cell strain to ionising radiation was characterised through fluorescence microscopy, as discussed in Materials and Methods, prior to more in-depth analysis through confocal microscopy. Cultures of DD1 fibroblasts were fixed 1h and 3h after exposure to 10 Gy of X-rays. Coverslips were then stained with an antibody for the stress granule marker G3BP1, and with an antibody for the DNA-damage response marker γH2AX. As seen in Fig. 8, exposure to a 10 Gy dose of ionising radiation did not result in the formation of G3BP1-

containing stress granules either 1h or 3h post-irradiation despite a significant increase in the number of γH2AX foci. This indicates that stress granule assembly is not an immediate response to X-ray irradiation, and further seems to suggest that stress granules do not form indirectly as a result of DNA damage response signalling or of an increase in reactive oxygen species, both of which would occur following irradiation. This result is contrary to a previous study examining cellular stress responses activated following UV exposure, wherein stress granules were found to form as early at 1h post-irradiation and peak after 4h, playing a crucial role in promoting cell survival (Pothof et al., 2009). This observed contrast between a concerted granule formation in response to UV radiation, and lack thereof following X-ray exposure, is likely the result of differential responses to different forms of ionising radiation, but suggests that stress granule formation is not a component of the DDR itself but may complement this response under certain conditions to help enhance survivability.

Whilst the majority of studies examining stress granule assembly detect their formation within the first hour after stress induction, a recent study using non-ionising radiation found that stress granules could form up to 18h following exposure to stress (Moutaoufik et al., 2014). Whilst this study utilised UV radiation as the class of ionising radiation, which as shown above can induce a different stress response in comparison to X-ray exposure, the finding of stress granules forming at later times following exposure lead to the possibility that X-rays may not induce stress granules in the short term, but may instead produce granules with a slight delay, potentially to aid in promoting survival and recovery only once the initial DNA damage was repaired. In order to determine whether such a delay in stress granule assembly was occurring in response to ionising radiation, DD1 cells were irradiated at a dose of 10 Gy and analysed 18h post-irradiation. However, the localisation of G3BP1 remained diffuse throughout the cytoplasm with no discernable difference from unirradiated cells (Fig. 8). The absence of stress granules after 18h was particularly notable considering the γH2AX foci had significantly decreased in number by this point in time. This suggests that, whilst the DNA damage response was still ongoing in irradiated cells with some foci likely to remain persistent as observed previously

(Fumagalli et al., 2012; Marková et al., 2011), the majority of DNA repair had occurred without the concordant assembly of stress granules in the cytoplasm. This further suggests that the DNA damage response itself does not require stress granules to function, instead suggesting that the UV-induced granules seen by Pothof et al. (2009) were additive in nature and limited to a specific type of irradiation, as opposed to being a key constituent of the universal DNA damage response.

Whilst these results indicate that a 10Gy X-ray does not induce formation of G3BP1-containing granules, it remained important to consider the possibility that dosimetry could play an important role in ionising radiation-induced stress granule formation. An increasing amount of evidence is now emerging that several responses to ionising radiation exposure differ in a nonlinear fashion dependent on dose, with the extent of mitochondrial protein import decreasing then subsequently increasing with progressively higher doses (Pandey et al., 2006), and with low-dose ionising radiation resulting in the formation of a proportionally greater number of DNA strand breaks compared with higher doses (Rombouts et al., 2013). Furthermore, DNA repair mechanisms, examined using γH2AX foci formation as a proxy, were more persistent in lens epithelial tissues following low-dose irradiation than following high doses, suggesting that cells exposed to lower doses experience a delay in the repairing of DNA breakages (Markiewicz et al., 2015). These studies indicate that a variety of cellular responses, including those associated with the repair and damage mitigation, do not follow a linear trend against dose and instead display more complex patterning likely brought about by the interplay between several different signalling pathways. Considering these complex trends and differential responses are becoming increasingly apparent, and further considering that low-dose exposures appear to require a proportionally greater and more sustained repair programme than in higher-dose counterparts (Markiewicz et al., 2015), it remained possible that low doses of ionising radiation could induce the formation of stress granules, but that this formation was lost with increasing doses. To test this hypothesis DD1 cells were cultured on coverslips and irradiated at 0.28 Gy, which falls below the currently accepted threshold for low doses, and at 2.26 Gy which, whilst above the threshold for low doses, still falls beneath the LD_{50} values for whole-body exposure calculated

in previous studies, which fall between 3-4.5 Gy (Mole, 1984; Strom, 2003), and therefore represents an intermediate value between low and high doses.

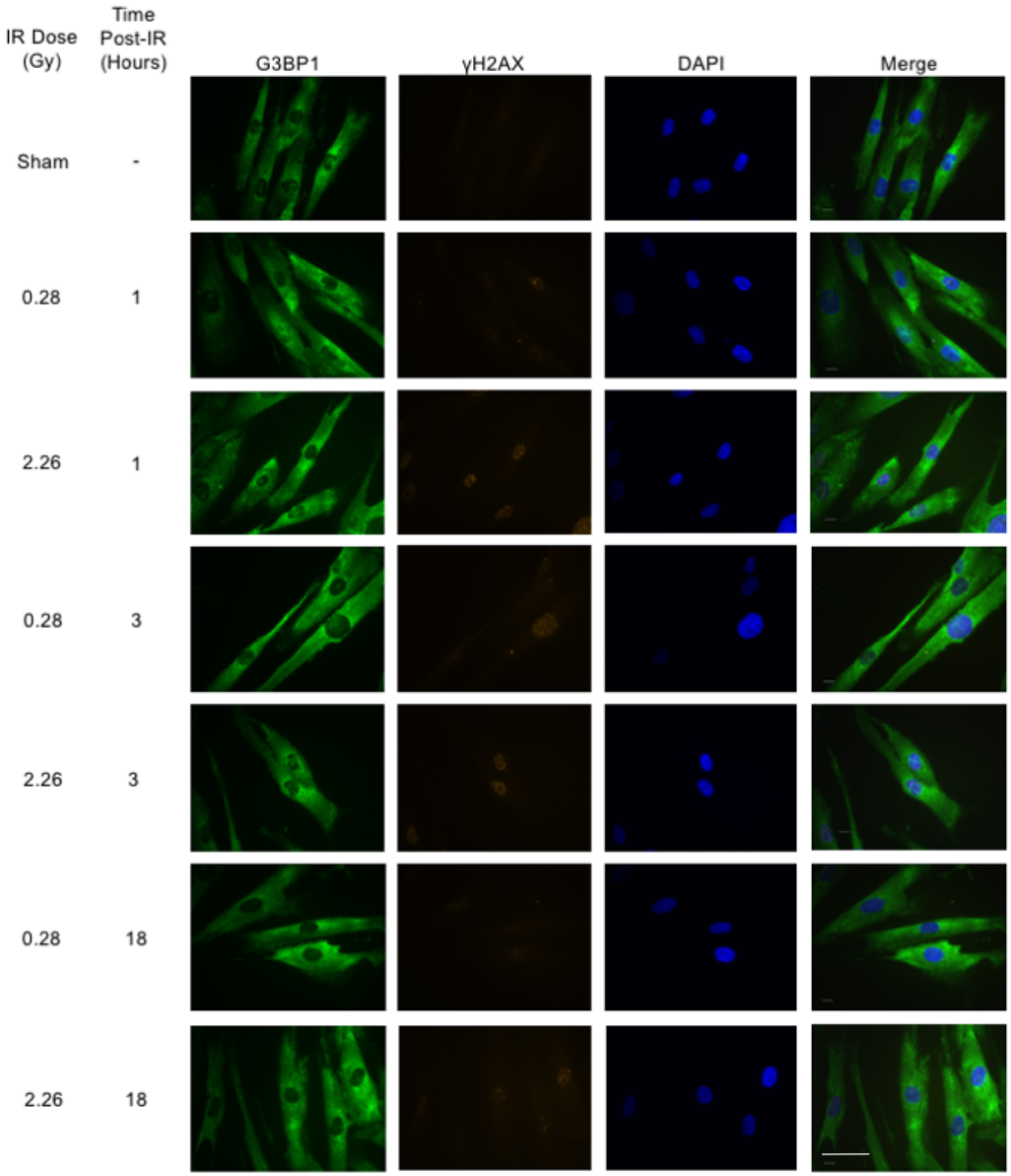

Figure 9. Low- and mid-dose ionising radiation do not induce stress granules. Fluorescence microscope images of phosphorylated H2AX (TRITC) and G3BP1 (FITC) at 40x magnification. Nuclear stain is DAPI. Scale bar=10μm.

Immunofluorescent analysis of γH2AX foci in stained samples exposed to the above three doses was used to validate the success of cell exposure to different doses. Concordant with exposure to lower doses of radiation, the number of foci per nucleus was visibly lower in both the low-dose and mid-dose exposed cultures

compared to those exposed to 10 Gy, as seen in Fig. 9, indicating a less extensive DNA damage response due to fewer DNA strand breaks. Considering the number of γH2AX foci formed per cell has been used in a number of previous studies to quantify radiation doses, the differential foci number observed in these irradiated DD1 cells acts as a robust indicator that cells have been exposed to different doses. With this confirmed, staining against G3BP1 was performed to determine to what extent low- and mid-dose radiation exposure resulted in short-term formation of stress granules. However, neither the 0.28 Gy nor 2.26 Gy-exposed cultures formed G3BP1-containing stress granules either 1h or 3h post-irradiation, as seen in Fig. 9. Whilst only three doses were examined, the initial assumption of this result is that stress granules are not induced by ionising radiation as a short-term stress response, regardless of dose. Furthermore, no G3BP1-containing stress granules formed 18h post-irradiation with either 0.28 Gy or 2.26 Gy, indicating that a delayed stress granule formation also seems unlikely following X-ray exposure. This result is in contrast to previous observations of UVC-induced stress granules (Moutaoufik et al., 2014), and therefore highlights a potential further difference in the response of cells to differing wavelengths of electromagnetic radiation.

Whilst these results were consistent with previous reports referring to the lack of stress granule formation following ionising radiation exposure, the result itself remains perplexing considering the extensive body of literature demonstrating that stress granules form to counteract the deleterious effects of oxidative stress. When further considering that a major cellular effect of ionising radiation is the production of reactive oxygen species due to the lysis of water molecules, it would seem logical for cells to form granules as part of a stress response.

Prior studies suggested that stresses could be categorised depending on the prevailing cellular response pathways, primarily whether a cell attempts to mitigate damage to survive or to activate apoptotic pathways to bring about cell death. Although emerging evidence suggests that ionising radiation can also induce survival pathways through Akt signalling in T-cells (Cataldi et al., 2009), the primary response to ionising radiation is the induction of apoptosis following both low- and high-dose

irradiation. Considering arsenite-induced stress granules have previously been shown to modulate MAPK signalling to inhibit apoptosis (Arimoto et al., 2008), providing an example of a survival mechanism halting cell death pathways, it is possible that signalling pathways could be induced following an apoptosis-inducing stress to inhibit stress granule formation.

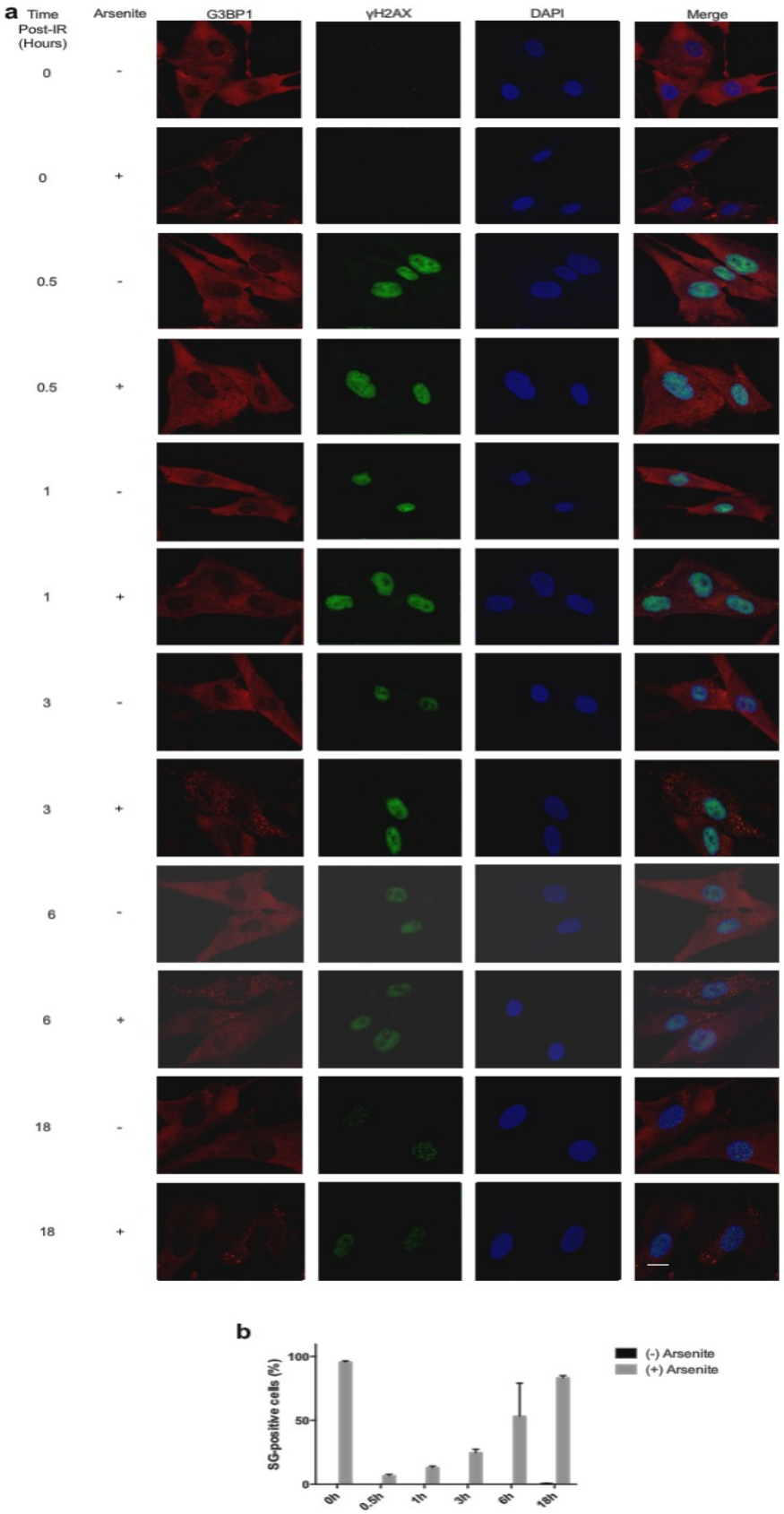

Figure 10. Ionising radiation ablates the granule-forming potential of fibroblasts. Confocal images of DD1 fibroblasts stained for phosphorylated H2AX (AF488) and G3BP1 (Cy3) before and after exposure to 10Gy X-ray radiation and/or sodium arsenite, at 63x magnification. Nuclear stain is DAPI. Scale bar=10μm.

Whilst the previous observations suggested that stress granules were not formed as part of either a short- or mid-term radioprotective response to X-rays, and therefore an early stress granule formation did not explain the abrogation of granule formation in stress-induced senescent cells, these results did not preclude the possibility that exposure to ionising radiation could in itself affect the capacity for cells to form granules. In order to test this, cells were exposed to 10Gy of X-rays, and were challenged with arsenite 30 mins, 1h, 3h, 6h and 18h post-irradiation to determine the number of granules formed under these conditions, as well as the percentage of cells capable of forming granules. In the first such experiment, cells were stained with antibodies for G3BP1 and γH2AX. This allowed granule activity to be assayed across a variety of time points, but also confirmed the efficacy of irradiation through the strength of γH2AX staining and demonstrates that images are taken at different points in time due to the progression of foci assembly and dispersal than can be seen across the time points. As soon as 30 minutes after exposure to 10Gy of ionising radiation, stress granule formation in response to arsenite treatment was almost entirely disrupted, with only a very small number of cells staining positive for granules containing G3BP1 (Fig. 10). Furthermore, any cells that did form granules indicated a much lower potential for formation, with only a few such granules forming per cell compared to the much larger numbers observed in unirradiated cells exposed to arsenite. This impaired ability to form granules persisted in the short-term, with cells after both 1h and 3h also showing significantly lower percentages of granule-positive cells. However, this inhibition of stress granule formation appeared to remedy over time, as indicated by the increase in the percentage of granule-positive cells with each successive time point post-irradation. The high variability in proportions of granule-positive cells following 6h (Fig. 10B) likely reflects a slight stochasticity in the recovery of normal granule function. However, challenging cells with arsenite 18h post-irradiation still did not result in as high a percentage of cells forming granules as in unirradiated cultures, with only 83% of cells staining positive for G3BP1-containing granules compared to 95% in sham-irradiated cultures. Therefore, whilst this inhibition of granule formation appears to be most pronounced immediately following radiation exposure, it appears that irradiated cultures contain a proportion of cells which require a greater amount of time to recover their granule-

forming potential, and may continue to lack this granule-forming capability over sustained periods. The extent to which this is the case would require further study with a greater number of time points, such as 48h, to determine whether there is any difference from unirradiated samples by this time.

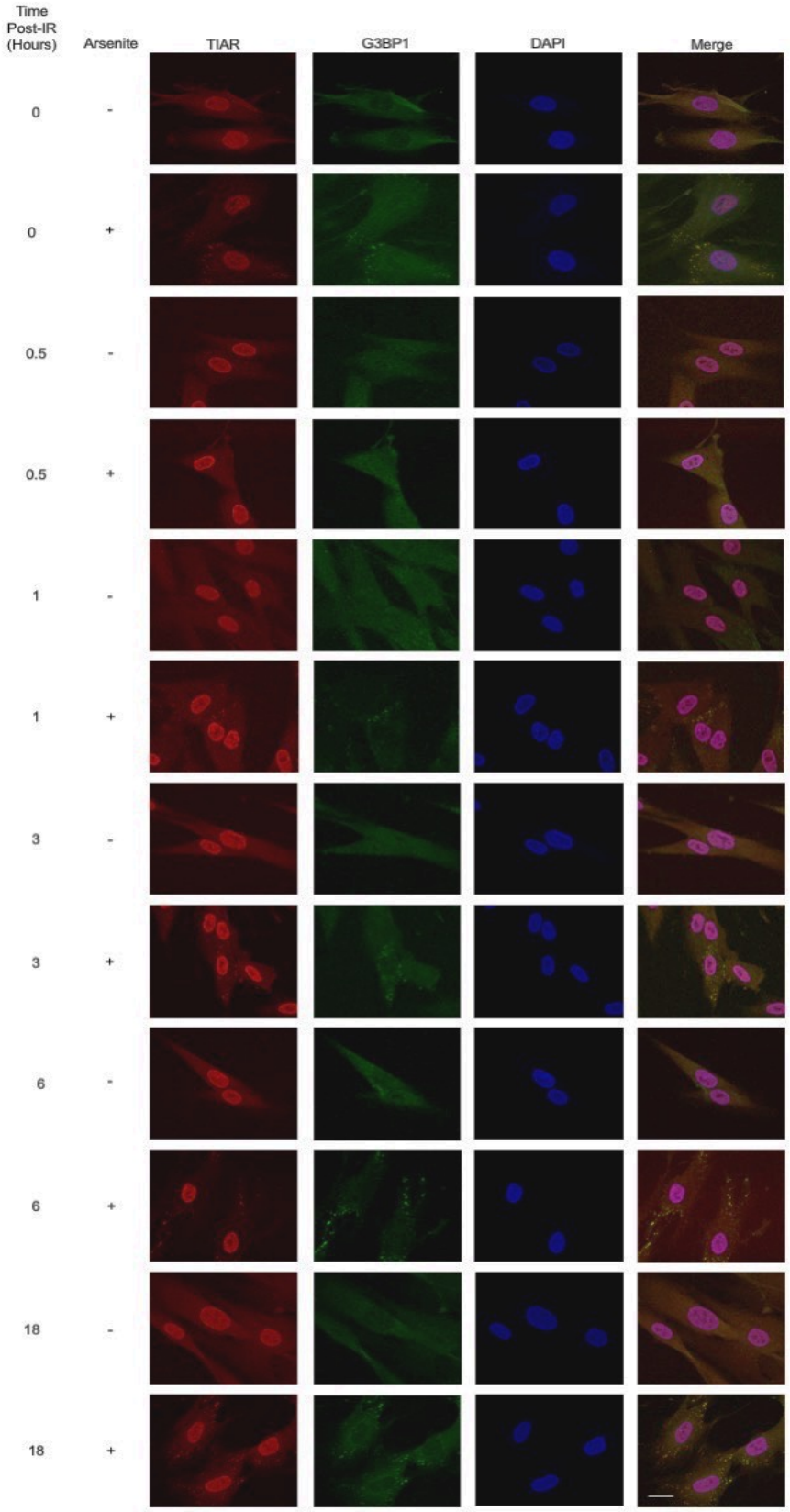

Figure 11. Neither G3BP1 nor TIAR assemble into stress granules immediately following ionising radiation and sodium arsenite. Confocal images of TIAR (Cy3) and G3BP1 (AF488) at 63x magnification. Nuclear stain is DAPI. Scale bar=10μm.

However, while these images do suggest a strong inhibition of G3BP1-containing granule formation, there was always a possibility that stress granules still formed through other mechanisms but that G3BP1 was not included. It has been previously established that G3BP1 can play other roles following ionising radiation exposure, such as being recruited to nuclear bodies which regulate the positioning and post-translational modification of a number of nuclear factors (Dellaire & Bazett-Jones, 2004; Liu et al., 2010), and could therefore be the case that G3BP1 was excluded from granules to allow for functions elsewhere in stress responses to occur. In order to assay whether this was the case, a G3BP1-TIAR co-stain was performed. As seen in Fig. 11, TIAR-containing granule formation was also decreased following irradiation, and the only granules that formed in cells contained both G3BP1 and TIAR, indicating that TIAR did not associate with any G3BP1-negative granules following ionising radiation. This further suggests that stress granules in their entirety were abrogated as a short-term effect, as opposed to the exclusion of one factor. However, as with the G3BP1-γH2AX staining, there appeared to be a recovery in terms of stress granule potential beginning at approximately 3h post-irradiation. Therefore, whilst it does appear to be the case that exposure to X-rays antagonises stress granule formation in DD1 cells in the short-term, this observed recovery of the majority of cells by 18h post-irradiation is inconsistent with the pronounced lack of stress granules in arsenite-treated cells 14 days post-irradiation. As a result, it is likely that other mechanisms are at play that result in this loss of granule formation, which may be related to differential signalling pathways activated during the development of a premature senescent phenotype compared to a replicative senescent onset. Were this to be the case, this impeded granule formation in response to stress would only be observed several days following the irradiation.

Canonical Wnt Signalling Activation Does Not Occur Following Ionising Radiation in Fibroblast Cultures

Whilst the discovery that a short-term response to X-ray exposure is the loss of stress granule-forming capabilities in the DD1 cell strain does not seem to provide a direct explanation for the lack of granules in stressed premature senescent cells due to the differences in time post-irradiation between these two results, the finding is still a novel result that merits further investigation. An important question to consider is how a disruption of stress granules could be brought about through known responses to X-ray exposure. The knockdown of G3BP1 using RNA interference reduced the number of granule-positive cells in the work of Matsuki et al. (2013). Whilst the level of impairment the authors observed was not as pronounced as in the present study, this could have been due to differences in cell type as their studies were carried out in embryonic liver cells as well as in HeLa cells. It therefore remained possible that G3BP1 protein levels were decreased as an early response to ionising radiation, and that fewer cells were able to nucleate stress granules as a result. Western blot analysis of whole cell lysates with a G3BP1 antibody was performed to test this possibility. However, G3BP1 protein levels remained relatively consistent between unirradiated and irradiated samples at 1h and 3h post-irradiation, indicating that G3BP1 degradation or downregulation was not responsible for the decrease in granule formation in these cells (Fig. 12). Furthermore, a larger G3BP1 immunoblot 18h following irradiation may indicate that G3BP1 upregulation occurs as a radiation response, although it remains difficult to draw definitive conclusions from this data in the absence of either technical or biological replicates.

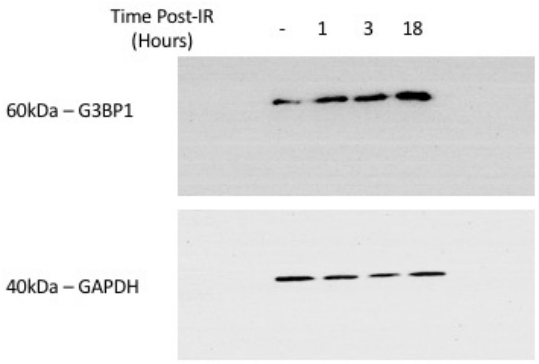

Figure 12. G3BP1 expression is not significantly altered following ionising radiation exposure. Western blot for G3BP1 following exposure to 10Gy X-ray radiation. GAPDH probing confirms equal loading of samples.

Although a number of proapopotic and prosurvival signalling pathways are activated in response to ionising radiation (Valerie et al., 2007), few of these have been observed to directly influence the formation of stress granules. Whilst the JNK pathway has previously been implicated in the localisation of the neuropathy-associated protein TDP-43 to stress granules, these granules were still able to form following JNK inhibition, suggesting that this pathway is not likely to play a role in granule formation itself (Meyerowitz et al., 2011). Furthermore, while the same study did show that inhibition of both the ERK1/2 and p38 pathways abrogated granule assembly, both of these pathways consistently show activation following irradiation (Jung et al., 2007; Wang et al., 2005; Wang, Liu, & Zhou, 2011), and would therefore be expected to either have no effect on granule dynamics or to enhance their formation and therefore seem unlikely to play a role in any putative blockage of stress granule activity.

Investigations in a number of cell lines have recently revealed that the Wnt signalling pathway can also be activated following irradiation, and studies in cancer models suggest that increased canonical Wnt signalling, as determined through

quantification of β-catenin expression, is correlated with increased radioresistance, whilst inhibition of Wnt signalling results in a concomitant reduction in cell viability post-radiation (Asuthkar et al., 2012; Kim et al., 2012). A number of protein components of both the canonical and non-canonical Wnt signalling pathways were also found to be acetylated following irradiation, further suggesting that modulation of the Wnt pathway is an early cellular response to exposure to ionising radiation (Barjaktarovic et al., 2015). Further to being identified as an increasingly important radiation-associated pathway, the Wnt pathway is also capable of antagonising stress granule formation through sequestration of the stress granule protein G3BP1 by Dishevelled-2 (Dvl-2), preventing it from nucleating other stress granule proteins (Sahoo et al., 2012). Wnt signalling is therefore an interesting candidate for further investigation in the context of the short-term radiation response, particularly in a fibroblast cell line, considering most work on Wnt in the context of radiation has used cancer cell models, and it was hypothesised that the activation of Wnt signalling following exposure to ionising radiation could result in the blockage of stress granule formation through a Dvl-2-dependent mechanism.

If the activation of Wnt signalling is involved in the stoppage of stress granule formation in response to ionising radiation then it would be expected that, following ionising radiation, the level of Wnt activation would correspond to the decrease in stress granule formation, most likely through an immediate activation that begins to subside after the first 3 hours. In order to determine whether this was occurring, cells were exposed to 10Gy of X-ray radiation prior to immunostaining with a β-catenin antibody. β-catenin is a well-established component of Wnt signalling (Kikuchi, 2003; MacDonald, Tamai, & He, 2009), and a major transducer of signals from the plasma membrane during the canonical Wnt pathway. When Wnt signalling is inactive, cytoplasmic β-catenin interacts with glycogen synthase kinase 3β (GSK3β) in a complex with an axin scaffold and adenomatous polyposis coli protein (Metcalfe & Bienz, 2011). This complex facilitates the phosphorylation of the β-catenin N-terminus by GSK3β, which in turn results in the ubiquitination of β-catenin via the F-box protein FWD1 and its subsequent degradation by the proteasome (Kitagawa et

al., 1999). Therefore, under normal conditions, β-catenin is cytoplasmic and maintained at low concentrations. However, upon binding of Wnt to the Frizzled transmembrane receptor and associated proteins, members of the Dishevelled protein family are recruited to the Frizzled complex (Gao & Chen, 2010; Wong et al., 2003) where they block the phosphorylation of β-catenin by GSK3β (Fiedler et al., 2011), inhibiting its degradation. As a result, β-catenin accumulates in the cytosol and is translocated into the nucleus, where it regulates binds to transcription factors including Lymphoid Enhancer Factor 1 to modulate gene expression (Eastman & Grosschedl, 1999). Considering this canonical Wnt pathway relies on the translocation of hypophosphorylated β-catenin to function, immunofluorescent probing would reveal a nuclear localisation for β-catenin following Wnt signalling. This staining has been used in several previous studies as a marker for canonical Wnt activation, and therefore was adopted for use in this study (Liu et al., 2011; Xie et al., 2008).

In order to quantify the extent of Wnt signalling activation through β-catenin accumulation, the colocalisation of β-catenin with the nuclear marker DAPI was examined. In sham-irradiated control cells, no β-catenin nuclear fraction was observed in any cell, indicating that under normal conditions, Wnt signalling was not active in DD1 cultures. However, the same result was observed in cells that were fixed 30 mins, 1h, 3h, 6h or 18h post-irradiation (Fig. 13). This result suggests that β-catenin accumulation was not occurring at any of these time

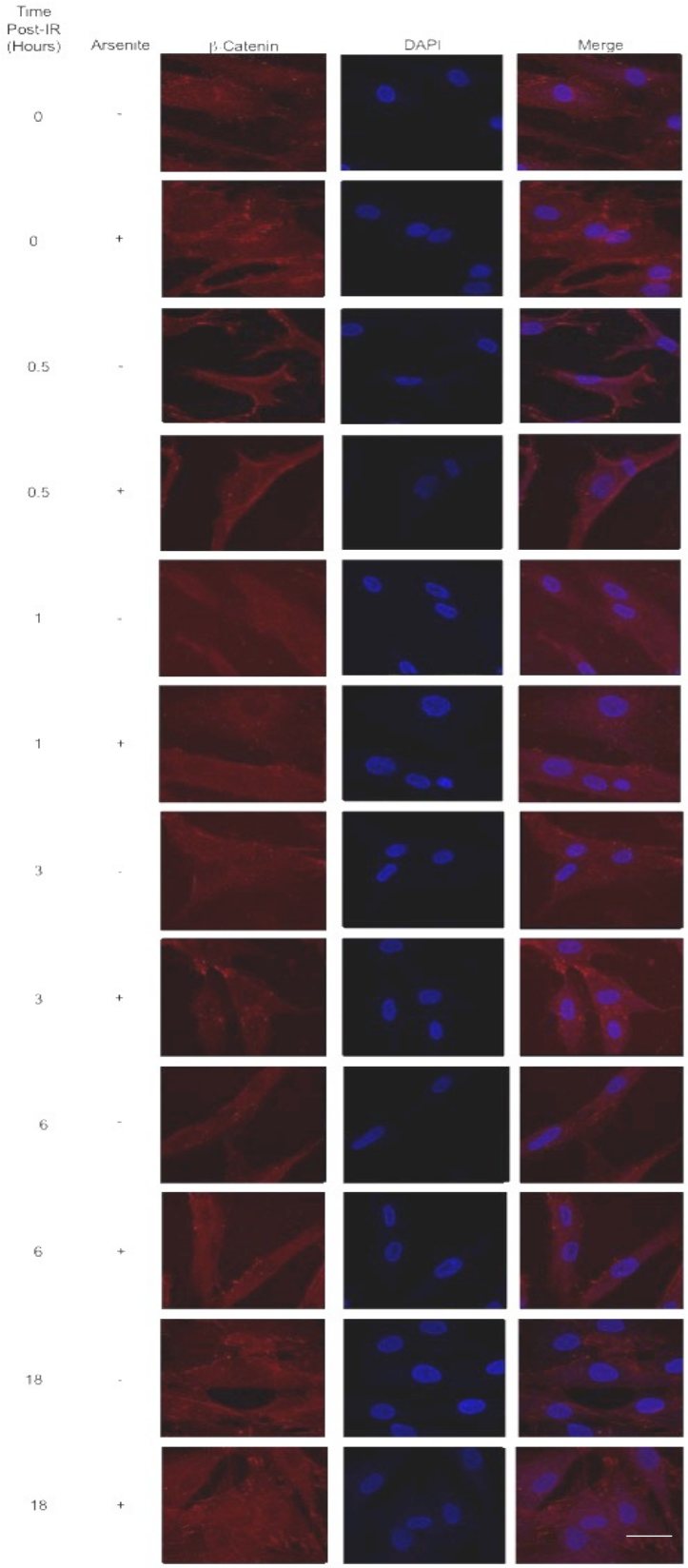

Figure 13. Wnt signalling is likely not activated in response to X-rays. Confocal image of fibroblasts stained with β-catenin (Cy3) and DAPI following exposure to 10Gy X-rays at 63x magnification. Scale bar=10μm.

points, and therefore that activation of canonical Wnt signalling was not utilised as a short-term response to ionising radiation in DD1 cells.

However, there still remained the possibility that a component of the response to ionising radiation could have primed the cells to activate the Wnt pathway upon the onset of a subsequent stress. This would result in cells only activating Wnt when necessary, preventing aberrant cell function until such a time. In order to test this, irradiated cells were treated with sodium arsenite and stained for β-catenin. However, exposure of irradiated cells to arsenite also had no effect on the proportion of cells containing nuclear β-catenin, with no nuclei appearing to contain β-catenin in culture at any time point. This result suggests that Wnt signalling is not activated in response to a second stress following ionising radiation, and is consistent with the existing literature that shows that a variety of stresses known to induce stress granule formation, including sodium arsenite and hydrogen peroxide, negatively modulate the canonical Wnt pathway through the downregulation of β-catenin in a number of embryonic stem cell lines (Hong & Bain, 2012; Shin et al., 2004), as well as through the diversion of β-catenin away from Wnt-associated transcription factors (Almeida et al., 2007), alongside observations in neuroblastoma cultures that Wnt pathway member overexpression provides a protective effect against such chemicals (Zhang, Bahety, & Ee, 2015).

Together, these results appear to suggest that the canonical Wnt pathway is not activated following X-ray exposure in DD1 cells, and furthermore is not activated by exposure to sodium arsenite in either irradiated or unirradiated cultures. However, it should be noted that several caveats related to both experimental design and to the nature of the Wnt signalling pathway render this result ultimately inconclusive. Firstly, the Wnt signalling pathway is not limited entirely to β-catenin dependent pathways. Several non-canonical Wnt pathways exist which utilise crosstalk with other signalling pathways in order to achieve a result independent of β-catenin accumulation, including through cooperation with JNK signalling via activation of

small GTPases such as Rho and Rac (Rao & Kühl, 2010; Yamanaka et al., 2002), as well as through activation of calcium signalling (Dejmek et al., 2006). These non-canonical pathways have been shown not only to function separately from the canonical signalling, but are also capable of modulated β-catenin in both a positive and negative manner, including through promoting degradation through the proteasome (Mikels & Nusse, 2006; Topol et al., 2003). When investigating the mechanisms through which Dishevelled-2 was capable of repressing stress granule assembly, Sahoo et al. (2012) determined that in addition to canonical Wnt signalling resulting in the inhibition of granule formation, treatment of cells with Wnt5a, which activates the non-canonical Wnt signalling pathway (Grumolato et al., 2010), was also capable of producing a reduction in stress granule formation. As a result, the apparent lack of β-catenin translocation and accumulation in irradiated cultures does not preclude the possibility that non-canonical Wnt pathways were instead activated, bypassing β-catenin as a signalling molecule. Further tests for Wnt signalling activation would confirm to what extent non-canonical activation was occurring, for example through the assaying of gene expression changes following irradiation using previously-developed luciferase assays (Ohkawara & Niehrs, 2011).

The second issue relating to the β-catenin nuclear translocation assay is the lack of a positive control. It is possible to induce the accumulation and subsequent nuclear import of β-catenin through the incubation of cell cultures with Frizzled ligands such as Wnt3a protein, which is readily detectable through immunofluorescence (Jiao Liu et al., 2011). However, considering neither arsenite treatment, ionising radiation exposure, or a combination of the two or lack thereof induced any discernable changes in β-catenin signal, it is not possible to determine whether it is the case that none of these treatments resulted in the activation of β-catenin signalling, or whether the immunofluorescence was unsuccessful, potentially due to the antibody being used, and that an alteration in β-catenin level and localisation was not detected as a result. In order to attempt to determine whether this was the case, immunoblotting for β-catenin levels in a whole cell lysate was attempted. Considering Wnt signalling results in the hypophosphorylation and stabilisation of β-

catenin, a common indicator for Wnt activity is an increased level of β-catenin protein (Gerlach et al., 2014; Khan, Bradstock, & Bendall, 2007). However, as seen in Fig. 14, this western blot was not successful, producing no detectable bands on gels despite the success of loading control detection. Whilst it could be argued that this lack of protein signal is due to the proteasomal degradation of β-catenin resulting in very small quantities present in lysates, it should be noted that previous publications still show a small amount of protein is present in cells even when Wnt is inactive. As a result, it is recommended that the experiment be repeated with a positive control in order to confirm the efficacy of the protocol and materials utilised before further conclusions are drawn from the data presented within this report.

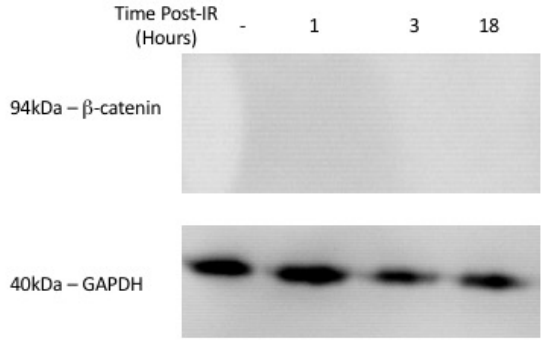

Figure 14. β-catenin is not detected during a western blotting attempt. GAPDH probing confirmed equal loading of samples, yet no β-catenin accumulation, or expression of any kind, is confirmed. Blot is representative of 3 attempts.

DISCUSSION AND CONCLUSIONS

The quantification of stress granule formation in replicative senescent cells by Lian & Gallouzi (2009) revealed that replicative senescence resulted in the production of a far higher number of stress granules per cell compared to in proliferating cells, and also demonstrated, using p21 mRNA as an example, that stress granule function also differed dependent on the proliferative capacity of the cell. However, to date this paper and the companion paper by Gallouzi (2009) have been the only ones to examine granule formation in the context of senescence.

In the present study, a validation of the works of Lian and Gallouzi (2009) was performed in a separate fibroblast cell line to confirm that greater numbers of stress granules form following oxidative stress in senescent cells. However, it is well-established that replicative senescence through telomeric shortening is not the only pathway through which a senescent phenotype can be established, as this growth arrest can also be established following the activation of oncogenes or upregulation of p21 due to oxidative stress (Muñoz-Espín & Serrano, 2014). Whilst this stress-induced premature senescence shares several hallmarks with replicative senescence (Toussaint et al., 2005), including increased sen-β-gal staining (Debacq-Chainiaux et al., 2009) and the adoption of post-mitotic morphotypes (Toussaint et al., 2000), more recent studies have revealed that differential gene expression influencing diverse cellular functions result in phenotypic differences between these two senescent programmes. (Aan et al., 2013; Pascal et al., 2005) As a result, it was not possible to conclude from the observations of Lian and Gallouzi (2009) that increase granule formation was universal across all senescence models. This project therefore also sought to expand the scope of the investigation to determine whether increased granule formation was also a hallmark of stress-induced premature senescence, or whether it remained limited to replicative senescence.

During this investigation the unexpected observation was made that, as opposed to a null hypothesis where stress-induced senescence showed no change in granule number from their proliferative counterparts, granule formation was almost entirely

abrogated in prematurely senescent populations, the opposite effect to that witnessed in replicative senescence. This finding builds on previous research demonstrating that these senescent programmes are biochemically distinct, and is the first time that such a difference has been observed to bring about a phenotypic change on the cellular level. Whilst this finding in itself is interesting in itself due to its underscoring of the contrasts between replicative and stress-induced senescence, it also helps to highlight other potential differences between these two systems.

Studies of cell death in senescent fibroblasts have previously shown that the onset of replicative senescence can result in the downregulation of apoptotic factors (Marcotte, Lacelle, & Wang, 2004; Murata et al., 2006), ultimately resulting in a resistance of senescent cells to apoptosis (Campisi & d'Adda di Fagagna, 2007). Considering stress granules are able to inhibit apoptotic cell death, both through the reduction of reactive oxygen species level (Takahashi et al., 2013) and through reduction in the activity of the mTOR signalling pathway (Thedieck et al., 2013), an increase in these granules would also likely result in the stoppage of apoptotis induction, and is therefore consistent with previous studies. However, the loss of granule-forming capacity in stress-induced senescent cells would presumably result in no decrease in mTOR activity, and a spike in reactive oxygen species levels in these cells. It is therefore possible that prematurely senescent fibroblasts are less resistant to apoptosis than their replicative senescent counterparts. This would likely represent a lower threshold of resistance to extracellular assaults by stress-induced senescent cells, and would be a broader and more significant difference between the two programmes. In order to test this, proliferating cells along with both senescent subtypes should be exposed to arsenite stress for 30 mins prior to incubation for a further 10h, and the extent of apoptosis in the population determined through the use of common apoptotic tests such as the DNA fragmentation assay (Gavrieli, Sherman, & Ben-Sasson, 1992), as well as through propidium iodide staining and flow cytometry (Riccardi & Nicoletti, 2006).

Another consideration is to what extent senescent stress granules can modulate the senescence-associated secretory phenotype. Following their examinations of stress

granule formation during senescence, Gallouzi (2009) postulated that the altered dynamics of stress granules, which found them assembling more readily in response to stress in senescent cells than in proliferating cells, when coupled with an alteration in function through differences in targets of translational regulation such as p21 (Lian & Gallouzi, 2009), could result in an exacerbation or increase in SASP, leading to the induction of senescence in nearby cells and increased level of chronic inflammation which is known to promote a number of age-related disorders such as angiogenesis (Tchkonia et al., 2013). However, recent studies of SASP regulation could suggest that, while stress granules may be able to modulate inflammatory secretion, their role may be the opposite to that predicted by Gallouzi (2009). A recent study by Laberge et al. (2015) identified the mTOR signalling pathway as a novel SASP regulator, through secretion of the pro-inflammatory cytokine IL1A, which has previously been shown to upregulate inflammation in neighbouring cells and induce senescence onset (Hubackova et al., 2012). A further study has also found that mTOR upregulates SASP by increasing the translation of the protein kinase MAPKAPK2, which in turn inhibits the degradation of SASP-related transcripts. These studies therefore identify the mTOR pathway as a crucial determinant of SASP levels in senescent systems, and the inhibition of mTOR through rapamycin treatment in both papers resulted in a decrease in SASP and a presumed ablation of its deleterious effects on tissues. However, treatment with the drug rapamycin can also have unintended side-effects, and has been implicated in the development of metabolic disorders such as diabetes due to the wide-ranging influences of the mTOR pathway (Li, Kim, & Blenis, 2014). Therefore, a more targeted therapy to downregulate mTOR activity in only senescent cells could result in decreased chronic inflammation, thereby alleviating some symptoms of age-related disease (Zhu et al., 2014).

Stress granule formation has recently been coupled to mTOR signalling in two main studies. Wippich et al. (2013) analysed the function of the stress-related kinase DYRK3, which regulates stress granule assembly, and found that, when active DYRK3 allows granule dissolution and simultaneously inactives mTOR inhibitors, suggesting a potential functional link between stress granules and mTOR activity. Thedieck et al. (2013) were able to further show that the mTOR complex 1 is sequestered in granules

by the component Astrin, resulting in an inhibition of mTOR signalling. Taken together, these papers suggest that stress granule condensation induces an inhibition of mTOR signalling in a multifaceted manner and therefore it is possible that stress granule formation may, contrary to the prediction of Gallouzi (2009), inhibit SASP through mTOR downregulation. This hypothesis that mild stress may play a beneficial role in senescence has some experimental evidence, as mild heat shock has previously been shown to induce a prolonged upregulation of heat shock proteins, assist in the maintenance of cellular function through late passage, and increase the capability of senescent cells to respond to stress (Fonager et al., 2002). Given that Lian & Gallouzi (2009) also showed that stress granule formation occurs more readily in response to stress stimuli in senescent cells than in proliferating cells, it could be possible to induce stress granules in only the senescent subpopulation through the administering of very low concentrations of stress granule-inducing compounds, presumably downregulating mTOR and therefore SASP without inducing the side-effects common to mTOR inhibiting drugs. A potential compound worthy of investigation is the granule-inducing pateamine A (Dang et al., 2006), which is already under consideration as a treatment for certain muscle-wasting disorders after having been shown to be non-toxic in low concentrations (Di Marco et al., 2012). However, it is recommended that a functional link between stress granules and mTOR should first be established in senescent cells prior to any clinically-orientated projects being undertaken.

However, although the experiments recommended above would provide interesting perspectives on how stress granule formation functions in the context of senescence, and more broadly how different senescent phenotypes differ both between themselves and in comparison to proliferating cells in terms of stress responses, it is also crucial to establish through which mechanisms the increased and decreased capacity for granule formation is brought about in replicative and stress-induced senescent cells, respectively. Whilst attempts were made to determine how this differential granule formation was established, none of the hypotheses examined were conclusive. Studies of immediate changes in stress granule formation following ionising radiation exposure, to determine whether the method of stress used to

induce senescence could in itself have brought about this granule disruption, did also reveal that a severe inhibition of stress granules occurs in the short term. However, this inhibition appeared to have been largely lifted by 18h post-irradiation, and therefore is unlikely to have been responsible for the lack of granules observed 14 days after exposure. It is more likely that the result of this granule inhibition occurs at a later timepoint, potentially during the onset of senescence, and a future study should perform a time course over 14 days to determine at which point stress granule potentially is lost in premature senescent cells. This would help to narrow down potential mechanisms that could be responsible for such an effect.

A further staining of senescent and proliferating cells with β-catenin, in order to attempt to determine whether the canonical Wnt pathway remained constitutively active in stress-induced cells – a result which would potentially explain the lack of granules formed – was ultimately unsuccessful due to photobleaching before a full image could be captured (data not shown). Although a qualitative analysis suggested that no nuclear staining had occurred, as discussed before there were still concerns relating to the quality of the β-catenin antibody, which were further strengthened by this bleaching, and a repeat of this immunofluorescent analysis is recommended to determine whether Wnt antagonisation of G3BP1-dependent granule formation is occurring. However, considering Wnt signalling is primarily associated with an increase in proliferation (Masckauchán et al., 2005; Pei et al., 2012) and is also downregulated in replicative senescence (Ye et al., 2007), it seems unlikely that Wnt activation will be identified in stress-induced senescent cells.

Furthermore, an initial examination of the vimentin networks in senescent cells showed that, in proliferating cells, stress granules appeared to colocalise to some extent with vimentin filaments following arsenite treatment. However, considering alterations to the vimentin networks occurred in both replicative and stress-induced senescent cells, likely as a result of changes in morphotype associated with senescent onset (Dumont et al., 2000), it seems unlikely that the vimentin cytoskeleton is a primary determinant of stress granule formation potential, as the difference between the two senescent networks is unlikely to be sufficient to induce the

wholesale loss of granules observed in Fig. 7. Furthermore, without any data from experiments analysing how the disruption of the vimentin cytoskeleton affects granule assembly, any potential mechanism involving vimentin remains groundless. However, this potential interaction between vimentin and stress granules does merit further investigation, as no such link has been determined previously, and it could be the case that alterations to the vimentin network ultrastructure play secondary roles in assembly of granules that could allow us to better understand their function or positioning. In order to do so, it is recommended that GFP-vimentin be transfected into cells to allow for live imaging to examine whether granules traffic or aggregate along vimentin filaments. Furthermore, considering the established role of microtubules in RNA granule formation (Ivanov et al., 2003), live imaging with an expressed fluorescently tagged tubulin could also provide further insight into whether microtubules are affected by senescence onset.

However, whilst many of the proposed experiments would provide insights into mechanisms influencing granule formation and the differences observed between proliferating and senescent stressed fibroblasts, it remains difficult to determine which hypotheses possess the most merit in explaining loss of granule-forming capacity during premature senescence. Perhaps an initial experiment to perform would be to induce senescence through the use of other subcytotoxic stresses, such as hydrogen peroxide (Kiyoshima et al., 2012; Pedro de Magalhães et al., 2004), followed by stress induction, to determine whether all cases of premature senescence result in the stoppage of granule formation or whether this is limited to radiation-exposed senescent cultures. Further experiments could be to use a variety of stresses such as heat shock or peroxide treatment to attempt granule induction, again to determine to what extent the loss of granules is universal as opposed to situational, as different stresses are known to induce granules with subtly different compositions which may affect to what extent their assembly is disrupted (Emara et al., 2012). These results would help to better conceptualise stress granule abrogation in stress-induced premature senescent cells, and – if any of these differing treatments resulted in no loss of granule formation – would also help to better

inform on potential mechanisms through which their assembly was inhibited in the present study.

The second novel finding of this research, that exposure to X-ray irradiation not only does not induce stress granules, but appears to actively antagonise their formation, is also highly interesting. This is firstly because this result is contrasted with other studies of UV irradiation-induced stress granules (Moutaoufik et al., 2014; Pothof et al., 2009), suggesting that exposure to different forms of ionising radiation can have contrasting effects on the cell. Considering this is not the first study to find differential responses to different ionising radiation forms (Allan & Fried, 1999), it is recommended, as a result, that greater care be taken when studying ionising radiation to ensure that data is not considered universal across all forms of ionising radiation, and this work also underscores the need to study radiation responses to all radiation subtypes. However, this research is also particularly interesting when contextualised with regards to known responses to X-ray exposure. X-rays have been well-characterised as activators of apoptosis across a variety of cell types (Jian et al., 2009; Nakano & Shinohara, 1994; Ortenzi et al., 2011), with this response likely occurring in order to minimise the risk of damaged DNA resulting in tumorigenesis in damaged cells. Stress granule formation is a known mechanism known to promote cell survival (Arimoto et al., 2008; Takahashi et al., 2013), and therefore their inhibition as an immediate response to X-rays would increase the likelihood of cells undergoing apoptosis. This research is therefore consistent with previous studies of cell death responses to X-ray irradiation, and provides a further putative mechanism through which this can occur, likely acting in an additive fashion to induce apoptosis. Whilst the mechanism through which this stress granule depletion is brought about remains unknown, the Wnt pathway remains a promising candidate due to its known modulation during ionising radiation, and due to previous observations that, upon pathway activation, the Wnt component Dvl2 can sequester G3BP1 (Bikkavilli & Malbon, 2011) and antagonise granule formation as a result (Sahoo et al., 2012). However, the capacity for G3BP1 to nucleate granules is not exclusively affected by Wnt, and therefore investigation of other pathways is also merited.

In particular, G3BP1 is reliant on dephosphorylation of the serine residue at position 149 in order to assemble granules (Tourrière et al., 2003). Therefore, it is possible that the removal of phosphate groups could be inhibited by an as-yet unidentified protein, or that a kinase activated as a result of X-ray radiation could phosphorylate G3BP1, therefore counteracting any dephosphorylation to effectively block granulation. Potential candidate kinases could be members of the mitogen-activated kinase (MAPK) family, whose signalling pathways are activated for short periods (less than 1h) following ionising radiation (Dent et al., 2003; Yacoub et al., 2001), are known to promote apoptosis (Wada & Penninger, 2004), and are inhibited by stress granules and therefore are already known to be linked (Arimoto et al., 2008). Furthermore, the short-term activation of MAPK pathways in response to radiation would explain why stress granule inhibition is only fully observed in the first 3 hours following exposure, as they would only be able to phosphorylate G3BP1 for a short period of time. All of these potential mechanisms would require further study in order to determine their validity, and it is possible that more than one such pathway is activated at once to provide redundancy in this inhibition.

Beyond mechanistic studies, it would also be interesting to determine whether the ablation of granule formation is linearly correlated to the radiation dose. Low-dose radiation is known to provoke a nonlinear response in many aspects, such as in the case of DNA damage foci persisting for comparatively longer in lens epithelial cells at lower doses than when exposed to high doses such as 10Gy (Markiewicz et al., 2015). It is therefore possible that the same nonlinear dosimetry is true of stress granule abrogation, and that even low doses are capable of eliciting the same response. A discovery that the same level of granule disruption can be induced through low doses would provide a potential mechanism through which apoptosis is activated early following radiation exposure (Furlong et al., 2013) despite not producing a great enough level of reactive oxygen species to cause direct damage to the cell (Smith, Willey, & Hancock, 2012), as it would indicate a lessened capability of these cells to cope with oxidative stress (Takahashi et al., 2013).

The results of the current study present two novel circumstances in which the assembly of cytoplasmic stress granules is inhibited despite cells being challenged with oxidative stress, and in doing so elucidate how cellular functions are altered in the contexts of proliferative arrest and responses to extracellular assault. Although the mechanisms through which this inhibition is established remain elusive, a number of proposals have been set forward through which these mechanisms could be determined, and further research should allow for the further narrowing of putative mechanisms to a subset of likely candidates based on analysis of further cellular alterations Subsequent studies to further characterise these changes to stress granules will therefore better integrate RNA granules into the responses of cells to stress and, more generally, to external cues.

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