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MEK drives cyclin D1 hyperelevation during geroconversion

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When the cell cycle becomes arrested, MTOR (mechanistic Target of Rapamycin) converts reversible arrest into senescence (geroconversion). Hyperexpression of cyclin D1 is a universal marker of senescence along with hypertrophy, beta-Gal staining and loss of replicative/regenerative potential (RP), namely, the ability to restart proliferation when the cell cycle is released. Inhibition of MTOR decelerates geroconversion, although only partially decreases cyclin D1. Here we show that in p21- and p16-induced senescence, inhibitors of mitogen-activated/extracellular signal-regulated kinase (MEK) (U0126, PD184352 and siRNA) completely prevented cyclin D1 accumulation, making it undetectable. We also used MEL10 cells in which MEK inhibitors do not inhibit MTOR. In such cells, U0126 by itself induced senescence that was remarkably cyclin D1 negative. In contrast, inhibition of cyclin-dependent kinase (CDK) 4/6 by PD0332991 caused cyclin D1-positive senescence in MEL10 cells. Both types of senescence were suppressed by rapamycin, converting it into reversible arrest. We confirmed that the inhibitor of CDK4/6 caused cyclin D1 positive senescence in normal RPE cells, whereas U0126 prevented cyclin D1 expression. Elimination of cyclin D1 by siRNA did not prevent other markers of senescence that are consistent with the lack of its effect on MTOR. Our data confirmed that a mere inhibition of the cell cycle was sufficient to cause senescence, providing MTOR was active, and inhibition of MEK partially inhibited MTOR in a cell-type-dependent manner. Second, hallmarks of senescence may be dissociated, and hyperelevated cyclin D1, a marker of hyperactivation of senescent cells, did not necessarily determine other markers of senescence. Third, inhibition of MEK was sufficient to eliminate cyclin D1, regardless of MTOR.
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In proliferating cells, MTOR (mechanistic Target of Rapamycin) stimulates cellular mass growth, whereas cyclins D and E initiate S-phase transition.[1–4] Withdrawal of growth factors causes reversible cell-cycle arrest (quiescence) with low levels of cyclins and MTOR activity, reversible by re-addition of growth factors. In contrast, cell-cycle arrest in the presence of growth stimuli, which activate MTOR, leads to cellular senescence.[5,6] In other words, when the cell cycle is blocked, while growth-promoting pathways such as MTOR remain active, cells continue to grow in size and undergo senescence. A process of conversion from normal reversible arrest into senescence was named as gerogenic conversion (geroconversion).[7] Cellular senescence is characterized by large flat morphology (hypertrophy), beta-Gal staining, very high levels of cyclins D1 and E, senescence-associated secretory phenotype and loss of replicative/regenerative potential (RP).[7–12] Loss of RP means that cells cannot proliferate even when cell-cycle block is removed and cells re-enter the cell cycle.[13,14] Rapamycin decreases hypertrophy and loss of RP, in a dose-dependent manner proportional to S6 dephosphorylation (a marker of MTORC1 activity).[15] Other agents (for example, hypoxia, mitogen-activated/extracellular signal-regulated kinase (MEK) and Mdm-2 inhibitors) that decelerate geroconversion indirectly (and partially) inhibit the MTOR pathway in various cell lines examined.[16–18]

We emphasize that our investigations are different from previous work on senescence. It has been known for over a decade that strong mitogenic/oncogenic signaling can cause senescence.[8,19–25] In all cases, this was due to induction of cell-cycle arrest. In some cases, inhibition of MEK/extracellular signal-regulated kinase (ERK) can prevent arrest induced by Ras, allowing cell proliferation to continue, leading to malignant phenotype.[20] Previous work demonstrated the role of the mitogen-activated protein kinase (MAPK) pathway in senescent type of cell-cycle arrest.[20] It is known that strong Ras signaling can cause induction of p21, p16 and other inhibitors of the cell cycle, thus eventually leading to senescent type of cell-cycle arrest.[26] Inhibitors of MEK/MAPK prevent cell-cycle arrest in response to oncogenic signals,[20,27,28] thereby blocking senescence and allowing cells to escape arrest and become proliferating and cancerous. The same effect can be achieved by induction of Ras/MAPK and simultaneous knockdown of inhibitors of cyclin-dependent kinases (CDKs) or deletion of p53.[20] The recent important study demonstrated that by decreasing cell-cycle activators, Ras caused senescent type of cell-cycle arrest,[28] and inhibitors of MEK (as well as depletion of CDK inhibitors) prevented senescence.[28] No arrest—no senescence.

We investigated a different topic. We examined how arrested cells became 'somehow' senescent instead of

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Abbreviations: RP, replicative/regenerative potential; IPTG, isopropyl-thio-galactosidase; LC, lactacystin; MTOR, mechanistic target of rapamycin; MEK, mitogen-activated/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; CDK, cyclin-dependent kinase
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quiescent. We did not investigate the mechanisms of arrest itself. Instead, we studied the next step: conversion of initially reversible arrest to senescence (geroconversion). In other words, we studied genuine aging. As an example, neurons are already arrested in a 5-year-old child but they are still 'young', and then during the lifetime, neurons undergo geroconversion. We demonstrated that when cell cycle is blocked, the active MTOR pathway drives geroconversion. Therefore, rapamycin and calorie restriction extend life span in all species examined from yeast to mice.[29] Rapamycin does not abrogate arrest or stimulate proliferation. By suppressing geroconversion, rapamycin preserves RP in the arrested cells. So cells remain arrested but not senescent, that is, lacking markers of senescence. In our experiments, MEK and MTOR inhibitors did not abrogate expression of ectopic p21/p16: our goal was to keep cells arrested. Thus, we could study geroconversion and its suppression (gerosupression). The word 'geroconversion' was coined 2 years ago.[7,17,18] It has become recognized that geroconversion is a basis of physiological aging in certain organisms.[30] Inhibition of MTOR suppresses aging in mice, worms, *Drosophila* and other species.[29] So suppression of cellular aging (gerosuppression), keeping cells 'young' but still arrested, is a new field of aging research. Cell-cycle arrest is not yet senescence.[7] In theory, strong mitogenic signaling such as Ras may cause both cell-cycle arrest (by inducing p21) and geroconversion (by activating MTOR).[5,31] Instead of investigating cell-cycle arrest and its abrogation in malignant transformation, we study the aging process, namely geroconversion and gerosuppression.[7]

Cyclins D1 and E are the driving force of cell-cycle transition from G1 to S phase in proliferating cells. Paradoxically, senescent cells have extremely high levels of cyclin D1.[14,32–40] The levels of cyclins D1 and E far exceeded their levels in proliferating cells.[14,41] Furthermore, hyperelevated cyclins were the earliest markers of geroconversion. Inhibition of MTOR prevented accumulation of cyclins D1 and E incompletely and rather transiently.[14] Despite inhibition of MTOR, levels of cyclin D1 were still highly elevated, even when compared with proliferating cells.[14] Hyperinduction of cyclin D1 seems to be the most persistent marker of senescence, and also the most mysterious one. Besides activation of CDK4/6, cyclin D1 exerts several other effects.[4,32,35,37,42–44]

Here we investigated whether other growth-promoting pathways rather than MTOR were involved in the hyperinduction of cyclin D1. There are several lines of reasoning that the MEK/ERK (MAPK) pathway may be a key driver. First, the MAPK pathway is the major inducer of cyclin D1 in proliferating cells.[3,45] Second, the MAPK pathway is activated in p21-induced senescent cells at similar or even higher levels than in proliferating cells.[46] Although inhibitors of MEK suppressed geroconversion in p21-arrested HT-p21 cells, this suppression was explained by indirect inhibition of the MTOR/pS6 pathway.[16] In fact, the MAPK pathway is known to affect phosphorylation of Raptor, p70S6K and RSK, thereby inducing S6 phosphorylation.[47–49] Incidentally, we observed that inhibition of MEK did not inhibit the MTOR pathway in some cell lines. This may provide the opportunity to elucidate MTOR-independent effects of MEK inhibition. In this study, we attempted to address several questions. Namely, are both the MTOR and MAPK pathways responsible for the high levels of cyclins observed? Can these markers be dissociated from other markers of senescence such as morphology and RP? Is cyclin D1 a universal marker of senescence? And finally can senescence be cyclin D1 negative?

Results

Inhibition of MEK abrogates hyperaccumulation of cyclin D1 in p21- and p16-induced senescence. In HT-p21 cells, IPTG-induced senescence is associated with dramatic induction of cyclins D1 and E.[14] In agreement with our previous findings,[14] both rapamycin and nutlin-3a decreased levels of cyclins D1 and E (Figure 1a). We also examined the effects of U0126, a commonly used inhibitor of MEK. Although all three agents inhibited phosphorylation of S6 (a marker of the MTOR activity), only U0126 completely eliminated cyclin D1 (Figure 1a). Although rapamycin and nutlin-3a decreased cyclin D1 levels, they were still detectable and even elevated compared with proliferating cells (not treated with IPTG) (Figure 1a). Next, we investigated the effect of U0126 on cyclin D1 in p16-induced senescence in the HT-p16 cell line, in which IPTG stimulated irreversible senescence by inducing p16.[50] We have previously observed that IPTG-induced cyclins D1 and E in HT-p16 cells.[14] In Figure 1b, we present the results from the same experiment, where we compare the effect of rapamycin (shown previously in Leontieva *et al.*[14]; Figure 1b) with U0126 (Figure 1b) in IPTG-treated HT-p16 cells. As expected, only rapamycin (not U0126) abrogated MTOR-dependent Thr-389 phosphorylation of p70S6K (Figure 1b), because the MEK/ERK pathway phosphorylates S6K on the other site(s) (Thr 421/Ser 424).[49,51,52] Unlike rapamycin, U0126 completely eliminated cyclin D1 (undetectable level, Figure 1b). Both rapamycin and U0126 suppressed geroconverssion in p21- and p16-induced senescence, rendering cells that were able to resume proliferation and form colonies after arrest was released (Figures 1c and d). Additive effects of rapamycin and U0126 were observed in p16-overexpressing cells (Figure 1d).

These results were strengthened by the use of an additional inhibitor of MEK (PD184352), which prevented the loss of RP in p21-induced cells (Supplementary Figure S1A). Similar to U0126, PD184352 decreased phosphorylation of p70S6K on Thr421/Ser424, inhibited S6 phosphorylation and eliminated cyclin D1 in p21-arrested HT-p21 cells (Supplementary Figure S1B).

MEK inhibitors were the most effective in decreasing the levels of cyclin D1. Could this observation be due to a drug-dosage effect? MEK inhibitors were used at standard doses that result in inhibition of phosphorylation of ERK1/2 (a MEK target). Furthermore, we also used a MEK inhibitor PD184352 at a wide range of concentrations (Figure 1e). PD184352 abolished cyclin D1 completely at the concentration as low as 1.25 μM, which also inhibited phosphorylation of ERK1/2 but not S6 phosphorylation, indicating that inhibition of MEK was indeed responsible for more potent effect of MEK inhibitors. In contrast, rapamycin was used at concentrations several fold higher than the concentrations that inhibit MTOR in most cell lines, in general, and in HT-p21 cells, in particular.[15,53]

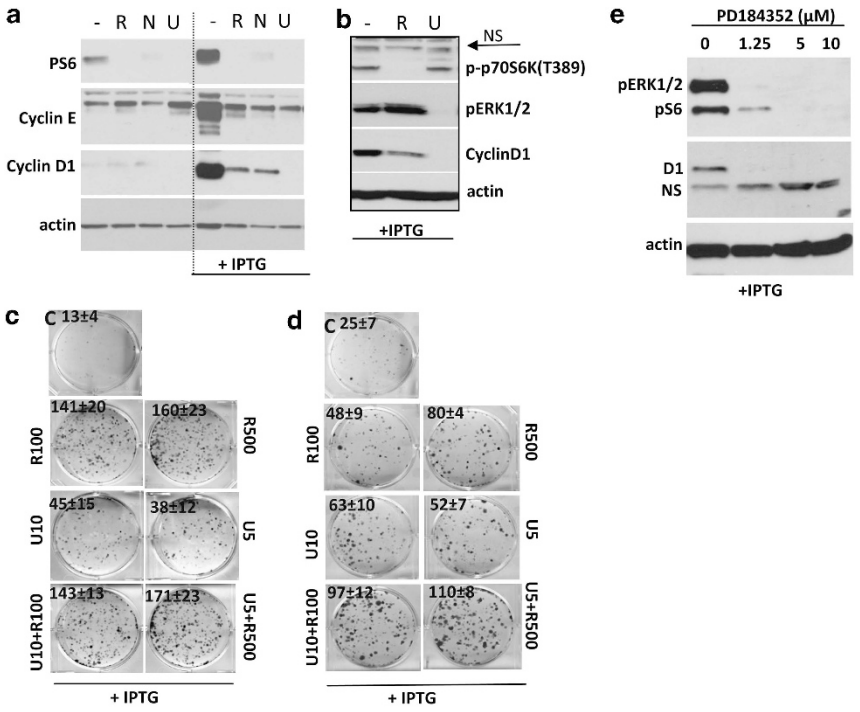

Figure 1 Effect of MEK inhibitors on senescence-associated cyclin D1 and RP. (**a** and **b**) Immunoblot analysis. (**a**) HT-p21 cells were treated with 500 nM rapamycin (R), 20 μM nutlin-3a (N) or 10 μM U0126 (U) in the presence or absence of IPTG for 24 h; (**b**) HT-p16 cells were treated with 500 nM R or 10 μM U in the presence or absence of IPTG for 24 h. Blot in **b** represents a part of experiment shown in Leontieva *et al.*[14] with all controls therein. (**c** and **d**) Regenerative/replicative potential. HT-p21 (**c**) and HT-p16 (**d**) cells were plated at low density, treated with IPTG in the presence of 100 or 500 nM of R, or 5 or 10 μM U, or with combination of R and U as indicated. After 3 days, the drugs were washed out and colonies were allowed to grow for 7 days, stained with crystal violet and counted. Data were presented as mean ± S.D. (**e**) Immunoblot analysis. HT-p21 cells were treated with a concentration range of MEK inhibitor PD184352 and IPTG for 24 h. NS: nonspecific band serves as additional loading control

In addition, siRNA directed to MEK1 (siMEK) decreased the levels of cyclin D1 in IPTG-treated HT-p21 cells (Figure 2a). A less prominent effect of siMEK in comparison with U0126 in this particular experiment could be due to incomplete knockdown of MEK as indicated by the presence of phosphorylated ERK1/2 upon a longer exposure of the blot (Figure 2a, see legend for experimental details). However, the decrease in levels of cyclin D1 was proportional to the decrease in phosphorylated ERK1/2 levels (Figure 2a). Most importantly, siMEK suppressed geroconversion of p21- arrested HT-p21 cells (Figure 2b). This may also be explained by inhibition of S6 phosphorylation, typical for MEK inhibitors in this cell line (Figure 2a).

To further imitate effects of MEK inhibitors, which were added simultaneously with IPTG, HT-p21 cells were transfected with siMEK for 2 days and then immediately treated with IPTG. After 24 h, cells were lysed. Complete inhibition of phosphorylated ERK1/2 and cyclin D1 was observed (Figure 2c).

Role of cyclin D1 in geroconversion. We next investigated the role of cyclin D1 in geroconversion. As shown in Figure 3a, siRNA directed to cyclin D1 (siD1) completely eliminated cyclin D1 in both proliferating and IPTG-treated HT-p21 cells. Noteworthy, it did not affect levels of cyclin E (Figure 3a). Furthermore, in the presence of siD1, IPTG could not induce cyclin D1 even to the lowest basal level (Figure 3a, lane 4 *versus* 1), indicating that post-translational mechanisms were not sufficient for hyperaccumulation of the protein. Whereas lactacystin (LC) increased levels of both cyclin D1 and p21 in proliferating cells, its addition after 24 h treatment with IPTG led to further increase in p21, indicating that p21 was degraded through the proteasome, as was shown previously.[54] Yet in the presence of IPTG, LC had no additional effect on cyclin D1 levels, implying the absence of its degradation through this pathway during geroconversion, and indicating that this may in part contribute to its accumulation (Supplementary Figure S3). Also, it was described previously that cyclin D1 is regulated by transcription, translation (MTOR-dependent), degradation by the proteasome and calpain proteases in cell type-specific manner;[3,55–61] therefore, it is difficult to evaluate the proportion of each mechanism and it could be suggested that all of the mechanisms are involved.

Elimination of cyclin D1 had no effect on S6 phosphorylation (Figure 3a). Therefore, it is not surprising that siD1 did not prevent senescent morphology, normally caused by IPTG (~90% of beta-gal-positive cells, Figure 3b). Elimination of cyclin D1 with siRNA also did not preserve RP of IPTG-treated HT-p21 cells (Figure 3c). However, depletion of cyclin D1 affected cell proliferation (Figure 3d). Interestingly, a comparable amount of the cells with senescent morphology appeared after 5 days in culture (Figure 3e). Thus, partial inhibition of cell proliferation by siD1 (Figure 3d) coupled with no effect on S6 phosphorylation resulted in senescent morphology in ~30% of the cells (Figure 3e). In fact, in proliferating cells,

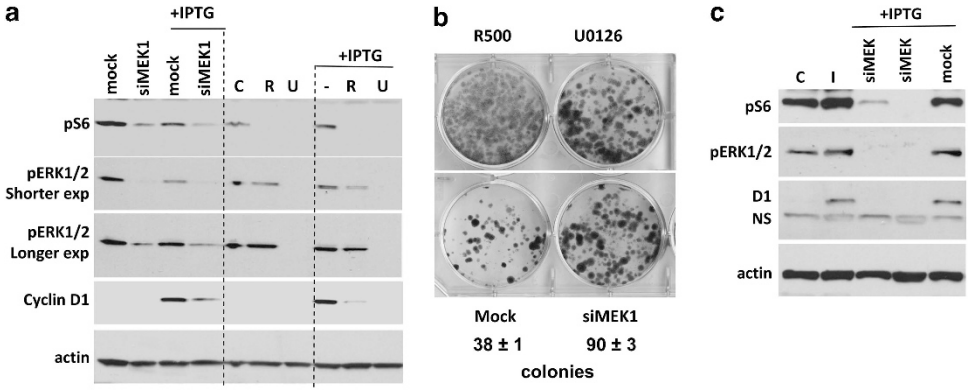

Figure 2 Effects of MEK1 siRNA on senescence-associated cyclin D1 and RP. (**a**) Immunoblot analysis. HT-p21 cells, transfected with MEK1 siRNA or with lipofectamine alone (Mock), were split 4 days after transfection, treated with IPTG for 24 h and lysed. Non-transfected cells were treated with 500 nM rapamycin (R) or 10 μM U0126 (U) in the presence or absence of IPTG for 24 h. Note: cells were lysed on the fifth day after transfection with siRNA. (**b**) Regenerative/replicative potential. HT-p21 cells, transfected with MEK1 siRNA or mock transfected as in panel **a**, were treated with IPTG for 3 days, then drugs were washed out and colonies were grown for 10 days and stained with crystal violet. The number of colonies is presented as mean ± S.D. Non-transfected cells were treated with IPTG with either 500 nM R or 10 μM U for 3 days, and then drugs were washed out and colonies were stained after 10 days of growth. (**c**) Immunoblot analysis. HT-p21 cells were transfected with 50 and 100 pmol of MEK1 siRNA, and 2 days after transfection, IPTG was added to the cells for 24 h. Note: cells were lysed on the third day after transfection

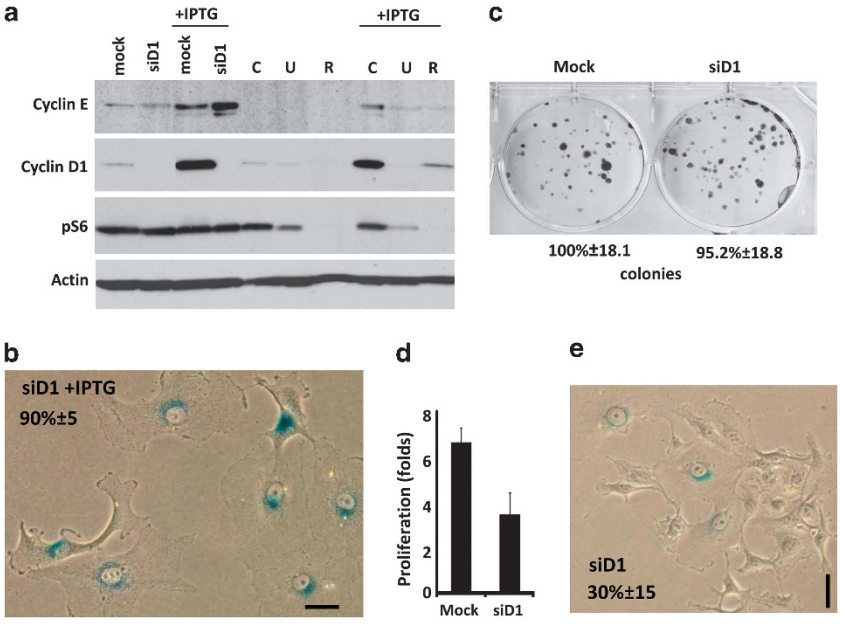

Figure 3 Effects of cyclin D1 siRNA on p21-induced senescence in HT-p21 cells. (**a**) Immunoblot analysis. HT-p21 cells were transfected with siD1 or transfection reagent alone (mock), split 1 day after transfection and treated with IPTG. Non-transfected cells were treated with 10 μM U0126 (U) or 500 nM rapamycin (R). Cells were lysed after 24 h treatment. Note: cells were lysed on the second day after transfection with siRNA. (**b**) Beta-gal staining. HT-p21 cells transfected with siD1 were treated with IPTG for 4 days and stained for beta-gal. Scale bar, 100 μm. Beta-gal-positive cells were counted and presented as percentage of total number of counted cells. Data were presented as mean ± S.D. of three independent experiments. (**c**) Regenerative/replicative potential. HT-p21 cells transfected with siD1 were plated at low density and treated with IPTG for 3 days, then drug was washed out and colonies were grown for 10 days and stained with crystal violet. Data were presented as number of colonies as percentage of control (mock-transfected cells) ± S.D. (**d**) Effect of siD1 on proliferation of HT-p21 cells. HT-p21 cells transfected with siD1 were cultured for 3 days. Increase in cell numbers are shown in folds relative to seeding numbers ± S.D. (**e**) Beta-gal staining. HT-p21 cells transfected with siD1 were stained for beta-gal after 5 days in culture. Scale bar, 100 μm. Beta-gal-positive cells were counted and presented as percentage of total numbers of counted cells. Data were presented as mean ± S.D. of three independent experiments

expression of cyclin D1 bypasses cell-cycle arrest and avoids senescence.[57,62] This further supports our notion that cell-cycle arrest plus active MTOR are sufficient for senescent type of the cell-cycle arrest.

Induction of MTOR-dependent senescence by MEK inhibitor. To exclude that the suppression of cyclin D1 by MEK inhibitors was due to inhibition of the MTOR pathway, we next employed MEL10 cells in which MEK inhibitor (U0126), such as nutlin-3a, did not inhibit MTOR (Figure 4a). As we described previously, while nutlin-3a inhibited MTOR and caused quiescence in HT-p21 cells, it did not inhibit S6 phosphorylation in MEL10 cells (Korotchkina *et al*.[63] and Figure 4a). By inducing p53 without inhibiting S6 phosphorylation,

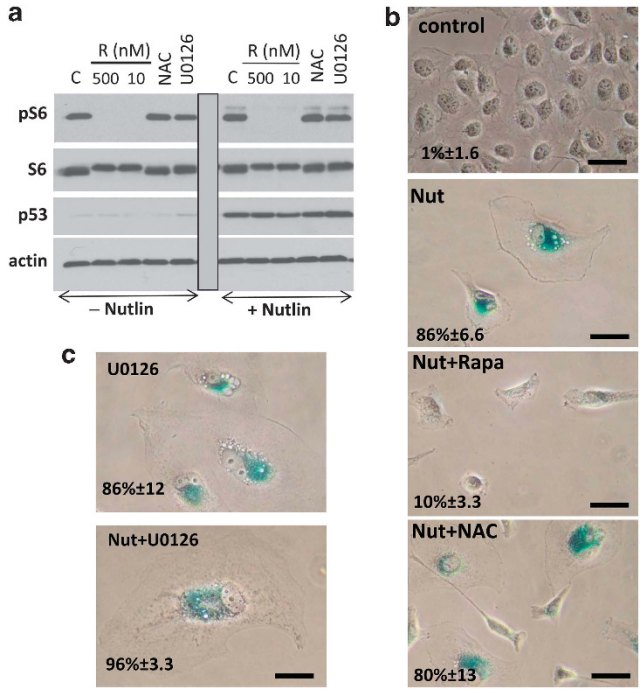

Figure 4 Induction of senescence in MEL10 cells. (**a**) Immunoblot analysis. MEL10 cells were treated with the indicated drugs in the presence or absence of 5 μM nutlin-3a for 24 h. R: rapamycin. NAC: *N*-acetyl-L-cysteine, an antioxidant. (**b** and **c**) Beta-gal staining. (**b**) Cells were treated with 5 μM nutlin-3a (Nut) alone or in combination with 5 nM R (Nut + Rapa) or 2 mM NAC for 3 days and stained for beta-gal. Scale bar, 50 μm. (**c**) Cells were treated with 5 μM U0126 (U) with or without 5 μM Nut for 3 days and stained for beta-gal. Scale bar, 50 μm. Numbers of beta-gal-positive cells are presented as percentage of total numbers of counted cells ± S.D

nutlin-3a treatment resulted in a senescent morphology (Figure 4b), which was prevented by rapamycin (Figure 4b). Importantly, *N*-acetyl-L-cysteine, an antioxidant, which did not inhibit MTOR in MEL10 cells (Figure 4a), did not affect the senescent morphology that resulted after nutlin-3a treatment (Figure 4b). Also U0126 did not suppress geroconversion caused by nutlin-3a treatment (Figure 4c). Furthermore, treatment with U0126 alone resulted in senescent morphology (Figure 4c).

U0126 and PD0332991 cause cyclin D1-negative and -positive senescence, respectively, which is suppressed by rapamycin. In order to develop an alternative model to IPTG-induced cyclin D1-positive senescence model (HT-p21 cells), we utilized PD0332991 that is a very selective inhibitor of CDK 4/6.[64] As expected, by inhibiting CDK4/6, PD0332991 inhibited proliferation of MEL10 cells (Figure 5a), but it did not inhibit phosphorylation of S6 (Figure 5b). Consistent with inhibition of proliferation plus active MTOR, these cells underwent geroconversion (became senescent). In particularly, the cells lost RP and poorly proliferated after PD0332991 was removed by washing it out (Figure 5c). Most importantly, the geroconversion was MTOR dependent, because co-treatment with PD0332991 and rapamycin inhibited phosphorylation of S6 (Figure 5b) and suppressed geroconversion, maintaining quiescence instead, with preserved RP (Figure 5c). In addition, PD0332991-treated cells acquired senescent morphology (Figure 5d), which became even more prominent when the drug was removed (Figure 5d). Most importantly, PD0332991 strongly induced cyclin D1 (Figure 5b). Although rapamycin moderately decreased PD0332991-induced cyclin D1, U0126 completely eliminated it (Figure 5b). In this particular cell line, U0126 treatment by itself did not block phosphorylation of S6 (Figure 5b) and therefore caused senescent morphology, which was prevented by rapamycin (Figures 5e and 4c). However, U0126, at nontoxic concentrations, did not inhibit proliferation of all the cells, and those cells, which were not arrested by U0126, retained normal morphology (Figure 5e). The existence of proliferating population precluded precise measurement of RP of the arrested cells. In order to measure RP specifically of U0126-arrested cells, we added nocodazole for the last 2 days of U0126 treatment to eliminate cells nonresponsive to U0126 (Figure 5f). This nocodazole test has been previously validated to distinguish MTOR-dependent senescence from quiescence.[65] U0126-resistant cells were eliminated by nocodazole and the remaining arrested (by U0126) cells showed decreased RP, which was increased by co-treatment with rapamycin (Figure 5f). We conclude that U0126 alone indeed caused MTOR-dependent senescence in U0126-arrested cells, and rapamycin prevented geroconversion and preserved RP (Figure 5f). Furthermore, we confirmed this result using morphological markers of senescence (Figure 5g). In the presence of U0126 and nocodazole only morphologically senescent cells were detected (as nocodazole kills all proliferating cells). The addition of rapamycin dramatically increased the number of morphologically quiescent cells (Figure 5g).

As U0126 (a MEK inhibitor) and PD0332991 (a CDK4/6 inhibitor) caused MTOR-dependent senescence in Mel10 cells, regardless of induction of cyclin D1, we suggest that cyclin D1 is an indicator of MEK activation in senescent cells rather than an obligatory marker of senescence. The combination of U0126 and PD0332991 was highly toxic. Owing to this toxicity, S6 was dephosphorylated (Figure 5b), and therefore we could expect that geroconversion might be suppressed. Although U0126 prevented senescent morphology caused by treatment with PD0332991 (Supplementary Figure S2), majority of the cells died. The toxicity precluded evaluation of RP. This may be exploited for potential cancer therapy but is irrelevant to the current study.

In summary, MEL10, a melanoma cell line, underwent senescence very easily, as all three agents (U0126, nutlin-3a and PD0332991) did not inhibit MTOR and therefore caused senescence in MEL10 cells. Rapamycin inhibited geroconversion caused by nutlin-3a, PD0332991 and U0126.

PD0332991 causes cyclin D1-positive senescence in normal RPE cells. Finally, we investigated whether geroconversion of normal cells would also be associated with MEK-dependent induction of cyclin D1. In RPE cells, PD0332991 (a CDK 4/6 inhibitor) inhibited cell proliferation (Figure 6a) and resulted in the senescent morphology (Figure 6b), typical for these cells.[18] Senescent cells were unable to proliferate following removal of PD0332991 (Figures 6a and b). The numbers of cells were increased only

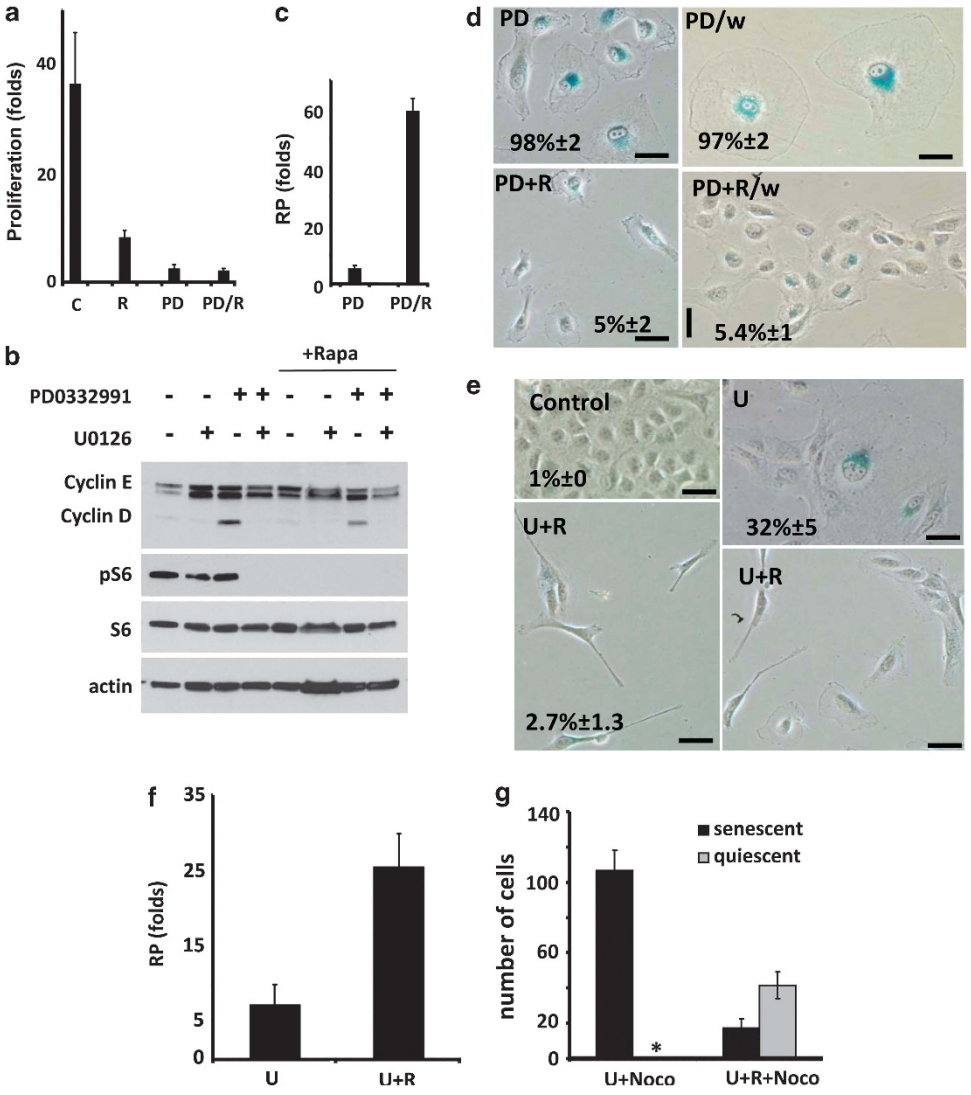

Figure 5 Cyclin D1-positive and -negative senescence in MEL10 cells. (**a**) Proliferation. Mel10 cells were treated with 1 μM PD0332991 (PD) with or without 5 nM rapamycin (R) for 5 days and counted. C: control (no treatment). Increase in cell numbers are shown in folds relative to seeding numbers ± S.D. (**b**) Immunoblot analysis. Cells were treated as indicated for 3 days. (**c**) Regenerative/replicative potential Cells were treated as in **a**. RP as fold increase in cell numbers was determined as described in Materials and Methods. Specifically, after 5 days, one set of cells was counted (numbers after treatment), and in the second set of cells, drugs were washed out and the cells were allowed to regrow for 8 days and counted. RP as fold increase in cell numbers was calculated ± S.D. (**d**) Beta-gal staining. Cells were treated with 1 μM PD alone or with 5 nM R (PD + R) for 5 days, and one set of cells was stained for beta-gal and in the second set of cells, the drugs were removed and the cells were allowed to regrow for 3 days and then stained for beta-gal (PD/W and PD + R/W). Scale bar,:100 μm. Numbers of beta-gal-positive cells are presented as percentage of total numbers of counted cells ± S.D. (**e**) Beta-gal staining. Cells were treated with 10 μM U alone or with 5 nM R (U + R) for 5 days and stained for beta-gal. Scale bar, 100 μm. Number of beta-gal-positive cells are presented as percentage of total numbers of counted cells ± S.D. (**f**) Regenerative/replicative potential. Cells were treated with 10 μM U alone or combination 10 μM U + 5 nM R (U + R). Nocodazole at 200 nM was added for the last 2 days of treatment to eliminate non-arrested cells. RP as a fold increase in cell numbers was determined as described in Materials and Methods. Specifically, after 5 days, one set of cells was counted (numbers after treatment), and in the second set of cells, the drugs were removed and the cells were allowed to regrow for 5 days and counted. RP as fold increase in cell numbers was calculated ± S.D. (**g**) Cells were treated with 10 μM U or U + R for 5 days. Nocodazole was added for the last 2 days of treatment. Large (senescent) and small (quiescent) cells were counted in three fields. Data were presented as number of cells as mean ± S.D

4–6 times during 6 days of culture in drug-free medium, compared with a 60–100-fold increase in untreated control cells (Figure 6a). In agreement, PD0332991 did not inhibit S6 phosphorylation in RPE cells (Figure 6c), explaining its ability to cause senescence. Most importantly, PD0332991 also induced cyclin D1, as early as by day 1 (Figure 6c). In these cells, rapamycin and U0126 both inhibited S6 phosphorylation, but only U0126 inhibited phosphorylation of ERK1/2 and completely blocked cyclin D1 accumulation (Figures 6c and d). This result was confirmed at a later time point (Figure 6d). Importantly, the levels of cyclin D1 were higher in senescent than in proliferating cells (Figures 6c and d). Rapamycin slightly decreased cyclin D1 hyperinduction caused by PD0332991 treatment, which remained higher than its levels in proliferating cells (Figure 6d, right lanes *versus* left lanes). In contrast, U0126 treatment completely depleted cyclin D1 protein.

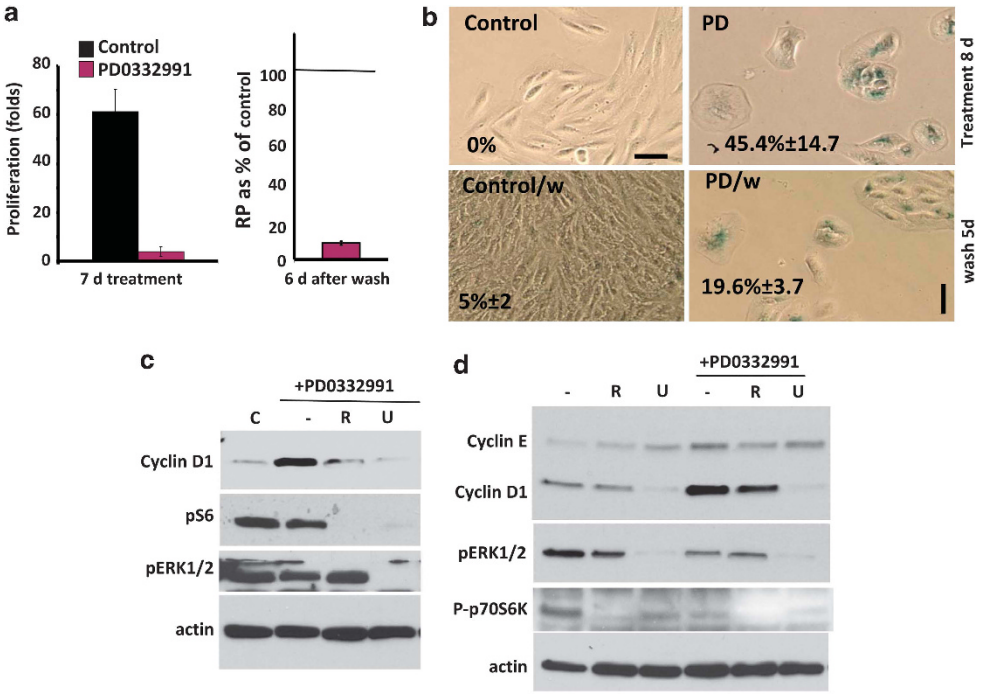

Figure 6 MEK is required for cyclin D1-positive senescence in normal RPE cells. (**a**) Proliferation and RP. RPE cells were treated with 1 μM PD0332991 for 7 days and one set of cells was counted. In the second set of cells, the drugs were washed out and the cells were allowed to regrow for 6 days and counted. RP was determined as described in the legend for Figure 5c. (**b**) Beta-gal staining. Cells were treated with 1 μM PD0332991 (PD) for 8 days, and one set of cells was stained for beta-gal and in the second set of cells, drugs were washed out and the cells were allowed to regrow for 5 days before beta-gal staining (PD/W). Scale bar, 100 μm. Number of beta-gal-positive cells are presented as percentage of total numbers of counted cells ± S.D. (**c** and **d**) Immunoblot analysis. Cells were treated as indicated: C, control (untreated), R, 5 nM rapamycin, U, 10 μM U0126, PD0332991 at 2 μM for 24 h (**c**) and 2 days (**d**), respectively

Although both rapamycin and U0126 affected the phosphorylation of p70S6K (Thr 389), only U0126 inhibited the phosphorylation of ERK1/2.

Discussion

Cellular senescence is manifested by conflicting hallmarks. For example, loss of RP is associated with very high levels of cyclins D1 and E, markers of hyperactivation and hyperfunction of senescent cells.[14] Rapamycin decelerates acquisition of senescent phenotype (gerosuppression by rapamycin), including hyperexpression of cyclin D1.[14] In this manuscript, we demonstrated that MEK inhibitors completely eliminated cyclin D1, whereas they were less effective in suppressing the MTOR pathway compared with rapamycin. Inhibitors of MEK can indirectly inhibit the MTOR pathway in a cell type-specific manner, because MEK/ERK phosphorylates and activates raptor, p70 S6K and RSK, thereby leading to phosphorylation of S6.[47–49] Importantly, inhibitors of MEK completely eliminated cyclin D1, regardless whether they suppressed MTOR or not (compare U0126 effects in HT-p21 *versus* MEL10 cells). In contrast to HT-p21 cells, U0126 treatment did not result in inhibition of MTOR in MEL10 cells, but inhibited cell proliferation, thus causing senescence in these cells, instead of suppressing it. This senescence was MTOR dependent and cyclin D1 negative. Thus, hallmarks of a senescent phenotype (morphology and RP) could be dissociated from such markers as cyclin D1 hyperaccumulation. Noteworthy, inhibition of MEK and disappearance of cyclin D1 did not affect cyclin E levels. Consistently, siD1 also did not decrease cyclin E levels. This indicates an absolutely different mode of regulation of cyclins in senescent cells, given that in normally proliferating cells induction of cyclin E follows the induction of cyclin D.[2] Most importantly, although cyclin D1 is a universal marker of cellular senescence, its elimination by siD1 did not affect at least three classical hallmarks of senescence: loss of RP, senescent morphology and beta-Gal staining. Perhaps, some markers of aging, such as signal resistance and cellular hyperfunctions, must be investigated in the future. In fact, cellular hyperfunction may link cellular senescence to organismal aging and age-related diseases.[30,66]

As rapamycin did not inhibit MEK, as evidenced by ERK phosphorylation, its effect on cyclin D1 levels was MEK independent. MTORC1 can increase cyclin D1 expression via inactivation of eukaryotic initiation factor 4E-binding protein 1.[60,61,67,68] Previously, the MTOR was the only pathway known to be involved in acquiring classic markers of a senescent phenotype, including cyclin D1 accumulation. For the first time, we added an additional pathway, the MAPK pathway that is required, for the acquisition of at least one hallmark of senescence: hyperaccumulation of cyclin D1. Although active MEK/ERK during cell-cycle arrest may contribute to geroconversion, some effects of MEK/ERK were due to its indirect activation of MTOR in a cell type-specific manner. Although MEK itself was required for hyperexpression of cyclin D1, MTOR had additional but not major roles in

this phenomenon. Noteworthy, it has been reported that cyclin D1 can, in turn, further activate MTOR,[69] and *vice versa* inhibition of MTOR can decrease cyclin D1 levels,[60,67] establishing positive feedback between them.

Materials and Methods

Cell lines and reagents. HT-p21 cells and HT-p16, derived from HT1080 human fibrosarcoma cells, (ATCC, Manassas, VA, USA) were previously described.[6,13,46,70] HT-p21 and HT-p16 cells were cultured in high-glucose DMEM without pyruvate supplemented with FC2 serum (HyClone FetalClone II from Thermo Scientific, Logan, UT, USA). In HT-p21 and HT-p16 cells, p21 and p16 expression, can be turned on or off using isopropyl-thio-galactosidase (IPTG).[13,70] Melanoma MEL10 (formally, SK-MEL-147) were obtained from Dr. Mikhail Nikiforov (RPCI, Buffalo, NY, USA), were described previously[71] and were cultured in DMEM plus 10% FBS. Normal retinal pigment epithelial RPE cell lines were obtained from ATCC and were maintained in MEM plus 10% FBS. Rapamycin was obtained from LC Laboratories (Wobun, MA, USA). IPTG (Invitrogen, Grand Island, NY, USA) was dissolved in water as 50 mg/ml stock solution and used in cell culture at final concentration 50 μg/ml. Nutlin-3, U0126 and PD184352 were purchased from Sigma-Aldrich (St. Louis, MO, USA). PD0332991 was purchased from Selleckchem (Houston, TX, USA).

Immunoblot analysis. Immunoblot analysis was performed as described previously.[18] The following rabbit antibodies for: phospho-S6 (Ser235/236), phospho ERK1/2, phospho-Thr 389 p70S6K and phospho-Thr421/Ser424 p70S6K, and mouse anti-S6 antibody were obtained from Cell Signaling Biotechnology (Boston, MA, USA). Rabbit anti-actin antibody was purchased from Sigma-Aldrich; mouse antibody for p21 was obtained from BD Biosciences; mouse anti-cyclins D1 and E were obtained from Santa Cruz Biotechnology. Secondary anti-rabbit and anti-mouse HRP-conjugated antibodies were obtained from Cell Signaling Biotechnology.

SA-β-Gal staining. Beta-Gal staining was performed using Senescence-galactosidase staining kit (Cell Signaling Biotechnology) according to manufacturer's protocol. Beta-gal-positive cells were counted in three different fields and presented as percentage of total number of counted cells.

siRNA technology. ON-TARGET plus SMART pool siRNAs for human MEK1 (MAP2K1) and cyclin D1 (CCND1) were purchased from Thermo Scientific. HT-p21 cells were ttransfected as described previously.[18,72]

RP assay. Cells were plated at low density, treated with senescence-inducing agents (IPTG, nutlin-3a or PD0332991) as indicated in figure legends. Cell numbers were determined at the end of treatment (initial cell numbers) and drugs were removed. Cells were incubated in fresh drug-free medium for a few days, as indicated in figure legends, and then final cell numbers were determined. RP was calculated as a ratio between final and initial cell numbers: a fold increase in cell numbers after the drugs were washed out.

Colony formation assay. HT-p21 or HT-p16 cells were plated at low density, treated with IPTG for 3–4 days with or without drugs as indicated in figure legends. Then, drugs were washed out and cells were incubated in fresh drug-free medium for a few days. Plates were fixed and stained with 1.0% crystal violet (Sigma-Aldrich). Colonies were counted using Adobe Photoshop.

Conflict of Interest

The authors declare no conflict of interest.

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