Senescent cells inhibit muscle differentiation via the lipid- SASP 15d-PGJ_2 mediated modification and control of HRas

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Abstract

Senescent cells which are characterized by multiple features such as increased expression of 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 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-PGJ_2, which is an electrophilic lipid produced by the non-enzymatic dehydration of the prostaglandin PGD_2. In this study, we show that 15d-PGJ_2 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 PGD_2, diminishes the release of 15d-PGJ_2 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- PGJ_2 to inhibit the differentiation of muscle cells and control the activity of HRas. This work shows that 15d-PGJ_2 released from senescent cells could be targeted to restore muscle homeostasis after chemotherapy.

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-PGJ_2(Wiley et al., 2021), a non-enzymatic dehydration product of prostaglandin PGD_2(Shibata et al., 2002). In the context of skeletal muscle, PGD_2 and 15d-PGJ_2 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-PGJ_2 as a member of SASP and identify mechanisms by which it might negatively affect muscle regeneration. 15d-PGJ_2 has been previously shown to covalently modify multiple proteins like MAPK1, MCM4, EIF4A-I, PKM1, GFAP etc. endothelial and neuronal cells(Marcone and Fitzgerald, 2013; Yamamoto et al., 2011). 15d-PGJ_2 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-PGJ_2 on muscle differentiation via covalent modification. We investigated HRas as a possible

effector of 15d-PGJ_2 because (i) HRas belongs to the Ras superfamily of small molecule GTPases and is known regulator of key cellular processes(BOHR Orthopredic Hospital et al., 1964; Davis et al., 1983; 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 Burgt 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 regulates 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-PGJ_2 is synthesized and released by senescent myoblasts upon treatment with Doxo. 15d-PGJ_2, 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-PGJ_2 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 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.

We then measured the induction of senescence *in vitro* in C2C12 mouse myoblasts after treatment with Doxo (Fig. S1B). We treated C2C12 myoblasts with Doxo (150 nM) and observed a flattened morphology and increased cell size (Fig. S1C). We also observed a significant increase in the size of nuclei in C2C12 cells after treatment with Doxo (Fig. 1D) and increased senescence-associated β galactosidase (SA-β gal) activity in cells treated with Doxo (Fig. S1D). These observations suggest the induction of senescence in C2C12 cells after treatment with Doxo.

Doxo-mediated senescence induces synthesis and release of 15d-PGJ_2 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 PGD_2 and its metabolite 15d-PGJ_2 have been shown to be significantly increased. Therefore, we measured the levels of mRNA of enzymes involved in the synthesis of PGD_2/15d-PGJ_2 (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. 1E). 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.

15d-PGJ_2 is a non-enzymatic dehydration product of PGD_2(Shibata et al., 2002). We observed an increase in the mRNA levels synthetic enzymes of 15d-PGJ_2 in senescent C2C12 cells. Therefore, we measured the levels of extracellular 15d-PGJ_2. We collected the conditioned medium from senescent and quiescent C2C12 cells and observed an increase in the concentration of 15d-PGJ_2 in the medium of senescent cells (Fig. 1F). This shows an increased release of 15d-PGJ_2 by senescent myoblasts.

Prostaglandin PGD_2 and its metabolites in the conditioned medium of senescent cells inhibit the differentiation of C2C12 myoblasts.

15d-PGJ_2 (the final non-enzymatic dehydration product of PGD_2) has been shown to inhibit the differentiation of myoblasts(Hunter et al., 2001). We observed the release of 15d-PGJ_2 by senescent cells, showing that 15d-PGJ_2 is a SASP factor (Fig. 1F). Conditioned medium of senescent cells inhibits the differentiation of myoblasts in myotonic dystrophy type 1(Conte et al., 2023). Therefore, we tested whether 15d-PGJ_2, the terminal dehydration product of PGD_2, 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; S. Hu et al., 2023; Shunfeng Hu et al., 2023; Irikura et al., 2009). We observed a decrease in the fusion index of the C2C12 myoblasts treated with the conditioned medium of senescent cells (Fig. 2A), suggesting that SASP factors decrease the differentiation of myoblasts. This decrease in inhibition was rescued in myoblasts treated with the conditioned medium of senescent cells treated with AT-56 (Fig. 2A). This suggests that prostaglandins PGD_2/15d-PGJ_2 released by senescent cells as SASP factors can inhibit the differentiation of myoblasts.

15d-PGJ_2 inhibits the proliferation and differentiation of mouse and human myoblasts.

15d-PGJ_2 has been shown to affect the proliferation of cancer cell lines, both positively and negatively (Chen et al., 2003; Choi et al., 2020; Slanovc et al., 2024; Yen et al., 2014). We measured the effect of 15d-PGJ_2 on the proliferation of C2C12 myoblasts. We treated C2C12 myoblasts with 15d-PGJ_2 (10 µM) or DMSO for 72 hours. We observed a decrease in the proliferation of C2C12 cells after treatment with 15d-PGJ_2 (Fig. 2B). The doubling time of C2C12 cells was also increased upon treatment with 15d-PGJ_2 (57.24 hours) compared to DMSO (13.76 hours). This suggests that 15d-PGJ_2 decreases the proliferation of C2C12 myoblasts.

We measured the differentiation of C2C12 mouse and primary human myoblasts after treatment with 15d-PGJ_2. C2C12 myoblasts showed cell death after 24 hours and 48 hours upon treatment with 15d-PGJ_2 (10 µM and 5 µM) respectively in the C2C12 differentiation medium (Fig. S2A). Therefore, we treated differentiating

myoblasts with 15d-PGJ_2 (1 µM, 2 µM, and 4 µM) for 5 days. We observed a dose-dependent decrease in the mRNA levels of MyoD, MyoG, and MHC in differentiating C2C12 cells after treatment with 15dPGJ_2 (Fig. 2C). We also observed a decrease in the fusion of myoblasts into myotubes in C2C12 cells treated with 15d-PGJ_2 (4 µM) compared to DMSO (Fig. 2D). We also observed a dose-dependent decrease in the protein levels of MHC in differentiating primary human myoblasts upon treatment with 15d-PGJ_2 (Fig. 2E). These observations suggest that 15d-PGJ_2 inhibits the differentiation of both mouse and human myoblasts.

Biotinylated 15d-PGJ_2 covalently modifies HRas at Cysteine 184.

15d-PGJ_2 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-PGJ_2 at cysteine 184 in NIH3T3 and Cos1 cells(Luis Oliva et al., 2003). Therefore, we tested whether 15d-PGJ_2 could covalently modify HRas in C2C12 cells. We treated C2C12 cells expressing the EGFP-tagged wild-type HRas with biotinylated 15d-PGJ_2 (5 µM). We then immunoprecipitated biotinylated 15d-PGJ_2 using streptavidin. We observed a significant increase in the pulldown of HRas upon treatment with 15d-PGJ_2-biotin compared to DMSO (Fig. 3A), suggesting an interaction between 15d-PGJ_2 and HRas. To measure the role of individual C-terminal cysteines in the binding of HRas with 15d-PGJ_2, we treated C2C12 cells expressing the EGFP-tagged C181S and C184S mutants of HRas with biotinylated 15d-PGJ_2 (5 µM), and immunoprecipitated using streptavidin. We observed that the intensity of EGFP-tagged HRas was significantly decreased in cells expressing the C184S mutant but not in those expressing the C181S mutant (Fig. 3A). This suggests that 15d-PGJ_2 covalently modifies HRas at cysteine 184 in C2C12 cells.

15d-PGJ_2 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-PGJ_2 on HRas GTPase activity using FRET. mCherry RAF RBD is a well characterized sensor of 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-tagged Ras Binding Domain of

RAF kinase (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 expressing EGFP-HRas WT before and after treatment of 15d-PGJ_2 (10 µM) for 1 hour. We observed an increase in the mean acceptor normalized FRET index upon treatment with 15d-PGJ_2 (Fig. 3C). This suggests that 15d-PGJ_2 activates HRas. To measure the role of individual C-terminal cysteines in 15d-PGJ_2 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-PGJ_2 (10 µM). We observed an increase in the mean acceptor normalized FRET index in cells expressing EGFP-HRas C181S upon treatment with 15d-PGJ_2 but not in cells expressing EGFP-HRas C184S (Fig. 3D). These observations suggest that activation of HRas by 15d-PGJ_2 occurs in a cysteine 184 dependent manner.

15d-PGJ_2 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-PGJ_2 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-PGJ_2 (5 µM and 10 µM) 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-PGJ_2 (Fig. S3C). These observations suggest that 15d-PGJ_2 activates the MAPK signaling pathway, but not the PI3K signaling pathway.

15d-PGJ_2 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-PGJ_2 (10 µM), a 15d-PGJ_2 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-PGJ_2 was significantly reduced as compared to the treatment with 15d-PGJ_2 (Fig. 3F). This shows that 15d-PGJ_2 activates the HRas-MAPK signaling pathway via the electrophilic center in its cyclopentenone ring.

15d-PGJ_2 increases the localization of EGFP-tagged HRas at the plasma membrane compared to the Golgi in a C-terminal cysteine-dependent manner.

15d-PGJ_2 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-PGJ_2 alters the intracellular distribution of HRas. We co-expressed the EGFP-tagged wild type and the C181S and the C184S mutants of HRas 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-PGJ_2 (10 μM) for 24 hours and observed a decrease in the R_{mean} of C2C12 cells expressing the wild-type HRas after treatment with 15d-PGJ_2 (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-PGJ_2 (Fig. 4C). These observations suggest that 15d-PGJ_2 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-PGJ_2 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-PGJ_2 covalently modifies cysteine 184 and

activates HRas (Fig. 3). Therefore, we tested whether the inhibition of myoblast differentiation by 15d-PGJ_2 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-PGJ_2 (4 µM) 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-PGJ_2. 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 and HRas C181S after treatment with 15d-PGJ_2 (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-PGJ_2 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, 2004; 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(Dimri et al., 1995; Lee et al., 2006), and altered metabolic state(Bittles and Harper, 1984; Jones et al., 2005; Wiley and Campisi, 2021, 2016; Zwerschke 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-associate 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 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 J_2 (15d-PGJ_2) (Fig. 1E and F), the terminal non-enzymatic dehydration product of prostaglandin PGD_2(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 PGD_2 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 (Fig. 2A). A study has shown that prostaglandin PGD_2 inhibits differentiation of C2C12 myoblasts(Veliça et al., 2010), but the authors noted that knockout of DP1 and DP2 (the known receptors of prostaglandins PGD_2) does not abrogate inhibition of differentiation of myoblasts by PGD_2. This observation suggested that PGD_2 might inhibit the differentiation of myoblasts by a receptor-independent mechanism, possibly by its spontaneous non-enzymatic dehydration to 15d-PGJ_2. 15d-PGJ_2 has been suggested to be an endogenous ligand of PPARγ(Li et al., 2019). However, the inhibition of PPARγ did not abrogate the inhibition of differentiation of C2C12 myoblasts by 15d-PGJ_2, suggesting existence other possible mechanisms(Hunter et al., 2001). 15d-PGJ_2 has varied effects on cell physiology in a context dependent manner. On one hand, 15d-PGJ_2 promotes tumorigenesis by inducing epithelial to mesenchymal transition in breast cancer cell line MCF7(Choi et al., 2020), 15d-PGJ_2 inhibits proliferation of

A549, H1299, and H23 lung adenocarcinoma cells via induction of ROS and activation of apoptosis(Slanovc et al., 2024). Here, we show that 15d-PGJ_2 inhibits the proliferation and the differentiation of C2C12 myoblasts (Fig. 2B, C and D).

15d-PGJ_2 contains an electrophilic cyclopentenone ring in its structure, allowing 15d-PGJ_2 to covalently modify and form a Michael adducts with cysteine residues of proteins(Shibata et al., 2002). A previous proteomic study in endothelial cells showed biotinylated 15d-PGJ_2 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 regulation (Elongation factor 1 and 2, EIF4A-I), intracellular transport (Importin subunit β1, Exportin 2, Kinesin 1 heavy chain)(Marcone and Fitzgerald, 2013). Another proteomic study in neuronal cells suggested that 15d-PGJ_2 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). This study also showed modification of a peptide fragments homologous to IκB kinase β, Thioredoxin, and a small molecule GTPase HRas. 15d-PGJ_2 modifies HRas in NIH3T3 and Cos1 cells(Luis Oliva et al., 2003) and IMR90 cells(Wiley et al., 2021). Modification by 15d-PGJ_2 led to activation of HRas, judged by an increase in GTP bound HRas. It is clear that 15d-PGJ_2 is capable of modifying numerous proteins in different contexts. In spite of these observations, the functional relevance of these modifications in numerous contexts remains to be mapped. Here we focused on the role of 15d-PGJ_2 in the context of senescence and skeletal muscle differentiation. In this study, we showed that 15d-PGJ_2 covalently modifies HRas at cysteine 184 but not cysteine 181 in C2C12 myoblasts (Fig. 3A). We showed by FRET microscopy that modification of HRas by 15d-PGJ_2 in HRas WT and HRas C181S activates HRas in C2C12 cells, but 15d-PGJ_2 is unable to activate HRas C184S in this context (Fig. 3C and D). This observation shows a direct link between modification of HRas by 15d-PGJ_2 and activation of HRas GTPase.

HRas activates two major downstream signaling pathways, the HRas-MAPK and the HRas-PI3K pathway(Pylayeva-Gupta et al., 2011). We showed that covalent modification of HRas by 15d-PGJ_2 via the electrophilic cyclopentenone ring activates HRas (Fig. 3C and D) and activates the HRas-MAPK pathway, demonstrated by an increase in the phosphorylation of Erk after treatment with 15d-PGJ_2 (Fig. 3E and

F). 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-PGJ_2 (Fig. S3C). MAPK and PI3K pathways are known regulators of muscle differentiation(Bennett and Tonks, 1997; Rommel et al., 1999), where inhibition of the RAF-MEK-Erk pathway or activation of PI3K pathway promotes differentiation of myoblasts. Preferential activation of HRas-MAPK over HRas-PI3K pathway after treatment with 15d-PGJ_2 can be a possible mechanism by which 15d-PGJ_2 can inhibit differentiation of myoblasts. HRas is known to regulate differentiation of myoblasts in different contexts. Constitutively active HRas signaling by oncogenic HRas mutant (HRas V12) leads to inhibition of differentiation of myoblasts(Konieczny et al., 1989; Lassar et al., 1989; Olson,' et al., 1987; Van Der Burgt et al., 2007). Here we showed that inhibition of differentiation of myoblasts after 15d-PGJ_2 is partially rescued in cells expressing C184S mutants of HRas but not the wild type or C181S mutant (Fig. 4D and E and S4E). HRas C184S did not get modified by 15d-PGJ_2 (Fig. 3A). These observations suggest that the inhibition of differentiation of myoblasts by 15d-PGJ_2 is partially dependent on modification of HRas by 15d-PGJ_2.

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 depalmitoylation(Rocks et al., 2005). Inhibition of palmitoylation of C-terminal cysteine 181, wither 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 modification of HRas by 15d-PGJ_2 alters the intracellular distribution of HRas. We showed that modification of HRas by 15d-PGJ_2 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-PGJ_2 (Fig. 4C). HRas C184S is not modified by 15d-PGJ_2, but HRas C181S is modified by 15d-PGJ_2 (Fig. 3A). This suggests that intracellular

redistribution of HRas due to modification by 15d-PGJ_2 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 strong correlation with expression of cell cycle genes, particularly the MAPK signaling pathway. Localization of HRas at the plasma membrane also showed 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 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 HRas mediated inhibition of differentiation of myoblasts.

Doxorubicin (Doxo) is a widely used chemotherapy agent for 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 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 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-PGJ_2 by senescent cells which

could be taken up by myoblasts in the microenvironment. The lipid could covalently modify and activates HRas at cysteine 184 to inhibit differentiation of myoblasts. Therefore, targeting the synthesis and release of 15d-PGJ_2 by senescent cells could serve as an important target to promote skeletal muscle homeostasis in cancer patients.

Figures

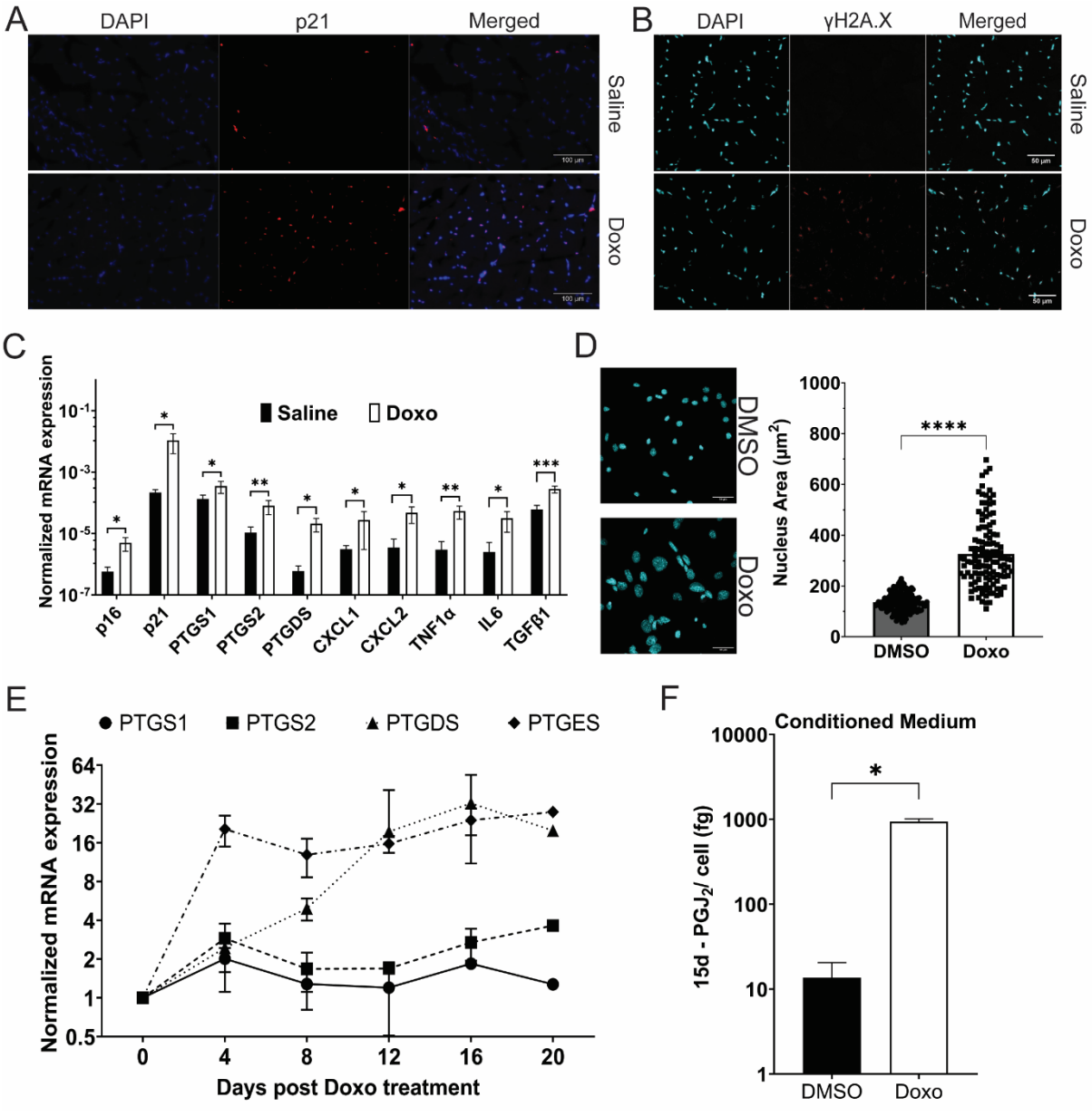


Figure 1. Prostaglandin synthesis and release by Doxo-induced senescent cells inhibits myoblast differentiation.

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, TNF1α, IL6, TGFβ1), and enzymes involved in the biosynthesis of prostaglandin PGD_2/15d-PGJ_2 (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. Expression of mRNAs of prostaglandin biosynthetic enzymes, measured by qPCR, in C2C12 myoblasts after treatment with Doxo (150 nM) or DMSO.
F. Concentration of 15d-PGJ_2 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)

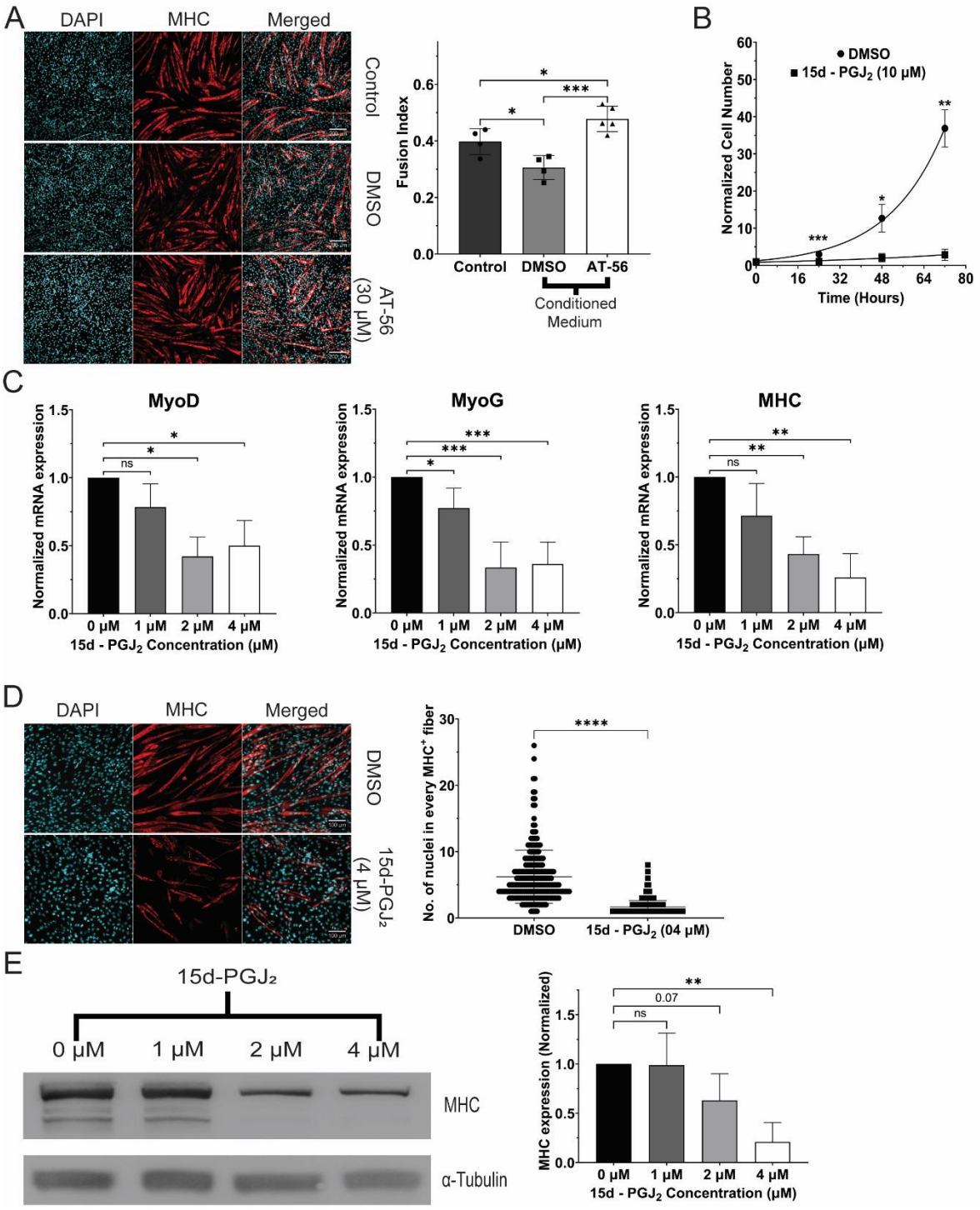


Figure 2. 15d-PGJ$_2$ inhibits differentiation of myoblasts.

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 μM) or DMSO

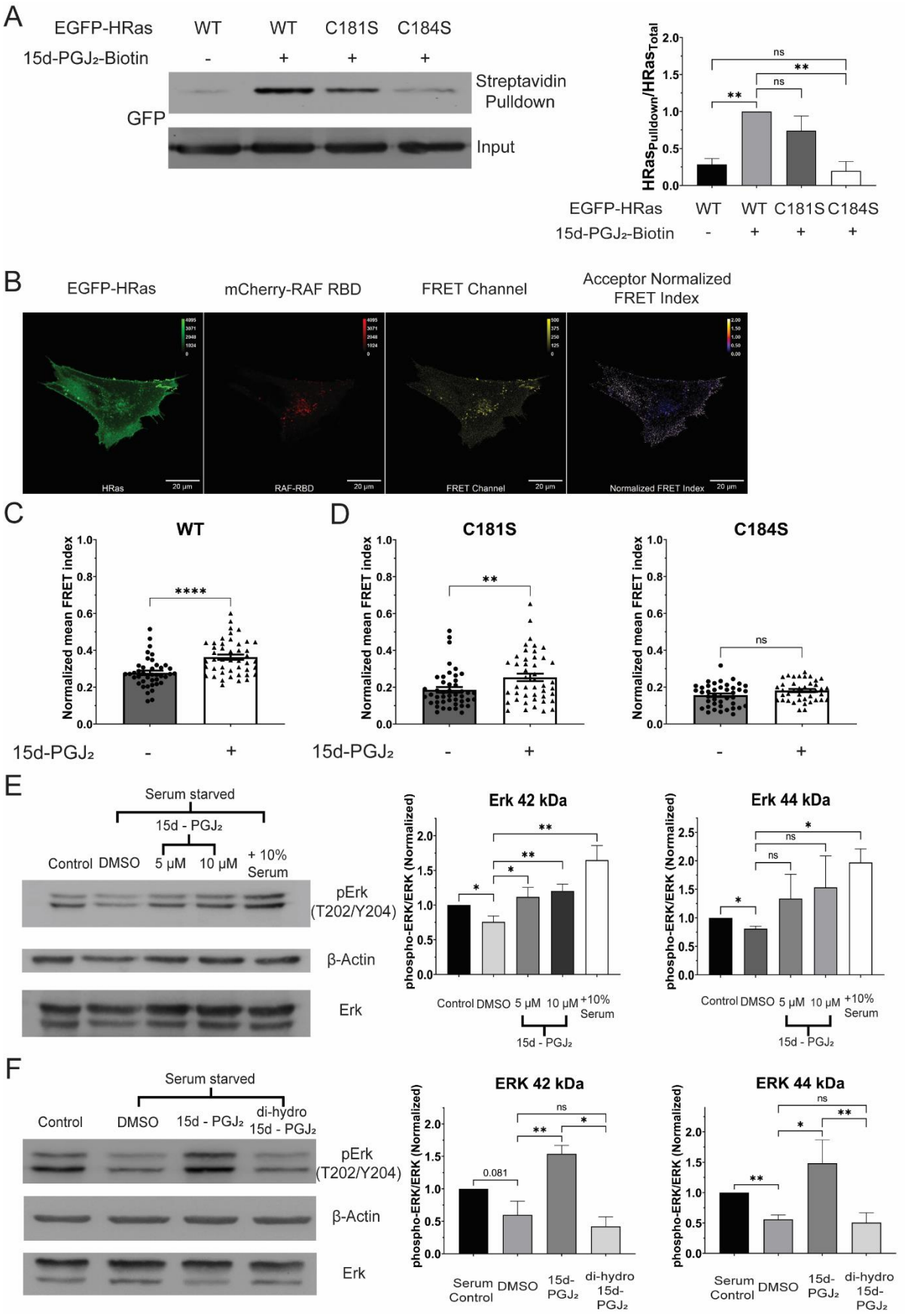
B. Normalized number of C2C12 myoblasts treated with 15d-PGJ$_2$ (10 μM) or DMSO

C. Expression of mRNAs of markers of differentiation (MyoD, MyoG, and MHC), measured by qPCR, in C2C12 myoblasts treated with 15d-PGJ_2 (1 µM, 2 µM, or 4 µM) or DMSO.

D. Expression of MHC protein and the fusion of myoblasts in syncytial myotubes, measured by immunofluorescence, after treatment with 15d-PGJ_2 (4 µM) or DMSO.

E. Expression of MHC protein, measured by immunoblotting, in primary human skeletal myoblasts after treatment with 15d-PGJ_2 (1 µM, 2 µM, or 4 µM) 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)


A
EGFP-HRas
WT
WT
C181S
C184S
15d-PGJ₂-Biotin
-
+
+
+
Streptavidin Pulldown
GFP
Input
HRas Pulldown/HRas Total
ns
**
ns
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B
EGFP-HRas
mCherry-RAF RBD
FRET Channel
Acceptor Normalized FRET Index
HRas
RAF-RBD
FRET Channel
Normalized FRET Index
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Normalized mean FRET index
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15d-PGJ₂
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+
D
C181S
C184S
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ns
E
Serum starved
15d - PGJ₂
Control
DMSO
5 µM
10 µM
+ 10% Serum
pErk (T202/Y204)
β-Actin
Erk
Erk 42 kDa
Erk 44 kDa
phospho-ERK/ERK (Normalized)
F
Serum starved
Control
DMSO
15d - PGJ₂
di-hydro 15d - PGJ₂
ERK 42 kDa
ERK 44 kDa
0.081
Serum Control
DMSO
15d-PGJ₂
di-hydro 15d-PGJ₂

460

Figure 3. 15d-PGJ$_2$ 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-PGJ$_2$-Biotin (5 µM).
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-PGJ$_2$ (10 µM) 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-PGJ$_2$ (10 µM) 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-PGJ$_2$ (5 µM, 10 µM) 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-PGJ$_2$ (10 µM)/ 9,10-dihydro-15d-PGJ$_2$ (10 µM) 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)

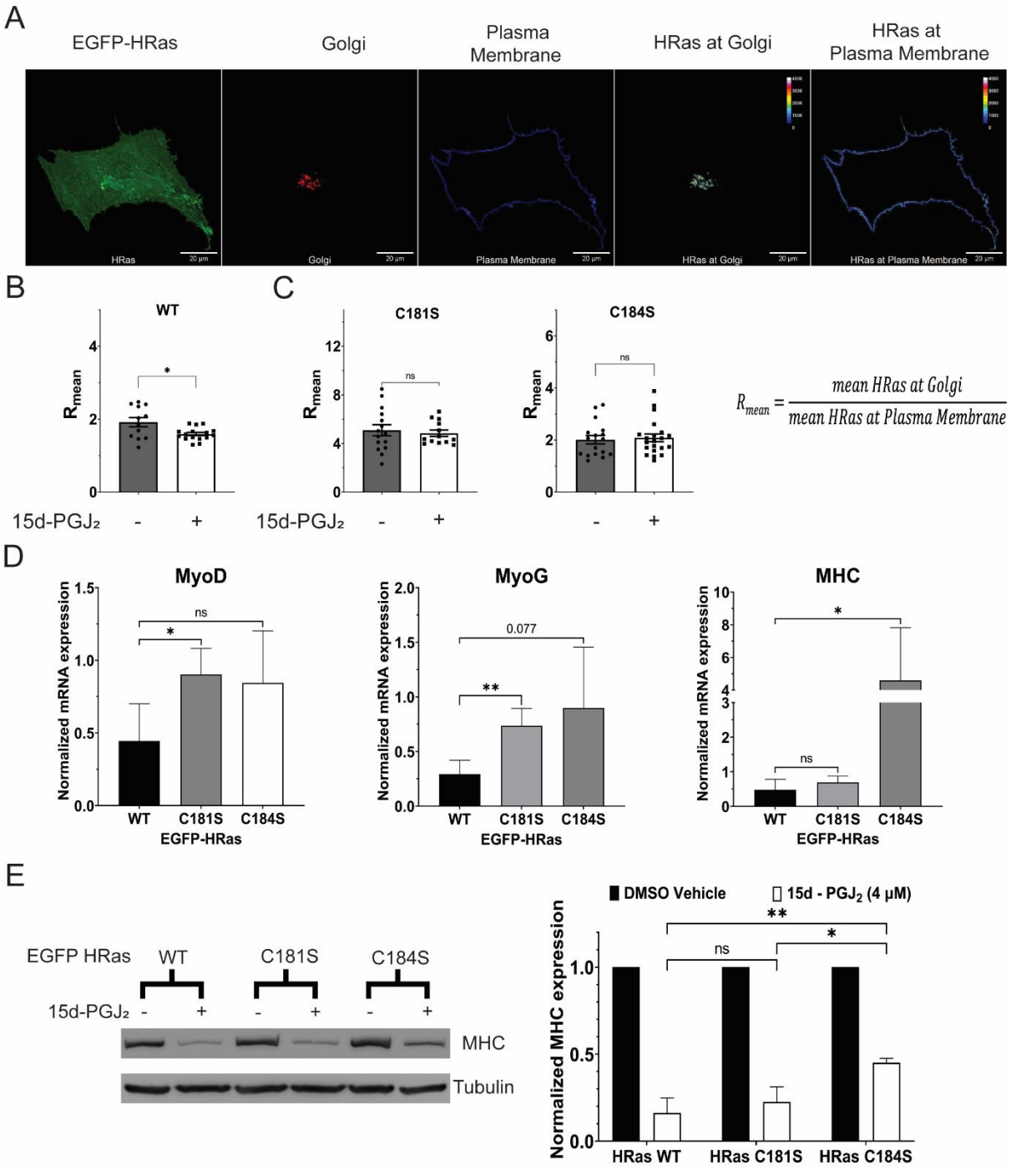

Figure 4. 15d-PGJ$_2$ controls the intracellular distribution of HRas and differentiation of C2C12 cells in an HRas C-terminal cysteine-dependent manner.

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-PGJ_2 (10 µM) 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-PGJ_2 (10 µM) 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-PGJ_2 (4 µM) 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-PGJ_2 (4 µM) 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)

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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.

SSS: Cell culture treatments and assays, Biochemistry, Writing: review and edits.

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

AV: Cell culture treatments and assays, Image processing and analysis, writing: review and edits.

MAJ: Image processing and analysis.

RGHM: Mass Spectrometry.

AR: Project Conceptualization, Writing: original draft, review, and edits. Corresponding Author.

Competing Interests

The authors declare no competing interests.

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Materials and Methods

Materials and Methods:

- **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 using Phusion High Fidelity DNA Polymerase (Thermo Scientific) followed by restriction digestion of HRas constructs and the pEGFPC1 vector using Xho1/EcoR1 enzymes (New England Biolabs Inc.). Ligation was set up using T4 DNA ligase (Takara Bio.). 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. Phillipe Bastiens and was used to measure the activity of HRas GTPase using FRET.

- **Cell Culture:**
  - **Cell Maintenance:**

C2C12 mouse myoblasts (CRL – 1772) were obtained from ATCC and were maintained in DMEM complete medium @ 37° C, 5% CO_2. For experiments, the cells were trypsinized with 0.25% trypsin – EDTA (Gibco): DPBS (Gibco) (1:1) @ 37° C, 3 mins. The cells were then resuspended in a triple volume of DMEM complete medium and were counted manually with a hemocytometer after diluting the cells with trypan blue (Gibco) (1:1) and were seeded in required numbers in cell culture dishes. Human Skeletal Muscle Myoblasts P2 (CC-2580) were obtained from Lonza and were maintained in DMEM Skeletal Muscle growth medium @ 37° C, 5% CO_2. For experiments, the cells were trypsinized with 0.25% trypsin – EDTA (Gibco): DPBS (Gibco) (1:1) @ 37° C, 2 mins. The cells were then resuspended in a double volume of DMEM complete medium and were counted manually with a hemocytometer after diluting the cells with trypan blue (Gibco) (1:1) and were seeded in required numbers in cell culture dishes. All cultures tested negative for mycoplasma checked by Mycoalert Mycoplasma Detection Kit (Lonza).

  - **Conditioned medium collection:**

C2C12 cells seeded in 35mm 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, centrifuged @1000g, R.T. for 5 minutes. The media was then stored at -80° C after flashfreezing in liq. N_2 till further requirement.

- **Treatments:**
  - **15d-PGJ$_2$:**

15d-PGJ$_2$ (Cayman Chemical Company) dissolved in methyl acetate was purged with N_2 stream @ R.T. till drying and was then redissolved in DMSO to make a 10 mM stock solution. 15d-PGJ$_2$ (10 mM) stock was then appropriately diluted in an appropriate volume of DMEM media for experiments. DMSO was used as vehicle control. A media change of the same composition was given every 24 hours.

  - **9,10-dihydro-15d-PGJ$_2$:**

9,10-dihydro-15d-PGJ$_2$ (Cayman Chemical Company) dissolved in methyl acetate was purged with N_2 stream @ R.T. till drying and was then redissolved in DMSO to make a 10 mM stock solution. 9,10-dihydro-15d-PGJ$_2$ (10 mM) stock was then appropriately diluted in an appropriate volume of DMEM media for experiments. DMSO was used as a control.

  - **Doxorubicin:**

C2C12 cells with 70-80% confluency in a 10 cm plate were treated with Doxorubicin (Doxo) for 3 days. After 72hr, Doxo was removed from the medium and the cells were kept for 10 more days with media change every 3 days. The cells were harvested on day 13 after Doxo treatment for experiments.

  - **15d-PGJ$_2$-Biotin:**

C2C12 cells transfected with EGFP-HRas WT/C181S/C184S in 35mm dishes were treated with 15d-PGJ$_2$-Biotin in DMEM Hi Glucose medium (Gibco) supplemented with 1% Penicilin-streptomycin-Glutamine (Gibco) without fetal bovine serum for 3 hours. The cells were then harvested for immunoprecipitation.

  - **Conditioned medium:**

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 seeded in 35mm dishes on coverslips were treated with the conditioned medium. A media change was given every 48 hours.

- **Transfections:**

C2C12 cells were seeded in 35 mm dishes at an intermediate density to achieve confluency of ~60 - 70%. For western blot, immunoprecipitation, and differentiation experiments, the cells were transfected with 1.5 µg of 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. Transfection efficiency was confirmed by checking for GFP fluorescence in cells under a Ti2 epifluorescence microscope (Nikon) using appropriate filters. For measuring the intracellular distribution of HRas, the cells were reverse transfected with 1µg each of 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 jetPRIME, where the DNA - jetPRIME mixture was incubated with the cell suspension while seeding the cells after trypsinization, and the transfection efficiency was confirmed by checking GFP and RFP fluorescence after 24 hours of transfection. For measuring the activity of HRas, the cells seeded in imaging dishes (iBidi) were transfected with 0.7 µg EGFP-HRas and 1.4 µg 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:**
  - **Untransfected cells:**

C2C12 cells were seeded in 35 mm dishes (Corning) at high density to achieve a confluency of ~90 – 95% the next day. The cells were then treated with either 15d-PGJ_2 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-PGJ_2 treatment for either RNA or protein isolation. For the Immunofluorescence experiment, the experiment was done in 35 mm dishes

(Corning) on glass coverslips (Blue star) coated with 0.2% Gelatin (Porcine, Sigma Aldrich), and the cells were fixed with the fixative solution at the end of the experiment and were immunostained. Human Skeletal Muscle Myoblast cells were seeded in 35 mm dishes in high density to achieve a confluency of ~90 – 95% the next day. The cells were then treated with DMSO or 15d – PGJ_2 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 in RIPA - PP.

- **Transfected cells:**

C2C12 cells were seeded in 35 mm dishes (Corning) at an intermediate density to achieve a confluency of ~60 – 70% confluency. The cells were transfected with EGFP-tagged HRas WT/ HRas-C181S/ HRas-C184S/HRas V12/HRas V12-C181S/ HRas V12-C184S using jetPRIME transfection reagent (Polyplus). After confirming a transfection efficiency of ~80% the next day, the cells were treated with DMSO vehicle or 15d – PGJ_2 (4 µM) 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 – PGJ_2 treatment for either RNA or protein isolation.

- **X-Gal staining:**

Proliferative and day-13 dox-treated cells were seeded at a density of $1x10^5$ cells /well into 35mm dishes. After overnight incubation, SA-β-gal activity was measured by using Senescence β-Galactosidase Staining Kit (Cell Signalling #9860) per manufacture's protocol. In brief, the cells were washed with PBS twice and were fixed for 10-15 min at RT. Following three washes of PBS, cells were incubated overnight in staining solution at 37℃ in a CO_2–free chamber. Development of blue colour was examined with Ti2 widefield inverted microscope (Nikon).

- **Immunoprecipitation:**

C2C12 cells transfected with EGFP-HRas and treated with 15d-PGJ_2-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 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 thrice with IP washing buffer and

then boiled in 20 µl Laemmlli buffer. 15 µl of the beads were loaded on 12% SDS – Polyacrylamide gel for detection of EGFP-HRas by immunoblotting using EGFP antibody.

- **Gene expression analysis:**
  - **Western blotting:**

For measuring Erk/Akt phosphorylation in C2C12 cells (untransfected/ transfected with EGFP-tagged HRas WT/ HRas-C181S/ HRas-C184S/ HRas V12/ HRas V12-C181S/ HRas V12-C184S) 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 - PGJ_2 after 24 hrs of starvation @37° C. The cells were harvested 1 hr 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 Laemmlli 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 Laemmlli 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 - PGJ_2, 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 (p.m. 43). Relative gene expression was quantified using the $\Delta\Delta C_T$ method[44] 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 MyHC 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 FV3000 inverted confocal laser scanning microscope (Olympus – Evident) using appropriate lasers and detectors.

- **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 – PGJ_2 (10 µM) 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., 5 mins, washed with PBS, and stained for plasma membrane with Alexa Fluor 633 conjugated Wheat Germ Agglutinin (WGA - 633) (Invitrogen) (10 µg/ml) @R. T. 15 mins. 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., 5 mins, washed with PBS, stained with WGA – 633, and mounted on slides in Prolong gold antifade medium (Thermo Scientific). The cells were imaged with FV 3000 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.

- **Intracellular activity of HRas:**

C2C12 cells expressing EGFP-tagged HRas WT/HRas C181S/HRas C184S + mCherry-RAF RBD were starved overnight in the DMEM starvation medium. The cells were imaged with FV 3000 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-PGJ_2 (10 µM) 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). 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-PGJ_2.

- **Animal care:**
  - **Maintenance:**

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).

  - **Treatment and tissue collection:**

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-PGJ$_2$ by mass spectrometry:**

For lipid extraction, cell pellets were resuspened 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 2ml of CM, making a uniform sample volume of 3 ml. Subsequently, 1ml of ethyl acetate was added to each sample and mixed vigorously. Phase separation was done by centrifuging the mixture (12000xg, 4℃ 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-PGJ$_2$ was performed using an electrospray ionisation 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-PGJ$_2$ were prepared and were 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.

Table 1: 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	TAAACGCAAGTGCCATTCCTG
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	GAGTACGCTCTGCTATTCAGC
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	AGTGGTATAGACAGGTCTGTTGG
mm TGF1β qPCR Fwd	CTTCAATACGTCAGACATTCGGG
mm TGF1β qPCR Rev	GTAACGCCAGGAATTGTTGCTA

Table 2: List of Reagents

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
cOmplete 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
WesternBrightTM ECL-spray Western blotting detection system	advansta	K-12049-D50
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

Table 3: List of Antibodies

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)	

p21	Monoclonal	Mouse	Santacruz Biotechnology	sc-6246
γH2A.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

Reagent Composition

- 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 KH_2PO_4, 137.93 mM NaCl, 8.06 mM Na_2HPO_4 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% NaN_3 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.

Supplementary Figures:

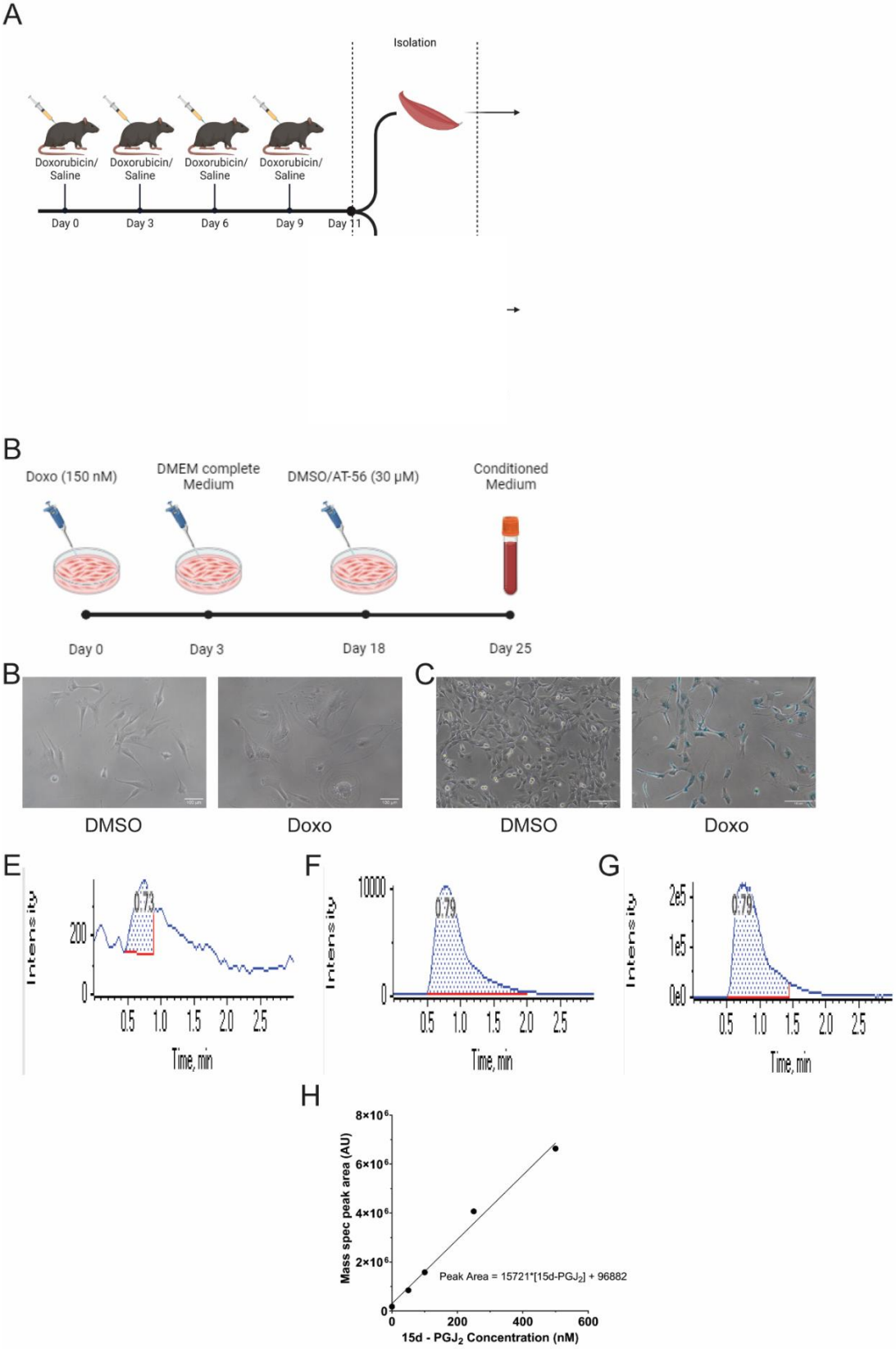

Figure S1. Treatment with Doxo induces senescence *in vivo* and *in vitro* and induces release of eicosanoid prostaglandin 15d-PGJ$_2$

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 μM) or DMSO.
C. Widefield micrograph of the morphology of C2C12 cells after treatment with Doxo (150 nM) or DMSO.
D. Widefield micrograph of expression of Senescence Associated β-galactosidase (SA β-gal), measured by X-gal staining, in C2C12 cells after 13 days of treatment with Doxo (150 nM) or DMSO.
E. Representative peaks from Blank sample.
F. Representative peaks from Conditioned medium of C2C12 myoblasts treated with DMSO.
G. Representative peaks from Conditioned medium of C2C12 myoblasts treated with Doxo (150 nM).
H. Standard curve of peak area vs known conc. of 15d-PGJ_2 detected using AB SCIEX QTRAP 6500 mass spectrometer.

Sample	15d-PGJ_2 (pmol)	Cell Number	15d-PGJ_2/Cell (fg)
Intracellular Quiescent C2C12 cells_1	27.087	462500	18.531
Intracellular Quiescent C2C12 cells_2	18.080	650000	8.801
Intracellular Senescent C2C12 cells_1	809.633	287500	891.018
Intracellular Senescent C2C12 cells_2	940.032	300000	991.420
Conditioned medium Quiescent C2C12 cells_1	8.271	462500	5.659
Conditioned medium Quiescent C2C12 cells_2	6.554	650000	3.190
Conditioned medium Senescent C2C12 cells_1	45.814	287500	50.419
Conditioned medium Senescent C2C12 cells_2	38.893	300000	41.019

Supplementary Table 1: 15d-PGJ_2 conc. (pmol and fg/cell) detected in samples by mass spectrometry.

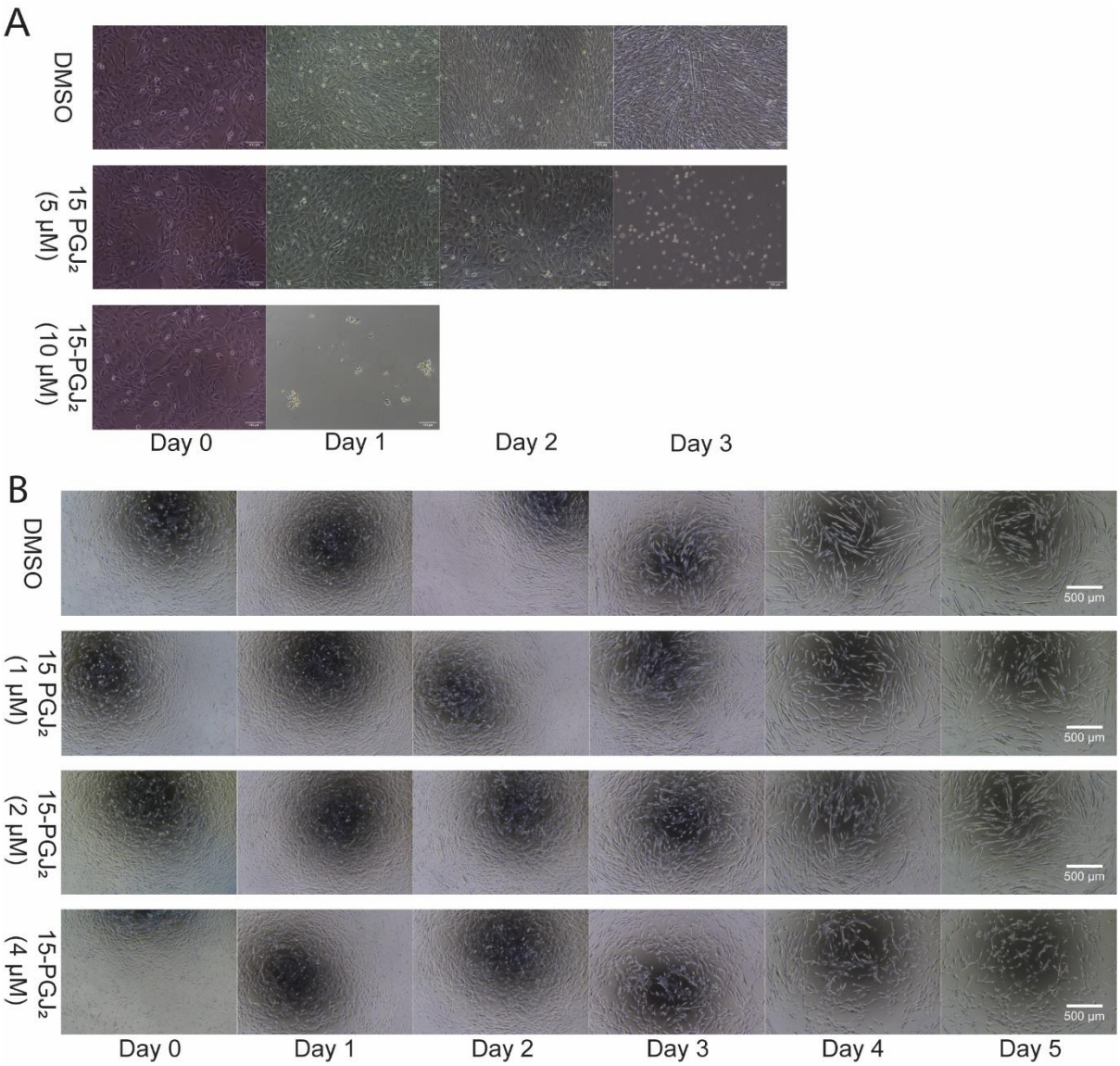

Figure S2. Reduced viability of C2C12 myoblasts after treatment with 15d-PGJ$_2$ (5 and 10 μM) in differentiating medium.

A. Representative images taken every 24 hours of C2C12 treated with 15d-PGJ$_2$ (5 and 10 μM) or DMSO in the C2C12 differentiation medium.
B. Representative images taken every 24 hours of C2C12 treated with 15d-PGJ$_2$ (1, 2, and 4 μM) or DMSO in the C2C12 differentiation medium.

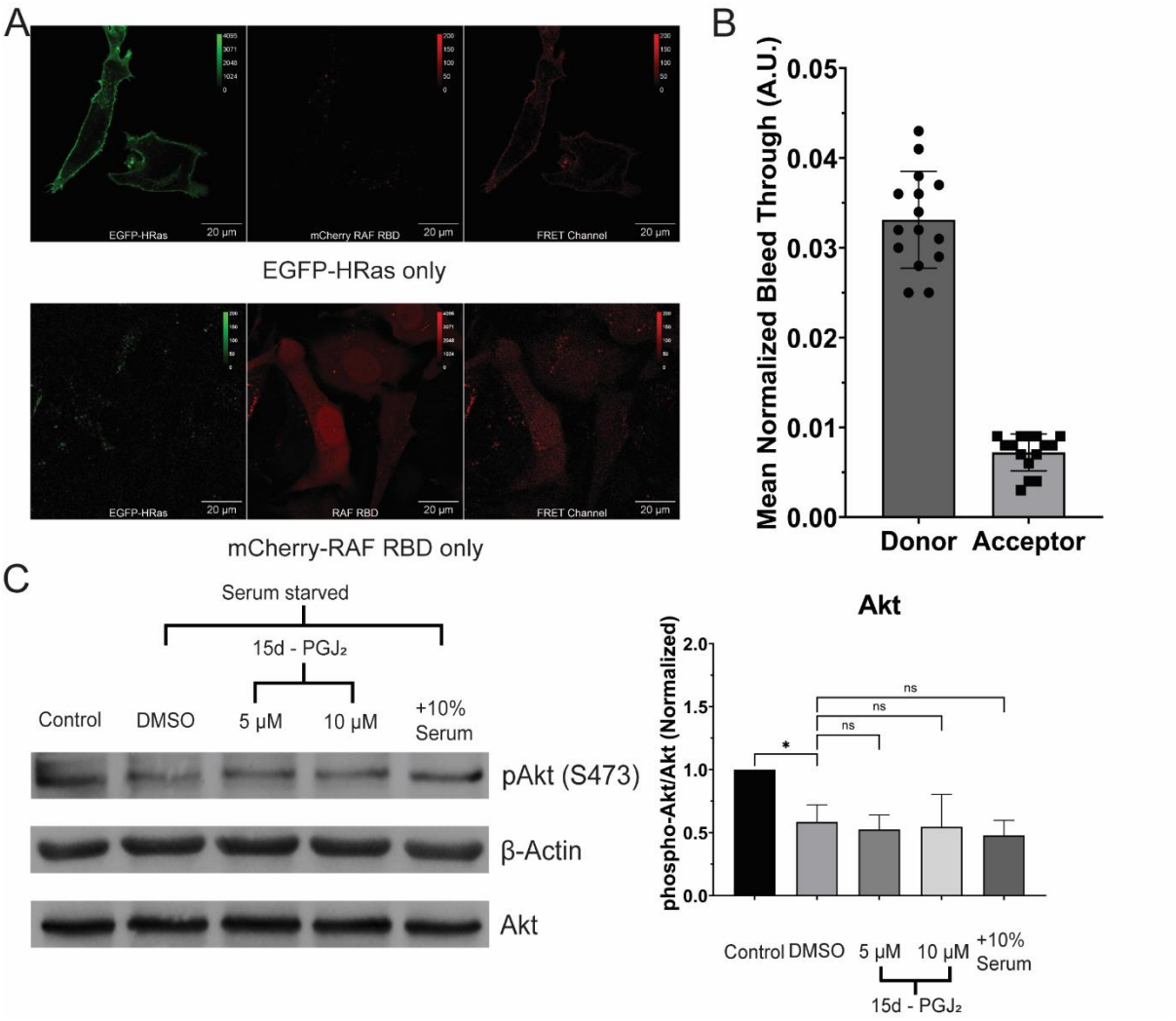

Figure S3. Activation of HRas after treatment with 15d-PGJ$_2$.

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-PGJ$_2$ (5, 10 µM) 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).

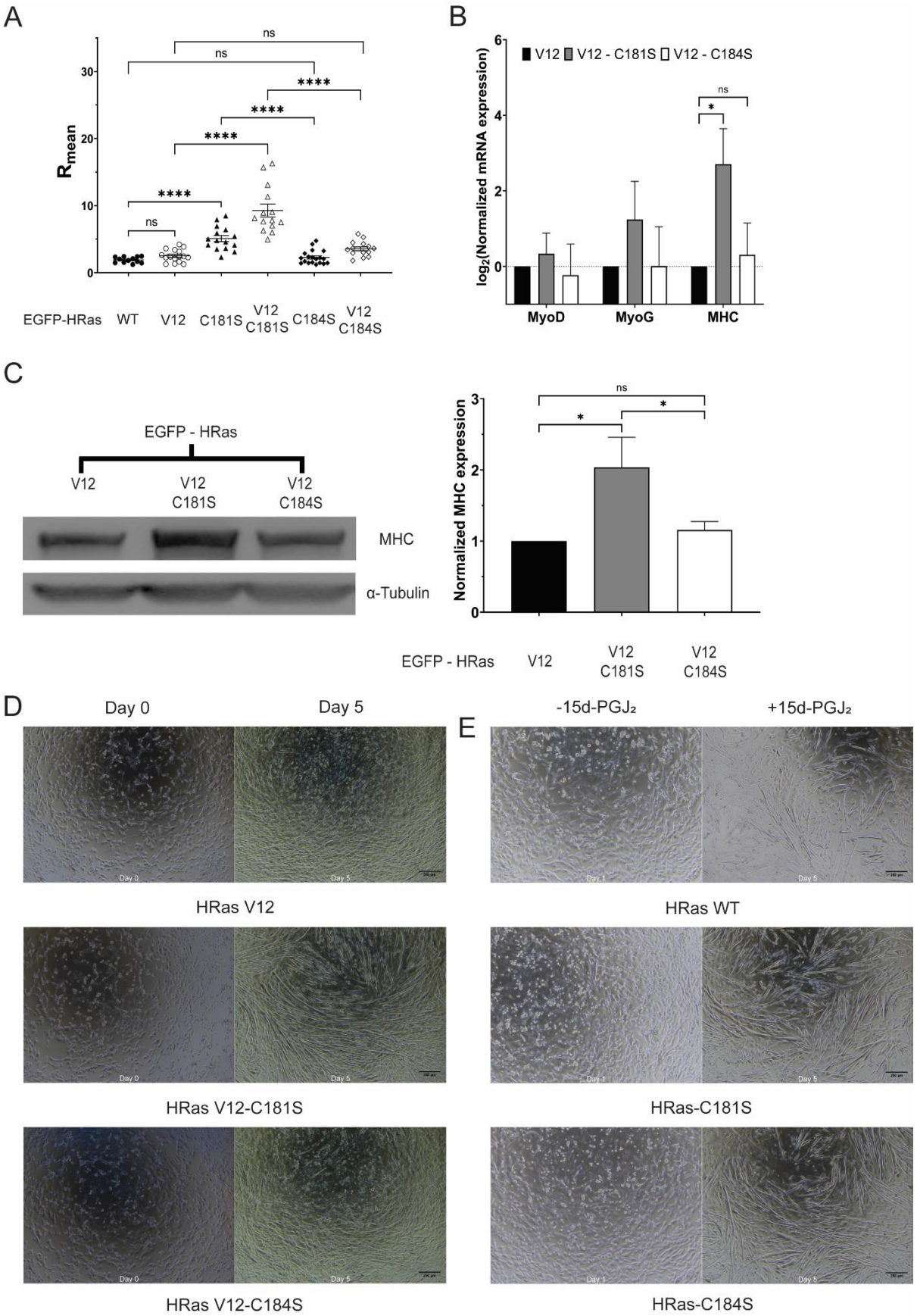
A
ns
ns
****
****
****
****
ns
R_{mean}
0
10
20
30
EGFP-HRas
WT
V12
C181S
V12 C181S
C184S
V12 C184S
B
V12
V12 - C181S
V12 - C184S
log_2(Normalized mRNA expression)
6
4
2
0
ns
*
MyoD
MyoG
MHC
C
EGFP - HRas
V12
V12 C181S
V12 C184S
MHC
α-Tubulin
Normalized MHC expression
3
2
1
0
ns
*
*
EGFP - HRas
V12
V12 C181S
V12 C184S
D
Day 0
Day 5
HRas V12
HRas V12-C181S
HRas V12-C184S
E
-15d-PGJ₂
+15d-PGJ₂
HRas WT
HRas-C181S
HRas-C184S

Figure S4. C-terminal cysteine mediated intracellular distribution of constitutively active HRas (HRas V12) regulates 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-PGJ_2 or DMSO.