

Senescent cells inhibit muscle differentiation via the SASP-lipid 15d-PGJ₂ mediated modification and control of HRas

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Abstract

Senescent cells, which are characterized by multiple features such as increased expression of Senescence-Associated β -galactosidase activity (SA β -gal) and cell cycle inhibitors such as p21 or p16, accumulate with tissue damage and dysregulate tissue homeostasis. In the context of skeletal muscle, it is known that agents used for chemotherapy such as Doxorubicin cause buildup of senescent cells, leading to the inhibition of tissue regeneration. Senescent cells influence the neighboring cells via numerous secreted factors which form the senescence-associated secreted phenotype (SASP). Lipids are emerging as a key component of SASP that can control tissue homeostasis. Arachidonic acid-derived lipids have been shown to accumulate within senescent cells, specifically 15d-PGJ₂, which is an electrophilic lipid produced by the non-enzymatic dehydration of the prostaglandin PGD₂. In this study, we show that 15d-PGJ₂ is also released by Doxorubicin-induced senescent cells as a SASP factor. Treatment of skeletal muscle myoblasts with the conditioned medium from these senescent cells inhibits myoblast fusion during differentiation. Inhibition of L-PTGDS, the enzyme that synthesizes PGD₂, diminishes the release of 15d-PGJ₂ by senescent cells and restores muscle differentiation. We further show that this lipid post-translationally modifies Cys184 of HRas in skeletal muscle cells, causing a reduction in the localization of HRas to the Golgi, increased HRas binding to RAF RBD, and activation of cellular MAPK-Erk signaling (but not the Akt signaling). Mutating C184 of HRas prevents the ability of 15d-PGJ₂ to inhibit the differentiation of muscle cells and control the activity of HRas. This work shows that 15d-PGJ₂ released from senescent cells could be targeted to restore muscle homeostasis after chemotherapy.

eLife assessment

This manuscript outlines an interaction between senescence-related 15d-PGJ₂ and the proliferation and differentiation of myoblasts, with potential implications for muscle health. This manuscript is **useful** in understanding the role of lipid metabolite 15d-PGJ₂ in myoblast proliferation and differentiation. However, in its current form, the manuscript is **incomplete** as there are several concerns in the statistical analysis, lack of clarity on the mechanistic details, and concerns about the use of an immortalized C2C12 myoblasts cell line to draw major conclusions related to senescence-associated secreted phenotype.

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Introduction

Senescent cells are important drivers of aging and damage-associated loss of tissue homeostasis (Childs et al., 2015). Anti-cancer chemotherapy presents an important context where treatment with chemotherapeutics such as Doxorubicin (Doxo) causes widespread cellular senescence which inhibits tissue homeostasis and regeneration, including in skeletal muscles (Francis et al., 2022). It has been shown that Doxo causes systemic inflammation and leads to the emergence of senescent cells across tissues (Di Leonardo et al., 1994; Hu and Zhang, 2019; Robles and Adami, 1998). Senescent cells negatively affect tissue homeostasis and regeneration by releasing factors including proteins like growth factors, matrix metalloproteases, cytokines, and chemokines, and small molecules like fatty acid derivatives (Campisi, 2005; Coppé et al., 2010; Dilley et al., 2003; Krtolica et al., 2001; Parrinello et al., 2005; Shelton et al., 1999; Yang et al., 2006). The release of these factors from senescent cells is called the Senescence-Associated Secretory Phenotype (SASP). It is expected that these SASP factors and their mechanisms of action will vary depending on cellular and tissue contexts. Identifying SASP factors and their underlying mechanistic targets will be critical for building an understanding of how senescent cells control tissue homeostasis (Coppé et al., 2010; Davalos et al., 2010). Lipids are a less explored family of SASP factors, and it is important to understand how they affect tissue regeneration (Hamsanathan and Gurkar, 2022). We have previously shown that senescent cells have increased intracellular levels of prostaglandin 15d-PGJ₂ (Wiley et al., 2021), a non-enzymatic dehydration product of prostaglandin PGD₂ (Shibata et al., 2002). In the context of skeletal muscle, PGD₂ and 15d-PGJ₂ have been shown to negatively regulate muscle differentiation via mechanisms that do not depend on a cognate receptor (Hunter et al., 2001; Veliça et al., 2010). Here we study the role of 15d-PGJ₂ as a member of the SASP and identify the mechanisms by which it might negatively affect muscle regeneration. 15d-PGJ₂ has been previously shown to covalently modify multiple proteins like MAPK1, MCM4, EIF4A-I, PKM1, GFAP etc. in endothelial and neuronal cells (Marcone and Fitzgerald, 2013; Yamamoto et al., 2011). 15d-PGJ₂ was shown to be covalently modifying HRas in NIH3T3, Cos1, and IMR90 cell lines (Luis Oliva et al., 2003; Wiley et al., 2021). We further studied HRas as an important target that might mediate the effects of 15d-PGJ₂ on muscle differentiation via covalent modification. We investigated HRas as a possible effector of 15d-PGJ₂ because (i) HRas belongs to the Ras superfamily of small molecule GTPases and is a known regulator of key cellular processes (Davis et al., 1983; Harvey, 1964; Kirsten and Mayer, 1967; Vetter and Wittinghofer, 2001). (ii) constitutively active HRas mutant (HRas V12) has been shown to inhibit the differentiation of myoblasts by inhibiting MyoD and Myogenin expression (Konieczny et al., 1989; Lassar et al., 1989; Olson, et al., 1987; Van Der Burg et al., 2007). (iii) Downstream signaling of HRas is important for muscle homeostasis as skeletal and cardiac myopathies are observed in individuals carrying constitutively active mutants

of HRas(Engler et al., 2021 [↗](#); Konieczny et al., 1989 [↗](#); Lee et al., 2010 [↗](#); Olson, et al., 1987; Scholz et al., 2009 [↗](#); Van Der Burgt et al., 2007 [↗](#)). HRas is highly regulated by lipid modifications, it undergoes reversible palmitoylation and de-palmitoylation at C-terminal cysteines, which regulate the intracellular distribution and activity of HRas(Gutierrez et al., 1989 [↗](#); Lu and Hofmann, 1995 [↗](#); Rocks et al., 2005 [↗](#)). In this study, we show that 15d-PGJ₂ is synthesized and released by senescent myoblasts upon treatment with Doxo. 15d-PGJ₂, taken up by the myoblasts, covalently modifies HRas at cysteine 184 and activates it. We also show that previously reported inhibition of differentiation of myoblasts by 15d-PGJ₂ depends on HRas C-terminal cysteines, notably cysteine 184. This study provides a mechanism by which prostaglandins secreted as SASP inhibit the differentiation of myoblasts, affecting muscle homeostasis in patients undergoing chemotherapy.

Results

Doxorubicin (Doxo) treatment induces senescence in mouse skeletal muscles and C2C12 mouse myoblasts

Doxorubicin-mediated DNA damage has been shown to induce senescence in cells(Di Leonardo et al., 1994 [↗](#); Hu and Zhang, 2019 [↗](#); Robles and Adami, 1998 [↗](#)). Therefore, we injected B6J mice intraperitoneally with Doxorubicin (Doxo) (5mg/kg) every 3 days for 9 days and observed induction of DNA damage-mediated senescence in hindlimb skeletal muscles (**Fig. S1A** [↗](#)). We observed an increase in the expression of p21 and increased nuclear levels of the DNA damage marker γH2A.X in mouse Gastrocnemius muscles (**Fig. 1A and B** [↗](#)). We also observed a significant increase in the mRNA levels of known senescence markers (p16 and p21), SASP factors (CXCL1, CXCL2, TNF1α, IL6, TGFβ1) in skeletal muscles of mice treated with Doxo compared to that of mice treated with saline (**Fig. 1C** [↗](#)). These observations suggest that there is induction of senescence in skeletal muscles of mice upon treatment with Doxo.

C2C12 cells have been shown to undergo senescence after DNA damage, as assessed by an increase in the levels of SA β-gal and known markers of SASP (IL1α, IL6, CCL2, CXCL2, CXCL10)(Moiseeva et al., 2022 [↗](#)). We treated C2C12 myoblasts with Doxo (150 nM) and observed a significant increase in the size of the nuclei (**Fig. 1D** [↗](#)), flattened cell morphology with an increase in the cell size (**Fig. 1E** [↗](#)), a significant increase in the mRNA levels of cell cycle inhibitor p21 and SASP factors IL6 and TGFβ (**Fig. 1F** [↗](#)), a significant increase in the protein levels of p21 (**Fig. 1G** [↗](#)), and an increase in the levels of SA β-gal (**Fig. 1H** [↗](#)) in C2C12 cells treated with Doxo. These observations suggest that C2C12 cells undergo senescence upon treatment with Doxo.

Doxo-mediated senescence induces synthesis and release of 15d-PGJ₂ in C2C12 myoblasts and mouse skeletal muscle

Synthesis of prostaglandins by senescent cells has previously been reported(Wiley et al., 2021 [↗](#); Wiley and Campisi, 2021 [↗](#)). Specifically, levels of PGD₂ and its metabolite 15d-PGJ₂ have been shown to be significantly increased in senescent cells. Therefore, we measured the levels of mRNA of enzymes involved in the synthesis of PGD₂/15d-PGJ₂ (PTGS1, PTGS2, and PTGDS), in the gastrocnemius muscle of mice after treatment with Doxo. We observed a significant increase in the mRNA levels of PTGS1, PTGS2, and PTGDS enzymes in the skeletal muscle of mice treated with Doxo (**Fig. 1C** [↗](#)). We also observed a time-dependent increase in the mRNA levels of PTGS1, PTGS2, PTGDS, and PTGES enzymes in C2C12 cells treated with Doxo compared to Day 0 (**Fig. 1I** [↗](#)). Expression of enzyme PTGES was elevated on Day 4, whereas the expression of Prostaglandin D synthase (PTGDS) increased only after Day 8, reaching maximum expression on Day 12. These observations suggest an increase in the synthesis of prostaglandins in senescent cells.

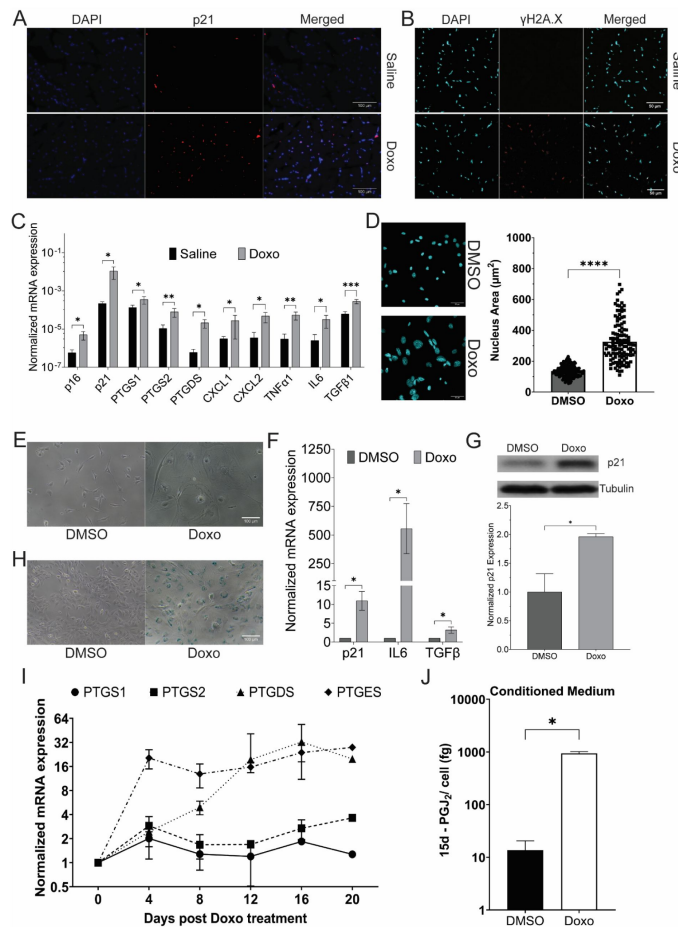


Figure 1.

Prostaglandin synthesis and release by Doxo-induced senescent cells inhibits myoblast differentiation.

- A. Expression and localization of tumor suppressor protein p21, measured by immunofluorescence, in hindlimb skeletal muscles of mice after 11 days of treatment with Doxo (5 mg/kg) or Saline.
- B. Representative confocal micrograph of expression of γH2A.X in the gastrocnemius muscle of mice treated with Doxo (5 mg/kg) or Saline.
- C. Expression of mRNAs of senescence markers (p16 and p21), SASP factors (CXCL1, CXCL2, TNFA1, IL6, TGFβ1), and enzymes involved in the biosynthesis of prostaglandin PGD₂/15d-PGJ₂ (PTGS1, PTGS2, PTGDS), measured by qPCR, in hindlimb skeletal muscles of mice after 11 days of treatment with Doxo (5 mg/kg) or Saline.
- D. A representative confocal micrograph and a scatter plot of the nuclear area of C2C12 myoblasts, measured by immunofluorescence, after 16 days of treatment with Doxo (150 nM) or DMSO.
- E. A representative widefield micrograph of cell morphology in C2C12 myoblasts after 16 days of treatment with Doxo (150 nM) or DMSO.
- F. Expression of mRNA of cell cycle inhibitor p21 and SASP factors (IL6 and TGFβ), measured by qPCR, in C2C12 myoblasts after 16 days of treatment with Doxo (150 nM) or DMSO.
- G. Expression of cell cycle inhibitor p21, measured by immunoblot, in C2C12 myoblasts after 16 days of treatment with Doxo (150 nM) or DMSO.
- H. Activity of Senescence Associated β-galactosidase (SA β-gal), measured by X-gal staining at pH~6, in C2C12 myoblasts after 16 days of treatment with Doxo (150 nM) or DMSO.
- I. Expression of mRNAs of prostaglandin biosynthetic enzymes, measured by qPCR, in C2C12 myoblasts after treatment with Doxo (150 nM) or DMSO.
- J. Concentration of 15d-PGJ₂ released from quiescent or senescent C2C12 cells.
- (Statistical significance was tested by the two-tailed student's t-test ns=p>0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001)

15d-PGJ₂ is a non-enzymatic dehydration product of PGD₂ (Shibata et al., 2002 [\[1\]](#)). We observed an increase in the mRNA levels of synthetic enzymes of 15d-PGJ₂ in senescent C2C12 cells. Therefore, we measured the levels of 15d-PGJ₂ released by senescent C2C12 cells using targeted mass spectrometry (Fig. S1C, D, E, and F). The concentration of 15d-PGJ₂ was quantified by monitoring the transition of the m/z of ions from 315.100 → 271.100 using a SCIEX 6500 mass spectrometer. We plotted a standard curve using purified 15d-PGJ₂ (Fig. S1F) to quantify the concentration of 15d-PGJ₂. We used the representative peaks from the conditioned medium collected from C2C12 cells incubated in 0.2% serum medium for 3 days (Quiescent cells) and C2C12 cells treated with Doxo (150 nM) (Senescent cells) to measure the concentration of 15d-PGJ₂ released by quiescent cells or senescent C2C12 cells. We observed a significant increase (~100 fold) in the concentration of 15d-PGJ₂ in the conditioned medium from senescent cells as compared to that in quiescent cells (Fig. 1J [\[1\]](#)). This suggests that senescent C2C12 cells release 15d-PGJ₂ in the medium.

Prostaglandin PGD₂ and its metabolites in the conditioned medium of senescent cells inhibit the differentiation of C2C12 myoblasts

15d-PGJ₂ (the final non-enzymatic dehydration product of PGD₂) has been shown to inhibit the differentiation of myoblasts (Hunter et al., 2001 [\[2\]](#)). We observed the release of 15d-PGJ₂ by senescent cells, showing that 15d-PGJ₂ is a SASP factor (Fig. 1F [\[1\]](#)). Conditioned medium of senescent cells inhibits the differentiation of myoblasts in myotonic dystrophy type 1 (Conte et al., 2023 [\[3\]](#)). Therefore, we tested whether 15d-PGJ₂, the terminal dehydration product of PGD₂, is required for the inhibitory effect of SASP on the differentiation of myoblasts. We treated C2C12 myoblasts with the conditioned medium of senescent cells or senescent cells treated with 30 μM of AT-56 (a well-characterized inhibitor of prostaglandin D synthase (PTGDS)) (Hu et al., 2021 [\[4\]](#); S. Hu et al., 2023 [\[5\]](#); Shunfeng Hu et al., 2023 [\[6\]](#); Irikura et al., 2009 [\[7\]](#)) and measured the differentiation of myoblasts by calculating the fusion index. We observed a significant decrease (~20%) in the fusion index of the C2C12 myoblasts treated with the conditioned medium of senescent cells (Fig. 2A [\[1\]](#)), suggesting that SASP factors decrease the differentiation of myoblasts. This decrease in the inhibition was rescued in myoblasts treated with the conditioned medium of senescent cells treated with AT-56 (Fig. 2A [\[1\]](#)). This suggests that prostaglandins PGD₂/15d-PGJ₂ released by senescent cells as SASP factors can inhibit the differentiation of myoblasts.

15d-PGJ₂ inhibits the proliferation and differentiation of mouse and human myoblasts

15d-PGJ₂ has been shown to affect the proliferation of cancer cell lines, both positively and negatively (Chen et al., 2003 [\[8\]](#); Choi et al., 2020 [\[9\]](#); Slanovc et al., 2024 [\[10\]](#); Yen et al., 2014 [\[11\]](#)). We measured the effect of 15d-PGJ₂ on the proliferation of C2C12 myoblasts. We treated C2C12 myoblasts with 15d-PGJ₂ (10 μM) or DMSO in DMEM 10% Serum medium for 72 hours and observed a significant decrease in the proliferation of C2C12 cells after treatment with 15d-PGJ₂ (Fig. 2B [\[1\]](#)). The doubling time of C2C12 cells was also increased upon treatment with 15d-PGJ₂ (57.24 hours) compared to DMSO (13.76 hours). This suggests that 15d-PGJ₂ decreases the proliferation of C2C12 myoblasts.

We measured the differentiation of C2C12 mouse and primary human myoblasts after treatment with 15d-PGJ₂. To rule out the toxic effects of 15d-PGJ₂ on cell physiology, we treated C2C12 cells with 15d-PGJ₂ (1 μM, 2 μM, 4 μM, 5 μM, and 10 μM) in the C2C12 differentiation medium, and measured the viability of cells after 24 hours of treatment, using an MTT viability assay. We judged that 15d-PGJ₂ was not cytotoxic up to 5 μM in the C2C12 differentiation medium (Fig. S2A [\[1\]](#)). Based on this, we treated differentiating myoblasts with 15d-PGJ₂ (1 μM, 2 μM, and 4 μM) for 5 days to measure the effects of 15d-PGJ₂ treatment on differentiation of myoblasts. We observed a dose-dependent decrease in the mRNA levels of MyoD, MyoG, and MHC in differentiating C2C12

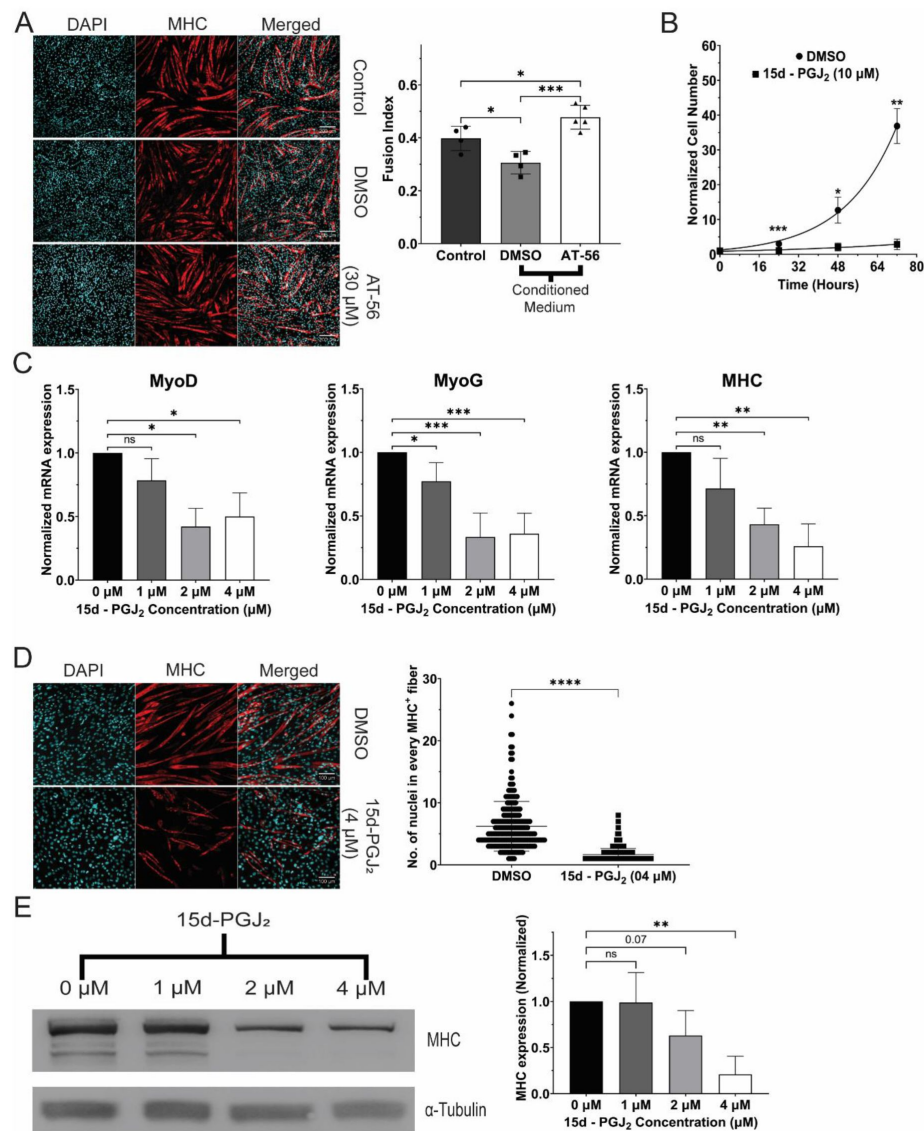


Figure 2.

15d-PGJ₂ inhibits differentiation of myoblasts.

A. Expression of MHC protein and the fusion of myoblasts in myotubes, measured by immunofluorescence, after treatment with conditioned medium of senescent cells treated with PTGDS inhibitor AT-56 (30 μ M) or DMSO

B. Normalized number of C2C12 myoblasts treated with 15d-PGJ₂ (10 μ M) or DMSO

C. Expression of mRNAs of markers of differentiation (MyoD, MyoG, and MHC), measured by qPCR, in C2C12 myoblasts treated with 15d-PGJ₂ (1 μ M, 2 μ M, or 4 μ M) or DMSO.

D. Expression of MHC protein and the fusion of myoblasts in syncytial myotubes, measured by immunofluorescence, after treatment with 15d-PGJ₂ (4 μ M) or DMSO.

E. Expression of MHC protein, measured by immunoblotting, in primary human skeletal myoblasts after treatment with 15d-PGJ₂ (1 μ M, 2 μ M, or 4 μ M) or DMSO for 5 days.

(Statistical significance was tested by the two-tailed student's t-test ns= $p > 0.05$, *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$)

cells after treatment with 15dPGJ₂ (**Fig. 2C**). There was a significant decrease in the no. of nuclei in individual MHC⁺ve fiber (~75%) in C2C12 cells treated with 15d-PGJ₂ (4 μM) compared to DMSO (**Fig. 2D**), suggesting a decrease in the fusion of myoblasts in myotubes. We also observed a dose-dependent decrease in the protein levels of MHC in differentiating primary human myoblasts upon treatment with 15d-PGJ₂ (**Fig. 2E**). Together, these observations suggest that 15d-PGJ₂ inhibits the differentiation of both mouse and human myoblasts.

Biotinylated 15d-PGJ₂ covalently modifies HRas at Cysteine 184

15d-PGJ₂ has been shown to covalently modify several proteins including p53 and NF-κB, which are involved in several key biological processes (Marcone and Fitzgerald, 2013). HRas was identified to be covalently modified by 15d-PGJ₂ at cysteine 184 in NIH3T3 and Cos1 cells (Luis Oliva et al., 2003). Therefore, we tested whether 15d-PGJ₂ could covalently modify HRas in C2C12 cells. We treated C2C12 cells expressing the EGFP-tagged wild-type HRas with biotinylated 15d-PGJ₂ (5 μM). We then immunoprecipitated biotinylated 15d-PGJ₂ using streptavidin. We observed a significant increase (~3.5 fold) in the pulldown of HRas upon treatment with 15d-PGJ₂ biotin compared to DMSO (**Fig. 3A**), suggesting an interaction between 15d-PGJ₂ and HRas. To measure the role of individual C-terminal cysteines in the binding of HRas with 15d-PGJ₂, we treated C2C12 cells expressing the EGFP-tagged C181S and C184S mutants of HRas with biotinylated 15d-PGJ₂ (5 μM), and immunoprecipitated using streptavidin. We observed that the intensity of EGFP-tagged HRas was significantly decreased in cells expressing the C184S mutant (~80% decrease compared to HRas WT) but not in those expressing the C181S mutant (**Fig. 3A**). This suggests that 15d-PGJ₂ covalently modifies HRas at cysteine 184 in C2C12 cells.

15d-PGJ₂ increases the FRET between EGFP-HRas and mCherry-RAF-RBD in wild-type and C181S mutant but not in the C184S mutant of HRas

We next tested the effect of covalent modification of HRas by 15d-PGJ₂ on HRas GTPase activity using FRET. mCherry-RAF-RBD is a well-characterized sensor of the activity of HRas. RAF-RBD binds to the activated HRas upon activation of HRas, allowing FRET between EGFP and mCherry (Rocks et al., 2005). We co-expressed EGFP-tagged HRas (EGFP-HRas) with mCherry-RAF-RBD in C2C12 myoblasts (**Fig. 3B**). We measured the efficiency of FRET between EGFP and mCherry using an ImageJ plugin, FRET analyzer (Hachet-Haas et al., 2006). We compared the mean acceptor normalized FRET index in C2C12 myoblasts co-expressing EGFP-HRas WT and mCherry-RAF-RBD before and after treatment of 15d-PGJ₂ (10 μM) for 1 hour. We observed a significant increase (~30%) in the mean acceptor normalized FRET index upon treatment with 15d-PGJ₂ (**Fig. 3C**). This suggests that 15d-PGJ₂ activates HRas. To measure the role of individual C-terminal cysteines in 15d-PGJ₂ mediated activation of HRas, we co-expressed EGFP-HRas C181S or C184S with mCherry-RAF-RBD in C2C12 myoblasts. We measured the mean acceptor normalized FRET index before and after 1 hour of treatment with 15d-PGJ₂ (10 μM). We observed a significant increase (~40%) in the mean acceptor normalized FRET index in cells expressing EGFP-HRas C181S upon treatment with 15d-PGJ₂ but not in cells expressing EGFP-HRas C184S (**Fig. 3D**). These observations suggest that activation of HRas by 15d-PGJ₂ occurs in a cysteine 184 dependent manner.

15d-PGJ₂ increases phosphorylation of Erk (Thr202/Tyr204) but not Akt (S473) in C2C12 myoblasts

HRas regulates two major downstream signaling pathways, the MAP kinase (MAPK) pathway and the PI3 kinase (PI3K) pathway (Pylayeva-Gupta et al., 2011). We tested the effects of treatment with 15d-PGJ₂ on these two downstream signaling pathways by measuring the phosphorylation of Erk (42 kDa and 44 kDa) and Akt proteins in C2C12 cells. We treated C2C12 cells with 15d-PGJ₂ (5

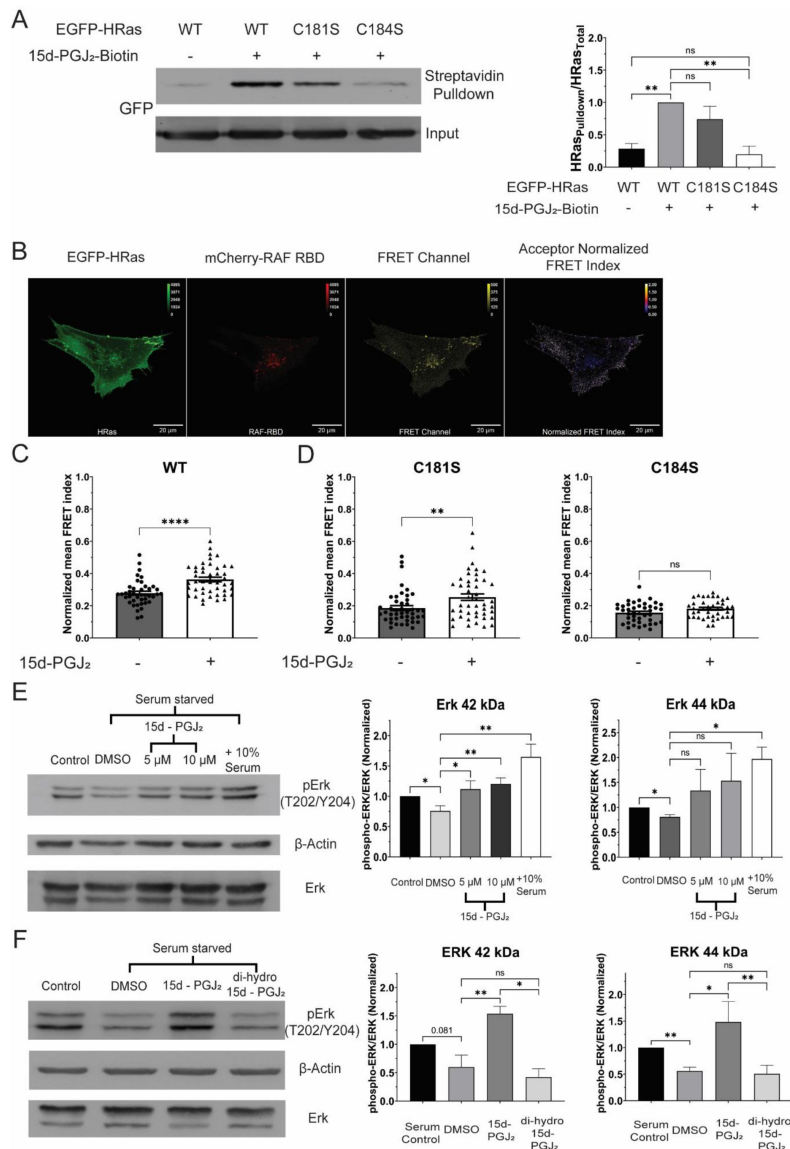


Figure 3.

15d-PGJ₂ covalently modifies HRas at Cysteine 184 and activates the HRas-MAPK pathway via the electrophilic cyclopentenone ring.

A. Streptavidin-immunoprecipitation of EGFP-HRas, measured by immunoblotting, in C2C12 cells after 3 hours of treatment with 15d-PGJ₂-Biotin (5 μ M).

B. Representative confocal micrograph of Fluorescence Resonance Energy Transfer (FRET) between EGFP-tagged HRas (EGFP-HRas) and mCherry-tagged Ras binding domain (RBD) of RAF kinase (mCherry-RAF RBD).

C. Activation of the EGFP-tagged wild type HRas (HRas WT), measured by FRET, before and after 1 hour of treatment with 15d-PGJ₂ (10 μ M) after starvation for 24 hours.

D. Activation of the EGFP-tagged C-terminal cysteine mutants of HRas (HRas C181S and HRas C184S), measured by FRET, before and after 1 hour of treatment with 15d-PGJ₂ (10 μ M) after starvation for 24 hours.

E. Phosphorylation of Erk (42 kDa and 44 kDa), measured by immunoblotting, in C2C12 cells after 1 hour of treatment with 15d-PGJ₂ (5 μ M, 10 μ M) or DMSO after starvation for 24 hours.

F. Phosphorylation of Erk (42 kDa and 44 kDa), measured by immunoblotting, in C2C12 cells after 1 hour of treatment with 15d-PGJ₂ (10 μ M)/ 9,10-dihydro-15d-PGJ₂ (10 μ M) or DMSO after starvation for 24 hours.

(Statistical significance was tested by the two-tailed student's t-test ns=p>0.05, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001)

μM and $10 \mu\text{M}$) or DMSO for 1 hr (after 24 hrs. of serum starvation) and observed a dose-dependent increase in the phosphorylation of Erk (T202/Y204) (42 kDa) but not of Erk (44 kDa) (Fig. 3E). We did not observe an increase in the phosphorylation of Akt (S473) in C2C12 cells after treatment with 15d-PGJ₂ (Fig. 53C). These observations suggest that 15d-PGJ₂ activates the MAPK signaling pathway, but not the PI3K signaling pathway.

15d-PGJ₂ contains a reactive electrophilic center in its cyclopentenone ring, that can react with cysteine residues of proteins (Luis Oliva et al., 2003). We tested its role in activating the MAPK signaling pathway. We measured the phosphorylation of Erk (42kDa and 44 kDa) in C2C12 cells after treatment with cells with 9,10-dihydro-15d-PGJ₂ ($10 \mu\text{M}$), a 15d-PGJ₂ analog which is devoid of the electrophilic center, for 1 hr (after 24 hr. of serum starvation). We observed that the phosphorylation of Erk (42 kDa and 44 kDa) in C2C12 cells treated with 9,10-dihydro-15d-PGJ₂ was significantly reduced ($\sim 70\%$) as compared to the treatment with 15d-PGJ₂ (Fig. 3F). This shows that 15d-PGJ₂ activates the HRas-MAPK signaling pathway via the electrophilic center in its cyclopentenone ring.

15d-PGJ₂ increases the localization of EGFP-tagged HRas at the plasma membrane compared to the Golgi in a C-terminal cysteine-dependent manner

15d-PGJ₂ covalently modifies cysteine 184 and activates HRas signaling (Fig. 3). Reversible palmitoylation of cysteine 181 and cysteine 184 in the C-terminal tail of HRas regulate intracellular distribution and signaling of HRas. Inhibition of palmitoylation of the C-terminal cysteine 181, either by a palmitoylation inhibitor 2-Bromopalmitate or by mutation to serine, causes accumulation of HRas at the Golgi compared to the plasma membrane and alters activity (Rocks et al., 2005). Therefore, we tested whether the modification of 15d-PGJ₂ alters the intracellular distribution of HRas. We co-expressed the EGFP-tagged wild type and the cysteine mutants of HRas (EGFP-HRas WT/C181S/C184S) with a previously reported marker of Golgi (Shaner et al., 2008) in C2C12 cells and stained the cells with plasma membrane marker WGA-633 (Fig. 4A). We compared R_{mean} , the ratio of mean EGFP-HRas intensity at the Golgi to the mean HRas intensity at the plasma membrane, to measure the distribution of HRas between the plasma membrane and the Golgi. We measured the intracellular distribution of HRas between the Golgi and the plasma membrane in C2C12 cells after treatment with 15d-PGJ₂ ($10 \mu\text{M}$) for 24 hours in DMEM 10% serum medium and observed a significant decrease ($\sim 20\%$) in the R_{mean} of C2C12 cells expressing the wild-type HRas after treatment with 15d-PGJ₂ (Fig. 4B). However, we did not observe a change in the R_{mean} of C2C12 cells expressing HRas C181S or HRas C184S after treatment with 15d-PGJ₂ (Fig. 4C). These observations suggest that 15d-PGJ₂ increases the localization of HRas at the plasma membrane as compared to that in the Golgi in an HRas C-terminal cysteine-dependent manner.

15d-PGJ₂ mediated inhibition of differentiation of C2C12 cells is rescued by C181S and C184S mutants of HRas

HRas inhibits the differentiation of C2C12 myoblasts (Engler et al., 2021; Konieczny et al., 1989; Lassar et al., 1989; Lee et al., 2010; Olson, et al., 1987; Scholz et al., 2009; Van Der Burgt et al., 2007). 15d-PGJ₂ covalently modifies cysteine 184 and activates HRas (Fig. 3). Therefore, we tested whether the inhibition of myoblast differentiation by 15d-PGJ₂ depends on the activation of HRas signaling by modification of the C-terminal cysteine 184. We expressed the wild-type and the cysteine mutants of HRas (EGFP-HRas WT/C181S/C184S) in C2C12 myoblasts and treated the cells with 15d-PGJ₂ ($4 \mu\text{M}$) or DMSO during differentiation. We observed a decrease in the levels of mRNA of MHC in C2C12 cells expressing HRas WT and HRas C181S after 5 days of treatment with 15d-PGJ₂. We did not observe this in expressing HRas C184S (Fig. 4D). We also observed a significant decrease in the protein levels of MHC in differentiating C2C12 cells expressing HRas WT

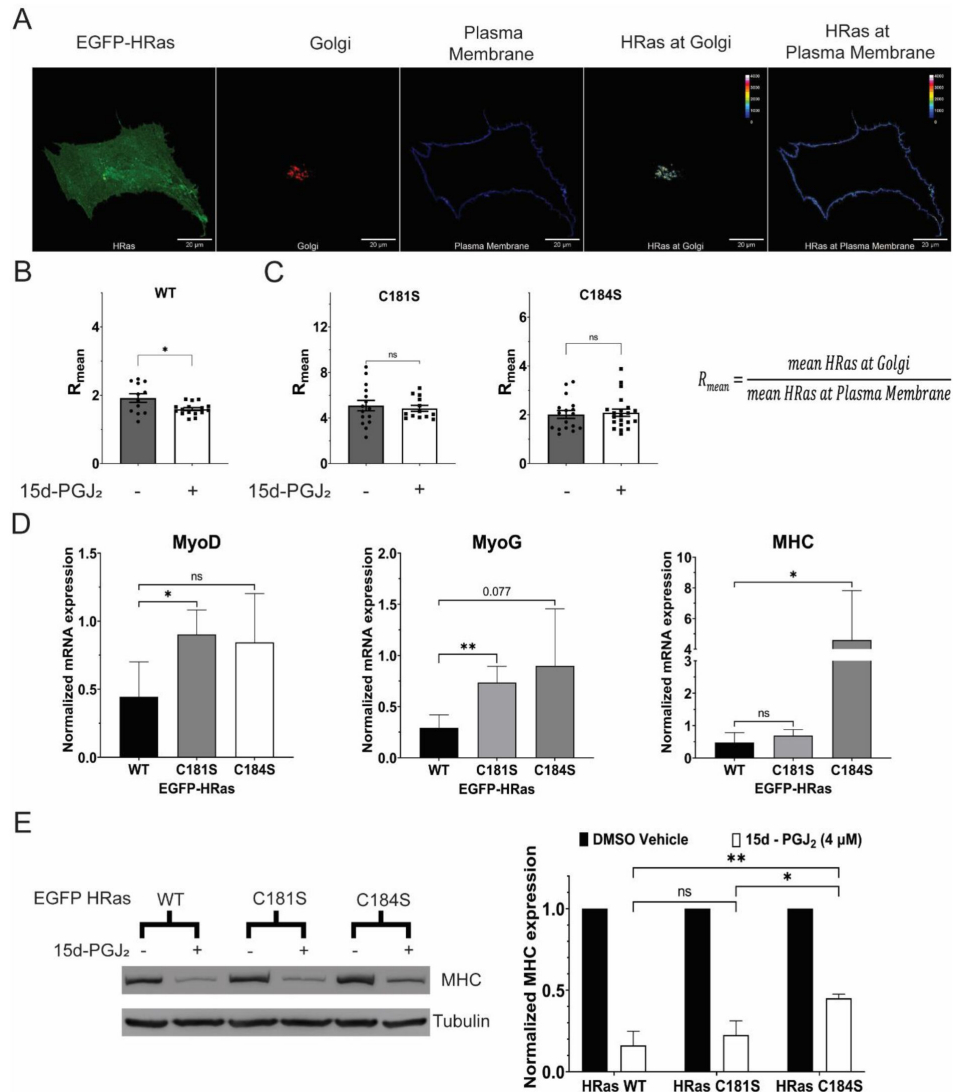


Figure 4.

15d-PGJ₂ controls the intracellular distribution of HRas and differentiation of C2C12 cells in an HRas C-terminal cysteine-dependent manner.

A. Representative confocal micrograph of C2C12 myoblasts showing localization of EGFP-tagged HRas between the plasma membrane (stained with Alexa Fluor 633 conjugated Wheat Germ Agglutinin) and the Golgi (labelled with TagRFP-tagged Golgi resident GalT protein). A statistic R_{mean} was defined as the ratio of mean HRas intensity at the Golgi to the mean HRas intensity at the plasma membrane.

B. Distribution of the wild-type HRas between the Golgi and the plasma membrane, measured by R_{mean} , in C2C12 myoblasts treated with 15d-PGJ₂ (10 μ M) or DMSO for 24 hours.

C. Distribution of the C-terminal cysteine mutants of HRas between the Golgi and the plasma membrane, measured by R_{mean} , in C2C12 myoblasts treated with 15d-PGJ₂ (10 μ M) or DMSO for 24 hours.

D. Expression of mRNAs of known markers of differentiation (MyoD, MyoG, and MHC), measured by qPCR, in differentiating C2C12 myoblasts expressing the EGFP-tagged wild-type and the C-terminal cysteine mutants of HRas after treatment with 15d-PGJ₂ (4 μ M) or DMSO for 5 days.

E. Expression of MHC protein, measured by immunoblotting, in differentiating C2C12 myoblasts expressing the EGFP-tagged wild-type and the C-terminal cysteine mutants of HRas after treatment with 15d-PGJ₂ (4 μ M) or DMSO for 5 days.

(Statistical significance was tested by the two-tailed student's t-test ns= $p > 0.05$, *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$)

and HRas C181S after treatment with 15d-PGJ₂ (Fig. 4E). This decrease was partially rescued in cells expressing HRas C184S (Fig. 4E). These observations suggest that the inhibition of myoblast differentiation by 15d-PGJ₂ depends on modification of HRas C-terminal cysteine 184.

Discussion

Senescence is characterized by an irreversible arrest in cell proliferation (Hayflick, 1965). Cells undergo senescence because of a myriad of stresses, including DNA damage, mitochondrial damage, and oncogene overexpression (Bihani et al., 2007; Casar et al., 2018; Chen and Ames, 1994; Chen et al., 1998; Coppé et al., 2008; D'Adda Di Fagagna, 2008; D'Adda Di Fagagna et al., 2003; Di Leonardo et al., 1994; Franza et al., 1986; Land et al., 1983; Robles and Adami, 1998; Serrano et al., 1997; Wiley et al., 2016; Woods et al., 1997). Senescent cells exhibit a multi-faceted physiological response, where they exhibit a flattened morphology, increase in cell size (Chen and Ames, 1994; Serrano et al., 1997), upregulation of tumor suppressor proteins (Calabrese et al., 2009; Lowe et al., 2004; Stein et al., 1990; Zindy et al., 2003), expression of neutral pH active β -galactosidase (SA β -gal) (Dimri et al., 1995; Lee et al., 2006), and altered metabolic state (Bittles and Harper, 1984; Jones et al., 2005; Wiley and Campisi, 2021; Zwierschke et al., 2003). Arachidonic acid metabolism is upregulated in senescent cells, which leads to increased synthesis of eicosanoid prostaglandins, which regulate the physiology of senescent cells (Wiley et al., 2021; Wiley and Campisi, 2021). Senescent cells exhibit a secretory phenotype (SASP) consisting of a variety of bioactive molecules including cytokines and chemokines, growth factors, matrix metalloproteases, etc (Coppé et al., 2008). Senescent cells influence the surrounding cells via the SASP factors, which regulate proliferation, migration, and other cell biological processes in the neighboring cells (Campisi, 2005). SASP-mediated perturbations in the microenvironment are implicated in several senescence-associated pathologies (Wiley and Campisi, 2021). Senescent fibroblasts increase the proliferation of premalignant and malignant epithelial cells (Krtolica et al., 2001). Conditioned medium of senescent fibroblasts promoted tumorigenesis in mouse keratinocytes (Dilley et al., 2003). Senescent fibroblasts transform pre-malignant breast cancer cells into invasive, tumor-forming cells (Parrinello et al., 2005). Senescence in muscle stem cells induces sarcopenia via activation of the p38 MAP kinase pathway and transient inhibition of the p38 MAP kinases rejuvenates aged muscle stem cells to ameliorate sarcopenia (Cosgrove et al., 2014). Senescent cells inhibit the differentiation of myoblasts by secretion of IL6 by senescent muscle stem cells in myotonic dystrophy (Conte et al., 2023).

In this study, we show that senescent myoblasts synthesize and release eicosanoid prostaglandin 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂) (Fig. 1I and J), the terminal non-enzymatic dehydration product of prostaglandin PGD₂ (Shibata et al., 2002). We used Doxorubicin (Doxo) to induce senescence in C2C12 myoblasts and showed that the conditioned medium of senescent C2C12 cells inhibits differentiation of C2C12 myoblasts (Fig. 2A). Inhibition of synthesis of PGD₂ by treatment of senescent cells with AT-56, a well-characterized inhibitor of prostaglandin D synthase (Hu et al., 2021; S. Hu et al., 2023; Shunfeng Hu et al., 2023; Irikura et al., 2009), rescued this inhibitory effect of the conditioned medium on the differentiation of myoblasts (Fig. 2A). A study has shown that prostaglandin PGD₂ inhibits differentiation of C2C12 myoblasts (Veliça et al., 2010), but the authors noted that knockout of DP1 and DP2 (the known receptors of prostaglandins PGD₂) does not abrogate inhibition of differentiation of myoblasts by PGD₂. This observation suggested that PGD₂ might inhibit the differentiation of myoblasts by a receptor-independent mechanism, possibly by its spontaneous non-enzymatic dehydration to 15d-PGJ₂. 15d-PGJ₂ has been suggested to be an endogenous ligand of PPAR γ (Li et al., 2019). However, the inhibition of PPAR γ did not abrogate the inhibition of differentiation of C2C12 myoblasts by 15d-PGJ₂, suggesting the existence of other possible mechanisms (Hunter et al., 2001). 15d-PGJ₂ has varied effects on cell physiology in a context-dependent manner. On one hand, 15d-PGJ₂ promotes tumorigenesis by inducing epithelial to mesenchymal transition in

breast cancer cell line MCF7(Choi et al., 2020 [DOI](#)), 15d-PGJ₂ inhibits the proliferation of A549, H1299, and H23 lung adenocarcinoma cells via induction of ROS and activation of apoptosis(Slanovc et al., 2024 [DOI](#)). Here, we show that 15d-PGJ₂ inhibits the proliferation and the differentiation of C2C12 myoblasts (**Fig. 2B, C** [DOI](#) and D).

15d-PGJ₂ contains an electrophilic cyclopentenone ring in its structure, allowing 15d-PGJ₂ to covalently modify and form Michael adducts with cysteine residues of proteins(Shibata et al., 2002 [DOI](#)). A previous proteomic study in endothelial cells showed biotinylated 15d-PGJ₂ covalently modified over 300 proteins, which regulate several physiological processes including cell cycle (MAPK1, MCM4), cell metabolism (Fatty acid synthase, Isocitrate dehydrogenase), apoptosis (PDCD6I), translation (Elongation factor 1 and 2, EIF4A-I), intracellular transport (Importin subunit β1, Exportin 2, Kinesin 1 heavy chain)(Marcone and Fitzgerald, 2013 [DOI](#)). Another proteomic study in neuronal cells suggested that 15d-PGJ₂ modifies several proteins including chaperone HSP8A, glycolytic proteins Enolase 1 and 2, GAPDH, PKM1, cytoskeleton proteins Tubulin β2b, β actin, GFAP, etc(Yamamoto et al., 2011 [DOI](#)). This study also showed modification of peptide fragments homologous to IκB kinase β, Thioredoxin, and a small molecule GTPase HRas. 15d-PGJ₂ has been shown to covalently modify HRas in NIH3T3 and Cos1 cells(Luis Oliva et al., 2003) and IMR90 cells(Wiley et al., 2021 [DOI](#)). Modification by 15d-PGJ₂ led to the activation of HRas, judged by an increase in GTP-bound HRas. It is clear that 15d-PGJ₂ is capable of modifying numerous proteins in different contexts. Despite these observations, the functional relevance of these modifications in numerous contexts remains to be mapped. Here we focused on the role of 15d-PGJ₂ in the context of senescence and skeletal muscle differentiation. In this study, we showed that 15d-PGJ₂ covalently modifies HRas at cysteine 184 but not cysteine 181 in C2C12 myoblasts (**Fig. 3A** [DOI](#)). We showed by FRET microscopy that modification of HRas by 15d-PGJ₂ in HRas WT and HRas C181S activates HRas in C2C12 cells, but 15d-PGJ₂ is unable to activate HRas C184S in this context (**Fig. 3B, C, and** [DOI](#) D and **Fig. S3A** [DOI](#) and B). This observation shows a direct link between the modification of HRas by 15d-PGJ₂ and the activation of HRas GTPase.

HRas activates two major downstream signaling pathways, the HRas-MAPK and the HRas-PI3K pathway(Pylayeva-Gupta et al., 2011 [DOI](#)). We showed that covalent modification of HRas by 15d-PGJ₂ via the electrophilic cyclopentenone ring activates HRas (**Fig. 3C and D** [DOI](#)) and activates the HRas-MAPK pathway, demonstrated by an increase in the phosphorylation of Erk after treatment with 15d-PGJ₂ (**Fig. 3E and F** [DOI](#)). However, we did not observe activation of the HRas-PI3K pathway, as we did not see an increase in the phosphorylation of Akt after treatment with 15d-PGJ₂ (**Fig. S3C** [DOI](#)). MAPK and PI3K pathways are known regulators of muscle differentiation(Bennett and Tonks, 1997 [DOI](#); Rommel et al., 1999 [DOI](#)), where inhibition of the RAF-MEK-Erk pathway or activation of the PI3K pathway promotes the differentiation of myoblasts. Preferential activation of the HRas-MAPK pathway over the HRas-PI3K pathway after treatment with 15d-PGJ₂ can be a possible mechanism by which 15d-PGJ₂ can inhibit the differentiation of myoblasts. HRas is known to regulate the differentiation of myoblasts in different contexts. Constitutively active HRas signaling by expression of oncogenic HRas mutant (HRas V12) leads to inhibition of differentiation of myoblasts(Konieczny et al., 1989 [DOI](#); Lassar et al., 1989 [DOI](#); Olson, et al., 1987; Van Der Burgt et al., 2007 [DOI](#)). Here we showed that the inhibition of differentiation of myoblasts after 15d-PGJ₂ is partially rescued in cells expressing the C184S mutant of HRas but not the wild type or the C181S mutant (**Fig. 4D and E** [DOI](#) and **S4E** [DOI](#)). HRas C184S did not get modified by 15d-PGJ₂ (**Fig. 3A** [DOI](#)). These observations suggest that the inhibition of differentiation of myoblasts by 15d-PGJ₂ is partially dependent on the covalent modification of HRas by 15d-PGJ₂.

Cysteine 181 and 184 in the C-terminal of HRas regulate the intracellular distribution of HRas between the plasma membrane and the Golgi by reversible palmitoylation and de-palmitoylation(Rocks et al., 2005 [DOI](#)). Inhibition of the palmitoylation of C-terminal cysteine 181, either by treatment with protein palmitoylation inhibitor 2-bromopalmitate or mutation of cysteine to serine, leads to accumulation of HRas at the Golgi. Intracellular localization of HRas maintains two distinct pools of HRas activity, where the plasma membrane pool shows a faster

activation followed by short kinetics and the Golgi pool shows a slower activation but a sustained activation (Agudo-Ibáñez et al., 2015 [↗](#); Busquets-Hernández and Triola, 2021 [↗](#); Lorentzen et al., 2010 [↗](#); Rocks et al., 2005 [↗](#)). We showed that the covalent modification of HRas by 15d-PGJ₂ alters the intracellular distribution of HRas. We showed that the covalent modification of HRas by 15d-PGJ₂ leads to an increase in the localization of the wild-type HRas at the plasma membrane compared to the Golgi (**Fig. 4B** [↗](#)). We did not observe any changes in the intracellular distribution of HRas C181S or HRas C184S after treatment with 15d-PGJ₂ (**Fig. 4C** [↗](#)). HRas C184S is not modified by 15d-PGJ₂, but HRas C181S is modified by 15d-PGJ₂ (**Fig. 3A** [↗](#)). This suggests that the intracellular redistribution of HRas due to covalent modification by 15d-PGJ₂ at cysteine 184 requires palmitoylation of cysteine 181.

Previous reports suggest that downstream signaling of HRas depends on the intracellular localization of HRas (Rocks et al., 2005 [↗](#); Santra et al., 2019 [↗](#)). For example, targeted localization of HRas at the ER membrane induced expression of cell-migration genes. Localization of HRas at the plasma membrane showed a strong correlation with the expression of cell cycle genes, particularly the MAPK signaling pathway. Localization of HRas at the plasma membrane also showed a negative correlation with genes associated with the PI3K-Akt pathway. Here we showed that the intracellular distribution of HRas regulates differentiation of myoblasts. In order to show this, we used the constitutively active mutant of HRas (HRas V12) which has been shown to inhibit the differentiation of myoblasts (Engler et al., 2021 [↗](#); Konieczny et al., 1989 [↗](#); Lassar et al., 1989 [↗](#); Olson, et al., 1987; Scholz et al., 2009 [↗](#); Van Der Burgt et al., 2007 [↗](#)). We expressed cysteine mutants of HRas V12 in C2C12 myoblasts and found that HRas V12 C181S localized predominantly at the Golgi whereas HRas V12 and HRas V12 C184S localized at both the plasma membrane and the Golgi (**Fig. S4A** [↗](#)). When differentiated, we observed that C2C12 cells expressing HRas V12 C181S differentiated but HRas V12 or HRas V12 C184S did not differentiate (**Fig. S4B, C** [↗](#), and D). These observations suggest alteration of intracellular distribution of HRas affects the HRas-mediated inhibition of the differentiation of myoblasts.

Doxorubicin (Doxo) is a widely used chemotherapy agent for the treatment of cancers (Johnson-Arbor and Dubey, 2022 [↗](#)). Treatment with Doxo induces senescence. Doxo-mediated DNA damage leads to p53, p16, and p21-dependent senescence in human fibroblasts (Di Leonardo et al., 1994 [↗](#); Robles and Adami, 1998 [↗](#)). On the other hand, treatment with doxorubicin leads to a decrease in muscle mass and cross-sectional area, leading to chemotherapy-induced cachexia (Hiensch et al., 2020 [↗](#)). Several mechanisms have been proposed behind chemotherapy-induced cachexia, including the generation of reactive oxygen species (Gilliam and St. Clair, 2011 [↗](#)), activation of proteases like calpain and caspases (Gilliam et al., 2012 [↗](#); Smuder et al., 2011 [↗](#)), and impaired insulin signaling (de Lima Junior et al., 2016 [↗](#)). This study provides a possible mechanism behind chemotherapy-induced loss of muscle mass and functioning. Induction of senescence in myoblasts by treatment with Doxo could lead to increased synthesis and release of 15d-PGJ₂ by senescent cells which could be taken up by myoblasts in the microenvironment. The lipid could covalently modify and activate HRas at cysteine 184 to inhibit the differentiation of myoblasts. Therefore, targeting the synthesis and release of 15d-PGJ₂ by senescent cells could serve as an important target to promote skeletal muscle homeostasis in cancer patients.

Supplementary Information

Materials and Methods

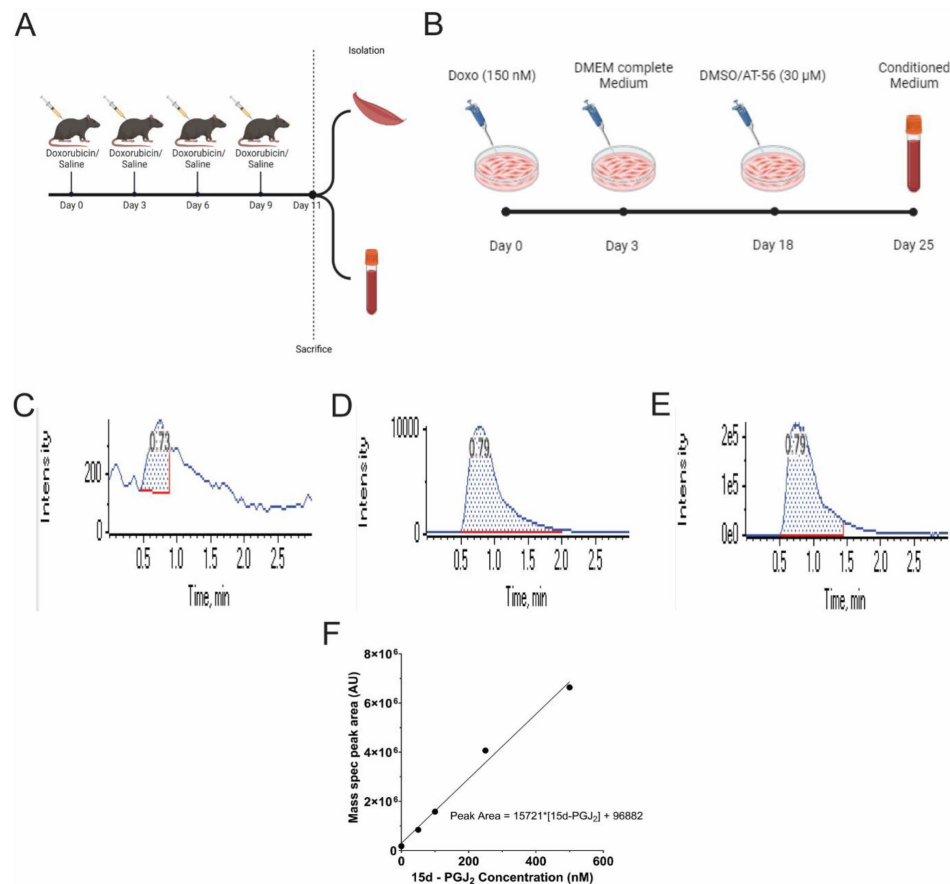


Figure S1.

Treatment with Doxo induces senescence *in vivo* and *in vitro* and induces release of eicosanoid prostaglandin 15d-PGJ₂

- A. Schematic Representation of the experimental flow for treatment of B6J mice with Doxo (5 mg/kg) or Saline.
- B. Schematic representation of the experimental flow for treatment of senescent C2C12 cells with prostaglandin D synthase (PTGDS) inhibitor AT-56 (30 μ M) or DMSO.
- C. Representative peak of quantification of m/z 315.100 $\rightarrow$ m/z 271 transitions from blank samples.
- D. Representative peak of quantification of m/z 315.100 $\rightarrow$ m/z 271 transitions from conditioned medium of C2C12 myoblasts treated with DMSO.
- E. Representative peak of quantification of m/z 315.100 $\rightarrow$ m/z 271 transitions from conditioned medium of C2C12 myoblasts treated with Doxo (150 nM).
- F. Standard curve of m/z 315.100 $\rightarrow$ m/z 271 fragment peak areas vs concentrations of 15d-PGJ₂

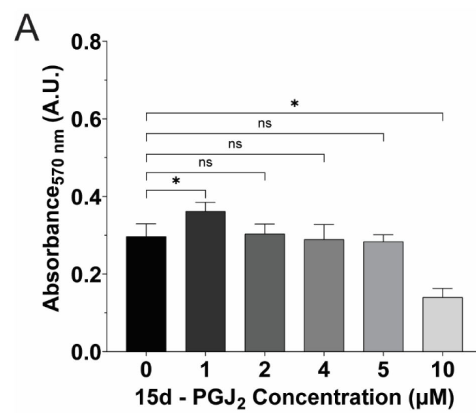


Figure S2.

Viability of C2C12 myoblasts after treatment with 15d-PGJ₂ (10 μM) in differentiating medium.

A. Viability of C2C12 cells, measured by MTT assay, after 24 hours of treatment with 15-PGJ₂ (0 μM, 1 μM, 2 μM, 4 μM, 5 μM, 10 μM).

(Statistical significance was tested by the two-tailed student's t-test ns=p>0.05, *=p<0.05, **=p<0.01, ***=p<0.001, ****=p<0.0001)

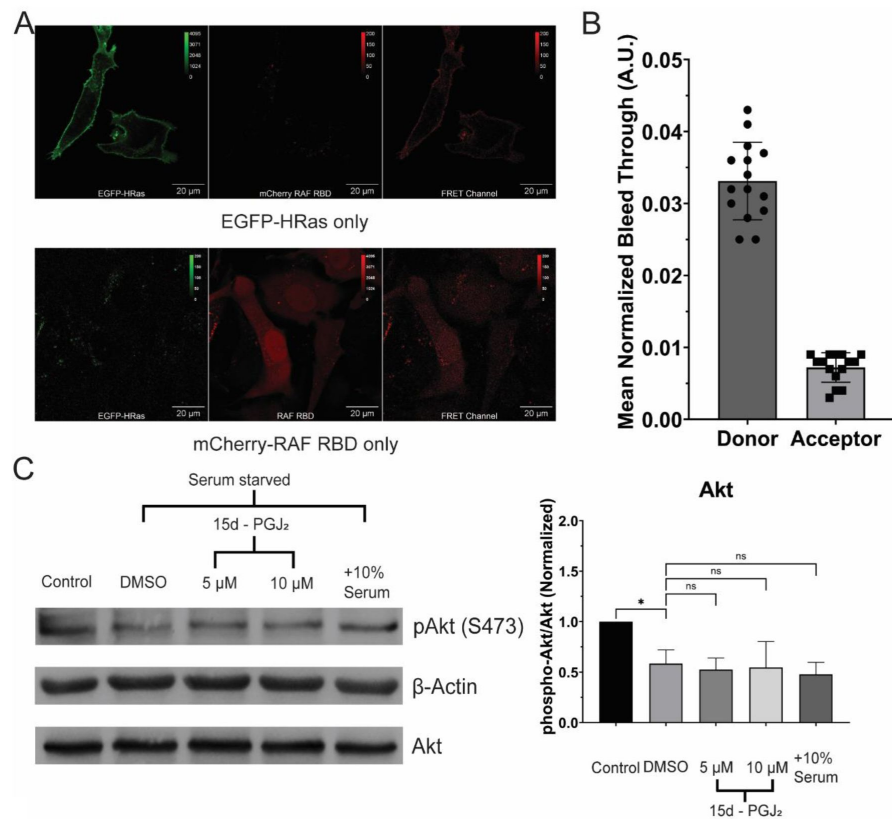


Figure S3.

Activation of HRas after treatment with 15d-PGJ₂.

A. Representative confocal micrograph of C2C12 cells expressing only EGFP-tagged HRas or mCherry-tagged RAF RBD alone for spectral overlap (bleed-through) calculations.

B. Mean bleed-through of EGFP-tagged HRas (Donor) and mCherry-tagged RAF RBD (Acceptor) in the FRET channel.

C. Phosphorylation of Akt (measured by immunoblotting) in C2C12 cells treated with 15d-PGJ₂ (5, 10 μ M) or DMSO for 1 hr after starving the cells in 0.2% serum medium for 24 hrs. The densitometric ratio of phosphorylated Akt (Ser473) to total Akt was normalized to non-starved C2C12 cells. (Statistical significance tested by two-tailed heteroscedastic student's t-test, N=3). (Statistical significance was tested by the two-tailed student's t-test ns= $p>0.05$, *= $p<0.05$, **= $p<0.01$, ***= $p<0.001$, ****= $p<0.0001$)

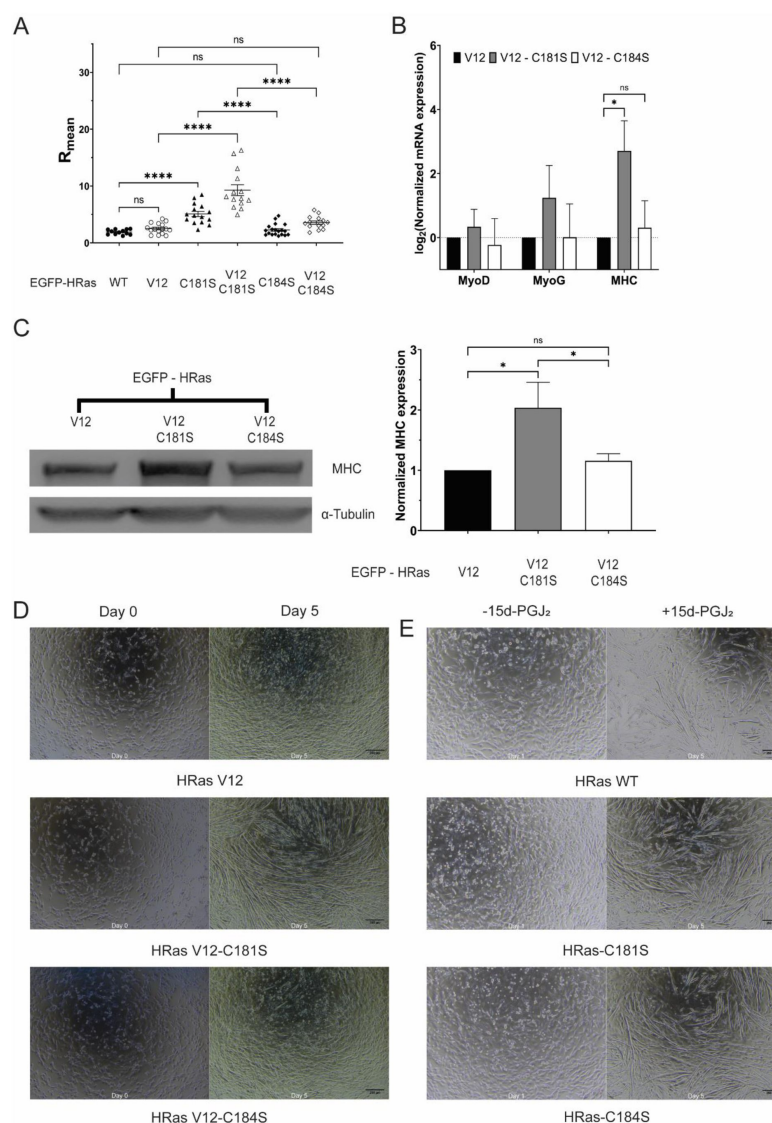


Figure S4.

C-terminal cysteine-mediated intracellular distribution of constitutively active HRas (HRas V12) regulates the differentiation of C2C12 myoblasts.

A. Distribution of EGFP-tagged HRas WT/ HRas V12/ HRas-C181S/ HRas V12-C181S/ HRas-C184S/ HRas V12-C184S between the Golgi complex and the plasma membrane, as seen by the scatter plot of R_{mean} , the ratio of the mean HRas intensity at the Golgi complex to that at the plasma membrane, in C2C12 cells. (Statistical significance tested by two-tailed heteroscedastic student's t-test, N=3)

B. mRNA levels of MyoD, MyoG, and MyHC relative to 18s rRNA (measured by quantitative PCR) in C2C12 cells expressing EGFP-tagged HRas V12/ HRas V12-C181S/ HRas V12-C184S in C2C12 differentiation medium for 5 days. (Statistical significance tested by two-tailed heteroscedastic student's t-test, N=3)

C. Protein levels of MyHC (measured by immunoblotting) in C2C12 cells expressing EGFP-tagged HRas V12/HRas V12-C181S/HRas V12-C184S in C2C12 differentiation medium for 5 days. The densitometric ratio of levels of MyHC to α -Tubulin was normalized to C2C12 cells expressing HRas V12. (Statistical significance tested by two-tailed heteroscedastic student's t-test, N=3).

D. Brightfield image of C2C12 cells expressing EGFP-HRas V12, V12 C181S, or V12 C184S on Day 0 and Day 5 of differentiation.

E. Brightfield image of C2C12 cells expressing EGFP-HRas WT, C181S, or C184S after 5 days of treatment with 15d-PGJ₂ or DMSO.

(Statistical significance was tested by the two-tailed student's t-test ns= $p > 0.05$, *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$, ****= $p < 0.0001$)

Sample	15d-PGJ ₂ (pmol)	Cell Number	15d-PGJ ₂ /Cell (fg)
Conditioned medium Quiescent C2C12 cells_1	27.087	462500	18.531
Conditioned medium Quiescent C2C12 cells_2	18.080	650000	8.801
Conditioned medium Senescent C2C12 cells_1	809.633	287500	891.018
Conditioned medium Senescent C2C12 cells_2	940.032	300000	991.420

Supplementary Table 1

15d-PGJ₂ conc. (pmol and fg/cell) detected in samples by mass spectrometry.

Supplementary Table 2

List of Primers:

Primer Name	Primer Sequence (5' – 3')
HRas WT Fwd	CCGCTCGAGCTATGACGGAATATAAGCTGG
HRas WT Rev	CCGGAATTCTCAGGAGAGCACACACTTGC
HRas C181S Rev	CCGGAATTCTCAGGAGAGCACACACTTGCAGCTCATGCTGCCGG
HRas C184S Rev	CCGGAATTCTCAGGAGAGCACACACTTGCTGCTCATG
mm 18s qPCR Fwd	CCCGTTGAACCCCATTCGTG
mm 18s qPCR Rev	GGGCCTCACTAAACCATCCA
mm MyoD qPCR Fwd	TCCGCTACATCGAAGGTCTG
mm MyoD qPCR Rev	GTCCAGGTGCGTAGAAGGC
mm MyoG qPCR Fwd	CGATCTCCGCTACAGAGGC
mm MyoG qPCR Rev	GTTGGGACCGAACTCCAGT
mm MyHC qPCR Fwd	TAAACGCAAGTGCCATTCCCTG
mm MyHC qPCR Rev	GGGTCCGGGTAATAAGCTGG
mm PTGS1 qPCR Fwd	TTACTATCCGTGCCAGAACCA
mm PTGS1 qPCR Rev	CCCGTGCGAGTACAATCACA
mm PTGS2 qPCR Fwd	TTCCAATCCATGTCAAAACCGT
mm PTGS2 qPCR Rev	AGTCCGGGTACAGTCACACTT
mm PTGDS qPCR Fwd	GAGTACGCTCTGCTATTTCAGC
mm PTGDS qPCR Rev	GGTTGGGGCAGGAAAACAATG
mm p16 qPCR Fwd	GCTCAACTACGGTGCAGATTC
mm p16 qPCR Rev	GCACGATGTCTTGATGTCCC
mm p21 qPCR Fwd	CCTGGTGATGTCCGACCTG
mm p21 qPCR Rev	CCATGAGCGCATCGCAATC
mm p53 qPCR Fwd	CCCCTGTCATCTTTTGTCCCT
mm p53 qPCR Rev	AGCTGGCAGAATAGCTTATTGAG
mm CXCL1 qPCR Fwd	ACTGCACCCAAACCGAAGTC
mm CXCL1 qPCR Rev	TGGGGACACCTTTTAGCATCTT
mm CXCL2 qPCR Fwd	CCAACCACCAGGCTACAGG
mm CXCL2 qPCR Rev	GCGTCACACTCAAGCTCTG
mm TNF1α qPCR Fwd	CCTGTAGCCCACGTCGTAG
mm TNF1α qPCR Rev	GGGAGTAGACAAGGTACAACCC
mm IL6 qPCR Fwd	CTGCAAGAGACTTCCATCCAG
mm IL6 qPCR Rev	AGTGGTATAGACAGGTCTGTGG
mm TGF1β qPCR Fwd	CTTCAATACGTCAGACATTCGGG
mm TGF1β qPCR Rev	GTAACGCCAGGAATTGTTGCTA

Item Description	Manufacturer	Catalog No.
pEGFP-C1	Clontech	
Phusion high fidelity DNA polymerase	Thermo Scientific	F 530
Xho1 restriction enzyme	New England Biolabs Inc.	R0146
EcoR1 restriction enzyme	New England Biolabs Inc.	R0101
T4 DNA Ligase	Takara Bio	2011
DMEM High Glucose	Gibco	11995065
FBS, Certified (Origin: United States)	Gibco	16000044
Horse Serum, Heat Inactivated (Origin: New Zealand)	Gibco	26050070
Penicillin – Streptomycin – Glutamine (100x)	Gibco	10378016
Penicillin – Streptomycin (100x)	Gibco	15140163
DPBS, no calcium, no magnesium	Gibco	14190144
0.25% Trypsin - EDTA	Gibco	25200056
jetPRIME transfection reagent	Polyplus	101000046
Doxorubicin	Sigma – Aldrich	D1515
15-deoxy- $\Delta^{12,14}$ -Prostaglandin J ₂	Cayman Chemical Company	18570
9,10-dihydro-15-deoxy- $\Delta^{12,14}$ -Prostaglandin J ₂	Cayman Chemical Company	18590
15-deoxy- $\Delta^{12,14}$ -Prostaglandin J ₂ -Biotin	Cayman Chemical Company	10141
Dynabeads™ MyOne™ Streptavidin C1	Invitrogen	65001
cComplete Protease inhibitor cocktail tablets	Roche	11697498001
Wheat germ agglutinin, Alexa fluor 633 conjugate	Invitrogen	2307289
ProLong Gold Antifade Mounting medium	Invitrogen	P36930
Paraformaldehyde	Sigma – Aldrich	158127
TRIzol reagent	Invitrogen	15596018
PrimeScript™ 1st strand cDNA Synthesis Kit	Takara Bio	6110A
PowerUp™ SYBR™ Green Master Mix	Applied Biosystems	A25742
WesternBright™ ECL-spray Western blotting detection system	advansta	K-12049-D50

Supplementary Table 3

List of Reagents

Fetuin (Bovine)	Sigma – Aldrich	F2379
hEGF	Sigma – Aldrich	E9644
N2 Supplement	Thermo Scientific	17502048
Dexamethasone	Sigma – Aldrich	D4902
DMEM Low Glucose	Thermo Scientific	10567014
Bovine Serum Albumin Fraction-V, Cell culture tested	HIMEDIA	9048468
Pierce™ Streptavidin Magnetic Beads	Thermo Scientific	88816

Supplementary Table 3 (continued)

Antibody Name	Clonality	Species of Origin	Manufacturer	Catalog No.
Phospho-Erk (Thr202/ Tyr204)	Polyclonal	Rabbit	Cell Signaling Technology	9101S
Erk	Polyclonal	Rabbit	Cell Signaling Technology	9102S
GAPDH	Monoclonal	Mouse	Puregene	PG23002
β -actin	Polyclonal	Rabbit	Cell Signaling Technology	4967S
Phospho-Akt (Ser473)	Monoclonal	Mouse	Cell Signaling Technology	4051S
Akt	Monoclonal	Rabbit	Cell Signaling Technology	4691S
GFP	Monoclonal	Rabbit	Cell Signaling Technology	2956S
Myosin Heavy Chain	Monoclonal	Mouse	Invitrogen	14-6503-82
Myosin Heavy Chain	Monoclonal	Mouse	Developmental Studies Hybridoma Bank (DSHB)	MF20
p21	Monoclonal	Mouse	Santacruz Biotechnology	sc-6246
γ H2A.X	Polyclonal	Rabbit	Novus Biologicals	NB100384
Tubulin	Monoclonal	Mouse	Cell Signaling Technology	3873S
HRP - Anti-Mouse		Horse	Cell Signaling Technology	7076S
HRP - Anti-Rabbit		Goat	Cell Signaling Technology	7074P2
Alexa Fluor 568 – Anti- Mouse	Polyclonal	Goat	Invitrogen	A11031

Supplementary table 4

List of Antibodies

Plasmids

Unmutated and cysteine mutants of HRas WT [HRas WT, HRas-C181S, and HRas-C184S] and HRas V12 [HRas V12, HRas V12-C181S, HRas V12-C184S] were cloned in the pEGFPC1 vector (Clontech) by restriction digestion-ligation method. Constructs of wild-type HRas were PCR amplified from a previously available HRas construct in the lab with construct-specific primers. Proper nucleotide additions were made to the forward primer to maintain the EGFP ORF, marking a 7 amino acid linker between the proteins. The construct sequences were confirmed by Sanger sequencing. GalT-TagRFP construct was a gift from Prof. Satyajit Mayor and was used to mark the Golgi. mCherry-RAF-RBD construct was a gift from Prof. Phillippe Bastiens and was used to measure the activity of HRas GTPase using FRET.

Cell Maintenance

C2C12 mouse myoblasts (CRL-1772) were obtained from ATCC and were maintained in DMEM complete medium @ 37° C, 5% CO₂. For experiments, the cells were trypsinized with 0.125% trypsin-EDTA (Gibco) and were seeded in required numbers in cell culture dishes. Human Skeletal Muscle Myoblast (CC-2580) were obtained from Lonza and were maintained in DMEM Skeletal Muscle growth medium @ 37° C, 5% CO₂. For experiments, the cells were trypsinized with 0.125% trypsin – EDTA (Gibco) and were seeded in required numbers in cell culture dishes. All cultures tested negative for mycoplasma checked by Mycoalert Mycoplasma Detection Kit (Lonza).

Conditioned media collection

C2C12 cells seeded in 60mm dishes were treated with Doxorubicin (150 nM) for 3 days. The media was then changed to DMEM complete medium without Doxorubicin for 19 days after treatment with Doxorubicin. The cells were treated with DMSO or AT-56 (30 µM) in the DMEM complete medium for 2 days. On Day 21, the cells were treated with DMSO or At-56 in DMEM Starvation medium for 3 days. The media was then collected and centrifuged @1000g, R.T. for 5 minutes. The media was then stored at -80° C after flash freezing in liq. N₂ till further requirement.

Treatments

15d-PGJ₂ (Cayman Chemical Company) dissolved in DMSO (10 mM) was diluted in DMEM media for experiments. 9,10-dihydro-15d-PGJ₂ (Cayman Chemical Company) dissolved in DMSO (10 mM) was appropriately diluted in DMEM media for experiments. DMSO was used as vehicle control. A media change of the same composition was given every 24 hours. C2C12 cells with 70-80% confluency were treated with Doxorubicin (Doxo) for 3 days. After 72 hours, Doxo was removed from the medium and the cells were kept for 10 more days with media change every 3 days till the end of the experiment. C2C12 cells transfected with EGFP-HRas WT/C181S/C184S in 35mm dishes were treated with 15d-PGJ₂-Biotin (5 µM) in DMEM Hi Glucose medium (Gibco) supplemented with 1% Penicillin-streptomycin-Glutamine (Gibco) without fetal bovine serum for 3 hours. Conditioned medium collected from senescent cells was thawed @ 37° C. The medium was then supplemented with 2% heat-inactivated horse serum and 1% penicillin-streptavidin-glutamine. C2C12 myoblasts were treated with the conditioned medium and were given a media change every 48 hours.

Transfections

C2C12 cells were seeded in 35 mm dishes to achieve confluency of ~60-70%. For western blot, immunoprecipitation, and differentiation experiments, the cells were transfected with EGFP-tagged HRas WT/ HRas-C181S/ HRas-C184S/ HRas V12/ HRas V12-C181S/ HRas V12-C184S using the jetPRIME transfection reagent (Polyplus) using the manufacturer's protocol. For measuring the intracellular distribution of HRas, the cells were reverse transfected with EGFP-tagged HRas WT/ HRas-C181S/ HRas-C184S/ HRas V12/ HRas V12-C181S/ HRas V12-C184S and GalT-TagRFP, a Golgi apparatus marker protein tagged with red fluorescent TagRFP protein using the jetPRIME transfection reagent using the manufacturer's protocol. For measuring the activity of HRas, the

cells seeded in imaging dishes (iBidi) were transfected with EGFP-HRas and mCherry-RAF-RBD using jetPRIME transfection reagent (Polyplus) using the manufacturer's protocol. Transfection efficiency was confirmed by checking for GFP and RFP fluorescence after 24 hours of transfection.

Myoblast differentiation

C2C12 cells were treated with either 15d-PGJ₂ or DMSO in the C2C12 differentiation medium. The cells were given a media change of the same composition every 24 hours. The cells were harvested after 5 days of 15d-PGJ₂ treatment for either RNA or protein isolation. Human Skeletal Muscle Myoblast cells were treated with DMSO or 15d-PGJ₂ in the Skeletal Muscle Differentiation medium. A media change of the same composition was given every 24 hours. The cells were harvested after 5 days of treatment for protein isolation.

X- Gal staining

Proliferative and Doxo-treated C2C12 cells were fixed with 0.25% glutaraldehyde, washed with PBS, and incubated overnight in X-gal staining solution at 37° C in a CO₂-free chamber. The presence of the Indigo blue product was confirmed using the Ti2 widefield inverted microscope (Nikon).

Immunoprecipitation

C2C12 cells transfected with EGFP-HRas and treated with 15d-PGJ₂-Biotin were harvested and lysed in RIPA-PP buffer and the lysate was centrifuged @15000 rpm, 4° C, 30 minutes. Protein estimation was done using the BCA assay kit (G Biosciences). 100 µg of protein was loaded on 10 µl MyOne Streptavidin C1 dynabeads blocked with 1% BSA in IP washing buffer. The lysate-streptavidin mix was incubated @4° C, 10 rpm overnight. The beads were then washed with IP washing buffer and then boiled in 20 µl Laemmli buffer. 15 µl of the beads were loaded on 12% SDS-Polyacrylamide gel for detection of EGFP-HRas by immunoblotting using EGFP antibody.

Western blotting

For measuring Erk/Akt phosphorylation in C2C12 cells were seeded in 35 mm dishes. 1x 35 mm dish was harvested in RIPA – PP the next day, while the rest were incubated in DMEM starvation medium @37° C. The cells were treated with 15d - PGJ₂ after 24 hrs of starvation @37° C. The cells were harvested at 1 hour after treatment in RIPA-PP. Protein quantification was done using BCA assay (G Biosciences) using the manufacturer's protocol. An equal mass of proteins was loaded onto a 12% SDS Polyacrylamide gel in Laemmli buffer. The proteins were transferred onto a PVDF membrane and were probed with phospho-Erk/Erk antibodies for measuring Erk phosphorylation and with phospho-Akt/Akt antibodies for measuring Akt phosphorylation. For measuring the expression of Myosin heavy chain, C2C12 cells expressing EGFP-tagged HRas WT/ HRas-C181S/ HRas-C184S/ HRas V12/ HRas V12-C181S/ HRas V12-C184S or Human Skeletal Muscle Myoblasts were seeded in 35 mm dishes and were harvested in RIPA-PP after 5 days of differentiation. Protein quantification was done using BCA assay (G Biosciences) using the manufacturer's protocol. An equal mass of proteins was loaded onto an 8% SDS Polyacrylamide gel in Laemmli buffer. The proteins were transferred onto a PVDF membrane and were probed with Myosin Heavy Chain Antibody.

qPCR

C2C12 cells, untransfected or expressing EGFP-tagged HRas WT/ HRas-C181S/ HRas-C184S and treated with DMSO/15d - PGJ₂, or expressing EGFP-tagged HRas V12/ HRas V12-C181S/ HRas V12-C184S were lysed in TRIzol at the end of the experiment (Invitrogen). RNA was isolated from the lysate by the chloroform-isopropanol method using the manufacturer's protocol. The RNA was quantified and 1.5 µg of RNA was used to prepare cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio) and random hexamer primer. Gene expression for differentiation

markers was measured by qPCR using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems) and previously reported qPCR primers (Wang et al., 2012 [DOI](#)). Relative gene expression was quantified using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001 [DOI](#)) with 18s rRNA as an internal loading control and DMSO vehicle as an experimental control.

Immunofluorescence

C2C12 cells were seeded in 35 mm dishes (Corning) on glass coverslips (Blue Star) coated with 0.2% Gelatin (Porcine, Sigma Aldrich) and were fixed with the fixative solution at the end of the experiment. The cells were then permeabilized and blocked with the blocking solution and were then incubated with Myosin Heavy Chain antibody in the blocking solution overnight. The cells were then washed with 1x PBS, incubated with fluorophore tagged secondary antibody, and were mounted in Prolong gold antifade medium with DAPI (Invitrogen). The cells were then imaged under the FV3000 inverted confocal laser scanning microscope (Olympus-Evident) using appropriate lasers and detectors.

Confocal microscopy for measuring HRas distribution between the Golgi and the plasma membrane

C2C12 cells expressing EGFP-tagged HRas WT/ HRas-C181S/ HRas-C184S + GalT-TagRFP were starved overnight in DMEM starvation medium and treated with DMSO or 15d-PGJ₂ (10 μ M) in DMEM complete medium for 24 hrs, with a medium change @ 12 hrs post-treatment. The cells were then fixed with the fixative solution @ R. T., washed with PBS, and stained for plasma membrane with Alexa Fluor 633 conjugated Wheat Germ Agglutinin (WGA-633) (Invitrogen). The cells were washed with PBS and were then mounted on glass slides in ProLong Gold Antifade Mounting medium (Invitrogen). C2C12 cells expressing EGFP-tagged HRas V12/ HRas V12-C181S/ HRas V12-C184S were also fixed with the fixative solution @R. T., washed with PBS, stained with WGA-633, and mounted on slides in Prolong gold antifade medium (Invitrogen). The cells were imaged with the FV3000 inverted confocal laser scanning microscope (Olympus-Evident) using appropriate lasers and detectors. Preliminary image processing was done using ImageJ (NIH), while batch analysis of HRas at the plasma membrane and the Golgi complex was done using a custom MATLAB script, where EGFP-HRas image was overlayed onto the GalT-TagRFP and WGA-633 image to obtain HRas localization at the Golgi complex and the Plasma Membrane respectively. A ratio of mean HRas intensity at the Golgi complex to that of at the Plasma membrane (R_{mean}) was calculated and was used to compare HRas distribution between treatments.

FRET confocal microscopy to measure the intracellular activity of HRas

C2C12 cells expressing EGFP-tagged HRas WT/HRas C181S/HRas C184S and mCherry-RAF-RBD were starved overnight in the DMEM starvation medium. The cells were imaged with the FV3000 inverted confocal laser scanning microscope (Olympus-Evident) using the following lasers and detectors:

1. Donor Channel: 488nm excitation, 510 (+/-) 20nm detection.
2. Acceptor Channel: 561nm excitation, 630 (+/-) 50nm detection.
3. FRET Channel: 488nm excitation, 630 (+/-) 50nm detection.

The cells were then treated with 15d-PGJ₂ (10 μ M) for 1 hour and were imaged using the same imaging parameters. C2C12 cells expressing EGFP-HRas or mCherry-RAF RBD only were used to calculate the bleed-through corrections (EGFP emission @ 630 (+/-) 50nm, and Excitation of mCherry by 488 nm laser). Preliminary processing was done using ImageJ (NIH). The FRET index was calculated using the FRET and co-localization analyzer plugin(Hachet-Haas et al., 2006 [DOI](#)). The

FRET index was then divided by the intensity of the Acceptor channel to normalize the variation in the expression of mCherry. We used the mean normalized FRET index to compare the activity of HRas before and after treatment with 15d-PGJ₂.

Quantification of myotube fusion index

Differentiated C2C12 myoblasts were immunostained for MHC and DAPI and were imaged on the FV3000 inverted confocal laser scanning microscope (Olympus-Evident). Analysis of the fusion index was done using the Myotube Analyzer Software (Noë et al., 2022 [DOI](#)). DAPI and MHC images were thresholded to remove background noise. The images were converted to binary masks and the channels were overlaid to obtain the no. of nuclei overlaying with MHC⁺ fibers. The fusion index was calculated as the percentage ratio of no. nuclei overlaying the MHC⁺ fibers to the total no. of nuclei in the field of view.

Quantification of cell doubling time

Cells were counted every 24 hours and the normalization was done to the number of cells counted on day 0 of the treatment (to consider attaching efficiency and other cell culture parameters). Doubling time was calculated as the reciprocal of the slope of the graph of log₂(normalized cell number) vs time.

MTT Assay

An equal number of C2C12 cells were seeded in 96 well plates in replicates. MTT assay was done at the end of the experiment using the manufacturer's protocol. MTT reagent (Sigma Aldrich) was dissolved in 1x DPBS (5 mg/ml) and was filter sterilized. MTT reagent was added to each well and the cells were incubated @37° C, 5% CO₂ for 3 hours. The medium was removed at the end of the incubation and the precipitated crystals were dissolved in DMSO @37° C, 5% CO₂ for 15 minutes. Absorbance @570 nm was recorded using the varioskan multimode plate reader (Thermo Scientific).

Animal Experiments

Mice were maintained at BLiSC Animal Care and Resource Centre (ACRC). All the procedures performed were approved by the Internal Animal Users Committee (IAUC) and the Institutional Animal Ethics Committee (IAEC). 12–15-week-old C57BL/6J (JAX#000664) mice were injected intraperitoneally (I.P.) with 5 mg/kg Doxorubicin (Doxo) four times, once every three days. Intraperitoneal injection of Saline was used as a control. The mice were sacrificed on Day 11 after the first injection. Hindlimb muscles from 4 animals (control and treated with Doxo each) were used for qPCR analysis and Hindlimb muscles from 3 animals (control and treated with Doxo each) were used for immunohistochemical analysis.

Lipid extraction and detection of 15d-PGJ₂ by mass spectrometry

For lipid extraction, cell pellets were resuspended in 3ml of a methanol solvent [water: methanol: 2:1, 1% formic acid (FA)] whereas only 1 ml of methanol with 3% FA was added to the 2 ml of CM, making a uniform sample volume of 3 ml. Subsequently, 1 ml of ethyl acetate was added to each sample and mixed vigorously. Phase separation was done by centrifuging the mixture (12000xg, 4°C for 10 mins), and the organic phase containing the lipid was collected. This process was repeated thrice in total and all the organic phases were combined and dried under a nitrogen stream at RT. The residues were resuspended in 100 µl of 50% acetonitrile in water with 0.1% FA and were subjected to mass spec analysis using the Waters® Acquity UPLC class I system. The detection of 15d-PGJ₂ was performed using an electrospray ionization source (ESI) operating in the negative ion mode and a quadrupole trap mass spectrometer (AB SCIEX QTRAP 6500) connected to a Waters® Acquity UPLC class I system (Waters, Germany) outfitted with a binary solvent delivery system with an online degasser and a column manager with a column oven coupled to a UPLC

autosampler. 5 µl samples were injected into the union for analysis. Solvent A consisted of 0.1% ammonium acetate in water and solvent B was 0.1% ammonium acetate in a mixture of acetonitrile/water (95:5). For each run, the LC gradient was: 0 min, 20% B; 0.5 min, 20% B; 1.5 min, 90% B; 2.5 min, 20% B; 3min, 20% B. Analyte detection was performed using multiple reaction monitoring (MRM), 315.100 → 271.100 and 315.100 → 203.100. Source parameters were set as follows: capillary voltage 3.8 kV, desolvation gas flow 25 L/h, source temperature 350 °C, ion source gas 1 flow 40 L/h, and ion source gas 2 flow 40 L/h. Acquisition and quantification were completed with Analyst 1.6.3 and Multiquant 3.0.3, respectively (method adopted from (Morgenstern et al., 2018)). For the standards, 2ml media of different known concentrations (50nM, 100nM, 250nM, and 500nM) of 15d-PGJ₂ were prepared and subjected to the same extraction procedure as that of CM. A standard curve was plotted with the known concentration and the mass spec peak area, and the concentration of the lipid in samples was calculated.

Reagents

- DMEM complete medium: DMEM Hi Glucose medium (Gibco) supplemented with 1% Penicillin – Streptomycin – Glutamine (Gibco) and heat-inactivated 10% Fetal Bovine Serum (US origin) (Gibco).
- Basal Conditioned medium: DMEM Hi Glucose medium (Gibco) supplemented with 1% Penicillin – Streptomycin – Glutamine (Gibco) and heat-inactivated 2% Fetal Bovine Serum (US origin) (Gibco).
- C2C12 differentiation medium: DMEM Hi Glucose medium (Gibco) supplemented with 2% Horse Serum (Gibco) and 1% Penicillin – Streptomycin – Glutamine (Gibco).
- DMEM Starvation medium: DMEM Hi Glucose medium (Gibco) supplemented with 0.2% heat-inactivated fetal bovine serum (US origin) (Gibco) and 1% Penicillin – Streptomycin – Glutamine (Gibco).
- RIPA – PP buffer: RIPA buffer (Invitrogen) supplemented with protease inhibitor cocktail (Roche) and 5 mM Sodium Fluoride and 5 mM Sodium Orthovanadate.
- TBS – T buffer: 50 mM Tris-Cl (pH = 7.5), 150 mM NaCl and 0.1% Tween – 20 in water.
- PBS: 2.67 mM KCl, 1.47 mM KH₂PO₄, 137.93 mM NaCl, 8.06 mM Na₂HPO₄ in water.
- IP Washing Buffer: 150 mM NaCl, 0.1% SDS, 1% NP-40 in 50 mM Tris-Cl (pH=7)
- Fixative Solution: 4% (w/v) Paraformaldehyde (Sigma – Aldrich) in PBS.
- Blocking Solution: 2% Heat Inactivated FBS, 0.2% BSA, 0.2% Triton - X, 0.05% NaN₃ in PBS.
- Skeletal Muscle Growth Medium: DMEM Low Glucose Medium (Gibco), supplemented with 1% Penicillin – Streptomycin – Glutamine (Gibco), heat-inactivated 10% Fetal Bovine Serum (US origin) (Gibco), Bovine Fetuin (50 µg/ml) (Sigma – Aldrich), Dexamethasone (0.4 µg/ml), and hEGF (10 ng/ml).
- Skeletal Muscle Differentiation Medium: DMEM low glucose medium (Gibco) supplemented with 2% Horse Serum, 1% Penicillin – Streptomycin (Gibco), and 1% N2 Supplement.

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Author Contribution

SSP: Project conceptualization, Cell culture treatments and assays, Biochemistry, Microscopy, Image Processing, and analysis, Writing: original draft, review, and edits.

AB: Animal work, Cell culture treatments and assays, Biochemistry, Mass Spectrometry, Writing: review and edits.

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Competing Interests

The authors declare no competing interests.

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Editors

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Reviewer #1 (Public Review):

Summary:

The authors show that upon treatment with Doxorubicin (Doxo), there is an increase in senescence and inflammatory markers in the muscles. They also show these genes get upregulated in C2C12 myoblasts when treated with conditioned media or 15d-PGJ2. 15dPGJ2 induces cell death in the myoblasts, decreases proliferation (measured by cell numbers), and decreases differentiation and fusion. 15d-PGJ2 modified Cys184 of HRas, which is required for its activation as indicated by the FRET analysis with RAF RBD. They also showed that 15d-PGJ2 activates ERK signaling, but not Akt signaling, through the electrophilic center. 15d-PGJ2 inhibits Golgi localization of HRAS (only WT, not C181 or C184 mutant). They also showed that expressing the WT HRas followed by 15d-PGJ2 treatment led to a decrease in the levels of MHC mRNA and protein, and this defect is dependent on C184. This is a well-written manuscript with interesting insights into the mechanism of action of 15d-PGJ2. However, some clarification and experiments will help the paper advance the field significantly.

Strengths:

The data clearly shows that 15d-PGJ2 has a negative role in the myoblast cells and that it leads to modification of HRas protein. Moreover, the induction of biosynthetic enzymes in the PGD2 pathway also supports the induction of 15d-PGJ2 in Doxorubicin-treated cells. Both conditioned media experiments and the 15d-PGJ2 experiments show that 15d-PGJ2 could be the active component secreted by the senescent myoblasts.

Weaknesses:

The genes that are upregulated in the muscles upon injection with Doxo are also markers for inflammation. Since Doxo is also known to induce systemic inflammation, it is important to delineate these two effects (Inflammatory cells vs senescent cells). The expression of beta Gal and other markers of senescence in the tissue sections will help to delineate these.

In Figure 2, where the defect in the differentiation of myoblasts upon treatment with 15d-PGJ2 is shown, most of the cells die within 48 hours at higher concentrations, making it difficult to perform the experiments. This also shows that 15d-PGJ2 was toxic to these cells. Lower concentrations show a decrease in the differentiation based on the lower number of nuclei in fibers and low expression of MyoD, MyoG, and MHC. However, it is unclear if this is due to increased cell death or defective differentiation. It would be a lot more informative if the cell count, cell division, and cell death could be plotted for these concentrations of the drug during the experiment. Also, in the myoblast experiments, are the effects of treatment with Dox reversible?

In Figure 3, most of the experiments are done at a high concentration, which induces almost complete cell death within 48 hours. Even at such a high concentration of 15dPGJ2, the increase in ERK phosphorylation is minimal.

The experiment Figure 4C shows that C181 and C84 mutants of the HRas show higher levels in Golgi compared with WT. However, this could very well be due to the defect in palmitoylation rather than the modification with 15d-PGJ2. Though the authors allude to the possibility that intracellular redistribution of HRas by 15d-PGJ2 requires C181 palmitoylation, the direct influence of C184 modification on C181 palmitoylation is not shown. To have a meaningful conclusion, the authors need to compare the palmitoylation and modification with 15d-PGJ2.

To test if the inhibition of myoblast differentiation depends on HRas, they overexpressed the HRas and mutants in the C2C12 lines. However, this experiment does not take the endogenous HRAs into consideration, especially when interpreting the C184 mutant. An appropriate experiment to test this would be to knock down or knock out HRas (or make knock-in mutations of C184) and show that the effect of 15d-PGJ2 disappears. Moreover, in this specific experiment, it is difficult to interpret without a control with no HRas construct and another without the 15d-PGJ2 treatment.

Moreover, the overall study does not delineate the toxic effects of 15d-PGJ2 from its effect on the differentiation.

<https://doi.org/10.7554/eLife.95229.2.sa1>

Reviewer #2 (Public Review):

Summary:

In this study, Swarang and colleagues identified the lipid metabolite 15d-PGJ2 as a potential component of senescent myoblasts. They proposed that 15d-PGJ2 inhibits myoblast

proliferation and differentiation by binding and regulating HRas, suggesting its potential as a target for restoring muscle homeostasis post-chemotherapy.

Strengths:

The regulation of HRas by 15d-PGJ2 is well controlled.

Weaknesses:

(1) I still think the novelty is limited by previous published findings. The authors themselves noted that the accumulation of 15d-PGJ2 in senescent cells has been reported in various cell types, including human fibroblasts, HEPG2 hepatocellular carcinoma cells, and HUVEC endothelial cells (PMCID: PMC8501892). Although the current study observed similar activation of 15d-PGJ2 in myoblasts, it appears to be additive rather than fundamentally novel. The covalent adduct of 15d-PGJ2 with Cys-184 of H-Ras was reported over 20 years ago (PMID: 12684535), and the biochemical principles of this interaction are likely universal across different cell types. The regulation of myogenesis by both HRas and 15d-PGJ2 has also been previously extensively reported (PMID: 2654809, 1714463, 17412879, 20109525, 11477074). The main conceptual novelty may lie in the connection between these points in myoblasts. But as discussed in another comment, the use of C2C12 cells as a model for senescence study is questionable due to the lack of the key regulator p16. The findings in C2C12 cells may not accurately represent physiological-relevant myoblasts. It is recommended that these findings be validated in primary myoblasts to strengthen the study's conclusions.

(2) The C2C12 cell line is not an ideal model for senescence study.

C2C12 cells are a well-established model for studying myogenesis. However, their suitability as a model for senescence studies is questionable. C2C12 cells are immortalized and do not undergo normal senescence like primary cells as C2C12 cells are known to have a deleted p16/p19 locus, a crucial regulator of senescence (PMID: 20682446). The use of C2C12 cells in published studies does not inherently validate them as a suitable senescence model. These studies may have limitations, and the appropriateness of the C2C12 model depends on the specific research goals.

In the study by Moustogiannis et al. (PMID: 33918414), they claimed to have aged C2C12 cells through multiple population doublings. However, the SA- β -gal staining in their data, which is often used to confirm senescence, showed almost fully confluent "aged" C2C12 cells. This confluent state could artificially increase SA- β -gal positivity, suggesting that these cells may not truly represent senescence. Moreover, the "aged" C2C12 cells exhibited normal proliferation, which contradicts the definition of senescence. Similar findings were reported in another study of C2C12 cells subjected to 58 population doublings (PMID: 21826704), where even at this late stage, the cells were still dividing every 2 or 3 days, similar to younger cells at early passages. More importantly, I do not know how the p16 was detected in that paper since the locus was already mutated. In terms of p21, there was no difference in the proliferative C2C12 cells at day 0.

In the study by Moiseeva et al. in 2023 (PMID: 36544018), C2C12 cells were used for senescence modeling for siRNA transfection. However, the most significant findings were obtained using primary satellite cells or confirmed with complementary data.

In conclusion, while molecular changes observed in studies using C2C12 cells may be valid, the use of primary myoblasts is highly recommended for senescence studies due to the limitations and questionable senescence characteristics of the C2C12 cell line.

(3) Regarding source of increased PGD in the conditioned medium, I want to emphasize that it's unclear whether the PGD or its metabolites increase in response to DNA damage or the senescence state. Thus, using a different senescent model to exclude the possibility of DNA damage-induced increase will be crucial.

(4) Similarly for the in vivo Doxorubicin (Doxo) injection, both reviewers have raised concerns about the potential side effects of Doxo, including inflammation, DNA damage, and ROS generation. These effects could potentially confound the results of the study. The physiological significance of this study will heavily rely on the in vivo data. However, the in vivo senescence component is confounded by the side effects of Doxo.

(5) Figure 2A lacks an important control from non-senescent cells during the measurement of C2C12 differentiation in the presence of conditioned medium. The author took it for granted that the conditioned medium from senescent cells would inhibit myogenesis, relying on previous publications (PMID: 37468473). However, that study was conducted in the context of myotonic dystrophy type 1. To support the inhibitory effect in the current experimental settings, direct evidence is required. It would be necessary to include another control with conditioned medium from normal, proliferative C2C12 cells.

(6) Statistical analyses problems.

Only t-test was used throughout the study even when there are more than two groups. Please have a statistician to evaluate the replicates and statistical analyses used.

For the 15d-PGJ2/cell concentration measurements in Figure 1F, there were only two replicates, which was provided in the supplementary table after required. Was that experiment repeated with more biological replicates?

For figure 1C, Fig 1F, 1G, 1J, 2C, 2E, 3A, 3E, 3F, 4D, 4E, please include each data points in bar graphs as used in Fig 1D, or at least provide how many biological replicates were used for each experiment?

There is no error bar in a lot of control groups (Fig 2C, 2E, 3EF, 4E, S4B).

For qPCR data in Figure 1C, the author responded in that the data in was plotted using $2^{-\Delta\Delta CT}$ instead of $2^{-\Delta\Delta CT}$ to show the variability in the expression of mRNAs isolated from animals treated with Saline. This statement does not align with the method section. Please revise.

(7) For Figure 1, the title may not be appropriate as there is insufficient data to support the inhibition of myoblast differentiation.

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Author response:

The following is the authors' response to the original reviews.

eLife assessment

This manuscript provides useful information about the lipid metabolite 15d-PGJ2 as a potential regulator of myoblast senescence. The authors provide experimental evidence that 15d-PGJ2 inhibits myoblast proliferation and differentiation by binding and regulating HRas. However, the manuscript is incomplete in its current form, as it lacks robust support from the data regarding the main conclusions related to senescence and technical concerns related to the senescence models used in this study.

We are grateful to the editors and the reviewers for their time and comments in sharpening the science and the writing of the manuscript. We have attached a detailed response to emphasize that the manuscript does include robust evidence regarding the claims, which could have been missed during the review process. We have provided a better context for these points now.

Public Reviews:

Reviewer #1 (Public Review):

Summary:

The authors show that upon treatment with Doxorubicin (Doxo), there is an increase in senescence and inflammatory markers in the muscles. They also show these genes get upregulated in C2C12 myoblasts when treated with conditioned media or 15d-PGJ2. 15dPGJ2 induces cell death in the myoblasts, decreases proliferation (measured by cell numbers), and decreases differentiation and fusion. 15d-PGJ2 modified Cys184 of HRas, which is required for its activation as indicated by the FRET analysis with RAF RBD. They also showed that 15d-PGJ2 activates ERK signaling, but not Akt signaling, through the electrophilic center. 15d-PGJ2 inhibits Golgi localization of HRAS (only WT, not C181 or C184 mutant). They also showed that expressing the WT HRas followed by 15d-PGJ2 treatment led to a decrease in the levels of MHC mRNA and protein, and this defect is dependent on C184. This is a well-written manuscript with interesting insights into the mechanism of action of 15d-PGJ2. However, some clarification and experiments will help the paper advance the field significantly.

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Weaknesses:

The genes that are upregulated in the muscles upon injection with Doxo are also markers for inflammation. Since Doxo is also known to induce systemic inflammation, it is important to delineate these two effects (inflammatory cells vs senescent cells). The expression of beta Gal and other markers of senescence in the tissue sections will help to delineate these.

As pointed out Doxo induces systemic inflammation along with inducing DNA damage-mediated senescence. Therefore, along with the inflammatory markers of the SASP (CXCL1/2, TNF1 α , IL6, PTGS1/2, PTGDS) we also observed an increase in the mRNA levels of canonical markers of DNA damage-mediated senescence. We observed an increase in the mRNA levels of cell cycle and senescence associated proteins p16 and p21 (Fig. 1C). We also observed an increased nuclear accumulation of p21 (Fig. 1A) and increased levels of phosphorylated H2A.X in the nucleus (Fig. 1B).

In Figure 2, where the defect in the differentiation of myoblasts upon treatment with 15d-PGJ2 is shown, most of the cells die within 48 hours at higher concentrations, making it difficult to perform the experiments. This also shows that 15d-PGJ2 was toxic to these cells. Lower concentrations show a decrease in the differentiation based on the lower number of nuclei in fibers and low expression of MyoD, MyoG, and MHC. However, it is unclear if this is due to increased cell death or defective differentiation. It would be a lot more informative if the cell count, cell division, and cell death could be plotted for these concentrations of the drug during the experiment.

We measured the viability of C2C12 cells after 24 hours of treatment with 15d-PGJ2 using the MTT assay and observed that the viability of cells was decreased after treatment with 15d-

PGJ2 (10 μ M) but not with 15d-PGJ2 (1 μ M, 2 μ M, 4 μ M, or 5 μ M) (see Fig. S2A of the updated manuscript). The results and figures of the manuscript have been updated accordingly.

Also, in the myoblast experiments, are the effects of treatment with Dox reversible?

The treatment with Doxorubicin is irreversible as the senescent phenotype was not reversed after withdrawal of Doxorubicin, even after 20 days.

In Figure 3, most of the experiments are done at a high concentration, which induces almost complete cell death within 48 hours.

Figure 3 is an acute experiment for only 1 hour, at which time no cell death was observed. Specifically, we measured the phosphorylation of Erk and Akt proteins after 1 hour of treatment with 15d-PGJ2 (10 μ M) during which we did not observe any cell death.

Even at such a high concentration of 15dPGJ2, the increase in ERK phosphorylation is minimal.

We observe a ~30% increase in the phosphorylation of Erk proteins after treatment with 15d-PGJ2 in 0.2% serum medium compared to treatment with vehicle (DMSO). This is reproducible and significant.

The experiment Figure 4C shows that C181 and C84 mutants of the HRas show higher levels in Golgi compared with WT. However, this could very well be due to the defect in palmitoylation rather than the modification with 15d-PGJ2.

Our data does not suggest higher levels of C184S mutant in the Golgi compared with WT (Fig. S4A). We observed that the ratio of HRas levels in the Golgi to the HRas levels in the plasma membrane were similar in C2C12 cells expressing HRas C184S and HRas WT (Fig. S4A graph columns 1 and 5).

Though the authors allude to the possibility that intracellular redistribution of HRas by 15d-PGJ2 requires C181 palmitoylation, the direct influence of C184 modification on C181 palmitoylation is not shown. To have a meaningful conclusion, the authors need to compare the palmitoylation and modification with 15d-PGJ2.

Palmitoylation of HRas C181S is required for the localization of HRas at the plasma membrane. The inhibition of palmitoylation of C181, either by mutation (C181S) or treatment with protein palmitoyl transferase inhibitor (2-Bromopalmitate), results in the accumulation of HRas at Golgi(Rocks et al., 2005) (Fig. S4A). Modification of HRas at C184 by 15d-PGJ2 (Fig. 3A) could inhibit the palmitoylation of HRas at C181. However, our data does not support this hypothesis as modification of HRas WT by 15d-PGJ2 does not increase the level of HRas at the Golgi, like in the case of inhibition of cysteine palmitoylation due to C181S mutation.

To test if the inhibition of myoblast differentiation depends on HRas, they overexpressed the HRas and mutants in the C2C12 lines. However, this experiment does not take the endogenous HRAs into consideration, especially when interpreting the C184 mutant. An appropriate experiment to test this would be to knock down or knock out HRas (or make knock-in mutations of C184) and show that the effect of 15d-PGJ2 disappears.

Endogenous HRas (wild type) is present in the C2C12 cells overexpressing the EGFP-tagged HRas constructs. Therefore, we only observe a partial rescue in the differentiation after 15d-PGJ2 treatment in C2C12 cells expressing the C184S mutant (Fig. 4D and E). However, since HRas is expressed under high expression CMV promoter and in the absence of other

regulatory elements, the overexpressed constructs do show a dominant effect over the endogenous HRas, showing cysteine mutant dependent inhibition of differentiation of myoblasts after treatment with 15dPGJ2 (Fig. 4D and E).

Moreover, in this specific experiment, it is difficult to interpret without a control with no HRas construct and another without the 15d-PGJ2 treatment.

The mRNA levels of MyoD, MyoG, and MHC in C2C12 cells expressing HRas constructs after treatment with 15d-PGJ2 were normalized to the mRNA levels in C2C12 cells expressing corresponding constructs and were treated with vehicle (DMSO). mRNA levels in C2C12 cells treated with vehicle were not shown as they were normalized to 1. MHC protein levels in C2C12 cells expressing HRas constructs after 15d-PGJ2 treatment were normalized to that in C2C12 cells treated with vehicle (DMSO). Since the hypothesis to study the effect of HRas cysteine mutations on the differentiation of myoblasts after treatment with 15d-PGJ2, C2C12 cells expressing HRas WT serve as adequate control. Fig. 2 shows the effect of 15dPGJ2 on muscle differentiation when HRas was not overexpressed.

Moreover, the overall study does not delineate the toxic effects of 15d-PGJ2 from its effect on the differentiation.

The inhibition of differentiation in C212 cells after treatment with 15d-PGJ2 cannot be attributed to the general toxicity of 15d-PGJ2 in cells. We show that the inhibition of differentiation of myoblasts after 15d-PGJ2 depends on modification of HRas at C184 i.e. failure to modify HRas at C184 (Fig. 3A) and resultant activation (Fig. 3B) by 15d-PGJ2 rescues this inhibition of differentiation of C2C12 cells (Fig. 4D and E), dissecting the inhibition of differentiation of myoblasts by 15d-PGJ2 from general toxic effects of 15d-PGJ2 on cell physiology.

Please note that the effect of 15d-PGJ2 on cell physiology is context-specific. On one hand, 15d-PGJ2 has been shown to exert tumor-suppressor effects by inhibiting the proliferation of ovarian cancer cells and lung adenocarcinoma cells (de Jong et al., 2011; Slanovc et al., 2024), 15d-PGJ2 also exerts pro-carcinogenic effects by induction of epithelial to mesenchymal transition in breast cancer cells MCF7 and inhibition of tumor-suppressor protein p53 in MCF7 and PC-3 cells (Choi et al., 2020; Kim et al., 2010).

Reviewer #2 (Public Review):

Summary:

In this study, Swarang and colleagues identified the lipid metabolite 15d-PGJ2 as a potential component of senescent myoblasts. They proposed that 15d-PGJ2 inhibits myoblast proliferation and differentiation by binding and regulating HRas, suggesting its potential as a target for restoring muscle homeostasis post-chemotherapy.

Strengths:

The regulation of HRas by 15d-PGJ2 is well controlled.

Weaknesses:

The novelty of the study is compromised as the activation of PGD and 15d-PGJ2, as well as the regulation of HRas and cell proliferation, have been previously reported.

Literature does not support this statement, and it is important to clarify this misimpression for the field as a whole.

Let us clarify-

Covalent modification of HRas by 15d-PGJ2 has been reported only twice in the literature (Luis Oliva et al., 2003; Yamamoto et al., 2011) in fibroblasts and neurons respectively.

Interaction between Hras and 15d-PGJ2 in skeletal muscles has not been shown before, even though both Hras and 15d-PGJ2 are shown to be key regulators of muscle homeostasis.

Activation of Hras by 15d-PGJ2 was reported first by Luis Oliva et al (Luis Oliva et al., 2003). However, this study does not comment on the functional implications of activation of Hras signaling.

Recently, our lab contributed to a study where the functional implication of activation of Hras signaling due to covalent modification by 15d-PGJ2 was shown in the maintenance of senescence phenotype (Wiley et al., 2021).

15d-PGJ2 was shown to inhibit the differentiation of myoblasts by Hunter *et al* (Hunter et al., 2001). This study hypothesized that the inhibition of myoblast differentiation is via 15d-PGJ2 mediated activation of the PPAR γ signaling, the study also showed inhibition of myoblast differentiation independent of PPAR γ activity, suggesting the presence of other mechanisms.

This is the first study to show a molecular mechanism where activation of Hras signaling in skeletal myoblasts due to covalent modification by 15d-PGJ2 at C184 of Hras inhibits the differentiation of skeletal myoblasts.

Additionally, there are major technical concerns related to the senescence models, limiting data interpretation regarding the relevance to senescent cells.

Major concerns:

(1) The C2C12 cell line is not an ideal model for senescence study due to its immortalized nature and lack of normal p16 expression. A more suitable myoblasts model is recommended, with a more comprehensive characterization of senescence features.

C2C12 is a good model for DNA damage-based senescence that is used in this manuscript. Several reports in the literature have shown the induction of senescence in C2C12 cells. Moiseeva et al 2023 show induction of senescence in C2C12 cells after etoposide-mediated DNA damage. Moustogiannis et al 2021 show the induction of replicative senescence in C2C12 cells. In this study, we show that C2C12 cells undergo DNA damage-mediated senescence after treatment with Doxo. We measured the induction of senescence in C2C12 cells upon DNA damage using several physiological (Nuclear Size, Cell Size, and SA β -gal) and molecular markers (mRNA levels of p21 and SASP factors (IL6 and TGF β), protein levels of p21) of senescence (see Fig. 1 of the updated manuscript). The results and the figures in the manuscript have been updated accordingly.

(2) The source of increased PGD or its metabolites in the conditioned medium is unclear. Including other senescence models, such as replicative or oncogene-induced senescence, would strengthen the study.

Fig. 1E shows time-dependent increase in the expression of PGD2 biosynthetic enzymes in senescent C2C12 cells. Fig. 1F shows an increase in the levels of 15dPGJ2 secreted by senescent C2C12 cells in the conditioned medium. This data shows that senescent C2C12 cells are the source of PGD and its metabolites in the conditioned medium.

Again, C2C12 is not suitable for replicative senescence due to its immortalized status.

We and others have shown that C2C12 cells undergo senescence, and this manuscript only used DNA damage induced senescence.

(3) In the in vivo part, it is unclear whether the increased expression of PTGS1, PTGS2, and PTGDS is due to senescence or other side effects of DOXO.

We concur that this is a limitation of this study and the subsequent work will demonstrate the origin of prostaglandin biosynthesis after treatment with Doxo *in vivo*.

(4) Figure 2A lacks an important control from non-senescent cells during the measurement of C2C12 differentiation in the presence of a conditioned medium.

Figure 2A tests the effect of prostaglandin PGD2 and its metabolites secreted by the senescent cells on the differentiation of myoblasts. Therefore, we inhibited the synthesis of PGD2 in senescent cells by treatment with AT-56, and then collected the conditioned medium. Conditioned medium collected from senescent C2C12 cells treated with vehicle (DMSO) served as a control for the experiment, whereas differentiation of C2C12 cells without any treatment serves as a positive control.

There is no explanation of how differentiation was quantified or how the fusion index was calculated.

The fusion index was calculated using a published myotube analyzer software (Noë et al., 2022). Appropriate information has been added to the materials and methods section of the manuscript.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

Line 3: Expand SA in "SA β -gal".

The manuscript has been updated accordingly (See line 3).

Line 68: HRas is highly regulated by lipid modifications.

The manuscript has been updated accordingly (See line 67).

Figures

Figure S1A seemed incomplete (maybe some processing issue).

The Figure has been updated in the revised manuscript (See Fig. S1A).

Figure S1B-H are mislabeled.

The figure has been updated in the revised manuscript (See Fig. S1C, D, E, and F).

Figures S1E-H are not mentioned in the manuscript.

The manuscript has been updated accordingly (See line 120).

Many supplementary figures are not cited in the article.

The manuscript has been updated accordingly. (See lines 85, 120, 123, 166, 225, 356, 364, 412, and 413)

Reviewer #2 (Recommendations For The Authors):

(1) Clarify the injection method for Doxorubicin in B6J mice on line 83 (IP or IM).

Mice were injected intraperitoneally with Doxorubicin (as mentioned in the materials and methods, see lines 83 and 794)

(2) Address missing information in figures or figure legends.

There is missing piece in Sup Fig 1A.

The figure has been updated in the revised manuscript (See Fig. S1A).

Correct labels in Sup Fig 1C and 1D.

The figure has been updated in the revised manuscript (See Fig. S1C, D, E, and F).

How would the authors explain the dramatic differences in the morphology of C2C12 cells treated with DOXO between bright field and SA-beta-gal staining images in Sup Fig 1B and 1C.

The SA β -gal image after treatment with Doxo does show a flattened cell morphology. Another field of view from the same experiment has been added in the figure to show the difference in the cell morphology more prominently in the revised manuscript (See Fig. 1H).

Provide explanations for Sup Fig 1E-1G, including the meaning of the y-axis and the blue dots and red lines.

We have provided an explanation for the multiple reaction monitoring mass spectrometry used to measure the concentration of 15d-PGJ2 in the conditioned medium in the revised manuscript (see lines 119-130 and the legends of Fig. S1C, D, and E)

(3) Please review the calculation of qPCR data in Figure 1C for correctness, ensuring reference samples with an average expression level of 1.

The data in Fig. 1C was plotted using $2^{-\Delta CT}$ instead of $2^{-\Delta\Delta CT}$ to show the variability in the expression of mRNAs isolated from animals treated with Saline.

(4) Please explain the calculation of 15d-PGJ2/cell concentration in Figure 1F and provide raw data for review, considering the substantial changes and small error bars. The method or result section lacks an explanation of how this calculation was performed. Additionally, there is no mention of the cell number count.

All the raw values (concentration of 15d-PGJ2 measured using mass spec and cell numbers counted at the time of collection of conditioned medium) are provided in the supplementary table 1. The standard curve to calculate the concentration of 15dPGJ2 in the conditioned medium is shown in Fig. S1F. The cell number was counted after trypsinization using a hemocytometer on the day of collection of the conditioned medium.

(5) Please clarify how cell number normalization and doubling time calculation were done in Fig 2B. Consider replacing the figure with a growth curve showing confluence on

| *the y-axis for easier interpretation.*

Cells were counted every 24 hours and the normalization was done to the number of cells counted on day 0 of the treatment (to consider attaching efficiency and other cell culture parameters). Doubling time was calculated as the reciprocal of the slope of the graph of $\log_2(\text{normalized cell number})$ vs time.

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