Modulation of Replicative Senescence of Diploid Human Cells by Nuclear ERK Signaling

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Normal somatic cells have a limited replicative lifespan, and serial subcultivation ultimately results in senescence. Senescent cells are irreversibly growth-arrested and show impaired responses to mitogens. Activation of the ERK signaling pathway, an absolute requirement for cell proliferation, results in nuclear relocalization of active ERKs, an event impaired in senescent fibroblasts. This impairment coincides with increased activity of the nuclear ERK phosphatase MKP2. Here we show that replicative lifespan can be altered by changes in nuclear ERK activity. Ectopic expression of MKP2 results in premature senescence. In contrast, knock-down of MKP2 expression, through transduction of MKP2 sequence-specific short hairpin RNA, or expression of the phosphatase resistant ERK2(D319N) mutant, abrogates the effects of increased endogenous MKP2 levels and senescence is postponed. Nuclear targeting of ERK2(D319N) significantly augments its effects and the transduced cultures show higher than 60% increase in replicative lifespan compared with cultures transduced with wt ERK2. Long-lived cultures senesce with altered molecular characteristics and retain the ability to express c-fos, and Rb is maintained in its inactive form. Our results support that MKP2-mediated inactivation of nuclear ERK2 represents a key event in the establishment of replicative senescence. Although it is evident that senescence can be imposed through multiple mechanisms, restoration of nuclear ERK activity can bypass a critical senescence checkpoint and, thus, extend replicative lifespan.

Somatic cells in culture can attain only a limited number of divisions in contrast to most cancer cells that can divide indefinitely. This limited replicative capacity, termed replicative senescence (1, 2), has been used extensively as a model in elucidating molecular mechanisms underlying organismic aging, as well as mechanisms that regulate normal cell growth. Consequently, the phenotype of senescent cells, characterized by enlarged and flattened morphology, increased sensitivity to contact inhibition and loss of responsiveness to mitogens, is well described although the molecular mechanisms responsible for this phenotype are not yet fully elucidated (reviewed in Refs. 3 and 4). The difference in proliferation potential between senescent and cancer cells has led some investigators to suggest that senescence is a cancer protection mechanism. Furthermore, it has been proposed that proteins that control cell cycle progression and that are inactivated in tumor cells, such as the restriction-point proteins Rb and p53 and various cyclin-dependent kinase inhibitors (CDIs)² play a role in the establishment of senescence (reviewed in Refs. 5–8). Although this view merits substantial experimental support, it is still unclear whether these are primary causes or, alternatively, if these proteins are activated in response to changes incompatible with normal cell growth that precede p53 and Rb activation. Studies on the cell cycle kinetics of senescent cells have shown that, although they display some characteristics of late G1 arrest, they are actually arrested in a distinct state. Early G1 events, such as proper activation of the ERK signal transduction pathway and expression of the immediate-early genes c-fos, erg-1, Id-1H, and Id-2H do not occur in senescent cells (3, 4). Hence, it is likely that signaling defects in early G1 result in phenotypic alteration of sufficient magnitude to block S phase progression.

The ERK pathway is a key signaling module that controls critical cell functions such as proliferation, differentiation, migration, and apoptosis (9–11). Central to this pathway, the ERK proteins are sites where diverse signals converge to elicit distinct biological responses via activation of the impressive spectrum of ERK effectors which modulate the rate of protein and pyrimidine-nucleotide biosynthesis, activate transcription and translation, regulate cytoskeletal remodeling and cell migration, and promote cell cycle progression (10–13). The wide range of signals that activate ERKs, their numerous substrates and their crucial role in determining antagonistic cell fates, suggest that precise spatiotemporal regulation of ERK activity is critical for cellular homeostasis. Loss of ERK regulation occurs in many pathological conditions, including age associated pathologies (e.g. cardiovascular disease (14), Alzheimer disease (15–17), and cancer (18–20)), as well as in normal aging (4). Interestingly, in contrast to carcinogenesis during which up-regulation of the ERK pathway has been linked to all

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1 The abbreviations used are: CDI, cyclin-dependant kinase inhibitor; ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; FBS, fetal bovine serum; PD, population doubling(s); SAβ-gal, senescence-associated β-galactosidase; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; wt, wild type; GFP, green fluorescent protein; DTT, dithiothreitol; NLS, nuclear localization signal; shRNA, short hairpin RNA.
stages of progression, from initial tumor growth to tumor vascularization and metastasis, aged tissues and in vitro senescent cells show impaired ERK signaling. For example, terminally arrested senescent fibroblasts show a significant loss of nuclear ERK activity (21–24), which results in impaired activation of certain transcription factors such as Elk-1 (21) which drives c-fos expression and cell cycle progression (25–29).

The biological outcome of ERK signaling is defined by the spatiotemporal control of ERK activity (30, 31), a key event of which is the catalytic activation of ERKs by dual phosphorylation of the tyrosine and threonine residues of the TEY motif in their activation loop (32, 33). ERK phosphorylation occurs in the cytoplasm, is catalyzed by members of the MEK family of dual specificity Thr/Tyr kinases (34), and depends on the sequential activation of the GTP-binding protein Ras and of a member of the Raf family of Ser/Thr kinases (35) that phosphorylate MEK (36–38). Catalytically active ERKs are either retained in the cytoplasm or re-localize in various subcellular compartments (30) such as endosomes (39), Golgi (30), mitochondria (40), and in the nucleus where ERKs modulate the activity of numerous transcription factors including Elk-1, Egr-1, Myc, Fos, Jun, C/EBPβ, STAT3, p53 SRC-1, and Pax6 (12, 29, 41). Precise control of the amplitude and duration of nuclear ERK signaling is fundamental for cell homeostasis and is achieved by the coordinated action of numerous proteins that participate in import, export, and ERK cytoplasmic and nuclear anchoring (11, 31, 42, 43). An additional degree of precision is attained by the subfamily of nuclear Dual Specificity MAP Kinases Phosphatases (DS-MKPs/DUSPs), a prominent member of which is MKP2/DUSP4 (44–47).

MKP2 exhibits high specificity toward ERK, is ubiquitously expressed, and, because of the tight ERK-mediated regulation of its levels and activity, is thought to play a critical role in the negative feedback control of nuclear ERK signaling (reviewed in Refs. 19 and 48). The accuracy of this autoregulatory mechanism is ensured by the multiple levels of MKP2 regulation by ERKs; mitogen-induced transcription of the immediate early response mkg2 gene depends on ERK pathway activation (49) and docking interactions between the two proteins, achieved through the MKP2 docking (D) domain and the ERK2 docking groove (DG), result in catalytic activation of MKP2 (50–53). Two acidic residues in the DG of ERK (Asp-316 and Asp-319) establish critical electrostatic interactions with the positively charged residues of the D domain of MKP2 and are critical for docking. Substitution of Asn for Asp at position 319 disrupts MKP2-ERK2 binding and results in a hyperactive ERK2 allele (ERK2(D319N)) analogous to Drosophila melanogaster Seven-maker (r1s) gain-of-function mutant (54–56). Thus, disruption of MKP2 docking to ERK2 disrupts both its catalytic activation and its inactivating capacity toward ERK.

Despite the significant advances in elucidating the mechanism of ERK2-MKP2 feedback regulation (48), the precise role of MKP2 in vivo is still poorly understood. There is some evidence that MKP2 dysregulation is involved with cancer progression in humans. Chromosomal abnormalities that result in loss of the MKP2 locus (8p12-p11) and protein, in primary malignant breast carcinomas and breast cancer lines, indicate that MKP2 may play a tumor suppression role (57). Human fibroblasts that reach terminal proliferative arrest have abnormally high MKP2 protein levels and activity (58) and reduced nuclear ERK activity (21–24). Thus in this physiologic setting high MKP2 levels may be implicated with the loss of proliferative capacity of senescent fibroblasts.

In the present study we examined the physiological role of MKP2 in ERK signaling and how subtle changes in nuclear ERK2 activity affect proliferation and longevity of diploid human fibroblasts. We provide evidence that nuclear targeting of the ERK2 phosphatase resistant mutant D319N restores nuclear ERK2 activity in senescent cells and significantly extends replicative lifespan. Significant lifespan extension was also achieved using short hairpin RNA (shRNA) to knock-down MKP2 expression. In contrast, ectopic expression of MKP2 in early passage cells results in inhibition of cell proliferation and a significant decrease in replicative lifespan. Although cultures with extended lifespan ultimately senesce, some of the molecular characteristics of the senescent phenotype are reversed; the cells retain the ability to express c-fos, and Rb is maintained in its inactive phosphorylated state. Our data provide evidence that replicative senescence is established, in part, through loss of nuclear ERK activity and support the hypothesis that defects in nuclear ERK signaling precede and impose other growth inhibitory changes in senescent cells.

**EXPERIMENTAL PROCEDURES**

*Materials*—All chemicals were purchased from Sigma unless stated otherwise. The retroviral expression vectors pLNCX, pLHCX, pLPCX, and peGFP-C3 were from BD Biosciences-Clontech Inc. (Palo Alto, CA). The Expression Arrest™ human retroviral MKP2 (DUSP4) shRNAmir cloned into pSHAG-MAGIC2 and the non-silencing shRNA control vector were purchased from OpenBiosystems (Huntsville, AL).

Hot start *Ta*q polymerase, dNTPs, and PCR buffer were obtained from Takara Bio Inc. (Japan), QIAquick PCR purification kit, QIAquick gel extraction kit, and plasmid DNA preparation kits were from Qiagen Inc. (Valencia, CA). Restriction enzymes, DNA ligase, and shrimp alkaline phosphatase were purchased from Roche Applied Science. The antibodies used were against ERK2 (D2), GFP (B2), MKP2 (S18), MKP2(F10), c-Fos (clone 4), p16 (F12), p27 (C19), p53 (DO-1), α-tubulin (TU-1) and were all from Santa Cruz Biotechnology Inc. (Santa Cruz, CA), anti-ERK1/2-CT was from Upstate Biotechnology Inc. (Lake Placid, NY), p19 (Ab-1) was from Calbiochem, total and hyperphosphorylated pRb were from Pharmingen, and p21 (clone 70) was from BD Transduction Laboratories (Lexington, KY). The phospho-p53 (Ser-15) (16G8) and horseradish peroxidase-conjugated anti-mouse and ant-rabbit IgGs were from Cell Signaling Technology (Beverly, MA). The anti-phospho ERK1/2 antibody was purchased from Sigma (MAPK-TY) and the fluorescein isothiocyanate-conjugated anti-mouse IgG was from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

*Cell Culture, Transfections, and Infections*—Stock cultures of human diploid WI-38 lung and AG07493 dermal fibroblasts were grown in Autopow minimum essential medium supplemented with basal medium Eagle’s vitamins, 2 mM L-glutamine, and 10% fetal bovine serum (FBS) and were subcultivated to
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senescence according to our standard procedure (3). Briefly, following trypsinization, cell numbers and size distributions were estimated using a Beckman Z2 coupler particle count and size analyzer (Beckman Coulter, Inc., Fullerton, CA), and 1 × 10^4 cells were seeded per cm^2 of growth surface area. At each subcultivation, the increase in population doubling (ΔPD) was calculated using the formula ΔPD = log_{10}(number of cells harvested/number of cells seeded)/log_{10}2; the obtained number was added to the previous PD to obtain the cumulative PD). Cultures were considered to be at the end of their replicative lifespan, when unable to complete one PD during a four-week period that included three consecutive weeks of re-feeding. The amphotropic packaging cell line Phoenix A was subcultivated using identical growth conditions as for WI-38 cells. For retrovirus production, Phoenix A cells were transfected with the various retroviral expression vectors using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Viral supernatants were harvested 48 h after transfection, filtered through 0.45-μm low protein-binding filters (Millipore Corp., Billerica MA) and used immediately to infect early passage WI-38 cultures in the presence of 5 μg/ml polybrene. Infected cells were selected with the appropriate antibiotic at experimentally determined concentrations that were as follows: 1 μg/ml puromycin, 75 μg/ml hygromycin, and 150 μg/ml G418. Selected cultures were subcultivated to senescence in antibiotic containing media to maintain selective pressure. For stimulation experiments subconfluent cultures were serum-deprived for 3 days in MCDB-104 medium (Invitrogen Corp.) to ensure G0 arrest and were subsequently stimulated for various time periods with FBS to a final concentration of 10% v/v to achieve maximal mitogenic stimulation. For senescence-associated β-galactosidase (SA-β-gal) assays (59), cells seeded at 1–2 × 10^5/cm^2 were grown for 24 h and fixed in 2% (v/v) formaldehyde, 0.2% (w/v) glutaraldehyde in phosphate-buffered saline (PBS). Activity assays were performed overnight at 37 °C in freshly prepared solution containing 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2, 150 mM NaCl, 30 mM citric acid/phosphate buffer, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Cell viability in response to apoptotic stimuli was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Roche Applied Science) as per the manufacturer’s instructions using a BioRad ELISA reader. [3H]Thymidine incorporation as a measure of cell proliferation potential was performed as described previously (60).

Cloning of Expression Plasmids—The oligonucleotide primers 5’-ccacacctgccacatggcccgcggaggcccggg-3’ and 5’-ccatcgcgggtttgtgtctctgggactgctgccctc-3’, which contain a HindIII and a Clal recognition sequence respectively, were used to PCR-amplify from pSRα-based plasmids, full-length wt ERK2, or ERK2(D319N), which were then subcloned between the HindIII and Clal sites of the pLNCX retroviral expression vector. To generate FLAG (DYKDDDDK) epitope-tagged ERKs, we used a reverse primer that in addition to the 3’ sequence of ERK2 contained an Agel restriction site inserted just prior to the termination codon. The Agel site was introduced to facilitate transfer and exchange of tags and fluorescent protein sequences. Thus, to generate ERK2:GFP the FLAG sequence was excised using Agel and Clal and replaced with PCR amplified full-length eGFP (minus the initiation codon). eGFP is a red-shifted variant of wt GFP optimized for higher fluorescence and expression in mammalian cells (Clontech Inc.). The pair of oligonucleotide primers 5’- cccacacctgccacatgtgctgctgctgcagctgccgaggcccgg-3’ and 5’-cccacacctgccacatgtgctgctgctgcagctgccgaggcccgg-3’, was used to PCR-amplify full-length human MKP2 from a pSRα based plasmid, which was then subcloned into HindIII and Agel sites of the pLNCX:FLAG and pLNCX:GFP expression plasmids to generate MKP2:FLAG and MKP2:GFP. wt ERK2, ERK2:FLAG, ERK2:GFP, MKP2:FLAG, and MKP2:GFP were also subcloned into plPCX (puromycin-resistant) and pLHCX (hygromycin-resistant) retroviral expression vectors between the HindIII and Clal restriction sites in their multiple cloning regions. To generate wt ERK2:nuclear localization signal (NLS) and ERK2(D319N):NLS a nucleotide sequence that encodes the nuclear localization signal of the SV-40 T antigen (PKKKRKV) (61, 62) was inserted at the 3’-end of ERK2 adjacent to the Agel site. All PCR amplifications were performed on a MJ Scientific, Inc., PTC-100 Thermocycler using hot-start Taq polymerase. Amplified DNAs were purified using the QIAquick PCR purification kit, and following restriction digestion of the inserts and vectors, shrimp alkaline phos- phatase treatment of the vectors, and agarose gel electrophore- sis, the gel-excised DNAs were purified using the QIAquick gel extraction kit. DNA concentrations were calculated by spectrophotometry, and inserts were ligated into vectors at a 3:1 molar ratio. Qiagen-purified plasmid DNAs were analyzed by restriction digestion and/or sequencing and were transfected into 293 cells using FuGENE 6 to confirm construct expression by immunoblotting.

Sample Preparation and Immunoblotting—For sample prep- aration, cells were lysed by brief sonication (2–4 s) in non- denaturing buffer containing phosphatase and protease inhibitors (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 5 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM Na3VO4, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin, 1 μM microcystin LR, and 1 mM phenylmethylsulfonyl fluoride). Particulate matter was removed by centrifugation and protein concentra- tions were determined using a modified Bradford method (Bio-Rad). Cytoplasmic and nuclear extracts were pre- pared as described previously (21). From each sample, equal protein amounts were size-fractionated on 5–20% gradient SDS-polyacrylamide gels (Cambrex Biochemicals, Balti- more, MD) and electrotransferred on nitrocellulose mem- branes (Schleicher & Schuell BioScience Inc.) using a Bio-Rad Mini-Protein electrophoresis system as per the manufacturers' instructions. Abundance of proteins of interest was assayed using antibodies at concentrations as recommended by the manufacturers. Binding of each primary antibody was performed overnight at 4 °C in Tween 20/Tris-buffered saline (20 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Tween 20) containing either 3% w/v nonfat dry milk or, 3% w/v bovine serum albumin when antibodies against phosphoproteins were used. Following binding of the appropriate anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary anti-
body and treatment with chemiluminescence substrate (Western Lightening Chemiluminescence Reagent Plus, PerkinElmer Life Sciences), light emission was detected on Kodak Biomax autoradiographic film (Kodak Scientific Imaging Systems, New Haven, CT). Densitometry was performed using a GE Healthcare Scanning Densitometer equipped with ImageQuant software (GE Healthcare). To control for loading, all membranes were stained with 0.5% w/v Ponceau S prior to antibody hybridization, and gels were stained with Coomassie Brilliant Blue G-250 immediately after transfer.

In-gel Kinase Assay for ERK Activity—In-gel kinase assays for ERK activity were performed essentially as described (63). Cell lysates containing 2 μg/ml total proteins were subjected to PAGE on standard Laemmli gels (64) with the exception that 0.5 μg/ml of Myelin Basic Protein (Invitrogen) were cross-linked in the gel. Following electrophoresis, SDS was removed from the gels by treatment with 20% isopropanol, 50 mM Tris, pH 8.0, followed by a wash in 50 mM Tris, pH 8.0, 5 mM DTT. Proteins in the gel were denatured in a buffer containing 50 mM Tris, pH 8.0, 5 mM DTT, 6 mM guanidine HCl, and 2 mM EDTA. After protein renaturation by extensive washing in several changes of buffer containing 50 mM Tris, pH 8.0, 5 mM DTT, 2 mM EDTA, and 0.004% Tween 20, the gels were equilibrated in 20 mM HEPES, pH 7.8, 20 mM MgCl₂, 2 mM DTT, 5 mM β-glycerophosphate, 0.1 mM Na₃VO₄, and the kinase reaction was performed in a buffer of similar composition in the presence of 30 μM ATP and 10 μCi/ml [γ-³²P]ATP. After extensive washes in 5% w/v trichloroacetic acid and 1% w/v Na pyrophosphate, the gels were dried and exposed on Kodak BioMax film at −70 °C using the appropriate intensifying screen.

RNA Isolation and Northern Analysis—Total RNA was isolated by the acid guanidinium thiocyanate/phenol-chloroform method (65), which was modified to remove contaminating carbohydrates (66). Northern analysis was performed using 5 μg of RNA from each sample, which were glyoxylated and fractionated in 1.2% agarose gels. Gels were subsequently denatured in 50 mM NaOH, neutralized in 100 mM Tris, pH 7.5, for 30 min, and RNA was electrotransferred to Nytran Plus nylon membranes in 1× TAE (40 mM Tris acetate, 1 mM EDTA, pH 7.8). Blots were hybridized under standard conditions using a ³²P-labeled full-length mkp-2 cDNA probe. As controls for loading, the membranes were probed using a ³²P-labeled, 28 S rRNA cDNA probe. All probes were radiolabeled using the High-Prime random-primer labeling reagent, according to the manufacturer’s instructions (Roche Applied Science).

Immunofluorescence Microscopy and Live Cell Imaging—Cells plated on glass coverslips were fixed with 4% paraformaldehyde, prepared in PBS, and permeabilized in 0.2% Triton X-100, PBS. After blocking of nonspecific antigens in 5% bovine serum albumin/PBS, hybridization with the primary antibody (mouse anti-phospho-ERK) at 1:200 dilution was performed overnight at 4 °C and with the secondary fluorescein isothiocyanate-conjugated anti-mouse antibody at 1:200 dilution for 30 min at room temperature. Following PBS washing, coverslips were mounted on glass slides using the 4.6-diamidino-2-phenylindole-containing ProLong Gold antifade reagent (Molecular Probes, Eugene, OR) and analyzed using a Zeiss Axiovert 220 M microscope powered by Axiowision 4.0 software with multi-channel, Z-stack acquisition, three-dimensional deconvolution, and four-dimensional rendering modules (Karl Zeiss Microimaging Inc., Thornwood, NY). For live cell imaging, cells were grown in glass-bottom 35-mm tissue culture dishes (MatTek Corp., Ashland, MA) and eGFP: tagged proteins were visualized using an inverted Zeiss Axiovert 220 M microscope.

RESULTS

MKP2 Inhibits Cell Proliferation and Induces Premature Senescence—In late passage human fibroblasts, terminal loss of proliferative capacity is accompanied by decreased mitogen-induced nuclear ERK activity that occurs concomitantly with an increase in basal MKP2 levels (21, 58). To investigate the importance of MKP2 in nuclear ERK regulation and its role in the establishment of senescence, we used retroviral-mediated gene transfer to overexpress MKP2 in WI-38 human fibroblasts. To this end, we cloned full-length human MKP2 alone, tagged with the FLAG epitope, or as a fusion with GFP, in the pLNCX constitutive expression vector. Viral supernatants from transfected Phoenix A packaging cells were used to infect early passage WI-38 fibroblasts.

In multiple experiments we found that high levels of MKP2 are incompatible with cell proliferation. Cells expressing either MKP2 or MKP2:GFP remained stationary for at least 2 weeks post selection. We monitored the MKP2:GFP cultures by live cell imaging, both during selection and while at the stationary phase, and observed that cells expressing high levels of MKP2 (more intense fluorescence) did not replicate. Eventually the cultures were taken over by low MKP2-expressing, slow proliferating cells. Cultures that expressed MKP2 at low enough levels to be compatible with cell proliferation, and control cultures, were subcultivated to senescence in antibiotic containing media to maintain selective pressure.

Replicative lifespan, measured as population doublings, of two MKP2-expressing lines and a vector control line are illustrated in Fig. 1A. Ectopic expression of either MKP2 or MKP2:GFP resulted in accelerated senescence and significantly decreased replicative lifespan relative to the control pLNCX line. As seen in Fig. 1A, throughout their lifespan, MKP2 cells had altered growth properties. This was confirmed by measuring growth rates of the cultures. Data from a growth curve experiment in which we used cells immediately after escape from MKP2-induced growth arrest are illustrated in the supplemental figure (graph and table). MKP2 and MKP2:GFP exhibited slower growth rates and increased sensitivity to contact inhibition (expressed as monolayer saturation density at confluence) relative to the control culture. Microscopic examination revealed increased numbers of cells with senescent morphology. In parallel experiments, we used FACS to sort cultures of MKP2:GFP cells into two subpopulations with higher and lower fluorescence levels. We found that growth inhibition correlated with the levels of ectopically expressed MKP2. The yields of these experiments were consistently low because of the low levels of expression, and consequently fluorescence, of MKP2:GFP. Thus, in each experiment we sorted only a subpopulation of cells (~20%) that had sufficiently high levels of MKP2:GFP to be detectable by the cell sorter. In the experiment shown in Fig. 1B, 1 × 10⁵ cells from each sorted group were
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**A**

**Lifespan In Vitro**

<table>
<thead>
<tr>
<th>Population Doubling (PD)</th>
<th>MKP2</th>
<th>MKP2:GFP</th>
<th>pLNCX</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
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<td></td>
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<tr>
<td>50</td>
<td></td>
<td></td>
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</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Sorted MKP2:GFP</th>
<th>In Vitro Age (PD)</th>
<th>Saturation Density (x10^4/cm²)</th>
<th>Cell Number (fold increase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High expression</td>
<td>33.85</td>
<td>1.99 ± 0.04</td>
<td>2.49 ± 0.04</td>
</tr>
<tr>
<td>Low expression</td>
<td>33.85</td>
<td>4.66 ± 0.25</td>
<td>5.83 ± 0.32</td>
</tr>
</tbody>
</table>

(*) Population doubling (PD) at the end of the experiment (day 21)

**C**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>In Vitro Age (PD)</th>
<th>Saturation Density (x10^4/cm²)</th>
<th>Particle Diameter (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKP2</td>
<td>38.27</td>
<td>1.74</td>
<td>19.80 ± 1.29</td>
</tr>
<tr>
<td>MKP2:GFP</td>
<td>39.67</td>
<td>2.03</td>
<td>19.66 ± 1.30</td>
</tr>
<tr>
<td>pLNCX</td>
<td>45.13</td>
<td>3.99</td>
<td>16.21 ± 1.24</td>
</tr>
</tbody>
</table>

**D**

**SA-β-galactosidase**

**E**

**Cell Viability**

FIGURE 1. A, constitutive expression of MKP2 results in accelerated senescence. Mass cultures of WI-38 cells transduced at early PD with MKP2 (FLAG or GFP-tagged) were subcultivated to senescence. The number of PD was plotted against time and each point represents one subcultivation. Cells transduced with vector alone (pLNCX) are shown as controls. MKP2 cultures show a 40% decrease in lifespan. B, inhibition of cell proliferation is proportional to the levels of ectopic MKP2. MKP2:GFP cells at early passage were sorted for cells with high and low fluorescence intensity which represent levels of MKP2:GFP expression. Because of the low levels of MKP2:GFP expression the fluorescence intensity of the majority of the cells was below the limits of detection, hence, only about the top 20% of expressors was used in this experiment. From each subpopulation 1 x 10^5 sorted cells were main-

seeded in T12.5 flasks and grown under standard culture conditions for 3 weeks with weekly re-feedings. The growth rates of these cells were extremely slow, especially of the high expressors, which doubled only once during the 3-week period. Thus, inhibition of cell proliferation by MKP2 is directly proportional to the levels of MKP2 expressed. It should be noted that growth inhibition in response to MKP2 overexpression is not specific to WI-38 fibroblasts. Although we performed the lifespan experiments only with WI-38 cells, in short term experiments we observed growth inhibitory effects of MKP2 in a variety of both diploid and tumor derived cells lines, including vascular smooth muscle cells, diploid dermal fibroblasts, HeLa, HS913T fibrosarcoma, and OV1063 ovarian carcinoma cells.

To confirm that the reduced cell numbers of the MKP2 expressing lines resulted from impaired proliferation and not from increased cell death, we monitored our cultures closely, both during selection and throughout their lifespan. Routine microscopic examinations and DNA staining with the nuclear dye 4,6-diamidino-2-phenylindole showed no evidence of chromatin condensation indicative of apoptotic cells (data not shown). Thus, we concluded that MKP2 inhibits cell proliferation rather than induce cell death. Furthermore, to ensure that expression of MKP2 does not increase the sensitivity of cells to environmental stress and thus predispose to death, we measured cell viability, using the MTT assay, 24 h after treatment with various apoptotic stimuli (Fig. 1E). As expected, we did not find any significant change in the susceptibility of the MKP2 cells to death induced by the compounds used. Tagging MKP2 with GFP did not affect the rate of cell death either. This was of concern initially, due to the GFP toxicity reported by other investigators. We have performed these experiments numerous times, as well as experiments in which we expressed other GFP tagged proteins or GFP alone (shown in Fig. 2C), and we are confident that WI-38 fibroblasts can readily tolerate high levels of GFP.

MKP2 lines near their lifespan-end, although at a much lower PD than control cultures (39 versus 45 PD), exhibited all the growth and morphological characteristics of senescence. Phase contrast images of the cells are shown in Fig. 1C and some of their growth properties in the accompanying table. In contrast to the vigorously proliferating, spindle-like control fibroblasts, MKP2 cells exhibit the distinct flattened, enlarged morphology of senescent cells, sensitivity to contact inhibition (i.e.
Reduced monolayer saturation densities, and loss of replicative capacity. Increased SA-β-gal activity is considered to be a marker of replicative senescence and under appropriate conditions, i.e. staining of healthy, subconfluent cultures correlates reasonably well with replicative age (59, 67). We routinely assayed SA-β-gal activity in subconfluent cultures (24 h after seeding at 1–2 × 10⁴ cells/cm²), and as expected, we found that throughout their lifespan, the percentage of stained cells was always higher in the cultures expressing MKP2. A representative SA-β-gal staining is shown in Fig. 1D. In every experiment, the number of SA-β-gal-positive cells in MKP2 cultures correlated with their reduced proliferation rate and increased numbers of senescent cells in the population, rather than with their actual PD.

The lifespan experiments were repeated multiple times and at least 10 different lines expressing MKP2 from various vectors, were created. All MKP2 lines underwent premature senescence. Furthermore, the decrease in lifespan, relative to control lines, was similar among experiments even though the maximum lifespan varied. Maximum lifespan was influenced by the culture age at infection, the toxicity of the antibiotic used for selection, and by the use of different WI-38 sublines between different experiments. To eliminate the possibility that observed differences between transduced lines were caused by experimental variations, in each experiment all lines were subcultivated simultaneously.

Expression levels of MKP2 (ectopic and endogenous proteins) are illustrated in Fig. 2A. Cells were assayed after they had escaped MKP2-induced proliferative arrest by immunoblotting using an anti-GFP monoclonal antibody (B2), which detected MKP2::GFP, or an anti-MKP2 polyclonal antibody (S19), which detected both endogenous and ectopically expressed MKP2. As seen in Fig. 2A, in G₀-synchronized and serum-stimulated cultures, MKP2 construct expression (MKP2 bands at 43 kDa molecular mass and MKP2::GFP bands at 70 kDa molecular mass) was only about 2-fold higher than endogenous levels. Interestingly, we observed that FBS stimulation results in a small but reproducible increase in abundance and size of the ectopically expressed MKP2::FLAG and MKP2::GFP. This increase may represent ERK-mediated stabilization of MKP2 as reported previously for MKP1 and MKP7 (68, 69). The immunoblots illustrated in Fig. 2B were prepared from samples derived from non-synchronized MKP2-transduced cells, immediately after selection (cultures were at the stationary phase) and after the cultures had escaped MKP2-induced growth arrest. As seen in the figure, cells capable of proliferation show a marked decrease in the abundance of ectopically expressed MKP2. Shown in Fig. 2C are live cell images from the MKP2::GFP line that was used to confirm the nuclear localization of ectopically expressed MKP2 and observe variations in MKP2::GFP abundance at the cellular level. Note the presence of fluorescent nuclei in MKP2::GFP-infected cells relative to cells infected with eGFP alone. Both images were obtained at identical conditions/-settings. Fig. 2D illustrates the effect of ectopic MKP2 expression on ERK protein levels and activity.

We expected that, because of the low levels of MKP2, its nuclear localization, and the fact that the cells were able to proliferate (albeit at reduced rates), total ERK2 activity (nuclear and cytoplasmic) would not be significantly affected. Indeed, neither the
levels of ERK1/2 nor their activating phosphorylation were different between the cell lines (Fig. 2D). We expected, however, that nuclear ERK2 activity would be reduced in cells expressing MKP2. Because c-fos serum induction dependents on the phosphorylation of the transcription factor Elk-1 by nuclear ERKs (26–29), we assayed protein abundance of c-Fos in serum stimulated cells as an indirect “measure” of nuclear ERK activity. Indeed, we found that the inducibility of c-fos is impaired in MKP2 expressing cells (Fig. 2D).

Transduction of Early Passage Cells with a Phosphatase-resistant ERK2 Mutant Results in Extension of Replicative Lifespan and Reversal of Senescence-associated Phenotypic Characteristics—MKP2-mediated ERK inactivation is not a simple catalytic reaction but depends on stable association interactions between the ERK docking groove and the N-terminal docking domain of MKP2 (52, 53). Disruption of these interactions, through substitution of the negatively charged aspartate to asparagine at position 316 of ERK2, results in a hyperactive ERK2 allele (ERK2 D319N) analogous to the gain-of-function Seven-maker (rl3F0) mutant of D. melanogaster (54–56). It is important to note that the D319N mutation does not impede ERK2 substrate recognition or activation by MEK (70) and, although it confers resistance against MKP-catalyzed inactivation, is not an absolute impediment to dephosphorylation. As a result, ERK2(D319N) is a cell cycle-regulated kinase that depends on MEK for its activation.

We have previously reported that senescent fibroblasts have increased MKP2 abundance and activity (58). Based on the premises that high nuclear MKP2 levels in senescent cells result in rapid ERK inactivation and that nuclear ERK activity is fundamental for cell cycle progression, we hypothesized that ectopic expression of the phosphatase resistant ERK2 Sevenmaker mutant would counter-balance the effect of MKP2 and, ultimately, increase lifespan. To test this hypothesis, we cloned in the retroviral pLNCX vector ERK2(D319N) as well as wt ERK2, which we used to control for possible ERK2 effects unrelated to its resistance to dephosphorylation. Cultures of the packaging cell line Phoenix A were transfected with the various constructs and viral supernatants were used to infect various sublines of early passage WI-38 fibroblasts. Because WI-38 cells have limited proliferative capacity, in all our experiments we used mass cultures. These were subcultivated to senescence in antibiotic containing media to maintain selective pressure.

In several repetitions of this experiment, we found that ERK2(D319N) extends culture lifespan. As illustrated in Fig. 3A, replicative lifespan (population doublings) of ERK2(D319N) expressing fibroblasts was significantly increased compared to cells expressing wild type ERK2 (~27%) or vector alone (~22%). The small difference in replicative lifespan between the two control cultures, wt ERK2 and vector, was not significant. We have often observed slightly better survival, during antibiotic selection, of cultures transduced with control vectors. Better tolerance to long term antibiotic exposure could account for the 1–1.5 PD difference we usually see between wt ERK2 and vector alone cultures. In contrast, ERK2(D319N) cells showed a significant increase in lifespan and maintained their morphological characteristics well past the point when control cultures had acquired the typical senescent phenotype (enlarged, flattened morphology and high sensitivity to contact inhibition). Phase contrast images and growth properties of cultures at similar PD are illustrated in Fig. 3B. As seen in the figure, in contrast to senescent ERK2 and pLNCX control cultures, the proliferation potential, morphology, and monolayer saturation density of ERK2(D319N) cells was similar to that of early passage cells.

Fig. 4 illustrates control experiments in which cultures were assayed for construct expression, proper activating phosphorylation, and activity of the expressed proteins. In parallel experiments, wt ERK2 and ERK2(D319N), cloned as fusion proteins to GFP, were used to assay the efficiency of infection, kinetics, and nuclear translocation of the expressed proteins using time-lapse microscopy. ERK2 expression and activity were also tested in these lines. Shown in Fig. 4A are immunoblotts and in Fig. 4B results from an in-gel ERK activity assay obtained using total cell lysates from G0-synchronized cells before and after serum stimulation. Bands at 42 and 69 kDa...
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This experiment was repeated numerous times and images were recorded every 30 s for up to 2 h post serum stimulation of G₀-synchronized cells. Shown are representative images of a typical response; only the 0, 2.5, 5, and 20 min time points are shown (Fig. 4D). Also, shown in Fig. 4C is the abundance of total and phosphorylated ERK2-GFP in nuclear extracts prepared from quiescent and serum stimulated cells. In quiescent cells, wild type ERK2-GFP and the phosphatase-resistant ERK2(D319N):GFP were both localized mainly in the cytoplasm. Upon serum stimulation there was a massive translocation into the nucleus that peaked 5-min post-stimulation. In the case of ERK2:GFP, there was a gradual decrease in nuclear accumulation although a portion remained nuclear for at least 2 h after stimulation. In contrast to ERK2-GFP, the nuclear accumulation of the phosphatase resistant ERK2(D319N):GFP was maintained at high levels for longer time periods. The lack of ERK2(D319N) phosphorylation in quiescent cells and the kinetics of its nuclear translocation are in agreement with published observations that ERK2(D319N) is not constitutively active; its basal activity is similar to that of wild type ERK2 but remains active for longer time after stimulation. In contrast to immortalized cells, uncontrolled activation of the ERK pathway in normal cells has been reported to inhibit, rather than stimulate, cell proliferation and to result in a senescent-like phenotype (see “Discussion”). Thus, the fact that ERK2(D319N) is not constitutively active was of particular importance for our experiments.

Although transduction of WI-38 cells with ERK2(D319N) increased lifespan, the cultures did senesce but with some phenotypic differences. ERK2(D319N) cells, even when they had completed 100% of their in vitro lifespan (phased out), had higher abundance of nuclear phospho-ERK, were able to express c-fos upon serum stimulation (Fig. 5A), and had lower activity of SA-β-galactosidase (Fig. 5B). Interestingly, senescent ERK2(D319N) cells that were maintained in culture for prolonged periods had unusually high numbers of binucleated cells (64% of phased out cells). The significance of this observation is at present unclear although a tempting hypothesis is that the cells are able to bypass the senescence/late G₁ block and are arrested at a later point of the cell cycle.

Enhancement of Nuclear Localization of ERK2(D319N) Results in 61% Increase of Replicative Lifespan—Because the phosphatase-resistant ERK2(D319N) mutant is expressed primarily in the cytoplasm while MKP2 localizes exclusively in the nucleus, we reasoned that the most effective strategy to neutralize the growth inhibitory effect of high MKP2 abundance would be through nuclear targeting of ERK2(D319N). For this purpose we generated constructs in which the nucleotide sequence encoding the nuclear localization signal of the SV-40 T antigen (61, 62) was cloned at the 3'-end of either wt ERK2 or ERK2(D319N). These constructs were subsequently transduced in early passage WI-38 cells and replicative lifespans were estimated by serial subcultivation. ERK2 and ERK2(D319N) were used as additional controls. In the experiment shown in Fig. 6 we used constructs based on the puromycin resistant pLPCX expression vector. Because in prior experiments we observed that ERK2(D319N)-NLS cells proliferated more rapidly than any other transduced line, we were con-
cerned that during a prolonged selection process, e.g. by G418, cell numbers during selection may be affected by changes in growth rates. We reasoned that because puromycin kills cells more rapidly, comparisons of the efficiency of infection would be more accurate. We found that the method of antibiotic selection had no significant impact; within each experiment the infection efficiencies were similar and, more importantly, changes in lifespan relative to controls were similar among independent experiments. As mentioned above, absolute maximum lifespans varied among experiments and were influenced by the culture age at infection, toxicity of the antibiotic used for selection, and by the use of different WI-38 sublines. Because these variations were anticipated, to eliminate the possibility that differences in lifespan were influenced by experimental variations, in each experiment all lines were subcultivated simultaneously.

To test the effect of increased nuclear ERK2 activity on replicative lifespan, we subcultivated ERK2(D319N):NLS and all the control cultures to senescence. Because in our initial studies ERK2 and vector alone lines grew identically and always achieved similar maximum PD; in subsequent experiments only ERK2 lines were subcultivated to senescence. The sequence of the SV-40 Tag NLS was incorporated at the C terminus of wt ERK2, ERK2(D319N), and pLNCX vector prior to replicative arrest (growing) and after they reached senescence (phased out). Subconfluent cultures were synchronized in G0, and subsequently stimulated with FBS (10% v/v) for 45 min. Total cell lysates were analyzed by immunoblotting using an anti c-Fos monoclonal antibody. Ponceau S staining of the membrane is shown as a control for loading. B, phased-out cultures (100% lifespan completed) transduced with either ERK2(D319N) or pLNCX vector alone, were seeded at 2 x 10^5/cm², incubated for 24 h, and stained for SA-β-galactosidase activity. Images shown are at ×40 and ×400 magnification. Early and late passage untransfected WI-38 cells that had completed 53% and 97% of their lifespan, respectively, are shown as controls. Intensity of SA-β-galactosidase staining in phased out ERK2(D319N) cultures is similar to that of early passage WI-38 cells.

FIGURE 5. A, senescent ERK2(D319N)-expressing cells retain molecular characteristics typical of early passage fibroblasts. Samples were prepared from cultures transduced with wt ERK2, ERK2(D319N), and pLNCX vector prior to replicative arrest (growing) and after they reached senescence (phased out). Subconfluent cultures were synchronized in G0, and subsequently stimulated with FBS (10% v/v) for 45 min. Total cell lysates were analyzed by immunoblotting using an anti c-Fos monoclonal antibody. Ponceau S staining of the membrane is shown as a control for loading. B, phased-out cultures (100% lifespan completed) transduced with either ERK2(D319N) or pLNCX vector alone, were seeded at 2 x 10^5/cm², incubated for 24 h, and stained for SA-β-galactosidase activity. Images shown are at ×40 and ×400 magnification. Early and late passage untransfected WI-38 cells that had completed 53% and 97% of their lifespan, respectively, are shown as controls. Intensity of SA-β-galactosidase staining in phased out ERK2(D319N) cultures is similar to that of early passage WI-38 cells.

FIGURE 6. A, nuclear targeting of the phosphatase-resistant ERK2(D319N) mutant results in 61% lifespan increase relative to control ERK2-transduced cultures. The sequence of the SV-40 Tag NLS was incorporated at the C terminus of wt and D319N ERK2, and the retroviral constructs ERK2:NLS and ERK2(D319N):NLS were transduced into early passage WI-38 cells. Sister flasks were also transduced with wt and D319N ERK2, which served as additional controls. Antibiotic-selected mass cultures were subcultivated to senescence. The number of PD was plotted against time and each point represents one subcultivation. B, morphological and growth characteristics of transduced cells. Phenotype of cells transduced with FLAG-tagged wt ERK2, ERK2(D319N), wt ERK2:NLS, and ERK2(D319N):NLS at similar PD. The arrow in the lifespan curve graph indicates the age of the cultures. Images were obtained from confluent cultures that were subsequently harvested by trypsinization, and cell number and size distribution were estimated using a Beckman Coulter Counter. C, SA-β-galactosidase activity in transduced lines at similar PD. Cultures shown in C were seeded at 2 x 10^5/cm², incubated for 24 h, and stained for SA-β-galactosidase activity. Images were obtained at ×100 magnification.
transduction with the phosphatase-resistant ERK2 is similar to that obtained in the experiment shown in Fig. 3. Some phenotypic characteristics of the cultures just prior to replicative arrest are illustrated in Fig. 6B. Staining for SA-β-galactosidase activity is shown in Fig. 6C. Note that, due to more vigorous growth rates throughout their lifespan, ERK2(D319N):NLS cells at the time of the comparisons had higher PD than the control cultures. Nevertheless, in contrast to the senescent morphology and reduced proliferation of the control ERK2 culture, ERK2(D319N):NLS cells maintained phenotypic characteristics of young cells (spindle-like morphology, normal size, high saturation density, and proliferation rate).

Expression levels and activating phosphorylation of the constructs were assayed by immunoblotting several times throughout lifespan, to ensure continued expression. Abundance and phosphorylation of endogenous and transduced wt and mutant proteins, at early and late passage cultures, are shown in Fig. 7A. Despite the slight size shift in transduced ERK2 and ERK2(D319N) due to the FLAG epitope (990 dalton) the bands appear to overlap with wt ERK (42 kDa). Cells expressing ERK2:NLS show a band at 43.76 (990-Da FLAG epitope and 770-Da NLS). Fig. 7A also illustrates the continuous expression of our constructs in terminally arrested cultures.

As in all our experiments, the nuclear-targeted proteins were also expressed as fusion to GFP, and their subcellular distribution was assayed by live cell imaging (Fig. 7B). We observed that while NLS could drive an increased number of ERK2 in the nucleus, the expressed proteins were localized in both the nuclear and cytoplasmic compartments. These results were anticipated. It is well established that ERK translocation is not sufficient for nuclear accumulation; ERK interaction with newly synthesized nuclear anchoring proteins is also required (71). Nuclear accumulation of the phosphatase-resistant ERK2(D319N):NLS was more efficient than wild type ERK2:NLS, consistent with the hypothesis that ERK2 dephosphorylation facilitates its nuclear export or possibly its degradation.

Extension of Replicative Lifespan of Dermal Fibroblasts in Response to Nuclear Targeting of ERK2(D319N)—To confirm that the effect of nuclear targeting of D319N ERK2 is neither tissue-specific nor affected by the developmental stage of the cell donor, we transduced ERK2(D319N):NLS into early passage dermal fibroblasts derived from a young donor. wt ERK2 and pLPCX vector transduced cultures were used as controls. As seen in Fig. 8A, lifespan extension of postnatal dermal fibroblasts in response to transduction with ERK2(D319N):NLS was similar to that observed with WI-38 fetal lung fibroblasts. ERK2 expression levels and activating phosphorylation (ectopic and endogenous) in the lines are illustrated in Fig. 8B.

Cultures with Extended Lifespan Senesce with Altered Phenotypic Characteristics—Genetic manipulation of nuclear ERK2 activity postulates but does not prevent senescence. In fact we have never seen any signs of spontaneous immortalization even after prolonged culture periods. It is likely that restoring nuclear ERK signaling in senescent fibroblasts is not sufficient to confer unlimited growth potential. Alternatively, late passage cultures may still lose nuclear ERK activity despite the ectopic expression of the phosphatase resistant ERK2 mutant. To address this possibility, terminally arrested cultures (100% lifespan completed) were assayed for nuclear ERK2 activity, at the cellular level, by immunofluorescence microscopy using a phospho-ERK-specific antibody (Sigma). Activating phosphorylation of ERK was assayed in G0-arrested cells before and after 5 min serum stimulation (10% v/v) were analyzed by immunoblotting by sequential probing for ERK2 and for active, phosphorylated ERK1/2. The asterisk indicates a sample loaded in both gels to control for transfer efficiency. Ponceau S staining of the membrane is shown as a control for loading.

FIGURE 7. A, expression levels and activating phosphorylation of wt and D319N ERK2, with and without the SV-40 NLS. Samples were prepared from cultures prior to replicative arrest (growing) and after they reached senescence. Total cell lysates prepared from quiescent subconfluent cultures before and after 5 min serum stimulation (10% v/v) were analyzed by immunoblotting by sequential probing of ERK2 and for active, phosphorylated ERK1/2. The asterisk indicates a sample loaded in both gels to control for transfer efficiency. Ponceau S staining of the membrane is shown as a control for loading. B, subcellular localization of ERK2, ERK2(D319N), ERK2:NLS, and ERK2(D319N) NLS proteins, fused to GFP. Nuclear localization of NLS-tagged wt and D319N ERK2 was assessed using live cell imaging of transduced WI-38 cells. Images shown were captured from subconfluent cultures (24 h after seeding) using an inverted Zeiss Axiovert 220M microscope and were obtained at ×200 magnification.
This observation supports the notion that restoring nuclear ERK signaling in senescent fibroblasts is not sufficient to confer immortality.

The ability of ERKs to indirectly control levels and activity of cell cycle regulators, including, cyclins, CDKs and their inhibitors (CDIs), and the checkpoint proteins p53 and Rb, is well documented (13, 72). Many of these proteins have also been linked with the senescent phenotype. For example gene expression of the CDIs p21 and p16 (Ink4a), protein stability of p21, p53 abundance, stability, and activity, as well as Rb phosphorylation by cyclinD1/CDK complexes can all be modulated by ERK-dependent signals (13). ERK can have opposing effects on p53; it can either stabilize p53 by up-regulating p19 (ARF) or enhance its degradation through the transcriptional activation of the negative p53 regulator mdm2 (72). Because of the numerous reports proposing a regulatory role in replicative senescence, we examined whether protein abundance of p53 and phosphorylation status of Rb are altered. For these experiments we compared contact inhibited lines at two different stages; pre-senescent and senescent (phased out). As seen in Fig. 10, protein abundance of p53 is identical between lines at both passages and shows no increase in the phased out cultures. The effect of the different ERK2 mutants was different on Rb; the most dramatic difference was the ability of ERK2(D319N):NLS cells to maintain Rb in its hyperphosphorylated state (inactive) even after reaching replicative arrest. Although we did not measure activity of cyclinD1/cdk2, we found no difference in the abundance of the CDIs p21, p27, p16, and p19 (data not shown) that could explain the Rb phosphorylation status in senescent ERK2(D319N):NLS cells. We also did not find any significant difference in the cyclin D1 levels under the same conditions in which we assayed Rb phosphorylation (non-synchronized cultures). A more detailed cell cycle analysis is warranted to identify the exact mechanism responsible for the Rb inactivation in senescent ERK2(D319N):NLS cells.

MKP2 Induces Premature Senescence by Specifically Inactivating Nuclear ERK2—MKP2 has high specificity toward ERK but can also inactivate the stress-induced p38 and the JNK members of the MAPK family (48, 51, 56, 73). To demonstrate that the decrease in lifespan caused by ectopic MKP2 expres-
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FIGURE 10. Abundance of p53 and Rb. Samples were prepared from cultures prior to replicative arrest (pre-senescent) and after they reached replicative arrest (senescent). Total cell lysates were prepared from contact-inhibited, confluent cultures and were analyzed by immunoblotting by sequential probing for p53, total Rb, and hyperphosphorylated Rb. Abundance of α-tubulin and Ponceau S staining of the membrane are shown as controls for loading.

Pre-Senescent

Senescent

- ERK2
- ERK2(D319N)
- ERK2:NLS
- ERK2(D319N):NLS
- ERK2
- ERK2(D319N)
- ERK2:NLS
- ERK2(D319N):NLS
- ERK2
- ERK2(D319N)
- ERK2:NLS
- ERK2(D319N):NLS
- ERK2
- ERK2(D319N)
- ERK2:NLS
- ERK2(D319N):NLS

p53

phosphoRb

Rb

αTubulin

IB: p53

IB: PhosphoRb

IB: Total Rb

IB: αTubulin

Ponceau S

FIGURE 11. A, MKP2-induced premature senescence is the result of nuclear ERK2 inactivation. The retroviral constructs ERK2:NLS and ERK2(D319N):NLS were transduced into early passage WI-38 cells. G418-selected mass cultures were subsequently transduced with either MKP2 (pLHCX) or pLHCX vector alone, and the hygromycin-selected cultures were subcultivated to senescence in the presence of both antibiotics. The number of PD was plotted against time and each point represents one subcultivation. MKP2-transduced cultures show no reduction of lifespan and had growth properties similar to pLHCX-transduced control ERK2(D319N):NLS cultures. In contrast, MKP2 results in dramatic lifespan reduction of the ERK2:NLS line. Note that PD were not normalized for cells that did not survive antibiotic selection. These were similar for all cultures and are estimated to account for approximately a 10 PD reduction per round of selection. B, images were obtained from confluent cultures that were subsequently harvested by trypsinization and cell numbers and size distributions were estimated using a Beckman Coulter Counter. Expression of ERK2(D319N):NLS prevents the premature appearance of MKP2-induced phenotypic characteristics of senescence.

shRNA-mediated Knock-down of MKP2 Levels Results in Lifespan Extension—To verify that the increased lifespan of cultures expressing ERK2(D319N) is related to their ability to withstand the effects of increased MKP2 activity at late passages (58), we knocked down MKP2 levels using RNA interference. To this end early passage cultures were transduced with a pSHAG-MAGiC2 retroviral vector expressing shRNAs targeted against MKP2. As a control we used cultures transduced with the non-silencing shRNA control vector. Both cultures were subcultivated to senescence in antibiotic containing media. The efficiency of MKP2 silencing was assayed by Northern blot analysis, shown in C, and immunoblotting, shown in D. As seen in the lifespan graph (Fig. 12A), despite the relatively modest knock-down of MKP2 expression, transduction with MKP2 shRNA resulted in 44% lifespan extension compared with the culture transduced with the non-silencing vector. Some phenotypic characteristics of the cultures just prior to replicative arrest are illustrated in Fig. 12B. Note that at the time of the comparisons, MKP2 shRNA-transduced cells had undergone more PDs than the controls, due to more vigorous growth rates throughout their lifespan. Nevertheless, in contrast to the senescent morphology and reduced proliferation of...
far less dramatic than the decrease in phosphorylated ERK. Thus, we concluded that in senescent cultures decreased nuclear ERK activity results mainly from ERK inactivation rather than impaired nuclear import. Our findings that senescent cells have high activity of the nuclear ERK phosphatase MKP2 support this interpretation (58). Because nuclear retention of ERKs entails interaction of phosphorylated ERK with nuclear anchoring proteins (31, 71), increased MKP2 activity can potentially affect both ERK activity and nuclear localization and, depending on the magnitude of inhibition, may impede cell cycle progression. Our current results support this hypothesis. Ectopically expressed MKP2 interferes with nuclear ERK signaling without altering total ERK activity and inhibits proliferation in a dose-dependent manner. Even marginal increases in MKP2 levels delay cell cycle progression and accelerate senescence. Prior expression of nuclear targeted phosphatase-resistant ERK2(D319N) abrogates the negative effects of ectopic MKP2, suggesting that they are mediated specifically by inhibition of nuclear ERK signaling and not of other MAPK members. The importance of MKP2 levels in the control of replicative senescence was further substantiated by our finding that shRNA-mediated knock-down of MKP2 expression significantly extends the lifespan of the transduced lines.

In view of the multitude of nuclear ERK effectors and their fundamental role in transcriptional regulation, the dramatic effect of even slightly increased MKP2 levels on cell proliferation is not surprising. Brunet et al. (75) have previously shown that ERK cytoplasmic anchoring (nuclear exclusion), by means of an inactive MKP3 mutant, inhibits AP-1-mediated cyclin D1 induction and S phase progression in fibroblasts. Our results are consistent with these findings. Moreover, they establish that nuclear import is not sufficient for proper ERK function; duration of ERK signaling in the nucleus is also important. Enhanced dephosphorylation of nuclear ERK, by ectopic expression of MKP2, has significant short and long term effects on cell proliferation.

In view of the pleiotropic effects of ERK and the capacity of MKP2 to induce premature senescence, a valid hypothesis is that certain phenotypic characteristics of senescent cells, and particularly loss of proliferative potential, result from impaired nuclear ERK signaling. Interestingly, in normal human cells constitutive activation of the ERK pathway has been shown to induce, rather than prevent, senescence. Use of oncogenic Ras (H-RasV12), constitutively active MEK1(Q56P), or Raf mutants (ΔRaf:ER, ΔRaf (Y340D, Y341D:ER)), all result in p53- and/or p16-dependent G1-arrest that displays phenotypic characteristics of replicative senescence (71, 76–79). Although this inhibition may be a stress response to unbalanced activation of signaling systems, it is well established that strict spatio-temporal control of ERK activity is fundamental for proliferation. In growth-regulated cells, ERK activation is an absolute requirement for exit from quiescence and cell cycle progression; however, ERK activity past late G1 results in cell cycle arrest (13, 80). Furthermore, since proper ERK localization requires complex interactions with scaffold and anchoring proteins, activation of the pathway does not always ensure proper localization. For example, it has been reported that during Ras-induced senescence, ERKs localize primarily in the cytoplasm due to a hyper-

FIGURE 12. A, lifespan extension as a result of MKP2 knock-down using RNA interference. Early passage WI-38 cells were transduced with retroviral vectors expressing shRNA targeted against MKP2 or non-silencing shRNA as a control. Antibiotic-selected mass cultures were subcultivated to senescence. The number of PD was plotted against time, and each point represents one subcultivation. Cultures expressing MKP2 shRNA show substantial increase in lifespan relative to those transduced with the non-silencing shRNA vector. B, morphological and growth characteristics of transduced cells. Phenotype of cells transduced with MKP2 shRNA or the non-silencing shRNA, at late PD. The arrow in the lifespan graph indicates the age of the cultures. Images were obtained from confluent cultures that were subsequently harvested by trypsinization, and cell number and size distribution were estimated using a Beckman Coulter Counter. Relative to the control MKP2, shRNA cells show improved growth properties and have the appearance of early passage cultures. C, steady state levels of MKP2 mRNA in transduced lines. MKP2 shRNA and control cultures were synchronized in G0, and subsequently stimulated with 10% v/v FBS for 2 h. Total RNA was subjected to Northern analysis using a radiolabeled MKP2 cDNA probe. Steady state levels of 28 S rRNA are shown as a control for loading. D, protein abundance of MKP2 in transduced lines. MKP2 shRNA and control cultures were synchronized in G0, and subsequently stimulated with 10% v/v FBS for the indicated times. Total cell lysates were analyzed by immunoblotting using a polyclonal antibody against MKP2. Abundance of α-tubulin is shown as loading control.

the control culture, MKP2 shRNA-expressing cells maintained phenotypic characteristics of young cells (spindle-like morphology, normal size, high saturation density, and proliferation rate).

DISCUSSION

We, and others (21–24), have shown that in senescent fibroblasts the most dramatic change in the ERK pathway is the impaired nuclear localization of active (phosphorylated) ERK. Although some investigators have proposed that this decrease stems from impaired nuclear import (23, 74), our experiments using live cell imaging with ERK2:GFP, immunofluorescence, and cell fractionations (data not shown) demonstrated a senescence-associated decrease in nuclear ERK abundance that was...
active nuclear export mechanism (81). Down-regulation of the ERK nuclear export factor PEA-15 by the adenoviral E1A oncoprotein or siRNA strategies rescues fibroblasts from Ras-induced senescence (81). In view of these observations, the approach we used was designed to increase nuclear ERK activity sufficiently to restore the reduced levels of senescent cells, while maintaining the normal cell cycle constraints of wt ERK2. These requirements were met by the Sevenmaker phosphatase-resistant ERK2(D319N) mutant, which in control experiments, in agreement with published reports, behaved very similarly to wt ERK2 with the exception that it had slightly higher and prolonged activity.

We found that constitutive expression of ERK2(D319N) could neutralize the inhibitory effect of increased endogenous MKP2 levels, so that nuclear ERK activity was restored to levels sufficient to postpone the onset of senescence and reverse some senescence-associated molecular changes. Lifespan extension of ERK2(D319N) cultures was small but significant, while nuclear targeting of this mutant resulted in more than 60% increase in lifespan. Interestingly, nuclear targeting of wt ERK2 also resulted in increased lifespan. At least two possibilities can explain this effect: continuous replenishment of the nuclear levels of phosphorylated ERK is sufficient to counterbalance the inhibitory effect of MKP2, or alternatively, there is a progressive age-associated decline in the efficiency of ERK nuclear translocation that is alleviated by the use of NLS. The later possibility assumes that the mechanism that regulates nuclear import of ERKs is distinct from that of proteins that contain typical nuclear localization signals. Nuclear-cytoplasmic shuttling of ERK is complex, and although ERK contains an atypical NLS (82), unlike typical NLS-containing proteins, ERK can directly associate with nucleoporins and pass through nuclear pores by importin β-independent mechanisms (43, 83–85). However, there is also evidence that nuclear localization of ERK is not controlled at the level of import through the nuclear pores but rather through docking interactions with nuclear or cytoplasmic proteins that participate in nuclear exclusion, retention, and shuttling (19, 30, 31); these should be identical for both ectopically expressed proteins, ERK2 and ERK2-NLS.

In our experiments, while we achieved marked lifespan extension, our genetic manipulations were not sufficient to confer unlimited proliferative potential. Several possibilities could explain the lack of immortalization of our cultures. It is possible that the levels of ERK2 activity attained were not sufficient to confer immortality. Endogenous MKP2, ultimately, reached high enough levels to inhibit the ectopically expressed ERK2. Terminally arrested cultures with extended lifespan showed high levels of phosphorylated nuclear ERK and maintained the ability to express c-Fos, albeit at reduced levels. Thus, in cells incapable of further proliferation, endogenous MKP2 was not sufficient to completely inhibit nuclear ERK activity. A possibility that warrants future examination is that potentially small decreases in nuclear ERK activity result in impaired activation of other nuclear ERK targets toward which ERK has lower affinity than Elk-1, the transcription factor responsible for ERK-induced c-fos up-regulation. Amplification of signaling is a well established mechanism employed by the ERK pathway. For example, activation of only 5% of Ras molecules is sufficient for complete activation of the ERK pathway (86). Similarly, we observed that even a small increase in nuclear ERK activity is sufficient to extend lifespan significantly. Thus, it is also possible that small decreases in nuclear ERK activity may be sufficient to inhibit activation of low affinity ERK effectors and have a significant impact in gene expression (41).

Another possibility for the lack of immortalization is that we restored only nuclear ERK2, but not ERK1, activity. Targeted disruption of the ERK1 and ERK2 genes has shown that despite their 84% amino acid sequence homology (87, 88), common mechanism of activation, and similar spatiotemporal patterns of expression during development, the two ERK isoforms have distinct biological functions. Mice lacking ERK1 are viable and fertile (89, 90), while ERK2 invalidation results in embryonic lethality at the time of gastrulation caused by defective placental angiogenesis, trophoblast and mesoderm development (91–93). Furthermore, Bost et al. (17) reported that loss of ERK1 does not affect cell growth whereas ERK2 inhibition blocks proliferation of ERK1(−/−) mouse embryonic fibroblasts, demonstrating that ERK2, but not ERK1, is indispensable for cell proliferation. Because WI-38 fibroblasts are of mesoderm origin, and ERK2 is indispensable for mesoderm development and cell proliferation, we chose to use ERK2 in our experiments. However, we cannot rule out that reduced nuclear ERK1 activity is also important in the establishment of senescence.

A third explanation, which we favor, is that nuclear ERK activity is necessary, but not sufficient, to confer immortality. Loss of this activity, either by increased endogenous MKP2 activity or by ectopic MKP2 expression, is critical for the establishment of senescence but most likely is not the only mechanism. This interpretation agrees with a generally accepted theory of senescence proposing that limited replicative potential of diploid cells is the result of “two barriers to immortality.” The one barrier is gradual stochastic accumulation of damage that results in “stress-induced proliferative arrest” (94, 95) and the other is the replication-driven progressive deterioration of telomeres. Cells surviving damage-induced stress, if they do not express the enzyme telomerase, will eventually arrest in response to signals originating at the chromosome ends. Our results are consistent with the two-barrier hypothesis and favor the notion that impaired signaling through the ERK pathway imposes the first barrier. Our data support the hypothesis that decreased proteasome activity, possibly in response to environmental damage, results in accumulation of MKP2 (58), which in turn inactivates nuclear ERKs. Thus, loss of nuclear ERK activity may establish the first barrier to immortality while reversal of this loss, by either shRNA-mediated MKP2 knock-down or ectopic expression of phosphatase resistant ERK2, temporarily prevents senescence.

The importance of nuclear ERKs in modulating gene expression, via the direct phosphorylation of numerous transcription factors and co-regulators, and their ability to indirectly control activity of cell cycle regulators including, cyclins, CDKs and their inhibitors, is well established (10, 13, 19). Nuclear ERKs contribute to the control of G1 and G1/S checkpoint progression by multiple mechanisms: 1) through control of AP1 and Ets transcriptional activity modulate cyclin D1 expression levels, cyclin D/cdk4 complex assembly, and consequently Rb
phosphorylation and activation of E2F and E2F-controlled promoters (including those of cyclin A and cyclin E); 2) facilitate cyclin A- and cyclin E-CDK2 complex assembly through the up-regulation of the CKIs p21 and p27, which act as assembly factors early in G1; and 3) affect the release of catalytically active CDK2 complexes by affecting the degradation of the inhibitory CKI p27 in late G1 (reviewed in ref. 13). While we are currently investigating the effect of ERK2(D319N) on these events, here we present evidence that expression of c-fos is partially restored in senescent ERK2(D319N) cultures. We also show that Rb phosphorylation is maintained at normal levels throughout their lifespan in contrast to senescent control cultures in which Rb is present mainly in its inhibitory, hypo-phosphorylated form. Thus, expression of the phosphatase-resistant ERK2 cannot only delay the onset but also inhibit some phenotypic characteristics of replicative senescence.

Chronologically, serum-induced ERK activation occurs early in the G1 phase of the cell cycle, prior to other changes required for cell cycle progression. Thus, nuclear ERK inactivation precedes, and may impose, cell cycle changes responsible for replicative arrest. Our results underscore the importance of nuclear ERK signaling in regulating aspects of the senescence phenotype and provide evidence that relatively minor genetic manipulations that restore nuclear ERK2 activity to normal levels can produce a dramatic increase in replicative lifespan and temporarily prevent the onset of senescence. Our data also underscore the physiological importance of MKP2 in the establishment of these changes and its role in the exquisite control of nuclear ERK activity and cell proliferation.

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Modulation of Replicative Senescence of Diploid Human Cells by Nuclear ERK Signaling
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